ROLE OF INSULIN AND INSULIN-LIKE PATHWAYS IN THE LEARNING AND MEMORY OF *Caenorhabditis elegans*

RASITHA SK

Ph.D. THESIS

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A THESIS SUBMITTED BY

RASITHA SK

TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM.

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

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DECLARATION BY THE STUDENT

I, Rasitha SK, hereby certify that I had personally carried out the work depicted in the thesis entitled, **"Role of Insulin and Insulin-like Pathways in the Learning and Memory of *Caenorhabditis elegans*"**. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: 06/11/2023

Rasitha SK

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This is to certify that Rasitha SK, 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 Insulin and Insulin-like Pathways in the Learning and Memory of *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.

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The thesis entitled

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Submitted by

Rasitha SK

for the degree of

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SYNOPSIS

Introduction

Learning and memory are two critical functions of the brain. Learning is typically defined as the activity or process of gaining knowledge or skill by studying, practising, being taught, or experiencing something. The net result is a change that increases the potential for improved performance in the future. On the other hand, memory is the process that involves encoding (acquisition and registration of information), storing (maintenance of information over time) and retrieving (bringing back stored information into conscious awareness) the learned information. Learning and memory play vital roles in how an individual reacts to the challenges in the face of changing environment. Otherwise, an individual's responses will be restricted to simple reflexes and stereotyped behaviour. Over the years, scientists around the globe have been trying to elucidate various aspects of learning and memory, and it is one of the most extensively studied in neuroscience.

Worldwide, disorders related to learning and memory are on the rise, and the affected population includes both the young and the old. Various factors contribute to its reasons, including neurodegenerative diseases, brain injury, genetic predisposition, and post-traumatic stress disorders. Various neurochemical changes are also linked to these disorders, one of them being disrupted insulin signaling in the brain. Until recently, the role of insulin in the brain was not well- understood. Recent studies have shown that along with governing fundamental behaviours like food intake, insulin signaling is involved in cognitive functions. At the synaptic level, insulin administration has been associated with modifying neurotransmitter release at various presynaptic terminals. Insulin and insulin-like signaling are also involved in neuronal growth, differentiation, and migration. Other beneficial roles of insulin in the brain include trophic effects on synapses and the promotion of dendritic spine formation.

Insulin can cross the blood-brain and blood-cerebrospinal fluid barrier to reach the brain. Insulin receptors are widely distributed in various regions of the brain and are expressed in both neurons and astrocytes. Insulin levels in the brain decrease with ageing and may be related to the decrease in cognitive functions, as reported in cases of Alzheimer's disease. Abnormal insulin/ insulin receptor levels have been observed in patients suffering from Alzheimer's dementia. Human post-mortem data, in several studies, has pointed to a link between brain insulin resistance and increased Alzheimer's pathology, including but not limited to increased amyloid-β deposition. A tau abundance in the cerebrospinal fluid and lower levels of cognition are also observed among diabetic patients. In several studies, the cognitive functions have shown drastic improvement when administered with exogenous insulin. The exact mechanism of alterations in learning and memory associated with insulin signaling is yet to be deciphered.

Insulin is also associated with release kinetics of many neurotransmitters and neuropeptides, among which the insulin- dopamine connection is of particular interest. This connection is also the basis for the higher risk of metabolic risks associated with various antipsychotic drugs. Mesolimbic-mesocortical dopamine pathways are involved in associative learning, and these regions have high insulin receptor expression. However, the association of insulin and dopamine in the learning and memory pathways is insufficiently addressed in the literature.

This study examined the roles of insulin and insulin-like pathways in the learning and memory of Caenorhabditis *elegans*. The effect of critical molecules of the pathway- the INS-1 ligand, the receptor DAF-2, and the DAF-16 transcription factor- in short- and long-term memories have been deciphered here. The effect of altered insulin signaling during early developmental stages in the learning and memory of worms has also been studied. I have also tried to elucidate the crosstalk between insulin and dopaminergic systems in the process.

Hypothesis

The fundamental hypothesis was that the insulin pathway is critical in the maintenance of learning and memory retrieval processes. It was also hypothesised that insulin affects learning and memory through alterations in the dopaminergic pathways.

Materials and Methods

C. *elegans* was used as the model organism to address the objectives of the study. The worms were maintained on Nematode Growth Medium with the Escherichia coli strain, OP50, as the food source. The worms were grown in their respective ambient temperatures as required. To obtain age-synchronised worms, gravid adults were washed to remove OP50 and treated with hypochlorite solution. Day 1 adults were used for all the experiments, unless otherwise mentioned.

Short- and long-term memory training paradigms were standardised using the wild-type control, Bristol N2 worms. An appetitive olfactory associative paradigm was used with the odorant 1/10 butanone and the bacterial food source, E. coli. After the training, worms were tested for their attraction towards the odorant in a chemotaxis assay. For the chemotaxis assay, worms were placed on the centre of an agar plate with control and test odorants spotted at the opposite ends. After 20 minutes, the chemotaxis index was calculated. To understand the roles of transcription, translation and calcium signaling, various chemical blockers were used in the desired concentrations during the conditioning phase of the training period. To understand the role of various molecules in the learning paradigm, mutant strains and siRNA-mediated silencing were used in the study. siRNA-mediated silencing was done against *daf-2* gene. Varying concentrations of exogenous insulin were added during the conditioning period to comprehend the effect of excess insulin on learning and memory formation.

For the large-scale culture of dauers, a modified egg white protocol was used. Dauers were extracted from the egg white plate by treating them with 1% sodium dodecyl sulphate. These dauers were then placed on OP50 plates to obtain post-dauer adults for the study.

In the local search assay, worms were washed from the food plate and starved for 30 minutes, and the number of omega turns taken by the worms on the foodless plate were counted and recorded. The insulin treatment in the worms was done 1 hour before the starvation in these experiments. To validate the avoidance behaviour of worms, glycerol was used as the repellent, and the fraction of worms taking omega reversals were noted.

Transgenic worms were created by microinjection of cloned plasmids having GCaMP-6 expressed under *dat-1* promoter in the wild-type and *daf-2* mutant [*daf-2(e1370) III*] worms. These worms were used in the calcium imaging experiments to elucidate insulin- dopamine interaction as well as to decipher the role of dopamine neurons in the process of learning and memory.

Major findings

An extended memory was observed in the insulin receptor mutant, *daf-2* [*daf-2(e1370) III*] after both short- and long- term trainings. Compared to the wild-type control, which retains short-term memory for 2 hours and long-term memory for 24 hours, the *daf-2* mutants [*daf-2(e1370) III*] were able to retain the memory for 5 hours following short-term training and 48 hours after the long-term training. To confirm if this altered memory was through the DAF-16 pathway, siRNA was designed towards *daf-2* and treated on the wild-type*, age-1* mutants and *daf-16* mutants followed by short-term memory assay. The results show that while the wild-type worms showed an extended memory upon the si-RNA treatment, the *age-1* and *daf-16* mutants failed to show similar behaviour. This result suggests us that AGE-1 and DAF-16 are

essential for the extended memory shown by *daf-2* mutants [*daf-2(e1370) III*], pointing to the fact that this pathway is through the DAF-16 transcription factor pathway. Because of the involvement of transcription factor in the process, the effect of general transcription and translation blockers were also checked. *daf-2* mutants [*daf-2(e1370) III*] also require new protein synthesis for short- and long-term memory acquisition like the wild-type N2 worms. Transcription is not necessary for short-term memory in both N2 and *daf-2* mutants [*daf-2(e1370) III*] alike. However, long-term memory is dependent on transcription. This is consistent with the earlier results obtained on *daf-16* mutants. Learning and memory also require calcium signaling.

Owing to the prolonged memory of *daf-2* mutants [*daf-2(e1370) III*], learning and memory of the mutants of downstream molecules were also checked. While mutations in the downstream molecules like AGE-1 [*age-1 (hx546) II*], AKT-1 [*akt-1(ok525) V*], and DAF-16 [*daf-16(m26) I*] did not affect short-term memory, these mutants showed defects in memory retention following long-term memory assays. *daf-16* mutants [*daf-16(m26) I*] also displayed poor learning at the 0th-hour post-training in the long-term assays compared to the wild-type worms.

To understand whether DAF-2 was required during the training or testing period, strain with a conditional mutation in the temperature-sensitive allele was used for short-term memory. This mutant expresses normal DAF-2 when placed at lower temperatures and a defective protein at higher temperatures. The worms were tested in different combinations of 15 ^{0}C and 23 ^{0}C during the training and/or testing period. While DAF-2 was found to be essential during memory retrieval in negative olfactory associative memory, DAF-2 was found to be required during the training period for the normal forgetting pathway in appetitive olfactory memory.

Post-dauer worms were used to comprehend the effect of downregulated insulin signaling during the developmental stages on the learning and memory of adult C. *elegans*. The data showed a severe defect in the long-term memory of these worms. On the other hand, only memory retention was affected in the case of short-term memory. Short- and long-term memory experiments on 24 hour starved worms and *eat-2* mutants [*eat-2(ad1116) II*] ruled out the possibility of dietary restriction in the altered memory of post-dauers. Furthermore, this defect was reverted by the administration of exogenous insulin.

INS-1 is a ligand of DAF-2, which is the most homologous to human insulin in the worm. Mutants [*ins-1(nj32) IV*] of this peptide also showed extended memory, which was abolished with the administration of exogenous insulin (0.1 IU and 0.5 IU concentration). Interestingly, the extended memory returned on the addition of higher concentrations of insulin (> 0.5 IU). However, in wild-type control worms, 0.1 IU insulin was sufficient to elicit an extended memory. From these results, it was concluded that excess insulin in the system significantly increases memory retention. The excess insulin antagonised the insulin pathway via the DAF-2 receptor. This was also confirmed in local search assays and DAF-16 nuclear localisation.

Insulin could also act as a reward signal similar to dopamine during training, and exogenous dopamine could revert the memory deficits observed in post-dauer worms. Memory deficits observed in the dopamine synthesis mutant *cat-2* [*cat-2(e1112*)] or the receptor mutants *dop-1* [*dop-1(vs101*)] and *dop-3* [*dop-3(vs106)*] did not show any significant changes upon conditioning with insulin. Dopamine could compensate for the lack of insulin signaling. Insulin, on the other end, could not compensate for downregulated dopamine pathway in the learning and memory paradigm. This observation was also on par with the results obtained in avoidance assays. Insulin could also cause dopaminergic neuronal activation in worms labelled with GCaMP-6 under *dat-1* promoter in the calcium imaging experiments.

Calcium imaging data of *dat-1p*::GCaMP-6 and *daf-2; dat-1p*::GCaMP-6 worms also revealed the role of dopamine neurons in the extended memory owing to *daf-2* mutation and administration of exogenous insulin. The role of RID interneuron that is mainly attributed to forward locomotion in learning and memory was also shown in the present study.

Significance of the study

This study incorporates the importance of insulin and insulin-like signaling in the learning and memory of C. *elegans*. The significance of proper insulin signaling during the critical period of early developmental stages was also studied. It was found that proper insulin signaling is vital for the maintenance of normal memory. Any alterations thereof would result in problems associated with memory. Results from this study strongly suggest that exogenous insulin administration could compensate for the downregulated insulin pathway of dauers in the learning and memory in their later stages could also be the basis for improved cognitive functions of dementia patients following intranasal insulin administration.

The present work also addresses the link between insulin and dopamine. Insulin-dopamine crosstalk is relevant in the metabolic effects of various antipsychotic drugs. Dopamine is one of the critical neurotransmitters known to modulate the learning and memory pathway. Another influential hypothesis posits that dopamine biases reinforcement learning. Based on the current study, it was found that insulin could also act as a reward signal during associative learning. Further, dopamine signaling was found to work downstream of insulin signaling in learning and memory. Put together, this study brings out the effect of insulin, dopamine, and their relationship, thereof, in the context of learning and memory.

Review of Literature

Insulin

Insulin was the first peptide hormone discovered (Weiss, Steiner and Philipson, 2000). In 1921, Frederick Banting and Charles Best were the first to isolate insulin from the pancreas of dogs at the University of Toronto. With the advent of DNA recombinant technology, insulin ranks as the first protein to be chemically synthesised (Johnson, 1983). When Frederick Sanger sequenced its amino acid structure (Figure 1) in 1951, insulin also became the first protein to be fully sequenced (Stretton, 2002).

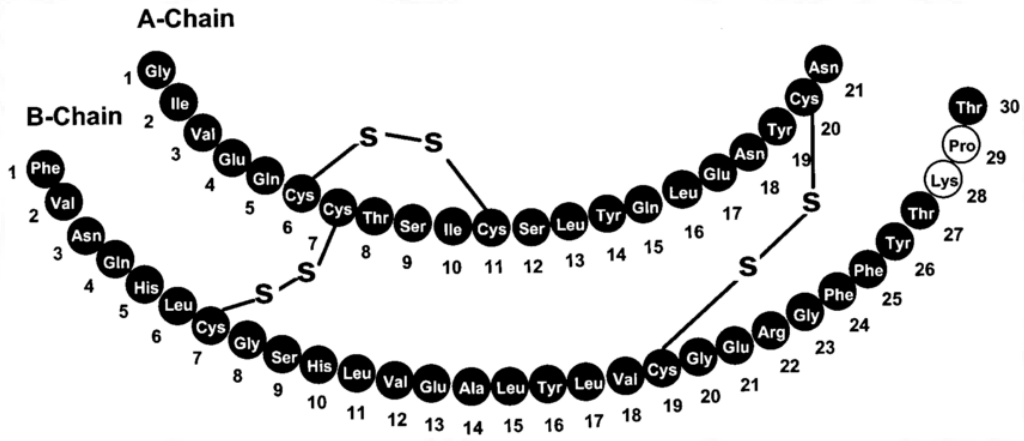

Figure 1. The amino acid sequence of human insulin. Insulin is composed of two separate peptide chains, the A chain and the B chain joined together by two disulphide bridges. A third disulfide is found in the A-chain joining the cysteine 6 and 11. Figure from Bowsher et al., 1999 and reprinted with permission from the Elsevier *Inc* © 1999

Insulin is regarded as the primary anabolic hormone of the body. It is involved in the metabolism of carbohydrates, fats, and protein by promoting the absorption of glucose from the blood into liver, fat and skeletal muscle cells. The absorbed glucose undergoes glycogenesis, converting the glucose into glycogen, or lipogenesis, converting glucose into triglycerides. Circulating insulin also affects proteins synthesis.

Although the primary function of insulin is considered to be peripheral glucose homeostasis, its role in the central nervous system (CNS) is also being studied recently. Recent reports on the brain, which was earlier considered to be an insulin insensitive organ, has revealed the presence of insulin, insulin-like peptides (ILPs), and insulin receptors (IRs) (Milstein and Ferris, 2021). It is now understood that brain insulin also mediates vital physiological functions like reproduction (Sliwowska *et al.*, 2014), obesity (Rebelos *et al.*, 2021), and cognition (McNay and Recknagel, 2011).

Insulin Signaling in the Central Nervous System

a. Insulin in the Central Nervous System

Till the late 1970s, brain was regarded as an insulin-insensitive organ. The first report on the evidence of insulin in brain came from Havrankova et al. (Havrankova *et al.*, 1978). They used radioimmunoassay to determine the levels of insulin in rat brain extracts. They found that insulin concentrations in acid/ethanol extracts of the whole rat brain were 25 times higher than plasma insulin levels. They observed that some regions had insulin concentrations as much as 100 times higher than in plasma; at least 10 times higher were found in other regions. Similarly, it was also reported that brain insulin levels were independent of peripheral insulin as circulating insulin did not affect brain insulin levels (Havrankova, Roth and Brownstein, 1979). They demonstrated that brain insulin, which is at least 10 times higher than plasma insulin levels, and the brain insulin receptor content, which is comparable to receptor content on peripheral tissues, are regulated independently of the hormone and receptor in the peripheral tissues. Likewise, insulin was reported in the brain of other organisms also (Dorn, Bernstein, *et al.*, 1983)

Since the evidence for the presence of insulin in the brain, questions regarding its source were raised. It was also regarded that, at least in part, cerebral insulin and glucagon are products

of the brain itself (Dorn, Bernstein, *et al.*, 1983). However, other sources for cerebral insulin were also considered, such as its peripheral origin and then crossing the BBB versus a central origin, or both (Havrankova *et al.*, 1978) (Figure 2).

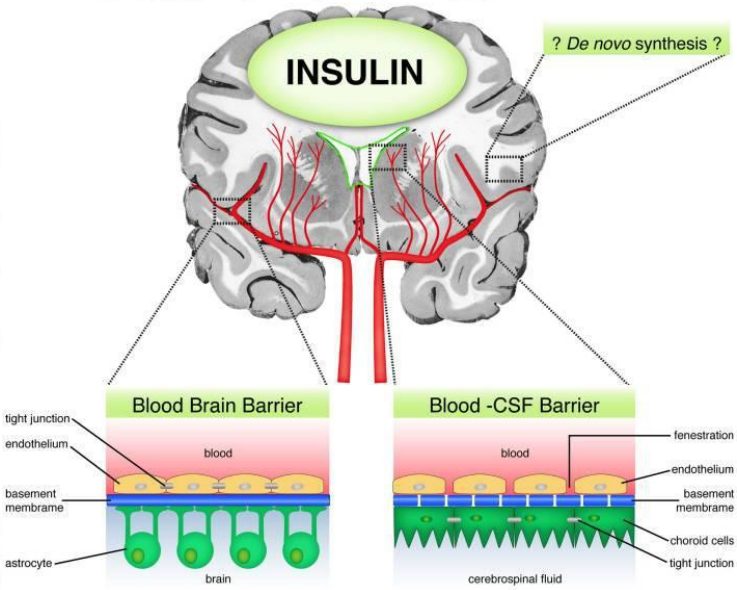

Figure 2. Sources of brain insulin. Peripheral insulin enters the brain through the blood brain barrier (BBB) by means of a selective, carrier-mediated transport system. Insulin can also diffuse through the blood–CSF barrier in circumventricular regions. Additionally, there is limited evidence suggesting a possibile *de novo* insulin synthesis in the brain. (Image courtesy: Akintola and van Heemst, 2015).

Peripheral sources of insulin

Margolis and Altszuler first proposed that insulin could cross the blood brain barrier (Margolis and Altszuler, 1967). Findings proving a non-linear correlation between plasma and CSF insulin levels provided the evidence for a saturable transport system for insulin from blood to the brain (Woods and Porte, 1977; Margolis and Altszuler, 1967). Regional differences in insulin permeability also corresponded to the hormone levels in these regions, with the highest values in the pons, medulla, and hypothalamus and the lowest in the occipital cortex and thalamus (Banks and Kastin, 1998). The rate of insulin transport depends on various factors like glucocorticoids (Baura *et al.*, 1996). In many physiological situations, such as obesity

(Kaiyala *et al.*, 2000), fasting and re-feeding (Strubbe, Porte and Woods, 1988), and hibernation (Florant *et al.*, 1991), insulin transport across the BBB is found to be affected. Likewise, it is also affected during ageing and in patients with diabetes mellitus (DM), and Alzheimer's disease (AD) (Banks et al., 2012; Ghasemi et al., 2013).

Central origin of Insulin

Detection of C-peptides in the brain led researchers into believing that at least a part of cerebral insulin was the product of the brain itself (Dorn et al., 1983b; Jezová et al., 1985; Frölich et al., 1998). In one of these studies, for example, C peptide was found in the cortical region of the post-mortem brain of the elderly and AD patients (Frölich *et al.*, 1998). They were also able to show for the first time that a direct correlation exists between -peptide concentrations and a decrease in the number of brain IRs.

In addition, insulin mRNAs have also been observed in various brain regions. For example, the presence of insulin mRNA was found in the periventricular nucleus of the rat hypothalamus by in situ hybridisation (Young, 1986). Additionally, insulin mRNA was also found in the brains of fetal, neonatal, and adult rats (Devaskar *et al.*, 1993), and also in the CA1 and CA3 regions of the hippocampus, in the dentate gyrus, and the granule cell layer of the olfactory bulbs of the neonatal rabbit brain (Devaskar *et al.*, 1994).

There are also evidence of *de novo* synthesis of insulin in the brain. In primary cultures of rat brain, incubation with [^{3}H] valine resulted in the incorporation of radioactivity into immunoprecipitable insulin (Raizada, 1983). Also, immunohistochemical and *in situ* hybridisation techniques has shown the ability of fetal neuron cell cultures to produce and secrete an insulin-like mRNA and an insulin-like substance (ILS) that was indistinguishable from real insulin (Schechter *et al.*, 1994). In another study, *in situ* synthesis and secretion of

insulin in the central mammalian neurons in neuron-enriched but not the glial-enriched postnatal rabbit brain cell cultures were reported (Devaskar *et al.*, 1994). These studies demonstrate that the brain synthesises a part of the insulin found in the CNS.

b. Brain insulin receptor signaling

Insulin Receptors in the Brain

The peripheral IRs and their role in mediating glucose transport into the cells are well-understood. However, till the 1970s, the existence of IRs within the brain was poorly understood, and their function seemed nothing short of an enigma. This was mainly because brain cells are not entirely reliant upon insulin for glucose supply as they have insulin-independent means of obtaining glucose (Schulingkamp *et al.*, 2000). Studies on the presence of insulin in the CNS began in the 1970s (Szabo and Szabo, 1972). IRs were first found and quantified in the CNS for the first time in 1978 (Havrankova, Roth and Brownstein, 1978). It was found to be present in the membrane preparations from the rat brain at all stages of the development studied (Lowe *et al.*, 1986). Since then, brain IRs have been widely studied, and its uneven distribution of IRs in the CNS has been reported.

IRs are unevenly distributed in the brain. For example, the receptor density is higher in the hypothalamus, especially the anterior hypothalamus, than the cortex and thalamus (Landau *et al.*, 1983). Similarly, IRs are densely located in the olfactory areas and closely-related limbic regions, neocortex, basal ganglia, cerebellum, and choroid plexus (Hill *et al.*, 1986). In autoradiography and computerised densitometry studies, the highest concentrations were observed in areas associated with olfaction, appetite, and autonomic functions (Werther *et al.*, 1987). Similarly, in situ hybridisation showed that the IR mRNA was the most abundant in olfactory bulb, cerebellum, dentate gyrus, piriform cortex, hippocampus, choroid plexus, and the hypothalamus (Marks *et al.*, 1990).

Insulin Signaling in the Brain

Insulin binds to the α-subunits of the IRs and triggers the activation of the β-subunit tyrosine-kinase activity by phosphorylation of its receptors in both neuronal and glial cells (Shemer *et al.*, 1989). The subsequent signal transduction is modulated through several insulin receptor substrates (IRSs) (Brummer, Schmitz-Peiffer and Daly, 2010) and other scaffold proteins (Taguchi and White, 2008) that initiate divergent molecular pathways (Saltiel and Pessin, 2002) (Figure 3). Insulin-binding triggers the internalisation of the aggregated IRs into the cells (Pilch *et al.*, 1983). This aggregation and internalisation are essential for insulin signaling (Heffetz and Zick, 1986). These internalised IRs can then be degraded or recycled back to the membrane (Di Guglielmo *et al.*, 1998).

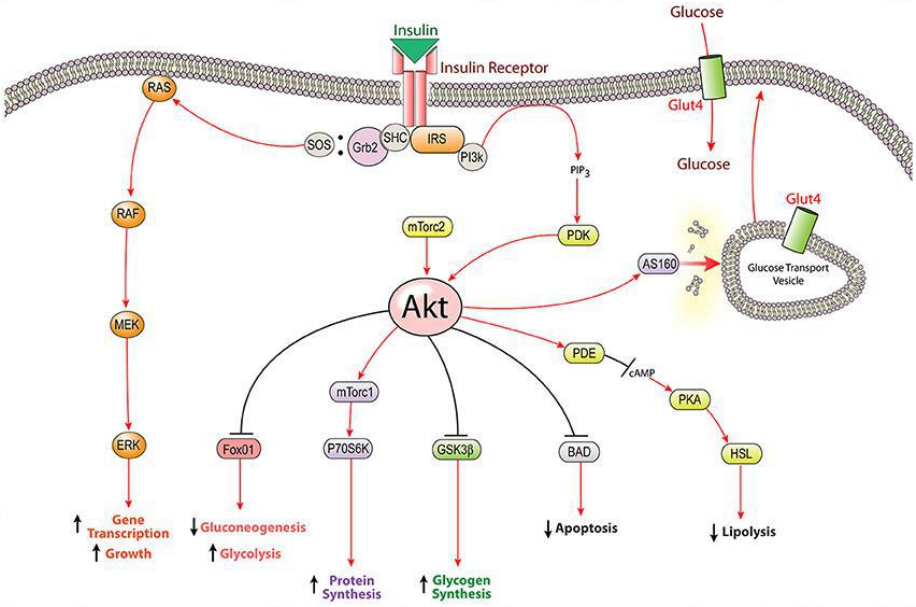

Figure 3. Insulin signaling in the brain. Binding of insulin to its receptor initiates a cascade of reactions downstream affecting gene transcription, glucose homeostasis, apoptosis, and biosynthesis of proteins and lipids. (Image courtesy: Grote and Wright, 2016)

The IRS proteins are a family of cytoplasmic adaptor proteins that transmit signals from the insulin and insulin growth factor-1 (IGF-1) receptors to elicit a cellular response. Insulin responses are mainly mediated by IRS-1 and IRS-2. IRS-1, the first member of the family to

be identified, controls body growth and peripheral insulin action (Aguirre *et al.*, 2002). On the contrary, IRS-2 regulates brain growth, body weight, glucose homeostasis, and female fertility (White, 2003). IRS proteins comprise an NH2-terminal pleckstrin homology (PH) domain adjacent to a phosphotyrosine-binding (PTB) domain which is followed by a tail containing many tyrosine and Ser/Thr phosphorylation sites (Myers, Sun and White, 1994). The Tyr phosphorylation sites orchestrate downstream signaling cascades by binding the SH2 domains in the common effector proteins (Taguchi and White, 2008; White, 2003). On the contrary, c-Jun N-terminal kinase (JNK-1)-mediated Ser^{307} phosphorylation of the IRS-1/2 inhibits insulin-stimulated tyrosine phosphorylation (Aguirre *et al.*, 2002). Similarly, ubiquitin-mediated degradation of IRS-1/2 inhibits insulin action (Krebs and Hilton, 2003). By contrast, the agonists that increase IRS-2 expression utilising cAMP production and CREB activation facilitate insulin signaling (Jhala *et al.*, 2003). The association of IRS proteins and PI3K triggers the phosphorylation of an inositol phospholipid in the plasma membrane, named PIP2, to PIP3, which recruits both the Ser/Thr kinase 3-phosphatidylinositol-dependent protein kinase (PDK) and protein kinase B (PKB or AKT) to the plasma membrane. The AKT is subsequently activated by PDK-1 and PDK-2-mediated phosphorylation (Lizcano and Alessi, 2002). The phospholipid phosphatases, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) or SH2-domain-containing inositol phosphatase 2 (SHIP-2) antagonises this pathway. AKT phosphorylates several substrates, such as tuberous sclerosis complex, tuberin (TSC-2), which finally activates the mammalian target of rapamycin (mTOR) and provides a direct link between insulin signaling and nutrient sensing (Taguchi and White, 2008; Jewell and Guan, 2013).

Additionally, a second signaling pathway has been reported in peripheral tissues for the translocation of the glucose transporter GLUT-4 by insulin that involves other substrates of IR. Following the recruitment of various proteins into the lipid raft, GLUT-4 vesicles fuse with the plasma membrane (Saltiel and Pessin, 2002; Lizcano and Alessi, 2002). Yet another signaling pathway activated by insulin is the one that involves Mitogen-activated protein kinase through

tyrosine phosphorylation of specific prototypical signaling adaptors, which activate the small G-protein Ras by stimulating GDP:GTP exchange. This activates Raf (Kolch *et al.*, 2002), initiating a cascade of several protein kinases that include MAPK/ERK kinase (MEK) and extracellular signal-regulated kinase (Evans *et al.*, 2004). ERK, in turn, phosphorylates various cytosolic and signaling proteins. ERK also controls the gene expression by phosphorylating transcription factors like ELK-1 and other ETS family proteins after entering the nucleus (Ghasemi et al., 2013; Taguchi and White, 2008).

c. Role of Insulin in Glucose Homeostasis

Though the brain utilises ketone bodies during starvation, glucose serves as its primary fuel that is required in a continuous and permanent supply (White and Venkatesh, 2011). In addition to being an energy substrate, it is also involved in glucoregulatory mechanisms (Figure 4). It provides an uninterrupted glucose supply to the CNS and meets the metabolic needs of peripheral tissues.

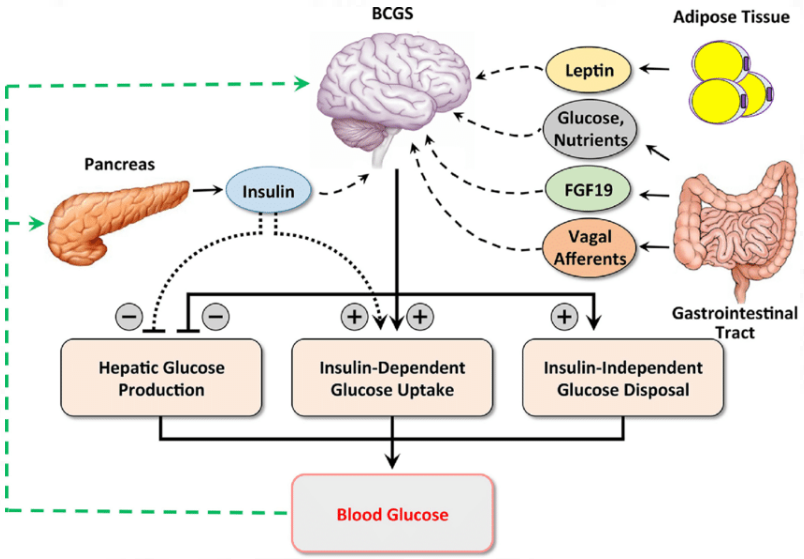

Figure 4. Role of insulin in glucose homeostasis. Glucose homeostasis involves cooperative and coordinated interactions between brain- and islet-centered regulatory systems. BCGS- brain-centered glucoregulatory system. Coordinated activation of both these

systems by nutrients helps maintain blood glucose levels within the narrow physiological range. (Image courtesy: Scarlett and Schwartz, 2015).

The glucoregulatory functions are generally secondary to glucose uptake, which is controlled by the level of glucose transporter and glucose sensor in most tissues (Thorens and Mueckler, 2010). GLUT-1 is the most abundant glucose transporter in the brain (Simpson *et al.*, 1999). They have tissue-specific functions, and some isoforms are sensitive to insulin (Schulingkamp *et al.*, 2000). GLUT-2 is co-expressed with glucokinase (Schulingkamp et al., 2000; Navarro et al., 2002) sulfonylurea receptor-1 (SUR1) (Li *et al.*, 2003) in several neuronal populations like in the paraventricular nucleus, the arcuate nucleus, and the lateral region (Kang et al., 2004; Levin et al., 2004) of the hypothalamus. GLUT-3 is the major glucose transporter in the neurons of the cerebellum, striatum, cortex, and hippocampus (Nagamatsu *et al.*, 1992). It has also been detected in brain glial and endothelial cells (Gould *et al.*, 1992). GLUT-4 is located in selective areas of the brain such as the olfactory bulb, dentate gyrus of the hippocampus, hypothalamus, and cortex (El Messari *et al.*, 1998). In cerebellar membranes, GLUT-4 is expressed in significant amounts, and its expression is insulin-dependent (Vannucci *et al.*, 1998). In addition, the trafficking of GLUT-4 to the plasma membrane is regulated in the cerebellum, cortex, and hippocampus under conditions that increase plasma insulin levels (Talbot *et al.*, 2012). GLUT-4, glucokinase (GK), and IR are co-expressed in both glucose- excitatory (GE) and glucose-inhibitory (GI) hypothalamic neurons suggesting stimulation of glucose uptake in response to insulin in these regions (Livingstone, Lyall and Gould, 1995). GLUT-8 is a neuron-specific glucose transporter. It is expressed in cell bodies and the most proximal apical dendrites of the brain (Ibberson *et al.*, 2002) in both excitatory and inhibitory neurons in the hippocampus (Reagan *et al.*, 2002). Since it is an insulin-responsive isoform, it may play a role in enhancing substrate delivery under conditions of increased demand (Sankar *et al.*, 2002). GLUT-8 contributes to glucose homeostasis in hippocampal neurons by transporting glucose out of the rough endoplasmic reticulum into the cytosol, which is impaired under hyperglycemic/insulinopenic conditions (McEwen and Reagan, 2004; Piroli et al., 2002).

Inhibition of insulin action in the hypothalamus, or the direct stimulation in the arcuate nucleus, induces a reduction in insulin's ability to block the glucose production in the liver (Obici, Feng, *et al.*, 2002). This action is mediated through the IRs in the liver and hypothalamus. Likewise, reduction in insulin sensitivity in the hypothalamus was found to be associated with inhibition of glucose formation (Obici, Zhang, *et al.*, 2002), which might, in turn, contribute to the hyperglycemia of diabetic patients (Demuro and Obici, 2006; Girard, 2006). The resection of the liver branch of the vagus nerve induces a decrease in the insulin inhibitory effect on hepatic glucose production (Girard, 2006). These findings are consistent with the report published as early as in 1855 that puncturing the fourth cerebral ventricle produced glycosuria in mice, which gives rise to the assumption that the brain is involved in glucose homeostasis (Blázquez *et al.*, 2014).

Insulin acts as an anabolic hormone in the periphery and a catabolic hormone in the brain because of its anorexigenic properties (Niswender *et al.*, 2003). Insulin is also known to strengthen leptinergic signals via Janus kinase-2 (JAK-2) and spinohypothalamic tract-3 (SHT-3) in the hypothalamus (Kim *et al.*, 2000).

d. Role of Insulin in Reproduction

Fertility is closely associated with reproduction. Alterations in the energy homeostasis cause changes in the regulation of reproduction (Castellano *et al.*, 2009) through the hypothalamic-pituitary-gonadal axis (Fernandez-Fernandez *et al.*, 2006). Many hormones, including insulin, are involved in the metabolic regulation of reproduction. These hormones act at several levels in the crosstalk between the hypothalamus, pituitary gland, and gonads. Perfusion of insulin at low concentrations in the hypothalamic pieces stimulates the secretion of luteinizing hormone releasing hormone (LHRH) (Arias *et al.*, 1992). Conversely, high levels of glucose cannot modify the release of LHRH (Arias *et al.*, 1992). Similarly, the intracerebral infusion of insulin increases the luteinizing hormone (LH) pulse frequency (Miller, Blache and Martin,

1995). Low levels of circulating insulin were accompanied by a reduction in the release of LH (Arias *et al.*, 1992), whereas the central or peripheral administration of insulin restored LH pulse frequency (Dong *et al.*, 1991). Other studies have established that low levels of circulating insulin in diabetic rats decrease gonadotropin-releasing hormone (GnRH) release from the hypothalamus and the response of pituitary LH-releasing cells to GnRH (Dong *et al.*, 1991). These results strengthened the hypothesis that intracerebral insulin is a key regulator of pulsatile GnRH secretion in diabetic sheep (Tanaka *et al.*, 2000). However, it has not been ruled out that the effects of glucose in LH secretion could not be entirely dependent on insulin activity because specialised glucodetectors in the hypothalamus can also modulate GnRH secretion, in an insulin-dependent or independent manner (Bucholtz *et al.*, 2000).

e. Role of Insulin in Cell Proliferation and Differentiation

The role of insulin in proliferation, differentiation, and neurite growth has been reported in developing nervous systems. One of the evidence of insulin promoting the development of the brain came from studies on neonatal rats in which the ornithine decarboxylase activity, which is an indicator of growth stimulation, in the brain was found to be increased in response to insulin (Roger and Fellows, 1980). Moreover, the expression of IRs increase during cell differentiation in the developing brain (Wozniak *et al.*, 1993). With the observations that IRS-2 mediated the effects of insulin on brain growth (Schubert *et al.*, 2003), and on the axonal outgrowth, maturation, and regeneration (Xu *et al.*, 2004), as well as on neurite growth (Ghasemi *et al.*, 2013), the notion that IR signaling plays a vital role in neuronal proliferation during development became stronger. Studies also pointed out that insulin stimulates nucleotide incorporation in the rat brain (Raizada, Yang and Fellows, 1980) and induces neuronal growth and differentiation in chick forebrain (Heidenreich, Vellis and Gilmore, 1988). This insulin-dependent growth and development have been reported in both neurons and glial cells (Clarke et al., 1985; Heidenreich and Toledo, 1989). Moreover, insulin and IGF-2 were found to be crucial for NGF to stimulate neurite outgrowth (Recio-Pinto, Lang and Ishii, 1984).

These effects were also found to be astrocyte-dependent (Ang, Bhaumick and Juurlink, 1993). Additionally, it has been reported that insulin promotes the proliferation of both cultured rat (Velázquez, Blázquez and Ruiz-Albusac, 2009) and human (Heni *et al.*, 2011) astrocytes, where the expression of many critical proteins involved in insulin signaling was shown to increase. Insulin increased ribosomal protein S6 phosphorylation (Heidenreich and Toledo, 1989) and PKC-epsilon activity in cultured fetal neurons, which could be closely related to neurite outgrowth (Vanhems et al., 1990; Heidenreich et al., 1991).

Insulin also activates other protein kinases, such as phosphatidylinositol 3-kinase (PI3K) (Patel *et al.*, 1993), thereby modulating the growth of neuronal cells. For example, by activating the PI3K/ mammalian target of rapamycin (mTOR) pathway, insulin increases the expression of dendritic scaffolding protein post-synaptic density-95 (PSD-95) in hippocampal area CA1, which explains the effect of insulin on synaptogenesis and the modulation of the synaptic function in CA1 (Lee *et al.*, 2005). This also explains the regulation of dendritic spine formation and excitatory synapse development in hippocampal neurons (Lee, Huang and Hsu, 2011) by insulin.

Another plausible mechanism of the regulation of neurite formation could be the upregulation of tau protein which is the main microtubule-associated protein in the CNS that participates in the axon/neurite growth. This also involves the PI3K/mTOR pathway, or the upregulation or stabilisation of tubulin mRNA and the subsequent increase in protein levels (Nemoto *et al.*, 2011). In the presence of IR inhibitors or insulin antibodies, endogenous insulin synthesised by neurons promotes neurofilament distribution (Schechter *et al.*, 1998). In addition, downregulation of the PI3K/AKT pathway was found to be critical to the cell survival signaling of differentiated human neurons and human-derived neural stem cells (hNSC) (Rhee *et al.*, 2013).

Thus, IR pathways function as an integrating factor that connects nutritional information with neuronal differentiation. In fact, the degree of differentiation correlates with the modifications of the nutritional state (Léopold, 2004).

f. Neuroprotective effects of Insulin

Insulin has potent neuroprotective effects and acts against apoptosis, β-amyloid toxicity oxidative stress, and ischemia. It has been also reported that the anti-apoptotic effect of insulin is through the PI3K pathway (Figure 5), and not through the mitogen-activated protein kinase (MAPK), because the inhibition of mTOR activity by rapamycin avoids the anti-apoptotic effects of insulin. This suggests that the protein p70SK, one of the downstream targets of the PI3K/AKT/mTOR pathway, may be one of the mechanisms through which insulin prevents apoptosis (Ryu *et al.*, 1999). Studies have reported insulin prevents the formation of Aβ fibrils (Rensink *et al.*, 2004).

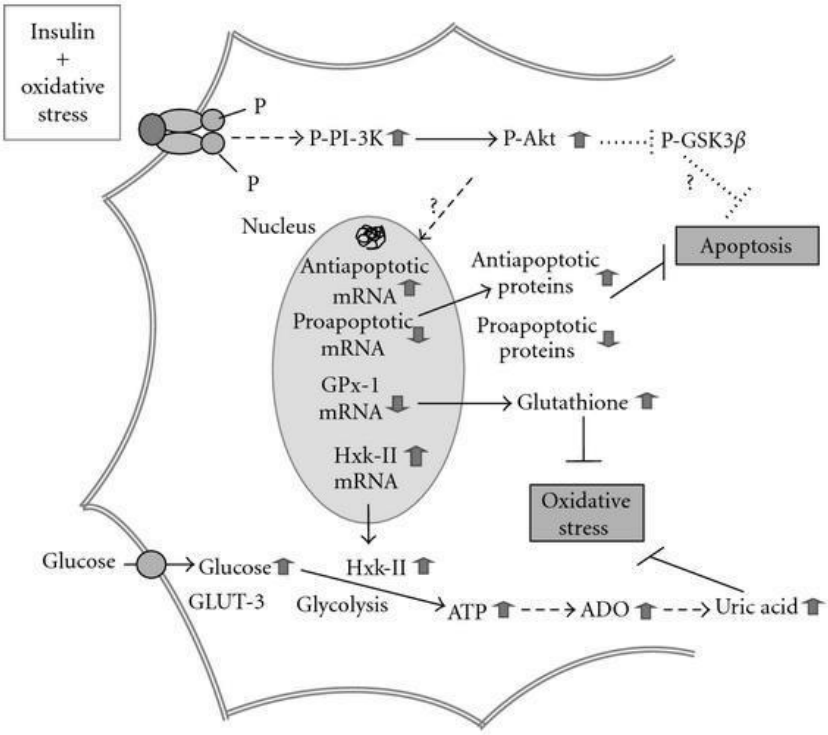

Figure 5. Insulin-mediated neuroprotection during oxidative stress. Insulin signaling regulatess the expression of "candidate" proteins, namely, glutathione peroxidase-1 (GPx-1), hexokinase-II (Hxk-II), and also the antiapoptotic Bcl-2 and the proapoptotic caspase-3. Insulin also interferes with GSK-3*β* signaling, decrease its activated form, and inhibit apoptotic neuronal death during oxidative stress. (Image courtesy: Duarte et al., 2012)

Insulin antagonises the adverse effects of oxidative stress in the CNS. As a result of oxidative stress, GLUT-3 is altered, which modifies glucose uptake and lactate accumulation, acidosis, and mitochondrial dysfunction (Garg *et al.*, 2006). Insulin restores intracellular ATP formation and reduces oxidative stress by stimulating glucose uptake and pyruvate formation (Duarte *et al.*, 2006). GABA and glutamate uptake are also decreased under oxidative stress, which, in turn, induces the accumulation of these neurotransmitters in the extrasynaptosomal space. Insulin restores these changes (Duarte *et al.*, 2003). Insulin also elevates uric acid in response to severe oxidative stress as uric acid, glutathione (GSH), and vitamins C and E are essential components of the neuronal antioxidant pool (Sevanian, Davies and Hochstein, 1991).

There are two mechanisms through which insulin protects against ischemia: the direct effect on brain tissues and by the reduction of peripheral glucose levels (Auer, 1998). Insulin increases GABA during transient ischemia, which inhibits pyramidal neurons, thereby protecting against ischemia in a manner independent of hypoglycaemia (Shuaib *et al.*, 1995). Likewise, insulin also alters glucose metabolism and decrease lactic acidosis (Grunstein *et al.*, 1985). Therefore, insulin has a stimulatory effect on the Na^+/K^+ ATP pump and reduces both extracellular K^+ and intracellular Na^+, which in turn, change the neuronal firing rate and its metabolic demands, all the while preventing water accumulation and the subsequent post-ischemic oedema (Voll and Auer, 1991). Insulin reverses the JNK-1/2 phosphorylation, B-cell lymphoma-2 (BCL-2) protein expression, and caspase-3 cleavage in the rat hippocampus induced by the cerebral ischemia-reperfusion (Sanderson *et al.*, 2008). Thus, it can be concluded that crosstalk exists between AKT and JNK-1/2 that could play a role in the anti-ischemic effects of insulin.

g. Neuromodulatory effects of Insulin

Insulin acts at the electrophysiological levels and also affects the concentration and function of certain neurotransmitters. Dose-dependent inhibitory response to insulin were observed with respect to GABA pretreatments blocked by co-administration of IR inhibitor (Voll and Auer, 1991). Insulin also modulates the activity of specific ion channels affecting intracellular ion concentrations. For example, insulin in hypothalamic neurons activates K^+ ATP channels causing membrane hyperpolarisation (Plum, Schubert and Brüning, 2005). Contrariwise, insulin also has stimulatory effects on Na^+/K^+ ATPase, resulting in an acute rise in intracellular Ca^{2+} concentration that triggers the release of neuropeptides (Jonas *et al.*, 1997).

Insulin modulates neurotransmitter concentration. For example, insulin induces the inhibition of norepinephrine while causing stimulation of serotonin reuptake in neuronal cells (Boyd et al., 1985; Masters et al., 1987). This increases glucose homeostasis through the crosstalk

between brain IRs and the neurotransmitter function (Wozniak *et al.*, 1993). Insulin also exerts its effect by regulating the neurotransmitter receptor density. Insulin reversed the increase in the number of dopamine receptors in the striatal membranes of alloxan or streptozotocin-induced diabetic rats (Lozovsky, Saller and Kopin, 1981) and rats treated with haloperidol (Lozovsky, Kopin and Saller, 1985). On the contrary, systemic administration of insulin caused an increase in dopamine and serotonin levels in the CSF, while this hormone downregulated the α_2-adrenergic receptors in hypothalamic neurons (Levin, Israel and Figlewicz Lattemann, 1998). Insulin also has a stimulatory effect on the amino acid uptake by the neurons required for neurotransmitter synthesis (Rhoads *et al.*, 1984).

h. Role of Insulin in Ageing and Longevity

Aging is defined as the time-dependent loss of fitness that begins after the organism attains its maximum reproductive competence (Vijg and Suh, 2005). Ageing increases susceptibility to stress (Miller, 2009) and culminates in an increased incidence of chronic diseases and an exponential increase in the chance of dying (Vijg and Suh, 2005). On the contrary, longevity is the property of approaching the species-specific maximum lifespan, to be precise, the oldest observed age of death in the species (Vijg and Suh, 2005).

In recent years, a parallel anti-ageing paradigm has been proposed, with the observations that genetic changes or caloric restriction in insulin signaling pathway slowed down the rate of living, thus decreasing metabolism and oxidative stress (Weindruch et al., 2001; Carter et al., 2002; Heilbronn et al., 2006; (Al-Regaiey *et al.*, 2007). This hypothesis has been supported further by studies demonstrating that dietary restriction inhibited insulin signaling that regulate glucose intake, prolonging rodent longevity (Masternak *et al.*, 2005). More recently, it has been proposed that epigenetic factors regulated by ageing (such as sirtuins, histone acetylases, and DNA methyltransferases) impose a metabolic shift towards an increased reliance on glycolysis and probably mediated age- and sedentarism-related insulin resistance (conditions

that require lower metabolic demands) (Brewer, 2010). As a result, aged tissue becomes incapable of coping with energy demands or stress, initiating a catastrophic cycle of oxidised membrane receptors, signaling molecules, transcription factors, and epigenetic transcriptional regulators, resulting in cell death and organ failure (Brewer, 2010). Insulin resistance also augments the formation of advanced glycation end products (AGEs), thereby creating a vicious cycle on ageing brain (Plum, Schubert and Brüning, 2005).

Changes in IR signaling associated with ageing may also arise due to increased cholesterol levels and decreased membrane fluidity or modification of IR internalisation, re-expression, or degradation by the proteasome (Fulop, Larbi and Douziech, 2003). Interestingly, ageing has also been implicated with decreased brain IR expression and binding capacity, especially in hippocampus, cortex, and choroid plexus (Zhao et al., 2004; Frölich et al., 1998; (Potau, Escofet and Martinez, 1991).

An increasing number of studies points towards the notion that downregulated insulin signaling pathway promotes longevity in yeast, worms, Drosophila, mice, and man (Carter et al., 2002; Pawlikowska et al., 2009; Taguchi et al., 2007; Kurosu et al., 2005; Van Heemst et al., 2005; Blüher et al., 2003). Increased expression of adiponectin, and peroxisome proliferator-activated receptor-γ-2 (PPARγ-2) gene (two well-known insulin sensitisers) is observed in centenarians and long-lived men, whose type-2 diabetes incidence was also dramatically decreased (Bartke, 2008). In addition, the mutation in the IR/IGF-1R homolog, DAF-2, causes an increased lifespan via the nuclear translocation of DAF-16, a protein belonging to the FOXO family of transcription factors (Hahm, Kim and Paik, 2009). Similar observations were also made in Drosophila, where null mutations in IR or IRS homologues were shown to extend the lifespan of females (Vijg and Suh, 2005). Likewise, overexpression of IRS-1 in mice was associated with a decrease in lifespan (Kurosu *et al.*, 2005), and overexpression of IRS-1, 2, 3, or 4 in adipose cells resulted in GLUT-4 translocation independently from insulin (Zhou *et*

al., 1999). Mice lacking IRS-2 was found to have an extended lifespan (Taguchi, Wartschow and White, 2007). On the other hand, IR over-expression in mammary epithelial cells resulted in a tumorigenic phenotype (Frittitta *et al.*, 1995), which suggests that the continuous activation of insulin signaling might not be beneficial to age-related oxidative stress. Some studies indicate that reversible control of cGMP might modulate insulin production and, thus, constitute a crucial regulatory messenger of lifespan extension (Hahm, Kim and Paik, 2009).

Studies on centenarian people, calorie-restricted rodents and non-human primates indicate that prerequisites for longevity include increased insulin sensitivity and subsequent normal IR signal transduction, decreased fasting glucose, and oxidative stress (Fulop et al., 2003; Barbieri et al., 2003). Consistent with these observations, a decrease in mitochondrial function also resulted in increased lifespan (Bishop, Lu and Yankner, 2010). This is further supported by studies in *clk-1* mutant worms, which display reduced respiratory, developmental and behavioural rates and longer lifespan (Bishop, Lu and Yankner, 2010). CLK-1 is essential for ubiquinone synthesis and is an essential component of the mitochondrial electron respiratory chain. Similar results have been observed in *Drosophila* mutants with reduced expression of electron transfer chain components in adult neurons (Copeland *et al.*, 2009) and in a mouse model with the downregulated activity of cytochrome c oxidase complex (Dell'Agnello *et al.*, 2007).

Taking these observations into account, Type-2 diabetes can be, at least partially, considered as a model for premature ageing. This is substantiated by a decrease in cellular replicative senescence in diabetic subjects (Fulop, Larbi and Douziech, 2003). Hence, the development of more efficient preventive and therapeutic strategies to overcome age-related diseases associated with diabetes is an emerging challenge.

i. Role of Insulin pathway in Learning, Memory, and Cognition

Treatment of high-fat-fed mice with an anti-diabetic drug restored the levels of insulin-induced neuronal IR, IRS1, and AKT activation and long-term depression, and enhanced cognitive function measured by the Morris water maze test (Pipatpiboon *et al.*, 2013). Diet-induced impaired neural insulin signaling in animal models of Alzheimer's disease-like neuropathology enhanced amyloidogenic Aβ1-40 and Aβ1-42 peptide generation, increased amyloid plaques in the brain, and impaired performance in spatial water maze task (Ho *et al.*, 2004). In hippocampal neuron cultures, Aβ caused loss of IR on dendrites and interfered with the insulin receptor signaling and long term potentiation (Zhao et al., 2008; Townsend et al., 2007).

Intranasal insulin administration improves short- and long-term object memory recognition in mice (Marks *et al.*, 2009). The effects of intranasal insulin on memory were dampened by diet-induced obesity (Marks *et al.*, 2009). Similarly, mice heterozygous for the IR demonstrate poor performance on both short-term (1 h) and long-term (24 h) memory tests (Das *et al.*, 2005). In yet another study on a rat model of type 2 diabetes, impaired brain insulin signaling and tau hyperphosphorylation were normalised by administration of intranasal insulin (Yang *et al.*, 2012). Several studies confirmed improvement in memory or cognitive functions in response to intranasal insulin, with the effect being more prominent in women than in men (Reger et al., 2006; Benedict et al., 2008; Krug et al., 2010; Hallschmid et al., 2008; Benedict et al., 2007; Novak et al., 2014; Reger et al., 2008; Benedict et al., 2007). These studies suggest that intranasal insulin has the potential to enhance working memory performance even in healthy non-diabetic subjects.

Many studies indicate that the peripheral or central administration of insulin to experimental animals positively affects memory and learning processes (Park *et al.*, 2000). This memory improvement is associated with increasing IR expression and its signal transduction pathways in the hippocampus (Zhao *et al.*, 1999). The loss of memory due to ischemic lesions in the

hippocampus can also be avoided by insulin administration (Voll, Whishaw and Auer, 1989). However, when chemicals inducing diabetes were injected directly at low doses into the brain, a central resistance to insulin was observed which was related to memory deficits and behavioural alterations (Lannert and Hoyer, 1998). Further, the systemic insulin administration to healthy humans under euglycemic hyperinsulinemic conditions improves verbal memory and selective attention.

Learning and memory functions require the remodelling of the dendritic spine morphology and modifications in the cytoskeleton produced during synaptic transmission. These processes involve the glutamate and two of its receptors, AMPA and NMDA. The neurotransmission is regulated by changing the expression levels of these receptors present in the membrane or by covalent modification of their subunit components. While LTP increases the post-synaptic density of the AMPA receptors, LTD is associated with a decrease in the receptors. Similarly, the phosphorylation of these receptors improves the efficiency of the ionic channel during LTP, while dephosphorylation during LTD decreases it (Song and Huganir, 2002). Insulin regulates glutamatergic neurotransmission at the synaptic level. It induces the LTD process by internalising the AMPA receptors in the post-synaptic membrane. Other studies demonstrate that insulin also promotes the phosphorylation of the GluR2 subunit in the AMPA receptors of hippocampal neurons, resulting in endocytosis and a decrease in the post-synaptic excitatory ability (Ahmadian *et al.*, 2004) . Insulin affects learning and memory through GABA receptors by promoting the translocation of these receptors to the plasma membrane. Insulin also increases the expression of GABA receptors on the post-synaptic and dendritic membranes of the CNS neurons (Ghasemi *et al.*, 2013).

Similarly, NO was also found to be involved in insulin-induced memory improvement since the administration of the NOS inhibitor was reported to disrupt insulin-induced memory

improvement (Choopani, Moosavi and Naghdi, 2008). Taken together, insulin's action in the CNS is important in maintaining cognitive function in animals.

Synaptic Basis of IR

Insulin/IR has been reported to modulate synaptic activities at both pre- and post-synaptic sites. At the presynaptic site, it modulates catecholamine neurotransmission. In dissociated rat brain cells, it inhibits the reuptake of norepinephrine (Raizada *et al.*, 1988) and also alters the kinetics of catecholamine in the hypothalamus (Raizada *et al.*, 1988). Furthermore, insulin enhances membrane phospholipid metabolism by enhancing endogenous adrenergic activity in hippocampal slices (Figlewicz and Szot, 1991). IR present on synaptosomes of the hippocampus provides a site for the action of insulin (Figlewicz et al., 1986; Marks et al., 1988; Matsumoto and Rhoads, 1990; Wei et al., 1990). The administration of insulin to the rat brain induces a substantial increase in acetylcholine and serotonin levels but a decrease in dopamine and noradrenaline (Bhattacharya and Saraswati, 1991). Moreover, insulin also participates in the clearance of neurotransmitters by regulating the synthesis and activity of the transporters for dopamine, serotonin and gamma-aminobutyric acid (GABA) (Figlewicz *et al.*, 1999).

Insulin/IR has been found to augment NMDA receptor activity shortly after insulin exposure of hippocampal slices at postsynaptic sites (Liu et al., 1995; Christie et al., 2001). This increased NMDA receptor activity is the result of tyrosine phosphorylation of the NMDA receptor subunits, NR2A and NR2B, induced by insulin in the hippocampal slices (Christie, Wenthold and Monaghan, 2001). Insulin also modulates NMDA receptor currents in a subunit-specific manner in the Xenopus oocyte and rat hippocampus (Liu et al., 1995; Liao and Leonard, 1999) through a process involving protein tyrosine kinases and protein kinase C (PKC).

It has also been demonstrated that insulin regulates the activities of the GABA receptor. For example, insulin administration induced recruitment of the type A GABA receptor to the plasma membrane in transfected HEK-293 cells (Wan *et al.*, 1997). Furthermore, insulin increased the expression of type A GABA receptors on the post-synaptic and dendritic membranes in CNS neurons (Wan *et al.*, 1997). Modifications in the biochemical structure of the GABA receptor and its functions have been associated with the origin of insulin resistance syndrome, characterised by a high incidence of death (Blasi and Jeanrenaud, 1993). Insulin also causes alterations of the dopaminergic system in the rat striatum resulting in an increased dopamine content (Šalković, Sabolić and Lacković, 1995).

Molecular Basis of IR

Phosphorylation of IRS1 initiates the activation of PI3K, which in turn activates PKC and the serine/threonine protein kinase, AKT/protein kinase B (PKB). Activation of this signaling pathway is necessary for glucose transport and glycogen and protein synthesis. It was demonstrated in a study that the PI3K signaling induced by insulin is responsible for the inhibition of apoptosis by NMDA in primary cultured rat cerebellar granule neurons (Zhang, Rubin and Rooney, 1998).

Another major signaling pathway of the IR is associated with the cytoplasmic intermediate protein SHC. Increased interactions between the IR and SHC-66 were detected in the hippocampal synaptic membranes of trained rats (Zhao *et al.*, 1999). Consistent with the changes in SHC phosphorylation and interaction with IR, the GRB-2 protein also demonstrated an increased expression in the hippocampal synaptic membranes after training. Consequently, MAPK phosphorylation is triggered at this membrane which in turn activates it. When the hippocampal synaptic membranes were treated with insulin, phosphorylation of MAPK was enhanced in trained animals but not in the control animals. However, the overall expression of MAPK in trained animals remained unchanged. Increasing evidence have been

accumulated over these years indicating the role of MAPK in learning and memory formation. For example, it is involved in associative learning (Atkins *et al.*, 1998), spatial learning in the Morris water-maze task and arm radial maze (Selcher et al., 1999; Zhao et al., 1999), inhibitory avoidance learning (Izquierdo *et al.*, 2000) and contextual and auditory fear conditioning (Schafe et al., 1999; Selcher et al., 1999). Additionally, MAPK has also been implicated in synaptic plasticity (English and Sweatt, 1997; Winder et al., 1999; Davis et al., 2000). It has been proposed that MAPK facilitates changes in the long-term storage of information in the brain, which requires gene regulation and expression. Furthermore, the Ras/Raf/MAPK pathway has been reported to act on PI3K and trigger its activation (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996; Rodriguez-Viciana et al., 1997). The molecular events proceeding this also play a role in memory storage, such as the activation of PKC and AKT/PKB Ser/Thr kinase. Interestingly, PI3K/AKT and/or PI3K/ PKC is also been known to act upstream to activate MEK and MAPK to regulate neuronal apoptosis and survival (Foulstone et al., 1999; Ryu et al., 1999; Yang and Raizada, 1999; Corbit et al., 2000).

Insulin and neurological diseases

a. Diabetic Neuropathy

Diabetic neuropathy (DN) refers to neurodegenerative disorders affecting the somatic and/or autonomic peripheral nervous system, in the context of diabetes mellitus (DM), in the absence of other secondary causes of peripheral neuropathy (Feldman et al., 2019; Bönhof et al., 2019). Apart from the typical forms of diabetic neuropathy, i.e., distal symmetrical sensorimotor polyneuropathy (DSPN) and autonomic neuropathy, atypical forms might also be seen in patients with DM, i.e., mononeuropathy, radiculo- or polyradiculopathy (Pop-Busui *et al.*, 2017).

DN is prevalent among Type 1 and Type 2 diabetic patients (Feldman *et al.*, 2019). Studies assessing the prevalence of DN in patients with type 1 DM (T1DM) have shown large variations (8–54%), possibly related to etiologic and/or methodologic factors (Hicks and Selvin, 2019; Dyck et al., 1993; Tesfaye et al., 1996; Jaiswal et al., 2013; Martin et al., 2014). Similar rates were also reported in patients with type 2 DM (T2DM) (16–51%) (Dyck et al., 1993; Jaiswal et al., 2013; Franklin et al., 1990; Pop-Busui et al., 2009; Partanen et al., 1995; Lu et al., 2020; Callaghan et al., 2016).

It has been demonstrated that low doses of insulin treatment in animals with DN can reverse many abnormal morphologic and behavioral changes associated with the disease without significantly altering glucose levels. Euglycemic- Streptozotocin (STZ)-treated rats display mechanical hyperalgesia in a paw-pressure withdrawal test (Romanovsky *et al.*, 2010). In another study, it was also reported that this observed change in paw-pressure threshold correlates with insulin deficiency in the euglycemic rats and could be reversed by low-dose insulin treatment (Romanovsky *et al.*, 2006). Additional biomarkers of neuropathy outside of peripheral nerves in the limbs have been shown to improve in response to insulin. A classic example is the prevention of nerve depletion in the cornea by the tropical application of insulin to the cornea (Chen *et al.*, 2013). In another study, it was reported that while no differences in PNS function were observed in the 2-month-old Goto-Kakizaki (GK) rat with impaired glucose tolerance (IGT) and hyperinsulinemia, 18- month-old GK rats with IGT and insulinopenia displayed classical features of DN (reduced nerve conduction velocity, loss of unmyelinated axons, and increased frequency of regenerating fibers). The authors suggested that these changes were related to the decrease in neuronal insulin support (Murakawa *et al.*, 2002). Moreover, in agreement with the increase in calcitonin gene-related peptide (CGRP) expression with insulin treatment observed by Toth et al., Murakawa et al. observed a significant decrease in CGRP expression in 18-month-old insulinopenic GK rats (Murakawa et al., 2002; Toth et al., 2006b). All these results put together point out that one of the

mechanisms through which insulin promotes proper sensory function is by regulating the synthesis of key neuromodulator proteins and peptides.

It has also been reported that sequestering of endogenous intrathecal insulin in non-diabetic rats by infusing anti-insulin antibodies slowed down the motor nerve conduction and atrophy of axonal fibers, as seen in models of DN (Brussee, Cunningham and Zochodne, 2004). This also suggests that non-glycemic triggers of DN exist and that the loss of PNS insulin signaling may be one of the initiating events. In one of the first publications investigating insulin signaling in the sciatic nerve, the authors revealed that the change in sciatic nerve insulin signaling explains the change in nociceptive behavior associated with DN (Sugimoto *et al.*, 2008). Both motor and sensory nerve conduction deficits were restored, and axonal atrophy was prevented upon intrathecal delivery of insulin or equimolar IGF-1 daily for 4 weeks in type 1 diabetic rats (Brussee, Cunningham and Zochodne, 2004). Administration of intrathecal insulin and IGF-1 were also able to rescue the loss of epidermal nerve fiber density and length in diabetic rats (Toth *et al.*, 2006). Likewise, positive effects of subcutaneous low dose insulin on reducing peripheral nerve dysfunction and MAP kinase activity were also reported in diabetic rats (Sugimoto et al., 2013). However, this is in contrast to the results obtained by Hoybergs and Meert. They reported that low-dose insulin could normalise diabetes-induced tactile allodynia and mechanical hyperalgesia, despite persistent hyperglycemia (Hoybergs and Meert, 2007). However, despite the controversy surrounding appropriate dosing regimen and delivery methods for beneficial effects on the PNS, it does appear that insulin treatment can ameliorate symptoms of DN through mechanisms other than reducing elevated blood glucose levels.

Insulin treatment has also been reported to improve many mitochondrial defects associated with DN. For example, Huang et al. showed that in an STZ model of type 1 diabetes, DRG neuronal mitochondria displayed increasing depolarisation (Huang *et al.*, 2003). Chowdhury et al. demonstrated that diabetes could induce impairments in mitochondrial and mitochondrial

protein expression (Chowdhury *et al.*, 2010). In both studies, insulin restored the mitochondrial parameters to the levels observed in control animals.

Furthermore, insulin treatment has also been shown to protect against late-stage diabetes-induced motor neuropathy (Francis *et al.*, 2011). Intranasal insulin showed beneficial effects on motor neuron morphology and function (Pang *et al.*, 2016). These results further substantiate the neurotrophic qualities of insulin and its beneficial role in neuronal function. Studies on *in vitro* models of chronic insulin treatment and type 2 diabetic mice have revealed blunted AKT activation in response to insulin in sensory neurons (Grote *et al.*, 2011). These results were correlated with reduced neurite outgrowth (Grote *et al.*, 2011) and changes in mitochondrial-associated proteins (Kim *et al.*, 2011). Insulin-induced neurite outgrowth stimulated was also sensitive to higher doses of insulin (Singh *et al.*, 2012). This notion of neuronal insulin resistance is on par with studies reporting reduced downstream insulin signaling *in vivo* in the PNS of insulin resistant mice in response to insulin (Grote *et al.*, 2013).

b. Alzheimer's disease

It has been observed that disturbances in brain insulin and IGF signaling mechanisms is characterised by early and progressive abnormalities and accounts for the majority of molecular, biochemical, and histopathological lesions in AD (de la Monte and Wands, 2005; Hoyer, 2002; Craft et al., 2003; Craft et al., 2000; Farris et al., 2004; Hoyer, 2004; Schubert et al., 2003; Schubert et al., 2004) (Figure 6). Insulin resistance shares many pathological features with AD, including cognitive impairment and disturbances in acetylcholine homeostasis (Rivera et al., 2005; Sims et al., 1980). AD is treatable with insulin sensitiser agents, i.e., drugs currently used to treat T2DM (Haan, 2006; Landreth et al., 2008; Landreth, 2007; Pedersen et al., 2006; Reger et al., 2006; Reger et al., 2008; Watson et al., 2006). These observations lead scientists to categorise AD as "type 3 diabetes", a form of diabetes

that selectively involves the brain and has molecular and biochemical features that overlap with T1DM and T2DM.

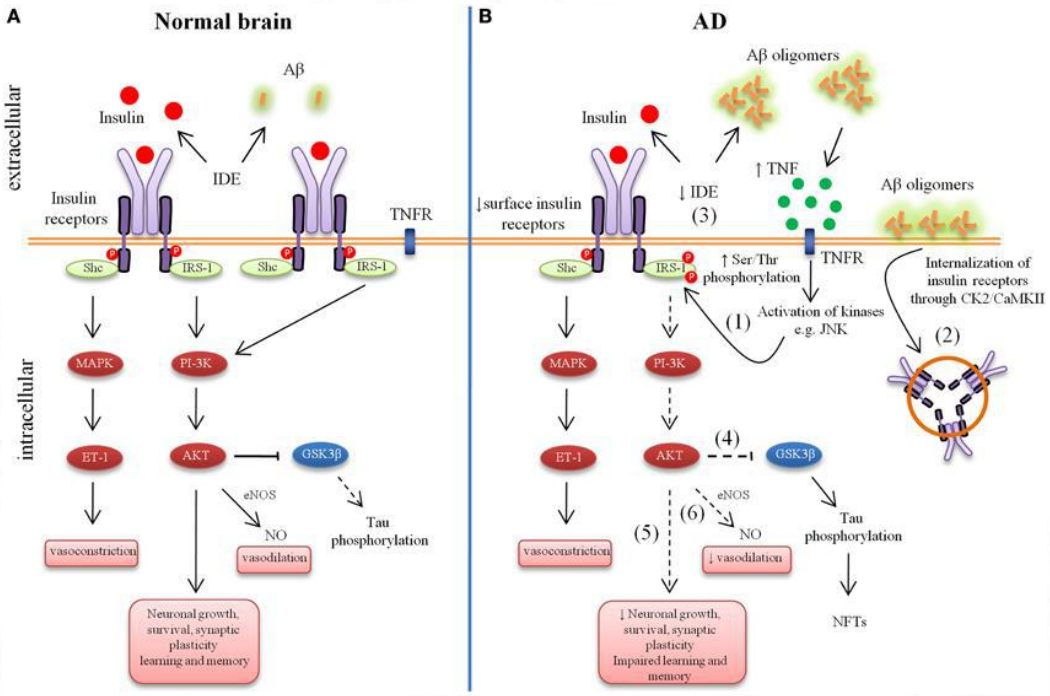

Figure 6. Schematic representation of brain insulin signaling under normal conditions and in patients with Alzheimer's Disease. A. Activation of the insulin pathway creates a balance between vasodilatation and vasoconstriction under physiological conditions to regulate the immediate metabolic requirements of various tissues. This facilitates neuronal growth, survival, synaptic plasticity, and learning and memory. B. In Alzheimer's Disease, Aβ oligomers cause internalisation of IRs from the cell surface, lowers the expression of Aβ-degrading insulin degrading enzyme (IDE) hindering Aβ degradation, and increases GSK-3β activity causing abnormal tau phosphorylation. This results in impaired nerve growth, synaptic plasticity, and learning and memory. (Image courtesy: Bedse et al., 2015)

Tau Phosphorylation

The Tau protein, first discovered in 1975, cause dysregulation of insulin signaling and pathogenic changes in the brain (Weingarten *et al.*, 1975). It has been identified as the main component of neurofibrillary tangles (NFT) associated with AD (Weingarten *et al.*, 1975). The Tau protein is involved in the assembly and stability of microtubules, and influences the synapses and nuclei of neurons (Arendt, Stieler and Holzer, 2016). It is important to note that

Tau is necessary for normal brain function. It is also released into the extracellular space (Pooler *et al.*, 2013). Although, its extracellular function remains largely unknown (Sergeant *et al.*, 2008). The expression and the phosphorylation of Tau protein are regulated by insulin and IGF stimulation (Schubert *et al.*, 2003).

AD brains are characterised by three times as much hyperphosphorylation of Tau than in normal brains (Burillo *et al.*, 2021) (Figure 7). Impaired insulin/IGF-1 signaling is reported to enhance Tau phosphorylation by inhibition of PI3K/AKT and enhance GSK3-β activation (Schubert *et al.*, 2003). Additionally, Tau hyperphosphorylation can also be enhanced by the downregulation of Tau O-GlcNAcylation in response to decreased glucose metabolism due to insulin resistance (Cassimeris and Spittle, 2001). Hyperphosphorylation causes conformational changes in Tau that impair its binding to microtubules, and as a result, misfolded Tau monomers cannot be transported into axons. Tau aggregates get deposited in the NFT, resulting in a group of diseases called tauopathies. Hyperphosphorylated Tau has been reported to affect cell morphology and growth and also the transport of organelles mediated by microtubule-dependent motor proteins (Burillo *et al.*, 2021). The accumulation of hyperphosphorylated Tau inside neurons enhances oxidative stress and triggers increased apoptosis, mitochondrial dysfunction, and necrosis (de la Monte, 2009). As the Alzheimer's disease symptoms progress, Tau pathology is observed first in the brainstem and entorhinal cortex and later in the hippocampus (Yuzwa and Vocadlo, 2014).

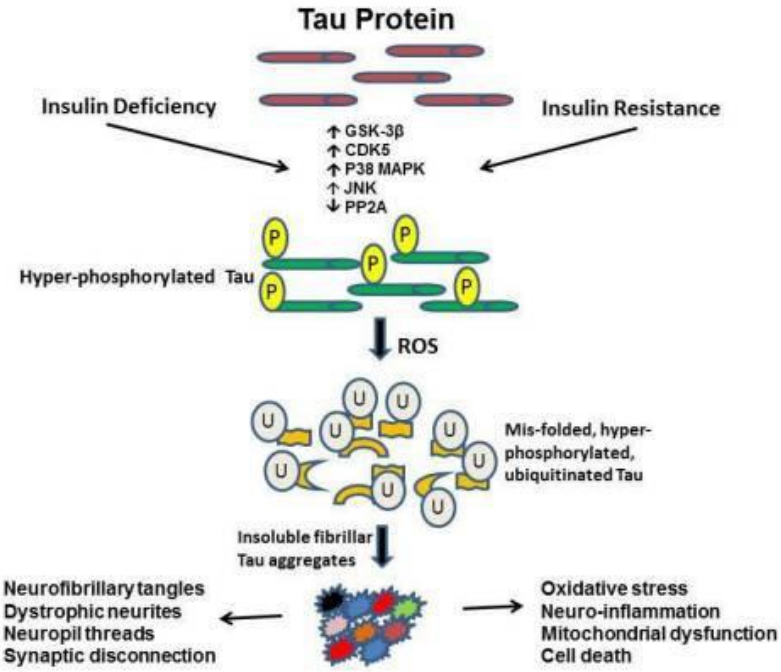

Figure 7. Role of brain insulin in tau pathology. Insulin deficiency and insulin resistance causes hyperphosphorylation of tau by activating kinases and inhibiting phosphatases. The resulting misfolded tau proteins are neurotoxic. (Image courtesy: Suzanne M de la Monte, 2012)

Interestingly, animal studies indicate that Tau protein can also regulate brain insulin signaling. Tau deletion impairs the hippocampal response to insulin (Marciniak *et al.*, 2017). Additional impairment is caused by IRS-1 and phosphatase and tensin homologue on chromosome 10 (PTEN), which is a negative regulator of the PI3-K/AKT pathway. Tau knockout mice were shown to exhibit an impaired hypothalamus. The study demonstrates that pathophysiological loss of Tau function is associated with brain insulin resistance and plays a critical role in cognitive and metabolic impairments associated with AD (Marciniak *et al.*, 2017). Furthermore, it has been observed that in AD and several other tauopathies, insulin accumulates as oligomers in neurons bearing hyperphosphorylated Tau. The accumulation of insulin in neurons was found to be directly related to the level of Tau hyperphosphorylation (Rodriguez-Rodriguez *et al.*, 2017).

Amyloid-β Pathology

Amyloid precursor protein (APP) is a transmembrane protein expressed in many tissues, including the CNS and is abundant at the synapses. It acts as a cell surface receptor and has been reported to regulate synapse formation, neural plasticity, antimicrobial activity, and iron export. Insulin stimulates the non-amyloidogenic processing of APP by regulating its phosphorylation. Hence, any impairments in insulin signaling will result in increased accumulation of pathological Aβ (Pandini *et al.*, 2013). Insulin degrading enzyme (IDE) acts on both insulin and Aβ. As insulin level increases the level of IDE, a defect in insulin signaling leads to reduced amyloid-β degradation (Zhao *et al.*, 2009). The Aβ fragments rapidly form oligomers, prefibrillar aggregates, and fibrils in the extracellular environment forming β-amyloid plaques (Burillo et al., 2021; Rad et al., 2018).

Insulin modulates APP metabolism, which regulates the balance between the anabolism and catabolism of Aβ. Downregulated insulin pathway increases NFT formation and causes oxidative damage to cells. Additionally, low insulin levels in the brain result in elevated Aβ levels and amyloid plaque formation. IDE modulates the metabolic and neurological pathways and causes hyperinsulinemia and AD (Rad *et al.*, 2018). Studies on transgenic mice found that overexpression of IDE results in hyperinsulinemia, glucose intolerance, and increased levels of Aβ in the brain. Therefore, it strengthens the idea that Aβ influences AD neurodegeneration by impairing insulin signaling and promoting insulin resistance (de la Monte, 2009).

The inhibition of $A\beta_{42}$ peptide synthesis may be a strategy for the treatment of brain insulin resistance (Zhang *et al.*, 2013). Intracerebral administration of Aβ oligomers results in a range of pathological states along with peripheral glucose intolerance, glucose resistance, inflammatory processes in adipose tissue, and disturbances in insulin-induced GLUT-4 translocation into the cell membranes of skeletal muscle (Clarke *et al.*, 2015). Increased Aβ

levels were observed in the temporal lobe in a monkey model of T1DM, especially in the hippocampus (Morales-Corraliza *et al.*, 2016). In addition, the brain also showed decreased levels of neprilysin (NEP), the Aβ-degrading enzyme, at the protein and mRNA levels (Morales-Corraliza *et al.*, 2016).

Insulin regulates the metabolism of the Aβ peptide by increasing its trafficking to the plasma membrane. In addition, insulin increases the extracellular levels of Aβ by promoting its secretion and inhibiting its degradation by IDE. The effects of insulin on APP metabolism are influenced by the MAPK pathway (Gasparini, 2002).

Taking all these reports together, insulin can affect Aβ pathology. Conversely, Aβ affects insulin signaling, either by competing with and inhibiting the binding of insulin to IR or reducing its affinity (Xie *et al.*, 2002). Aβ also exerts neurodegenerative effects through its neurotoxic activities (Doré, Kar and Quirion, 1997). Intracellular Aβ interferes with the PI3-K activation of AKT while increasing GSK-3α activation and Tau hyperphosphorylation. Increased levels of GSK-3α, in turn, promote APP processing and Aβ accumulation (Phiel *et al.*, 2003).

Inflammation

The effect of inflammation on the pathogenesis of AD is well-documented. Aβ is known to play a central role in the pathogenesis according to the neuroinflammation hypothesis of AD. This hypothesis states that the accumulation of Aβ increases inflammatory cytokines, chemokines, and complement proteins synthesised and released by chronically-activated glia (Gupta *et al.*, 2020). This is supported by the evidences of elevated levels of inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) in the cerebrospinal fluid of AD patients (Swardfager *et al.*, 2010).

“Neuroinflammation” is the term given to the presence of activated microglia and astrocytes, which causes injury through the expression and release of pro-inflammatory cytokines, chemokines, and increased production of membrane fatty acids, eicosanoids, lipid peroxidation products, and reactive oxygen and nitrogen species (de la Monte, 2017). Astrocytes and microglia mediate the innate immunity in the brain, and activation of these cells is a hallmark of central inflammation and potential brain pathology (Nimmerjahn, Kirchhoff and Helmchen, 2005).

Neuroinflammation is an important propagator of the neurodegeneration observed in AD and plays a critical role in the progression and prognosis of AD (Dandrea *et al.*, 2001). Earlier studies report that it also causes neuronal injury and cholinergic dysfunction (Giovannini *et al.*, 2002). Yet another study demonstrates that it causes oxidative stress, increased production of reactive oxygen (ROS) and reactive nitrogen species (RNS), which in turn damages nerve terminals, causing synapse dysfunction (Agostinho, A. Cunha and Oliveira, 2010).

Chronic inflammation exacerbates insulin resistance which increases the expression and activation of IDE and stimulates the accumulation of advanced glycation end products (AGEs) (Suzanne M. de la Monte, 2012). The accumulated AGEs negatively affect tissues and have been found in amyloid-containing senile plaques and Tau-containing NFTs. Glycation is also known to promote Aβ aggregation. In addition, AGEs also stimulate vascular pathology in the brain (Ramasamy *et al.*, 2005). Aβ peptides bind with the receptors of AGE (RAGE) present in the neuronal cells, microglia, astrocytes and brain endothelial cells (Verdile *et al.*, 2015), with high affinity, and it can transport brain-derived Aβ across the BBB into the peripheral circulation (Cai *et al.*, 2016) and back again (Deane *et al.*, 2003), promoting neurodegeneration. The binding of Aβ peptides promotes the release of cytokines (such as TNF-α and IL-6) from microglia, inducing neuronal damage (Deane et al., 2003; Matrone et al., 2014). Additionally, this interaction may also increase the expression of beta secretase-1, which promotes the generation of Aβ by the amyloidogenic processing of APP (Hartlage-

Rübsamen *et al.*, 2003). In response to Aβ, RAGE makes the vascular system permeable to macromolecular invasion and mediates the migration of monocytes across human brain endothelial cells (Giri *et al.*, 2000).

The inflammatory mediators impair insulin signaling via JNK pathway activation (De Felice et al., 2014; Zhou et al., 2017). Insulin also suppresses the activity of pro-inflammatory proteins and through the AKT-mTOR signaling pathway, induces the synthesis of postsynaptic density protein 95 (PSD-95) in Dendron's and hippocampal area of inflammation (Rad *et al.*, 2018). Several other polypeptides and neuroinflammatory mediators may also be involved in insulin resistance in the brain in the pathogenesis and progression of AD (Robbins et al., 2020).

c. Parkinson's disease

Analysis of epidemiological studies by Sandyk in the year 1993 proposed that there is an association between T2DM and PD, and that PD is associated with a significant prevalence of DM (Sandyk, 1993). Studies in different ethnic groups have demonstrated that T2DM is a risk factor of developing PD (Hu et al., 2007; Schernhammer et al., 2011; Bosco et al., 2012; Sun et al., 2012; Yue et al., 2016; Yang et al., 2017; De Pablo-Fernandez et al., 2018), and that majority of the PD patients are insulin-resistant (Bosco *et al.*, 2012). Over the years, there has been a rapid increase in the investigations regarding the connection between T2DM and PD (Figure 8), leading to the hypothesis that new drugs for diabetes mellitus might alter the natural history of PD itself. Interestingly, a new formulation of bromocriptine, an old antiparkinsonian drug, has been specifically approved by the FDA to treat T2DM. In one of the studies, Ramalingam and Kim demonstrated that insulin reduces the hydrogen peroxide-induced oxidative damage to neuronal and glial cells (Ramalingam and Kim, 2014; Ramalingam and Kim, 2016b). They also observed the neuroprotective action of insulin in cell cultures treated with a neurotoxin which causes oxidative stress, mitochondrial dysfunction, and ultimately apoptotic cell death in vitro. Pre-treatment with insulin was shown to prevent

cell death and reduce the release of nitric oxide (NO) and reactive oxygen species (ROS) (Ramalingam and S. Kim, 2016). The neuroprotective role of insulin was also confirmed in PD model of rats treated with 6-hydroxydopamine (6-OHDA). In this study, intranasal insulin was found to rescue 6-OHDA-induced motor impairments, providing potent protection of DA neurons against neurotoxicity (Pang *et al.*, 2016). This neuroprotective action of insulin is the result of the suppression of GSK-3 activity, which is a crucial multi-target kinase whose dysregulation is implicated in PD pathogenesis and augmentation of Lewy body formation, a pathological hallmark of PD (van der Heide et al., 2006; Albeely et al., 2018). The insulin signaling pathway affects two proteins, whose mutations are closely involved in the pathogenesis of PD: α-synuclein (Polymeropoulos *et al.*, 1997) and Leucine-Rich Repeat Kinase-2 (LRRK-2) (Paisán-Ruíz *et al.*, 2004).

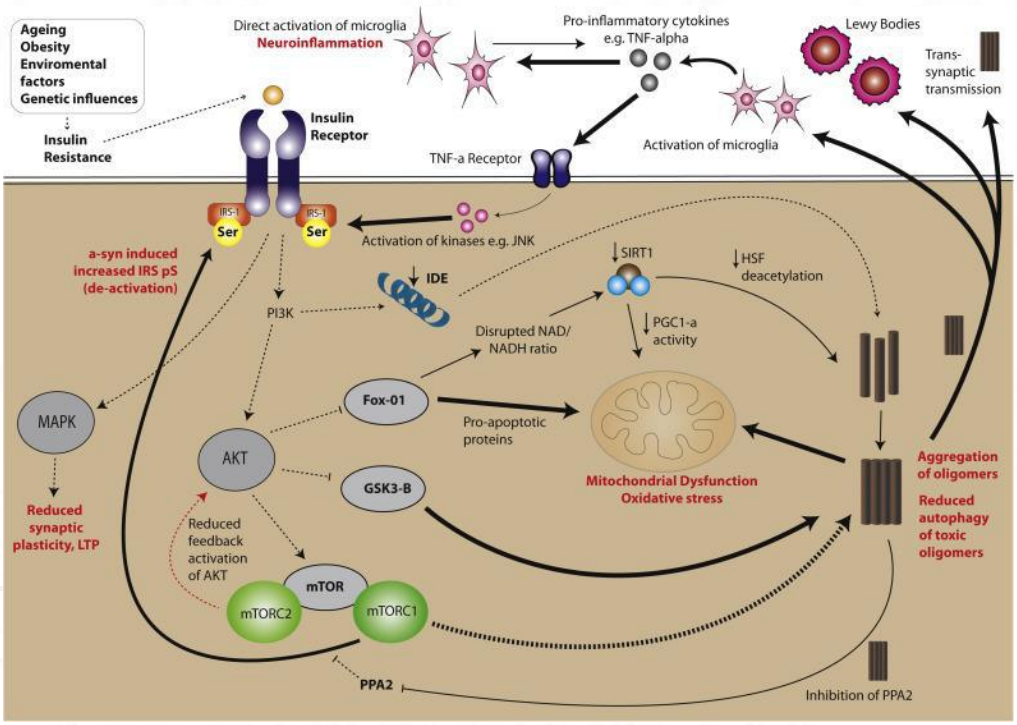

Figure 8. Insulin signaling in the pathogenesis of Parkinson's Disease. Disturbances in the insulin signaling pathway reduce the activation of AKT, which promote mitochondrial dysfunction, inflammation, oxidative stress and accumulation of alpha-synuclein. Accumulation of α-syn activates MTORC1 and in turn, induce insulin resistance. Figure from Athauda and Foltynie, 2016 and reprinted with permission from Elsevier *Inc* © 2016.

α-synuclein

α-synuclein is the main component of Lewy bodies and Lewy neurites in the PD brain. Its physiological roles range from chaperone activity to management of synaptic vesicle trafficking (Bendor, Logan and Edwards, 2013). It is expressed in pre-synaptic sites and several non-neuronal tissues (Askanas et al., 2000; Baltic et al., 2004; Barbour et al., 2008). The possibility of α-synuclein spreading through different organs in PD has been an unresolved debate among researchers (Braak et al., 2003; Olanow and Prusiner, 2009; Bendor et al., 2013).

This protein decreases insulin secretion (Geng *et al.*, 2011). Overexpression of α-synuclein negatively regulates the IRS-1, impairing the PI3/AKT signaling and promoting insulin resistance (Gao *et al.*, 2015). Low plasma levels of α-synuclein in the serum of 1152 subjects from medical check-ups were found to be associated with insulin resistance. In the same study, mice deficient in α-synuclein displayed alterations in glucose and insulin response during diet-induced insulin resistance (Rodriguez-Araujo *et al.*, 2015). IGF-1, together with insulin and IGF-2 (Fernandez and Torres-Alemán, 2012) and IDE (Duckworth, Bennett and Hamel, 1998) were found to affect the expression of α-synuclein. Protective effects of IGF-1 against DAergic neuronal loss have been proved in cell culture and animal models of PD (Offen *et al.*, 2001). IGF-1 rescues α-synuclein toxicity and suppresses α-synuclein aggregation by activating PI3K/AKT pathways (Chung *et al.*, 2011). It has also been hypothesised that increased serum IGF-1 levels in early PD may be a compensatory effort to protect dopaminergic neurons (Godau et al., 2010; Godau, 2011). Another study confirmed that IDE *in vitro* prevented the formation of α-synuclein (Sharma *et al.*, 2015).

Leucine-Rich Repeat Kinase-2

The effect of insulin signaling on LRRK-2 (Maas, Yang and Edwards, 2017) and DA receptor trafficking (Rassu *et al.*, 2017), has been shown in LRRK-2 deficient cells from experimental

animals and fibroblasts of PD patients with G2019S mutated-LRRK-2. In the absence of LRRK-2, insulin-induced translocation of GLUT-4-containing vesicles to the cell surface appeared to fail. This study highlighted the essential role of LRRK-2 in the phosphorylation of Rab10 required in the PI3/AKT signaling pathway for the insulin-induced translocation of GLUT-4 (Funk *et al.*, 2019).

d. Depression

Their observations that diabetic children show marked symptoms of depression and that insulin improved these symptoms led Cowie and his team to investigate the possible effect of insulin in states of true mental depression (Cowie DM et al., 1924). Intriguingly, this observation predates the modern diagnostic conceptualisation for either disorder. The British physician Thomas Willis was one of the first to recognise glycosuria as a symptom of diabetes and also to postulate that diabetes was caused by 'sadness or long sorrow and other depressions and disorders' (Rubin and Peyrot, 2002).

Major depressive disorder (MDD) was regarded as a common co-morbidity of diabetes (Rubin and Peyrot, 2002; Musselman et al., 2003). However, the biological basis of MDD was not clearly understood. It is now known that the relationship between MDD and diabetes is bidirectional (Musselman et al., 2003; Silva et al., 2012). The mechanistic links between the two are Insulin resistance, hypothalamic-pituitary-adrenal (HPA) axis dysregulation, and inflammation (Silva, Atlantis and Ismail, 2012). As in diabetes (Baker *et al.*, 2011) and dementia (Chen and Zhong, 2013), cerebral glucose metabolism is significantly altered in MDD (Su *et al.*, 2014). Furthermore, systemic insulin resistance is a pathogenic feature of MDD (Silva, Atlantis and Ismail, 2012). In addition, in a plasma proteomics study aimed at identifying peripheral markers of MDD, the analyte with the most significant differential expression and statistical significance between healthy and MDD subjects was found to be insulin (Domenici *et al.*, 2010).

In rats exposed to chronic unpredictable mild stress to induce a depression-like state, IRS2 tyrosine phosphorylation and PI3K activation (stimulated by insulin) are suppressed, and suppressor of cytokine signaling 3 (SOCS3) is overexpressed in the arcuate nucleus of the hypothalamus. These effects were reversed by treatment with the antidepressant fluoxetine (Pan *et al.*, 2013). Also, lentivirus-mediated hypothalamic insulin receptor-knockdown promoted anhedonia and behavioral despair in rats, as measured by the sucrose preference test and forced swim test, respectively (Grillo *et al.*, 2011). Furthermore, NIRKO mice develop age-related anxiety and depression-like behaviors accompanied by central mitochondrial dysfunction, increased oxidative stress, and increased lipid and protein oxidation in the striatum and nucleus accumbens (Kleinridders *et al.*, 2015). Collectively, these observations support the idea that impairments in CNS insulin receptor signaling contribute to depression-like behaviors in animals.

In healthy subjects, intranasal insulin administration prior to a psychosocial stressor decreased secretion of saliva and plasma cortisol without affecting heart rate or blood pressure stress reactivity, suggesting that intranasal insulin diminishes the responsiveness of the stress-induced HPA axis (Bohringer *et al.*, 2008). Similarly, intranasal insulin therapy also lowered plasma adrenocorticotropic hormone (ACTH) and serum cortisol in obese subjects, suggesting that HPA axis activity was reduced (Hallschmid *et al.*, 2008). It was found that while obese subjects were resistant to the effects of longer-term intranasal insulin to reduce weight or adiposity, they remained sensitive to improve declarative memory and mood (Hallschmid *et al.*, 2008). Moreover, emerging experimental and clinical data demonstrate that insulin in the brain may play a direct role in controlling mood regulation and cognition in MDD.

e. Traumatic Brain Injury

Co-morbidities associated with traumatic brain injury (TBI) include increased risk of neurodegenerative diseases such as AD and chronic traumatic encephalopathy (Plassman *et*

al., 2000). Furthermore, patients with diabetes are at higher risk of developing neurodegenerative diseases after TBI (Zimering, Patel and Bahn, 2019).

Since learning and memory function are hippocampal-dependent functions (Scoville and Milner, 1957; Lazarov and Hollands, 2016) these deficits that accompany TBI can be attributed to the significant hippocampal atrophy often observed following TBI (Kotapka et al., 1992; Bigler et al., 1996; Vakil, 2005; McKee and Daneshvar, 2015). These cognitive deficits can also result from cellular and metabolic dysfunction after injuries, such as inflammation, insulin resistance, and decreased cerebral glucose uptake. Moreover, hyperglycemia accompanied by hyperglycolysis is observed following TBI as cells utilise ATP-requiring membrane ionic pumps in an effort to restore ionic and cellular homeostasis (Yoshino et al., 1991; Giza and Hovda, 2014). After this initial burst of energy, the brain enters a hypometabolic state that can last for days to weeks after injury (Yoshino *et al.*, 1991).

Many patients with TBI demonstrate hyperglycemia (Young et al., 1989; Terzioglu et al., 2015). Additionally, diabetic patients who can have both hyperglycemia and insulin resistance are at higher risk of mortality after TBI (Ley *et al.*, 2011). Reduced insulin sensitivity and signaling at synapses has been observed in animals with TBI; the mechanism behind which is still not understood (Karelina et al., 2016; Franklin et al., 2019).

Insulin and Dopamine Crosstalks

The interplay between insulin and dopaminergic systems has long been studied. Insulin affects dopaminergic signaling through the dopamine receptors and the dopamine transporter, and shared components between dopaminergic and other signaling pathways.

a. **Insulin and Dopamine Transporter**

Studies on hypoinsulinemic rats demonstrated changes in the availability and activity of the dopamine transporter (DAT) (Williams *et al.*, 2007). Another study has noted reduced DAT mRNA levels in the ventral tegmental/substantia nigra areas of rats treated with STZ, a diabetogenic agent (Figlewicz *et al.*, 1996). This effect was reversed by intracerebroventricular insulin infusion (Figlewicz *et al.*, 1994). Similar decreases in DAT mRNA levels and DA reuptake kinetics were also reported in a rat model of hypoinsulinemia caused by acute fasting (Patterson *et al.*, 1998). In tissue cultured HEK cells stably expressing DAT, an increased DA uptake was observed in response to increasing concentrations of insulin (Carvelli *et al.*, 2002). The results were further reproduced in the ventral tegmental area of mice brains (Mebel *et al.*, 2012). This effect is mediated through PI3K signaling, which increases DAT cell surface expression (Carvelli et al., 2002; Doolen and Zahniser, 2001).

On the contrary, *in vivo* studies in diet-induced obese rats with neuronal insulin resistance revealed delayed synaptic DA clearance concomitant with reduced DAT expression in the striatum (Speed *et al.*, 2011). This contrasting result might be due to the duration effect for insulin signaling, whereby prolonged insulin exposure, leading to insulin resistance, ultimately down-regulates DAT. Dopamine receptor D2 (D2R) acts through the ERK1/2 pathway to alter DAT surface levels (Bolan et al., 2007; Lee et al., 2007; Morón et al., 2003).

In rats treated with STZ, serial administration of AMPH increased activation of ERK1/2. Application of ERK1/2 inhibitor resulted in delayed DA clearance through decreased DAT surface expression (Owens *et al.*, 2012). On incubation with exogenous insulin, *ex vivo* striatal cell preparations from euinsulinemic rats, demonstrated increased ERK1/2 activation, and rats made hyperinsulinemic through insulin-releasing surgical implants exhibited faster DA clearance rates (Owens *et al.*, 2012). Interestingly, in euinsulinemic rats repeated AMPH administration did not alter ERK1/2 activity or significantly enhance AMPH-stimulated DA

efflux (Owens *et al.*, 2012). Thus, AMPH has differential effects on healthy controls compared to insulin-dysregulated rats. Insulin reversed the effects of AMPH-mediated cell surface redistribution of DAT in cultured striatal synaptosomes even when insulin was present with AMPH in the bath solution (Carvelli et al., 2002; Lute et al., 2008). These effects were attenuated by PI3K inhibition, while transient expression of constitutively active PI3K antagonised AMPH effects (Carvelli et al., 2002; Lute et al., 2008).

b. **Insulin and Dopamine levels**

As expected from rodent experiments, it was shown that peripheral administration of a DA agonist reduced insulin levels (Kok *et al.*, 2006). Nevertheless, another study revealed that peripheral DA infusion acutely increased serum insulin (Contreras *et al.*, 2008). Furthermore, depending on the dose of DA to which they were exposed, isolated pancreatic islet β-cells demonstrated differential effects on insulin release (Shankar, Santhosh and Paulose, 2006). Physiological studies have demonstrated that higher plasma DA levels correspond to lower circulating insulin levels in non-diabetic subjects (Tomaschitz *et al.*, 2012). In the imaging studies done on the CNS of healthy controls, decreased insulin levels corresponded to increased DA levels and acute DA depletion corresponded to an increase in insulin levels (Caravaggio *et al.*, 2015).

Insulin resistance relates to lower dopaminergic tone even in healthy subjects (Brunerova *et al.*, 2013). Schizophrenia is thought of as a naturally occurring hyperdopaminergic state (Sonnenschein, Gomes and Grace, 2020). In new-onset schizophrenia patients, a greater glucose intolerance prior to and independent of treatment with antipsychotics has been observed (Fernandez-Egea et al., 2009; Henneman, 1954; Ryan et al., 2003; Spelman et al., 2007). In addition, there is evidence for pre-existing DA/insulin dysregulation in schizophrenia patients as reported from sibling studies which have shown higher plasma insulin levels and increased risk for diabetes in unaffected family members (Kohen, 2004; van Beveren et al.,

2014). All these studies have prompted the understanding of the long-term effects of dopamine dysregulation on insulin signaling.

c. Insulin and Dopamine Receptors

Administration of neuroleptic drugs, which block dopamine receptors, causes hyperinsulinemia in normal human subjects (Sowell *et al.*, 2002), and is associated with diabetes in psychiatric patients (Pijl, 2003; Citrome et al., 2004; Marder et al., 2004). Several reports suggested that treatment with antipsychotics agents that affect D2R is associated with an increase in obesity, the clinical presentation of new-onset diabetes mellitus or diabetic ketoacidosis, and the development of hypertriglyceridemia (Cohen, 2004; Guenette et al., 2013; Dibben et al., 2005).

Additionally, *in vitro* studies performed in isolated pancreatic islets demonstrated the participation of the pancreatic D2R in insulin secretion and β-cell proliferation and apoptosis (Farino et al., 2020; Sakano et al., 2016). Furthermore, siRNA-mediated knockdown of D2Rs, but not other dopamine receptor subtypes, affected glucose-stimulated insulin secretion in cell lines, such as insulin-secreting INS-1E cells (Han *et al.*, 2019). In contrast, global D2R knockout mouse exhibit impaired insulin secretion and causes glucose intolerance (García-Tornadú *et al.*, 2010). Selective pharmacologic inhibition of each receptor showed that D3R is the only dopaminergic mediator of inhibition of insulin secretion in the mouse, and D3 inhibition resulted in increased insulin secretion (Ustione and Piston, 2012). There are no observed effects of D3 receptor activation on the redox state of the β-cells, but a significant reduction in the frequency of the intracellular [Ca^{2+}] oscillations is reported (Ustione and Piston, 2012).

D2-like receptor activation does not affect cAMP levels (Rubí *et al.*, 2005). Thus, it was proposed that D3 receptors signal mainly via the Gβγ subunit to directly affect the L-type

calcium channels (Ivanina *et al.*, 2000), or indirectly by activating the potassium channels (Kuzhikandathil, Yu and Oxford, 1998). Given the overlap in dopaminergic inhibition of insulin secretion between the mouse and human islet data, it can be speculated that a similar mechanism is responsible for this effect in the human β-cells as well.

Caenorhabditis elegans as a Model in Neurobiology

The use of invertebrates in neurobiology was popularised by Eric Kandel and Seymour Benzer (Kandel and Tauc, 1965; Quinn et al., 1974). Sydney Brenner used *Caenorhabditis elegans* as a model organism to study animal development and behavior (Brenner, 1973). C. *elegans* is a soil nematode of about 1 mm in length (Figure 9). Over the years, it has become an increasingly popular model organism among neurobiologists.

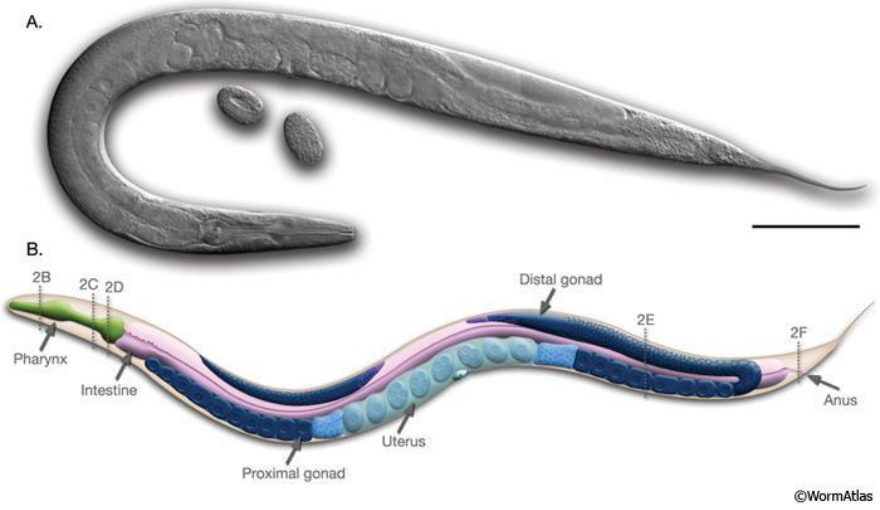

Figure 9. Anatomy of an adult hermaphrodite. **A.** Microscopic image of an adult C. *elegans* hermaphrodite, left lateral side. Scale bar 0.1 mm. **B.** Schematic representation of the anatomical structures (Image courtesy: wormatlas.org).

There are many advantages associated with C. *elegans* that are responsible for its extensive use in various studies. It is easy to grow this nematode in bulk populations. A few hundred nematodes can be kept on a single agar plate and suitable growth medium with OP50 as the food source. OP50 is a uracil-requiring organism, and its deficiency in the plate prevents the overgrowth of bacteria, which would obscure the worms (Brenner, 1974). OP50 also does not demand any major laboratory safety measures as it is non-pathogenic and can be easily grown in Luria-Bertani (LB) media overnight (Hart, 2006). The worms have a lifespan of about 21 days and a reproductive cycle of 3 days. They also have a stress-resistant larval stage called the dauer larvae (Figure 10).

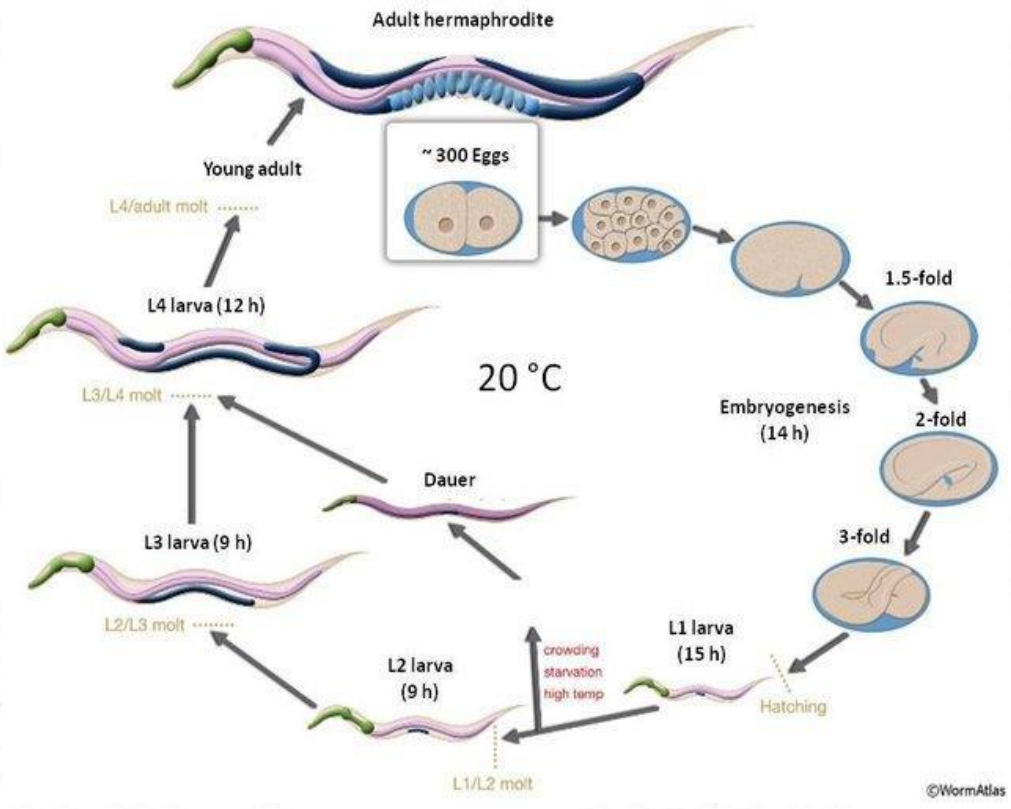

Figure 10: Life cycle of *C. elegans* showing the stress-resistant dauer stage. C. *elegans* follow four larval stages before attaining reproductive maturity under favorable conditions. These stages include L1, L2, L3, and L4 larval molts. However, under unfavorable conditions the development is arrested after the L1 molt, resulting in a stress-resistant dauer larval stage. The dauers resume its development when conditions become favorable (Image courtesy: wormatlas.org).

Further, C. *elegans* can be frozen, and on subsequent thawing, they remain viable, allowing long-term storage (Brenner, 1974).The transparency of C. *elegans* enables the study of cellular differentiation and other developmental processes in the intact organism. Moreover, the developmental fate of every single somatic cell (959 in the adult hermaphrodite; 1031 in the adult male) has been mapped (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979).

The whole genome of this organism has been sequenced (C. *elegans* Sequencing Consortium, 1998). C. *elegans* protein sequences revealed that human gene homologs exist for about 83% of the C. *elegans* proteome (Lai *et al.*, 2000). Furthermore, C. *elegans* homologs exist for 60–80% of human protein-coding genes (Harris et al., 2004; Kuwabara and O'Neil, 2001; Lai et al., 2000; Sonnhammer and Durbin, 1997). Remarkably, human genes have been shown repeatedly to replace the C. *elegans* homologs when introduced into them. Conversely, many C. *elegans* genes function similarly to mammalian genes.

Genetic manipulation can also be done in C. *elegans*. RNAi is a powerful tool in the study of functional genomics. C. *elegans* can be soaked in (Tabara et al., 1998), injected with (Kamath, 2003), or fed with (Timmons and Fire, 1998) genetically transformed bacteria that express the double-stranded RNA of interest complementary to the sequence of the gene that the researcher wishes to disable.

It is one of the simplest organisms with a nervous system (Figure 11). The structure of the C. *elegans* nervous system has been described in detail by electron microscopic reconstruction (White *et al.*, 1986a). The high-resolution electron microscopic images helped identify all the synapses, map all the connections, and work out the entire neuronal circuit. In the hermaphrodite, this system comprises 302 neurons (Kosinski and Zaremba, 2007), which has been comprehensively mapped (Cook *et al.*, 2019). Regardless of its simplicity, the C. *elegans*

nervous system is can control several complex behaviors, in addition to the basics such as locomotion, foraging, feeding, and defecation (Bono and Villu Maricq, 2005). C. *elegans* can discriminate and move towards or away from chemicals, odorants, temperatures, and food sources. It can also detect the presence of nearby nematodes by short-range diffusible signals, by a pheromone (Golden and Riddle, 1982; Jeong et al., 2005), and by changes in oxygen levels (Cheung et al., 2004; Gray et al., 2004). Most of these behaviors are plastic and, therefore, subject to change through learning and memory (Giles, Rose and Rankin, 2005). Learning and memory were first characterised in C. *elegans* by Rankin (Rankin, Beck and Chiba, 1990). The following sections will entail the aspects of learning and memory in C. *elegans*.

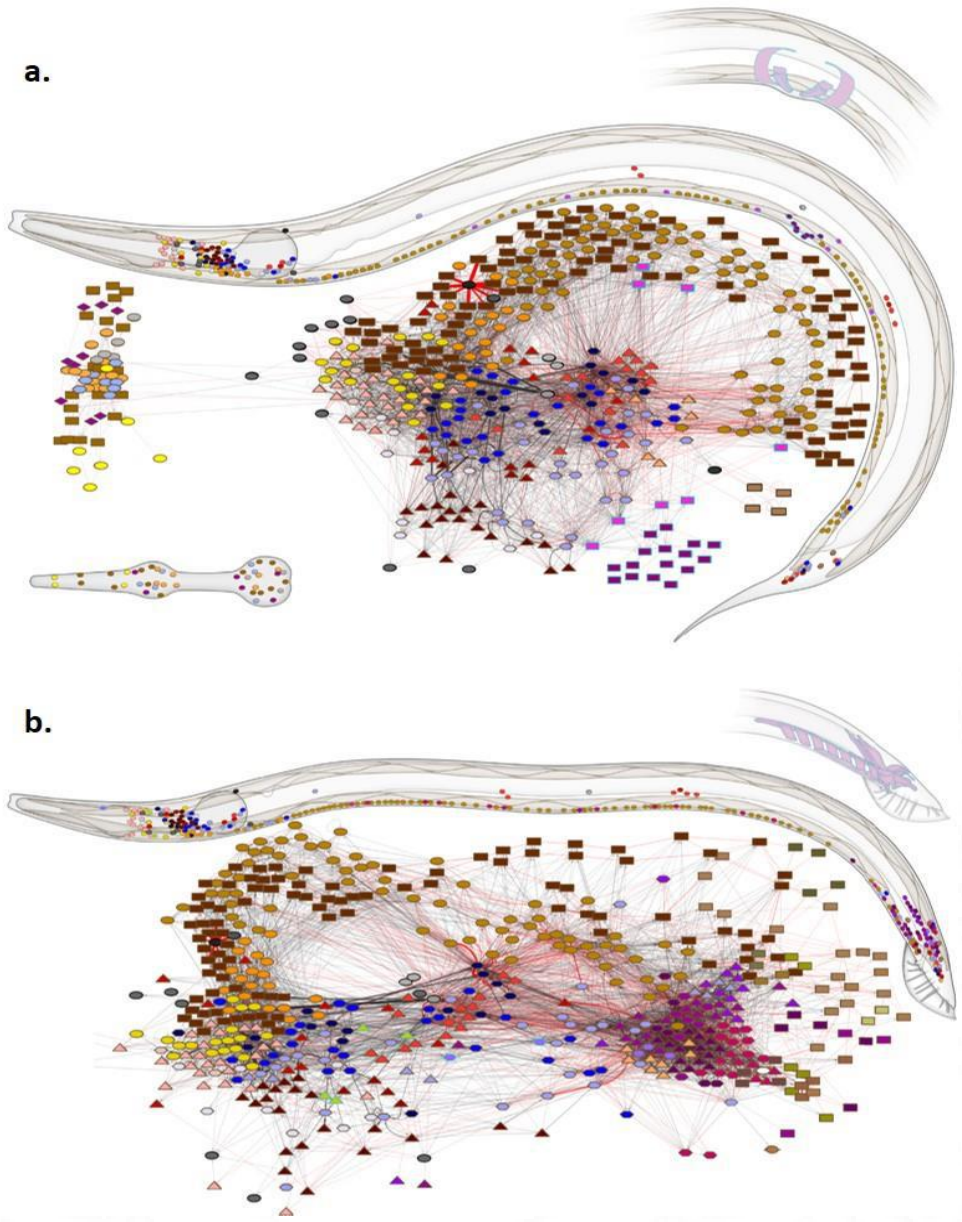

Figure 11. Nervous system of C. elegans hermaphrodite (a) and male (b). The primary centres of connectivity in both the sexes are the nerve ring. In the male, an additional ganglions is present at the pre-anal region. The major sex difference is a larger number of neurons and muscles in the male tail that facilitate copulation (Image courtesy: Cook et al., 2019)

a. Learning and Memory in *Caenorhabditis elegans*

Learning and memory are fundamental biological properties necessary for the survival of an organism in the changing environment. Learning can be defined as the acquisition of

knowledge or skills (through study, experience, or being taught), and memory is the faculty by which the brain stores and retrieves the learnt information. C. *elegans* has been used as a model system to study learning and memory processes. Both associative and non-associative memories have been characterised in C. *elegans*.

Non-associative memory

It is the simplest form of memory and comprises habituation and sensitisation. In this category of learning, the animal learns to reduce (habituate) or enhance (sensitise) its behavioural response to a single stimulus (Rahmani and Chew, 2021a). Studies have shown that habituation and sensitisation are mirror images of each other (Blumstein, 2016). Here, I briefly explain mechanosensory habituation as an example of non-associative memory.

Mechanosensory habituation

Rankin et al. (1990) studied the plasticity of the "tap-withdrawal response" (TWR) in C. *elegans*, a behavior whereby worms swim backwards in response to a non-localised mechanical stimulus. They observed that repeated administration of the tap resulted in a decrement of the amplitude and the frequency of the response. They also demonstrated that a dishabituating stimulus (brief electric shock) facilitated the decremented response, suggesting that it was due to habituation and not sensory/motor fatigue or adaptation. Habituation and spontaneous recovery from habituation were found to be dependent upon the interstimulus interval (ISI), prompting his group to hypothesise that habituation was governed by multiple molecular mechanisms (Rankin and Broster, 1992).

In 1985, Chalfie et al. used laser ablation in C. *elegans* to understand the neural circuitry responsible for the reversal response to anterior touch and the forward acceleration response

to posterior touch. The non-localised mechanical stimulus from a tap activates the anterior and posterior mechanosensory neurons. Combining the circuits described by Chalfie and his colleagues (Chalfie *et al.*, 1985) with the neural wiring diagram (White *et al.*, 1986b), the mechanosensory cells (ALM, AVM, PLM, and PVD) and interneurons (AVD, AVA, AVB, PVC, and DVA) mediating the TWR were identified Wicks and Rankin (Wicks and Rankin, 1995). Downstream to the mechanosensory neurons, the interneurons and motor neurons involved in the TWR largely overlap with those mediating spontaneous reversals for exploration and reversals induced by a thermal stimulus (Gray, Hill and Bargmann, 2005).

Repeated activation could alter the response properties of the mechanosensory neurons (Suzuki *et al.*, 2003). They found a cell-wide reduction in calcium response in the anterior touch cell, ALM, on repeatedly poking the anterior of the worm with a glass probe. A similar reduction in calcium response was also reported in the posterior touch cell, PLM, following repeated stimulation (Kindt *et al.*, 2007). To test if the habituation arose as a result of desensitisation of the mechanoreceptor, a whole-cell patch-clamp recording was performed to measure mechanoreceptor currents in the posterior touch cell, PLM (O'Hagan, Chalfie and Goodman, 2005). It concluded that repeatedly poking the cell body with a glass probe had no effect on the touch-evoked mechanoreceptor current. This finding suggests that the site of mechanosensory habituation is downstream from mechanotransduction.

Another group identified a K+ channel (*shw-3*) and an accessory subunit (*mps-1*) with a role in regulating touch sensitivity (Cai *et al.*, 2009). They proposed that repeated activation of the touch cells results in auto-phosphorylation of the SHW-3–MPS-1 complex, thereby diminishing K+ flux and prolonging the duration of mechanoreceptor potentials. Disrupting glutamate neurotransmission also altered habituation to tap (Rankin and Wicks, 2000). *eat-4* encodes the C. *elegans* ortholog of the mammalian vesicular glutamate transporter (VGLUT1) and is expressed in the touch cells underlying the TWR (Lee *et al.*, 1999). *eat-4* mutants had

normal TWR but habituated to tap more quickly and failed to dishabituate following a brief electric shock (Rankin and Wicks, 2000). This suggests that modulation of glutamate release is essential for mechanosensory habituation, perhaps downstream from cell excitability or as part of a parallel pathway.

C. *elegans* also show long-term memory for tap habituation (Beck and Rankin, 1997). It was reported that the AMPA-type glutamate receptor subunit, GLR-1, was required for long-term habituation. *glr-1* mutants exhibited habituation but did not retain decremented responses (Rose *et al.*, 2003). It was hypothesised that short-term habituation is mediated by multiple mechanisms in the touch cells, and long-term memory is linked to changes in the strength of the glutamatergic synapses in the interneurons (Rose et al., 2003; Rongo et al., 1998; Burbea et al., 2002). It was also reported that larval TWR stimulation could affect adult behavior, but the effect depends on the timing and pattern of stimulation (Ebrahimi and Rankin, 2007; Emtage et al., 2009).

Associative memory

C. *elegans* exhibit a remarkable capacity to learn and remember the environmental cues that predict good food, bad food, no food, or aversive stimuli. Associating various cues allows the worm to chemotax, thermotax, or aerotax to favorable environments. Associative learning is a fundamental component of adaptive learning and is referred to as the ability of living organisms to perceive or learn the relation between two or more events (Jozefowiez, 2012). As my study involves associative learning, this section will discuss this paradigm.

Taste as a conditioned stimulus

ASE gustatory neurons mediate chemotaxis to various salts and water-soluble attractants (Bargmann and Horvitz 1991). Partial responses to NaCl in the absence of ASE neurons can

be attributed to ASI, ASG, and ADF neurons (Bargmann and Horvitz, 1991). Although Na^+ and Cl^- are chemoattractants, they are sensed by different neurons. Na^+ is sensed by the ASEL, and Cl^- primarily by the ASER neurons (Pierce-Shimomura *et al.*, 2001). In a choice assay between specific concentrations of Na^+ and Cl^-, untrained worms do not show any preference and migrate to both the ions equally. Nonetheless, if one of the ions was paired with food or an aversive stimulus, the preferences change (Wen *et al.*, 1997). When trained in the presence of food, the worms migrated towards the ions paired with it (appetitive associative learning), but in the presence of aversive taste like garlic extract, the worms moved towards the other ion (aversive associative learning). The same group also conducted a genetic screen and isolated the first learning mutants in C. *elegans*, *lrn-1* and *lrn-2*.

Oxygen as the conditioned stimulus

Oxygen preference could also be altered by experience in C. *elegans* (Cheung *et al.*, 2005). AQR, PQR, and URX are the principal sensory neurons mediating aerotaxis. They co-express five soluble guanylate cyclases (GCY-32, -34, -35, -36, and -37) (Yu et al., 1997; Cheung et al., 2004; Gray et al., 2005). These heme-binding proteins interact with gases via heme iron. The naive *gcy-35* and *gcy-36* mutants showed altered aerotaxis behaviour. However, *gcy-32* and *gcy-34* mutants displayed normal aerotaxis compared to the WT controls, except that they could not change their behavior with experience. This implicates that GCY-32 and GCY-34 are involved in oxygen-mediated learning.

Temperature as the conditioned stimulus

Well-fed worms thermotax to their cultivation temperature and move isothermally at that temperature when placed at a temperature gradient (Hedgecock and Russell, 1975). Laser ablation studies reported that AFD was the primary thermosensory neuron, AIY interneuron

mediates thermophilic movement, AIZ interneuron mediates cryophilic movement, and RIA interneuron integrates the thermo- and cryophilic drives (Mori and Ohshima, 1995).

Temperature preferences can be changed using classical conditioning methods. It is reported that worms in starved plates tend to disperse more randomly away from their cultivation temperature through a learned understanding of the temperature and feeding status of the worm (Hedgecock and Russell, 1975). Calcium imaging experiments showed that INS-1 and TAX-6 are required for the starvation-induced inhibition of AIZ (Kodama et al., 2006; Kuhara and Mori, 2006).

Smell as the conditioned stimulus

C. *elegans* chemotax towards or away from volatile organic compounds. Diacetyl is usually a chemoattractant, but worms could learn to avoid it if it was previously presented with an aversive signal (Morrison *et al.*, 1999). Conversely, C. *elegans* chemotax more towards butanone after pre-exposure to it in the presence of food (Torayama, Ishihara and Katsura, 2007). C. *elegans* learn to avoid odors associated with pathogenic bacteria and prefer those associated with familiar non-pathogenic strains (Zhang, Lu and Bargmann, 2005). Serotonin-deficient *tph-1* (tryptophan hydroxylase) mutants do not learn bacterial preference, and worms exposed to exogenous serotonin learned to avoid pathogens faster (Zhang, Lu and Bargmann, 2005). Rescuing *tph-1* expression in the serotonergic neuron, ADF, was sufficient to rescue learned aversion behavior, but not learned attraction behavior. Rescuing the expression of *tph-1* in ADF and NSM restored wild-type behavior, suggesting ADF controls food aversion behavior and NSM controls food attraction behavior.

b. Role of Insulin Pathway in the Learning and Memory of *Caenorhabditis* elegans

Insulin and insulin-like pathways have been well-documented in C. *elegans* (Figure 12). Binding of insulin peptides to the DAF-2 insulin receptor activates the phosphoinositide 3-kinase AGE-1/PI3K (Tomioka et al. 2006). This results in the activation of the serine/threonine kinases PDK-1, AKT-1, and AKT-2, which causes phosphorylation of the DAF-16/FoxO transcription factor (Paradis et al. 1999; Paradis & Ruvkun 1998).

In C. *elegans*, a mutation in the insulin-like peptide-encoding *ins-1* gene disrupts temperature aversive learning and salt aversive learning (Kodama et al. 2006; Mohri et al. 2005; Tomioka et al. 2006; Ohno et al. 2017), but not butanone appetitive learning (Lin et al. 2010). These defects can be reversed by the expression of INS-1 in a subset of neurons. For example, expression of INS-1 in ADF neurons rescued the temperature aversive learning defect (Kodama et al. 2006). INS-1 is required in the AIA interneurons for salt aversive learning (Tomioka et al. 2006).

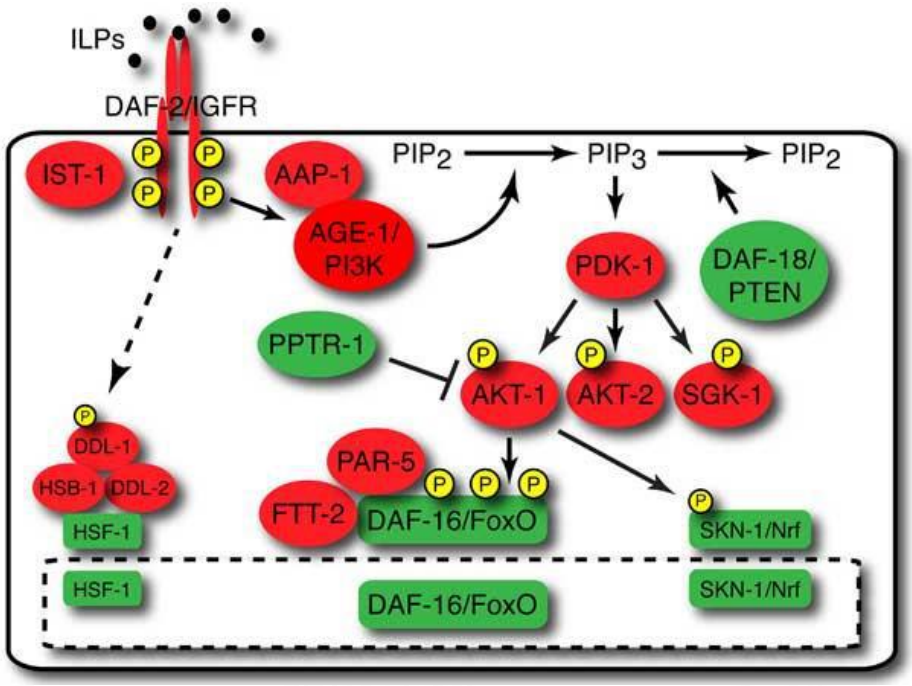

Figure 12. Schematic diagram of Insulin and Insulin-like pathway in C. *elegans*. Activation of insulin signaling promotes phosphorylation-dependent cytoplasmic sequestration of the transcription factors DAF-16/FoxO, HSF-1, and SKN-1/Nrf. (Image courtesy: wormbook.org)

INS-1 signals through the insulin receptor DAF-2 (Tomioka et al. 2006). *Pseudomonas aeruginosa* aversive learning and salt aversive learning were defective in *daf-2* mutants (Tomioka et al. 2006; Chen et al. 2013). *daf-2* mutants regained the salt aversive learning upon ASER-specific expression of DAF-2 (Tomioka et al., 2006). On the other hand, the expression of DAF-2 in the RIA interneurons restored *P. aeruginosa* aversive learning in *daf-2* mutants (Chen et al. 2013). Interestingly, daf-2 mutation enhanced the butanone appetitive learning in C. *elegans* (Kauffman et al. 2010). DAF-2c translocation and salt attractive behaviour are dependent on the Ras/MAPK pathway, and suppression of this pathway is proposed to affect salt aversive learning through CASY-1/KLC-2 mediated transport of DAF-2c to the axon terminal of ASER (Ohno et al. 2014).

age-1 mutants show enhanced temperature aversive learning (Kodama et al. 2006). Contrariwise, these mutants are defective in salt aversive learning, but this defect could be rescued by ASER-specific re-expression of AGE-1 (Tomioka et al. 2006). PDK1, and AKT-1 are required for salt aversive learning (Tomioka et al. 2006; Paradis et al. 1999). Temperature aversive learning is enhanced in age-1 mutants but is defective in *daf-16* null mutants (Kodama et al. 2006). Likewise, daf-16 mutants display defective salt aversive learning Nagashima et al. 2019; Tomioka et al. 2006). This is particularly interesting as DAF-16 activity is normally suppressed by insulin signaling (Lee et al. 2001). However, activation of the insulin pathway is required for salt aversive learning (Tomioka et al. 2006), suggesting that this pathway affects this behavioural paradigm through a different downstream mechanism. DAF-16 acts in the nucleus of ASER to promote salt aversive learning (Nagashima et al. 2019). DAF-16 is also implicated in butanone appetitive learning as the enhanced memory in *daf-2* mutants was suppressed in *daf-2;daf-16* double mutants (Kauffman et al. 2010). These results indicate that although insulin pathway does not target DAF-16 to affect salt aversive learning (Tomioka et al. 2006), butanone appetitive learning likely requires the negative regulation of DAF-16 by DAF-2 (Kauffman et al. 2010).

c. Other Neurotransmitters and Neuropeptides in the Learning and Memory of *Caenorhabditis* elegans

Glutamate

Glutamate is an excitatory neurotransmitter. It is transported into synaptic vesicles by vesicular glutamate transporter 1 (VGLUT1) (Bellocchio et al., 2000; Takamori et al., 2000). In C. *elegans*, the *eat-4* gene encodes a VGLUT and is involved in glutamate release (Lee *et al.*, 1999). EAT-4 is expressed in the ALM, AVM, and PLM mechanosensory neurons that are activated by tap stimulation (Chalfie et al., 1985; Lee et al., 1999). Since *eat-4* is implicated in

glutamate release, it was proposed that *eat-4* mutants cannot maintain a prolonged habituation response because of reduced levels of releasable glutamate (Rankin and Wicks, 2000). EAT-4 is also implicated in temperature aversive learning (Takeishi et al., 2020).

GLR-1 is a putative AMPA-type glutamate receptor in C. *elegans* (Hart et al., 1995; Maricq et al., 1995). *glr-1* null mutation impaired the diacetyl avoidance learning ability of worms (associative learning). Diacetyl habituation (non-associative learning) was also impaired in these mutants (Morrison and Kooy, 2001). Glutamate mediates associative learning through its action on NMDA receptors in other organisms (de Oliveira Coelho et al., 2013; Rosenegger and Lukowiak, 2010; Xia et al., 2005). *nmr-1* and *nmr-2* are the putative NMDA receptors in C. *elegans* (Brockie et al., 2001; Kano et al., 2008). Deletion mutants of *nmr-1* and *nmr-2* exhibit defective short-term memory of salt aversive learning. Since the defects are non-additive in *nmr-1;nmr-2* double mutants, these genes possibly regulate this form of learning by the same molecular pathway. In contrast, the short-term memory of salt aversive learning is intact in *glr-1* mutants (Kano *et al.*, 2008). This suggests that glutamate acts through different receptors to affect specific behavioural paradigms. In addition to salt aversive learning, nmr-1 mutants also display impaired diacetyl aversive learning and *Pseudomonas aeruginosa* aversive learning (Choi et al., 2020; Fadda et al., 2020).

Dopamine

Dopamine neurotransmitter and its role in reward and motivation have been well-studied (Floresco, Tse and Ghods-Sharifi, 2008) (Mohebi *et al.*, 2019) (Roitman et al., 2004). CAT-2 is a protein with tyrosine hydroxylase activity (Calvo et al., 2011), expressed in the dopaminergic neurons of C. *elegans* (Lints and Emmons, 1999). *cat-2* mutation abolishes gustatory plasticity and mechanosensory, and these learning defects can be rescued by exposure to exogenous dopamine (Beets et al., 2012; Hukema et al., 2008; Sanyal et al., 2004).

Habituation via plate-tap assay is enhanced by a *dop-1* receptor mutation and could be rescued by the expression of DOP-1 in the ALM, AVM, PLM, and PVM mechanosensory neurons in these worms (Sanyal *et al.*, 2004). D2-like dopamine receptor DOP-2 also modulate mechanosensory habituation (Pandey and Harbinder, 2012). This enhanced rate of habituation in these mutants were suppressed by exposure to exogenous dopamine during habituation (Mersha *et al.*, 2013).

Neurotransmitter release from dopaminergic neurons is enhanced by salt aversive learning in an acid-sensing ion channel (ASIC)-1-dependent manner. Additionally, this form of learning was impaired in worms with a null mutation in *asic-1* (Voglis and Tavernarakis, 2008).

Serotonin

In C. *elegans*, temperature aversive learning and salt aversive learning are diminished by exposure to exogenous serotonin during the conditioning phase (Mohri et al., 2005; Saeki et al., 2001). Furthermore, gustatory plasticity, diacetyl aversive learning, and *Pseudomonas aeruginosa* aversive learning were impaired in *tph-1* mutants that are defective in serotonin synthesis (Beets et al., 2012; Fadda et al., 2020; Sze et al., 2000; Zhang et al., 2005). Contrariwise, *tph-1* mutants exhibit intact butanone appetitive learning (Torayama, Ishihara and Katsura, 2007).

Neuropeptide Y (NPY)/neuropeptide F (NPF)-related Neuropeptides

In C. *elegans*, the flp-34 gene has been predicted to encode a neuropeptide in the NPY/NPF system. FLP-34 is involved in diacetyl aversive learning. This learning paradigm is impaired in *flp-34* null mutants (Fadda et al. 2020). NPR-11 is an NPY/NPF-related neuropeptide receptor that can be activated by FLP-34 *in vitro* (Fadda et al. 2020; Fadda et al. 2019;

Gershkovich et al. 2019). npr-11 null mutants exhibit defective diacetyl aversive learning that could be rescued by AIA-specific re-expression of NPR-11.

NPR-11 is activated by peptides encoded by nlp-1 (Gershkovich et al. 2019; Li et al. 1999). Mutations in nlp-1 cause impaired isopentanol adaptation, which could be restored by NLP-1 re-expression in isopentanol-sensing AWC neurons (Colbert & Bargmann 1995; Chalasani et al. 2010). Isopentanol adaptation was also impaired in *npr-11* mutants (Chalasani et al. 2010).

FMRFamide-related neuropeptides

C. *elegans* codes for at least 31 genes in the FMRFamide-related peptide gene family (Nelson et al. 1998; Li & Kim 2008), of which FLP-20 peptides were found to be associated with mechanosensory habituation (Li et al. 2013). It also affects touch sensitisation (Chew et al. 2018). In the habituation paradigm, FLP-20 modulates neurotransmitter release from PLM mechanosensory neurons (Li et al. 2013). In the sensitisation paradigm, the site of FLP-20 action is the neuroendocrine cell RID (Chew et al. 2018), which triggers ASH sensory facilitation (Chew et al. 2018).

Chapter-1

Introduction

The brain is one of the most complex organs and regulates various functions necessary for survival. Importantly, it controls learning abilities, houses our memory and thoughts (Stangor and Walinga, 2014). Learning is the acquisition of knowledge or skills through study, experience, or being taught, and memory is the faculty by which the brain stores and retrieves the learned information. Together, learning and memory serve as essential tools for the organism to adapt to changing environment (Van Damme *et al.*, 2021). Understanding the anatomical and physical bases of learning and memory is one of the challenges of modern neuroscience.

Numerous mechanisms have been postulated regarding the consolidation of memory. Many neurotransmitters and neuropeptides have also been implicated in learning and memory processes (Kandel, 2001). The role of insulin as a neuropeptide was recognised long after its discovery (Havrankova *et al.*, 1978). For several years the role of insulin on the brain remained largely unknown because the central nervous system was considered a non-insulin-dependent tissue. The discovery of insulin receptors (IR) and insulin and insulin-like peptides in the brain opened a new area of interest- the role of insulin signaling in the brain. Their roles range from feeding control to learning and memory. This discovery has revolutionised and paved the way for understanding how the brain is a highly insulin-sensitive organ (Hill et al., 1986; Zhao and Alkon, 2001).

Over the years, insulin has been found to affect various brain functions. MAPK pathway functioning downstream of the insulin is involved in learning and memory formation in different species. These include, but are not limited to, associative learning (Atkins *et al.*, 1998), spatial learning in the Morris water maze task and arm radial maze (Selcher et al., 1999; Zhao et al., 1999), inhibitory avoidance learning (Izquierdo *et al.*, 2000) and contextual and auditory fear

conditioning (Schafe et al., 1999; Selcher et al., 1999). Deficits in peripheral and CNS insulin/IR action are associated with clinical disorders, such as Alzheimer's disease (AD) and Parkinson's disease, where memory impairment is a prominent symptom.

Historically, it was believed that invertebrates lack cognitive functions. However, pioneers in the field such as Eric Kandel popularised invertebrate model systems. Studying the marine mollusc *Aplysia*, his group was able to relate behavioral plasticity to changes at specific synapses of identified neurons and began a biochemical analysis of these neuronal changes, uncovering a role for cAMP, PKA, and CREB (Kandel and Tauc, 1965). Benzer and his colleagues worked on the genetic dissection of learning in the fruit fly Drosophila melanogaster, established an associative learning assay (Quinn, Harris and Benzer, 1974), and identified the first learning mutant, *dunce* (Dudai *et al.*, 1976). Sydney Brenner introduced *Caenorhabditis elegans* as the organism to study development and the nervous system in 1974 (Brenner, 1974). It has become one of the most well-understood animal models through the years. Neurobiologists around the globe have accepted this model organism for its relatively simple nervous system that makes it easier to study. The nervous system of C. *elegans* comprises 302 neurons, about 5000 chemical synapses, 600 gap junctions, and 2000 neuromuscular junctions, the location of which are reasonably consistent between animals (White *et al.*, 1986b). Associative and non-associative memories and imprinting have been described in C. *elegans* (Ardiel and Rankin, 2010).

Rankin and his group were the first to characterise learning and memory in C. *elegans* (Rankin, Beck and Chiba, 1990). Since then, scientists have been working on elucidating molecular pathways and neural circuits of learning and memory in this model organism. The insulin pathway is also well-established in C. *elegans* (Murphy and Hu, 2018). In this study, primary focus is on the effect of the insulin pathway on the butanone-associated associative memory. Murphy and her team have shown that mutants of the insulin receptor, *daf-2*, have

daf-16-dependent extended learning and memory (Kauffman *et al.*, 2010). However, the exact mechanism and the connectome involved have not been discussed. Thus, this study attempts to understand the role of the insulin pathway in learning and memory in a more elaborate manner.

In the present study, I used various mutants and siRNA-mediated silencing to map out the essential genes of the insulin pathway involved in the learning and memory of C. *elegans*. Additionally, I have looked into the translational and transcriptional requirements in the process. Exogenous administration of huminsulin was carried out to elucidate the effect of excess insulin on learning and memory. Furthermore, this study deals with the impact of altered insulin pathways during the early developmental stages on the learning and memory of adult C. *elegans*.

Materials and Methods

Caenorhabditis elegans Strains

The following strains were used in this study: The WT strain N2 (Bristol); CB1387 [*che-3(e1378) I (daf-10)*]; CB1370 [*daf-2 (e1370) III*]; TJ1052 [*age-1 (hx546) II*]; DR26 [*daf-16(m26) I*]; RB759 [*akt-1(ok525) V*]; DA1116 [*eat-2(ad1116) II*]; IK581 [*ins-1(nj32) IV*]; TJ356 [*daf-16p::daf-16a/b::GFP + rol-6 (su1006)*]. All strains, unless otherwise mentioned, were provided by the Caenorhabditis Genetic Centre (CGC, Minnesota, St. Paul).

C. elegans maintenance

The C. *elegans* strains were reared in petri dishes with Nematode Growth Medium (NGM) and OP50, an auxotroph of *Escherichia coli* (E. *coli*), as a food source. The strains CB1370 [*daf-2 (e1370) III*] was grown at 15 ^{0}C. All other strains were maintained at 20 ^{0}C (Brenner, 1974).

Synchronisation of C. elegans

A slightly modified version of the standard hypochlorite treatment described by Stiernagle (Stiernagle, 2006) was used to obtain an age-matched population of worms. Gravid adult worms were treated with bleach solution (20% NaOCl, 1 M NaOH) in a 1.7 ml centrifuge tube. When worm debris turned invisible to the naked eye (≈10 minutes), the bleach solution was discarded after centrifugation at 8000 rpm for 1 minute. The resulting pellet containing the eggs was washed three times with autoclaved distilled water and carefully stored in M9 Buffer at 20 ^{0}C overnight to have all the eggs hatch to the L1 stage. The L1 larvae were then transferred to NGM plates seeded with OP50 and stored at 20 ^{0}C. Day 1 adult worms were recovered from the plate after 48 hours.

Short-Term Adaptive Memory Training

The standard protocol (Kauffman *et al.*, 2011) was used with minor modifications to train worms for Short-Term Adaptive Memory (STAM). Butanone (Himedia, product code-AS053) was used as the conditional stimulus (CS) and liquid OP50 (OD_{600}=0.4-0.6) as the unconditional stimulus (US). Day 1 adult worms were collected from the NGM-OP50 plates in a centrifuge tube and washed three times with M9 buffer to remove excess OP50 adhered to the worms. Worms were then starved for 1 hour in a centrifuge tube with 1 mL M9 buffer at 20 ^{0}C. After starvation, worms were transferred to a fresh NGM plate after removing excess M9 buffer with a Kimwipe. Immediately afterwards, 400 µL of OP50 culture was added to the NGM plate and 5 µL of 10% butanone (prepared in 95% ethanol) was smeared on the lid, and the plate was closed and sealed with parafilm. The plate was then kept for 1 hour conditioning at 20 ^{0}C. After the conditioning period, the trained worms were collected in an NGM-OP50 plate till the completion of the chemotaxis assay (Figure A).

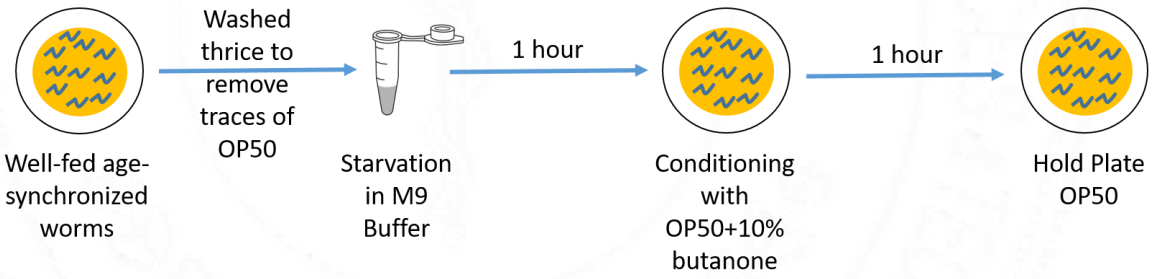

Figure A. Short-term olfactory adaptive training paradigm. Well-fed day-1 adult worms were collected and starved for 1 hour and then conditioned for the next hour with 10% butanone and food. From then on, the worms were kept on a hold plate till the assay was performed.

Long-Term Adaptive Memory Training

The protocol for Long-Term Adaptive Memory (LTAM) training is similar to that of STAM. The training starts with 1 hour of starvation followed by 30 min of conditioning phase. The starvation and conditioning stages were subsequently repeated for seven more cycles, 30

minutes each. Trained worms are then collected in an NGM-OP50 plate till the completion of the chemotaxis assay (Figure B).

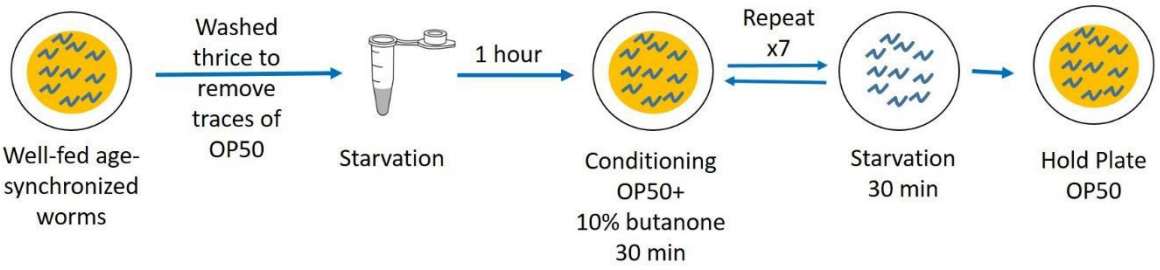

Figure B. Long term olfactory adaptive training paradigm. Well-fed day 1 adult worms were collected and washed to remove any traces of OP50. The worms were starved for 1 hour, followed by a 30 min conditioning step. The worms were starved and conditioned 30 minutes each seven more times and kept in hold plates till the assay.

Chemotaxis Assay

The attraction of the trained worms to the conditioned solvent was assessed by chemotaxis assay as previously described (Kauffman *et al.*, 2011) with minor modifications. Naïve (untrained) and conditioned (trained) worms from short- or long-term adaptive memory trained population were collected from the hold plates and washed thrice to remove any traces of OP50. They were then collected worms were then placed on the centre of a chemotaxis plate (), as shown in Figure C. Excess buffer was dried off using a Kim wipe. The opposite ends of the plates were spotted with 3 µL each of the control (95% ethanol) and test (10% butanone) odorants. The plates were sealed immediately afterwards and kept at 20°C for 20 min. After 20 minutes, the number of worms reaching the test and control area were counted and carefully recorded. While counting the total number of worms, those that did not cross circle D was excluded. Chemotaxis index (CI) was calculated using the formula (Bargmann, Hartwieg and Horvitz, 1993). The assay was repeated at different time intervals.

$$Chemotaxis\ index\ (CI) = \frac{Number\ of\ worms\ at\ the\ test\ area - Number\ of\ worms\ at\ the\ control\ area}{Total\ number\ of\ worms}$$

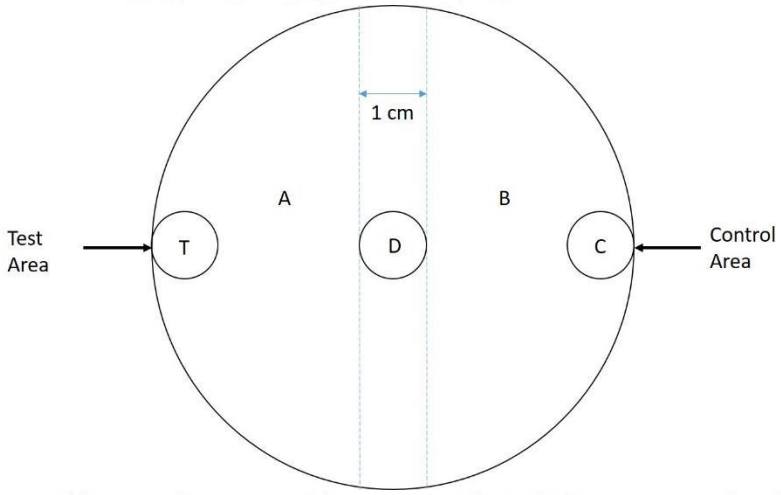

Figure C. Chemotaxis assay plate. The worms are washed to remove OP50 and placed on the centre, D. Excess buffer is wiped off with a Kim wipe and solvents are spotted on the areas marked for control and test solvents, C and T, respectively. The plate is sealed for 20 minutes, the number of worms in each area is counted, and the chemotaxis index is calculated.

siRNA mediated silencing

siRNA-mediated silencing of *daf*-2 gene was performed using an earlier well-established protocol (Ashe *et al.*, 2012). siRNA- mediated silencing of the genes were done by feeding RNase III resistant E. *coli* HT115 (DE3), which express a double-stranded RNA (dsRNA) against a portion of the gene. The sequences were chosen such that their sequence did not match any other C. *elegans* genes or GFP. 357 bp of *daf*-2 gene was sub-cloned into the plasmid pL4440. pL4440 plasmid has two opposable T7 polymerase promoter sites. Furthermore, this promoter also provides an Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible expression of the phage T7 RNA polymerase (Conte *et al.*, 2015). L1 larvae were plated onto the RNAi plates seeded with either the empty vector, pL4440 or the dsRNA-expressing HT115 bacteria. Day 1 adults were then used for the siRNA studies. In the case

of memory experiments, OP50 was substituted with either pL4440 or the dsRNA-expressing HT115 during the conditioning and in the hold plates (See Annexure for more details).

Local search Assay

The assay followed the protocol previously described in the literature (Gray, Hill and Bargmann, 2005). Number of omega turns taken by the worms in food plate and plates supplemented with 10 U exogenous insulin was recorded for 5 minutes after an acclimatisation period of 20 minutes. The values were then plotted as the number of omega turns/ minute.

Chemical treatments

Cycloheximide and Actinomycin D treatment: 200 µg/mL Actinomycin D ≥ 95% was added to M9 buffer during starvation and along with OP50 during the conditioning. Cycloheximide ≥ 94% was added at 0.8 mg/mL concentration during the starvation and conditioning.

Insulin treatment: Huminsulin was used at 0.1, 0.5, or 1 IU/mL concentrations during the conditioning phase of STAM experiments. In the local search assay and DAF-16 localisation assays, worms were incubated in OP50 plates with 1 IU/mL huminsulin for 1 hour before performing the assay.

Microscopy

A stereo microscope (Magnus Analytics, India) with 10X zooming was used for viewing and general handling like picking, transferring, and washing of C. *elegans* strains. For fluorescence imaging, Olympus IX51 inverted microscope, (Olympus Imaging, Center Valley, PA, USA) objective lens 40X/0.60 Ph2/∞/0-2/FN22, which works with image acquisition software NIS

Elements-Advanced Research (NIKON) and Rolera XR monochrome camera (QImaging, Canada) was used. Nuclear localisation of DAF-16 was analysed using Leica DMi8 fluorescence microscope.

Software

Graphpad Prism version 6 (GraphPad Software Inc.) was used for graphical representation and statistical analysis of the data. Image analysis was carried out using Fiji (an open-source image processing package based on ImageJ).

Statistical Analysis

To determine the performance of three or more groups as in case of short-term adaptive memory formation, we conducted two-way ANOVA with Tukey's multiple comparison test to compare the means of CI values of different experimental groups and at different time points. The number of experiments (n) are represented as the number of trials, and each trial contains more than 50 animals unless otherwise mentioned. Significance is represented as follows *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Results

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

It is well-established that C. *elegans* sense odors through three sets of chemosensory neurons in the amphid sensory region, namely, AWA, AWB and AWC (Bargmann and Horvitz, 1991; Bargmann et al., 1993). These neurons express multiple chemosensory receptors that aid in recognising various molecules. AWA and AWC neurons mediate responses to various attractive odorant stimuli like butanone (Bargmann, Hartwieg and Horvitz, 1993). AWB neuron is responsible for sensing repulsive odorants such as nonanone (Troemel, Kimmel and Bargmann, 1997).

The Short-Term Associative Memory (STAM) paradigm used in the study involved one hour of starvation followed by another hour of conditioning phase (Figure 1 in Materials and Methods). During the starvation, Day 1 adult worms are washed from the food plate and rinsed thrice with the M9 buffer to remove any traces of food. Subsequently, the worms are incubated in M9 buffer for 1 hour. During the conditioning, the worms are transferred to an NGM plate with 400 µL of OP50 (food) and with 5 µL of 1/10 butanone streaked on the lid of the plate. The plate is then sealed with parafilm and kept for 1 hour. Contrariwise, Long-Term Associative Memory (LTAM) involved an hour of starvation followed by seven bouts of intermittent conditioning and starvation periods, each for 30 minutes (Figure 2 in Materials and Methods). 1/10 dilution of butanone as the conditioned stimulus (CS) and food (OP50) as the unconditional stimulus (US) were used in in these experiments. Control Bristol N2 strain (WT), amphid-defective CB1387 [*che-3(e1378) I (daf-10)]* strain, and AWC-defective JC2209 [olrn-1(ut306)] were used to standardise the STAM training paradigm.

As was expected, the WT worms exhibited a significant increase ($p \leq 0.0001$, n=6) in the chemotaxis index (CI) values following STAM training which revealed that the worms were able to learn the association between the CS, butanone and the unconditional stimulus (US), OP50 (Figure 1a). The memory lasted for 2 hours following the training and the CI values reverted to naïve levels at the 3rd hour (Figure 1a). Hence, the STAM in the worms can last up to 2 hours after the training. On LTAM training, the WT worms learnt the association between the food and butanone, shown by the significant increase ($p \leq 0.0001$, n=6) in CI value at the 0th hour. The CI remained significant ($p \leq 0.0001$, n=6) even at the 24th hour but returned to naïve levels ($p > 0.05$, n=6) at the 48th hour (Figure 1b). This prove that WT worms could retain the LTAM for 24 hours after the training.

The amphid-defective strain, CB1387 [*che-3(e1378) I (daf-10)]*, however, displayed an impairment in learning when compared to the WT worms at the 0th, 1st, and 2nd hours post-training $p \leq 0.0001$, n=6) (Figure 2a). Although this strain showed an increase in CI value at the 0th hour compared to its naïve ($p \leq 0.0001$, n=6), the worms were unable to maintain the memory recall after this time point (Figure 2a). On the other hand, the AWC-defective JC2209 [olrn-1(ut306)] strain showed a significant learning defect even at the 0th hour ($p \leq 0.0001$, n=6) (Figure 2a). This result was anticipated as the AWC neuron recognises the odorant, butanone (Bargmann, Hartwieg and Horvitz, 1993). On the other side, CB1387 [*che-3(e1378) I (daf-10)]* and AWC-defective JC2209 [olrn-1(ut306)] worms were defective in LTAM owing to which they displayed significantly lower CI values at 0th, 24th, and 48th hours ($p \leq 0.0001$, n=6 at all time points) (Figure 2b).

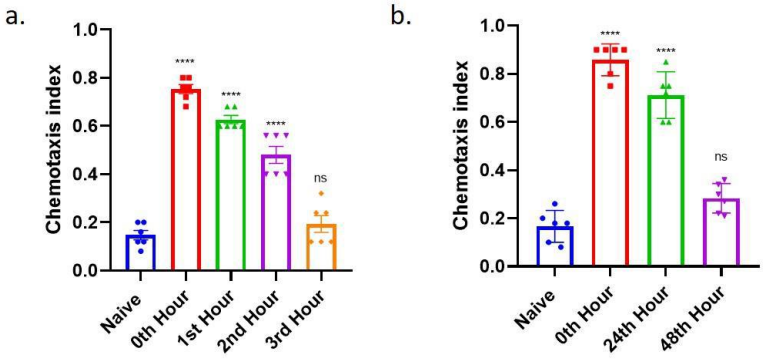

Figure 1. Short-Term (a) and Long-Term (b) Associative Memory formation in wild-type worms. **a**. WT worms trained towards butanone (Conditional Stimulus, CS) in the presence of OP50 (Unconditional Stimulus, US) showed an increase ($p \leq 0.0001$) in attraction to the odorant butanone when compared to the untrained naïve WT worms. This attraction remained significant ($p \leq 0.0001$) till the 2nd hour post-training. At the 3rd hour the CI value of the trained worms decreased to naïve level ($p > 0.05$). **b**. The WT worms showed a significant increase ($p \leq 0.0001$) in CI values at 0th and 24th hours post Long-term associative memory training. The memory is lost by the 48th hour at which point the CI value becomes comparable to the untrained worms. n=6 trials; each trial contains more than 50 worms; statistical analysis was done by one-way ANOVA with Tukey's multiple comparisons test; Data represented as the mean + S.E.M. Significance indicated. ns- non-significant and ****$p \leq 0.0001$.

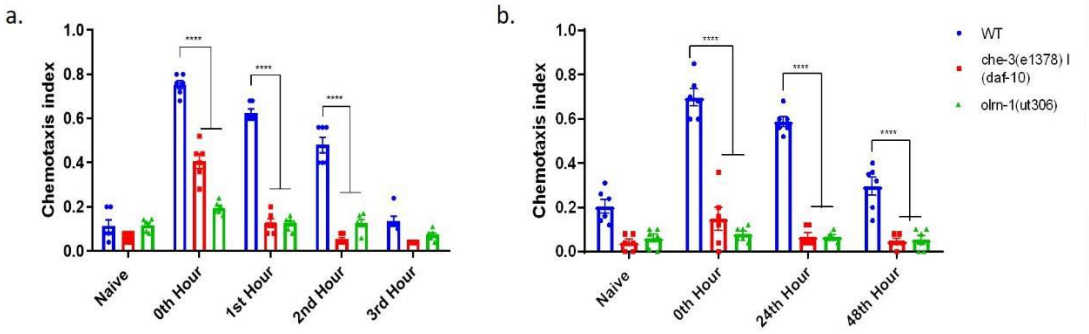

Figure 2. Short-Term (a) and Long-Term (b) Associative Memory formation in amphid neuron defective CB1387 (*che-3(e1378) I [daf-10]*) and AWC defective JC2209 (*olrn-1[ut306]*) worms. **a**. The CI values of the amphid-defective CB1387 [*che-3(e1378) I (daf-10)]* and the AWC neuron defective JC2209 [*olrn-1(ut306)]* at 0th, 1st, and 2nd hours were significantly ($p \leq 0.0001$) lesser than that of the WT values at respective time points. **b**. The amphid-defective CB1387 [*che-3(e1378) I (daf-10)]* and AWC-defective JC2209 [olrn-1(ut306)] mutants showed no significant change at any time-points compared to the WT worms in LTAM paradigm. n=6 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. ****$p \leq 0.0001$.

daf-2 mutants have extended Short-Term and Long-Term Associative Memory

To understand how insulin pathway affects the butanone associated memory, a strain (CB1370 [*daf-2(e1370) III]*) deficient in the insulin receptor, DAF-2, was used. The DAF-2 gene encodes for the insulin growth factor receptor (IGFR) in the nematode *C. elegans.* It is expressed in various organs like nervous system, the intestine and the gonads. Apart from metabolic effects, *daf-2* is also associated with ageing in C. *elegans* (Dorman *et al.*, 1995). It has also been noted that *daf-2* mutations are associated with extended memory in butanone-associated appetitive learning and that these mutants also maintain learning ability better with age (Kauffman *et al.*, 2010).

On STAM training*,* these *daf-2* mutants retained memory for 5 hours ($p \leq 0.001$, $n \geq 3$) compared to the untrained naïve group (Figure 3a). At all time points, the CI values of CB1370 [*daf-2(e1370) III]* remained significantly higher (naïve $p \leq 0.0001$, 0^{th} hour $p \leq 0.05$, 1^{st} to 6^{th} hour $p \leq 0.0001$; $n \geq 3$) than the WT worms (Figure 3a). These results indicated that CB1370 [*daf-2(e1370) III]* worms display an abnormal extended memory following STAM appetitive training towards 1/10 butanone; similar to the results obtained by Murphy and her team (Kauffman *et al.*, 2010).

To understand whether the extended memory was also observed in the LTAM context, CB1370 [*daf-2(e1370) III]* worms were tested for chemotaxis following LTAM training towards butanone. The CI values of trained CB1370 [*daf-2(e1370) III]* worms were lower ($p \leq 0.01$, $n \geq 5$) at 0^{th} and 24^{th} hours when compared to WT worms (Figure 3b). At the 48^{th} hour following LTAM training, the CI value of CB1370 [*daf-2(e1370) III]* worms was maintained significantly ($p \leq 0.01$, $n \geq 5$) compared to the WT worms (Figure 3b). This demonstrates that CB1370 [*daf-2(e1370) III]* worms also exhibit extended LTAM after appetitive conditioning towards butanone- confirming the observations of Murphy and her team (Kauffman *et al.*, 2010).

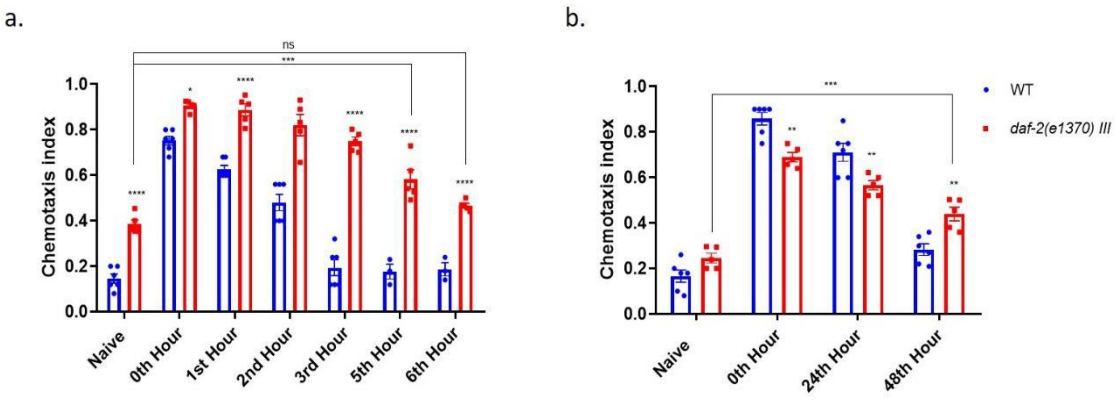

Figure 3. Short-Term (a) and Long-Term (b) Associative Memory Formation in CB1370 [*daf-2(e1370) III*] Insulin Receptor mutants. **a**. *daf-2(e1370)* mutants trained for STAM showed a significantly higher CI at all time-points (naïve $p \leq 0.0001$, 0^{th} hour $p \leq 0.05$, 1^{st} to 6^{th} hour $p \leq 0.0001$). The memory lasted till the 5^{th} hour post-training ($p \leq 0.001$). The CI value returned to naïve levels at the 6^{th} hour. **b**. Though the CI values of CB1370 [*daf-2(e1370) III*] were significantly lower ($p \leq 0.01$) compared to the CI values of WT worms at 0^{th} and 24^{th} hours post-training, the CI value at the 48^{th} hour showed a marked increase ($p \leq 0.01$) in value. At 48^{th} hour, the CI value of *daf-2(e1370)* mutants remained significantly higher ($p \leq 0.001$) compared to its naïve value. 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, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

Mutants of molecules downstream to DAF-2 have normal Short-Term Associative Memory but defective Long-Term Associated Memory

A cascade of downstream phosphorylation reactions occur after the activation of the DAF-2 receptor. Tyrosine-phosphorylated IRS proteins endorse the recruitment and activation of components of downstream cascades such as the AGE-1 phosphoinositide 3-kinase and AKT (Taniguchi, Emanuelli and Kahn, 2006). This, in turn, results in the phosphorylation of the DAF-16/FoxO transcription factor and its subsequent cytoplasmic sequestration.

To understand the role of molecules downstream to the DAF-2 receptor, such as AGE-1, AKT and DAF-16, in the learning and memory process, the following mutants of the pathway were used: TJ1052 *[age-1 (hx546) II]*, RB759 *[akt-1(ok525) V]*, and DR26 [*daf-16(m26) I]*. All these mutants showed normal STAM in comparison with the WT. At all given time points, the CI

values were similar to that of WT worms ($p>0.05$, $n\geq3$) and the memory was lost by the 3rd hour of training (Figure 4a). Also, the strains TJ1052 *[age-1 (hx546) II]* and RB759 *[akt-1(ok525) V]* exhibited normal LTAM at 0th hour post-training and the CI values were comparable ($p>0.05$, $n\geq3$) with that of the WT worms (Figure 4b). At 24th hour, the CI values of both these mutants were significantly lower ($p\leq0.0001$, $n\geq3$) than that of the WT within the same time group. The DR26 [*daf-16(m26) I]* worm, on the other hand, was defective in LTAM and displayed lower ($p\leq0.0001$, $n\geq3$) CI values at both 0th and 24th hour time points (Figure 4b).

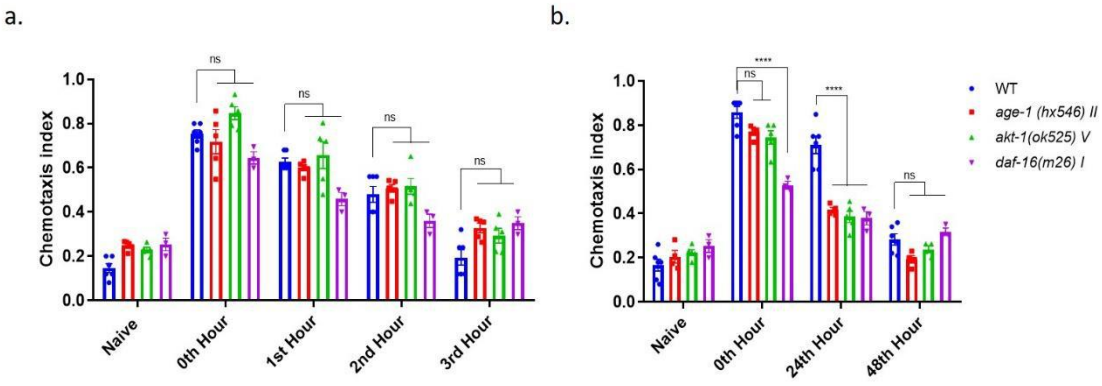

Figure 4. Short-Term (a) and Long-Term (b) Associated Memory of TJ1052 *[age-1 (hx546) II]*, RB759 *[akt-1(ok525) V]*, and DR26 *[daf-16(m26) I]*. a. Following STAM training, the mutants showed normal associative memory similar ($p> 0.05$) to that of the WT worms at all time points. The worms retained the memory till the 2nd hour, after which the CI value reached the naïve levels. **b.** After LTAM, TJ1052 *[age-1 (hx546) II]* and RB759 *[akt-1(ok525) V]* exhibited normal learning in comparison with the WT worms at 0th hour. Conversely, at 24th hour, they showed a significant reduction ($p\leq0.0001$) in CI values compared to the WT worms. DR26 *[daf-16(m26) I]* worms, on the other hand were found to be defective in LTAM. $n\geq3$ trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Tukey's multiple comparisons test; Data represented as the mean + S.E.M. Significance indicated: ns- non-significant and ****$p\leq0.0001$.

The extended memory of daf-2 mutants is through the daf-16 pathway

In C. *elegans*, the insulin receptor DAF-2 controls the activity of a conserved AGE-1 phosphoinositide 3-kinase (PI3K)/AKT kinase cascade, culminating in the regulation of DAF-

16 transcription factor that governs most of the functions of this pathway (Brunet *et al.*, 1999). DAF-16 is a member of the FoxO family of Forkhead transcription factors. Insulin-regulated DAF-16 FoxO transcription factor is the key transcription factor responsible for insulin action on gene expression. They are crucial regulators of metabolism (Kimura *et al.*, 1997a), stress responses (Honda and Honda, 1999), cell cycle control (Dottermusch *et al.*, 2016), growth, and longevity (Kenyon *et al.*, 1993).

To understand whether the extended memory is through the *daf-16* pathway, an siRNA clone targeting the *daf-2* gene was created. WT, TJ1052 [*age-1 (hx546) II*], and DR26 [*daf-16(m26) I*] worms were grown in the presence of control (pL-4440) and test (pL-daf-2) vectors and trained for STAM. All sets of worms showed normal memory at 0^{th} hour following STAM training, and the CI values were significantly higher ($p \leq 0.0001$, n=4) than that of their untrained counterparts (Figure 5). As expected, the WT worms treated with siRNA of *daf-2* (pL-daf-2) showed a significantly higher ($p \leq 0.001$, n=4) CI value compared to its naïve, which is consistent with the results obtained in CB1370 [*daf-2(e1370) III]* worms (Figure 3). The TJ1052 [*age-1 (hx546) II*] and DR26 [*daf-16(m26) I*] worms, however, failed to elicit extended STAM following treatment with either the control (pL-4440) or the siRNA of *daf-2* (pL-daf-2) ($p>0.05$ compared to the respective naïve values, n=4) (Figure 5). These results demonstrate that functional AGE-1 and DAF-16 molecules are essential for the maintenance of extended memory associated with the *daf*-2 mutation. The extended memory is through the *daf-16* FOXO pathway.

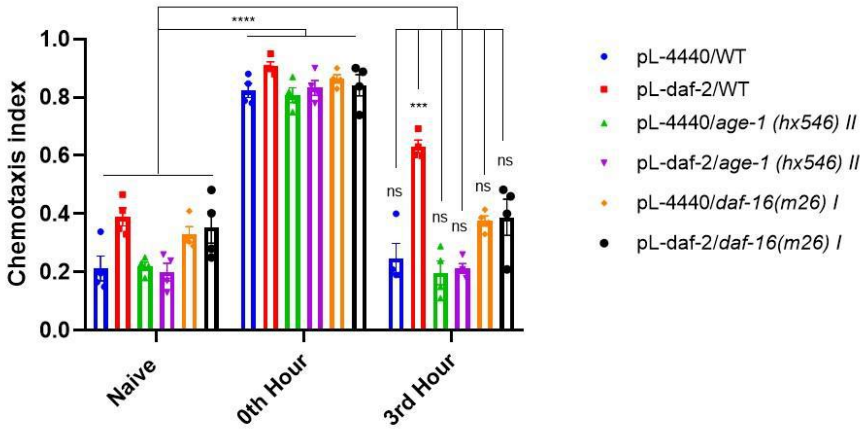

Figure 5. Short-Term Associated Memory of WT, TJ1052 [*age-1 (hx546) II*] and DR26 [*daf-16(m26) I*] mutants treated with siRNA of *daf-2*. Following STAM, worms treated with either of the control (pL4440) or test (pL-*daf-2*) vectors, the worms displayed normal learning ability and the CI values were significantly higher ($p \leq 0.0001$) than their untrained equivalents. At the 3rd hour, the CI values became comparable ($p > 0.05$) with their corresponding naïve value except in the WT worms treated with siRNA of daf-2. The worms on treatment with siRNA of *daf-2* showed significantly higher ($p \leq 0.001$) CI value when compared to its naïve. n=4 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, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

Translational machinery is Essential for both Short-Term and Long-Term Associated Memory

To understand if new protein synthesis is essential for establishing memory, WT and CB1370 *[daf-2(e1370) III]* worms were conditioned for STAM and LTAM in the presence of 0.8 mg/mL cycloheximide (Stein and Murphy, 2014), which is a known translation blocker. Cycloheximide exerts its effects by interfering with the translocation step in protein synthesis, thus blocking eukaryotic translational elongation (Schneider-Poetsch *et al.*, 2010).

The learning and memory of both the WT and CB1370 [*daf-2(e1370) III]* worms showed a marked decrease ($p \leq 0.0001$, $n \geq 4$) in the chemotaxis after training for STAM in the presence of cycloheximide (Figure 6a and c). This establishes the fact that new protein synthesis is required for consolidating STAM. Moreover, the extended memory of CB1370 [*daf-2(e1370) III]* was also lost when the translational machinery was hindered (Figure 6c). In the case of

LTAM, the WT was had impaired learning and memory when compared to the untreated control and had significantly lower CI values at 0th ($p \leq 0.01$, n=3) and 24th ($p \leq 0.001$, n=3) hours after the training (Figure 6b). Likewise, CB1370 [*daf-2(e1370) III],* when treated with cycloheximide showed a marked decrease in CI values at all time points ($p \leq 0.0001$ at 0th hour and 24th hour, $p \leq 0.05$ at the 48th hour; n=5) (Figure 6d). These observations establish that despite the superior memory performance in CB1370 [*daf-2(e1370) III]* worms compared to the WT worms, the translation process remained a vital factor in the establishment of STAM and LTAM. This is consistent with the previous notion that new protein synthesis is involved in memory formation across species (Hernandez and Abel, 2008).

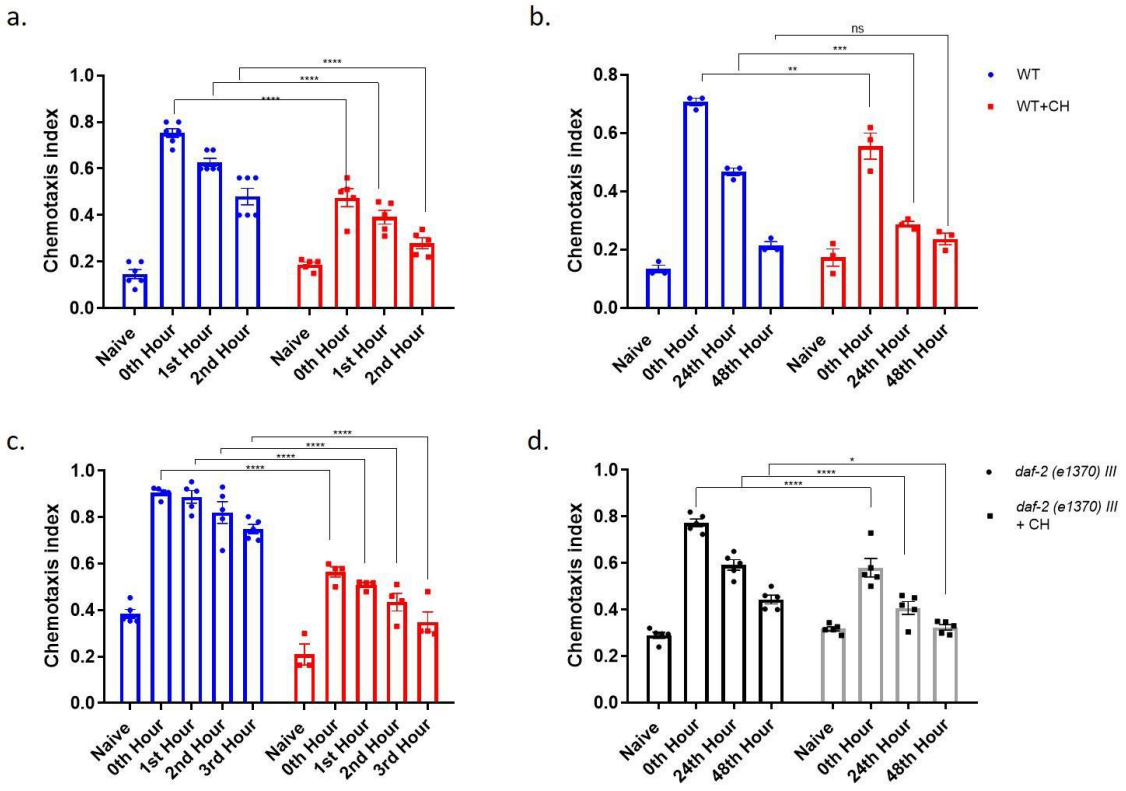

Figure 6. Wild-type and CB1370 [*daf-2(e1370) III]* worms Conditioned with cycloheximide during Short- and Long-Term Associated Memory Training. **a**. WT conditioned in the presence of cycloheximide during STAM training. The CI values following the treatment were significantly lower ($p \leq 0.0001$) than that of the untreated controls at all time-points. **b**. WT conditioned in the presence of cycloheximide during LTAM training. 0th hour learning of the worms treated with cycloheximide displayed a marked reduction ($p \leq 0.01$) in the CI value. CI at 24th hour also exhibited lower values $p \leq 0.001$) compared to that of the untreated control. **c.** CB1370 [*daf-2(e1370) III]* worms treated with cycloheximide during the conditioning phase of STAM. Worms treated with cycloheximide show a significant difference ($p \leq 0.0001$) from that of the untreated control. **d**. CB1370 [*daf-2(e1370) III]* worms treated with

cycloheximide during the conditioning phase of LTAM. The CI values corresponding to 0^{th} and 24^{th} hour CI values were comparatively lower than the untreated controls. The CI at the 48^{th} hour was also significantly reduced ($p \leq 0.05$) in worms conditioned in the presence of cycloheximide. CH- cycloheximide. $n \geq 3$ 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, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.

Transcriptional machinery is essential for Long-Term but not Short-Term Associated Memory

After establishing that *de novo* protein synthesis is pivotal for short- and long-term memories in WT and CB1370 [*daf-2(e1370) III]*, investigating whether the existing mRNA pool was sufficient for the process was considered essential for the study. To deduce this, actinomycin D was used as the chemical blocker of transcription. The mechanism of action of actinomycin D is such that it binds to a premelted DNA conformation existing within the transcriptional complex (Perry and Kelley, 1970). The binding subsequently immobilises the complex, interfering with the elongation of growing RNA chains, thus blocking transcription.

When worms were conditioned for STAM in the presence of 200 µg/mL actinomycin D (Stein and Murphy, 2014), no distinct difference ($p > 0.05$, $n \geq 3$) in the learning and memory was observed in the case of either the WT (Figure 7a) or CB1370 [*daf-2(e1370) III*] worms (Figure 7c). On the contrary, LTAM in both the WT and CB1370 [*daf-2(e1370) III*] worms was severely affected following actinomycin treatment. The CI of WT worms after conditioning with actinomycin D was significantly lower at both the 0^{th} ($p \leq 0.01$, $n \geq 3$) and 24^{th} ($p \leq 0.001$, $n \geq 3$) hour time points (Figure 7b). Similarly, the CB1370 [*daf-2(e1370) III*] worms showed a significantly lower CI value after conditioning for LTAM in the presence of actinomycin D at 0^{th} ($p \leq 0.0001$, $n = 5$) and 24^{th} ($p \leq 0.001$, $n = 5$) hour time points (Figure 7d). These data substantiate that transcription is not required for STAM but is vital for LTAM. The results also indicate that the *daf-2* mutation has no transcription-associated advantage over the WT worms in the learning and memory process. Also, the results substantiate the memory impairment observed in the mutants of the transcription factor, DAF-16.

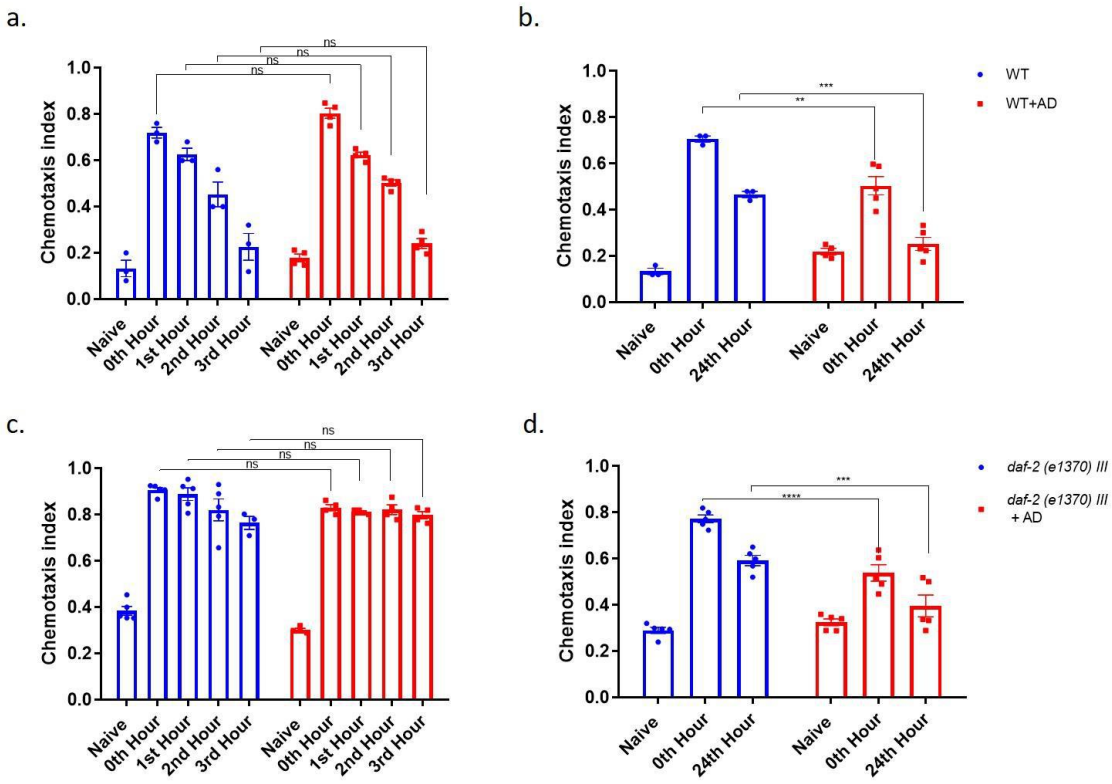

Figure 7. Wild-type and CB1370 [*daf-2(e1370) III]* worms Conditioned with Actinomycin D during Short- and Long-Term Associated Memory Training. **a**. WT conditioned in the presence of actinomycin D during STAM training. The CI values following the treatment were not significantly different ($p>0.05$) from that of the untreated controls at any time points. **b.** WT conditioned in the presence of actinomycin D during LTAM training. 0^{th} hour learning of the worms treated with actinomycin D displayed marked reduction ($p\leq0.01$) in the CI value. CI at 24^{th} hour also exhibited lower values $p\leq0.001$) compared to that of the untreated control. **c**. CB1370 [*daf-2(e1370) III]* worms treated with actinomycin D during the conditioning phase of STAM. Actinomycin D did not alter ($p>0.05$) the CI values of the worms after training for STAM compared to that of the untreated controls at any time points. **d**. CB1370 [*daf-2(e1370) III]* worms treated with actinomycin D during the conditioning phase of LTAM. The CI values corresponding to 0^{th} and 24^{th} hour CI values were comparatively lower (0^{th} hour- $p\leq0.0001$ and 24^{th} hour- $p\leq0.001$) than that of the untreated controls. AD- actinomycin D. 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, **$p\leq0.01$, ***$p\leq0.001$, and ****$p\leq0.0001$.

Functional DAF-2 is essential during the Training for Normal Short-Term Associative Appetitive Memory

CB1370 [*daf-2(e1370) III*] worms are conditional mutants in which the *daf-2* remains functional at lower temperatures (15 ^{0}C) and becomes defective at higher temperatures (≈23 ^{0}C) (Lin *et*

al., 2010). This conditional mutation was exploited to elucidate the role of *daf-2* during training and testing periods. The WT and CB1370 [*daf-2(e1370) III*] worms were kept at different combinations (15 ^{0}C and 23 ^{0}C) of temperatures during training and testing sessions (Figure 8a). In all the combinations, the CI value at the 0th hour post-STAM training remained significantly higher ($p \leq 0.0001$, n=5) when compared to the untrained set of worms (Figure 8b). The learning ability of the worms remained unaltered in the presence and or absence of the *daf-2* mutation. This ascertained that *daf-2* does not affect the learning pathway in these worms.

Similarly, the 3rd hour CI values were analysed after STAM training and testing the worms at both temperatures for the extended memory formation. At the 3rd hour, in the standard STAM experiments, the memory is lost in WT worms and CB1370 [*daf-2(e1370) III*], on the other hand, show an extended memory (Figure 8c). When the worms were trained at 15 ^{0}C and tested at either 15 ^{0}C or 23 ^{0}C, the memory was lost ($p>0.05$, n=6) by the 3rd hour in WT worms (Figure 8c). The abnormal extended memory exhibited by CB1370 [*daf-2(e1370) III*] was also suppressed ($p>0.05$, n=6) in these cases (Figure 8c). Interestingly, the extended memory remained intact in CB1370 [daf-2(e1370) *III*] worms when trained at 23 ^{0}C and tested at either 15 ^{0}C ($p \leq 0.0001$, n=6) or 23 ^{0}C ($p \leq 0.05$, n=6) (Figure 8c). This evidences that functional DAF-2 is essential during the training for normal forgetting pathway as observed in WT worms.

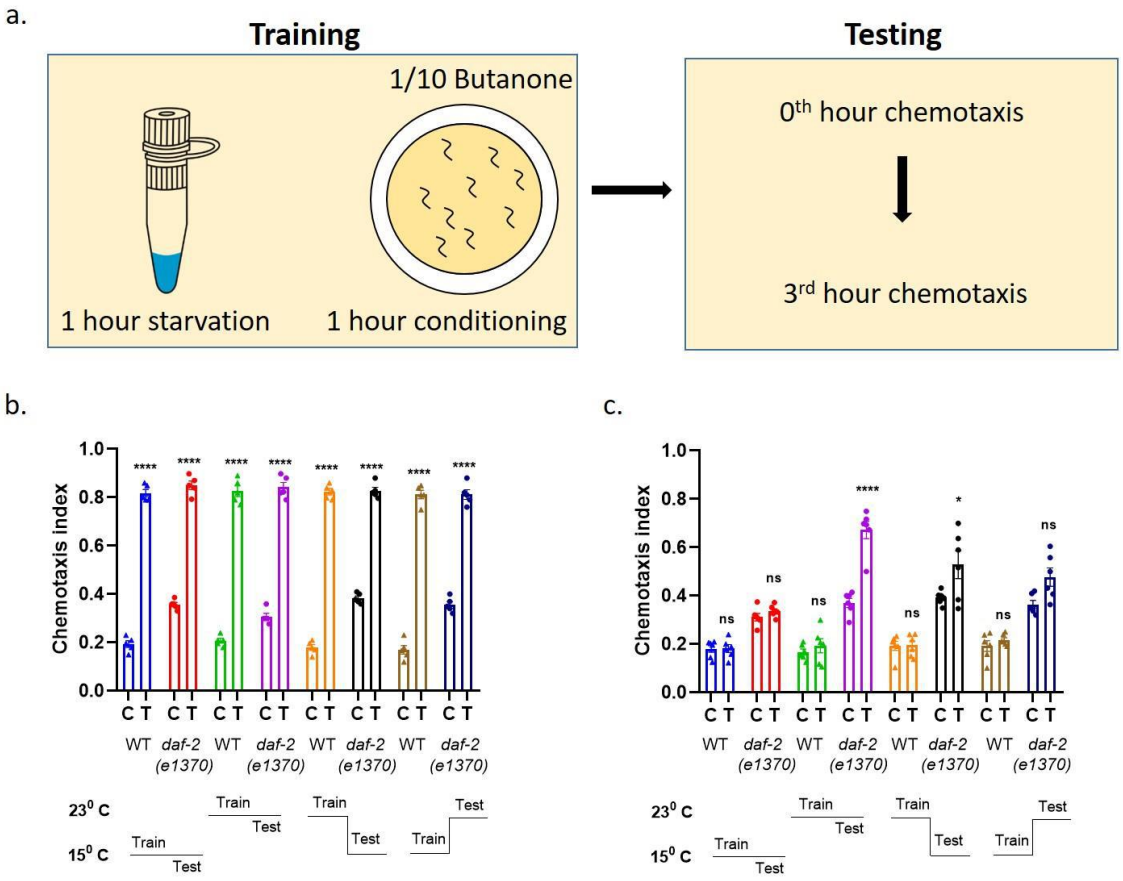

Figure.8. *daf-2* mutated at different combinations of training and testing periods in appetitive short-term associative memory paradigm. **a**. Graphical representation of the experiment. The "Training" phase of the experiment involved starvation and conditioning (with butanone in the presence of food, OP50) and the "Testing" phase included the chemotaxis. The worms would be placed at either 15 ^{0}C or 23 ^{0}C during these different phases and the 0th and 3rd hour CI would be analysed and compared with the naïve worms. *daf-2* would remain functional in the CB1370 [*daf-2(e1370) III*] worms when placed at 15 ^{0}C but would be mutated at 23 ^{0}C. **b**. The learning was independent of daf-2 mutations in the CB1370 [*daf-2(e1370) III*] worms. In all the training and testing conditions, the 0th hour CI value remained significantly ($p \leq 0.0001$) higher than the untrained naïve. **c**. The CB1370 [*daf-2(e1370) III*] worms trained at 15 ^{0}C and tested at either 15 ^{0}C or 23 ^{0}C displayed no significant difference ($p > 0.05$) from the naïve controls. The CB1370 [*daf-2(e1370) III*] worms showed significantly higher CI values when trained at 23 ^{0}C and tested at either 15 ^{0}C ($p \leq 0.0001$) or 23 ^{0}C ($p \leq 0.05$). C- Untrained control worms, T- Trained worms at the 0th (Figure 8b) and 3rd (Figure 8c) hours. n≥5 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, *$p \leq 0.05$ and ****$p \leq 0.0001$

Functional DAF-2 is essential during the Memory Retrieval for Starvation-associated Short-Term Olfactory Memory

CB1370 [*daf-2(e1370) III*] mutants demonstrate a substantial defect in benzaldehyde–starvation associative plasticity at 23°C (when the DAF-2 is dysfunctional). It has also been shown that DAF-2 is essential during memory retrieval in benzaldehyde-associated starvation memory (Lin *et al.*, 2010). Since it contrasts with the results obtained in appetitive training paradigm (Figure 8) which is used in this study, another odorant, isoamyl alcohol, was used to create a negative Short-Term Associative Olfactory Memory (Figure 9a).

WT showed a marked decrease ($p \leq 0.0001$, n=3) in CI value when the odor was associated with the starvation condition. *daf-2(e1370)* mutants were defective in associating the odor isoamyl alcohol with starvation. This was observed in cases when CB1370 [*daf-2(e1370) III*] worms are trained at either 15 ^{0}C or 23 ^{0}C and tested at 23 ^{0}C ($p>0.05$, n=3) (Figure 9b). In other instances, when these worms were trained at 15 ^{0}C or 23 ^{0}C and tested at 15 ^{0}C, the worms behaved as normal WT, exhibiting normal starvation associated plasticity (Figure 9b). Albeit the use of another odorant (1/300 isoamyl alcohol) these results are consistent with the observations made by Lin *et al*., 2010 who had used benzaldehyde in their assays. Thus, DAF-2 is essential during memory retrieval in starvation associated memory paradigm. This, together with the results obtained in similar experiments in the appetitive paradigm, suggests that starvation and appetitive memory functions in distinct pathways.

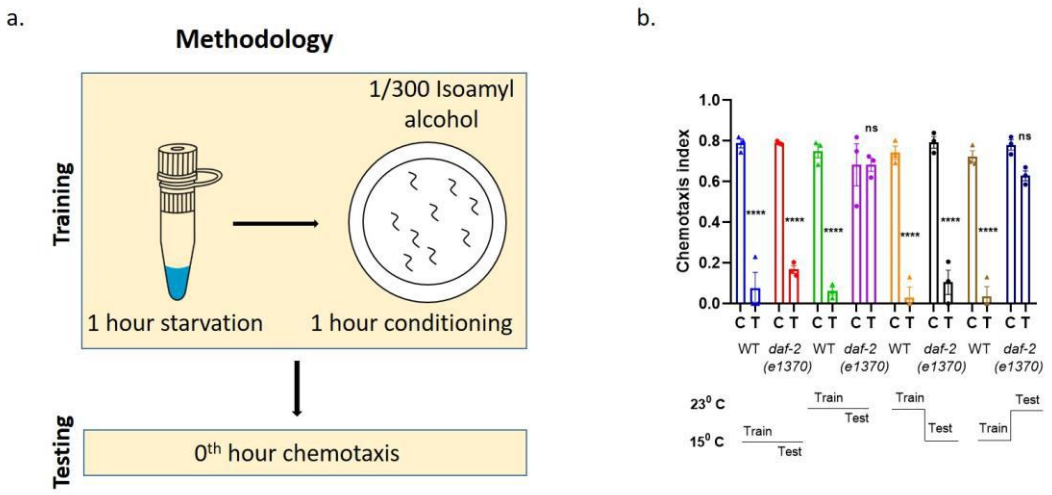

Figure 9. *daf-2* mutated at different combinations of training and testing periods in negative olfactory short-term associative memory paradigm. **a**. Graphical representation of the experiment. The "Training" phase of the experiment involved starvation and conditioning with 1/300 isoamyl alcohol, and the "Testing" phase included the chemotaxis. The worms would be placed at either 15 ^{0}C or 23 ^{0}C during these different phases and the 0th hour CI would be analysed and compared with the naïve worms. *daf-2* would remain functional in the CB1370 [*daf-2(e1370) III*] worms when placed at 15 ^{0}C but would be mutated at 23 ^{0}C. **b**. The WT worms, when conditioned with 1/300 isoamyl alcohol, would show a reduction in chemotaxis following the association with starvation. This behaviour was consistent in all the combinations of temperatures during the Training and Testing phase. However, CB1370 [*daf-2(e1370) III*] worms showed this associative memory only when trained at either 15 ^{0}C or 23^{0}C and tested at 15 ^{0}C. The CB1370 [*daf-2(e1370) III*] worms when trained at either 15 ^{0}C or 23 ^{0}C and Tested at 23 ^{0}C. This indicates that DAF-2 is essential during memory retrieval in isoamyl alcohol-associated starvation. C- Untrained control worms, T- Trained worms at the 0th hour. 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 and ****p≤0.0001

Ins-1 mutants have Extended Short- and Long-Term Associative Memories

C. *elegans* have about 37 insulin-like peptides (ILPs) ranging from INS-1 to INS-37, of which INS-1 is the most closely related to insulin based on structural predictions and likely C-peptide cleavage sites typical of mammalian insulin (Pierce *et al.*, 2001). *Ins-1* mutations have been shown to cause significant deficits in the benzaldehyde-starvation associative plasticity (Lin *et al.*, 2010). However, its role in the appetitive memory paradigm is not known. As INS-1 is one of the ligands of the DAF-2 receptor, we employed IK581 [*ins-1(nj32) IV*] worms to

comprehend the role of INS-1 peptide in the short- and long-term associative appetitive memory in C. *elegans*.

The IK581 [*ins-1(nj32) IV*] worms showed an extended memory similar to the CB1370 [*daf-2(e1370) III*] worms in both STAM and LTAM. The CI value of naïve IK581 [*ins-1(nj32) IV*] was significantly higher ($p \leq 0.0001$, $n \geq 3$) than the WT worms, and showed substantially higher memory retention at all the time points tested (Figure 10a). The 0^{th} hour learning of IK581 [*ins-1(nj32) IV*] worms was not significantly ($p > 0.05$, $n \geq 3$) different from that of the WT worms. However, at every other time points observed, the CI values of IK581 [*ins-1(nj32) IV*] worms remained significantly higher (1^{st} hour- $p \leq 0.001$, 2^{nd}-6^{th} hours- $p \leq 0.0001$; $n \geq 3$) than the WT counterparts (Figure 10a). The CI value reduced only at the 6^{th} hour when there was no significant difference ($p > 0.05$, $n \geq 3$) between the naïve and trained IK581 [*ins-1(nj32) IV*] worms (Figure 10a). The STAM, therefore, lasted till the 5^{th} hour, unlike that of the WT worms (Figure 10a). IK581 [*ins-1(nj32) IV*] worms, when trained for LTAM, did not show any substantial difference ($p > 0.05$, $n \geq 3$) in CI values at 0^{th} and 24^{th} hour compared to the WT worms (Figure 10b). However, it displayed a marked difference ($p \leq 0.0001$, $n \geq 3$) in the 48^{th} hour CI value from the WT (Figure 10b). These results demonstrate that IK581 [*ins-1(nj32) IV*] worms, like CB1370 [*daf-2(e1370) III*] worms, display an extended butanone-associated appetitive memory. This could be the result of downregulated insulin pathway in these worms.

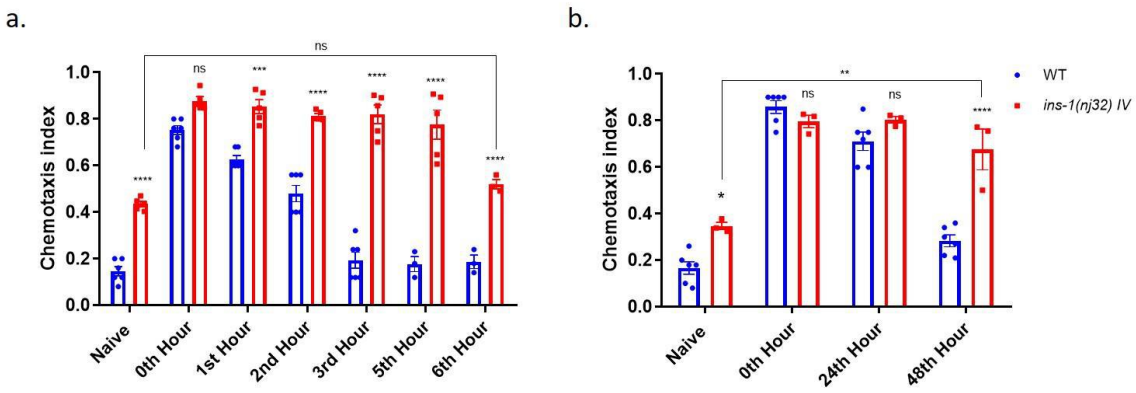

Figure 10. Short- (a) and Long-Term (b) Associated Memory in IK581 [*ins-1(nj32) IV*] worms. a. After STAM training, the 0th hour learning of IK581 [*ins-1(nj32) IV*] worms were insignificant ($p>0.05$) compared to that of the WT worms. The CI values of IK581 [*ins-1(nj32) IV*] naïve worms ($p\leq0.0001$) and after 1st ($p\leq0.001$), 2nd ($p\leq0.0001$), 3rd ($p\leq0.0001$), 5th ($p\leq0.0001$), and 6th hours ($p\leq0.0001$) post-training were maintained higher than that of the WT worms. The memory remained intact till the 5th hour after the training and reached naïve level ($p>0.05$) at the 6th hour. **b**. Although the 0th and 24th hour CI values of IK581 [*ins-1(nj32) IV*] worms displayed no marked difference ($p>0.05$) from that of the WT worms after LTAM training, at the 48th hour there was a significant difference ($p\leq0.0001$) in the CI values corresponding to the WT worms. The memory of IK581 [*ins-1(nj32) IV*] worms remained intact even at the 48th hour after LTAM training as the CI value corresponding to the 48th hour was significantly greater than the naïve worms. $n\geq3$ trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Tukey's multiple comparisons test; Data represented as the mean + S.E.M. Significance indicated: ns- non-significant, **$p\leq0.01$, ***$p\leq0.001$, and ****$p\leq0.0001$.

Excess Insulin Antagonises the Insulin Pathway

Since INS-1 is the most homologous to that of human insulin, the ability of exogenous insulin to compensate for the lack of functional *ins-1* gene in IK581 [*ins-1(nj32) IV*] was tested in the STAM paradigm. The IK581 [*ins-1(nj32) IV*] worms were conditioned with three different concentrations of huminsulin (0.1, 0.5, and 1 IU/mL). The CI was calculated for untrained naïve worms treated with insulin for 1 hour and worms conditioned in the presence of insulin during the STAM training (Figure 11a).

Treatment with insulin did not affect the CI of the naïve worms at any of the three concentrations used. The 0th hour learning was as observed even upon the administration of exogenous huminsulin and showed a significant increase ($p\leq0.0001$, $n\geq3$) in CI values from that of their respective naïve values (Figure 11a). The CI at the 0th hour also remained

unaltered compared to the untreated controls. As we have observed earlier, IK581 [*ins-1(nj32) IV*] worms showed an extended memory ($p \leq 0.0001$ compared to naïve, $n \geq 3$) at the 3rd hour. However, this extended memory at 3rd hour was abolished by the addition of 0.1 and 0.5 IU/mL insulin. However, the addition of 1 IU/mL of huminsulin brought back the extended memory as seen in the untreated control (Figure 11a). It was hypothesised that this could be the consequence of excess insulin in the system when treated with 1 IU/mL huminsulin. This excess insulin might have antagonised the DAF-2 receptor, resulting in *daf-2* mutant-like behaviour.

To test the hypothesis whether excess insulin downregulated the insulin pathway, WT worms were conditioned with 0.1, 0.5, and 1 IU/mL insulin in the STAM paradigm. The learning behaviour was not altered compared to the untreated controls, and the CI values remained significantly higher ($p \leq 0.0001$, $n \geq 3$) than their corresponding naïve values (Figure 11b). As was expected, the WT worms also exhibited extended memory in response to exogenous insulin at all the given concentrations. The CI at the 3rd after the training remained significantly higher than that of the respective naïve (Figure 11b). These results also substantiate our hypothesis that excess insulin cause antagonisation of the DAF-2 receptor.

Local search assay (Gray, Hill and Bargmann, 2005) was employed as another approach to confirm the effect of excess insulin on the insulin pathway. It was observed that CB1370 [*daf-2(e1370) III*] worms took significantly greater frequency ($p \leq 0.05$, $n=5$) of omega turns compared to WT worms (Figure 11c). Similarly, when WT worms with functional DAF-2 were treated with 1 IU/mL exogenous insulin, the worms took significantly greater number ($p>0.05$, $n=5$) of omega turns/ minute compared to the untreated control (Figure 11c). This suggest that the exogenous addition of huminsulin has hindered the function of the DAF-2 receptor. The addition of exogenous insulin did not have any effect ($p>0.05$, $n=5$) on the local search

behavior of CB1370 [*daf-2(e1370) III*] worms compared to the untreated control (Figure 11c). This was expected since these worms lack DAF-2 to receive the insulin signal.

To further ascertain the DAF-2 antagonistic hypothesis, the DAF-16 localisation pattern was observed following insulin treatment (Figure 11d). It is known that the insulin pathway retains the DAF-16 in the cytoplasm and the downregulation of insulin pathway causes the DAF-16 to translocate to the nucleus (Yen, Narasimhan and Tissenbaum, 2011).TJ356 [*daf-16p::daf-16a/b::GFP + rol-6 (su1006)*] worms were used to observe the localisation of DAF-16. These worms express GFP under the promoter of *daf-16*. Observing the GFP intensity in these worms would enable the visualisation of DAF-16 distribution in the worm. Under normal conditions, the GFP in these worms is uniformly distributed throughout the body. However, when insulin pathway is disrupted, it causes the localisation of DAF-16 into the nucleus which can be observed as small pockets of GFP in the worms. It was observed that exogenous insulin caused nuclear translocation of DAF-16 (Figure 11d). Therefore, it was concluded that excess insulin could antagonise the DAF-2 pathway resulting in nuclear translocation of DAF-16 and, thereby, causing extended memory retention in the worms.

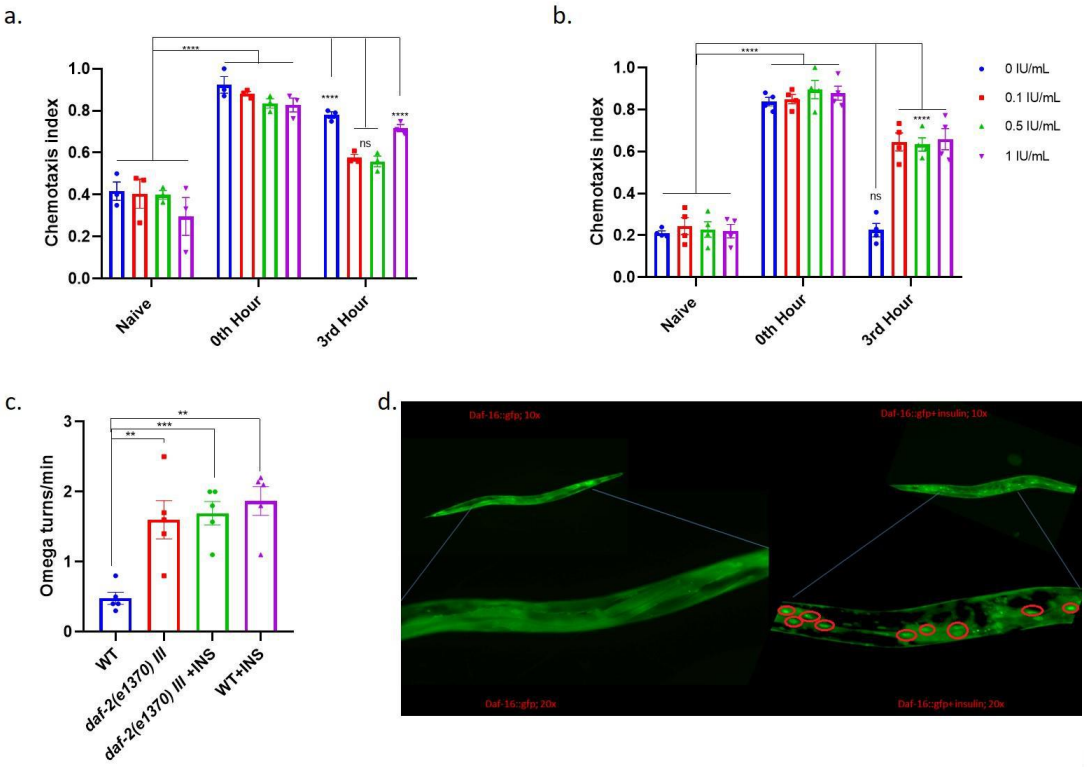

Figure 11. Effect of exogenous insulin on IK581 [*ins-1(nj32) IV*], CB1370 [*daf-2(e1370) III*], Wild-type and TJ356 [*daf-16p::daf-16a/b::GFP + rol-6 (su1006)]* worms. a. The extended STAM of IK581 [*ins-1(nj32) IV*] worms is lost when conditioned with lower concentrations of insulin and is regained back when conditioned with a higher concentration. The 0th hour CI value of IK581 [*ins-1(nj32) IV*] worms remained significantly higher ($p \leq 0.0001$) than the corresponding CI values of naïve worms after conditioning with 0.1, 0.5 and 1 IU/mL of huminsulin. At the 3rd hour, CI of IK581 [*ins-1(nj32) IV*] worms treated with 0.1 and 0.5 IU/mL of huminsulin reached their respective naïve values ($p > 0.05$). The extended memory of IK581 [*ins-1(nj32) IV*] worms was lost at the 3rd hour when the worms were conditioned with 0.1 and 0.5 IU/mL of huminsulin. The 3rd hour CI value of IK581 [*ins-1(nj32) IV*] worms showed a marked increase ($p \leq 0.0001$) compared to the corresponding naïve values when the worms were treated with 1 IU/mL of huminsulin. The extended memory was restored in IK581 [*ins-1(nj32) IV*] worms when conditioned with higher huminsulin concentration. **b**. Extended memory is observed in WT worms after STAM training when conditioned with different concentrations of exogenous insulin. CI value of the WT worms was significantly higher ($p \leq 0.0001$) than their naïve counterparts without and after treatment with 0.1, 0.5, and 1 IU/mL of huminsulin. At the 3rd hour following the STAM training, the CI value of the untreated worms showed no difference ($p > 0.05$) from its naïve control. On the other hand, the worms conditioned with exogenous huminsulin at 0.1, 0.5, and 1 IU/mL concentrations exhibited a significant difference ($p \leq 0.0001$) from their corresponding naïve values. **c**. Local search assay shows no effect of exogenous insulin on CB1370 [*daf-2(e1370) III*] worms but an altered behaviour in the WT worms. The number of omega turns/minute in the CB1370 [*daf-2(e1370) III*] worms is greater ($p \leq 0.01$) than the WT worms. As the DAF-2 receptor is mutated in these worms, exogenous insulin does not affect the local search behavior of these worms, and the frequency of omega turns remain higher ($p \leq 0.001$) compared to the WT worms. When the WT worms were treated with exogenous insulin, the worms took more number of omega turns ($p \leq 0.01$) when compared to its untreated control. **d**. TJ356 [*daf-16p::daf-16a/b::GFP + rol-6 (su1006)*] worms show nuclear translocation (marked with red arrows) of DAF-16 after treatment with exogenous insulin. DAF-16 is uniformly distributed in the body of TJ356 [*daf-*

16p::daf-16a/b::GFP + rol-6 (su1006)] worms. After treatment with huminsulin, DAF-16 is localised to the nucleus which is a hallmark of downregulated insulin pathway. INS- 1 IU/mL huminsulin. n≥3 trials for STAM experiments (each trial contains more than 50 worms), n=5 for the local search assay, and n≥15 for GFP localisation analysis; 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≤0.01, ***p≤0.001, and ****p≤0.0001.

Downregulated insulin signaling during the early developmental stage affects Short- and Long-Term Associative Memory

During the developmental cycle, L1 larvae of C. *elegans* can select one of two developmental pathways depending upon the environmental conditions. Under favourable conditions, they select the reproductive pathway and develop directly through the L2, L3 and L4 stages and reach the adult stage in 2-3 days. On the contrary, harsh environments trigger the larvae to enter diapause and developmental arrest. This is an alternative larval stage in C. *elegans* and is called the dauer larvae. The dauers resume their reproductive development when the environmental conditions become favorable (Cassada and Russell, 1975). The worms that have reached reproductive adulthood after passing through the dauer stage is termed as post-dauers (PDs). The insulin-like pathway is found to inhibit dauer arrest through activation of the DAF-2 insulin receptor homolog, AGE-1 phosphoinositide 3-kinase (PI3K), and the protein kinases PDK-1, AKT-1, and AKT-2 (Kimura et al., 1997; Morris et al., 1996; Paradis *et al.*, 1999; Paradis and Ruvkun, 1998). Dauer development is associated with a downregulated insulin pathway, and thus, PDs can be taken as a model having a developmental history of downregulated insulin pathway.

To understand how alterations in insulin signaling during the early developmental stages affect learning and memory, post-dauers of WT worms were trained for STAM and LTAM. WT worms grown to adulthood under normal reproductive growth conditions were taken as the control. PDs of WT, when trained for STAM showed no change in the 0^{th} hour learning compared to the worms with normal reproductive development (Figure 12a). Conversely, the memory retention at the later time points displayed a marked reduction from the respective control set

of worms ($p \leq 0.0001$, n=6) (Figure 12a). Interestingly, PDs trained for LTAM exhibited a sharp decline in learning and memory represented by a lower CI value than the control at 0th and 24th hours. ($p \leq 0.0001$, n=6) (Figure 12b). Therefore, in the case of STAM, PDs were found to have defective memory retention despite having normal learning, whereas they were defective in LTAM. These results demonstrate that alterations in the insulin signaling during the early developmental stages has detrimental effects on the learning and memory.

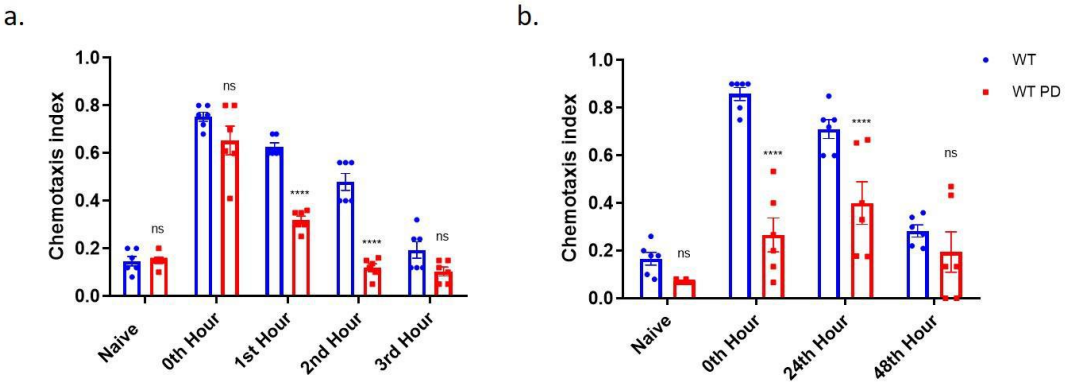

Figure 12. Short- (a) and Long-Term (b) Associative Memory of Wild-type Post-Dauers. **a**. The CI value of PDs were similar ($p>0.05$) to that of normal adults at the 0th hour. A significant difference ($p \leq 0.0001$) was observed at the 1st and 2nd hours of PDs compared to the normal adults. **b**. The CI values of PDs after LTAM training were significantly lower ($p \leq 0.0001$) than the normal adults at the 0th, 24th, and 48th hours. n=6 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 and ****$p \leq 0.0001$.

The Learning and Memory Defect in Post-dauers is not due to Dietary Restriction

Since the dauer formation involves a period of starvation, the defect in its learning and memory could be attributed to dietary restriction during the larval stages. To rule out this possibility, two dietary restriction models were used. The first model was a set of WT worms starved for 24 hours and subsequently tested for STAM and LTAM, and the second was a mutant DA1116 [*eat-2(ad1116) II*]. This mutant has reduced pharyngeal pumping and is a commonly used dietary restriction model (Kauffman *et al.*, 2010).

The starved WT worms were STAM-defective and showed significantly lower ($p \leq 0.0001$, $n \geq 3$) CI values at the 0th, 1st, and 2nd hours after training, whereas the DA1116 [*eat-2(ad1116) II*] worms showed a normal STAM (Figure 13a). Both these patterns of CI values differed significantly from the PDs, which had normal learning but impaired memory retention (Figure 13a). The starved WT and DA1116 [*eat-2(ad1116) II*] worms showed a normal 0th hour learning compared to the normal WT control following LTAM training, while the learning in PDs of the WT was impaired (Figure 13b). Nevertheless, the memory retention in these worms was also compromised and was reflected in their comparatively lower CI values of starved WT and DA1116 [*eat-2(ad1116) II*] ($p \leq 0.0001$ and $p \leq 0.001$ respectively; $n \geq 3$) at the 24th hour (Figure 13b). The differences in CI values in eat-2 and starved WT worms compared to that of PDs confirmed that the learning and memory deficits in the PDs were independent of dietary restriction.

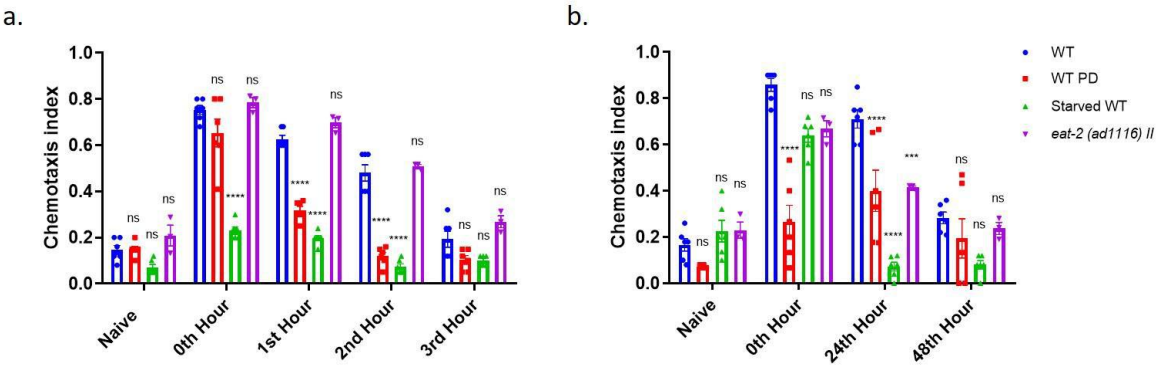

Figure 13. Short- (a) and Long-Term (b) Memories of starved Wild-type and DA1116 [*eat-2(ad1116) II*] worms. a. The CI values of starved worms were significantly lower ($p \leq 0.0001$) than the normal WT worms at all time points. DA1116 [*eat-2(ad1116) II*] worms showed normal STAM, and the CI values did not differ ($p > 0.05$) from that of the normal WT control worms. **b.** The 0th hour CI of both the starved WT and DA1116 [*eat-2(ad1116) II*] worms was not altered with respect to that of the normal WT worms. Significantly lower CI values were observed in the starved WT ($p \leq 0.0001$) and DA1116 [*eat-2(ad1116) II*] ($p \leq 0.001$) worms after LTAM training. $n \geq 3$ 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 ***$p \leq 0.001$,and ****$p \leq 0.0001$.

The Short-Term Memory Deficit of Post-Dauers are reversed by Exogenous Insulin

To ascertain whether the defect in PDs resulted from the downregulated insulin pathway, WT PDs were conditioned with 1 IU/mL huminsulin and tested for STAM and LTAM. Interestingly, the STAM retention capacity of the PDs was recovered back similar to normal WT ($p>0.5$, $n\geq4$) following insulin administration (Figure 14a). The short-term memory retention was, thus, able to be reversed by the exogenous addition of insulin. This indicates that the memory deficit in the STAM of PDs results from the altered insulin pathway in these worms. Nonetheless, the LTAM did not improve substantially in response to insulin administration. The CI values at 0^{th} and 24^{th} hour of the PDs treated with insulin were comparable with the untreated PDs. The CI values of both the PDs treated with and without insulin remained significantly lower ($p\leq0.0001$, $n\geq4$) compared to the normal WT (Figure 14b). The differences in the recovery of memory following insulin administration in STAM and LTAM could be the result of the fact that both these memory pathways differ from each other in many respects.

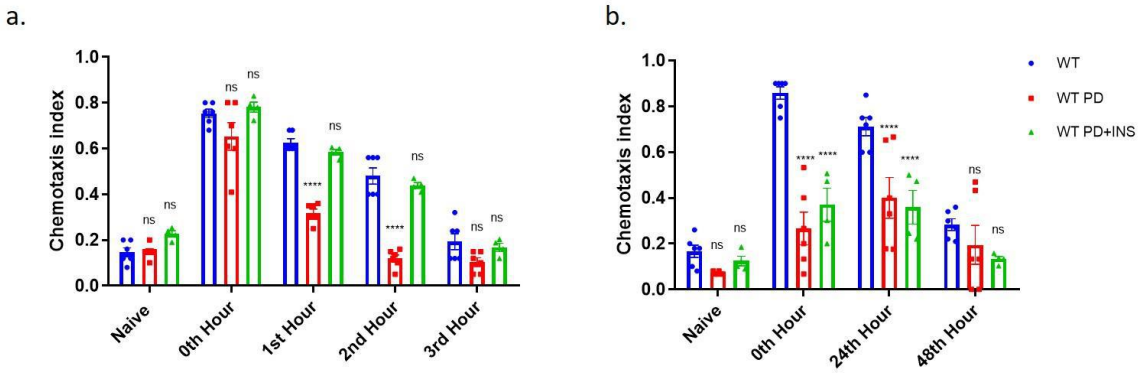

Figure 14. Memory deficits of post-dauers can be reduced to some extent by conditioning with insulin. a. PDs exhibit normal STAM if conditioned in the presence of exogenous insulin. When PDs were STAM conditioned in the presence of exogenous huminsulin, the CI values improved from that of their untreated control and showed no marked difference ($p>0.05$) from the normal WT adults. **b**. LTAM of PDs does not improve upon the addition of insulin during the conditioning. The CI values of the PDs remained significantly lower ($p\leq0.0001$) than the normal WT adults even after conditioning in the presence of exogenous huminsulin. INS- 1 IU/mL huminsulin. $n\geq4$ 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 and ****$p\leq0.0001$.

Discussion

Modifying animal behavior to adapt to various environmental cues is a vital skill necessary for their survival. Associative learning encompasses changes in behavior due to specific temporal contingencies between stimuli and a behavioral response. In contrast, non-associative learning involves altering a behavior due to the presentation of a single cue (Brown, 1998; Carew and Sahley, 1986). C. *elegans* can form both associative and non-associative memories (Ardiel and Rankin, 2010).

This present work involves short- and long-term associative learning paradigms in C. *elegans* in which food (conditional stimulus -CS) was associated with the odor, butanone (unconditional stimulus- US). The wild-type (WT) worms retain the memory of this positive appetitive association for 2 hours after short-term memory training. On the other hand, after long-term memory training, the memory can last for 24 hours. Our results also demonstrate that the AWC amphid neurons are essential for this association. This could result from the role of AWC neurons in recognising the US, butanone (Bargmann, Hartwieg and Horvitz, 1993).

Insulin and insulin-like pathways have been associated with various diseases affecting the memory (Zhao and Alkon, 2001b). Insulin insensitivity has been linked to memory deficits, cognitive decline, and many of the characteristic symptoms of Alzheimer's disease (AD) (Arvanitakis *et al.*, 2020). In Type 3 diabetes, insulin insensitivity in the brain and increased risk of developing AD have been observed (Caberlotto et al., 2019; Nguyen et al., 2020). This metabolic syndrome may cause abnormalities associated with progressive brain insulin resistance with subsequent impairment of central insulin signaling, accumulation of neurotoxins, neuronal stress, and neurodegeneration (Caberlotto et al., 2019; Nguyen et al., 2020). There are also reports on the effectiveness of intranasal insulin in delaying the

progression of Alzheimer's disease (Benedict *et al.*, 2007). Intranasal insulin was also found to improve the memory deficits associated with aging in rats (Maimaiti *et al.*, 2016).

We used various insulin pathway mutants and checked for the impact on learning and memory in *C. elegans*. While in the absence of the initial molecules of the pathway (the ligand, INS-1, and the receptor, DAF-2), the memory of the worms increased substantially, mutations in the downstream molecules affected memory differently. The *daf-2* mutants, first classified as long-lived mutants (Kimura *et al.*, 1997b), also exhibit extended memory in butanone-associated appetitive memory (Kauffman *et al.*, 2010). There is supporting evidence that these mutants can retain their memory better with age (Kauffman *et al.*, 2010). In benzaldehyde–starvation associative plasticity, DAF-2 has a significant role in memory retrieval than in the memory acquisition (Lin *et al.*, 2010). However, our results suggest that DAF-2 is essential during the memory acquisition pathway in butanone-associated positive olfactory learning. These results indicate that starvation and appetitive learning occur through independent molecular pathways.

Various studies have shown conflicting results on the effect of insulin on memory (Santucci et al., 1990; Kopf et al., 1998; Kopf and Baratti, 1996). It was observed that intraperitoneal injection of insulin immediately after fear conditioning (electric shock) in rodents resulted in impaired retention of memory (Kopf et al., 1998; Kopf and Baratti, 1996). This effect was reversed by AF-DX 11, a selective M2 muscarinic receptor antagonist, probably by enhancing acetylcholine and glutamate release (Kopf et al., 1998). These neurotransmitters play critical roles in memory formation (Atri et al., 2004; Green et al., 2005; Buccafusco et al., 2005; Riedel, 2003). Besides AF-DX 11, glucose administration also enhances memory retrieval (Kopf and Baratti, 1995). Their results show that hyperinsulinemic effects on memory could be reversed by raising plasma glucose levels. This also supports the notion that hypoglycemia induced by hyperinsulinemia contributed to the memory deficits in these studies. Conversely, studies

suggest that insulin facilitates memory (Park et al., 2000; Marks et al., 2009; Reger et al., 2006). The rats that received an intra-cerebroventricular injection of insulin after training (in a step-through passive-avoidance task) showed enhanced memory (Park *et al.*, 2000). Intranasal insulin administration improved the recall of two measures of verbal memory in memory-impaired APOE-epsilon4- adults (Reger *et al.*, 2006). Intranasal insulin treatment enhanced short- and long-term object memory recognition and increased odor recognition in mice (Marks *et al.*, 2009).

Our studies have shown that *ins-1* mutants lacking insulin peptides affected memory. INS-1 is the most homologous to that of human insulin in C. *elegans* (Pierce *et al.*, 2001). Studies involving insulin-like peptides suggest that they signal through DAF-2/IGFR (Matsunaga *et al.*, 2018). INS-1 could also regulate the activity of AWC sensory neurons (one of the critical neurons in the olfactory pathway) independent of the DAF-2/IGFR (Chalasani *et al.*, 2010). INS-1 is necessary for the benzaldehyde-starvation associative learning (Lin *et al.*, 2010). However, these mutants displayed an extended memory similar to the *daf-2* receptor mutants in the butanone-associated adaptive learning. We hypothesise that this could be due to the downregulation of the insulin pathway in the absence of the INS-1 ligand. The administration of exogenous insulin alleviated the extended memory. We observed that excess insulin also resulted in extended memory. Our results implicate that insulin has a concentration-dependent effect on memory retrieval pathways. Our results suggest that deviations from the normal physiological insulin level would lead to abnormal memory recall. The antagonisation of the insulin pathway by human insulin has been reported earlier in C. *elegans* (Haque *et al.*, 2020). Their results demonstrated that the administration of human insulin antagonises the DAF-2 receptor and decreases α-synuclein aggregation in worms with the human α-synuclein::YFP transgene.

Consistent with the earlier observations that new protein synthesis is involved in memory formation across species (Ghirardi et al., 1995; Hernandez and Abel, 2008; Jin et al., 2011; Parvez et al., 2005; Vukojevic et al., 2012), blocking translation by the administration of cycloheximide also lead to deficits in short- and long-term memories. *daf*-2 mutants, despite their extended memory, also required translational machinery for memory formation. It was also observed that blocking transcription using the chemical actinomycin D did not affect the short-term memory in the WT and *daf*-2 mutants. However, the long-term memory in these worms was negatively affected on treatment with actinomycin D. Since the *daf-2* mutants also exhibit similar deficits in response to actinomycin D, it can be assumed that the extended memory observed in these worms is not the result of any transcriptional advantage over the WT worms. The transcriptional requirement for the LTAM, but not STAM, agrees with observations made earlier in several studies (Kauffman et al., 2010; Stein and Murphy, 2014).

The effect of the transcriptional blocker was similar in the worms devoid of the DAF-16/FOXO transcription factor having the normal STAM and defective LTAM. Since short-term memory does not involve transcriptional machinery, *daf-16* mutants did not show any defect in STAM. However, *daf-16* mutants display a defective LTAM since transcription is essential for long-term memory. In humans, the FOXO transcription factor (FoxO6) promotes memory consolidation by regulating a program coordinating neuronal connectivity in the hippocampus (Salih *et al.*, 2012).

DAF-16 pathway has a critical role in the extended memory recall pathway. *daf-16* mutants do not have extended memory even in the background of *daf-2* mutation. This suggests that the extended memory is established by the set of genes controlled by the DAF-16. Genes regulated by DAF-16 include many longevity-associated genes like *daf-7*, *aco-1*, *din-1,* etc. (Li and Zhang, 2016). Some of these genes are implicated in various brain functions. For example, the gene *lgg-1*, an orthologue of the human GABARAP (GABA type A receptor-

associated protein) that is associated with GABAergic synaptic currents (Ye *et al.*, 2021), is under the transcriptional regulation of DAF-16 (Li and Zhang, 2016). The DAF-16-target genes associated with brain and cognition are listed in Table 1. Studies focussing on these genes might shed more light on the extended memory observed in *daf-2* mutants.

DAF-16 TARGET IN **C. *elegans***	**FUNCTIONS**	**REFERENCE**
lgg-1 (orthologue of GABARAP)	Associated with GABAergic synaptic currents	Ye et al., 2021
nnt-1 (nicotinamide nucleotide transhydrogenase)	Maintain brain mitochondrial redox balance (Mice)	Francisco et al., 2018
prdx-3 (peroxiredoxin-3)	Spatial memory performance (Rats); Spatial memory, plasticity (Mice); Regulation of fear memory (Mice)	Lubec et al., 2019 Phasuk et al., 2021a Phasuk et al., 2021b
aco-2 (aconitase-2)	Age-related memory impairment (Flies)	Cho et al., 2021
gpd-2 (glyceraldehyde-3-phosphate dehydrogenase)	Implicated in Alzheimer's disease (Humans)	Butterfield et al., 2010; Bertram et al., 2007
ubh-4 (orthologue of human ubiquitin C-terminal hydrolase L5)	Implicated in Parkinson's disease (Humans); Implicated in Alzheimer's disease (Humans); For normal synaptic and cognitive functions (Mice)	Wang et al., 2002 Öhrfelt et al., 2016 Gong et al., 2006
*pck-2 (*Orthologue of human phosphoenolpyruvate carboxykinase-2, mitochondrial)	Implicated in Alzheimer's disease (Humans)	Wang et al., 2020
sod-2 (orthologue of human superoxide dismutase-2)	Implicated in Alzheimer's disease (Humans); Prevents memory deficits in Alzheimer's disease (Mice)	Wiener et al., 2007 Massaad et al., 2009
srp-2 (human orthologue of SERPINI1)	Regulation of synapse density (Mice); Regulation of synaptic plasticity and learning (Mice)	Madani et al., 2003 Reumann et al., 2017

Table 1. DAF-16-associated genes involved in brain functions and pathologies. A list of genes under the control of DAF-16 transcription factor and its functions in brain functions and pathologies as deduced from various animal studies

This study also looked into the effect of downregulated insulin signaling during the early developmental stage on learning and memory. The post-dauers have a developmental history of downregulated insulin pathway, but the insulin pathway is restored when it recovers and becomes adults (Hung *et al.*, 2014). Post-dauers were found to have reduced short-term memory retention. These worms also were found to be defective in LTAM. The involvement of dietary restriction in this abnormal memory was ruled out.

Further, insulin administration in these worms during training recovered the memory deficit. This indicates that normal insulin signaling is essential during early developmental stages for the formation of cognitive memory. This has also been noted in children with diabetes onset before five years of age (Kail *et al.*, 2000). In these children, there is an increased risk of developing learning deficits and performing poorly in tasks of general intelligence during the later stages of their life. However, no such cognitive impairment is observed if the children develop diabetes between 5 to 10 years of age. These results demonstrate the role of the insulin pathway during the critical developmental stages in the maintenance of learning and memory.

Our results substantiate that normal insulin pathway and peptide levels are critical in learning and memory functions.

Chapter-2

Introduction

Memory helps the organism to make better choices based on past experiences (Bornstein *et al.*, 2017). This usually includes learning to integrate various cues with the changes in reward contingencies (Cuthbert, 2014). A "reward" is defined as any pleasant or positive experience, such as the availability of food (White, 2011). Thus, food is used as the unconditional stimulus in most associative learning paradigms (Martin-Soelch, Linthicum and Ernst, 2007). Dopamine is the principal biomolecule aiding the consolidation of reward cues (Yokel and Wise, 1975). It is already well-known that ingestion of a meal is associated with increased insulin levels (Strubbe and Steffens, 1975). Insulin is also often referred to as the prandial hormone for satiety (Vanderweele, 1994). Since the present study primarily involves appetitive olfactory training, the role of dopamine in the learning paradigm of worms with altered insulin signaling was studied. We hypothesised that insulin and dopamine could act as a reward signal in the worms. This also implicates that insulin and dopamine might function in a common pathway to regulate appetitive memory.

Insulin's action on the dopaminergic system is well-documented. Insulin increases the tyrosine hydroxylase mRNA levels in rats (Rusnák *et al.*, 1998). Insulin also amplifies the dopamine release in the brain via striatal cholinergic neurons expressing InsRs (Stouffer *et al.*, 2015). Conversely, dopamine also alters insulin signaling. For example, patients who have Parkinson's disease, which is characterised by dopaminergic neuronal death, show an augmented autoimmune reactivity to insulin (Wilhelm *et al.*, 2007). It has been reported that dopamine D-2-like receptors in the pancreatic islets regulate the release of insulin (Aslanoglou *et al.*, 2021). Besides acting as a reward signal, dopamine also modulates associative learning and memory processes in the frontostriatal systems in the brain (Puig, Antzoulatos and Miller, 2014). Thus, the possibility of insulin signaling altering the dopaminergic pathway to modulate

the learning and memory process cannot be overlooked. However, their relationship in the context of learning and memory is poorly understood. Hence, one of my objectives for the study is to evaluate the existence of a reciprocal relationship, if any, between insulin and dopamine in the learning and memory paradigm of C. *elegans*.

Insulin also affects various other neurotransmitters and neuropeptides (Blázquez *et al.*, 2014). Insulin inhibits the reuptake of norepinephrine in the neuronal cells (Boyd *et al.*, 1985). Contrariwise, it stimulates the reuptake of glutamic acid, aspartic acid, proline, and GABA from the synapses in a dose-dependent manner (Rhoads *et al.*, 1984). Insulin also promotes the delivery of NMDA receptors to the cell surface (Skeberdis *et al.*, 2001). Many of these neurotransmitters have been known to affect learning and memory. For example, the role of GABA (Gasbarri and Pompili, 2014) and NMDA receptors (Traynelis *et al.*, 2010) in memory consolidation is well-documented. Insulin signaling also affects cognitive memory (Zhao and Alkon, 2001a). Another study in C. *elegans* shows that INS-1 released from cholinergic AIA acts on glutamatergic AWC sensory neurons, and thereby, mediates benzaldehyde–starvation associative plasticity (Lin *et al.*, 2010). In C. *elegans*, *daf-2*/IGFR mutation is reported to be associated with enhanced memory retention (Kauffman *et al.*, 2010). Nevertheless, the effect of altered insulin signaling in specific subsets of neurons (i.e. glutamatergic, GABAergic, dopaminergic or cholinergic) on learning and memory has not been studied.

This study attempted to understand the possible relationship between insulin and dopaminergic systems using various mutants and transgenic worms expressing GCaMP in dopamine neurons. Furthermore, this study also aims to provide a list of possible neurons involved in the extended memory of insulin pathway mutants. RNAi targeted against *daf-2* in subsets neurons in C. *elegans* are analysed to understand the role of the insulin pathway in these neurons in the altered learning and memory.

Materials and Methods

Caenorhabditis elegans Strains

The following strains were used in this study: The WT strain N2 (Bristol)]; CB1112 [*cat-2(e1112)*]; MT15434 [*tph-1 (mg2820) II*]; Dat-1::ICE [*dat-1::GFP; dat-1::ICE*]; LX636 [*dop-1(vs101)]; LX703 [dop-3(vs106)*]; UA57 [*baIs4 (dat-1p::GFP + dat-1p::CAT-2)];* BZ555 [*egIs1 (dat-1p::GFP)];* DR26 *[daf-16(m26) I];* AT7437 [*dat-1p::GCaMP-6*]; AT7438 [*daf-2; dat-1p::GCaMP-6*]; ZM9078 [*hpIs587 (flp-14p::GCaMP6::wCherry + lin-15(+))]*;XE1474 [(*lin-15B(n744) X; eri-1(mg366) IV; rde-1(ne219) V; wpSi6[Pdat-1::rde-1:SL2:sid-1, Cbunc-119(+)] II*]; XE1582 [(*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219)* V; *wpSi11(eat-4p::rde-1:SL2:sid-1, Cbunc-119(+)* II)]; XE1375 [(*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219*) V; *wpSi1(unc-47p::rde-1:SL2:sid-1, Cbunc-119(+)* II; *wpIs36(unc-47p::mCherry)* I]; XE1581 [(*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219)* V; *wpSi10[unc-17p::rde-1:SL2:sid-1, Cbunc-119(+)* II)]. The strain CB1112 [*cat-2(e1112)II*] was a gift from Dr. Gert Jansen; Dat-1::ICE [*dat-1::GFP; dat-1::ICE*] was a gift from Dr. Andres Villu Marique, University of UTAH; UA57 [*baIs4 [dat-1p::GFP + dat-1p::CAT-2*] was gifted by Dr. Cladwell . AT7437 [*dat-1p::GCaMP-6*] was a gifted by Dr. Vishnu Raj, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. AT7438 [*daf-2; dat-1p::GCaMP-6*] was created using microinjection. All strains, unless otherwise mentioned, were provided by the Caenorhabditis Genetic Centre (CGC, Minnesota, St. Paul).

C. elegans maintenance

The C. *elegans* strains were reared in petri dishes with Nematode Growth Medium (NGM) and OP50, an auxotroph of *Escherichia coli* (E. *coli*), as a food source. The strain AT7438 [*daf-2; dat-1p::GCaMP*-6] was grown at 15 ^{0}C. All other strains were maintained at 20 ^{0}C (Brenner, 1974).

Synchronisation of C. elegans

Age-synchronised worms were obtained by hypochlorite treatment (for details, refer Materials and Methods section of Chapter 1). Day 1 adult worms were recovered from the plate after 48 hours.

Short-Term and Long-Term Adaptive Memory Training

Worms were trained for STAM or LTAM and chemotaxis assay was performed (for details, refer Materials and Methods of Chapter 1).

Fluorescence intensity analysis

Worms were placed on a glass slide containing 7 µL of 25 mM sodium azide solution. The final volume was made up to 15 µL using M9 buffer. The movement of the worms was monitored periodically under a stereomicroscope. When the worms were paralysed, they were covered with a cover glass with petroleum jelly applied along the edges to avoid dessication. The images were taken using Olympus IX51 equipped with Rolera-XR CCD camera (Q Imaging) with the image acquisition software NIS Elements-Advanced Research (NIKON) (λex/λem 460-490/520). Image analysis was done using the open software Fiji (Raj et al, 2021). CEP and ADE neurons were the region of interest while measuring the DAT-1 intensity. Background correction was done by subtracting the average of five readings from different areas of the background from the actual intensity obtained at the region of interest. The final values were then plotted in the graph.

Transgenic strains

To study dopaminergic neuronal activity, a strain that carries GCaMP-6 under the control of *dat-1* promoter, which ensures the expression specifically on dopaminergic neurons was created using microinjection. The plasmid *dat-1p::GCaMP-6* (*dat-1p::GCaMP-6::mCherry*) having Dat-1 promoter in frame with GCaMP-6 ensures its efficient expression in dopaminergic neurons. This plasmid (Sequence and details attached in Annexure) was a gift from Dr. Vishnu Raj at Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. Transgenic strains were generated using the microinjection method (Mello *et al.*, 1991). The transgenic lines having GCaMP-6 under Dat-1 promoter sequence in wild- type and daf-2 backgrounds were created by injecting plasmid concentration of 70ng/µL of *dat-1p::GCaMP-6* to the distal arm of the gonads of respective young adult worms. After successful injection, the surviving worms were maintained on a food plate at 15 ^{0}C (Mello *et al.*, 1991). The genes pass on to the next generation extrachromosomally. The F2 generation carrying RFP and GFP signals on dopaminergic neurons were selected by viewing under a fluorescence microscope and propagated further. The strain under the wild type background was named as AT7437 [*dat-1p::GCaMP-6::mCherry*] and the one under the *daf-2* mutant background was named AT7438 [*daf-2*; *dat-1p::GCaMP-6::mCherry*].

Calcium imaging

A drop of 5% sodium alginate was placed on top of a glass slide and spread into a thin layer. The worms were placed on the thin film of alginate and 100 µL of $CaCl_2$ was added on top of it to immobilise the worms on the sodium alginate. The calcium alginate resulting thereof is a water-insoluble, gelatinous, cream-coloured substance that entraps the worm in its matrix, allowing minimal movement. This, however, does not interfere with the imaging under high magnification. The olfactory stimulus butanone was introduced to the worm employing a small sheet of Whatman® filter paper like a flag. Microinjection set-up was used to bring the flag containing 10 µL of 10% butanone near the worm. The flag was subsequently retracted after

10 seconds. Time-lapse images were taken at an interval of 0.5 seconds (2 frames/ second) using a Leica Microscope Model DMi8 (Leica Microsystems. Wetzlar & Mannheim, Germany) and objective lens 20X/0.40 (∞/O/C N PLAN). The GFP intensity of the dopaminergic neurons was then analysed using the open software Fiji. The average intensity of 20 frames before the exposure to the solvent were then subtracted from the original value. This value was designated as ΔF. The value ΔF/F was plotted in the graph where F is the original intensity observed in each frame.

siRNA mediated silencing

siRNA-mediated silencing of *daf*-2 gene was performed (details given in the Materials and Methods of Chapter 1). L1 larvae were plated onto the RNAi plates seeded with either the empty vector, pL4440 or the dsRNA-expressing HT115 bacteria. Day 1 adults were then used for the siRNA studies. In the case of memory experiments, OP50 was substituted with either pL4440 or the dsRNA-expressing HT115 during the conditioning and in the hold plates. (See Annexure for more details)

Avoidance assay

The assay was carried out as previously described by Hilliard, Bargmann and Bazzicalupo, 2002. 0.5 M glycerol (prepared in distilled water) was used as the repellent. A small drop of the repellent was placed near the tail of the worms using an insulin syringe. To have considerably smaller drops of glycerol for the assay, the needle of the syringe was finely polished with a piece of sandpaper. An acclimatisation time of 20 minutes was given to the worms in each of the plates used before the assay. The assay was performed at 20 ^{0}C. As the drop touch the tail of the worms, the liquid reaches the amphid sensory regions of the head through capillary action. The worm recognises the stimuli as been given from the anterior region and takes a reversal. A complete omega turn was taken as a positive response. The

results were scored as positive (worms taking omega turns) and negative (worms that do not take omega turns) and plotted as avoidance index. Avoidance index was calculated using the formula:

$$Avoidance\ Index = \frac{Number\ of\ worms\ taking\ omega\ turns}{Total\ number\ of\ worms}$$

The assay was performed on NGM plates, OP50 plates, insulin plates, and Dopamine plates.

Chemical treatments

Insulin treatment: Huminsulin was used at 1 IU/mL concentrations during the conditioning phase of STAM experiments.

DA treatment: 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 minimise oxidation of DA. 10 mM DA was used in the STAM experiments during starvation and conditioning. During the drug treatment, care was taken to limit the exposure to light. In the avoidance assays, to make DA-treated plates for worm incubation, 200 µl 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. The solution was 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 thrice with M9 buffer, and then added to the plates for the experimental condition.

Serotonin treatment: 15 mM serotonin was used in the STAM experiments during starvation and conditioning.

Microscopy

A stereo microscope (Magnus Analytics, India) with 10X zooming was used for viewing and general handling like picking, transferring, and washing of C. elegans strains. For fluorescence imaging, Olympus IX51 inverted microscope, (Olympus Imaging, Center Valley, PA, USA) objective lens 40X/0.60 Ph2/∞/0-2/FN22, which works with image acquisition software NIS Elements-Advanced Research (NIKON) and Rolera XR monochrome camera (QImaging, Canada) was used. The kinetics of fluorescence changes in the calcium imaging experiments was analysed using Leica DMi8 fluorescence microscope.

Software

Graphpad Prism version 6 (GraphPad Software Inc.) was used for graphical representation and statistical analysis of the data. Image analysis was carried out using Fiji (an open-source image processing package based on ImageJ).

Statistical Analysis

To determine the performance of three or more groups as in case of short-term adaptive memory formation, we conducted two-way ANOVA with Tukey's multiple comparison test to compare the means of CI values of different experimental groups and at different time points. The number of experiments (n) are represented as the number of trials, and each trial contains more than 50 animals unless otherwise mentioned. Significance is represented as follows *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Results

Insulin Acts as Reward Signal during Conditioning

In the usual STAM experiments mentioned earlier, OP50 was used as the US. Since insulin is a signal concomitant with food, the prospective of insulin to act as the reward signal in worms was tested. The WT worms were trained for STAM with Butanone as the CS and OP50, 1 IU/mL huminsulin, or 10 mM DA (Ezcurra *et al.*, 2011) as the US. DA was used as a control since DA is generally known as the reward signal in the biological system (Wise, 1998; Koob, 2001).

Interestingly, both DA and huminsulin were able to evoke STAM in the worms, similar to OP50. However, the CI values following the STAM training were lower ($p \leq 0.0001$, $n=6$) compared to the worms conditioned with OP50 at all time points (Figure 1). Though the association of Butanone with insulin and DA is weaker compared to that with the food, OP50, the worms were still able to learn the association and retain the memory. This demonstrates possible crosstalk between insulin and DA pathways in its potential to act as a reward signal. This was an interesting perspective on how the insulin pathway modulates learning and memory. The attention was then shifted to understand more about this underlying relationship between insulin and DA.

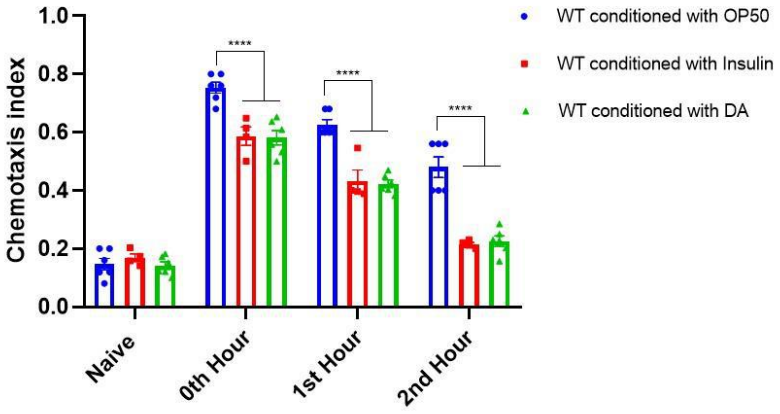

Figure 1. Short-term Memory of Wild-type worms conditioned with OP50, huminsulin and dopamine. WT worms were conditioned with Butanone and OP50 (standard STAM experiment), exogenous insulin (huminsulin), and DA. Worms conditioned with both huminsulin and DA were able to learn the association and retain its memory. However, the CI values were significantly lower ($p \leq 0.0001$) than the worms trained to associate OP50 and Butanone. N=6 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: ****$p \leq 0.0001$.

Exogenous Dopamine Rescues the Memory Defect in Post-Dauers

Based on the earlier observation that insulin can act as the reward signal like DA (Figure 1), the possibility of DA compensating for insulin in recovering the memory retention capacity of WT PDs was tested. PDs were conditioned with butanone and OP50 in the presence of 10mM DA (Ezcurra *et al.*, 2011) or 15 mM Serotonin (5-HT) (Chen, Seyedsayamdost and Ringstad, 2020) in the STAM paradigm. Like insulin, DA was able to ameliorate the memory defects of PDs. The 1st hour CI value of PDs, which was significantly lower ($p \leq 0.0001$, n=6) than the normal WT, became comparable to the normal WT when conditioned in the presence of DA. The same was observed in the 2nd hour also (Figure 2).

On the other hand, when the PDs were conditioned in the presence of 5-HT, the worms showed no improvement in learning or memory. On the contrary, these worms exhibited marked reduction ($p \leq 0.0001$, n=6) in CI values compared to the normal WT at the 0th, 1st, and

2nd hours after STAM training (Figure 2). This establishes that DA, and not 5-HT, can compensate for the developmental history of downregulated insulin pathway in the PDs in the context of learning and memory.

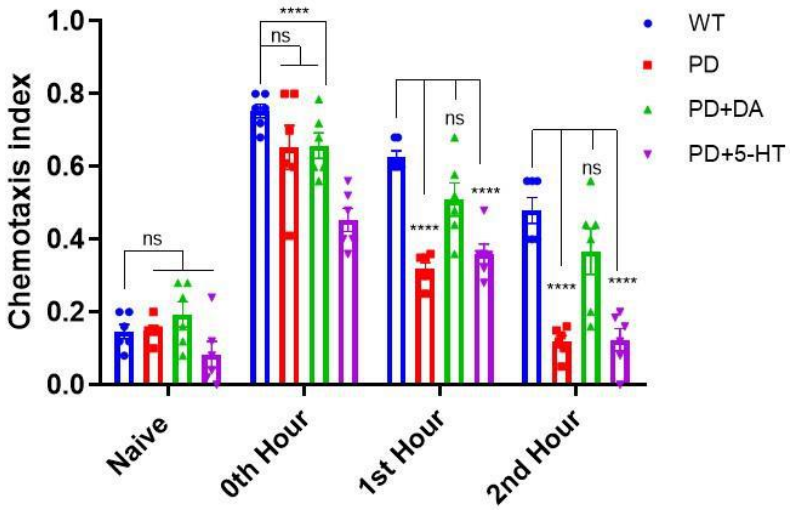

Figure 2. Short-term Memory of Post-dauers conditioned in the presence of Dopamine and Serotonin. PDs were conditioned in the presence of exogenous DA or 5-HT. Exogenous DA recovers PDs from the memory retention defect. The PDs conditioned in the presence of exogenous DA show no significant ($p>0.05$) difference from the normal WT worms. 5-HT, on the contrary, does not alleviate the memory deficits of PDs. The CI values of PDs trained in the presence of exogenous 5-HT are, in fact, significantly lower ($p\leq0.0001$) than the normal WT worms at all time points. n=6 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- not significant and ****$p\leq0.0001$.

Insulin-dependent Extended Memory of the Wild-Type N2 worms does not Require Serotonin

To further verify whether 5-HT was involved in the memory of the worms, the STAM of worms deficient in the Tryptophan hydroxylase gene (*tph-1*) was tested. TPH-1 catalyses the biopterin-dependent monooxygenation of tryptophan to 5-hydroxytryptophan, which is successively decarboxylated to form 5-HT. TPH-1 is the rate-limiting enzyme in the biosynthesis of 5-HT neurotransmitter.

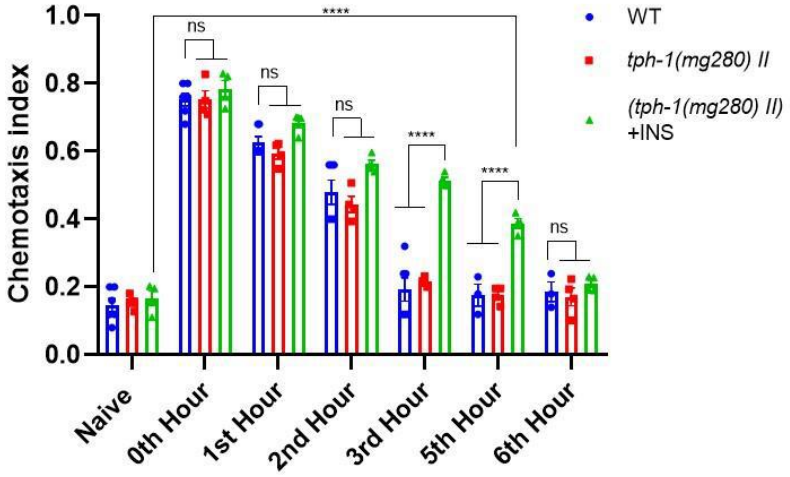

Figure 3. Short-term Memory of MT15434 *[tph-1(mg280) II]* worm conditioned in the Presence of exogenous Insulin. Serotonin mutant, MT15434 *[tph-1(mg280) II]* showed no marked difference in the CI values from the WT worms when trained for STAM. When conditioned in the presence of exogenous insulin, MT15434 *[tph-1(mg280) II]* worms displayed extended memory retention. The CI of these worms were significantly higher ($p \leq 0.0001$) than the untreated worms at the 3rd and 5th hours after training in the presence of huminsulin. The difference in the CI value became non-significant ($p > 0.05$) at the 6th hour. $n \geq 3$ 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- not significant and ****$p \leq 0.0001$.

The strain MT15434 *[tph-1(mg280) II]* was found to have a normal STAM compared to the WT worms. They showed no difference ($p > 0.05$, $n \geq 3$) in their CI values compared to the WT worms. They also did not show the atypical extended memory as CB1370 *[daf-2(e1370) II]* worms (Figure 3). To understand the role of 5-HT in the extended memory in response to excess insulin, MT15434 *[tph-1(mg280) II]* worms were conditioned in the presence of huminsulin, and the STAM was analysed. Surprisingly, the lack of TPH-1 did not affect the extended memory. The CI values at the 3rd and 5th hours following the STAM training in the presence of huminsulin were significantly higher ($p \leq 0.0001$, $n \geq 3$) than the untreated MT15434 *[tph-1(mg280) II]* worms (Figure 3). This corroborates that 5-HT has no role in the aberrant memory associated with altered insulin signaling.

Exogenous Insulin Fail to Improve the Memory of Worms Overexpressing Dopamine

To further understand the role of DA in the learning and memory pathways, UA57 *[baIs4 (dat-1p::gfp + dat-1p::cat-2)]*, were conditioned and tested for STAM. CAT-2 encodes for tyrosine 3-monooxygenase, the rate-limiting enzyme in the DA synthesis from Tyrosine. UA57 *[baIs4 (dat-1p::gfp + dat-1p::cat-2)]* worms overexpress CAT-2 under the promoter of *dat-1* which ensures expression specific to DAergic neurons. Thus, this strain has excess DA in the system.

These worms were defective in learning and memory in the STAM assay. Following the training, the UA57 *[baIs4 (dat-1p::gfp + dat-1p::cat-2)]* worms displayed a comparatively lower ($p \leq 0.01$, n=6) CI value than the WT worms at the 0^{th} hour. This defect was retained even at the 1^{st} ($p \leq 0.0001$, n=6) and 2^{nd} ($p \leq 0.0001$, n=6) hours after the training (Figure 4). This demonstrates that overexpression of DA, instead of being beneficial to learning and memory in the worms, is, in fact, detrimental to them.

To assess the effect of exogenous insulin in this system, UA57 *[baIs4 (dat-1p::gfp + dat-1p::cat-2)]* worms were conditioned in the presence of huminsulin. When conditioned with exogenous insulin, the learning defect of UA57 *[baIs4 (dat-1p::gfp + dat-1p::cat-2)]* worms, shown by the 0^{th} hour CI readings, were overcome. These worms displayed normal ($p > 0.05$, n=6) CI values similar to the WT worms. However, the exogenous insulin failed to recover the memory deficit shown at the 1^{st} and 2^{nd} hour post-training. The CI values remained significantly lower ($p \leq 0.0001$, n=6) than the WT worms at these time points (Figure 4). This substantiates that excess insulin cannot compensate for the aberrant DA signaling in the learning and memory model.

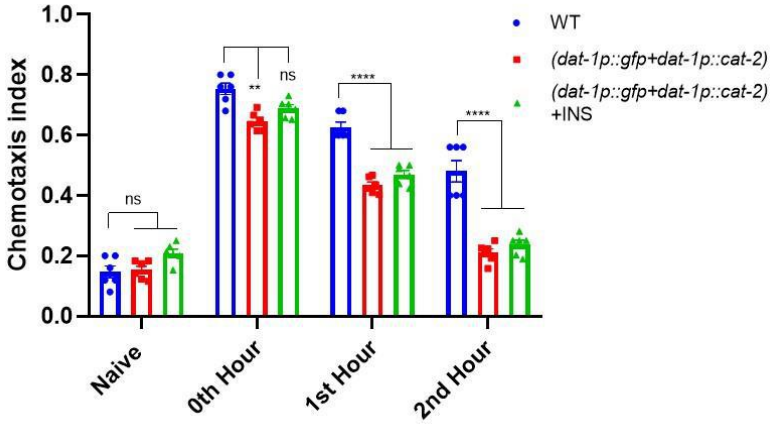

Figure.4. Short-term Memory of UA57 *[bals4 (dat-1p::gfp + dat-1p::cat-2)]* worms conditioned in the absence and presence of huminsulin. UA57 *[bals4 (dat-1p::gfp + dat-1p::cat-2)]* worms over-expressing DA neurotransmitter show learning and memory defect. The CI values were significantly lower than the WT worms at the 0th ($p \leq 0.01$), 1st ($p \leq 0.0001$), and 2nd ($p \leq 0.0001$) hours. On conditioning in the presence of exogenous insulin, the 0th hr CI became comparable ($p > 0.05$) to the WT, whereas the CI at the 1st and 2nd hours remained significantly lower ($p \leq 0.0001$). n=6 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- not significant, **$p \leq 0.01$, and ****$p \leq 0.0001$.

Exogenous Dopamine and Insulin Ameliorates the Learning Deficiency of worms devoid of Dopaminergic neuron but Fail to Improve the Memory Retention

To investigate the role of dopaminergic (DAergic) neurons in learning and memory, Dat-1::ICE [*dat-1p::GFP; dat-1p::ICE*] worms were used. C. *elegans* neurons that express human caspase interleukin-1β-converting enzyme (ICE) have been shown to undergo premature cell death (Zheng *et al.*, 1999). Dat-1::ICE [*dat-1p::GFP; dat-1p::ICE*] worms express ICE under the *dat-1* promoter which ensures its expression solely in the DAergic neurons. The DA neuronal degeneration in adult worms can be monitored by the change in GFP expression.

Dat-1::ICE [*dat-1p::GFP; dat-1p::ICE*] worms exhibit a marked reduction in learning and memory. The CI values at the 0th, 1st, and 2nd hours are significantly lower ($p \leq 0.0001$, n=6) than the WT worms (Figure 5a).This demonstrates that dopaminergic neurons are essential

in the learning and memory processes. Exogenous DA could not alter the memory remarkably. However, the 0th hour CI value after conditioning in the presence of DA exhibited a significant increase ($p \leq 0.0001$, n=6) than the untreated control. This DA-dependent increase was not observed at the time points that followed. The CI values at the 1st and 2nd hours did not alter much as compared ($p > 0.05$, n=6) to the untreated control (Figure 5b). This corroborates an earlier observation from our laboratory that DA neuronal connections are essential for the maintenance of memory.

To explore whether exogenous insulin could cause any effect on the learning and memory of dat-1::ICE [*dat-1p::GFP; dat-1p::ICE*] worms, huminsulin was presented during the conditioning phase of the STAM assay. Interestingly, exogenous insulin displayed the same effect as DA in these worms. Huminsulin was able to restore the learning defect to some extent. The 0th hour CI value following the insulin treatment was significantly higher ($p \leq 0.001$, n=6) than that of the untreated dat-1::ICE [*dat-1p::GFP; dat-1p::ICE*] worms. Nevertheless, the memory deficits were not restored upon the treatment with huminsulin. The CI value at the subsequent time points (1st and 2nd hours) remained unaltered ($p > 0.05$, n=6) compared to that of the untreated worms (Figure 5b). This validates that insulin and DA act alike in the learning and memory pathways which further validates our hypothesis that there might be substantial crosstalk between these molecules in these pathways.

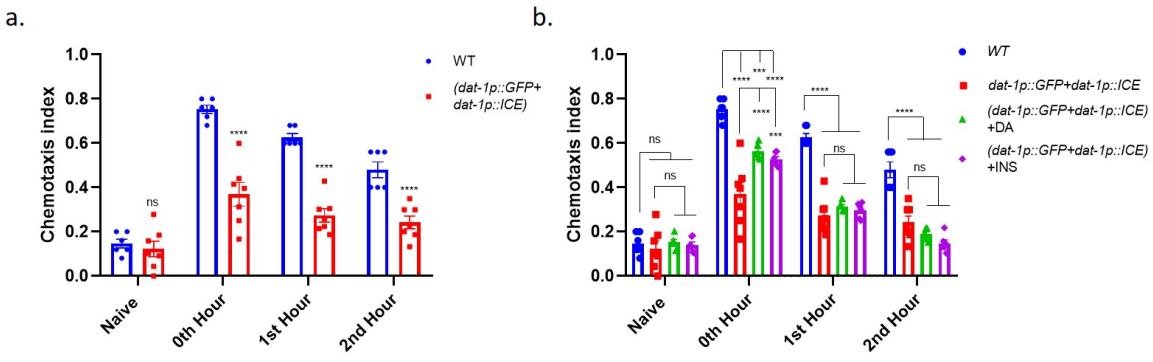

Figure 5. Short-term Memory of Dat-1::ICE [*dat-1p::GFP; dat-1p::ICE*] worms conditioned in the Absence and Presence of Exogenous DA and Insulin. a. Short-term memory of Dat-1::ICE [*dat-1p::GFP; dat-1p::ICE*] worms. These worms have impaired

learning and memory as substantiated by the significantly lower CI values than the WT worms at the 0^{th} ($p \leq 0.0001$), 1^{st} ($p \leq 0.0001$), and 2^{nd} ($p \leq 0.0001$) hours. **b**. Short-term memory of Dat-1::ICE [*dat-1p::GFP; dat-1p::ICE*] worms conditioned in the presence of exogenous DA and huminsulin. The worms exhibited a significant improvement in learning but not in memory retention after conditioning in the presence of exogenous DA or insulin. The CI values were significantly higher than the untreated worms at the 0^{th} hour when conditioned in the presence of DA ($p \leq 0.0001$) or huminsulin ($p \leq 0.001$). However, no marked change ($p > 0.05$) was observed at the 1^{st} and 2^{nd} hours following the treatment. n=6 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- not significant and ****$p \leq 0.0001$.

Exogenous Insulin Cannot Rescue Memory Deficit of dop-1 and dop-3 mutants

In C. *elegans*, DOP-1 and DOP-3 encode DA neurotransmitter receptors. Mutants of *dop-1* and *dop-3* were used to elucidate the role of these receptors in learning and memory. LX636 [*dop-1(vs101)*] worms with *dop-1* gene mutated showed learning and memory defects following STAM training. The CI value of these worms were significantly lower ($p \leq 0.0001$, $n \geq 4$) than the WT worms at all time points after the training (Figure 6a). The strain, LX703 [*dop-3(vs106)*], devoid of *dop-3* receptor gene also showed a lower ($p \leq 0.05$, $n \geq 4$) CI value at the 0^{th} hour after STAM training. These worms also displayed memory impairment at the subsequent time points. The CI values at the 1^{st} and 2^{nd} hours of LX703 [*dop-3(vs106)*] worms were significantly lower ($p \leq 0.0001$, $n \geq 4$) than the WT control (Figure 6b). This demonstrates that DA signaling is crucial for learning and memory.

To investigate the effect of Insulin on the STAM of these worms, LX636 [*dop-1(vs101)*] and LX703 [*dop-3(vs106)*] worms were conditioned in the presence of huminsulin. Exogenous insulin could not restore the learning and memory deficit in these worms. LX636 [*dop-1(vs101)*] worms when conditioned along with huminsulin showed no considerable improvement from the untreated group and displayed lower CI values at the 0^{th} ($p \leq 0.05$, $n \geq 4$), 1^{st} ($p \leq 0.001$, $n \geq 4$), and 2^{nd} ($p \leq 0.0001$, $n \geq 4$) hours after STAM training compared to the WT (Figure 6a). Similarly, LX703 [*dop-3(vs106)*] worms, when accompanied with huminsulin

during the conditioning phase also showed no significant improvement from the untreated worms. Even after being conditioned in the presence of exogenous insulin, the CI values of these worms remained significantly lower than the WT worms at the 0th ($p \leq 0.0001$, n=6), 1st ($p \leq 0.001$, n=6), and 2nd ($p \leq 0.0001$, n=6) hours (Figure 6b). This substantiates that insulin cannot compensate for the downregulated DA signaling in the learning and memory of the worms.

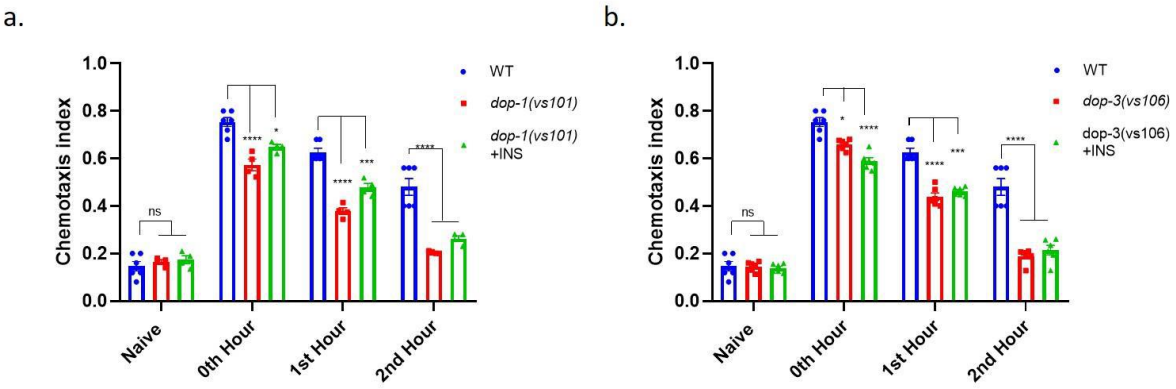

Figure 6. Short-term Memory of LX636 [*dop-1(vs101)*] and LX703 [*dop-3(vs106)*] worms conditioned in the absence and presence of exogenous insulin. a. Short-term memory of LX636 [*dop-1(vs101)*] worms conditioned in the absence and presence of exogenous insulin. The strain LX636 [*dop-1(vs101)*] is defective in STAM, as is evident from the significantly lower CI values than the WT worms at the 0th ($p \leq 0.0001$), 1st ($p \leq 0.0001$), and 2nd ($p \leq 0.0001$) hours. The learning and memory defect did not reverse by the addition of huminsulin. LX636 [*dop-1(vs101)*] worms conditioned in the presence of huminsulin still displayed lower CI values than the WT worms at the 0th ($p \leq 0.05$), 1st ($p \leq 0.001$), and the 2nd ($p \leq 0.0001$) hours. **b.** Short-term memory of LX703 [*dop-3(vs106)*] worms conditioned in the absence and presence of exogenous insulin. The strain LX703 [*dop-3(vs106)*] is defective in STAM as evidenced by the significantly lower CI values than the WT worms at the 0th ($p \leq 0.05$), 1st ($p \leq 0.0001$), and 2nd ($p \leq 0.0001$) hours. The addition of huminsulin did not reverse the learning and memory defect. LX703 [*dop-3(vs106)*] worms conditioned in the presence of huminsulin still displayed lower CI values than the WT worms at the 0th ($p \leq 0.0001$), 1st ($p \leq 0.001$), and the 2nd ($p \leq 0.0001$) hours. n≥4 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- not significant, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

Learning and Memory Defects in the Dopamine Synthesis Mutant can be Rescued by Exogenous Dopamine but not Insulin

CAT-2 encodes Tyrosine monooxygenase in the C. *elegans*. It is the rate-limiting enzyme in the biosynthesis of DA. The strain CB1112 [*cat-2(e1112) II*] lack functional *cat-2* and, thus, can be used as a system lacking in DA (Nagatsu, 1995). These worms also show learning and memory impairment. The CI values following the STAM training are significantly lower ($p \leq 0.0001$, $n \geq 6$) than the WT worms. This defect is evident at the 0^{th}, 1^{st}, and 2^{nd} hours post-training (Figure 7a). This observation validates the importance of DA in the learning and memory of C. *elegans*. DA is an essential neurotransmitter in determining the learning and memory capacities of the worm.

If the memory deficit results from a lack of DA, exogenous administration of DA should be able to rescue the behavior. To test this, the CB1112 [*cat-2(e1112) II*] worms were conditioned in the presence of exogenous DA, and the CI was analysed after STAM training. As expected, exogenous DA alleviated the learning and memory defects in these worms. The CI increased dramatically in worms conditioned in the presence of DA at the 0^{th} ($p \leq 0.001$, $n \geq 7$), 1^{st} ($p \leq 0.0001$, $n \geq 7$), and 2^{nd} ($p \leq 0.0001$, $n \geq 7$) hours compared to the untreated control worms (Figure 7b). To check whether insulin could substitute for DA in CB1112 [*cat-2(e1112) II*] worms, exogenous huminsulin was added during the conditioning phase. Interestingly, insulin was unable to compensate for DA deficiency in these worms. The CI did not show any significant change ($p > 0.05$, $n \geq 7$) from that of the untreated control (Figure 7b). This again substantiates our earlier results that insulin cannot compensate for DA.

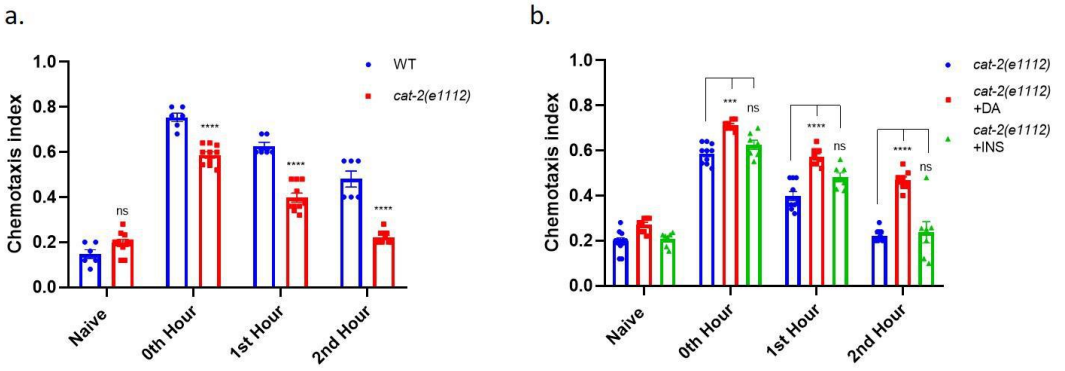

Figure 7. Short-term Memory of CB1112 [*cat-2(e1112) II*] worms conditioned in the absence and presence of exogenous DA and huminsulin. **a.** Short-term Memory of CB1112 [*cat-2(e112)II*] worms. The strain CB1112 [*cat-2(e1112) II*] shows a significant reduction in learning and memory compared to the WT worms. The CI values of CB1112 [*cat-2(e1112) II*] worms were significantly lower than the WT worms at the 0th ($p \leq 0.0001$), 1st ($p \leq 0.0001$), and 2nd ($p \leq 0.0001$) hours. **b.** Short-term Memory of CB1112 [*cat-2(e1112) II*] worms conditioned in the presence of exogenous DA and huminsulin. n≥6 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: ****$p \leq 0.0001$.

Insulin promotes DA secretion but cannot Substitute for its Deficiency

DA is one of the major neurotransmitters modulating the locomotory behaviour in the presence and absence of food (Sawin, Ranganathan and Horvitz, 2000). In our previous results, we have also seen that insulin is can mimic the food signal (Figure 1). To understand the insulin-DA crosstalk in-depth, an avoidance assay (Hilliard, Bargmann and Bazzicalupo, 2002) was performed on the worms on foodless NGM, OP50, insulin, and DA plates. Avoidance assay is a standardised method to assess DA-associated locomotory behaviour. This assay analysed the number of worms taking omega turns in response to a drop of 0.5 M glycerol, a known repellent.

WT worms show a significant increase ($p \leq 0.0001$, n=3) in avoidance index in food plate (OP50). This is because the presence of food caused a surge in DA neurotransmitter in the system. Even in the absence of food, this behaviour was reproduced in insulin and DA plates. The avoidance index increased significantly in insulin ($p \leq 0.01$, n=3) and DA plates ($p \leq 0.001$,

n=3) (Figure 8). This is mainly because, insulin mimics food signal in the insulin plates and indirectly increases the DA. On the other hand, DA is provided to the worms in the form of exogenous DA in the DA plate, resulting in a higher avoidance index in these plates than the foodless plate (Figure 8). This increase in response to exogenous DA is observed in all the strains- IK581 [*ins-1(nj32) IV*] ($p \leq 0.05$, n=3), CB1370 [*daf-2(e1370) III]* ($p \leq 0.01$, n=3), DR26 *[daf-16(m26) I]* ($p \leq 0.05$), and CB1112 [*cat-2(e112) II*] ($p \leq 0.05$, n=3) (Figure 8).

The increase in avoidance index compared to that in food plate is also observed in IK581 [*ins-1(nj32) IV*] ($p \leq 0.05$, n=3), CB1370 [*daf-2(e1370) III]* ($p \leq 0.01$, n=3), DR26 *[daf-16(m26) I]* ($p \leq 0.01$, n=3) worms (Figure 8). The surge in DA was also found in IK581 [*ins-1(nj32) IV*] worms assayed on insulin ($p \leq 0.05$, n=3) plate. However, there was no significant ($p > 0.05$, n=3) change in the avoidance index of CB1370 [*daf-2(e1370) III]* worms in the insulin plate due to the lack of insulin receptors (Figure 8). As a result, the strain CB1370 [*daf-2(e1370) III]* could not recognise insulin in the media and, therefore, cannot cause DA surge, to induce avoidance behaviour. Effect of exogenous insulin on the avoidance index of DR26 *[daf-16(m26) I]* worms was also negligible ($p > 0.05$, n=3) compared to that in NGM plate as the downstream molecule of the insulin pathway, *daf-16*, is absent in these worms (Figure 8). In the strain CB1112 [*cat-2(e112) II*] devoid of *cat-2*, the protein which is essential for DA biosynthesis, there was no subsequent increase in the avoidance index in OP50 or insulin plates (Figure 8). Put together with the previous results, this data also proves that DA functions downstream to Insulin as in the absence of *cat-2* (CB1112 [*cat-2(e112) II*] worms), insulin cannot increase the avoidance index, which is a hallmark of DA surge.

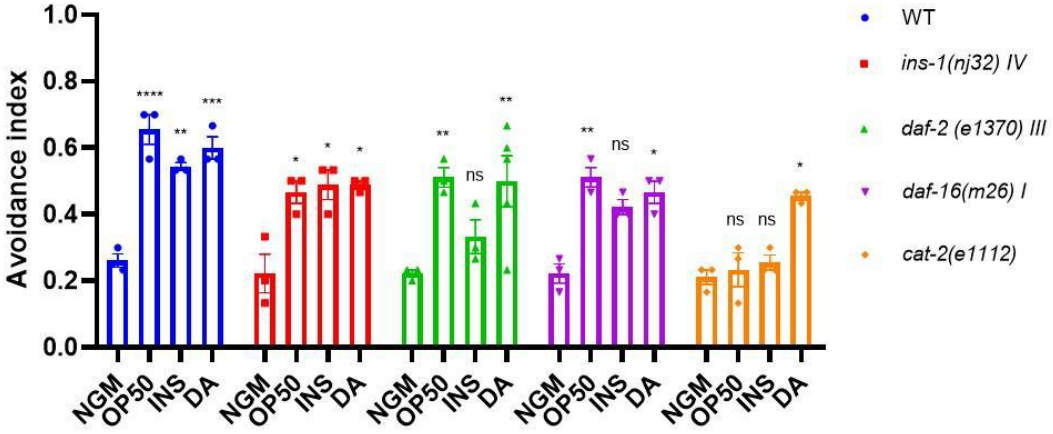

Figure 8. Avoidance index of Wild-type, IK581 [*ins-1(nj32) IV*], CB1370 [*daf-2(e1370) III],* DR26 *[daf-16(m26) I]*), and CB1112 [*cat-2(e112) II*] worms in NGM, OP50, Insulin, and DA plates. The avoidance index increases in OP50 food plate due to surge in the internal DA content. A significant increase in the avoidance index is seen in WT ($p \leq 0.0001$), IK581 [*ins-1(nj32) IV*] ($p \leq 0.05$), CB1370 [*daf-2(e1370) III]* ($p \leq 0.01$), and DR26 *[daf-16(m26) I]* ($p \leq 0.01$). There is no significant change in the avoidance index of CB1112 [*cat-2(e112) II*] worms in the OP50 plate due to the lack of enzyme responsible for the synthesis of DA. The avoidance index also increases in response to external insulin in the WT ($p \leq 0.01$) and IK581 [*ins-1(nj32) IV*] ($p \leq 0.05$) worms as insulin might cause indirect rise in the DA content. This significant change is absent ($p > 0.05$) in the CB1370 [*daf-2(e1370) III]* and DR26 *[daf-16(m26) I]* worms due to disruption in the insulin signaling. In the case of CB1112 [*cat-2(e112) II*] worms, insulin was unable to increase the DA content due to the lack of CAT-2 enzyme. Avoidance index increases in all the worms supplied with exogenous DA (Wild-type- $p \leq 0.001$, IK581 [*ins-1(nj32) IV*]- $p \leq 0.05$, CB1370 [*daf-2(e1370) III]*- $p \leq 0.01$, DR26 *[daf-16(m26) I]*- $p \leq 0.05$, and CB1112 [*cat-2(e112) II*]- $p \leq 0.01$). n=3 trials; each trial contains 30 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- not significant, *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, and ****$p \leq 0.0001$.

Insulin causes calcium influx in the dopaminergic neurons, causing them to fire

To evaluate the response of DAergic neurons towards exogenous insulin, worms expressing GCaMP under the promoter of DA transporter *dat-1* were created using microinjection. The worms were named AT7437 (*dat-1p::GCaMP-6::mCherry*) and AT7438 (*daf-2; dat-1p::GCaMP-6::mCherry*). AT7437 express GCaMP-6 and mCherry under *dat-1* promoter in an otherwise WT background, whereas, AT7438 (*daf-2; dat-1p::GCaMP-6::mCherry*) express GCaMP-6 and mCherry in CB1370 [*daf-2(e1370) III*] background. The worms were exposed to either insulin or buffer at the 10th second, and the change in fluorescence in the ADE and

CEP neurons was recorded. The increase in fluorescence accounts for the calcium-mediated activation of DAergic neurons.

The transgenic worm AT7437 (*dat-1p::GCaMP-6::mCherry*) showed an increase in calcium flux, represented by the rise in the fluorescence intensity, in the DAergic neurons following the addition of huminsulin (n=7) (Figure 9a). This response to the stimulus was continued for a short period and subsequently decreased with time. This response was absent when the worms were exposed to buffer solution (n=6) (Figure 9a). Insulin-induced calcium influx in the DAergic neurons was also not observed in the strain AT7438 (*daf-2*; *dat-1p::GCaMP-6::mCherry*) due to the absence of functional insulin receptor, DAF-2 (n=7) (Figure 9 b). These *in vivo* calcium transients are evidence of the crosstalk between insulin and DA. Insulin signaling can, thus, cause transient changes in the calcium influx in DAergic neurons.

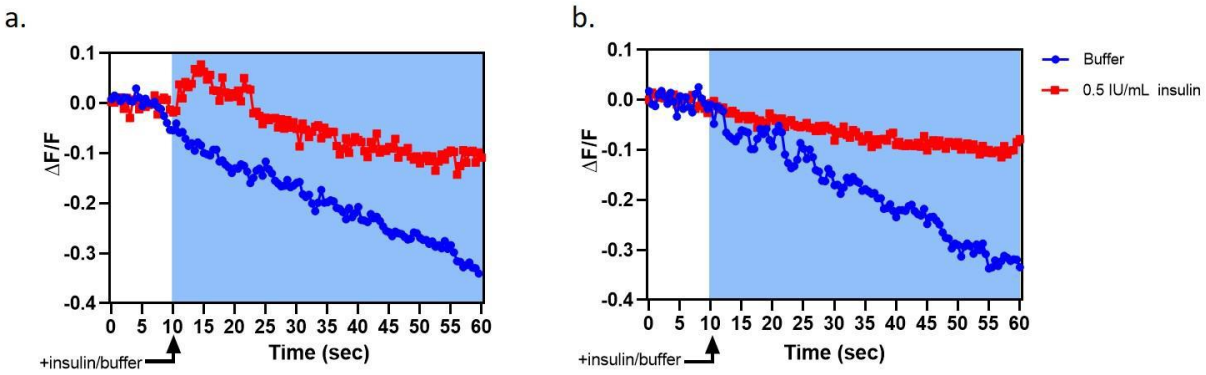

Figure. 9. Change in dopaminergic neuronal fluorescence in AT7437 (*dat-1p::GCaMP-6::mCherry*) and AT7438 (*daf-2; dat-1p::GCaMP-6::mCherry*) in response to exogenous insulin and buffer. a. Change in fluorescence intensity of AT7437 (*dat-1p::GCaMP-6::mCherry*) before (0-10 sec) and after (10-60 sec) being presented with exogenous insulin or buffer. There is an increase in the fluorescence intensity in response to exogenous insulin, but not the buffer, which decreases after some time. **b**. Change in fluorescence intensity of AT7438 (*daf-2; dat-1p::GCaMP-6::mCherry*) before (0-10 sec) and after (10-60 sec) being presented with exogenous insulin or buffer. There is no insulin-mediated activation of DAergic neurons because of the absence of a functional insulin receptor, DAF-2. n≥6 trials. Data represented as the mean of all the trials.

Increased Calcium influx in Dopaminergic neurons is observed at the 3rd hour after training in AT7438 (daf-2; dat-1p::GCaMP-6::mCherry)

AT7437 (*dat-1p::GCaMP-6::mCherry*) and AT7438 (*daf-2; dat-1p::GCaMP-6::mCherry*) worms were trained for STAM, and the calcium transients of naïve worms and the trained worms at the 0^{th} and 3^{rd} hours were analysed. The naïve AT7437 (*dat-1p::GCaMP-6::mCherry*) worms (n=6) did not show any increase in fluorescence intensity when presented with the odor, butanone (10^{th} sec), but these worms exhibited an increase in fluorescence at the 0^{th} hour post-training (n=6) (Figure 10a). This response to the conditioned stimulus continued for a short period even after the withdrawal of the stimulus (20^{th} sec) and subsequently decreased with time. However, the increase in fluorescence was not seen at the 3^{rd} hour. This substantiates the loss of memory by the 3^{rd} hour in WT worms (Figure 10a). This is on par with an earlier result from our laboratory (unpublished data).

On the contrary, AT7438 (*daf-2; dat-1p::GCaMP-6::mCherry*) worms which have the background of CB1370 [*daf-2(e1370) III*], shows an increase in fluorescence at both the 0^{th} (n=6) and the 3^{rd} hours (n=7) (Figure 10b). This corroborates our results that the strain CB1370 [*daf-2(e1370) III*] has extended memory. Thus, there is an increased calcium influx in the dopaminergic neurons during both the learning and memory recall. This enhanced calcium influx observed in the 3^{rd} hour is due to the *daf-2* mutation that was introduced (Figure 10b). These results underscore the critical role of dopamine neurons in memory recall.

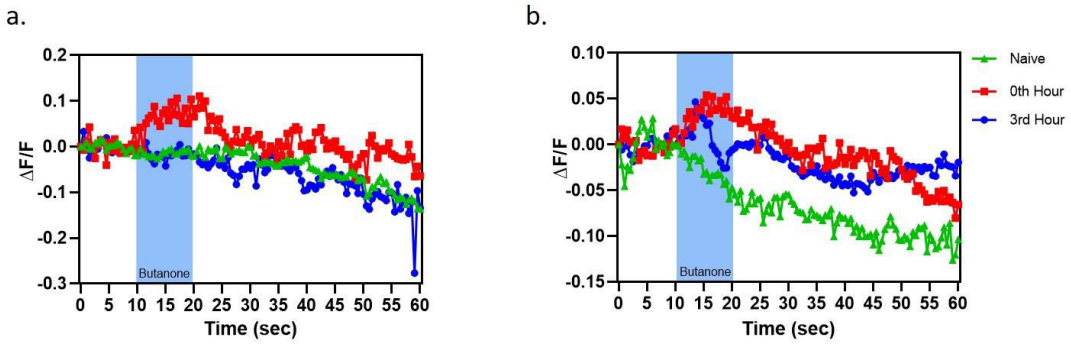

Figure 10. Calcium transients in AT7437 (*dat-1p::GCaMP-6::mCherry*) and AT7438 (*daf-2; dat-1p::GCaMP-6::mCherry*) worms after Short-Term Associative Training. a. Calcium imaging of AT7437 (*dat-1P::GCaMP6::mCherry*) shows calcium-mediated activation of DAergic neurons at the 0th hour when the odor butanone was presented (10th sec), compared to the naïve. This response to the conditioned stimulus (CS) continued for a short period even after the withdrawal of the stimulus (20th sec) and successively decreased with time. Untrained naïve worms and trained worms at the 3rd hour did not show any increase in response to the CS butanone. **b.** Calcium imaging of AT7438 (*daf-2; dat-1P::GCaMP6::mCherry*) shows calcium-mediated activation of DAergic neurons at the 0th and 3rd hours, when the odor butanone was presented (10th sec), compared to the naïve. This response to the conditioned stimulus (CS) continued for a short period even after the withdrawal of the stimulus (20th sec) and successively decreased with time. Untrained naïve worms did not show any increase in response to the CS butanone. n≥6 trials. Data represented as the mean of all the trials.

Increased Calcium influx in the Dopaminergic neuron is observed at the 3rd hour after training in the presence of exogenous insulin

In the previous results, it was noticed that excess insulin results in antagonisation of the DAF-2 receptor. The excess insulin was also able to elicit extended memory in the worms. To investigate the role of DAergic neurons in the extended memory resulting from excess insulin, AT7437 (*dat-1p::GCaMP-6::mCherry*) worms were conditioned in the presence of huminsulin. These worms also exhibited extended memory retention. The CI were significantly higher than the untreated worms at the 2nd ($p \leq 0.001$, n=6) and 3rd hours ($p \leq 0.0001$, n=6) (Figure 11a). This was also supported by the calcium imaging of DAergic neurons in the AT7437 (*dat-1p::GCaMP-6::mCherry*) worms. There was an increase in fluorescent intensity corresponding to the worms conditioned in the presence of huminsulin at the 0th (n=8) hour and 3rd (n=8) hours (Figure 11b). The data suggests an increase in calcium flux in the DAergic neurons in response to the extended memory associated with excess insulin.

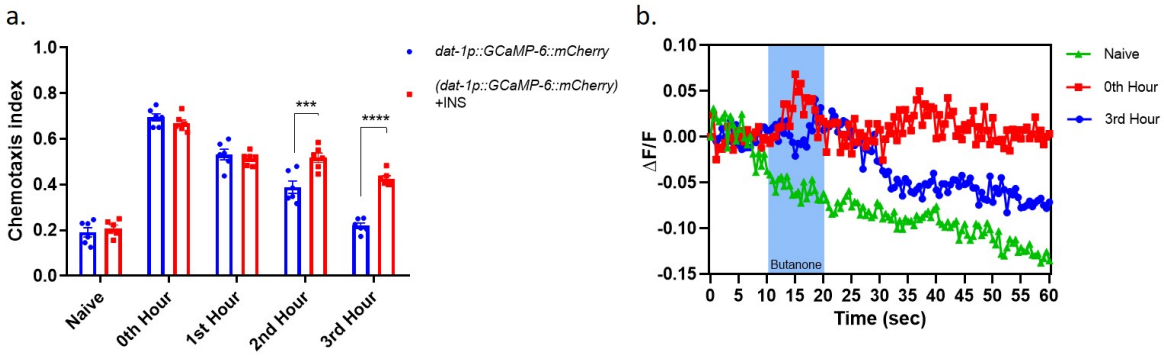

Figure 11. STAM and calcium influx in the dopaminergic neurons of AT7437 (*dat-1p::GCaMP-6::mCherry*) following conditioning in the presence of huminsulin. a. AT7437 (*dat-1p::GCaMP-6::mCherry*) worms conditioned in the presence of huminsulin show extended memory at the 3rd hour compared to the untreated worms. The CI of the worms conditioned in the presence of huminsulin is significantly greater than the untreated controls at the 2nd ($p \leq 0.001$) and 3rd ($p \leq 0.0001$) hours. **b.** Calcium imaging of naïve and STAM-trained (0th, 3rd hours) AT7437 (*dat-1p::GCaMP6::mCherry*). Calcium-mediated activation of DAergic neurons is observed at the 0th and 3rd hours when the odor butanone was presented (10th sec), compared to the naïve. This response to the conditioned stimulus (CS) continued for a short period even after the withdrawal of the stimulus (20th sec). $n \geq 3$ trials and at least 50 worms per trial in the STAM experiment; $n \geq 6$ in the calcium imaging experiment. Data represented as the mean of all the trials.

Calcium Surge is observed in RID Neurons in Response to Higher Chemotaxis Following STAM

RID is a peptidergic neuron that modulates the *C. elegans* motor state to sustain forward movement (Lim *et al.*, 2016). Since the present study focuses on positive chemotaxis to the test solvent following the associative memory training and largely involves forward movement, the role of RID neurons in memory retrieval was investigated. The strain ZM9078 *(hpIs587 [flp-14p::GCaMP6::wCherry + lin-15(+)]* was used to study the calcium transients following STAM training. The naïve worms (n=6) did not show any increase in fluorescent intensity upon being presented with butanone, the test solvent. After the training, the worms showed an increase in the fluorescent intensity at the 0th hour (n=6), which disappeared by the 3rd hour (n=4) (Figure 12a). This can be substantiated by the increase in chemotaxis following the training at the 0th hour which finally decreases to the naïve level by the 3rd hour. To extend the role of RID neurons in the abnormal memory exhibited by worms treated with excess insulin,

ZM9078 *(hpIs587 [flp-14p::GCaMP6::wCherry + lin-15(+)]* worms were conditioned in the presence of huminsulin and the calcium transients were analysed following the training. In these strains of worms, though the naïve (n=6) did not show a marked increase when presented with butanone, there was substantial increase in fluorescent intensity at the 0th (n=6) and 3rd hours (n=6). A second peak of intensity change can be observed in the worms at the 0th hour even after removing butanone because RID neurons fire in response to forward movements (Figure 12b).

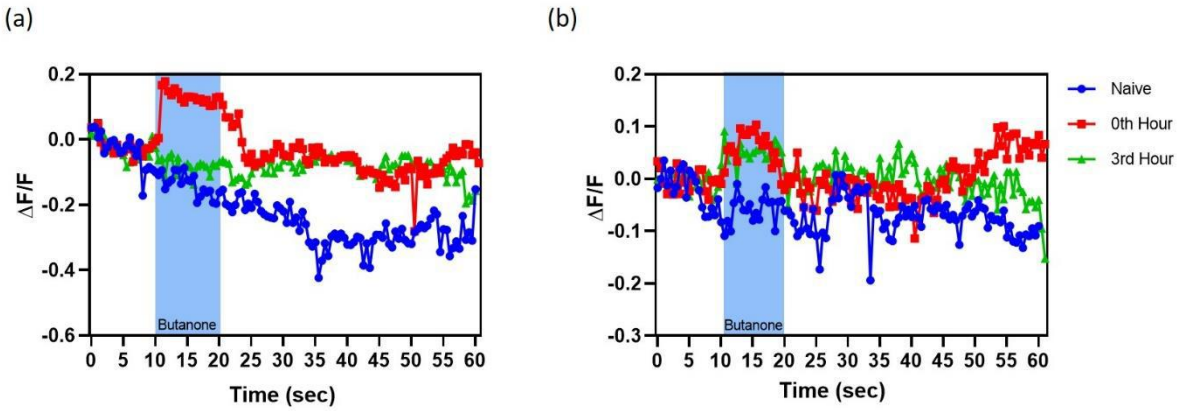

Figure 12. Calcium transients in the RID neurons after STAM training in the absence and presence of exogenous Insulin. a. Calcium imaging of ZM9078 *(hpIs587 [flp-14p::GCaMP6::wCherry + lin-15(+)]* worms reveals calcium-mediated activation of RID neurons at the 0th hour when the odor butanone was presented (10th sec), compared to the naïve. This response to the conditioned stimulus (CS) continued for a short period even after the withdrawal of the stimulus (20th sec). Untrained naïve worms and trained worms at the 3rd hour did not show any increase in response to the CS butanone. **b.** Calcium imaging of naïve and ZM9078 *(hpIs587 [flp-14p::GCaMP6::wCherry + lin-15(+)]* worms trained in the presence of exogenous insulin. Calcium-mediated activation of RID neurons is observed at the 0th and 3rd hours when the odor butanone was presented (10th sec), compared to the naïve. n=6 trials. Data represented as the mean of all the trials.

Role of dopaminergic neuronal daf-2 gene in the extended memory

ADE, CEP and PDE are the DAergic neurons in C. *elegans* hermaphrodite. Among these, daf-2 gene expression has been observed in the ADE and PDE neurons (Taylor *et al.*, 2021). In the CB1370 [*daf-2(e1370) III]* worms, the daf-2 gene is mutated throughout the organism. To get a clearer idea of the role of the dopaminergic neuronal *daf-2* gene in the extended memory

process, a mosaic mutant designed to target siRNA towards dopaminergic neurons was used. The strain XE1474 (*lin-15B(n744) X; eri-1(mg366) IV; rde-1(ne219) V; wpSi6[Pdat-1::rde-1:SL2:sid-1, Cbunc-119(+)] II*] restrict RNAi sensitivity to dopaminergic neurons through the *dat-1* promoter.

These worms were trained for STAM and LTAM after treatment with siRNA towards *daf-2* and their CI values were analysed (Figure 13 a and b). After STAM, the extended memory was observed despite the siRNA being targeted only towards the dopaminergic neurons. The CI value of the worms treated with pL-*daf-2* were significantly higher post the 1st ($p \leq 0.05$, n=6), 2nd ($p \leq 0.001$, n=6), 3rd ($p \leq 0.0001$, n=6), and 5th ($p \leq 0.0001$, n=6) hours of training when compared to the worms treated with the control vector, pL4440 (Figure 13a). The CI value remained significantly higher ($p \leq 0.0001$, n=6) than the control even at the 5th hour after training after which it became insignificant ($p > 0.05$, n=6) (Figure 13a). The memory of this strain, when treated with pL-*daf-2* persisted till the 5th hour, exhibiting an extended memory.

After LTAM in the presence of pL-*daf-2*, though the CI values remained insignificant ($p > 0.05$, n=6) at the 0th and 24th hours compared to the worms treated with control vehicle, pL-4440, a significant increase ($p \leq 0.0001$, n=6) in CI was observed at the 48th hour (Figure 13b). These worms also showed an extended memory at the 48th hour as deduced from the significantly higher ($p \leq 0.05$, n=6) CI value at the 48th hour compared to its naïve (Figure 13b).

Together, these results suggest that knocking down the *daf-2* gene in the dopaminergic neurons leads to extended STAM and LTAM. This highlights the importance of DAergic neurons and the insulin pathway in these neurons in the extended memory.

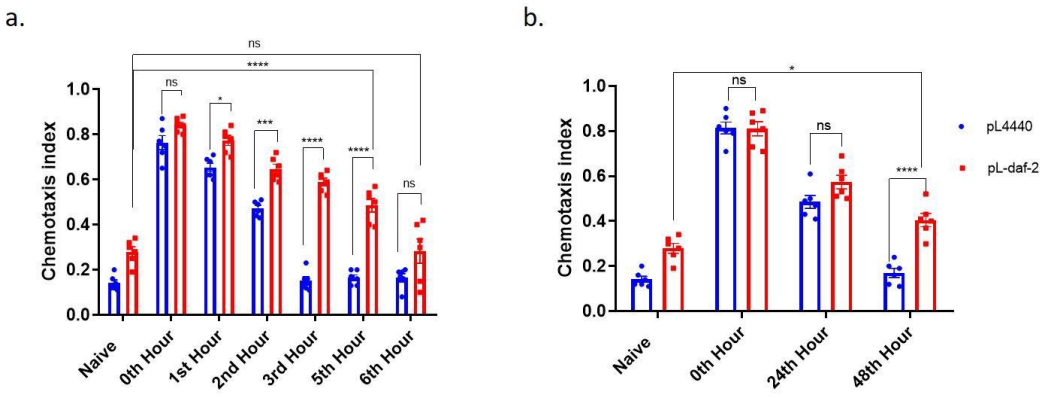

Figure 13. Short- (a) Long-term (b) memory of worms with *daf-2* siRNA targeted against DAergic neurons. **a**. Short-term memory of worms with *daf-2* siRNA targeted against DAergic neurons. The siRNA-treated worms show significantly higher CI at all time points except the 0^{th} and 6^{th} hours (1^{st} hour- $p \leq 0.05$; 2^{nd} hour- $p \leq 0.001$; 3^{rd} hour- $p \leq 0.0001$; 5^{th} hour- $p \leq 0.0001$) compared to the worms treated with pL4440. In the siRNA-treated worms, the CI at the 5^{th} hour is significantly higher ($p \leq 0.0001$) than the naïve worms. **b**. Long-term memory of worms with *daf-2* siRNA targeted against DAergic neurons. There is no significant difference ($p > 0.05$) in the CI values of pL-*daf-2* and pL4440-treated worms after LTAM training. However, the CI value at the 48^{th} hour of the siRNA-treated worms is significantly greater ($p \leq 0.05$) than the naïve worms. n=6 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- not significant, *$p \leq 0.05$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

Role of other Neurons in the daf-2-mediated Extended Memory

Strains with siRNA sensitivity towards glutmatergic [XE1582 (*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219)* V; *wpSi11(eat-4p::rde-1:SL2:sid-1, Cbunc-119(+)* II)], GABAergic [XE1375 (*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219*) V; *wpSi1(unc-47p::rde-1:SL2:sid-1, Cbunc-119(+)* II; *wpIs36(unc-47p::mCherry)* I] and cholinergic [XE1581 (*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219)* V; *wpSi10[unc-17p::rde-1:SL2:sid-1, Cbunc-119(+)* II)] neurons were used to uncover the possible role of *daf-2* in these neurons in the extended memory. The STAM and LTAM of the worms were noted following the treatment of siRNA vector designed against the *daf-2* gene.

Glutamatergic neurotransmission is observed in many head neurons like the ADL, AFD, AIB, AIM, AIZ, ASH, ASE, ASG, ASK, AWB, AWC, RIA, etc. When the *daf-2* siRNA was targeted against the glutamatergic neurons, a significantly higher CI was observed at the 2nd ($p \leq 0.05$, n=6), 3rd ($p \leq 0.0001$, n=6), and 5th ($p \leq 0.001$, n=6) hours after STAM training compared to the worms treated with pL4440 (Figure 14a). The CI value remained significantly higher ($p \leq 0.01$, n=6) at the 5th hour compared to its naïve, indicating an extended memory (Figure 14a). After LTAM training, the CI of the worms treated with pL-*daf-2* did not show any marked ($p > 0.05$, n=6) difference from the control pL4440-treated worms (Figure 14b). However, the 48th hour CI value of these worms were significantly higher ($p \leq 0.05$, n=6) than its naïve worms, suggesting an extended LTAM in these worms (Figure 14b). These results indicate that glutamatergic neurons are involved in the extended memory associated with the *daf-2* mutation.

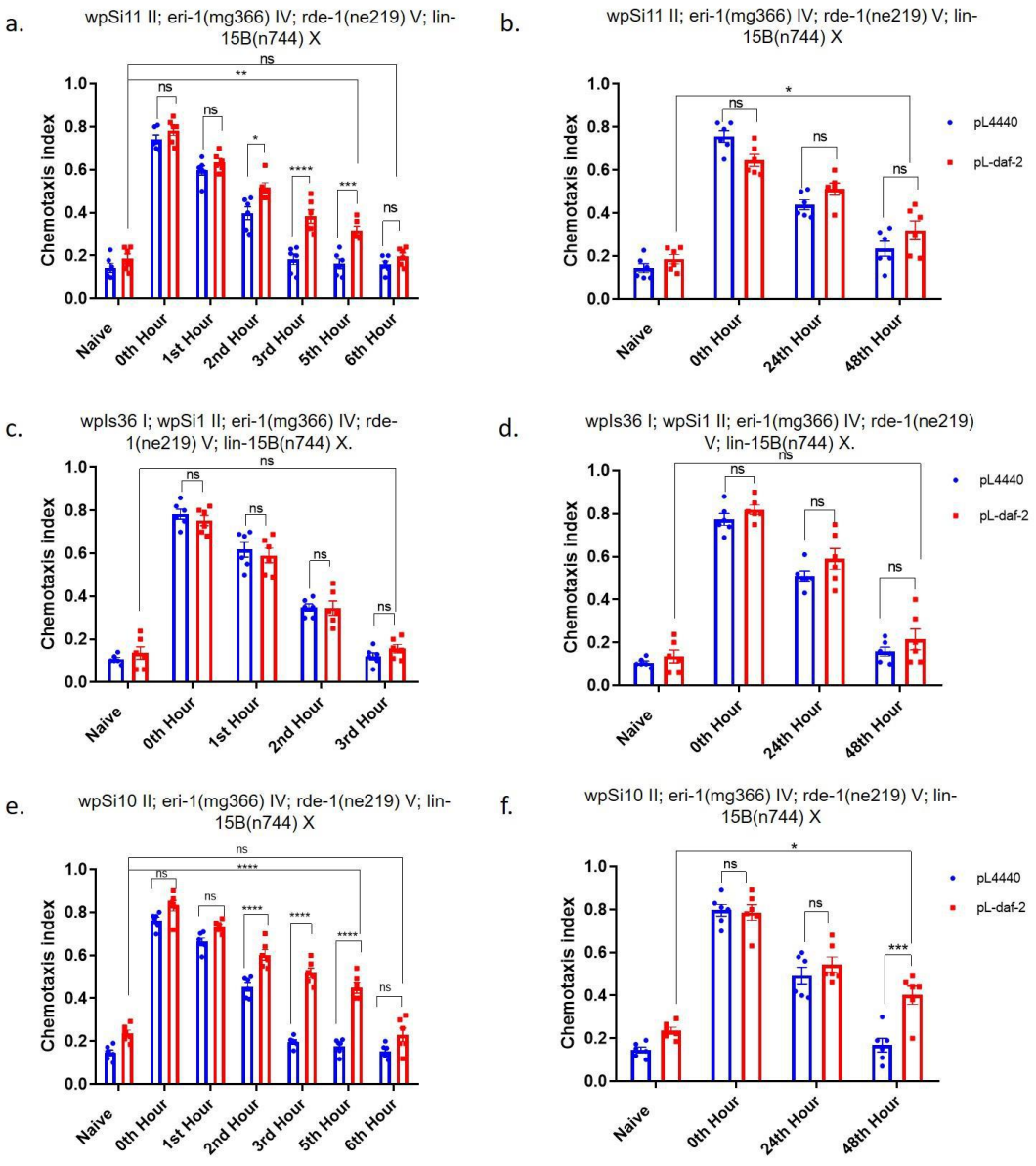

Figure 14. Short- (a, c, and e) Long-term (b, d, and f) memory of worms with *daf-2* siRNA targeted against glutamatergic (a and b), GABAergic (c and d) and cholinergic (e and f) neurons. a. STAM of [*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219)* V; *wpSi11(eat-4p::rde-1:SL2:sid-1, Cbunc-119(+)* II] worms with *daf-2* siRNA targeted against glutamatergic neurons. pL-*daf-2*-treated worms exhibit higher CI values at the 2nd ($p \leq 0.05$), 3rd ($p \leq 0.0001$), and 5th ($p \leq 0.001$) hours than the worms treated with pL4440 at the same time point. The worms treated with pL-*daf-2* also show significantly higher CI at the 48th hour compared to the naïve worms. **b.** LTAM of [*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219)* V; *wpSi11(eat-4p::rde-1:SL2:sid-1, Cbunc-119(+)* II] worms with *daf-2* siRNA targeted against glutamatergic neurons. There is no significant difference ($p>0.05$) in the CI values of pL-*daf-2* and pL4440-treated worms after LTAM training. However, the CI value at the 48th hour of the siRNA-treated worms is significantly greater ($p \leq 0.05$) than the naïve worms. **c.** STAM of [*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219*) V; *wpSi1[unc-47p::rde-1:SL2:sid-1, Cbunc-119(+)]* II; *wpIs36(unc-47p::mCherry)* I] worms with *daf-2* siRNA targeted against GABAergic neurons. The CI values of the siRNA-treated and pL4440-treated worms do not show any significant difference ($p>0.05$). The difference in CI value of the siRNA-treated worms at the 3rd hour is insignificant ($p>0.05$) compared to its naïve. **d.** LTAM of [*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219*)

V; *wpSi1[unc-47p::rde-1:SL2:sid-1, Cbunc-119(+)]* II; *wpIs36(unc-47p::mCherry)* I] worms with *daf-2* siRNA targeted against GABAergic neurons. The CI values of the siRNA-treated and pL4440-treated worms do not show any significant difference ($p>0.05$). The difference in CI value of the siRNA-treated worms at the 48th hour is also not significant ($p>0.05$) compared to its naïve **e.** STAM of [*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219)* V; *wpSi10[unc-17p::rde-1:SL2:sid-1, Cbunc-119(+)]* II)] worms with *daf-2* siRNA targeted against cholinergic neurons. pL-*daf-2*-treated worms exhibit higher CI values at the 2nd, 3rd, and 5th hours ($p\leq0.0001$) than the worms treated with pL4440 at the same time point. The worms treated with pL-*daf-2* also show significantly higher CI at the 48th hour compared to the naïve worms. **f.** LTAM of [*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219)* V; *wpSi10[unc-17p::rde-1:SL2:sid-1, Cbunc-119(+)]* II)] worms with *daf-2* siRNA targeted against cholinergic neurons. There is no significant difference ($p>0.05$) in the CI values of pL-*daf-2* and pL4440-treated worms following LTAM training. Although, the CI value at the 48th hour of the siRNA-treated worms is significantly greater ($p\leq0.05$) than the naïve worms. n=6 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- not significant, *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, and ****$p\leq0.0001$.

GABA expression is reported in the intestine, rectal gland cells, hypodermis, nervous system, head neurons of C. *elegans*. Unlike the DAergic and glutamatergic neuronal siRNA of *daf*-2, the worms with *daf-2* siRNA targeted against GABAergic neurons did not show extended memory. Following STAM, the worms treated with pL-*daf*-2 did not show any difference ($p>0.05$, n=6) in CI compared to the ones treated with pL4440 at any time points (Figure 14c). The CI value dropped to the naïve levels ($p>0.05$, n=6) by the 3rd hour (Figure 14c). These worms also failed to exhibit extended LTAM (Figure 14d). These results demonstrate that the disrupted insulin signaling in the GABAergic neurons might not be sufficient to cause an extended memory in the worms.

To extend our knowledge on the role of cholinergic neurons in the extended memory attributed to *daf-2* mutation, siRNA of *daf-2* was targeted against these neurons. AIA, AIM, AIY, ASJ, and AWB are the cholinergic head neurons. The strain XE1581 [*lin-15B(n744)* X; *eri-1(mg366)* IV; *rde-1(ne219)* V; *wpSi10[unc-17p::rde-1:SL2:sid-1, Cbunc-119(+)* II)] targets these neurons and also makes it prone to siRNA. When treated with pL-daf-2, these worms also exhibit extended STAM (Figure 14e) and LTAM (Figure 14f). After STAM, a significantly higher CI value is observed in worms treated with pL-*daf-2* at the 2nd ($p\leq0.0001$, n=6), 3rd ($p\leq0.0001$, n=6), and 5th ($p\leq0.0001$, n=6) hours compared to the vehicle control (Figure 14e).

Also, the memory is extended till the 5th hour as the CI at this hour is significantly greater ($p \leq 0.0001$, n=6) than the corresponding naïve worms (Figure 14e). These worms when treated with pL-*daf-2* during LTAM show significantly higher ($p \leq 0.0001$, n=6) CI than the worms treated with pL4440 at the 48th hour (Figure 14f). Similarly, the CI at the 48th hour was significantly higher than the control naïve worms (Figure 14f). The LTAM is also extended in these worms following siRNA treatment against the *daf-2* gene. This data provides evidence that GABAergic neuronal DAF-2 is critical in the extended memory displayed by the *daf-2* mutants.

Put together, these results establish the importance of glutamatergic and cholinergic neurons along with the dopaminergic neurons in the *daf-2*-associated extended memory. DAF-2 in these neurons is essential for normal memory and forgetting pathways.

Discussion

Learning can be categorised into two: associative and non-associative. Associative learning occurs when an animal learns to relate two stimuli, whereas, in non-associative learning, an animal learns to habituate or sensitise to a single stimulus (Rahmani and Chew, 2021b). Both these forms of learning are essential for the animal to adapt to changing environments (Van Damme *et al.*, 2021). Animals must learn to integrate various cues with the changes in reward contingencies (Cuthbert, 2014). Associative memories can be subdivided into aversive and appetitive (Itzhak, Perez-Lanza and Liddie, 2014). In aversive learning, the animal learns to avoid or escape from the associated stimulus (Itzhak, Perez-Lanza and Liddie, 2014). On the contrary, appetitive conditioning procedures enable a positive association of stimulus with a natural reward such as food (Reichelt and Lee, 2013).

A "reward" is defined as a pleasant or positive experience, such as the availability of food (White, 2011). Dopamine (DA) is one of the neurotransmitters that act as reward signals in the brain (Di Chiara and Imperato, 1988). However, the role of the dopaminergic system in the reward pathway is not limited to the effects of psychostimulants and other drugs of abuse but also natural rewards, such as food (Baik, 2013). DA release in various brain structures like the substantia nigra and ventral tegmental area (VTA) is increased in response to palatable food (Wang *et al.*, 2009) (Saper, Chou and Elmquist, 2002) (Chambers, Sandoval and Seeley, 2013), addictive drugs (Robinson and Berridge, 2000), or sexual activity (Robbins and Everitt, 1996) all of which act as reward signals.

The training protocol followed in this study associates the availability of food (unconditional stimulus -US) with the odor, butanone (conditional stimulus -CS) in C. *elegans*. Here, food (OP50) is the reward signal during the training paradigm. I tested whether substituting OP50 with DA could also elicit associative learning in worms. Interestingly, the worms were able to

learn this association and displayed increased chemotaxis to butanone following the training. Thus, exogenous dopamine can mimic the appetitive signal and elicit a positive association between the cues. This was not surprising as the presentation of rewards is associated with a surge in dopamine content, as discussed above.

In addition, insulin is another hormone that is secreted in response to food (Nauck and Meier, 2018). It has been established that plasma insulin levels increase during and after a meal, activating insulin receptors (IRs) in the hypothalamus (Stouffer *et al.*, 2015). Thus, the potential of insulin to act as a food cue could not be overlooked. To test this hypothesis, worms were conditioned with insulin and butanone. As expected, the results showed increased chemotaxis to butanone, establishing that insulin could also act as a reward in the associative learning paradigm similar to DA. However, there are contradicting reports on the effect of insulin signaling on reward contingency. One study has reported that increasing insulin receptor substrate-2 activity in the dopaminergic neurons within VTA increases cocaine sensitivity, resulting in enhanced reward response (Iñiguez *et al.*, 2008). In another study, insulin augmented reward signals by enhancing striatal dopamine release (Stouffer *et al.*, 2015). On the contrary, acute intraventricular insulin administration was found to decrease the food reward signals in the CNS (Figlewicz *et al.*, 2006).

The present study also addresses the crosstalk between the insulin and dopaminergic system. The memory defects of post-dauers could be reversed by exogenous insulin, which demonstrates that the memory defect resulted from downregulated insulin pathway during its developmental period. Interestingly, this defect could also be reversed by exogenous DA. DA compensates for the downregulated insulin pathway in the post-dauers. These changes might be due to a DA-dependent increase in insulin levels in the system. A similar observation has been found in humans, where DA administration elevated insulin levels in diabetic and healthy patients (Contreras *et al.*, 2008). In a mice model of downregulated DA signaling, disruption

of D2 receptors impaired pancreatic insulin secretion (García-Tornadú et al., 2010). However, the relationship between the insulin and DA systems is controversial. *In vitro* studies reveal inhibition of glucose-stimulated insulin secretion in the pancreas in response to DA (Shankar et al., 2006). Contrariwise, another group has shown that DA infusion did not adversely affect insulin secretion (Underland et al., 2018). Nevertheless, this study revealed a DA-dependant improvement in memory in worms with downregulated insulin pathway during early development.

Despite the evidence that insulin decreases striatal DA (Kullmann *et al.*, 2021), the present study revealed that exogenous insulin could not alleviate the memory defects observed in DA over-expressing worms. Although a reciprocal relationship between the insulin and DA pathways has been postulated (Nash, 2017a), insulin could not substitute for the defective dopamine pathway in learning and memory. This is evidenced by the inability of insulin to reverse memory deficits observed in dopamine synthesis mutant, *cat-2* or the receptor mutants, *dop-1* and *dop-3*. Nevertheless, on an important note, this study revealed that exogenous insulin increased the calcium influx in DA neurons. In the worms with DA neuronal degeneration, insulin could rescue the learning deficit but did not improve memory. Similar results were observed when the worms were trained in the presence of exogenous DA. These results validate that insulin and DA function in the learning and memory pathways. Collectively, this study provides evidence that DA functions downstream of the insulin pathway in learning and memory. This hierarchy was also observed in the avoidance behaviour. Exogenous DA augmented the avoidance behaviour of insulin pathway mutants, but insulin could not substitute for the lack of DA in this assay.

The role of DA neurons in learning and forgetting is well-documented in humans (Nobili *et al.*, 2017) and animal models (Berry et al., 2018; Kempadoo et al., 2016). When presented with the test odor, increased calcium influx was observed in dopaminergic neurons in the trained

worms at the 0th hour. This increase was not observed in naïve worms and trained worms at the 3rd hour. Further studies were performed to deduce the role of DA neurons in the altered memory of insulin pathway mutants. The enhanced memory retention at the 3rd hour observed in the *daf-2* mutants and WT worms treated with exogenous insulin was supported by the increased calcium transients in DA neurons of these worms at this time point. This data strongly supports the hypothesis that insulin affects the dopaminergic system to modulate learning and memory.

Neurotransmitter	**Effect of *daf-2* RNAi**	**List of neurons with the neurotransmitter and expressing *daf-2* receptor**
Dopamine	Extended memory	ADE, PDE
GABA	Normal memory	DD, DD
Glutamate	Extended memory	ADA, AFD, AIM, ALM, AQR, ASG, ASH, AUA, AVM, BAG, DVC, M4, MI, PLM, PVQ, RIG
Acetyl choline	Extended memory	AIM, AIY, AS, AVD, AVE, AVG, DA, HSN, I3, IL2, M1, M4, M5, MC, PDA, RIB, RIR, RMD, RMH, SAB, URB, VC4, VC5

Table 1. Effect of *daf-2* RNAi on extended memory. Effect of *daf-2* RNAi in the dopaminergic, GABAergic, glutamatergic, and cholinergic neurons in the butanone-associated learning and memory and list of neurons co-expressing the respective neurotransmitters and *daf-2*.

Neurotransmission is not just limited to synapses. With the accumulating evidence of extrasynaptic neurotransmission (De-Miguel and Fuxe, 2012), the current study also expanded beyond synapse and neuronal wiring data to deduce the neurons involved in the learning and memory of worms with altered insulin pathways. Extrasynaptic neurotransmission enables neurons to transmit information at variable distances depending on the release and diffusibility of the neurotransmitter (De-Miguel and Trueta, 2005). This form of communication is also observed in C. *elegans* (Chan et al., 2013; Bentley et al., 2016). RNAi worms targeted against *daf-2* in subsets neurons were analysed to understand the role

of the insulin pathway in these neurons in altered learning and memory. Insulin signaling was found to be critical in the dopaminergic, glutamatergic and cholinergic neurons for normal learning and memory in worms (see Table 1). However, when the *daf-2* siRNA was targeted against the GABAergic neurons (see Table1), worms displayed normal memory, suggesting that the insulin pathway in these neurons has a limited role in the learning and memory processes. This study will facilitate elucidation of the precise neuronal connectomes involved in adaptive learning in C. *elegans*.

Conclusion

The present study demonstrates that insulin signaling is crucial in olfactory learning and memory. Downregulation of insulin pathway and excess insulin alter the learning and memory in C. *elegans* in a differential manner. On the one hand, post-dauers with developmental history of downregulated insulin pathway show defects in short-term memory retention and defective long-term memory. On the other, insulin ligand and receptor mutants show extended memory. The observations that post-dauers have low memory retention highlight the importance of insulin signaling in the cognitive development of an organism during its early developmental period. Similar observations have also been made in diabetic human children (Kail *et al.*, 2000). It was reported that children who develop diabetes before 5 years of age are more prone to cognitive deficits at later stages than those who develop diabetes after 5 years. These observations and our results suggest a possible role of the insulin pathway in early synaptogenesis.

Insulin pathway mutants, *ins-1* and *daf-2*, exhibited extended memory, whereas the mutations in the downstream *age-1*, *akt-1*, and *daf-16* did not affect short-term memory. *age-1* and *akt-1* mutants did not exhibit learning defects after long-term training. However, this learning was impaired in *daf-16* mutants. These observations can be attributed to the necessity of transcriptional machinery for long-term memory consolidation but not short-term memory (Kauffman *et al.*, 2010); (Stein and Murphy, 2014). While extended memory of *daf-2* mutants has been reported earlier in the butanone-associated learning paradigm (Kauffman *et al.*, 2010), my study shows *ins-1* mutation also can result in extended memory retention. Exogenous insulin and its role in learning, *daf-16* nuclear localisation, and local search behaviour demonstrate that excess insulin antagonises the endogenous insulin pathway. These results explains the extended memory retention observed in WT worms trained in the

presence of excess insulin. Thus, this work clearly illustrates that the absence of insulin signaling and excess insulin creates abnormal extended memory retention in C. *elegans*.

The observations that insulin could mimic reward signals similar to DA lead to further research into the possible crosstalk between these two systems in maintaining normal learning and memory. Reports on possible dialogues between insulin and DA have been discussed previously (Nash, 2017b). Increased calcium transients in the DA neurons in response to exogenous insulin observed in this present study indicate the relationship between insulin and DA signaling. The extended memory observed in response to altered insulin signaling is also corroborated in the calcium imaging of DA neurons in the 3rd hour after training. The current study demonstrates a non-reciprocal relationship between DA and insulin signaling in the context of learning and memory. Additionally, the present study strongly suggests that DA functions downstream of insulin in learning and memory.

RNAi was carried out against the *daf-2* gene in neurotransmitter-specific neurons to understand the role of other neurotransmitters in learning and memory concerning altered insulin signaling. In contrast, knockdown of the *daf-2* gene in GABAergic neurons did not affect memory but resulted in extended memory in the cases of dopaminergic, glutamatergic, and cholinergic neurons. These results substantiate the importance of insulin signaling in these individual neurons in learning and memory processes.

Based on these results, further investigations should be considered on the effect of insulin on other neurotransmitter systems and the role of these relationships in learning and memory archetypes. The work also leans towards the extrasynaptic model of functional connectomes. The role of neurons other than the RID neurons discussed in this work also needs to be studied in future work. While the research limits the generalisability of the result due to the analysis of

only one type of learning paradigm, it provides new insights into the role of insulin signaling in the learning and memory of C. *elegans*. In summary, the present thesis research clearly and concisely addressed and validated the importance of insulin signaling on learning and memory by using transgenic strains and exogenous insulin. Further studies should be carried out to understand the implications of these results in other vertebrate model systems.

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Annexure

Appendices

1. Construction of plasmid vector for creating siRNA mediated silencing of daf-2 mRNA

- **The mRNA sequence taken for generation of primers for *daf-2* silencing. Genbank Accession ID: AY382557.2**

- **Primers used for the amplification of 357 bp region of *daf-2* gene.**

DAF-2_ Fwd	ATTTGCGAACATTCACACGA
DAF-2_ Rev	GCTGTGATGCTCACGTTGAT

- **PCR amplified product of 357 bp. The PCR product is gel purified using Qiagen Gel purification Kit (Cat No./ID: 28115).**

Amplicon:

ATTTGCGAACATTCACACGAtcaccggctacctgttggtacgtcaatcgtcaccgtttatctcgttgaacat
gttccggaatttacgacgtattgaggcaaagtcactgttcagaaatctatatgctatcacagtttttgaaaatccgaatttta
aaaaagctattcgattcaacgacggatttgacgcttgatcgtggaactgtgtcaattgccaataacaagatgttatgctt
caagtatatcaagcagctaatgtcaaagttaaatataccactcgatccgatagatcaatcagaagggacaaatggtg
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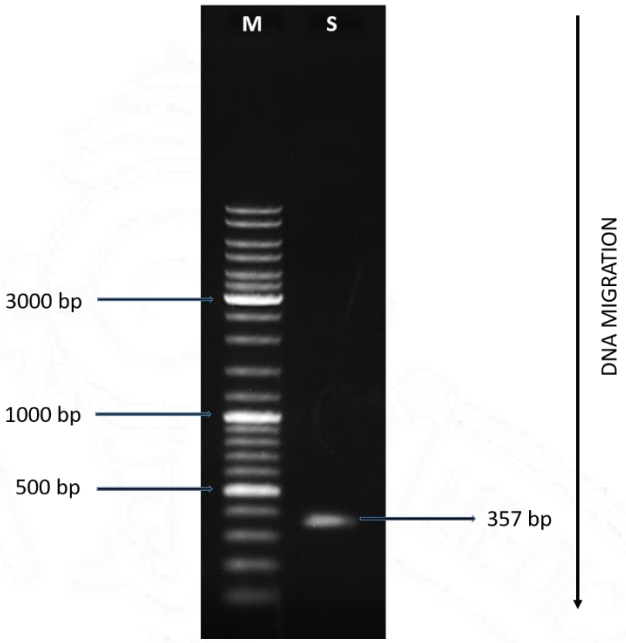
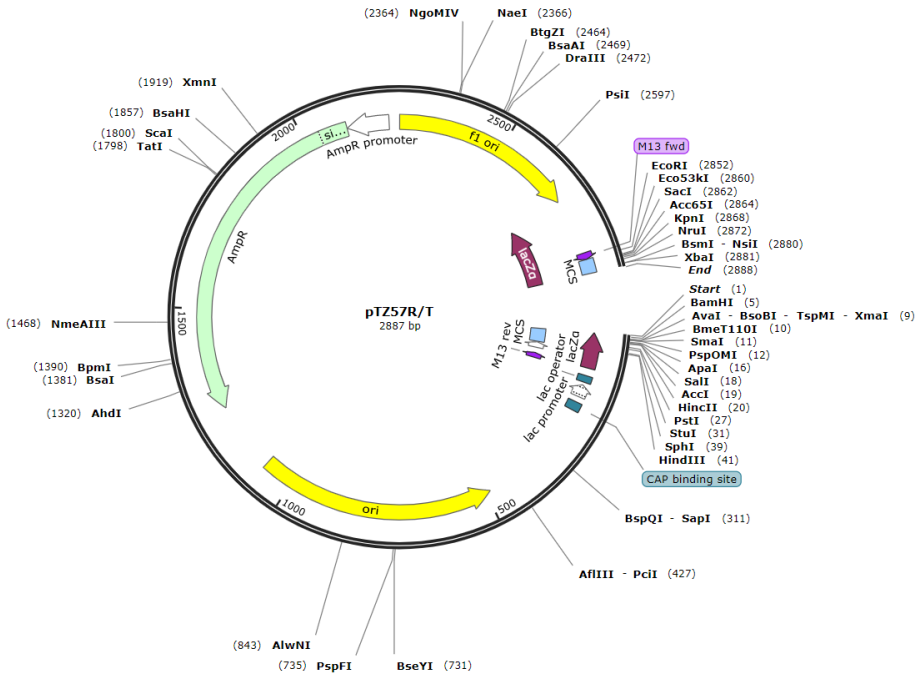
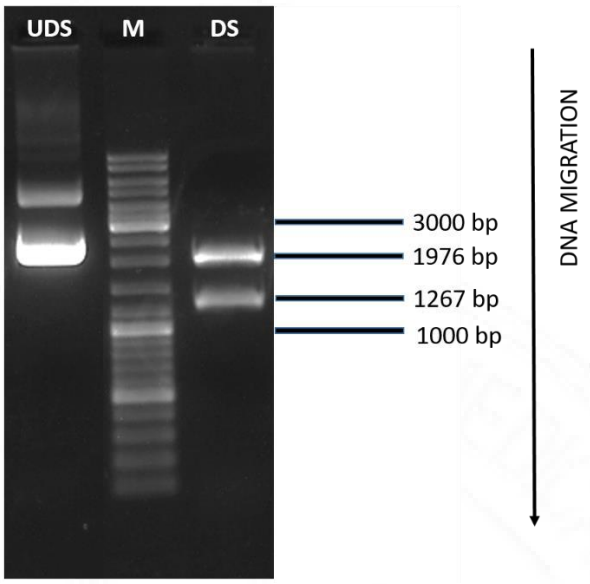


M- marker; **S-** sample

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

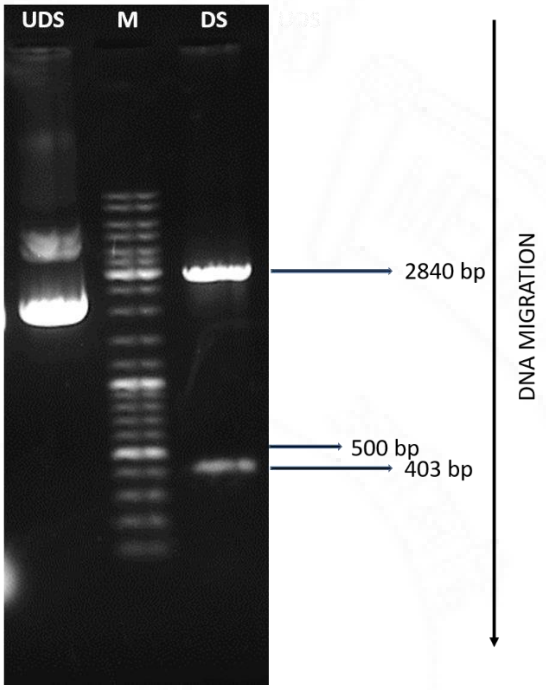


❖ **pTZ::daf-2 digestion with BglI restriction enzyme.**



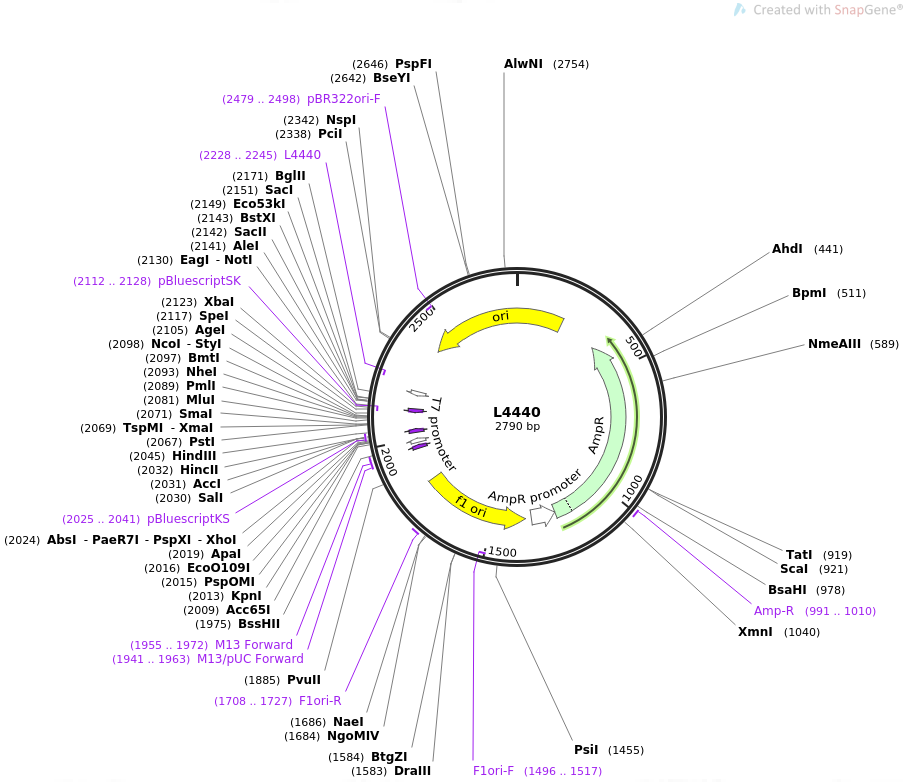
UDS- Undigested sample**; M-** Marker**; DS-** Digested sample

- **The plasmid pTZ::daf-2 was then double digested using restriction enzymes Xba-1 and HindIII to release the daf-2 gene fragment. The expected size of the daf-2 gene fragment is 403 bp and 2840 bp. The daf-2 gene fragment (403 bp) was gel purified using Qiagen Gel purification Kit (Cat No./ID: 28115).**

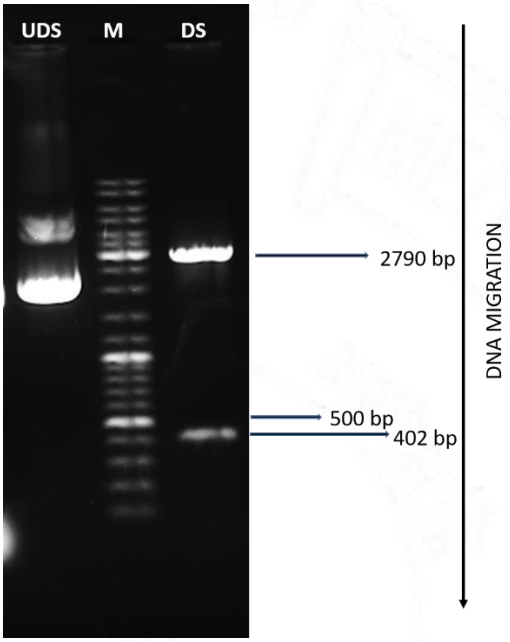


UDS- Undigested sample**; M-** Marker**; DS-** Digested sample

❖ **The plasmid pL4440 was used to express dsRNA of daf-2 gene**

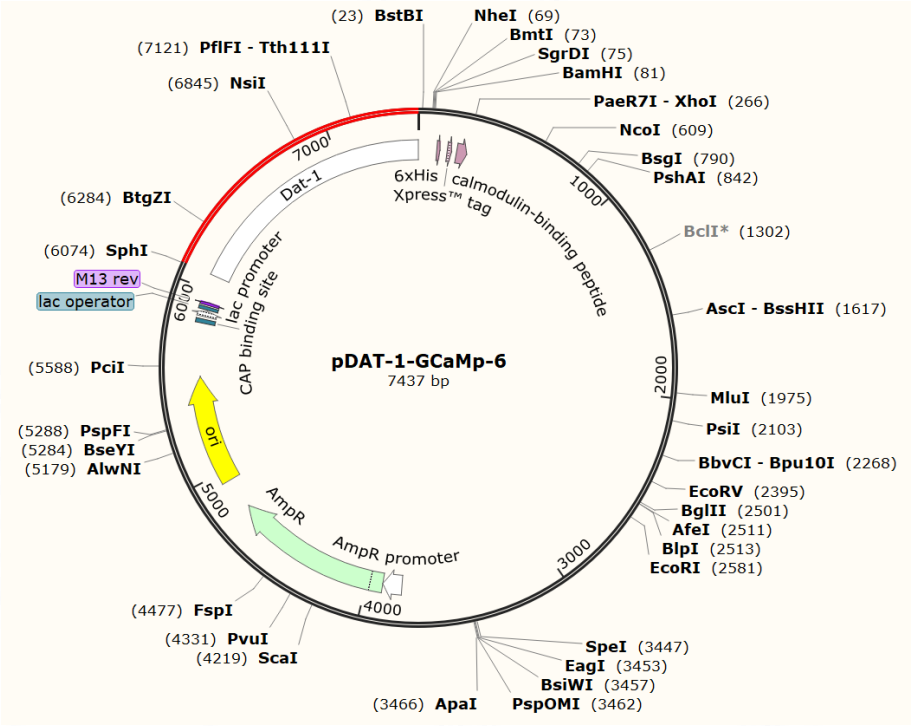


- **The daf-2 gene fragment was cloned pL4440 plasmid that was digested using restriction enzymes, Xba-1 and HindIII. The resultant plasmid pL::daf-2 was confirmed using double digestion with Xba-1 and HindIII restriction enzymes. This pL::daf-2 plasmid were used for the siRNA mediated silencing of daf-2 gene.**



UDS- Undigested sample**; M-** Marker**; DS-** Digested sample

2. Plasmid used for the microinjection to create the transgenic strain AT7438 (daf-2; dat-1p::GCaMP-6)



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