Abstract of Ph.D. Thesis

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

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Introduction

A living organism like human being contains trillions of cells. Each cell is surrounded by membrane which forms a physical barrier to retain the cell components. The membrane also regulates that conditions are ideal for cell to maintain its structure and to respond to the surrounding environment. To maintain an optimal environment inside a cell, charged molecules have to move in and out of the cell through specialized pores or ion channels.

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 multiple genes. In *Drosophila*, there are four subfamily of K^+ channel. Two of these subfamilies encode the A- type channels.

In neuron, the A- type channels play a role in regulating the frequency of repetitive firing. In the heart, it contributes to fast or early phase of action potential repolarization and determines the amplitude and duration of cardiac action potential. They are also suggested to play a 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.

Aim

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 Shal/Kv4 α-subunit forms tetramer only with its subfamily of Shal/Kv4 α-subunits, it hampers the channel's 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 Shal/Kv4 ion channel plays in motor activity, longevity and learning/ memory in *Drosophila*.

Methods and Materials

Generation of Dominant-Negative (DNKv4) construct

Shal/Kv4 dominant negative α-subunit construct was generated 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] and sub cloned into pUAST expression vector.

Insert size determination

The size of DNKv4 α-subunit fragment was verified by comparing the pUAST-DNKV4 plasmid *EcoR* I- *Hind* III restriction enzyme digestion product with the bands in the DNA ladder (1KB) following DNA agarose gel electrophoresis.

Generation of transgenic line

To generate DNKv4 transgenic lines, pUAST-DNKV4 vector was mixed with *pπ 25.7*wc (wing clipped delta 2-3 transposase) helper plasmid and injected into dechorionated W^{1118}; +; + (white eyed) *Drosophila* embryos. The DNKv4 insertion site was determined and then balanced using second and third chromosome double balanced lines.

Protein extraction and Western blot analysis

In order to purify DNKv4 protein, heads of transgenic lines were severed and sonicated in 20 µl 2X SDS sample running buffer. Anti- α-HA.11primary and anti-mouse IgG secondary antibodies were used to determine the DNKv4 expression level.

Embryonic cells immunostaining

Synchronized 5 hours old single DNKv4 embryo's content was dissociated in 20 µl Schneider *Drosophila* culture media and grown for about 7 days in a 60% humidified chamber at room temperature. The cells were then fixed with 4% formaldehyde in PBS, hybridized with anti α-HA:11 primary antibody overnight at 4 ^{0}C, washed with 0.1% sapponin in PBS and then incubated with FITC–conjugated or rhodamine-conjugated secondary antibody and mounted in glycerol.

Embryotic cell culture for electrophysiology

Developmentally stage 9-10 single DNKv4 embryo's content was removed by sucking with a sharp glass micropipette and transferred onto glass cover slips to dissociate in Schneider *Drosophila* culture media. The cells were placed in 20 ^{0}C and 60% humidified chamber for about 3- 5 days to age.

Longevity test of adult flies

For longevity test, ninety to hundred freshly hatched adult flies were collected from cultures grown at 25^{o}C and grouped into ten. Each group consisted of 10 flies were transferred into new vials every five days until the last fly died. Sigma plot was used to calculate the median survival age for adult flies.

Larva locomotion assay

For larva locomotion, room temperature (21 ^{0}C) raised individual 3rd instar DNKv4 larva was placed on 1% pure agarose plate where a 0.5 x 0.5 cm square paper grid was taped at the bottom. The number of squares crossed by the larva in 5 minutes was counted. The number of grids crossed by multiple individual larvae was averaged and then translated into number of squares crossed per minute.

Larva learning and memory assay

In order to test learning and memory, room temperature raised age synchronized 3rd instar DNKv4 larvae were trained on a 60 mm Petri dish to associate fructose (gustatory) with an odorant OCT (AM-/OCT+). Larvae with the same genotype were also trained similarly to learn to associate fructose with AM (AM+/OCT-) odorant. Learning index value is calculated

as the difference in odor preference between reciprocally trained larvae divided by two

[LI= PREF (AM+/OCT) - PREF (AM-/OCT+) / 2].

Results and conclusions

The transiently fast activating and inactivating A-type 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. In mouse cortical pyramidal neuron, for example, three transient I_A are encoded 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 (Kv4.2)* gene, encodes Shal/Kv4 current in neural somatodendritic region while the other gene, *Shaker (Kv1.4)* gene, encodes Shaker/Kv1 current in the axon and muscle.

Here, we generated more than twenty independent *Drosophila* transgenic lines that express the Shal/Kv4 dominant-negative α–subunit transmembrane protein (DNKv4). Following SDS-PAGE analysis of these DNKv4 α–subunit transgenic lines, we identified a 52 kD protein, DNKv4 α–subunit protein, that expressed at different level, and correspond to the size of Shal/Kv4 protein. With the application of voltage-clamp recording, we showed that Shal/ Kv4 dominant-negative α–subunit eliminated the I_A current and Shal/Kv4 channel function without compensating or changing other ion channels function, for instance, the delayed rectifier K ion channel. Elimination of the endogenous I_A current by DNKv4 α–subunit therefore 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. In these transgenic lines, we also showed DNKv4 α–subunit subcellular localization to the cell body and puncta along neural processes following immunostaining of embryonic neurons for HA (HA- DNKv4). Animals with DNKv4 channel revealed anesthesia-dependent leg and body shaking phenotype (hyperexitablity) similar but milder than *Shaker*^{ks133} mutant line. We also identified shorter

life span in the DNKv4 lines compared to control lines; however, we did not find change in the duration of their life cycle suggesting *Kv4/Shal* role in *Drosophila* longevity. DNKv4 lines also displayed abnormalities in larvae crawling, adult fly locomotion and grooming and defect in larvae odor-associated learning. During larvae and adult flies locomotion assay we showed that both DNKv4 larvae and adult flies exhibited reduction in the rate of crawling and lower score in wall climbing, respectively, in contrast to control lines. Adult DNKv4 flies also showed inefficient body cleaning.

During larvae odor-associated learning test, we first confirmed that DNKv4 has no effect on DNKv4 larvae gustatory perception, i.e. similar to the wild type larvae, the DNKv4 larvae were able to choose from two conditions given (preferred fructose than agarose). We also confirmed that DNKv4 larvae did not show preference for neutral odorants (Amyl acetate and Octanol). However, during learning test, DNKv4 larvae failed to associate gustatory (fructose) with the odorant (amyl acetate or Octanol) suggesting *Shal/Kv4* involvement in 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 and 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.

Present thesis is primarily based on the author's research data and the article below:

*Ping, Y., ***Waro, G.**, Licursi, A., Smith, S., Vo-Ba, D. A., & Tsunoda, S. (2011). Shal/Kv4 channels are required for maintaining excitability during repetitive firing and normal locomotion. *PloS One. Vol.6.*

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Publications

1. Ping, Y., **Waro, G.**, Licursi, A., Smith, S., Vo-Ba, D. A., & Tsunoda, S. (2011). Shal/Kv4 channels are required for maintaining excitability during repetitive firing and normal locomotion. *PloS One. Vol.6.* Impact factor = 4.092.
2. Diao, F., Chaufty, J., **Waro, G.**, Tsunoda, S. (2010). SIDL Interacts with the Dendritic Targeting Motif of Shal (Kv4) K^+ channels in Drosophila. *Molecular and Cellular Neuroscience 45, 2010.* Impact factor = 3.861.
3. Diao, F., **Waro, G.**, Tsunoda, S. (2009). Fast inactivation of Shal (Kv4) K^+ channels is regulated by the novel interactor SKIP3 in Drosophila neurons. *Molecular and Cellular Neuroscience 42 (2009).* Impact factor = 3.569.
4. Sanxaridis, P. D., Cronin, M. A., Rawat, S. S., **Waro, G.,** Acharya, U. & Tsunoda, S. (2007). Light-induced recruitment of INAD-Signaling complexes to Detergent-Resistant Lipid Rafts in Drosophila Photoreceptors. *Molecular and Cellular Neuroscience 36 (2007).* Impact factor = 3.994.
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Conferences and meetings

1. Ping, Y., **Waro, G.**, Licursi, A., Smith, S., Vo-Ba, D. A., & Tsunoda, S (2010). Kv4 is essential for maintaining excitability during repetitive firing of Drosophila neurons and normal locomotion *[Annual Meetings/Society of Neuroscience, 2010]*
2. Ping, Y., **Waro, G.**, Licursi, A., Smith, S., Vo-Ba, D. A., & Tsunoda, S (2010). Shal/Kv4 channels are required for maintaining excitability during repetitive firing and normal locomotion and grooming in *Drosophila. [Colorado State University, Fort Collins, CO/ Front Range Neuroscience Group, 2010]*.
3. Diao, F., Chaufty, J., **Waro**, G., Tsunoda, S. L. (2007). (Boston Univ., Boston, MA) SIDLS and SKIP, two newly identified proteins required for the trafficking and localization of Shal (Kv4) channels in Drosophila neurons *[Annual Meetings/ Society of Neuroscience, 2007]*.
4. Chaufty, J., Diao, F., **Waro**, G., Tsunoda, S. L. (2008). (Boston Univ., Boston, MA). Dendritic localization of Shal (Kv4) K^+ channels is mediated by the novel interactor SIDL *[Annual Meetings /Society of Neuroscience, 2008]*.