Roles of voltage-gated Shal/ Kv4 ion channels in *D. melanogaster* life span, motor activity, learning and memory

Ph.D. Dissertation

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Introduction

A living organism like human being contains trillions of cells, and each cell is surrounded by membrane which forms a physical barrier to retain the cell components such as nucleus, mitochondria, endoplasmatic reticulum, Golgi apparatus and lysosome. The membrane also allows the environment inside a cell to be regulated so that conditions are ideal for cell to grow and reproduce, maintain their structures, and respond to their environment. In order to maintain an optimal environment inside a cell, charged molecules such as Sodium (Na^+), Potassium (K^+), Calcium (Ca^{2+}) and Chlorine (Cl^-) have to move in and out of the cell through specialized pores or ion channels.

Ion channels are integrated membrane proteins. Although they are present in the membrane of all cells, ion channels have been well studied in nerve and muscle cells, where their regulation is responsible for the transmission of electrical signals. Depending on gating, i.e. what stimulates to open and close, ion channels are grouped into two classes: the ligand gated ion channels and voltage gated ion channels. The ligand gated ion channels such as the nicotinic acetylcholine receptor, gamma aminobutyric acid (GABA), and glycine receptor are regulated in response to the binding of ligands to the channel. In contrast to ligand gated ion channels, the voltage-gated ion channels are regulated by membrane potential i.e. the voltage difference that exists between the inside and the outside of the cell membrane.

The voltage-gated ion channels include the superfamily Na^+ channel, K^+ channel, Ca^{2+} channel and Cl^- channel. Among voltage-gated ion channels, the K^+ voltage gated ion channels are the most diverse ion channels with more than 60 members, which further divide into 12 distinct subfamilies as a consequence of alternative splicing (Catterall et al, 2007, Coetzee et al, 2006), and this research study aims to investigate one of the voltage gated K^+ ion channel subfamilies, the A- type voltage gated potassium channel (Kv channel) and more

specifically Shal/Kv4 A-type voltage gated potassium channel, by which it plays a role in repetitive behavior such as locomotion as well as longevity and learning and memory.

A-type channel structure, localization, kinetics and function

The A-type voltage-gated channel is the first K^+ ion channel to be identified and cloned by chromosome walking from the behavioral mutant of *Drosophila* line known as *Shaker*. Subsequent to *Shaker (Kv1)* gene cloning and its cDNA isolation other voltage gated K^+ ion channel genes such as *Shab (Kv2), Shal (K4)* and *Shaw (Kv3)* were also cloned from *Drosophila*. While the *Shab (Kv2)* and *Shaw (Kv3)* genes code for a delayed rectifier type potassium channel, the *Shal (K4)* gene encodes for the A-type potassium channel (3, 11, and 19).

The Shal (Kv4) A-type channel which is also known as fast transient, transient outward or rapidly inactivating K^+ channel was first observed by Hagiwara et al (1961) in mollusk neuron. It was later described in detail by Conner and Stevens (1971b) in marine gastropod, *Anisdoris*, and termed as I_A or A-type. It was also characterized in detail by Neher (1971) in snail *Helix Pomatia*, and Thompson (1977) in mollusk Nudibranch *Tritonia diomedia* neuron.

The Shal (Kv4) A-type channel like other voltage-gated ion channels is made of four α-subunits. Each α-subunit is composed of cytoplasmic amino-terminal region, six transmembrane domains (S1-S6) interlinked by intra-and extracellular loops, and the cytoplasmic carboxy- terminal region (8). Similar to other voltage-gated K^+ channels, the amino-terminal region (N terminal) of the Shal (Kv4) channel's α-subunit also comprises a sequence called the T1 domain (tetramerization-1 domain) which plays role in tetramerization of four α-subunits to form a functional Shal (Kv4) channels. At the N-terminus, proximal to T1 domain of Shal (Kv4) α-subunit, there is a region where auxiliary subunits (Kvβ subunits) and K^+ channel interacting proteins (KChIP) bind. At the C-terminal region of the Shal (Kv4) α- subunit there is a motif, the dileucine motif, which is critical for

the I_A channels dendritic localization. Similar to other voltage-gated K^+ channels, the fourth transmembrane domain (S4) of the Shal (Kv4) α-subunit is also composed of positively charged amino acid, arginine or lysine, at every third position of its sequence, and serves as a voltage-sensor by moving the charges across the electric field during voltage-dependent gating (9). The fifth and sixth transmembrane domains of Shal (Kv4) α-subunit are linked by a loop known as the S5-S6 loop or H5 loop or p-loop (pore loop) and form the innermost structure of the channel. Following tetramerization of Shal (Kv4) α- subunits, the pore loops form the pore domain that serves as site for K^+ conductance and selectivity. The S6 domains converge to constrict the end of the channel to provide site for controlling gating of the K^+ channel (10). The six transmembrane domains, S1-S6, which form the "core region" of the Shal (Kv4) channel, are highly conserved structurally and functionally among the animal kingdom, vertebrate and invertebrates. For example, sequence analysis of this core region showed 82% amino acid identity between insect (*Drosophila*) and mammal (rodent) Shal (Kv4) channels. However, there is sequence variability in the amino terminal and carboxyi terminal regions (3, 11-14).

Immunohistochemistry studies have revealed the subcellular localization of the transient A-type channels in both mammal (rat) and insect (*Drosophila*) neuronal and muscle cells. The gene that codes for Shal (Kv4) channel localized at somatodendritic compartmentalized region of a neuron while *Shaker (Kv1)* localized at the axonal region of a neuron (15-18). Diao, Chaufty, Waro, and Tsunoda (2010) also showed Shal (Kv4) A-type channel localization in Drosophila to neural dendrite. *Shaker (Kv4)* has also been identified to localize in the muscle cells (19). The expression of *Shal (Kv4)* and *Shaker (Kv4)* channels at different regions of neuron suggest their distinct role in neural cell activity.

The Shal (Kv4) A-type channel activates rapidly at a subthreshold membrane potential and also inactivates rapidly within tens to hundreds of milliseconds following

depolarization of the cell compared to other voltage gated ion channels. It also recovers from inactivation quickly. Full recovery from inactivation requires repolarization to hyperpolarization potentials, usually more negative than the resting potential (5-7, 20-22, 24, 25). The activity of Shal (Kv4) A-type channels [I_A] can be blocked with the application of drugs or toxins. It is sensitive to organic compound drugs such as 4-amino pyridine (4-AP) and congeners like 3, 4-diaminopyridine (DAP) and catechol (25, 26). These drugs also block the activity of Shaker (Kv1) A-type channel. However, *Shal (Kv4)* can be discriminated against *Shaker (Kv1)* with the application of Shal (Kv4) channel specific peptide toxins, phrixotoxins-1 and –2, derived from the venom of the Chilean copper tarantula (27).

In neuron, the I_A channel plays role in regulating the frequency of repetitive firing i.e. they prolong interval between spikes in such a way that following depolarizing stimulus the I_A current activates but rapidly inactivates so that the cell depolarizes, and then afterhyperpolarizes (AHP), leading to the removal of I_A current inactivation and production of an outward current that slows down the return of membrane potential to the threshold for initiation of another action potential (28-30). In the heart it is responsible for shaping the I_{to} (transient outward K^+ current). The I_{to} also contributes to fast or early phase of action potential repolarization and determines the amplitude and duration of cardiac action potential (31, 32). I_A contribution to repolarization was also shown following intracellular recording from thoracic ganglion neurons and giant fiber nerve of I_A mutant *Drosophila* resulting in cells delayed repolarization which in turn led to prolongation of action potential (33-35). The I_A is also implicated in limiting back propagation of dendritic action potential, and learning and memory (36-38).

A-type ion channel disorders (Channelopathy)

A distinctive characteristic of neurons is they enable to transmit and process information in the form of electrical impulses. However, alteration of the normal function of

membrane neural ion channels result in change of their electrical behavior leading to ion channel disease, channelopathy.

Mutations in A-type channels have been shown to result in neurological disorder. For example, truncation of the Shal (Kv4) A-type protein in mammals has been linked to epilepsy (39, 40). Shal (Kv4) channel has also been associated with chronic pain and heart arrhythmia (23). Functional knockout of I_{to} in mammal has been shown to lead to marked increases in action potential durations and to prolongation of the QT interval (41). Since presenilins (a protein that processes the amyloid precursor protein) interact with KChIP and, intern, with Kv4 channel, Shal (Kv4) has been suspected to play role in Alzheimer's disease. It has also been shown that mutation in male C. *elegans* A-type channel gene, Shl-1, to be the cause for reduction in mating efficiency since the mutant male worms have difficulty in locating the hermaphrodite vulva (42). Elimination of Shal (Kv4) channel from mouse dorsal horn neurons has also been shown to result in an increase in dorsal horn neurons excitability and therefore in enhanced sensitivity to tactile and thermal stimuli ((43).

Drosophila gene mutations such as *ether a go-go* (*eag*) gene, not an A-type channel but its subunit contributes to voltage-dependent K^+ channels, and *Shaker* gene were identified on the basis of flies' leg-shaking behavioral phenotype while etherized. Voltage-clamp experiments by Trout and Kaplan (1970) revealed that disruption of *Shaker* gene in *Drosophila* to result in change in A-type channel (Kv1) function. Jan et al (1977) and Ganetzky and Wu (1982, 1985) studies on both *Shaker* and *ether a go-go* mutant *Drosophila* have revealed that mutations in these genes to cause prolongation of a nerve to repolarize. Mutant adult Shaker (Kv1) flies such as Sh^{14} and Sh^5 not only shake their legs, but also scissor their wings and shake their bodies vigorously while etherized (48). The above information clearly suggest the involvement of A- type channels in different physiological processes in different organisms ranging from worm to fly to mammals. Since disruption of

Shal (Kv4) A- type channel function impacts signaling, we hypothesize that manipulation of *Drosophila* Shal (Kv4) A- type channel might result in change in neural activity which in turn leads to expression of Shal (Kv4) mutant behavioral phenotype.

A-type channels role in motor activity (locomotion)

Rhythmic movements such as walking, crawling, respiration, chewing, swimming, grooming and scratching are cyclic, and involve repetitive movement of muscles (29). Neural networks underlying the generation of a coordinated rhythmic motor patterns have been identified and characterized in detail in different organisms. It has also been proposed that generation of these rhythmic behaviors depend on the intrinsic electrical properties of neurons that form the neural network (50, 51).

Kuenzi, F and Dale, N. (1998) were able to record disruption in swimming pattern and abolishment of swimming activity in *Xenopus* embryo after blocking the K^+ current with dendrotoxin indicating the role I_{KS} current plays in motor pattern generation in *Xenopus* embryo. Hess, D. and El Manira (2001) also recorded change (an increase) in the frequency of locomotor rhythm and an irregular cycle duration of an alternating ventral root bursts in lamprey's spinal cord neuron following the blockade of the transient A-type K^+ current with catacol suggesting that the I_A current plays significant role in the production of coordinated locomotor behavior in lamprey. Following prolonged repeated current injections into Shal (Kv4) dominant negative mutant cells, our colleague Ping (Ping, Waro et al, 2011), has recorded a decrement in action potential firing that followed by fast adaptation. This fast action potential firing adaptation is most likely the result of over-excitation of mutant cells and suggests Shal (Kv4) channel's requirement for maintaining repetitive firing. Since our electrophysiological work on Shal/ Kv4 dominant negative *Drosophila* lines have shown defects in neural repetitive firing and normal rhythmic activities depend on maintained

repetitive firing of neurons, we propose that Shal (Kv4) mutation affects *Drosophila* larval and adult fly rhythmic behavior such as crawling and adult flies wall climbing.

A-type channels' impact on insect developmental process and life span

Development of an organism begins with fertilization followed by series of changes in the egg. For instance, *Drosophiala* egg cultured at 25 ^{0}C undergoes two more developmental stages, larva and pupa, before it emerges as an adult fly ten days after fertilization. During the developmental process, the zygotic nucleus of a fertilized egg of *Drosophila* subjected to series of nine divisions to form a multinucleated syncytial blastoderm while they migrate to the periphery and continue to divide four more times to form the cellular blastoderm. Immediately after cellularization gastrulation establishes. Twenty-four hours following fertilization the embryo hatches out as 1st instar larva. Due to an increase in cell size the larva continues to increase in size and then molts to 2nd and 3rd instar larva. During pupa stage some of the larval structures will lyse and a cluster of undifferentiated epithelial cells known as imaginal discs differentiate in order to form the structure of the adult fly. Normally, the life cycle from egg to adult stage in wild type *Drosophila*, Oregon-R or Canton-S, takes about 8-10 days at 25 ^{0}C and 60% humidity chamber. It has been reported that a wild type fruit flies can live up to 60 days; however on average they can live up to 30 to 35 days.

Genetic alteration of ion channel has been shown to influence the development of an organism. For example, mutation in structurally voltage-gated potassium channel related gene in *Drosophila*, *dKCNQ* also referred as Kvα7 or KvLQT gene, has been shown to affect the developmental process of an embryo. Embryos from homozygous *dKCNQ* deletion females failed to hatch into larvae, i.e. embryo lethal (55). Therefore, the result suggests that in *Drosophila* the *dKCNQ* voltage gated ion channel is necessary during embryonic developmental process. Mutation in neural cell components, ion channels, has also been implicated in affecting the longevity of an organism. Trout and Kaplan (1970) have described mutations that affect

Drosophila Shaker (Kv1) gene to result in abnormally active flies while they are awake and enhanced sensitivity to environmental stimuli such as movement of other flies. These abnormal physical activities of *Shaker* mutant flies suspected to result in an increase in metabolic rate that in turn resulted in shorter life span than wild type flies. They also described the effect of *eag* gene mutation to result in earlier death of *Drosophila* line. Tim Fergestad et al (56) also described the genetic interaction of *Shaker-eag* ($Sh^{133}eag^1$) double mutation to display *Drosophila* with severe shaking phenotype as well as an uncoordinated movement with age as a consequence of severe motor defect that in turn lead to shorter life span than in either single mutant.

While culturing I observed change in the Shal/Kv4 dominant negative lines life span and a follow up work using electrophysiology also showed change in excitability, we hypothesize that DNKv4 mutation might have effect on the longevity of DNKv4 mutant lines.

A-type ion channels role in learning and memory

In order to survive, living organisms like insects and other animals have to learn, remember and discriminate distinct environmental cues associated with harm or reward, and ion channels have been proposed to play a role in this process.

Early studies on food-deprived blowfly (*pheormia regina)* have shown that repeated application of sugar solution (chemostimulus) on the tarsi has resulted in conditioned proboscis extension to water (Nelson, 1971). It had also been shown that *Drosophila* learns many cues. The studies by Quinn et al (1974) and Dudai (1977) on *Drosophila* larvae and adult flies have shown that both larvae and adult flies learn quickly and remember to avoid a specific odor after it has been paired with electric shock. Tempel and co-workers (1983) have also reported on *Drosophila* larvae and adult flies abilities to learn to approach an odor that has previously been paired with sugar. An odor shock paired associative learning studies by Quinn and colleague (1974) and Dudai et al (1976) have also revealed *Drosophila* mutant lines such as *dunce, rutabaga and amnesic* inability to learn and remember to avoid the odorant

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paired with an electric shock. Follow up studies on these lines linked the learning defect to mutations in these specific genes that play role in biochemical signaling cascade (62-64).

Alteration of neural membrane ion channels activity has been proposed to alter organisms' learning and memory behavior. Following a repeated light and rotation conditioning (associative learning) experiment on mollusk *Hermissenda* (sea slug), Alkon et al (1982) reported an increase in the latency of sea slug movement towards light (phototaxis) as a consequence of an increase in the excitability (enhanced depolarization) of type B photoreceptor cells caused by a decrease in the A-type K^+ current. Cowan and Siegel (1984) have shown disruption in courtship behavior (associative learning behavior) in *Drosophila* because of change in the A- type K^+ channel, *Shaker*. For example, when a wild type male fruit fly is introduced with a previously mated female, he rejected to court. However, Sh^5 mutant fruit flies have failed to depress the level of courtship activity with virgin females as a consequence of prior courtship experience (conditioning) with fertilized females. *Drosophila* mutant for *Shaker* gene, Sh^5, has also failed in associative olfactory learning (Cowan and Siegel, 1986). During Morris water maze task *Shal/Kv4* knocked out mice have shown defect in learning and memory (Lockridge and Yuan (2010) and Joaquin et al (2012). These behavioral changes both in sea slug, *Drosophila* and mammal suggest that K^+ ion channel is essential for normal learning and/or memory process. Although A-type *Shal/Kv4* gene localization is somatodendric, we hypothesize that change in its activity in *Drosophila* also might exhibit associative learning and memory defect.

Learning and memory site in *Drosophila*

Since a variety of sensory information (input) from sensory systems converges on insects mushroom bodies (cluster of ~ 2500 Kenyon neural cells), where their perikarya (cell body) is located in the dorsal and posterior cortex of the brain lobe while extending their dendrite into the calyx and splitting their axons further into α and β / γ lobes to the anterior,

have been considered as a site for learning and memory. Study by Erber and Menzel (1980) have reported that cooling of honeybee's mushroom bodies after olfactory reward conditioning resulted in memory loss. Mushroom body structural mutations in *Drosophila* such as *mushroom body miniature* and *mushroom body defect* have been shown to result in defect in negatively reinforced olfactory learning (Heisenberg et al, 1985). Ablation of mushroom bodies from newly hatched *Drosophila* larvae by feeding hydroxyurea (HU) resulted in failure of adult flies to perform in classical conditioning paradigm that tests associative learning of odor cues and electric shock (deBelle and Heisenberg, 1994). Taken all together, both learning defect and memory loss in flies indicate that mushroom body as a site for learning and memory processes.

A proper distribution and localization of ion channels govern the normal neural function including the mushroom body. Immunological study by Schwarz et al (1990) have shown expression of *Drosophila* voltage- gated K^+ channels, *Shaker,* in the α, β / γ lobes of the mushroom body indicating its physiological role in learning and memory. Experiment by Cowan and Siegel (1986) revealed that a *Drosophila* mutant for *Shaker* gene, *Sh^5*, has failed to learn and remember to avoid an odor that was previously paired with electric shock indicating the requirement of functional integrity of ion channels for learning and memory. Electrophysiological and pharmacological experiments by Gasque et al (2005) on *Drosophila* mushroom body have revealed the expression of I_A currents by both Shal and Shaker channels. However, *Shal* channels are the main contributors to the somatic A- type current. Since *Shal* also localizes in mushroom body, we predict that genetic alteration of its function might result in defect in *Drosophila* associative learning and memory.

Objective

Ion channels are membrane spanning pore forming proteins. Among ion channels, the voltage gated K+ ion channel is the most abundant and diverse ion channel. In mammals, the K^+ ion channel family which further divides into subfamilies is encoded by more than 60 different genes. The subfamily of A- type K^+ channel, for example in mouse visual cortex, is encoded by three genes, Kv1.4, Kv4.2, and Kv4.3. In contrast to mammals *Drosophila* has four subfamily of K^+ channel. Two of these subfamilies, Kv1.4 and Kv4.2, each with a single member encode the A- type channels. The A- type channels regulate action potential repolarization, onset of neural firing, the length of interspike interval, neural repetitive firing and dendritic action potential back propagation. They are also suggested to play role in rhythmic behavior such as locomotion, longevity and synaptic plasticity a mechanism that underlying the induction of long lasting potentation (LTP). Since the *Drosophila's* motoneurons and projections to their target muscle, and its learning and memory center in the brain (mushroom bodies) and its neural cells input and output is well established, it serves as a good model organism to unravel the neural transient A- type channel's role it plays in organism's rhythmic behavior such as locomotion, grooming, and learning and memory. In this study, the goal is to carry out a comprehensive examination of the role of one of the I_A currents, Shal/kv4 related, found in *Drosophila* neuron following generation of *Shal/ Kv4* transgenic lines on which the channel's pore forming α- subunit is mutated. Since this mutant α- subunit forms tetramer only with its subfamily of α- subunits, it hampers the channel function Dominant-Negatively and therefore allows us to examine how the loss of I_A affects the behavior of *Drosophila*. With these studies, we aim to gain a better understanding of the role of Shal/Kv4 ion channel in rhythmic behavior, longevity and learning/ memory in *Drosophila*.

Abbreviation

AHP	Afterhyperpolarization
AP	Action potential
APS	Ammonium persulphate
cDNA	Complementary deoxyribonucleic acid
DNKv4	Dominant-Negative voltage potassium channel
Elav	embryonic lethal abnormal visual system
GAL	Gene involved in galactose metabolism
HA	Hemagglutinin
kB	kilo Base
kD	kilo Dalton
LB	Luria-Bertani broth
NaCl	Sodium Chloride
NaOAc	Sodium acetate
PBS	phosphate buffer solution
S.cerevisiae	Saccharomyces cerevisiae
SDS-PAGE	Sodium Dedocyl Sulphate- polyacrylamide gel electrophoresis
SM Cy	Second Multiple Curly
SOB	Super Optimal Broth
Sp	Sternopleural
TEMED	Tetramethylethylenediamine
TM6B,Hu, Tb	Third Multiple 6 B, Humeral Tubby
UAS	Upstream Activating Sequence

Methods and Materials

Materials

Fly stocks

Canton- S

W^{1118}; +; +

Shaker

UAS-HA-DNKv4

W^{1118}; Sp/ SMCy; hs-Gal4/ TM6B, Hu-Tb

W^{1118}; Sp/ SMCy; MKRS/ TM6B, Hu-Tb

elav-Gal4; Sp/ SMCy; MKRS/ TM6B,Hu-Tb

c164-GAL4:: UAS-HA-DNKv4

109(80)-GAL4:: UAS-HA-DNKv4

RRa-Gal4; Sp/ SMCy; MKRS/ TM6B, Hu-Tb

10% Separation gel	**Stacking gel**
3.33 ml 30% Acryl/Bis	1.5ml 30% Acryl/Bis
2.5 ml 1.5 M Tris Ph= 8.8	2.5 ml 0.5 M Tris Ph= 8.8
100 µl 10% SDS	100 µl 10% SDS
4 ml ddH$_2$O	6 ml ddH$_2$O
10 µl TEMED	10 µl TEMED
100 µl 10% APS	100 µl 10% APS
Western Running Buffer	**Block solution**
100ml 10X SDS	5 gm dry milk
900 ml ddH^{2}O	100 ml PBS- Tween solution

PBS-Tween solution

100ml 1X PBS

500 μl Tween

900 ml ddH$_2$0

Injection buffer

0.1 mM Sodium Phosphate, PH=7.8

5 mM Potassium Chloride (KCl)

Schneider Drosohila Media

18% fetal bovine albumin

100 U/ ml Penicillin

100 μg/ ml Streptomycin

10X PBS

76.1 gm NaCl

18.8 gm sodium phosphate, dibasic

4.1 gm sodium phosphate, monobasic

1 L dH$_2$O

4X SDS sample buffer

5 ml 0.5M Tris Ph= 6.8

400μl 10% SDS

4ml glycerol

0.25 mg Bromphenol blue

660 mg Dithiothreitol (DDT)

10 ml dH$_2$0

10X SDS running buffer

60 gm Tris base

288 gm Glycine

20 gm SDS

2 L dH$_2$0

Western Transfer Buffer

45 gm Glycine

9.69 gm Tris base

800 ml methanol

4 L dH$_2$O

50X TAE

48.4 gm Tris base

11.4 ml acetic acid

40 ml 250 mM EDTA

200 ml ddH20 PH=8

Fly food media

Corn meal

Agar

Dry yeast granule

Molasses

Propionic acid

Tegosept (Methyl 4-hydroxybenzoate)

Water

3M NaOAc

408.3 gm $C_2N_3O_2Na.3H_2O$ (sodium acetate)

1 L ddH2O PH= 5.2

Methods

Insert size determination

A - pUAST-DNKv4 plasmid construct restriction enzyme digestion

To determine the size of DNKv4 α-subunit fragment, 4 μg lyophilized pUAST-DNKV4 plasmid construct was dissolved in 20 μl ddH$_2$O. To set up restriction digestion reaction, 5 μl pUAST-DNKv4 plasmid DNA, 1 μl *Eco*R I (20,000 u/ml) and 1 μl *Xba* I (20,000 u/ml) restriction enzymes (New England Biolabs), 2 μl 10X Buffer 2 (New England Biolabs) was pipette and mixed in 20 μl total volume reaction. The reaction mixture incubated in a 37 ^{0}C water bath overnight.

B - DNKV4 size determination by gel electrophoresis.

Following overnight restriction digestion of pUAST-DNKV4 plasmid construct, a DNA ladder (3 μl) and the digestion products (4μl) were loaded on 0.8% agarose gel and run at 55 mV for 40 min in agarose gel electrophoresis min-gel tank (Bio-Rad laboratories) filled with TAE buffer (1X). Picture of the gel (Bio-Rad laboratories) was then taken and the size of DNKv4 α-subunit fragment was determined by comparing with the bands in the DNA ladder.

Amplification and isolation of plasmid DNA

A - Amplification of pUAST-DNKv4 Plasmid DNA

To amplify pUAST-DNKv4 plasmid DNA, 40 μl *E*. coli DHα5a competent cells and 2 μl pUAST-DNKv4 plasmid DNA construct was transferred to a prechilled 0.1 cm cuvette (Bio-Rad laboratories), mixed gently by pipetting up and down and incubated on ice for 2 min. The cuvette was then placed in an electroporation apparatus and an electric pulse triggered to transform the cells (Bio-Rad laboratories). To revive the cells from shock, 1 ml LB broth was pipetted immediately into the cuvette. The cells were transferred from the cuvette to a sterile Eppendorf tube, cultured at 37 ^{0}C by shaking at 225 RPM for 1 hour and

plated on LB+ Ampicillin (50 μg/ ml, IBI scientific) agar medium and then placed in a 37 ^{0}C incubator to grow overnight.

B - pUAST-DNKv4 Plasmid DNA Miniprep

Among over night grown colonies single pUAST-DNKv4 colonies were selected and inoculated into 5 ml LB+Ampicillin (50 μg/ml, IBI scientific) broth medium and cultured at 37 ^{0}C overnight with shaking at 225-RPM. These overnight-cultured bacteria were then transferred to a 1.5 ml Eppendorf tube and centrifuged for 2 min at 3000 RPM in 4 ^{0}C Eppendorf centrifuge (Brinkman Instruments Inc.). The upper phase solution discarded and the pellets resuspended in 250 μl Buffer P1 (resuspension buffer, Qiagen). Additional 250 μl of Buffer P2 (Lysis buffer, Qiagen) pipetted and mixed thoroughly by inverting the tubes 4-6 times. Another 350 μl of Buffer N3 (neutralization buffer, Qiagen) was pipetted and mixed thoroughly by inverting the tubes 4-6 times. Lysis cells were then centrifuged at 13,000 RPM for 10 min. The supernatant decanted to separate spin columns and centrifuged at 13,000 RPM for 30 sec. The flow-through discarded. To wash the columns, 750 μl of Buffer PE added and centrifuged at 13,000 RPM for 30 sec. The flow-through discarded. The plasmid DNA eluted by pipetting 50 μl of Buffer EB (elution buffer) and centrifuging at 13,000 RPM for 1 min. The size of DNKv4 checked by double digesting the plasmid DNA with *EcoR* I- *Xba* I and running the product in agarose gel electrophoresis min-gel tank filled with TAE buffer (1X).

C - pUAST-DNKv4 and pπ 25.7 wc plasmid DNA Maxiprep

1 - Initiating "Starter culture" for large scale plasmid isolation

Frozen stocks of pUAST-DNKv4 and pπ 25.7 wc ("wing-clipped"▲2-3 transposase) plasmids were thawed. 5 μl plasmid DNA was pipetted from each stock into two separate sterile 10 ml LB+Ampicillin (50 μg/ml, IBI scientific, IA) broth medium and cultured overnight at 37 ^{0}C on 225 RPM shaker as a starter.

2 - pUAST-DNKv4 and pπ 25.7 wc plasmid DNA large scale isolation

100-200 μl of overnight-cultured starter transferred to a 250 ml sterile Erlenmeyer flask filled with 100 ml LB+ Ampicillin (50 μg/ml, IBI scientific, IA) broth medium and cultured at 37 ^{0}C overnight on 225-RPM shaker. The overnight-cultured cells were then transferred to a 50 ml conical centrifuge tubes and centrifuged at 6500 RMP in 4 ^{0}C for 15 min (Beckman Coulter). The upper phase solution was discarded. Pelleted cells were vortexed to resuspend in 10 ml of Buffer P1 (Qiagen). Additional 10 ml of Buffer P2 (Lysis Buffer Qiagen) was pipetted and mixed thoroughly by vortexing the tube 4-6 times. Cells were then incubated at room temperature for 5 min. Another additional 10 ml of chilled (4 ^{0}C) Buffer P3 (neutralization buffer, Qiagen) was pipette and mixed thoroughly by vortexing 4-6 times. Lysis cells were incubated for 20 min on ice and centrifuged for 30 min at 20,000 x g in a 4 ^{0}C centrifuge (Beckman Coulter). The supernatant was loaded on to columns to flow-through by gravitational force. To wash the columns, 30 ml of QC Buffer (wash buffer, Qiagen) pipetted and allowed to flow-through by gravitational force. 15 ml QF Buffer (elution buffer,Qiagen) and 10.5 ml Isopropanol (Sigma) was transferred to the columns to precipitate the plasmid DNA. The flow-through collected in tube and soon after centrifuged at 15, 000 xg in a 4 ^{0}C centrifuge for 30 min. Pellets were washed with 5 ml 70% ethanol and centrifuge in a 4 ^{0}C centrifuge at 15, 000 xg for 10 min. Air dried pellets were resuspended in 100 μl ddH$_2$O and the size of DNKV4 checked by double digestion (*EcoR* I and *Xba* I) and agarose gel electrophoresis.

Microinjection of plasmid DNA into *Drosophila* embryo

A - Preparation of embryos for microinjection

To collect eggs for microinjection, flies with white-eye phenotype (W^{1118}, +, +) were placed on a room temperature pre-warmed apple juice agar plate for 15 min. The synchronized embryos were transferred on to a double-sided Scottish tape which was taped

on glass slide (VWR Inc). The embryos were dechorionated under a stereomicroscope (Olympus SZ60, OOA Corp.) by gently rolling on the double-sided tape with the help of a tip of clean forceps. The dechorionated embryos were aligned in the same orientation where the tail of the embryos could face the microinjection needle, and then desiccated for 10 min in a Petri dish that contained desiccating pellets (anhydrous calcium sulfate, Hammond Drierite Company LTD).

B - pUAST-DNKv4 plasmid DNA preparation for microinjection

Maxipreped 50 μg pUAST-DNKv4 and 15 μg "wing-clipped"▲2-3 transposase plasmid DNA were mixed in a 1.5 ml Eppendorf tube, precipitated in 1/10 volume of 3M NaOAc PH= 5.2 and 2 volume of 100% cold ethanol for 30 min in -80 ^{0}C refrigerator and centrifuged for 20 min at 14,000 RPM in 4 ^{0}C bench top Eppendorf centrifuge (Brinkman Instruments Inc.). The pellet was washed with 500 μl 70% cold ethanol and centrifuge for 5 min at 14,000 RPM in 4 ^{0}C bench top Eppendorf centrifuge. The pellet air dried for 5 min. and resuspended with 50 μl injection buffer dyed with 1 μl green food coloring dye.

C - Embryo microinjection

In order to microinject, the desiccated embryos were covered with halocarbon oil (Sigma) and then placed on the stage of microinjection microscope. A glass microinjection needle filled with a mixture of green-dyed pUAST-DNKv4 and "wing-clipped"▲2-3 transposase plasmids (2 μl) was hooked to the injector. The tip of the needle was broken by pressing gently against the edge of the microscope stage. To inject the plasmid, the stage of the microscope was moved slowly towards the tip of the broken needle until the tail of the embryo penetrated by the needle. Once the tip of the needle was confirmed to be inside the embryo, the microinjector's "push" button was triggered to apply pressure on the plasmid DNAs so that the plasmids could flow into the embryo. The microinjection needle was then withdrawn quickly from the embryo once the green dye was traced in the embryo. The remaining aligned

embryos were also injected one after the other in a similar manner. The microinjected embryos were then kept in 18 ^{0}C and 60% humid chamber to develop. First instar larvae were collected every day and transfer into *Drosophila* instant media (Carolina Biological Supply Company, NC). Immerging adult flies were collected every day for further studies.

Protein purification and SDS- PAGE analysis

A - Establishing *elav-GAL4 :: UAS-HA-DNKv4* stock.

To establish transgenic lines that express DNKv4 α-subunit ectopically, the GAL/UAS system developed by Brand and Perrimon was adapted. Virgin double-balanced lines with elav-GAL4 insertion on X- chromosome were crossed to double balanced UAS-HA-DNKv4 transgenic lines. F1 male progenies with 2nd or 3rd DNKv4 insertion, *elav-GAL4* / Y; UAS-HA-DNKv4/CyO; MKRS/TM6B, Hu-Tb and *elav-GAL4* / Y; Sp / CyO; UAS-HA-DNKv4/ TM6B, Hu-Tb, were back crossed to elav-GAL4 double balanced lines to establish a stock.

B - Purification of DNKv4 protein from *elav-GAL4 :: UAS-HA-DNKv4* stock

To purify DNKv4 protein, a total of four heads were severed from F1 male transient *elav-GAL4::UAS-HA-DNKv4* transgenic line and sonicated in 20 μl 2X SDS sample buffer in a 200 μl Eppendorf tube. The homogenate was centrifuged at 14,000 RPM in Eppendorf (5417C) bench top centrifuge for 2 min. The supernatant was transferred to new 200 μl Eppendorf tube and placed in –20 ^{0}C until SDS-PAGE gel was ready.

C - Casting SDS-PAGE gel and DNKv4 protein analysis

To cast SDS-PAGE gel, first 10% separation gel (Bio-Rad labs) was prepared, poured between two glass plates (Bio-Rad lab) that were clamped with clamper. Following polymerization of the separation gel, 10% staking gel (Bio-Rad labs) was prepared and poured on top of the separating gel. A 1.5 mm 10 wells comb (Bio-Rad Lab) was placed immediately on top of the staking gel and allowed polymerizing for 30 min. Shortly, the

casted gel was placed into a buffer tank (Bio-Rad lab) filled with 10X SDS running buffer and then comb removed from the staking gel by pulling. Precision standard protein molecular weight marker (7 μl) and DNKv4 protein samples (18 μl) were loaded into the wells and 180 V was applied for 30 min to run the samples. Following protein electrophoresis, the glass plates casting the gel were removed from the buffer tank and separated from each other with the help of spatula (Bio-Rad lab). The gel was then transferred from the 1.5 mm glass plate to Whatman 3MM blotting paper (VWR Inc). A 0.45 μm thick nitrocellulose membrane (PerkinElmer) was placed on top of the gel. On top of the nitrocellulose membrane another Whatman 3MM blotting paper was placed. To transfer the protein, the sandwiched nitrocellulose membrane was transferred to a gel holder cassette (Bio-Rad), immersed in to a buffer tank filled with Western transfer buffer and 100 V applied for one hour.

D - **DNKv4 protein hybridization and detection**

In order to detect the DNKv4 protein, the nitrocellulose membrane was removed from the sandwich, placed in a plastic dish and blocked with blocking solution for 20 min at room temperature by swirling on nutator. The nitrocellulose membrane was then transferred to a new plastic bag and probed with monoclonal anti-HA: 11 primary antibodies (1 μl antibody to 500 μl block solution, Covance Research Product) at room temperature overnight on rocker. Following overnight incubation, the nitrocellulose membrane was washed 5X for 5 min with PBS-Tween solution and transferred into a new plastic bag to hybridize with Peroxidase- Conjugated Affinitive Goat Anti-mouse IgG secondary antibody (1 μl secondary antibody to 5000 μl block solution, Jackson Immuno Research Lab) at room temperature on a rocker for 1 hour. The nitrocellulose membrane was then washed 5X for 5 min with PBS-Tween solution and stained with a mixture of 500 μl SuperSignal west and 500 μl pico stable peroxide substrate in 12 ml total volume for 1 min (Thermo Scientific). The Supersignal solution was discarded. The nitrocellulose membrane was wrapped with Saran wrap and

placed in a photographic film-developing cassette. In a dark photo lab an X-omat photographic film (Kodak) was placed on top of the Saran wrapped nitrocellulose membrane and exposed for 30 sec and developed for 2 min.

Embryonic cells immunostaining

Adult *elav-Gal4:: UAS-DNKv4* flies were placed on room temperature preheated apple juice agar plate to lay egg. Synchronized 5 hours old (developmentally stage 9-10) single *elav-Gal4:: UAS-DNKv4* embryo's vitelline membrane was broken with a sharp broken tip of borosilicate glass micropipette (Sutter Instruments Co). The content of the embryo was removed by mouth suction using the same glass micropipette and transferred onto glass cover slips to dissociate in 20 μl Schneider *Drosophila* culture media. The cells were placed to grow in a 60% humidified chamber at room temperature for about 7 days and then fixed for 10 min with 4% formaldehyde in PBS. Following fixation the embryonic cell cultures were incubated overnight at 4 ^{0}C with 1:100 ratio of anti α-HA:11 (Covance Research Products, Emeryville, CA) primary antibody. Cultured cells were washed with 0.1% sapponin in PBS and then incubated with 1: 500 ratio of fluorescein isothiocyanate (FITC)–conjugated or rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). To remove the secondary antibody, cells were washed with 0.1% sapponin in PBS and then mounted in 90% glycerol and inspected under fluorescence microscope (Olympus BX51, OOA Corp.).

Longevity test of adult flies

To test the life span of DNKv4 transgenic lines, adult *elav-GAL4::UAS-HA-DNKv4* were cultured in multiple bottles in a 25 ^{0}C and 60% humidity fly incubator (Percival). Hundred freshly hatched adult flies were collected, grouped into ten and transferred to vials that contained fly food media and labeled 1-10. Each group consisting of 10 flies were transferred every five days into new vials until the last fly died. The number survived flies

during transfer to a new vial were recorded. Sigma plot was used to calculate the median survival age of the transgenics.

Embryo cell culture for electrophysiology

Adult *elav-Gal4:: UAS-DNKv4* flies were allowed to lay synchronized egg on apple juice agar plate at room temperature. Developmentally stage 9-10 (5 hours old) single *elav-Gal4:: UAS-DNKv4* embryo's vitelline membrane was broken with the aid of the sharp tip end of a borosilicate capillary glass micropipette (Sutter Instruments Co). Using the same sharp capillary glass micropipette the content of the embryo was removed by mouth suction and transferred onto glass cover slip to dissociate in 20 μl Schneider *Drosophila* culture media. The glass cover slip with embryonic cells was placed in 20 ^{0}C and 60% humidified chamber for about 3- 5 days to age.

Electrophysiological recording of embryonic cell cultured neurons

All whole-cell recording was done in the perforated patch configuration, using 400 μg/ ml Amphotericin-B in the patch pipette. For recording K+ currents, a K-internal solution of 140 mM KCl, 2 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPEs, PH=7.2 and a Choline-external solution of 140 mM Choline-Cl, 2 mM KCl, 6 mM $MgCl_2$, 5 mM HEPEs, PH=7.2 was used. For recording only Na^+ currents, a Cs- internal solution of 140 mM CsCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 1.1 mM EGTA, 10 mM HEPEs, PH=7.2 with NaOH and external solution of 140 mM NaCl, 2 mM CsCl, 6 mM $MgCl_2$, 5 mM HEPEs, PH=7.2 was used. For current-clamp recordings, we used an internal solution of 20 mM K- Gluconate, 20 mM KCl 10 mM HEPEs, 1.1 mM EGTA, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, PH=7.2, and external solution of 140 mMNaCl, 2 mM KCl, 6 mM Mg Cl_2, 5 Mm HEPEs, PH=7.2 used. Electrode resistance for all voltage and current-clamp experiments was 3-8 MΩ. Data were recorded using an Axopatch200B amplifier (Molecular Devices Corp.). Recordings were digitized at 5 kHz and filtered at 2 kHz, using a lowpass Bessel filter.

Shaking behavior analysis of adult flies

Twenty to thirty one day old synchronized adult flies with *UAS-DNKv4#14, elav-Gal4::UAS-DNKv4#14, hs -Gal4:: UAS-DNKv4#14,* Shaker and Canton S genotypes were collected and heat shocked in a 37 ^{0}C incubator for one week for one hour per day. Two hours after the last day of heat shock, day 7, flies were etherized (Sigma) for about 30 sec and monitored under stereomicroscope (Olympus, OOA Corp.) for body or extremities shaking phenotype. Flies degree of shaking were scored. With out prior heat shock treatment flies with the same genotypes were also treated with ether and tested for shaking phenotype.

Larva locomotion assay

Room temperature (21 ^{0}C) raised individual 3rd instar larva of Canton S, *UAS-DNKv4#20, elav-Gal4:: UAS-DNKV4# 20, c164-Gal4:: UAS-DNKv4# 20 and 109(80) - Gal4:: UAS-DNKv4#20* was placed separately at the center of 60 mm 1% agarose plate where a 0.5 x 0.5 cm square paper grid was taped at the bottom. The number of squares crossed by a larva in 5 min period was scored. The test was repeated 15 more times with new individual larva for each genotype. The number of squares crossed by 15 larvae in 5 minutes was averaged and translated into number of squares crossed per minute.

Grooming assay

Twenty to thirty two days old adult Canton S, *UAS-DNKv4#14, elav-Gal4:: UAS-DNKV4-*14 flies were collected and placed into a clean vial that contains 30 mg overnight backed (50 ^{0}C) and mesh filtered Reactive Yellow 86 dust. Ten seconds following dusting, the legs, eyes and Scutoid of each fly line was monitored every 15 min under microscope for their cleanness. The percentage of clean fly population was then calculated.

Locomotion assay of adult flies

A total of 35, two to three days old male Canton S, *UAS-DNKv4-14, elav-Gal4:: UAS-DNKV4* adult flies were placed separately at the bottom of a 12.4 cm tall glass test tube

(VWR international) and given 30 sec to climb to the top of the tube and move into the second test tube inverted on top of the 1st test tube. The flies that moved into the 2nd test tube were allowed to settle down at the bottom of the tube by hitting the test tube against the top of a working bench. In a similar fashion to the 1st test tube the flies were given 30 sec to climb and move into a 3rd tube inverted on top of the 2nd test tube. This process continued for each genotype through ten successive tubes. A total of 10 assays with 35 flies each were performed for each genotype. The flies were each given one point for every two tubes they climbed out of and the mean score of flies from group was recorded.

Larva learning and memory assay

A - Media preparation and gustatory preference test of *elav-Gal4::UAS-HA-DNKv4* lines

For gustatory preference test, 0.5 gm agarose and 18 gm fructose (Sigma) was dissolved in 25 ml H_2O in 100 ml beaker at 100 ^{0}C hot plate stirrer. The solution was then adjusted to 50 ml total volume to make a 2 M fructose in 1% agarose media plate. In another beaker 0.5 gm agarose only was dissolved completely in 25 ml H_2O at 100 ^{0}C hot plate stirrer and also adjusted to 50 ml total volume to make a 1% pure agarose media plate. In order to separate the 1% agarose media from the 2 M fructose media, thin X-ray film was placed at the middle of a 60 mm Petri dish. The 1% agarose media was poured to one-half of the Petri dish. On the other one-half of the Petri dish the 2 M fructose media was also poured. The X-ray film was removed thirty seconds after pouring the two media. The media was then allowed to harden at room temperature. To perform gustatory test, ten 3rd instar larvae of Canton S, background *UAS-HA-DNKv4#14* and *ela-Gal4::UAS-HA-DNKv4#14* genotypes were placed in the middle of separate plates where the two media were divided. Then, the larvae were allowed to crawl freely for 20 min. The number of larvae on each side of the medium was counted and their preference score was calculated as:

Preference = (# of larvae on half side of the plate) - (# of larvae on the other half side of the plate)
Total number of larvae

B - Media preparation and odor preference test of *elav-Gal4::UAS-HA-DNKv4* lines

For odor preference test, 0.5 gm agarose was dissolved completely in 25 ml H_2O by boiling at 100 ^0C hot plate stirrer and then adjusted to 50 ml total volume to make a 1% pure agarose media plate. The agarose solution poured into a 60 mm Petri dish to harden. In a 7 mm plastic cap 10 μl of 1- octanol (Sigma) was pipetted and covered with a perforated parafilm and placed on one side of the plate. In another 7 mm plastic cap 10 μl of Amyl acetate diluted in paraffin oil (1 amyl: 50 paraffin, Sigma) was pipetted, covered with parafilm perforated and placed on the opposite side of the plate. Ten 3rd instar larvae of Canton S, background *UAS-HA-DNKv4#14* and *ela-Gal4::UAS-HA-DNKv4#14* genotypes were placed in the middle of separate media plates and allowed to wonder around freely for 20 min. The number of larvae on each side of plastic cap was counted and their preference score calculated as:

$$\text{Preference} = \frac{(\text{\# of larvae on one side of cap}) - (\text{\# of larvae on the other side of cap})}{\text{Total number of larvae}}$$

C - Associative learning test of *elav-Gal4::UAS-HA-DNKv4* transgenic lines

For odor associated learning test, five age synchronized 3rd instar larvae which were cultured at 25 ^oC and 60% humidity were placed for 1 min on 1% pure agarose media plate where two 7 mm ventilated plastic caps with 10 μl of 1-octanol odorant were planted on the opposite side of the plate. All five larvae were transferred to 1% pure agarose plate to rest for one min and then transferred to 2 M fructose in 1% agarose plate on which two ventilated plastic caps with 10 μl Amyl acetate were positioned at the opposite side of the plate. The larvae were allowed to migrate for 1 min and then transferred again to another 1% pure agarose media plate to rest for 1 min and then transferred to 1% pure agarose media plate where two 7 mm ventilated plastic caps with 10 μl of 1-octanol odorant were planted. Larvae training were repeated for 10 consecutive times. At the end of the 10th cycle larvae were

transferred to a test plate (1% pure agarose only media plate) where ventilated cap with 1-octanol odorant was placed on one side of the plate and another ventilated cap with Amyl acetate odorant was placed on the opposite side of the plate. The larvae were then given 20 min to associate 1- octanol with fructose.

Larvae were also trained to associate Amyl acetate odorant with fructose, reciprocally. A total of 30 larvae batched into six groups were examined form each line, Canton S, UAS-HA-DNKv4#14 and *elav-Gal4:: UASDNKv4#14*, separately. A learning index was calculated taking both amyl acetate and 1-octanol training and performance into account as previously described by Hendel et al (87).

$$\text{Learning index} = \frac{(\text{Amyl acetate Preference}) - (\text{1-octanol preference})}{2}$$

Results

Generation of Shal/Kv4 Dominant-Negative (DNKv4) construct

The normal function of a given gene can be studied following induction of mutation in a given organism by a chemical agent for example ethyl methyl sulfonate (EMS) or methyl ethyl sulfonate (MES) or physical agent like Röntgen (X-rays) or an insertional element (P element and viruses). To investigate the significance of Shal/Kv4 channels function in *Drosophila*, we generated transgenic lines that express the mutant form of Shal/Kv4 α-subunit following *in vitro* substitution of the amino acid tryptophan (W) for the amino acid phenylalanine (F) at position 362 (W362F) in the pore forming region of Shal2 α-subunit cDNA [GenScript, Inc, [Piscataway, NJ] as shown in figure 1.
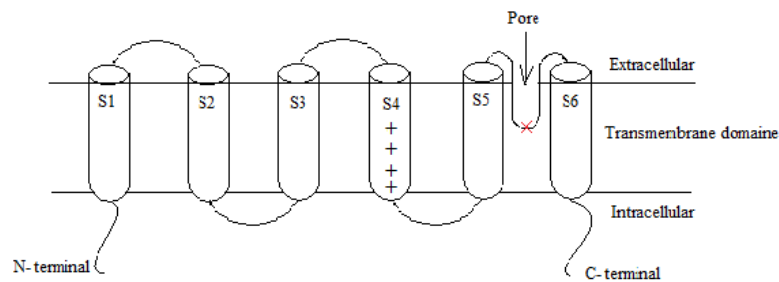
M A S V A A W L P F A R A A A I G W V P I A T H P L P P P P M P K D R R K T D D E K L L I N V S G R R F E T W R N T L E K Y P D T L L G S N E R E F F Y D E DC K E Y F F D R D P D I F R H I L N Y Y R T G K L H Y P K H E C L T S Y D E E L A F F G I M P D V I G D C C Y E D Y R D R K R E N A E R L M D D K L S E N G D Q N L Q O L T N M R Q K M W R A F E N P H T S T S A **L V F Y Y V T G F F I A VS V M A N V V** E T V P C G N R P G R A G T L P C G E R Y K I V **F F C L D T A C V M I F T A E Y L L R L F A** A P D R C K F V R S **V M S I I D V V A I M P Y Y I G L G I T D N** D D V S G A F **V T L** R **V F** R **V F** R **I F K F S** R **H S Q** G L R I L G Y T L K S C A S E **L G F L V F S L A M A I I I F A T V M F Y A** E K N V N G T N F T S I P A A F F Y T I V T M T T L G Y G D M V P E T I A G K **I V G G V C S L S G V L V I A L P V P V I V** S N F S R I Y H Q N Q R A D K R K A Q R K A R L A R I R I A K AS S G A A F V S K K K A A E A R W A A Q E S G I E L D D N Y R D E D I F E L O H H H L L R C L E K T T M U

Figure1. Top: Structure of single Shal/Kv4 α-subunit composed of cytoplasmic N- terminal, six transmembrane domains, pore-loop and cytoplasmic C-terminal regions. Bottom: Shal/Kv4 channel forming transmembrane amino acid sequence. Residue change (F) at position 362 of the pore-forming region (blue) of the Shal α-subunit is highlighted in red. The six transmembrane domains (S1-S6) of the α-subunit are underlined and highlighted in bold. Amino acids highlighted with green color within S4 transmembrane domain indicate

positively charged serine amino acids that serve as voltage sensor. The loop (sequence between S5 and S6) that forms the Shal/Kv4 channel is also highlighted in blue color.

The Shal/Kv4 (W362F) α-subunit functions in a dominant-negative manner following tetramerization and hence hinders the permeation of potassium ions resulting in a non-functional Shal/ Kv4 channel as previously described by Barry (41) This *in vitro* modified Shal2 α-subunit is referred to as DNKv4 α-subunit was constructed and sub-cloned into P element based pUAST transformation vector (GenScript, Inc.) , figure 2A, and therefore GAL/ UAS binary expression system could be applied in order to express the DNKv4 α-subunit in our transgenic *Drosophila* lines (Brand and Perrimon (1993), Fischer et al (1988), Duffy, JB (2002) and Traven et al (2006). Since the DNKv4 α-subunit construct is under the control of yeast (*S. cerevisiae*) *cis-* acting regulatory sequence (DNA sequence serving as binding site) known as *upstream activating sequences (UAS),* our *Drosophila* transgenic lines express the DNKv4 α-subunit ectopically in a tissue-specific and inducible manner in the presence of several types of yeast transcription factor, such as galactose-induced transcription factor,GAL4, as depicted in figure 3A on page 32. In order to detect the localization and expression level of the DNKv4 α-subunit protein in transgenic lines, its N- terminal end was tagged to the N-terminal amino acid sequences of heamagglutinin (HA), YPYDVPDYA, protein resulting in HA-DNKv4 construct.

Generation of *Drosophila* DNKv4 α-subunit transgenic lines

Prior to microinjection of the HA-DNKv4 construct into embryos from a white eyed, w[1118]; +; +, *Drosophila* lines and the generation of *Drosophila* DNKv4 transgenic lines, we first determined the size of the HA-DNKv4 α-subunit cDNA by double digesting the pUAST-HA-DNKv4 plasmid with *Eco*R I-*Hind* III restriction enzymes and DNA gel electrophoresis analysis. More than 1.4 KB fragments detected confirming the size of the fragment as shown in figure 2B below.
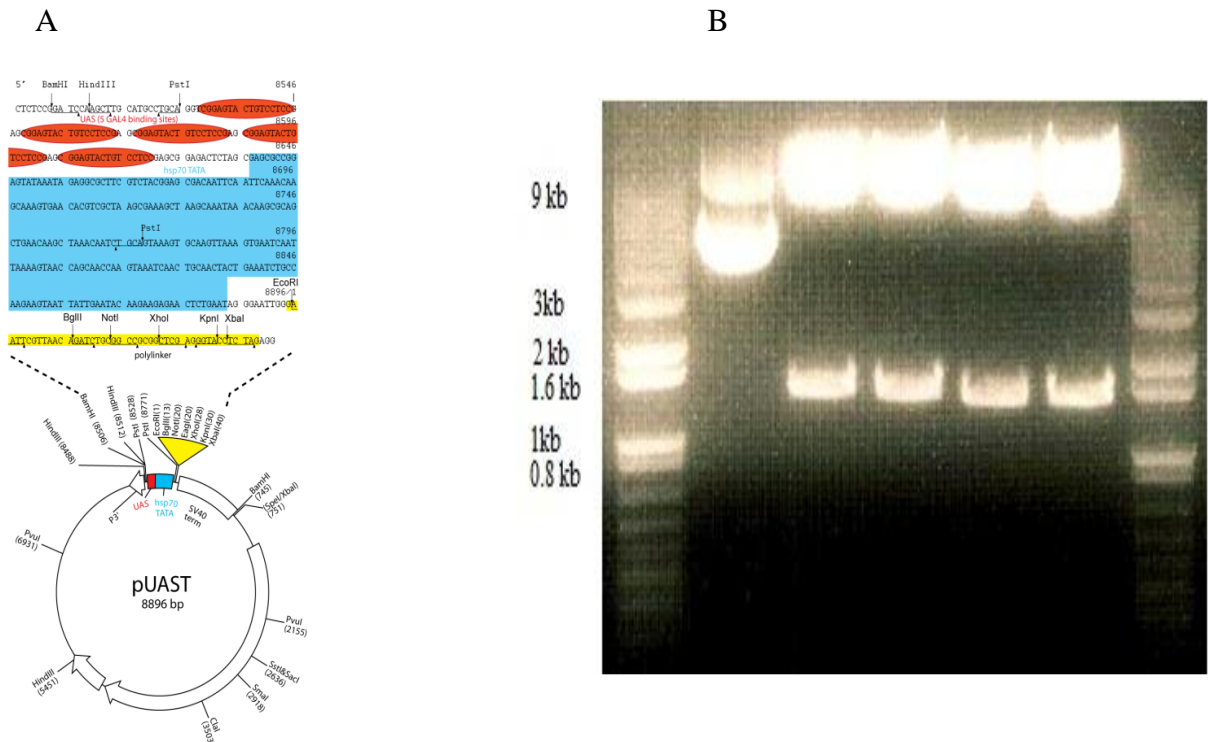
Figure 2. DNKv4 cDNA size determination by gel electrophoresis

The drawing on the left (A) shows the structure of pUAST expression vector on which the UAS sequence and multiple cloning sites is zoomed. The picture on the right (B) indicates the product of *Eco*R I-*Hind* III digested pUAST-HA-DNKv4 plasmid following DNA gel electrophoresis. Lane 1, *Eco*R I and *Hind* III digested λ-DNA marker; lane 2, uncut pUAST-HA-DNKv4 plasmid; lane 3-6, *Eco*R I-*Hind* III double digestion from four single pUAST-HA-DNKv4 plasmid colonies. Note the size of Shal cDNA and pUAST fragments at about 1.5 kb and 9 kb, respectively, after running the digestion product at 50 mV.

To generate DNKv4 transgenic lines, a mixture of pUAST-HA-DNKv4 and *pπ 25.7*wc (wing clipped delta 2-3 transposase) helper plasmid (77) was microinjected into W^{1118}; +; + (white eyed) *Drosophila* embryos. From about five hundred W^{1118}; +; + microinjected embryos, approximately two hundred fifty-five first instar larvae collected. Among these larvae seventy-eight independent *UAS-HA-DNKv4* transgenic mutant flies were identified. Twenty of these were chosen and the *UAS-HA-DNKv4* insertion site was mapped as described briefly in the crossing scheme below and table 1 (see appendix). Of these, two

insertions were mapped to the X-chromosome, seven to the 2nd and another seven to the 3rd, and for the remaining four lines the insertion was mapped both on 2nd and 3rd chromosomes. Chromosomes of these transgenic lines were balanced by crossing to double balanced fly lines for the follow-up studies.

♀♀ $\dfrac{W^{1118}}{W^{1118}}; \dfrac{Sp;}{CyORoi} \dfrac{MKRS}{TM6B,Hu,Tb}$ X $\dfrac{W^{1118}▼;}{Y} \dfrac{p▼}{+}; \dfrac{p▼}{+}$ ♂

F1 ♂ $\dfrac{W^{1118}▼}{Y}; \dfrac{p▼}{CyORoi}; \dfrac{p^{+}▼}{TM6B,Hu,Tb}$ X $\dfrac{W^{1118}}{w^{1118}}; \dfrac{+}{+}; \dfrac{+}{+}$ ♀♀

F2 $\dfrac{W^{1118}}{W^{1118}} \dfrac{p▼}{P▼}; \dfrac{+}{+} \dfrac{±}{TM6B Hu-Tb}$ $\dfrac{±}{CyORoi}; \dfrac{p▼}{+}$ $\dfrac{p▼}{+}; \dfrac{▼}{+}$

1   **2**   **3**   **4**
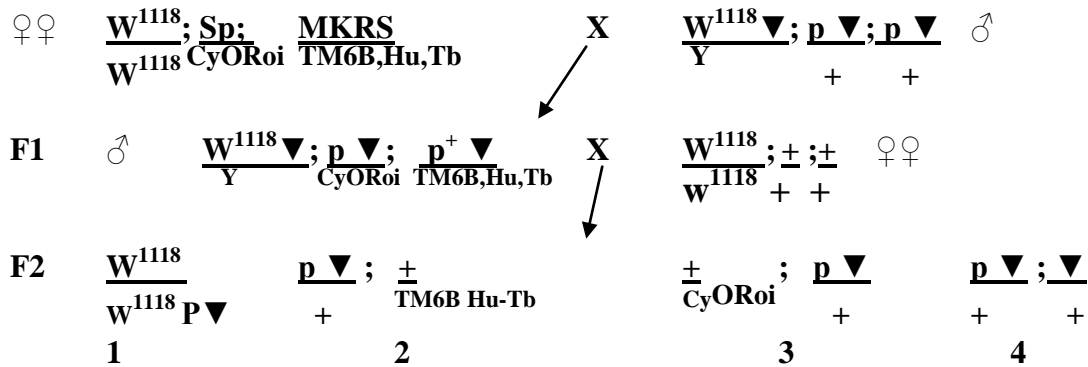
DNKv4 insertion site determination scheme

Four W^{1118} / W^{1118}; Sp/ CyRoi; MKRS/TM6B, Hu,Tb (white-eyed double balanced) virgins were crossed to a single red-eyed *UAS-HA-DNKv4* (p▼) male transformant line. F1 male progeny with insertion on any of the four chromosomes (p▼w^{1118} / Y; p ▼ / CyoRoi; p ▼ / TM6B, Hu, Tb) was crossed to multiple W^{1118} / W^{1118}; +/+; +/+ (white-eyed) virgins. F2 progenies were then scored for red eyed color (p▼) and segregation from dominant markers, Cyo Roi or TM6B, Hu, Tb. If the insertion of p▼ was on the X-chromosome, all female flies scored for red eye while all male flies scored for white eye. Segregation of p▼ from CyoRoi indicated insertion on the 2nd chromosome (2) while segregation of p▼ from TM6B, Hu, Tb indicated insertion on 3rd chromosome (3). If the insertion of p▼ is both on 2nd and 3rd chromosome, the UAS-HA- DNKv4 fails to segregate from either dominant marker (Cy Roi and TM6B, Hu, Tb).

Detection of HA-DNKv4/ Shal protein expression.

Since HA-DNKV4 is placed under the control of the yeast *upstream activating sequences (UAS),* we crossed HA-DNKV4 transformants to another transgenic line where the GAL4 is driven by embryonic lethal abnormal vision promoter, ELAV, to express the HA-DNKv4 α-subunit ectopically in *elav-GAL4::UAS-HA-DNKv4* lines, as demonstrated in figure 3A below. A total of four heads of transient male progenies with *elav-GAL4::UAS-HA-DNKv4* genotype were chopped off from each transgenic lines and tested for HA-DNKv4 expression by Western blot analysis. Of the twenty *elav-GAL4::UAS-HA-DNKv4* transgenic lines tested, two lines (one with an insertion on the 2nd chromosome and labeled as DNKv4#14, and the other on 3rd chromosome and labeled as DNKv4#20) were identified to express HA-DNKv4 α-subunit protein with anti-HA immunoreactivity; the 52 kDa protein, as seen on figure 3B on page 33 is consistent with the expected size of HA-DNKv4.
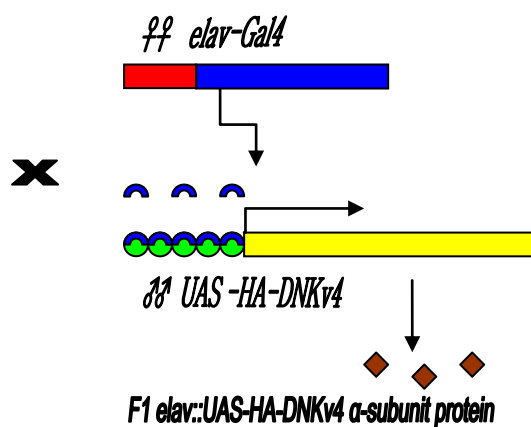
Figure 3A. Gal4/ UAS bipartite system is applied to express and localize HA-DNKv4 α-subunit in the *elav-Gal4::UAS-HA-DNKv4* transgenic lines. The two components, the *UAS-HA-DNKv4*, which is in an inactive state and the *elav-Gal4* (driver) that expresses the Gal4 constitutively in all neurons are maintained in separate parental lines. Following a cross between the two parental lines an ectopic production of dominant-negative HA-DNKv4 α-subunit protein takes place in the elav-*Gal4:: UAS-HA-DNKv4* F1 progenies and in turn inactivate the wild type Kv4 α-subunit activity.
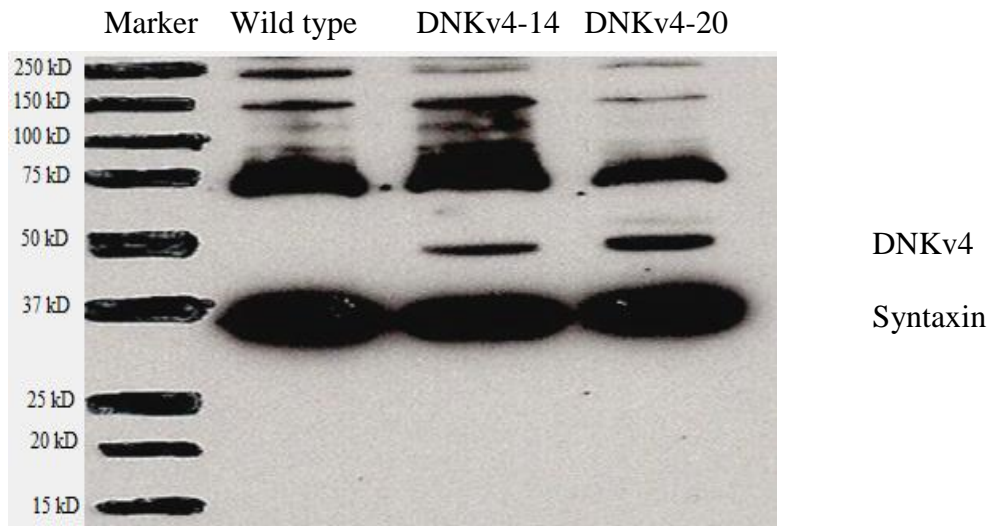
Figure 3B Western blot analysis for DNKv4 α-subunit expression in *elav-Gal4:: UAS-HA-DNKv4* transient transgenic lines. Lane 1, Prestained protein molecular weight marker; Lane 2 Canton S (Control); lane 3 and 4 the two *elav-Gal4:: UAS-HA-DNKv4* lines (#14 and #20) are indicated. Bands with a molecular weight of 52 kDa that correspond to HA- DNKv4 are detected by anti- α-HA.11 antibody. A 37 kD loading control protein (syntaxin) is also indicated.

Verification of DNKv4 α-subunit effect on Shal/Kv4 current

To test the effect of the dominant-negative α-subunit (DNKv4 α-subunit) on inhibiting the wild type Shal current, Drs. Susan Tsunoda and Yong Ping (2011) employed whole-cell voltage-clamp recordings from embryonic neurons expressing HA-DNKv4. As shown in figure 4, depolarization of wild-type embryonic cell cultured neurons to + 50 mV, preceded by a 500 ms hyperpolarized prepulse of –125 mV, evoked total whole cell K^+ current that constitute the Shal/ Kv4 (I_A) and delayed rectifier (I_{DR}) potassium currents. In these wild-type neurons, depolarizing prepulse that precede voltage jump to + 50 mV resulted in inactivation of the Shal/Kv4 current while evoking the delayed rectifier current intact. Subtracting the delayed rectifier K^+ current trace from the total whole cell K^+ current trace isolate the Shal/Kv4 (I_A) current (inset). Similar voltage-clamp recording (to + 50 mV) from

both transgenic lines, *elav-Gal4: UAS-HA- DNKv4 -14 and elav-Gal4: UAS-HA- DNKv4 -20,* resulted in complete loss of the I_A current indicating that the endogenous Shal/Kv4 current was indeed inhibited by expression of HA-DNKv4. No changes in the delayed rectifier current or other ion currents were observed in these transgenic lines.
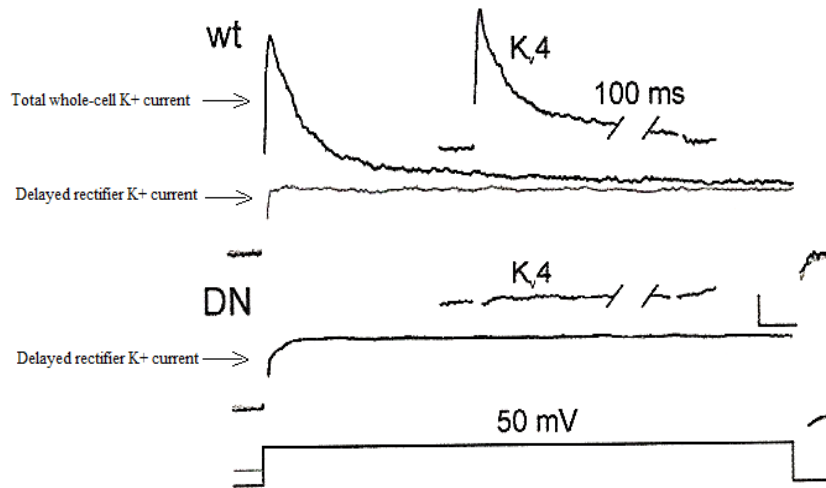
Figure 4. Voltage-clamp analysis of *elav-Gal4; UAS:: HA-DNKv4* line.

Top: Seen is total whole cell K^+ current that constitute the I_A and I_{DR} following voltage – clamp recording from wild-type embryonic neurons. Middle: The I_{DR} evoked from depolarization of the cell to + 50 mV, proceeded by – 45 mV prepulse, which inactivates the I_A current. Bottom: Voltage-clamp recordings executed for *elav-Gal4: UAS-HA-DNKv4* line. Note the absence of the I_A current, with no effect on the delayed rectifier K^+ current.

Detection of DNKv4 α subunit subcellular localization

In order to determine the DNKv4 α subunit's subcellular localization, the *UAS-HA-DNKv4* transgenic line was crossed to a line where the Gal4 protein is driven by a neuron-specific promotor ELAV which in turn express the HA-DNKv4 throughout the nervous system. Progeny with *elav-Gal4::UAS-HA-DNKv4* genotype was scored and a stock established as illustrated in figure 3A on page 32. Following embryonic cell culture of this line and immunostaining for HA tag with α-HA:11 primary antibodies we observed the DNKv4

expression and localization in neuronal cell body and puncta of the neuronal processes which is in agreement with our previous study for *Shal/Kv4* localization in *elav-Gal4::UAS-GFP-Shal* line using anti-GFP primary antibodies (Diao, Chaufty, Waro and Tsunoda (2010).

Figure 5.Subcellular localization of HA tagged DNKv4 α subunit in *elav-Gal4:: UAS-HA-DNKv4* line. Note that neural immunostaining for heamagglutinin tag (HA) localizes the DNKv4 α- subunit to the cell bodies (white oval shape) and in the puncta along the neural processes (white dots).

DNKv4 effect on *Drosophila* development and longevity

Since voltage-gated K^+ channel has been suggested to play a role in *Drosophila* development (55), we examined Canton S and the two transgenic lines, *elav-GAL4:: UAS-HA-DNKv4#14* and *elav-GAL4::UAS-HA-DNKv4#20,* for any visible change during the course of their development. Thirty wild type and *elav-GAL4::UAS-HA-DNKv4#14* and twenty-five *elav-GAL4:: UAS-HA-DNKv4#20* age-synchronized embryos were collected and their developmental progress at 25 ^{0}C were monitored by counting the number of hatched embryos and molted larvae (L1-L3) every 24 hours until they reached pupae stage. The numbers of emerged adult flies from pupae stage were also recorded. Comparing to the wild type, we did not find big observable change in the developmental process i.e. in the number of hatched embryos, molted larvae and emerged adult flies or in the duration of life cycle (8-10 days) both in the wild type and the two transgenic lines (*elav- GAL4:: UAS-HA-DNKv4-14 and elav-GAL4:: UAS-HA-DNKv4-20*) as seen on figure 6.

Developmental study of DNKv4 lines
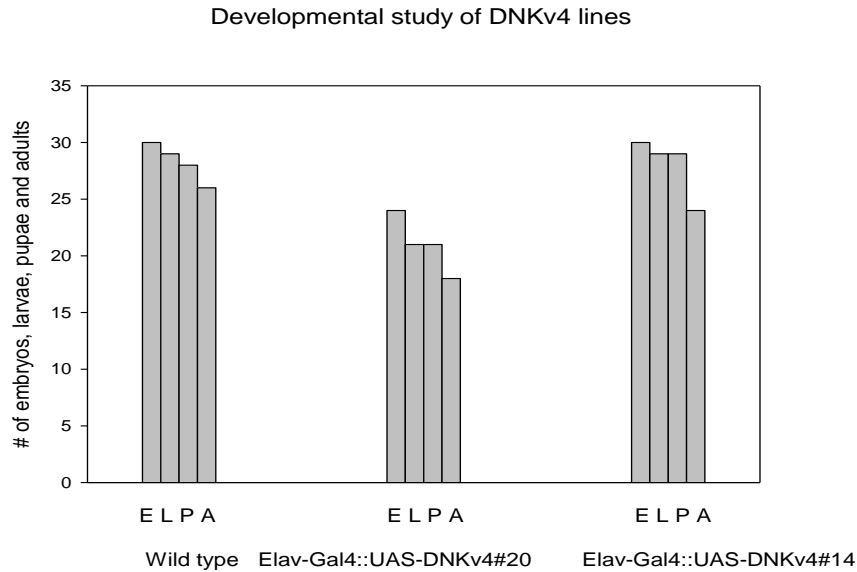
Figure 6. DNKv4 has no effect on the developmental process.

Shown is the number of embryo, larva, pupa and adult flies of the wild type and the two transgenic lines, *elav- GAL4:: UAS-HA-DNKv4#14* and *#20* recorded (Y-axis) during each stage (E=embryo, L=larva, P=pupa and A=adult) of the developmental process (X-axis). Note that no significant change observed on the process.

Since mutation in the A- type voltage-gated ion channel has also been implicated in affecting the life span of *Drosophila,* we ought to test the longevity of *UAS-HA-DNKv4* transgenic lines. Ninety to hundred freshly hatched adult flies of Canton S, the two background, *UAS-HA-DNKv4#14* and *UAS-HA-DNKv4#20*, and the two transgenic lines, *elav-GAL4::UAS-HA-DNKv4#14* and *elav-GAL4::UAS-HA-DNKv4#20,* were collected from cultures grown at $25^{o}C$ and grouped into ten. Each group consisting of 10 flies were transferred to ten separate vials which were labeled 1-10. These flies were then transferred every five days into new vials until the last fly died (see table 2 on appendix). We observed that both *elav-GAL4::UAS-HA-DNKv4#20* and *elav-GAL4::UAS-HA-DNKv4#14* transgenic displayed a shorter lifespan with median survival age of 23 days and 28 days, respectively, while the median survival age for Canton S was 42 days. The median survival age for the two

background lines, *UAS-HA-DNKv4#14* and *UAS-HA-DNKv4#20,* were found to be 44 and 45 days, respectively, as seen in figure 7 below. Statistical analysis have shown significance difference between *elav-GAL4::UAS-HA-DNKv4#20* and Canton S (*t*-test, P=.003), *elav-GAL4::UAS-HA-DNKv4#20* and *UAS-HA-DNKv4#20* lines, *t*-test, P=0.009(see appendix). Significance difference also observed between *elav-GAL4::UAS-HA-DNKv4#14* and Canton S lines (*t*-test, P=0.035). There is also significance difference between the *elav-GAL4::UAS-HA-DNKv4#14 and UAS-HA-DNKv4#14* lines (*t*-test, P= 0.42). However, there is no significance difference between *elav-GAL4::UAS-HA-DNKv4#14* and *elav-GAL4::UAS-HA-DNKv4#20* (Mann-Whitney Rank Sum Test, P =0.394), Canton S and *UAS-HA-DNKv4#14* (*t*-test, P= 0.10), and Canton S and *UAS-HA-DNKv4#20* (*t*-test, P=0.353) lines. Shorter median survival age of the two transgenic lines, *elav-GAL4:: UAS-HA-DNKv4#14* and *elav-GAL4::UAS-HA-DNKv4#20,* suggest that *Shal/K*v4 plays a role in *Drosophila* longevity.

DNKv4 lines longevity test at 25°C
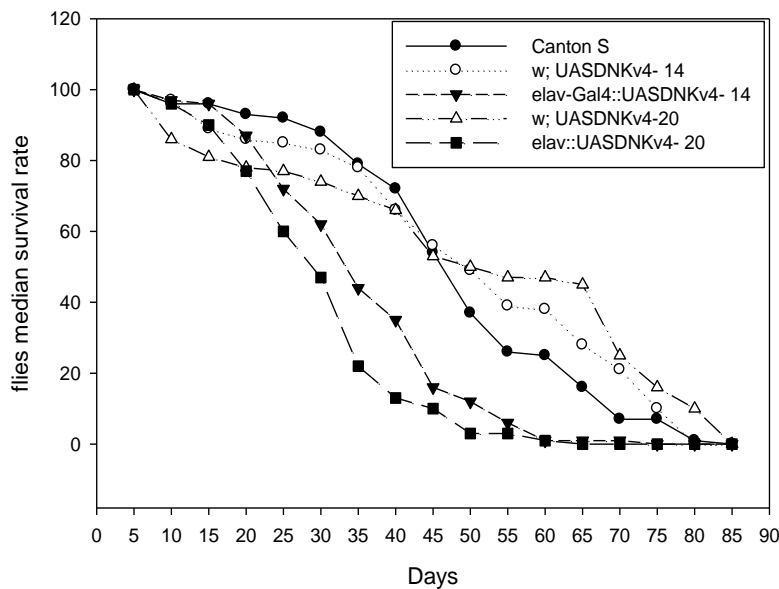
Figure 7 DNKv4 expression in *elav-GAL4::UAS-HA-DNKv4* flies results in defect in longevity. The median survival age (Y-axis) of adult flies at time points from 0 to 90 days (X-axis) is plotted using Sigma plot. Note a reduction in median survival age for *elav-*

GAL4::UAS-HA-DNKv4#14(--▼--) and *elav-GAL4::UAS-HA-DNKv4#20*(--■--) transgenic lines comparing to the median survival age of Wild type(--●--), background *UAS-HA-DNKv4#14* (--○--) and *UAS-HA-DNKv4#20 (*--Δ--) transgenic lines.

Since *elav*-Gal4 produces constitutively and to nullify its effect on the development of the two transgenic lines, *elav-GAL4::UAS-HA-DNKv4,* and therefore on their longevity, one of the transgenic line, *elav-GAL4::UAS-HA-DNKv4#20,* was crossed to $Gal80^{ts}$ transgenic line to establish *elav-Gal4::Gal80^{ts}::DNKv4#20* stock. Embryos of the W^{1118}; +; +, *UAS-DNKv4#20* background, and *elav-Gal4::Gal80^{ts}::DNKv4#20* lines were allowed to develop at 18 ^{0}C till they emerged as adult. The bounding of $Gal80^{ts}$ protein to the *Gal4* at 18 ^{0}C suppressed the *Gal4* activity and as a result the embryos developed to adulthood without the effect of *Gal4*. Two hundred adult flies were collected from each line and grouped into 20 on which each group consisting of 10 flies. The flies were then transferred to 20 vials and shifted to 30 ^{0}C. While transferring the flies every two days, the numbers of survived flies were recorded as seen on table 3 (see appendix). Similar to the *elav-Gal4::UAS-HA-DNKv4* transgenic lines tested at 25 ^{0}C, the *elav-Gal4::Gal80^{ts}::DNKv4# 20* line has also shown shorter life span than W^{1118}; +; + and *UAS-DNKv4#20* lines with its median survival age of 26 days. The median survival age for W^{1118}; +; + and *UAS-DNKv4#20* background lines was 33 days and 30 days respectively, as shown on figure 8 below. Statistical analysis has indicated that there is significance difference between W^{1118}; +; + and *elav-Gal4::Gal80^{ts} ::DNKv4#20* lines (*t-test*, P =0.002). There is also significance difference between *elav-Gal4::Gal80^{ts}::DNKv4#20* and *UAS-DNKv4#20* background lines, *t-test*, P= 0.001 (see appendix). There is no significance difference between W^{1118}; +; + and *UAS-DNKv4#20* (*t-test*, P=0.054). This result suggest that the early death of *elav-GAL4::UAS-HA-DNKv4#14* and *elav-GAL4::UAS-HA-DNKv4#20* flies is not due to the effect of constitutive *elav-Gal4* on their development but it is due to the effect of DNKv4 α-subunit on the wild type Shal/Kv4 α-subunit. It also suggests the role of *Shal/Kv4* on *Drosophila* longevity.

DNKv4 lines longavity test at 30°C
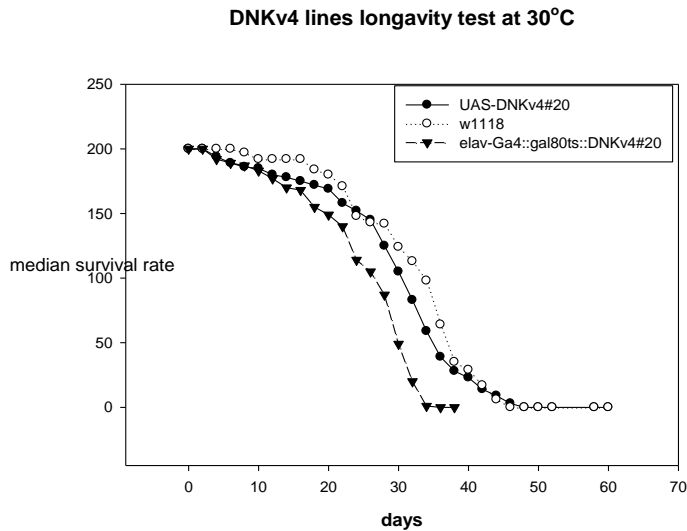
Figure 8. Expression of DNKv4 in transgenic lines following shift up to 30 °C also exhibited shorter life span supporting the involvement of *Shal/Kv4* in longevity.

The median survival age of adult flies (Y- axis) at time points from 0 to 70 days (X- axis) is plotted using Sigma plot. Note a reduction in median survival age for *elav-GAL4:: Gal80*[ts];*UAS-HA-DNKv4#20*(--▼--) compared to the median survival age of the back ground(--●--) and W[1118], +, + (--○--).

DNKv4 results in hyperexitablity

Alterations of neuronal ion channels have been implicated to result in change in animals' behavior. In *Drosophila*, modifications in the A-type channel gene, *Shaker (Kv1),* has been shown to manifest hyperexcitible phenotype while etherisation. Using ether as anesthesia is a common procedure to screen flies with behavioral phenotype caused by mutations in ion channels. Mutation in *Drosophila eag* gene, a member of K^+ ion channel gene, has also displayed anesthesia-induced leg-shaking behavior. Electrophysiological recording of this mutant line also displayed spontaneous motor neuron firing in the absence of stimulation (45, 78). Since our DNKv4 neurons have displayed larger mEPSC and increased spontaneous activity (Ping, Waro et al, 2011), we examined the shaking behavior of *elav-GAL4:: UAS-HA-DNKv4#14* and the background *UAS-HA-DNKv4#14* transgenic lines following ether treatment. Similar to the two transgenic lines, the two positive controls, the

wild type and *Shaker* [ks133] flies, which were cultured at 25 ^{O}C, were also treated with ether for shaking phenotype analysis. Based on our experimental conditions, we did not observe any behavioral change, shaking phenotype, in either the wild type or the transgenic lines, *elav-GAL4::UAS-HA-DNKv4#14* and the background *UAS-HA-DNKv4#14,* while flies were under the influence of anesthesia. The absence of shaking phenotype in the *elav-GAL4:: UAS-HA-DNKv4#14* line could be the result of constitutive inhibition of Kv4 function and/or compensatory remodeling that in turn masks hyperexcitability. We then swapped the *elav-GAL4* driver to the heat shock inducible *Gal4* driver (*hs-Gal4*) to allow us to express the DNKv4 transiently and hinder the Shal/Kv4 function following heat shock treatment. Both the wild type, *UAS-HA-DNKv4#14* background*, hs-Gal4:: UAS-HA-DNKv4#14* and *Shaker*[ks133] lines were heat-shocked for 1 hour per day at 37 ^{0}C for one week and assayed for shaking phenotype after ether treatment. During microscopic examination of shaking behavior of all four lines following etherization, we found that the *hs-Gal4:: UAS-HA-DNKv4#14* transgenic lines manifested shaking phenotype which is similar but milder than *Shaker*[ks133] mutant line, while the background and wild type flies failed to display mutant phenotype (figure 9). Statistical analysis have shown that there is significance difference between *hs-Gal4:: UAS-HA-DNKv4* and *Shaker*[ks133] flies (*t*-test**,** P = 0.002). There is also significance difference between the wild type and *hs-Gal4:: UAS-HA-DNKv4***,** *t*-test*,* P = < 0.001 (see appendix). There is no significance difference between the wild type and background *UAS-HA-DNKv4* lines (*t*-test, P = 0.158). Therefore, we suggest that acute, but not constitutive, loss of Kv4 function results in leg and body shaking phenotype, hyperexitability.

DNKv4 behavioral test
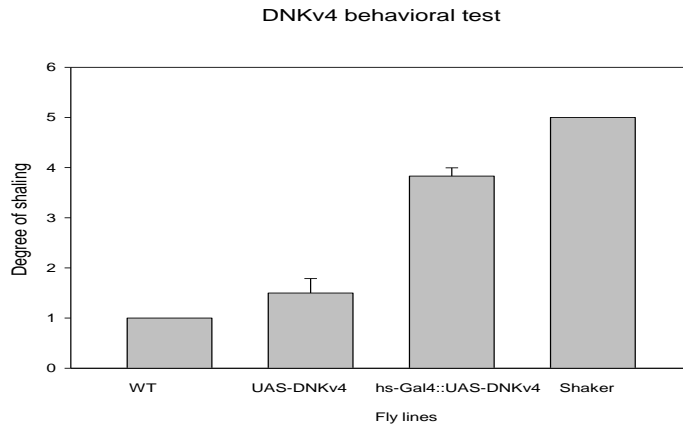
Figure 9. Blockade of Shal/Kv4 function in DNKv4 transgenic lines results in shaking phenotype.

The plot indicates the degree of shaking (Y-axis) of four lines (X-axis): the WT (wild type), *UAS-HA-DNKv4, hs-Gal4::UAS–HA-DNKv4* and *Shaker[ks133]* lines. From each line three independent groups of 15-20 flies were tested for shaking following 2-3 second ether treatment. The value of degree of shaking for each line was averaged and scored 1 to 5. Error bars are mean SE. Note that the two control lines, wild type and *Shaker*[ks133], scored 1 and 5, respectively. The *UAS-HA-DNKv4* line scored 1.5 while the *hs-Gal4-HA-DNKv4* scored 4.

DNKv4 effect on motor activity: larva crawling and adult locomotion

Rhythmic activity such as walking is the result of repetitive movement of muscles (49, 79). These rhythmic movements depend upon the repetitive firing of neurons. Since DNKv4 neurons of our transgenic lines displayed defects in repetitive firing (Ping, Waro et al 2011), we tested the behavior of DNKv4 larvae and adult flies during locomotion i.e. larva crawling and adult fly wall climbing. For larval crawling, individual third instar larvae of five different genotypes were tested: wild type, background *UAS-HA-DNKv4#20* and the three transgenic lines *elav-GAL4::UAS-HA-DNKv4#20*, *c164-GAL4::UAS-HA-DNKv4#20* and *109(80)-GAL4::UAS-HA-DNKv4#20* where the DNKv4 subunits express throughout the nervous system, in all motor neurons (80) and in multidendritic sensory neurons (81), respectively. Larvae from each individual line were allowed to crawl freely on separate 60

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mm 1% agarose plate placed over a 0.5 x 0.5 cm paper grid. From each line we tested 30 individual larvae. As Xu et al have demonstrated previously, we scored the numbers of grids crossed by each individual larva in 5 min period, averaged and then translated to the number of squires crossed in 1 min. We found that *elav-GAL4::UAS-HA-DNKv4#20, c164-GAL4::UAS-HA-DNKv4#20* and *109(80)-GAL4::UAS-HA-DNKv4#20* larvae crawled with slower speed than the wild type and background larvae. The *elav-Gal4:: UAS-HA-DNKv4#20* larvae crossed an average of 4.3 grids per minute while the wild type and the background *UAS-HA-DNKv4#20* larvae crossed 6.4 and 6 grids per minute, respectively. Larvae of *c164-GAL4::UAS-HA-DNKv4#20* and *109(80)-GAL4::UAS-HA-DNKv4#20* lines were also scored slower locomotion rate of 5 and 5.4 grids per minute, respectively, than the wild type and the background *UAS-HA-DNKv4#20* but not as slow as *elav-Gal4:: UAS-HA-DNKv4#20* larvae (figure 10 and table 4). Based on statistical analysis, we found significant difference between *elav-GAL4::UAS-HA-DNKv4#20* transgenic and the wild type and background *UAS-HA-DNKv4* lines (*t*-test, p= < 0.001). However, there is no significance difference between the wild type and back ground *UAS-HA-DNKv4* line, *t*-test, p=1.000 (see appendix). Similarly, we observed significant difference between *elav-GAL4::UAS-HA-DNKv4#20* and the two transgenic lines, *c164-GAL4:: UAS-HA-DNKv4#20* and *109(80)-GAL4:: UAS-HA-DNKv4#20,* (*t*-test, p=0.010 and p= 0.004). No significance difference was observed between line *c164-GAL4:: UAS-HA-DNKv4#20* and *109(80)-GAL4:: UAS-HA-DNKv4#20 (t*-test, p=1.000).These results clearly suggest that the loss of repetitive firing of neurons in DNKv4 lead to defects in larva locomotion i.e. reduction in the speed of larva crawling.

Larval locomotion test
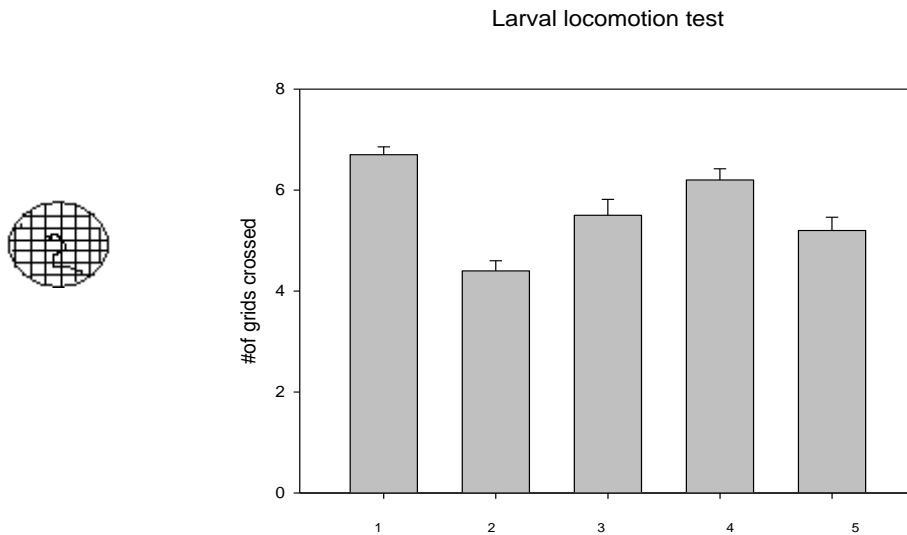
Figure10. DNKv4 expression in transgenic larvae results in change in the rate of locomotion. The bars 1 to 5 represent the Wild type, *elav-GAL4:: UAS-HA-DNKv4#20, 109(80)-GAL4:: UAS-HA-DNKv4#20, UAS-HA-DNKv4-20* and *c164-GAL4::UAS-HA-DNKv4#20* lines, respectively. Inset illustrates larva locomotion on agarose grid media. The number of grids crossed by larvae from each individual line was averaged to determine the rate of locomotion.

Since adult fly wall climbing is also likely to depend on repetitive firing of neurons, we examined climbing behavior in adult DNKv4 transgenic lines using a climbing assay developed by Benzer et al and Xu et al (1967, 2006). For this assay, 35 two day old adult flies were placed at the bottom of a 12-cm test tube and allowed to climb upwards for 30 seconds into a second tube that was inverted on top of the first tube. Flies that climbed into the second tube were allowed to climb upward for 30 seconds to third tube inverted on top of the second tube. The operation proceeded for ten successive tubes. Each fly was given one point for every two tubes climbed out of. Mean score of the flies from each group was calculated and this experiment was repeated for ten groups for each genotype with average determined. We have found that *elav-GAL4::UAS-HA-DNKv4#14* transgenic lines showed lower climbing score compared to the wild type. We also tested *c164-GAL4::UAS-HA-DNKv4#14* transgenic lines and found lower climbing score compared to the background and wild type (Ping, Wao et al, 2011). These results suggest that loss of Shal/ Kv4 function leads to defect in motor neuron out put

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which in turn resulting in defect in locomotion. Overall, we suggest that the normal functioning of Kv4 channels in neurons are required for repetitive firing behaviors such as larval crawling and adult climbing.

DNKv4 effect on larval odor-associative learning behavior

Kv4 channels have been suggested to play a role in synaptic plasticity mechanism and long-term potentiation (84, 85). Since mutation in *Drosophila* A-type channel gene has also been suggested to lead to disruption in normal associative learning and/or memory process, we examined learning performance of our DNKv4 transgenic lines. In order to test the learning performance of these DNKv4 transgenic lines, we trained *elav-Gal4::UAS-HA-DNKv4* third instar larvae to associate a gustatory with odors, as previously demonstrated by Scherer et al and later by Hendel et al (2003, 2005). *Drosophila* larvae have been shown to have a natural tendency to prefer fructose (gustatory). Prior to gustatory odor associative learning test of the *elav-GAL4:: UAS-HA-DNKv4* line, we first performed control test to confirm that the wild type and background *UAS-HA-DNKv4#14* and *elav-GAL4::UAS-HA-DNKv4#14* lines show preference for fructose. To proceed with the test, three groups of 10 third instar larvae from each line (N= 30) were placed separately in the center of a 60 mm Petri dish plate on which one-half was filled with 1% pure agarose and the other one-half filled with 1% agarose supplemented with 2 M fructose as presented on figure 11 (inset). Since our *elav-GAL4::UAS-HA-DNKv4* line showed locomotion defect as a consequence of neural firing impairment (Ping, Waro et al 2011), larvae were given 20 min to crawl freely on the plate and to choose one of the two gustatory. Larvae preference for fructose was determined by subtracting the number of larvae found on one-half side of the plate from the number of larvae found on the other one-half side of the same plate divided by the total number of larvae placed on the plate. Since larvae were given to choose from two conditions, preference score range from −1 to + 1. Zero preference score indicates larvae lack of preference to any of the two conditions while +1/-1 indicates larvae highest preference to one

of the two conditions as shown in figure 11. If DNKv4 larvae fail to prefer fructose then DNKv4 should be accounted for. However, we found that all three genotypes displayed variable level of preference for fructose with a positive score of 0.56, 0.71 and 0.36 for Canton S, background *UAS-HA-DNKv4* and *elav-Gal4::UAS-HA-DNKv4*, respectively. Statistical analysis has indicated that there is no significant difference between these three lines Canton S and *UAS-HA-DNKv4* (*t*-test, P = 0.738), Canton S and *elav-Gal4::UAS-HA-DNKv4* (*t*-test, P = 0.325) and *UAS-HA-DNKv4* and *elav-Gal4::UAS-HA-DNKv4* (*t*-test, P = 0.752) related to fructose preference. These results suggest that DNKv4 does not affect larvae sense of taste.
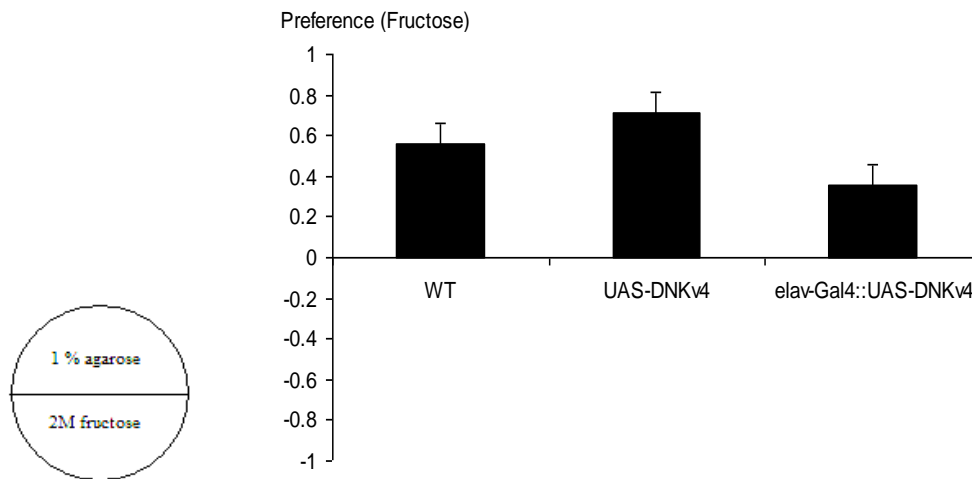
Figure 11 DNKv4 does not affect gustatory perception (sense of test) of transgenic larvae. The inset on the left represents a 60 mm Petri dish plate divided into two parts where half was filled with 1% pure agarose and other half filled with 2 M fructose in 1% agarose. Note that all three genotypes, WT (Canton S), background UAS (*UAS-HA- DNKv4*) and DN (*elav-Gal4::UAS-HA-DNKv4*, indicate positive score representing preference to 2M fructose, and there is no substantial preference difference among the genotypes.

We also carried out another control test on DNKv4 third instar larvae for their preference of two odorants, Octanol (OCT) and Amyl acetate (AM). Normally larvae do not display preference for the two odorants i.e. odorants act inert to attract or repeal larvae. To test for odor preference, 10 third instar larvae from DNKv4 lines were placed in the middle of

1% pure agarose media or 2 molar fructose in 1% pure agarose media for 20 min where two ventilated plastic caps dipped with both odorants positioned on the opposite sides (one cap pipetted with AM odorant and placed on one side of the plate and another cap pipetted with OCT odorant was placed opposite to the AM side) of a 60 mm Petri dish as shown in the inset in figure 12. The number of larvae on each side of the odorant cap (AM and OCT cap) was counted and a preference score calculated as the difference in the number of larvae found on each side of odorant cap divided by the total number of larvae placed on agarose or fructose plate. As shown in the figure below, we found larvae preference scores very close to zero indicating that all three genotypes have no preference for any of the odorants either on agarose or fructose plate.
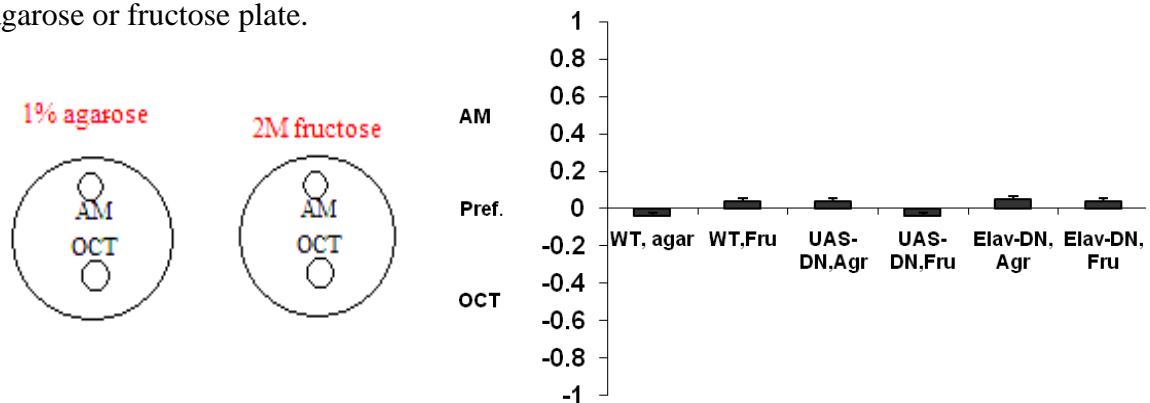
Figure 12 DNKv4 larvae do not display preference for both neutral odorants, Octanol (OCT) and Amyl acetate (AM). Note that preference score for both lines, WT (Canton S), UAS (*UAS-HA-DNKv4)* and DN (*elav-Gal4:: UAS-HA-DNKv4)*, is close to zero.

To rule out any loss of sense of smell due to DNKv4 mutation we also set up control test on *elav-Gal4::UAS-HA-DNKv4-14* larvae to two odorants, Acetone (ACE) and Methyl acetate (MA), to which larvae normally attracted to. We found that *elav-Gal4:: UAS-HA-DNKv4#14* larvae showed a preference for both Acetone (ACE) and Methyl acetate (MA). As the plot shows on figure 13 below ACE preference score for the wild type was 0.86 and for *elav-Gal4::UAS-HA-DNKv4#14* 0.8, while MA preference score for Canton S and *elav-*

Gal4::UAS-HA-DNKv4#14 was 0.4 and 0.5, respectively. This result suggested that *elav-Gal4::UAS-HA-DNKv4#14* larvae have not lost their sense of smell.
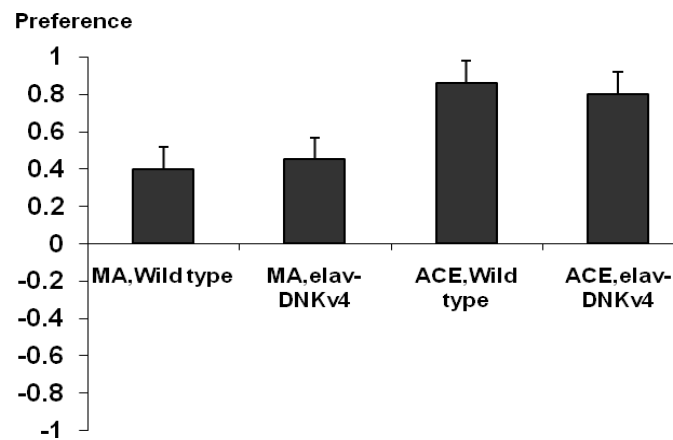
Figure 13. DNKv4 transgenic lines do not display anosmia for attractant odorants, acetone (ACE) and methyl acetate (MA). Note that both the wild type (Canton S) and *elav-Gal4::UAS-HA-DNKv4#14* prefer acetone and methyl acetate indicating that *elav-Gal4::UAS-HA-DNKv4#14* does not suffer in loss of smell.

Following determination of transgenic larvae performance for gustatory and odor (neutral and attractant) preference, we finally trained and tested third instar larvae of the same genotypes, Canton S, *elav-Gal4::UAS-HA- DNKv4* and background UAS-HA-DNKv4 , for their learning abilities to associate fructose with OCT (AM-/OCT+) odorant. In order to proceed with the test, larvae to be trained were placed on 1% pure agarose plate with ventilated plastic caps pipetted with OCT odorant and allowed to crawl for 1 min. and then transferred to 1% pure agarose media to rest for 1 min. The larvae were then transferred to 2M fructose in 1% agarose media where a ventilated plastic cap was filled with AM (AM +/OCT-) odorant and given 1 min to wonder. The training was repeated for 10 cycles. At the end of 10 cycles of training, the larvae were transferred and placed in the middle of a 60 mm Petri dish test plate, 1% pure agarose only plate, where two 0.5 cm in diameter ventilated plastic caps with both AM and OCT odorants were placed on opposite side facing each other as diagramed in figure 14 below. Four groups of five larvae were tested for each genotype.

Following 20 min free movement on the test plate, larvae odor preference was calculated as the difference in the number of larvae on each side of the odorant cap divide by the total number of larvae.
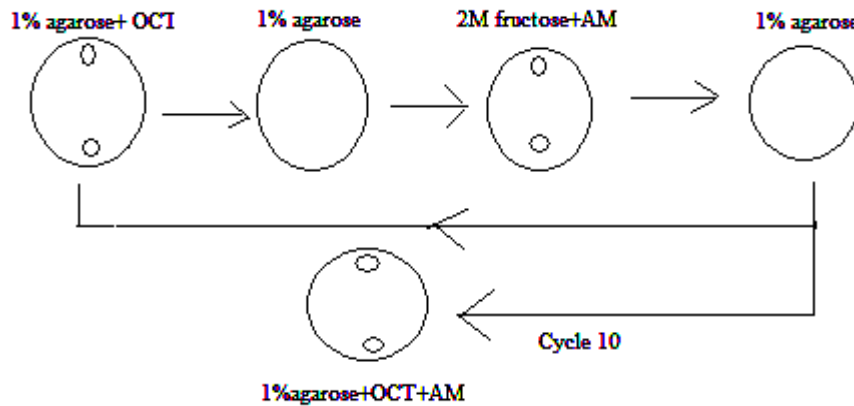
Figure 14. DNKv4 transgenic larvae odor- associative learning assay

The three genotypes, Canton S, *elav-Gal4:: UAS-HA- DNKv4* and background *UAS-HA-DNKv4,* were trained to associate fructose (gustatory) with OCT (AM-/OCT+) odorant. The two small circles in the opposite side of a 60 mm Petri dish plate represent odorant containers with the appropriate odorants i.e. OCT, AM and OCT and AM.

To calculate odor preference for reciprocal test new 3rd instar larvae of the same genotype were also trained similarly to learn to associate fructose with AM (AM+/OCT-) odorant. We found that wild type and back ground *UAS-HA-DNKv4#14* larvae exhibited preference for the odor associated with the 2 M fructose in 1% agarose media during training, while the *elav-Gal4:: UAS-HA-DNKv4#14* did not exhibit preference for either odorant. To quantify larvae learning performance, we calculated a learning index (LI) value. Learning index value is calculated as the difference in odor preference between reciprocally trained larvae (the larvae trained on AM+/OCT- and the larvae trained on AM-/OCT+) divided by two [LI= PREF(AM+/OCT) - PREF (AM-/OCT+) / 2]. We found both the wild type and *UAS-HA-DNKv4#14* larvae have shown positive learning index value of 0.4. These positive index values indicated that larvae of the wild type and the back ground were able to learn to

associate odor with fructose, while *elav-Gal4::UAS-HA-DNKv4#14* larvae with their negative

index value of –0.2 showed their inability to associate odor with fructose as shown in figure

15 . This result suggests that the loss of functional Shal/Kv4 channel leads to associative-

learning defects.
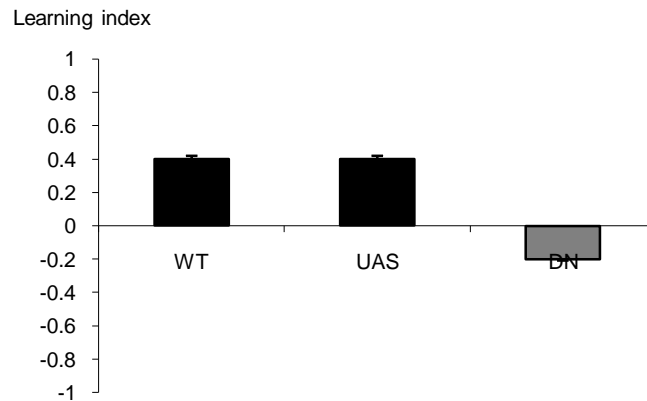
Figure 15 DNKv4 affects larvae odor-associative learning behavior.

While the positive learning index values for WT (Canton S) and (UAS) *UAS-HA-DNKv4-14*

show larvae's ability to learn to associate odor with fructose, the negative learning index

value for DN (*elav-Gal4:: UAS-HA-DNKv4*) indicating larvae's failure to learn to associate

odor with gustatory.

Discussion

This study provides insight into the role the transient voltage-activated K+ current, caused by Shal/Kv4 channels, plays in rhythmic behaviors, such as larval crawling and adult climbing, longevity and in a more complex behavior, such as learning/memory, in *Drosophila*. In order to eliminate Shal/Kv4 function, we introduced a residue change, tryptophan to phenyalanine, at position 362 in the pore-forming region of the Shal/Kv4 α-subunit to generate a Dominan–Negative Shal/Kv4 α-subunit (DNKv4 α-subunit). To establish *Drosophila* transgenic lines the mutant Shal/ Kv4 α-subunit cDNA inserted into a P element based vector, pUAST. Genetically, we determined the insertion site of pUAST-DNKv4 vector in the genome of the transgenic lines. Following application of the GAL-UAS binary system, we expressed a 52 KD protein, HA-DNKv4 α- subunit protein, ectopically in the transgenic lines, which corresponds to the Shal/Kv4 molecular weight. We showed DNKv4 subcellular localization to cell body and puncta along neural processes following embryonic cell culture immunostaining for HA. We confirmed by voltage clamp analysis that the I_A/Shal current was specifically eliminated as a result of the DNKv4 α- subunit effect similar to previous studies by Tsunoda et al (1995) in which the *Shal/Kv4* gene was removed using a large deletion. However, elimination of one specific ion channel activity as a result of genetics or pharmacological agents have been shown to leading to compensation/electrical modeling of another ion channel through homeostasis mechanism so that neurons can maintain their excitable properties and firing activities. Recent study by Norris et al (2010) have shown electrical modeling of other potassium ion currents in rat following targeted deletion of individual K ion channel α-subunits encoding components of I_A current. Nebornne (2008) and Chen (2006) respectively showed normal excitable phenotypes and a milder than expected defects in excitability in mice where the Kv4.2 deleted. This normal excitable phenotype might be due to compensatory up regulation of another potassium ion current that

is similar to the lost I_A current. Schaefer et al (2010) have also been able to record incomplete I_A current following application of RNAi to *Drosophila* motor neuron to alter Shal/Kv4 activity. In *Drosophila* line where the *Shal* gene is deleted, Bergquist and others (2010) reported an increase in *Shaker/Kv1* gene transcription level that can be translated to more Shaker/Kv1 A-type channel and targeted to axon and neural terminal. Therefore, the increase in surface Shaker/Kv1 channel at neural terminus might stabilize neural synaptic function (homeostasis). In this study, however, we did not observe compensation of another *Drosophila* K^+ channel/ current, for example Shab and Shaw, or incomplete Shal/ Kv4 loss of current as a consequence of dominant negative mutation effect. Future additional studies by RT-PCR analysis and electrical physiology recording on axon might give us a clue about any change in the expression level of other ion channels and/or compensation/electrical modeling of the Shaker/ Kv1 channel or other channels due to the effect of DNKv4 α- subunit on the Shal/Kv4 α- subunit.

Mutations in neural ion channels have been implicated in neural hyperexcitability. A decrease in the availability of I_A due to loss of A- type channel or increased channel phosphorylation by kinase has been reported to increases the excitability of neuronal dendrites (Hoffman, 1997). Reduced expression level of A-type channel (Kv4.2) in heterotopic cell region of the hippocampus in rat also shown to exhibit increased neuronal firing, epilepsy (Castro et al, 2001). Since we did not observe compensation of other potassium or other current and the I_A/Shal current was eliminated completely in our study as a consequence of DNKv4, we looked into behavioral defects such as ether-induced shaking phenotype behavior in the *UAS-DNKv4* transgenic lines we generated. When DNKv4 expressing flies, *elav-Gal4: UAS-DNKv4*, were treated with ether they did not exhibit *Shaker* behavioral phenotype such as abnormal wing scissoring and leg shaking similar to *Shaker* mutant flies. When we induced expression of DNKv4 protein in *hs-Gal4:: UAS-HA-DNKv4*

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adult flies, abnormal body and leg shaking and wing scissoring phenotypes were mimicked suggesting that acute but not constitutive loss of Kv4 function resulted in hyperexitability. Absence of shaking behavioral phenotypes in *elav-Gal4::UAS-DNKv4* transgenic flies might be the result of compensation since DNKv4 was expressed constitutively. In addition to hyperexcitibility, ion channels mutants have been shown to result in shorter life span. For instance, mutations in *Drosophila ether ago-go* (*eag)* and *Shaker* (*Sh)* gene have been shown to result in shorter life span. Although the life cycle of DNKv4 channel expressing adult flies is normal and are viable, we observed a shorter life span (longevity) in *elav-Gal4: UAS-DNKv4* compared to wild type and background adult flies. DNKv4 adult flies' shorter life span might be the consequence of the secondary effect of other behavioral defects, for example disruption of coordinated locomotion of flies due to defect in motor neuron activity that in turn leading to shorter lifespan.

Neural networks underlying the generation of coordinated rhythmic motor pattern have been identified and characterized in different organisms, and maintaining of these rhythmic patterns depend on the cellular components such as ion current/channel. Subsequent to stimulation of rat's visual cortex pyramidal neuron that express the dominant negative Kv4.2 subunits, Yuan, Burkhalter and Nebornne (2005) have shown an increase in neural firing rates and adaptation of neural firing indicating the role of ion currents (I_A) in maintaining of neural activity. Following a prolonged and repeated larger current stimulation of identified DNKv4 motor neuron (an input on which a larvae motor neurons receive to crawl), we, Ping, Waro et al (2011), showed an increase in firing frequency with a progressive decrement in action potential amplitude leading to adaptation and then cession of repetitive firing. When we expressed DNKv4 throughout the entire nervous system, larvae and adult flies exhibited defects in locomotion. When we specifically expressed the DNKv4 in the motor neurons in the whole animal, defect in larvae crawling and adult fly's wall

climbing observed. Defect in body cleaning, a type of rhythmic behavior, was also observed by colleague, Sarah, in this DNKv4 flies (Ping, Waro et al, 2011). Together, these results suggest that Shal/ Kv4 channel is essential for repetitive firing that controls repetitive behavior such as larva crawling, wall climbing and a non-locomotive behavior grooming.

For animals to move and carry out purposeful behaviors require neural network (circuitry) and its target, muscle. The Central Pattern Generators (CPGs) are neural networks which produce rhythmic patterns even in isolation from motor and sensory feedback from muscle targets. Studies on mammals such as cat and dog have shown that rhythmic behaviors like locomotion can function without sensory feedback (input) from peripheral nervous system (PNS) but a sensory feed back is essential in order to refine the correct rhythmic movement in response to environmental stimuli (Brown.TG (1911), Shik, Severin and Orlovsky (1966), and Grillner (1986). The role of sensory feedback has also been shown during *Drosophila* larval crawling, a patterned muscle contractions moving stereotypically along the body segments. Wei and others (2007) have shown the significance of sensory feedback for neural circuits within CNS to maintain firing pattern following conditional blockade, shifting to 37 ^{0}C, of an input from multidendritic sensory neurons in *Gal4::UAS-Shibire^{ts} Drosophila* larvae that resulted in altering in crawling speed. Hughes and Thomas (2007) have also reached similar result after expression of *Gal4:: UAS-Shibire^{ts}* to block neuros in PNS that sends feed back signals to CNS. In this study, when we specifically express the DNKv4 in multidendritic sensory neuron using the *109(80)-Gal4* promoter, reduction in larvae crawling speed was also exhibited. The sensory feedback from these multidendritic sensory neurons to CPG neurons in the CNS that then drives motor neurons output might have failed to properly facilitate propagation of the peristaltic wave and to maintain the speed of larva crawling. It is therefore most likely that Shal/Kv4 also functions in sensory feedback part of the locomotor CPG circuitry in larvae to integrate signals in

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dendrites. Since the *109(80)-Gal4* driver expresses the DNKv4 in all multidendritic sensory neurons which are subdivided and further classified into classes of neurons, future investigation using a variety of different *Gal4* driver lines is needed to specifically drive DNKv4 in these classes of multidendritic sensory neurons and to locate to specific class of neuron and determine as a site for feedback signal integration that facilitate normal larval crawling.

The localization of specific ion channels at distinct sites (compartments) in neuron is fundamental for neural distinct function. Neuronal dendrites provide area where excitatory and inhibitory inputs form synapses. Dendrites contain a variety of voltage-gated channels such as sodium, calcium and potassium and play role in the integration and propagation of action potentials activity throughout the neuron. Shal/Kv4.2 channels are localized to soma and dendrites of neuron. The density of Shal/Kv4.2 channels increases with distance from the soma in apical dendrites of hippocampal pyramidal neurons. These increase in Shal/Kv4.2 channels density therefore play role in regulating back propagating action potential (bAP) and thus regulate the induction of long-term potentiation (LTP) that underlay learning and memory (Hoffman, 1997, Chen 2006). Previous studies by Chen (2006) and Kim (2005) have shown that elimination of the *Shal/ Kv4.2* from mice to alter Ca^{2+} influx in the dendrites of hippocampal CA1 pyramidal neural cells leading to the induction of LTP. Lockridge and Yuan (2010) have shown the poor performance of the CA1 neurons of Kv4.2 channel knocked out mice in spatial learning during Morris water maze task. Most recently Joaquin et al (2012) have also shown hippocampal-dependent learning and memory defect in a Kv4.2 channel knocked out mice following Morris water maze task. In *Drosophila*, Cowan and Siegel (1984 and 1986) have shown that genetic ablation of the voltage gated A- type K^+ channel to result in poor performance of conditioned courtship and reduced acquisition in olfactory classical conditioning. In this study we also found that the *elav-Gal4:: UAS-DNKv4*

flies, that express the *DNKv4* in all neurons, exhibited defect in odor-association assignment. Our findings support the role of Shal/Kv4 channels in learning and memory. However, thorough and further investigation is needed to locate this DNKv4 defect to specific neuron or specific brain region using different brain specific promoters; meanwhile the effect on learning/memory can be observed through behavioral performance and perhaps link to underlying mechanism. Since Shal/Kv4 channels have been shown to express in the mushroom bodies (71), application of immunostaining of brain will locate DNKv4 to particular neuron at specific brain region. Furthermore it would be interesting to remobilize the *UAS::DNKv4* insertion from our *elav-GAL4::UAS-HA-DNKv4* transgenic lines and test the revertant lines for Shal/ Kv4 channel/current activity, longevity, locomotion as well as learning and memory. How can this test be achieved? Since the DNKv4 construct is cloned into P-element based vector, precise excision of the UAS::DNKv4 from its insertional site in *elav-GAL4::UAS-HA-DNKv4* reverts the transgenic lines to wild-type lines and therefore restores the function of Shal/Kv4 channel. In order to excise the DNKv4 from the genome, *elav-GAL4::UAS-HA-DNKv4* mutant line will be crossed to a different insertional line, P{*ry*$^+$ Δ2-3} (99B) or P{W$^+$Δ 2-3}(99B), that expresses the enzyme transposase. The presence of transposase in *pUAS-HA-DNKv4* cells leads the *pUAS-HA-DNKv4* to excise. DNKv4 mobilization can be traced phenotypically i.e. searching for flies that lost the dominant marker carried by the insertional element. A single revertant fly will be further crossed to another line to establish excision mutant line. These stable revertant progenies will be then tested for the restoration of Shal/ Kv4 channel/current activity, and their longevity, larval locomotion and learning and memory. The manifestation of normal phenotypic behaviors by DNKv4excision lines following the test will therefore confirm our suggestion that Shal/ Kv4 plays a role in longevity, locomotion and learning and memory in *Drosophila*.

Summary

Membranes of living cells are composed of different kinds of ion channels on which their opening and closing are regulated by ligands and voltage difference across the membrane. The transiently fast activating and inactivating A-type voltage gated channel/outward current (I_A) has been implicated in action potential repolarization, regulating action potential inter spike interval, delaying onset of AP and limiting back propagation of dendritic action potential. In mammals, the transient outward I_A current is encode by multiple K^+ channel genes. For example mouse cortical pyramidal neuron encodes three transient I_A by three K^+ channel genes, Kv1.4, Kv4.2 and Kv4.3. Inactivation of one or all of these I_A current by drug or toxin or genetic means in mammals results in change/ compensation in the function or expression of another ion channel gene. Hence, identifying the role the transient I_A current plays in mammals has been problematic. In contrast to mammals *Drosophila* has two genes that express the transient I_A current. One of the genes, *Shal* gene, encodes Shal/ Kv4 current in neural somatodendritic region while the other gene, Shaker gene, encodes Shaker/ Kv1 current in the axon and muscle. Shal (Kv4) channel is the most highly conserved channel structurally as well as functionally among the animal kingdom. For instance sequence analysis of the Shal (Kv4) showed 82% amino acid identity between *Drosophila* insect and rodent.

Here, we generated twenty independent *Drosophila* transgenic lines expressing the Shal/ Kv4 dominant-negative α–subunit transmembrane protein (DNKv4) that has a substitution mutation (tryptophan for phenylalanine) at position 362 in the pore forming region of the channel. With the application of GAL/UAS system and Western blot analysis we identified two lines that express a 52 kD protein, DNKv4 α–subunit protein, which correspond to the Shal/Kv4 protein molecular weight.

Tetramerization of the DNKv4 α–subunit with Shal/Kv4 α–subunit would block the passage of I_A current, and therefore results in non-functional Shal/ Kv4 channel. With voltage-clamp analysis we verified that Shal/Kv4 dominant-negative α–subunit eliminated the Shal/ Kv4 channel function and I_A current without compensating/ changing other ion K channels function such as the delayed rectifier K ion channel or expression level, and this allowed us to perform comprehensive study on neural firing patterns and repetitive behaviors such as larval locomotion and adult flies wall climbing, longevity and on a more complex behavior larva odor-associated learning.

Following immunostaining of embryonic neuron for HA, we showed the subcellular localization of DNKv4 α–subunit to the cell body and puncta along neural processes. Animals with DNKv4 channel revealed ether dependent shaking phenotype (hyperexitability) which is similar but milder than *Shaker*[ks133] mutant line. In addition to hyperexitability these DNKv4 animals have also shown shorter life span compared to the wild type animals; however, we did not find change in the developmental process and duration of life cycle (8-10 days). This result suggests that *Shal/ Kv4* play a role in *Drosophila* longevity.

Flies with DNKv4 insertion also displayed abnormalities in repetitive behaviors, for instance, defects in larva locomotion and adult flies wall climbing. During larvae locomotion test, the DNKv4 larvae manifested reduction in the speed of crawling comparing to control larvae. In addition, DNKv4 adult flies also exhibited lower score during wall climbing assignment and inefficient body cleaning (grooming) compared to the wild type flies. Together, these results suggest that Shal/Kv4 channel is required for repetitive firing that controls repetitive behavior such as larval crawling, wall climbing and a non-locomotive behavior grooming.

In this study we also showed that DNKv4 larvae that express the DNKv4 in all neurons exhibit defect in larvae odor-associated learning, i.e. larvae were unable to associate

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gustatory (fructose) with odorants (amyl acetate and octanol), suggesting that Shal/Kv4 channel plays a role in larvae learning and memory.

DNKv4 neuron displayed prolonged action potential, smaller afterhyperpolarization, short latencies to 1st spike and defects in repetitive firing that adapts quickly (Ping, Waro et al, 2011).These behavioral phenotypes correlate with the observed defects in repetitive firing, and give a better understanding about significance of I_A/ Kv4.

Összefoglaló

Az élő szervezet, mint az emberé, trillió sejtet tartalmaz. A sejtet a sejtmembrán (sejthártya) veszi körül. A sejtmembrán feladata, hogy a sejten belül levő komponenseket, például a sejtmagot, mitokondriumot, endoplazmatikus reticulumot, Golgi készüléket és lizoszómát a külső környezettől elkülönítse. Ezen kívül a sejten belüli környezetet is szabályozhatóvá teszi, hogy ideális legyen a sejt szaporodására, a sejtstruktúra fenntartására és a környezetre való reagálásra. Hogy a sejten belüli környezet ideális legyen, töltéssel rendelkező ionoknak - mint például Nátrium (Na^+), Kálium (K^+), Kalcium (Ca^{2+}) és Klór (Cl^-) - a sejtből ki és be kell áramlaniuk. Ezt a kétirányú áramlást a membránon található pórusok vagy ioncsatornák (kapuk) biztosítják.

A ioncsatornák integráns fehérjék. Annak ellenére, hogy minden sejtmembránon megtalálhatók, az ioncsatornák az ideg- és izomsejtekben jobban tanulmányozhatók, ahol ezek szabályozása felelős a elektromos jelek (signal) továbbításáért. Szabályozásuk (nyitás és zárás) alapján az ioncsatornák két osztályba csoportosíthatók: ligandummal kapuzott ioncsatornák és feszültséggel kapuzott ioncsatornák. A ligand-vezérelt ioncsatornák - mint például a nikotinik acetilkolin receptor, gamma aminovajsav (GABA) és glicin receptor - nyitása és zárása a ligandnak a receptorhoz való megkötésétől függ. A ligandum által szabályozott ioncsatornákkal ellentétben, a feszültségfüggő ioncsatornák akkor nyitódnak és záródnak, ha a membránon potenciálváltozás következik be, azaz feszültségkülönbség keletkezik a külső és belső sejtmembrán között. A feszültségfüggő ioncsatornák magukba foglalják a Na^+, K^+, Ca^{2+} és Cl^- ioncsatorna szuperfamiliákat. A feszültséggel szabályozható ioncsatornák szuperfamiliái közül a K^+ ioncsatorna a legváltozatosabb ioncsatorna, amely több mint 60 tagból áll és az alternatív splicing során további 12, egymástól eltérő szubfamiliára osztható.

Ez a disszertáció az egyik K^+ ioncsatorna szubfamiliáját, specifikusan az A-típusú feszültségfüggő Shal/Kv4 ioncsatornát tárgyalja, valamint ebben a témakörben végzett kutatásaimat és kísérleteimet foglalja össze.

Az A-típusú feszültségfüggő Shal/Kv4 ioncsatorna a tranziensen gyors aktiválású és inaktiválású A-típusú kifelé haladó áramot (I_A) kódolja. Az A-típusú feszültségfüggő ioncsatorna az akciópotenciál repolarizációjában, az akciópotenciális kisülés (firing) közötti intervallum szabályozásában, az akciópotenciál kezdetének késleltetésében és a dendrite akciópotenciáljának visszafelé történő terjedésének korlátozásában játszik szerepet. Az A-típusú feszültségfüggő ioncsatornáról feltételezik, hogy kritikus szerepet játszik az élő szervezet mozgásában, élethosszúságában és a szinaptikus plasztikusságban (a szinaptikus kapcsolatok hatékonyságának megváltozása során történő neuron-struktúra és/vagy funkcióváltozásban), ami a tanulás és memória mechanizmusának alapja. Emlősökben a tranziens kifelé áramló áramot több K^+ csatorna gén kódolja. Például az egér cortical piramis neuronjában három kálium csatorna gén található, amely három tranziens I_A típusú csatornát - Kv1.4, Kv4.2, Kv4.3 - kódol. Az egyik vagy mind három I_A típusú csatorna gyógyszerrel, méreggel vagy genetikai módszerekkel (deleció) történő inaktiválása az emlősökben egy másik ioncsatorna gén expresszálódásához, funkcióváltozásához vagy kompenzálásához vezet, ezért az emlősökben a tranziens A-típusú csatornák szerepét értelmezni problematikus. Az emlősökkel ellentétben a muslicának két olyan génje van ami a tranziens I_A típusú csatornát kódolja. Az egyik I_A típusú gén, a Shal gén, az ideg szomatodendrit régiójában található és a Shal/Kv4 csatornát kódolja, míg a másik gén, a Shaker gén, az axonban és az izomban elhelyezkedő Shaker/Kv1 csatorna kódolásáért felelős.

Mivel a muslica motorneuronjainak és idegnyúlványainak a célsejthez eljutása, a tanulási és memória központja (mushroom body - az agyban található idegköteg) és idegsejtjeinek a jelbemenete (input) és jelkimenete (output) jól ismert, ezért kiváló

modellszervként szolgál arra, hogy az idegmembránján található tranziens A-típusú csatornák szerepét feltárjuk a repetitív (ismétlődő) viselkedésben, például helyváltoztatásban, test tisztogatásban, élethoszúságban, tanulásban és memóriában.

Erre a célra a Shal/Kv4 csatornát alkotó α-alegység régiójának a 362-es pozíción található triptofan aminosavat fenil-alaninnal (W362F) helyettesítve az úgynevezett Shal/Kv4 domináns-negatív α-alegység (DNKv4) konstrukciót hoztunk létre. A pDNKv4 konstrukciót a fehérszemű embriókba (W^{1118}, +, +) való injektálást követően húsz független transzgenikus *Drosophila* vonalat állítottunk elő, amelyeken az inszerciót az X, második, harmadik, illetve mind a második és harmadik kromoszómán térképeztük. Ezek a mutáns legyek egy 52 kDa-os méretű fehérjét expresszálnak, amely megegyezik a várt Shal/Kv4 domináns-negatív α-alegység transzmembrán fehérje méretével (DNKv4). Mivel a DNKv4 α-alegység N-terminálisa hozzá van fűzve egy kilenc peptidből (YPYDVPDYA) álló hemagglutinin epitóphoz (HA- DNKv4) a DNKv4 α-alegység sejten belüli lokalizációját az anti-HA antitest segítségével a neuron testsejtben és idegnyúlványban figyeltük meg. Ez a domináns negatív α-alegység transzmembrán fehérje a Shal/Kv4 csatorna funkcióját és I_A áramot megszüntette anélkül, hogy egy másik ioncsatorna funkcióját vagy expressziós szintjét megváltoztatta vagy kompenzálta volna. Ez lehetőséget biztosított arra, hogy széleskörű kutatásokat végezhessünk a *Drosophillá*ban az idegsejt-kisülésről (spike) és a repetitív viselkedésről − például a lárva és a felnőtt muslica mozgásáról, a felnőt muslica test-tisztogatásáról, ezen kívül a lárva illat-asszociált tanulásáról. A DNKv4 csatornával rendelkező muslicák az éter-kezelés során a Shaker mutánsokhoz hasonló tüneteket − mint test- és lábrázkódást − mutatnak. Azoknak a legyeknek, amelyek a Shal/Kv4 domináns negatív mutációt tartalmazzák az élettartama rövidebb, mint a vad típusúaknak. Ezen kívül, a DNKv4 legyekben abnormális viselkedést is megfigyelhetünk, mint például zavarok a lárva és légy mozgásában, a felnőtt légy test-tisztogatásában és a lárva illat-asszociált tanulásában.

A DNKv4 neuronnal rendelkező legyek hosszabb akciós potenciált, kisebb utóhiperpolarizációt (afterhyperpolarization), rövidebb latenciával rendelkező első akciós potenciált és gyorsan adaptáló hiányos repetitív kisülést (spike) mutatnak. Ezek a viselkedési fenotípusok összefüggésben vannak a megfigyelt ismétlődő idegimpulzusi (spike) hibával, és ez lehetőséget ad arra, hogy a I_A/Kv4 csatorna fontosságát, jelentőségét jobban megérthessük.

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Appendix

Table 1. Determination of HA-DNKv4 insertion site.

Line	Red-eyed CyORoi;	Red-eyed;Tb	Red-eyed	Total number of flies scored	Chromosomal Insertion site
1	0	19	26	45	2nd
2	11	17	22	50	2nd and 3rd
3	28	16	53	97	2nd and 3rd
4	15	7	13	35	2nd and 3rd
5	0	6	25	31	2nd
6	0	20	19	39	2nd
7	25	0	28	53	3rd
8	10	0	9	19	3rd
9	0	24	20	44	2nd
10					X
11	7	16	25	48	
12	0	5	26	31	2nd
13					X
14	0	24	33	57	2nd
15	26	0	13	49	**3rd**
16	0	9	17	26	2nd
17	29	0	19	48	3rd
18	9	0	27	36	3rd
19	24	0	29	53	3rd
20	24	0	39	63	3rd

Table1 Shown is *pUAS-HA- DNKv4* insertion site determined in 20 independent transgenic lines. *pUAS-HA- DNKv4* insertion on X-chromosome results in only red eye females and white eyed males. The absence of red eyed flies with CyoRoi marker maps the *pUAS-HA-DNKv4* insertion on the 2nd chromosome. The absence of red eyed flies with TM6B, Hu, Tb marker indicated insertion on 3rd chromosome. The presence of red eye and CyoRoi and red eye and TM6B, Hu, Tb flies in a single line indicate insertion of *pUAS-HA- DNKv4* both in 2nd and 3rd chromosome.

Table 2. Longevity test of DNKv4 transgenic lines at 25 ^{0}C

CantonS	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30	Day 35	Day 40	Day 45	Day 50	Day 55	Day 60	Day 65	Day 70	Day 75
Vial 1	10	10	10	8	8	8	5	3	2	1	0	0	0	0	0	0
Vial 2	10	10	10	10	10	10	10	10	6	1	0	0	0	0	0	0
Vial 3	10	10	10	10	10	10	9	8	5	2	0	0	0	0	0	0
Vial 4	10	9	9	9	9	7	7	7	6	2	0	0	0	0	0	0
Vial 5	10	9	9	8	7	6	5	5	3	1	0	0	0	0	0	0
Vial 6	10	9	9	9	9	9	9	8	5	5	5	5	3	0	0	0
Vial 7	10	9	9	9	9	9	8	8	7	7	6	6	3	0	0	0
Vial 8	10	10	10	10	10	10	9	6	6	5	4	4	4	2	2	0
Vial 9	10	10	10	10	10	10	9	9	7	6	5	4	4	3	3	1
Vial10	10	10	10	10	10	9	8	8	7	7	6	6	2	2	2	0
Total	100	96	96	93	92	88	79	72	54	37	26	25	16	7	7	1

elav-Gal4; UAS 14;+	Day 0	Day 5	Day 10	Day 15	Day 20	Day2 5	Day 30	Day 35	Day 40	Day 45	Day 50	Day 55	Day 60	Day 65
Vial 1	10	8	8	3	3	3	2	1	0	0	0	0	0	0
Vial 2	10	10	10	10	7	4	1	0	0	0	0	0	0	0
Vial 3	10	10	10	10	10	9	6	4	0	0	0	0	0	0
Vial 4	10	10	10	9	9	8	6	5	2	0	0	0	0	0
Vial 5	10	10	10	9	9	8	6	6	5	3	1	0	0	0
Vial 6	10	10	10	10	8	8	7	7	3	3	1	0	0	0
Vial 7	10	10	10	10	7	7	5	5	3	3	3	1	1	1
Vial 8	10	10	9	8	6	3	2	1	0	0	0	0	0	0
Vial 9	10	10	10	10	6	6	5	3	2	2	1	0	0	0
Total	90	88	87	79	65	56	40	32	15	11	6	1	1	1

w;UAS#14; +	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30	Day 35	Day 40	Day 45	Day 50	Day 55	Day 60	Day 65	Day 70	Day 75
Vial 1	10	9	9	9	8	8	8	8	5	6	4	3	1	0	0	0
Vial 2	10	10	10	10	10	10	10	8	6	6	6	6	2	1	0	0
Vial 3	10	9	9	8	8	8	8	8	6	6	3	3	1	0	0	0
Vial 4	10	10	9	9	9	9	7	7	6	5	3	3	3	3	1	0
Vial 5	10	10	9	9	9	9	8	8	6	6	4	4	4	3	1	0
Vial 6	10	10	7	6	6	6	5	4	4	2	2	2	2	2	1	0
Vial 7	10	10	8	8	8	6	6	5	5	4	4	4	4	4	3	0
Vial 8	10	10	10	10	10	10	9	9	9	6	6	6	5	4	0	0
Vial 9	10	10	10	10	10	10	10	4	4	3	3	3	3	3	3	0
Vial10	10	9	8	7	7	7	7	5	5	5	4	4	3	1	1	0
Total	100	97	89	86	85	83	78	66	56	49	39	38	28	21	10	0

elav-Gal4; ;UAS#20	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30	Day 35	Day 40	Day 45	Day 50	Day 55	Day 60
Vial 1	10	10	10	10	6	5	0	0	0	0	0	0	0
Vial 2	10	10	10	10	7	6	4	0	0	0	0	0	0
Vial 3	10	8	8	6	5	4	0	0	0	0	0	0	0
Vial 4	10	9	8	5	3	3	0	0	0	0	0	0	0
Vial 5	10	10	10	6	6	6	6	4	2	0	0	0	0
Vial 6	10	10	7	5	4	3	1	1	1	0	0	0	0
Vial 7	10	10	9	9	8	2	0	0	0	0	0	0	0
Vial 8	10	9	9	8	8	8	5	5	4	3	3	1	0
Vial 9	10	10	10	9	8	6	6	3	3	0	0	0	0
Vial10	10	10	9	9	5	4	0	0	0	0	0	0	0
Total	100	96	90	77	60	47	22	13	10	3	3	1	0

w; ;UAS # 20	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30	Day 35	Day 40	Day 45	Day 50	Day 55	Day 60	Day 65	Day 70	Day 75
Vial1	10	8	7	7	7	6	6	6	6	4	4	4	4	0	0	0
Vial2	10	9	9	9	9	9	9	9	8	8	7	7	6	1	0	0
Vial3	10	8	8	8	8	7	7	7	0	0	0	0	0	0	0	0
Vial4	10	8	6	6	6	6	4	4	3	3	3	3	3	0	0	0
Vial5	10	5	5	5	5	5	4	4	4	4	4	4	4	0	0	0
Vial6	10	10	10	9	9	9	9	9	8	8	8	8	7	5	3	0
Vial7	10	10	10	10	9	8	8	7	6	6	6	6	6	6	2	0
Vial8	10	10	9	9	9	9	9	8	7	6	6	6	6	6	5	4
Vial9	10	10	9	8	8	8	7	6	6	6	5	5	5	5	5	5
Total	90	78	73	71	70	67	63	60	48	45	43	43	41	23	15	9

Table 2. The tables illustrate how the median survival age of Wild type, background *UAS-HA-DNKv4 and elav-Gal4::UAS-HA-DNKv4* transgenic lines determined at room temperature. Top upper most rows indicate: transferring of flies to fresh media every five days. Least bottom rows indicate: total number of flies that were counted every five days. These numbers inserted to Sigma plot to determine the median survival age of the five genotypes mentioned above.

Table 3 DNKv4 longevity test at 30 ^{0}C

Elav-gal80^{ts} pore 20

10	10	9	9	9	9	9	9	9	8	8	7	5	4	4	2	1	0	0
10	10	8	8	8	7	6	6	6	6	5	4	4	4	3	2	1	0	0
10	10	10	10	10	10	9	9	9	9	9	8	7	7	4	2	1	0	0
10	10	10	10	10	10	10	10	9	8	8	7	7	7	7	5	3	2	0
10	10	10	10	10	10	9	9	9	9	7	7	6	6	6	3	1	0	0
10	10	10	10	10	10	10	9	8	7	7	6	5	4	4	2	1	1	0
10	10	10	10	10	7	7	7	7	6	6	6	5	4	3	2	0	0	0
10	10	10	10	10	10	9	8	8	8	8	8	6	6	5	2	1	0	0
10	10	10	10	9	9	9	8	8	8	7	7	5	5	3	1	0	0	0
10	10	10	10	9	9	9	9	9	8	8	7	7	5	2	1	1	0
10	10	8	7	7	7	7	7	7	7	7	7	6	5	4	3	2	1	0
10	10	9	9	9	9	9	9	9	9	9	9	7	6	5	3	2	2	0
10	10	9	9	9	9	9	8	8	7	7	6	5	5	5	2	0	0	0
10	10	10	9	9	9	8	8	8	8	7	7	5	5	5	3	1	1	0
10	10	10	10	10	10	10	10	10	9	9	9	6	5	4	2	1	0	0
10	10	10	10	10	10	10	10	10	8	8	7	5	5	4	2	1	1	0
10	10	10	10	10	10	10	9	9	7	7	6	5	5	4	3	1	0	0
10	10	10	9	9	9	9	9	9	8	8	7	6	4	3	3	0	0	0
10	10	10	10	10	10	10	8	8	7	7	7	7	6	5	3	1	1	0
190	190	183	180	178	174	169	162	160	148	142	133	109	100	83	47	19	10	0

W^{1118}

10	10	10	10	10	10	10	10	10	10	10	8	7	7	7	5	5	5	4	4	2	0	1	0
10	10	10	10	10	8	8	8	8	8	7	7	7	7	7	6	5	4	2	1	1	0	0	0
10	10	10	10	10	10	10	10	10	10	10	10	8	7	7	4	3	3	3	0	0	0	0	0
10	10	10	10	10	10	10	10	10	10	10	7	6	6	6	4	3	3	3	0	0	1	0	0
10	10	10	10	10	10	10	10	10	8	8	8	7	7	7	7	6	5	4	2	2	1	0	0
10	10	10	10	9	9	9	9	9	9	9	9	8	7	7	7	7	5	4	2	2	0	0	0
10	10	10	10	10	10	10	10	10	9	8	8	7	7	7	5	4	1	0	0	0	0	0	0
10	10	10	10	10	10	10	10	10	8	8	7	7	7	7	6	4	3	1	0	0	0	0	0
10	10	10	10	10	9	9	9	9	9	8	7	6	6	6	6	5	4	0	0	0	0	0	0
10	10	10	10	10	10	10	10	10	10	10	10	8	8	8	6	5	4	2	0	0	1	0	0
10	10	10	10	10	10	10	10	10	10	10	9	7	7	7	6	6	6	4	2	2	1	1	0
10	10	10	10	9	9	9	9	9	8	8	7	7	6	6	5	5	4	3	3	3	0	0	0
10	10	10	10	9	9	9	9	9	9	9	9	7	7	7	6	6	6	3	0	0	2	0	0
10	10	10	10	10	10	10	10	10	10	10	10	7	7	7	7	6	5	3	3	2	1	1	0
10	10	10	10	10	10	10	10	10	10	10	10	9	8	8	7	7	7	4	2	1	2	0	0
10	10	10	10	10	10	10	10	10	10	9	9	8	8	8	7	7	7	5	3	3	1	1	0
10	10	10	10	10	8	8	8	8	7	7	5	5	5	5	4	4	4	3	3	2	2	0	0
10	10	10	10	10	10	10	10	10	10	10	9	9	9	9	9	9	8	7	4	4	1	1	0
10	10	10	10	10	10	10	10	10	10	10	10	10	10	9	8	8	7	4	2	1	2	1	0
10	10	10	10	10	10	10	10	10	9	9	9	7	7	7	7	7	7	5	4	4	2	0	0
200	200	200	200	197	192	192	192	192	184	180	171	148	143	142	124	113	98	64	35	29	17	6	0

UAS-HA-DNKv4# 20

10	10	9	9	9	8	8	8	8	8	8	7	7	5	5	4	2	0	0	0	0	0	0	0
10	10	9	9	9	9	9	9	9	9	9	9	9	9	8	8	6	5	4	4	2	1	0	0
10	10	10	10	10	10	10	10	10	10	10	8	8	6	5	5	4	2	2	2	1	0	0	0
10	10	10	10	9	9	9	9	8	8	7	7	7	7	5	5	3	2	0	0	0	0	0	0
10	10	9	9	9	9	9	8	8	8	7	7	7	7	5	4	3	3	2	2	1	0	0	0
10	10	10	10	9	9	9	9	9	9	9	8	7	7	5	5	2	1	1	1	1	1	1	0
10	10	10	10	9	9	9	8	8	8	8	8	8	7	6	6	3	2	1	1	1	0	0	0
10	10	10	10	10	10	10	10	10	10	10	8	8	8	6	5	3	2	1	1	1	1	0	0
10	10	10	9	9	9	9	9	9	9	9	8	8	8	6	6	5	3	1	0	0	0	0	0
10	10	9	9	9	9	9	9	9	9	8	7	7	6	5	5	5	3	2	2	1	1	1	1
10	10	10	10	10	10	9	9	8	8	8	8	7	7	7	5	3	2	1	0	0	0	0	0
10	10	10	10	10	10	9	9	9	8	8	8	8	8	8	6	5	3	2	2	2	2	1	1
10	10	10	8	8	8	8	8	8	8	8	7	7	7	7	6	6	5	4	4	4	3	2	0
10	10	8	8	8	8	8	8	8	8	8	7	7	7	6	4	4	2	0	0	0	0	0	0
10	10	10	10	10	10	9	9	8	8	8	8	7	7	6	4	4	2	0	0	0	0	0	0
10	10	10	9	9	9	9	9	9	9	9	8	8	8	8	6	6	4	4	2	2	2	2	1
10	10	10	10	10	10	8	8	8	7	7	7	7	7	6	5	4	4	3	0	0	0	0	0
10	10	10	10	10	10	10	10	10	9	9	9	8	8	7	6	6	5	4	3	3	1	1	0
10	10	10	9	9	9	9	9	9	9	9	9	8	7	6	3	3	3	3	2	2	0	0	0
200	200	194	189	186	185	180	178	175	172	169	158	152	145	125	105	83	59	39	28	23	14	9	3

Table 3 The above three tables illustrate how the median survival age of W^{1118} , background *UAS-HA-DNKv4#20 and elav-gal80^{ts} UAS-HA-DNKv4 #20* lines determined. One hundred ninety or two hundred adult flies were collected from each line, transferred to 20 vials, on which each line consisting of 10 flies and then shifted to 30^0 C. These flies were transferred every two days and the number of survived flies was recorded to determine the median survival rate of the genotypes by Sigma plot.

Table 4 Determination DNKv4 larva locomotion

Stock	$N^{\underline{0}}$ of larvae assayed	$N^{\underline{0}}$ of squares crossed by 30 larvae	$N^{\underline{0}}$ of squares crossed in 5 minutes by 30 larvae	$N^{\underline{0}}$ of squares crossed in one minute
Wild type	30	965	965/ 30 = 32	6.4
UAS-DNKv4-20	30	880	880/ 30 = 29	6
C164-Gal4::UAS-DNKv4-20	30	792	792/ 30 = 26	5
Elav-Gal4::UAS-NKv4-20	30	645	645/ 30 = 21	4.3
109-80-Gal4::UAS-DNKv4-20	15	407	407/ 15 = 27	5.4

Table 4 Shown is the rate of larval locomotion determination. Fifteen to thirty individual transgenic lines with a genotype of wild type, UAS-DNKv4-20, C164-Gal4:: UAS-DNKv4-20, Elav-Gal4::UAS-NKv4-20 and 109-80-Gal4::UAS-DNKv4-20 were tested for the number of squires crossed per minute.

Statistical analysis of adult DNKv4 flies shaking behavior

1- t-test

Data source: Data 1 in DNKv4 behavioral test. JNB

Normality Test: Passed   (P = 0.101)

Equal Variance Test: Passed   (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
wild type	4	1	1.000	0.000	0.000
UAS-HA-DNK	4	1	1.500	0.500	0.289

Difference   -0.500

t = -1.732 with 4 degrees of freedom. (P = 0.158)

95 percent confidence interval for difference of means: -1.301 to 0.301

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.158).

Power of performed test with alpha = 0.050: 0.191

The power of the performed test (0.191) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

2- t-test

Data source: Data 1 in DNKv4 behavioral test. JNB

Normality Test: Passed   (P = 0.078)

Equal Variance Test: Passed   (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
wild type	4	1	1.000	0.000	0.000
hs::UAS-HA-DNK	4	1	3.833	0.289	0.167

Difference   -2.833

t = -17.000 with 4 degrees of freedom. (P = <0.001)

95 percent confidence interval for difference of means: -3.296 to -2.371

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

3- t-test

Data source: Data 1 in DNKv4 behavioral test.JNB

Normality Test: Passed   (P = 0.078)

Equal Variance Test: Passed   (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
hs::UAS-HA-DNK	4	1	3.833	0.289	0.167
Shaker[ks133]	4	1	5.000	0.000	0.000

Difference   -1.167

t = -7.000 with 4 degrees of freedom. (P = 0.002)

95 percent confidence interval for difference of means: -1.629 to -0.704

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.002).

Power of performed test with alpha = 0.050: 0.999

DNKv4 larva locomotion statistical analysis

1-t-test

Data source: Data 1 in locomotion test.JNB

Normality Test: Passed   (P = 0.316)

Equal Variance Test: Passed   (P = 0.827)

Group Name	N	Missing	Mean	Std Dev	SEM

wild type	11	1	32.900	2.470	0.781
elav- DNKV4 20	11	1	21.300	3.164	1.001

Difference   11.600

t = 9.139 with 18 degrees of freedom. (P = <0.001)

95 percent confidence interval for difference of means: 8.933 to 14.267

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P= <0.001).

Power of performed test with alpha = 0.050: 1.000

2- t-test

Data source: Data 1 in locomotion test.JNB

Normality Test: Passed   (P = 0.234)

Equal Variance Test: Passed   (P = 0.092)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
elav- DNKV4 20	11	1	21.300	3.164	1.001
c164-DNKV4 20	11	1	26.700	4.990	1.578

Difference   -5.400

t = -2.890 with 18 degrees of freedom. (P = 0.010)

95 percent confidence interval for difference of means: -9.325 to -1.475

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.010).

Power of performed test with alpha = 0.050: 0.735

3- t-test

Data source: Data 1 in locomotion test.JNB

Normality Test: Passed   (P = 0.302)

Equal Variance Test: Passed   (P = 0.479)

Group Name	N	Missing	Mean	Std Dev	SEM
elav- DNKV4 20	21	11	21.300	3.164	1.001
UAS- DNKV4 20	21	11	30.600	3.502	1.108

Difference   -9.300

t = -6.231 with 18 degrees of freedom. (P = <0.001)

95 percent confidence interval for difference of means: -12.436 to -6.164

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P= <0.001).

4- t-test

Data source: Data 1 in locomotion test.JNB

Normality Test: Passed   (P = 0.898)

Equal Variance Test: Passed   (P = 0.386)

Group Name	N	Missing	Mean	Std Dev	SEM
elav- DNKV4 20	21	11	21.300	3.164	1.001
109(80)UAS DNKv420	21	11	26.700	4.138	1.309

Difference   -5.400

t = -3.278 with 18 degrees of freedom. (P = 0.004)

95 percent confidence interval for difference of means: -8.861 to -1.939

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.004).

Power of performed test with alpha = 0.050: 0.854

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

5- t-test

Data source: Data 1 in locomotion test.JNB

Normality Test: Passed (P = 0.732)

Equal Variance Test: Passed (P = 0.775)

Group Name	N	Missing	Mean	Std Dev	SEM
UAS DNKv4 20	14	4	30.600	3.502	1.108
109(80)UAS DNKv4 20	14	4	26.700	4.138	1.309

Difference   3.900

t = 2.275 with 18 degrees of freedom. (P = 0.035)

95 percent confidence interval for difference of means: 0.298 to 7.502

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.035).

6- t-test

Data source: Data 1 in locomotion test.JNB

Normality Test: Passed (P = 0.356)

Equal Variance Test: Passed (P = 0.408)

Group Name	N	Missing	Mean	Std Dev	SEM
c164 UAS DNKv420	15	5	26.700	4.990	1.578
109(80) UAS DNKv420	15	5	26.700	4.138	1.309

Difference   0.000

t = 0.000 with 18 degrees of freedom. (P = 1.000)

95 percent confidence interval for difference of means: -4.307 to 4.307

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 1.000).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

7- t-test

Data source: Data 1 in locomotion test.JNB

Normality Test: Passed   (P = 0.079)

Equal Variance Test: Passed   (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
wild type	25	5	25.950	5.772	1.291
UAS DNKv420	25	5	25.950	5.772	1.291

Difference   0.000

t = 0.000 with 38 degrees of freedom. (P = 1.000)

95 percent confidence interval for difference of means: -3.695 to 3.695

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 1.000).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

8- t-test

Data source: Data 1 in locomotion test.JNB

Normality Test: Passed (P = 0.265)

Equal Variance Test: Passed (P = 0.363)

Group Name	N	Missing	Mean	Std Dev	SEM
c164UAS DNKv420	24	4	29.800	4.980	1.114
UAS- DNKv420	24	4	25.950	5.772	1.291

Difference   3.850

t = 2.259 with 38 degrees of freedom. (P = 0.030)

95 percent confidence interval for difference of means: 0.399 to 7.301

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.030).

Power of performed test with alpha = 0.050: 0.496

9-t-test

Data source: Data 1 in locomotion test.JNB

Normality Test: Passed   (P = 0.365)

Equal Variance Test: Passed   (P = 0.261)

Group Name	N	Missing	Mean	Std Dev	SEM
109(80)UAS DNKv420	24	4	29.800	4.595	1.028
UAS DNKv420	24	4	25.950	5.772	1.291

Difference   3.850

t = 2.334 with 38 degrees of freedom. (P = 0.025)

95 percent confidence interval for difference of means: 0.510 to 7.190

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.025).

Power of performed test with alpha = 0.050: 0.531

Statistical analysis of associative learning behavior

wild type	UAS-DNKv4	elav-Gal4::UAS-DNKv4
6.0000	7.0000	7.0000
8.0000	3.0000	4.0000
7.0000	9.0000	6.0000

1- t-test

Data source: Data 1 in fructose preference test

Normality Test:　　　Passed　　　(P = 0.895)

Equal Variance Test:　　　Passed　　　(P = 0.184)

Group Name	N	Missing	Mean	Std Dev	SEM
wild-type	4	1	7.000	1.000	0.577
UAS-DNKv4	4	1	6.333	3.055	1.764

Difference　　　0.667

t = 0.359 with 4 degrees of freedom. (P = 0.738)

95 percent confidence interval for difference of means: -4.486 to 5.820

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.738).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

2- t-test

Data source: Data 1 in fructose preference test

Normality Test:　　　Passed　　　(P = 0.659)

Equal Variance Test: Passed   (P = 0.117)

Group Name	N	Missing	Mean	Std Dev	SEM
wild- type	8	2	7.000	0.894	0.365
elav-UAS-DNkv4	8	2	6.000	2.191	0.894

Difference   1.000

t = 1.035 with 10 degrees of freedom. (P = 0.325)

95 percent confidence interval for difference of means: -1.153 to 3.153

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.325).

Power of performed test with alpha = 0.050: 0.055

The power of the performed test (0.055) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

3- t-test

Data source: Data 1 in fructose preference test

Normality Test: Passed   (P = 0.809)

Equal Variance Test: Passed   (P = 0.312)

Group Name	N	Missing	Mean	Std Dev	SEM
UAS-DNKv414	1	6.333	3.055	1.764	7.0000
Elav- DNKv4	1	5.667	1.528	0.882	7.0000
		3.0000	4.0000	9.0000	6.0000

Difference   0.667

t = 0.338 with 4 degrees of freedom. (P = 0.752)

95 percent confidence interval for difference of means: -4.809 to 6.142

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.752).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.