The Role of Rugose, a Drosophila AKAP and Loco, a RGS Protein in Synaptogenesis and Development

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The Role of Rugose, a Drosophila AKAP and Loco, a RGS Protein in Synaptogenesis and Development

By

Georgina Kemehe

MASTERS’ THESIS

Mentor: DR. Tadmiri Venkatesh
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Summary

Development of a synapse involves the formation of a neurotransmitter release site, which consists of a presynaptic neuron and a receptive field at the postsynaptic partners. The molecular and genetic mechanisms underlying synaptogenesis of the neuromuscular junction (NMJ), as well as the formation of functional nerve connections are critical to the proper functioning of the nervous system. Synaptogenesis is a highly regulated process which involves the synthesis, assembly and degradation of many proteins. Previous studies have shown that A kinase anchoring proteins (AKAPs) and regulator of G-protein signaling (RGS) proteins are critical in the regulation of synaptic activity through the cAMP-dependent PKA pathway. Therefore we hypothesized that there may be an interaction between rugose, a Drosophila AKAP and loco, a RGS protein at the NMJ. To better understand the mechanisms underlying synaptogenesis we studied the interactions between rugose and loco and their role in the development of functional neuromuscular synapses. We employed adult locomotor activity as an assay for these studies. We crossed rugose gamma and p-element mutants with loco mutants and tested the first generation offspring males for activity. Results showed that rugose gamma and loco double mutants had an activity greater than that of the rugose gamma mutant alone. In contrast, the rugose p-element and loco double mutants had an activity lower than that of the rugose p-element alone. Results from this experimentation suggest that loco interacts with rugose and the interaction is allele specific.
Introduction

Synaptogenesis is a process involving the formation of a neurotransmitter release site in the presynaptic neuron, and a receptive field at the postsynaptic partners (fig.1). In studying the molecular and genetic mechanisms underlying synaptogenesis of the Neuromuscular Junction (NMJ), the formation of functional nerve connections are critical to the proper functioning of the nervous system and understanding neurological diseases. The event of forming and maintaining functional synapses at the NMJ requires the precise temporal and spatial regulation of the assembly of protein complexes during synaptogenesis. Synapse formation during nervous system development and synapse degeneration in the pathogenesis of human neurological diseases are highly regulated processes.

Synaptogenesis can be divided into a sequence of progressive stages. (1) Motor axon filopodia begin neurotransmitter expression and concurrent exploration of the myotube surface. (2) Myotubes uncouple to form single-cell units soon after motor axon contact. (3) A small number of transmitter receptors are homogeneously displayed on the myotube surface immediately following myotube uncoupling. (4) Endogenous transmitter release from pioneering growth cones is detected; nerve stimulation elicits postsynaptic EJC response. (5) Motor axon filopodia and transmitter receptors are localized to the mature synaptic zone; filopodial localization is complete in advance of receptor localization. (6) A functional neuromuscular synapse is formed; endogenous muscular activity begins; nerve stimulation leads to muscle contraction. (7) Morphological presynaptic specializations develop; synapse develops mature morphology. (6) A second motor axon synapses on the myotube at the pre-established synaptic
zone. (9) Vigorous neuromuscular activity, characteristic of larval locomotory movements, begins. (10) A second stage of receptor expression begins and continues through the end of embryogenesis (Broadie et al., 1993).

Synapse formation occurs in two general manners: en passant—synaptic boutons are formed along the axon shaft; or terminaux—synaptic boutons are formed at the end of axon branches (fig. 2). C. elegans synapses occur en passant between neighboring parallel nerve processes, or between nerve processes and muscle arms (White et al., 1986). The presynaptic terminal is characterized by a cluster of synaptic vesicles surrounding the electron-dense membrane specializations; and the postsynaptic site contains densely packed ion channels and signal transduction molecules. Both the pre- and post-synaptic specializations display variable appearances depending on the organisms and neuronal types (De Camilli et al., 2001; Sorra and Harris, 2000; Zhai and Bellen, 2004).

Drosophila is a favorite model system for studying the development and function of the nervous system. In Drosophila melanogaster, mutations in specific ion channel genes can increase or decrease the level of neural/synaptic activity. Broadie et al found that electrical activity is required for the neural induction of transmitter receptor expression during synaptogenesis. Likewise, neural electrical activity is required to localize transmitter receptors to the synaptic site and presynaptic electrical activity is required to mediate the neural induction of the transmitter receptor field in the postsynaptic membrane. During the development of the NMJ, many elements must interact to form an efficient communication system: the motor neuron must find the appropriate target muscle and arborize correctly on that muscle, signaling mechanisms (i.e., transmitter synthesis, vesicle synthesis and localization, excitation-transmitter release coupling, etc.) must develop in
the presynaptic nerve terminals, and receptive mechanisms (i.e., transmitter receptor synthesis and localization, excitation, contraction coupling, etc.) must develop in the postsynaptic muscle. All of these developmental processes must be temporally and spatially coordinated to generate the precise, stereotyped, mature NMJ (Broadie et al, 1993).

Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system (CNS) and at Drosophila NMJs but Drosophila relies on many of the same neurotransmitters as vertebrates, including acetylcholine, dopamine, serotonin, γ-aminobutyric acid (GABA), and glutamate. As researchers map out neural circuits in the Drosophila CNS and begin to understand their function, it will be necessary to know the neurotransmitter identity of the relevant neurons. Acetylcholine is thought to be the most prevalent excitatory neurotransmitter used in the insect CNS, and cholinergic neurons are well described in the Drosophila CNS (Yasuyama and Salvaterra, 1999). Multiple glutamate receptor subunits are present in the embryonic brain including DGluR-IA, DGluR-IB, DNMDAR-I, DNMDA-II (Völkner et al., 2000), and DGluR-IID (Featherstone et al., 2005). The vesicular glutamate transporter (VGLUT) is an integral membrane synaptic vesicle protein that pumps glutamate into the vesicle lumen (Bellocchio et al., 2000; Takamori et al., 2000). Three VGLUT isoforms exist in mammals; at maturity, a glutamatergic neuron will typically express only one isoform. In Drosophila, a single VGLUT ortholog, DVGLUT, has been identified (Daniels et al., 2004), and the next nine genes with the closest sequence identity are not expressed in neurons (Mahr and Aberle, 2006). DVGLUT is found on synaptic vesicles and is required for vesicles to fill with glutamate (Daniels et al., 2004, 2006). VGLUTs are both necessary and sufficient for vesicular glutamate release. Therefore, all cells expressing DVGLUT as glutamatergic (Daniels et al., 2008).
Postsynaptically, the Drosophila MAGUKs (membrane associated guanylate kinases) also serve as the key organizing proteins at the synapse (Budnik V. 1996). MAGUK family are proteins that contain three or more post-synaptic density (PDZ) domains, which are involved in scaffolding organization and ion channel organization, namely Discs-Large (dlg), bears homology to the mammalian scaffold protein, PSD95 (Woods DF et al. 1993; Tejedor et al. 1997). The process ultimately forms a stable synapse and septate junction. They function in stabilizing the growing filopodia interaction with myopodia that extend from the muscle cell, forming prevaricosities. Through selective elimination and stabilization, these prevaricosities mature into boutons (Goda Y and Davis G.W. 2003).

Drosophila larvae are unusual in the high degree of stereotypy present in the organization of its musculature and neuromuscular junctions. A musculature composed of a small and apparently invariant number of muscle fibers suggests that each muscle fiber has a distinct identity and a specialized functional role. This implies that for a motor system made up of very few pre- and postsynaptic elements there must be a high degree of precision in the establishment of correct neuromuscular connections. Each muscle cell possesses regional cell-surface specializations or recognition molecules that are necessary for the different innervation patterns to be established Fig 1.

Information about the NMJ can tell us more about how communication occurs normally and during disease. Subtle changes in the setting of the complex network can either breakdown or create synaptic connections. Once synaptic function is disrupted by natural or manmade neurotoxic substances, it could lead to long-lasting and often irreversible neuronal damage. For example, a toxic insult to the nervous system can cause neuronal synapses to deteriorate in the early phase of neurotoxicity, leading to neurite degeneration and neuronal cell death, if the
damage is severe. Synaptic damage is often the first sign of neurodegeneration in many different pathological conditions, including traumatic nerve injury or ischemic stroke, and many neurodegenerative disorders, such as Motor Neuron Disease, Alzheimer’s, Parkinson’s and Huntington’s diseases.

In 1971, Ron Konopka and Seymour Benzer published "Clock mutants of Drosophila melanogaster", a paper describing the first mutations that affected an animal’s behavior. Wild-type flies show an activity rhythm with a frequency of about a day (24 hours). They found mutants with faster and slower rhythms as well as broken rhythms; flies that move and rest in random spurts. Work over the following 30 years has shown that these mutations (and others like them) affect a group of genes and their products that comprise a biochemical or biological clock. This clock is found in a wide range of fly cells, but the clock-bearing cells that control activity are several dozen neurons in the fly's central brain. Mutations inducing changes in the circadian period have been isolated for several components of the core clock, namely per, tim, dbt, ck2alpha, ck2beta (Akten et al., 2003; Konopka and Benzer, 1971; Lin et al., 2002; Rothenfluh et al., 2000; Suri et al., 2000). Behavioral screens have been used to isolate genes involved in vision, olfaction, audition, learning/memory, courtship, pain and other processes, such as longevity. Memory genes encode components of an intracellular signaling pathway involving cyclic AMP, protein kinase A and a transcription factor known as CREB. These molecules were shown to be also involved in synaptic plasticity in Aplysia and mammals.

One of the systems, which act as major regulatory components of synaptogenesis and synaptic transmission, is the cAMP pathway. The cAMP pathway activation allows for amplification of ligand binding to g-protein coupled receptor causing a cascade that induces kinase activation and regulation of gene transcription, thus leading to both dynamic and
permanent structural changes in neurons. Another system of regulation comes from the regulator of G-protein signaling (RGS) activity. We have focused on the role and activity of two functionally dynamic proteins, Rugose and Locomotion Defects (Loco) during development, and their role in synaptic structure and function. They are both involved in cAMP pathway to regulate various cellular processes that are critical for normal neuronal function.

Loco is a RGS Protein

RGS are multi-functional, GTPase-accelerating proteins that promote GTP hydrolysis by the alpha subunit of heterotrimeric G proteins, thereby inactivating the G protein and rapidly switching off G protein-coupled receptor signaling pathways. G proteins are involved in the PKA pathway. Two RGS proteins have been discovered in Drosophila, dRGS7 and Loco (an RGS12 ortholog in Drosophila). RGS12 plays a significant role in specific tissues and periods of mouse embryogenesis as well as pharmacotherapy of central nervous system disorders. RGS12 have been associated with lung cancer survival. Recent studies show that reduced expression of Loco, a Drosophila RGS protein, resulted in a longer lifespan of flies with stronger resistance to stress, higher MnSOD activity and increased fat content. In contrast, overexpression of the loco gene shortened the fly lifespan significantly, lowered stress resistance and reduced fat content, also indicating that the RGS domain containing GTPase-activating protein (GAP) activity is related to the regulation of longevity. (Yuh-Ru et al, 2011).

Loco functions as an RGS protein, inactivating the inhibitory α subunit of g-coupled protein receptor and can be located both pre- and post-synaptically. This interaction may lead to changes in synaptic transmission that was previously observed (Wise et al., in press). Gi regulates cAMP levels through inhibiting adenylate cyclase, ultimately regulating levels of
cAMP. Studies have shown that in individuals with Fragile X Syndrome and autism, have presented low levels of cAMP (Berry-Kravis, 1990; Berry-Kravis and Ciurlionis, 1998). This has be shown to lead to a decrease in evoked synaptic potential, dendritic architecture and actin “clumping” in areas near the post synapse (Kelley et al., 2007; Medrihan et al., 2009; Niesmann et al., 2011). Our lab employed a genetic modifier screen to identify genes that interact with Rap/Fzr (Kaplow et al. 2007). One of the genes that stood out among the thirty-three was locomotion defects (loco). loco has significant involvement in nervous system development: it is involved in the regulation of hetero-trimeric G protein signal (Han et al. 2006) and loco encodes a protein of the RGS (regulators of G-protein signaling) family that (Han et al. 2006). In the genetic modifier screen, loco was identified as a dominant suppressor of rough eye phenotype of rap/fzr. In loco mutants, glia cells fail to properly ensheath longitudinal axon tracts (Schwabe et al., 2005). Rap/Fzr targets Loco for ubiquitination, thereby regulating glial differentiation in the developing nervous system (Kaplow et al 2008). A delicate change in loco expression is important for the regulation of longevity. When loco expression was reduced using a UAS-loco-dsRNAi transgene under the control of Gal4 drivers (locoP283 /+ flies), the flies expressing 78% of loco transcripts extended mean lifespan by 32%. Concomitant with a longer lifespan, the flies also exhibited higher stress resistance and elevated fat content. Reduced expression of Loco, a RGS protein of Drosophila melanogaster, resulted in a longer lifespan for both male and female flies, also exhibiting stronger resistance to three different stressors (starvation, oxidation, and heat) and higher manganese-containing superoxide dismutase (MnSOD) activity (Park et. al, 2011).
Rugose is a Drosophila AKAP

Rugose (rg) encodes a Drosophila AKAP (DAKAP550), which has been previously shown to be required in normal pattern formation in the developing eye (Shamloula et al. 2002). AKAPs are a large family of proteins originally identified in mammals which modulate the specificity of protein kinase A (PKA) function by targeting and classifying PKA to various subcellular structures. AKAPs contain binding sites for other proteins that require PKA phosphorylation and thus provide a framework for the coordination of phosphorylation and dephosphorylation events by sequestering enzymes such as protein kinases and phosphatases with appropriate substrates. This allows integration of cAMP-PKA pathway with other pathways. AKAPs also bring together signal transduction and signal termination molecules in a convergence of signaling pathways. It has been shown by many labs that, in addition to directing the action of PKA, AKAPs engage other signaling molecules (Colledge and Scott, 1999; Tasken and Aandahl, 2004). Kim and Wu (1994) have shown that disruption in the cAMP pathway can greatly impact the ability of the filopodia to make contact with its target and create presynaptic boutons. Anchoring of the kinase facilitates localized activation of the PKA catalytic subunit (C) following elevation of the second messenger cyclic AMP (cAMP). Alterations in synaptic activity and cAMP function are known to modulate the synaptic terminal growth at Drosophila larval NMJs (Budnik et al., 1990; Davis et al., 1996; Schuster et al., 1996; Zhong & Wu, 2004). Ht31, a molecule with affinity to bind to PKA RII, has been shown to uncouple PKA from amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptors which reduce the postsynaptic AMPA receptor currents and synaptic potential (Rosenmund et al., 1994). Activation of Protein kinase A (PKA) at discrete intracellular sites facilitates oogenesis, establishment of tissue polarity in the embryo, imaginal disc morphogenesis, synaptic function.
and development in Drosophila. In the developing eye, PKA plays an important role in the initiation of pattern formation and morphogenesis through its interactions with Hedgehog (Hh), DPP, and Wingless (Wg). Signaling by Sev, Egfr, N, and the cAMP-PKA pathways leads to the activation of a variety of downstream transcription factors. Because these signaling pathways regulate a multitude of cellular functions in different tissues and due to the number of transcription factors involved, it became apparent that a single signaling pathway alone could not specify particular cell fates. Thus, PKA-anchor protein complexes may be involved in controlling these crucial biological processes. Evaluation of this proposition requires knowledge of PKA binding/targeting proteins in the fly. DAKAP550/Rugose is a large (>2300 amino acids) acidic protein that is maximally expressed in anterior tissues. Later in development it is found in neuroblasts and is enriched in neurons. It binds regulatory subunits (RII) of both mammalian and Drosophila PKAIi isoforms. The anchor protein is expressed in many cells in nearly all tissues throughout the lifespan of the fly. However, DAKAP550 is highly enriched and asymmetrically positioned in subpopulations of neurons and in apical portions of cells in gut and trachea. The combination of RII (PKAIi) binding activity with differential expression and polarized localization is consistent with a role for DAKAP550 in creating target loci for the reception of signals carried by cAMP (Han, 1997).

In the developing Drosophila eye, cell fate determination and pattern formation are directed by cell-cell interactions mediated by signal transduction cascades. Mutations at the rugose locus (rg) result in a rough eye phenotype due to a disorganized retina and aberrant cone cell differentiation, which leads to reduction or complete loss of cone cells. The cone cell phenotype is sensitive to the level of rugose gene function. Molecular analyses show that rugose encodes a Drosophila A kinase anchor protein (DAKAP 550). Genetic interaction studies show
that rugose interacts with the components of the Egfr- and Notch-mediated signaling pathways. These results suggest that rg is required for correct retinal pattern formation and may function in cell fate determination through its interactions with the Egfr and Notch signaling pathways. rugose has also been identified in a genetic screen for modifiers of Hairless (H), a Notch pathway antagonist (Schreiber, 2002) and rugose interacts with Egfr and N signaling pathways (Shamloula, 2002).

In addition to phenotypes in the eye, rugose mutants exhibit wing vein defects and embryonic lethality with partial penetrance. The penetrance is variable depending on the particular rugose allele. Loss of rugose function leads to an incomplete wing vein L5. rugose mutants also exhibit an embryonic semilethal phenotype. The extent of lethality varies with the strength of the rugose allele. In the null alleles rg gamma6 and rg gamma3, for example, 20%–40% of the fertilized eggs fail to hatch, suggesting defects in the embryonic stages. The cellular and molecular basis for the lethality is not clear at this point. The pleiotropic nature of rugose mutations is consistent with the multiple roles of cAMP-PKA-mediated signaling in Drosophila (Shamloula, 2002).

Studies on neurobeachin (nbea) gene, the mammalian homolog of rugose, lend further support for a role for AKAPs at the synapse. Genetic studies in Drosophila have shown that cAMP and PKA (protein kinase A) signaling pathway mediate many processes in cells such as oogenesis, tissue polarity, and synaptic organization. rugose encodes for a Drosophila A kinase anchor protein (DAKAP) which is necessary for functional PKA signaling. DKAPs are included in the family of proteins found in mammals called AKAP and modulate the specificity of PKA function by targeting and compartmentalizing PKA to various sub-cellular structures.
The protein’s isoforms can vary depending on the nature and location and of the organelle, thus modifying the molecular weight.

Neurobeachin, a protein implicated in membrane protein traffic and autism, is required for the formation and functioning of central synapses. Loss-of-function of nbea completely blocks evoked synaptic transmission at neuromuscular junctions while nerve conduction, synaptic structure and spontaneous neurotransmitter release are completely normal (Su et. Al 2004). Nbea has also been implicated in vesicular traffic at the synapse and has been shown to be required for normal development of the synapses (Medrihan et al., 2009). Recent studies have shown that nbea gene is disrupted in individuals with Autism spectrum Disorder and the nbea gene spans the common Fragile site FRA 13A in human (Savelyeva et al., 2006; Medrihan, 2009). In addition, nbea gene is also disrupted in the human Chediak-Higash syndrome, which presents as mental retardation and can cause fatal fetal developmental complications (Wang et al., 2000; Medrihan, 2009). Research in our lab has shown adverse effects on social behavior, especially on social interaction and innate avoidance.

Shamloula et al. research has shown that DAKAP 550 drosophila was mutated with p-element and gamma ray exposure. These mutants were shown to have substantial deregulated effects that were seen in each stage of development and in adults. These mutants show a gradient of phenotypic defects. As a result, data has shown morphological changes in bouton size, length and branching, and activity.
Methods

To identify proteins that regulate synaptic development, we began to test rugose (rg) and loco mutants for changes in NMJ morphology. The cAMP-PKA pathway is necessary to regulate many different cellular development processes in the nervous system. To test whether Drosophila A kinase anchoring proteins (DAKAP), which are cAMP-PKA organizing centers that provide scaffolding for allowing PKA protein phosphorylation, are necessary in synaptogenesis, we used rugose loss-of-function mutants (DAKAP 550) to examine the morphological structure of the NMJ. We also examined the morphological structure of the NMJ in the loco mutants. The we assessed the activity and circadian rhythm of both rugose and loco flies using the Drosophila activity monitor (DAM) from Trikinetics. The locomotor activity assay can identify altered behavior patterns over the course of several days in small populations, or even individual flies. Commercially available, highly efficient automated systems allow for continuous data collection from large numbers of individuals, and analytical tools make it possible to quickly analyze multiple aspects of circadian behavior from each experiment.

Immunohistochemistry

To identify changes in immunofluorescence intensity, control and mutant larvae were dissected and stained together. Wandering third instar lava was dissected in hemolymph-like 3.1 Ca-free insect saline by a dorsal incision through the bodywall and pinned out flat in Sylgard-coated dishes. All internal organs were removed, exposing the inner surface of the bodywall muscles. The preparations were fixed in 4% paraformaldehyde, for 25 minutes. Washes and antibody incubations were performed in Phosphate Buffer Solution with Triton-X (0.1%). Males
were chosen due to the presence of rugose allele mutation on the X-chromosome. The preparations were washed three times for 5 minutes with 0.2 M Phosphate Buffer Solution containing 0.2% Triton-X 100 (PBST). Specimen were kept overnight in 4°C. the next day, the preparations were washed again; three times for 5 minutes with 0.2 M Phosphate Buffer Solution containing 0.2% Triton-X 100 (PBST). Secondary antibodies were applied for 2 hours: Cy5-Alexa Fluor 635 conjugated anti-Phalloidin at 1:1000 (Invitrogen), goat anti-mouse FITC (Jackson Laboratories) at 1:100 and TRITC conjugated goat anti-Horseradish Peroxidase (Jackson Laboratories) at 1:100. Sections then were washed twice for 5 min and mounted using Vectashield (Vector Laboratories). All imaging was performed using a LSM 510 and LSM 710 Confocal Laser Scanning System (Carl Zeiss). Dlg and HRP positively stained boutons counts were performed on muscle 6 and 7, abdominal segment A3.

**Drosophila Locomotor Activity Assay**

We tested to see if there were changes in synaptic transmission by testing their activity and circadian rhythm. Both the wild type and mutant 1-3 day old male flies, were grown on standard fly food and standard room temperature, under a 12 h:12 h light dark cycle (lights on at 9:00 h). Experiments included one day of adaptation, and four days of a 12 h:12 h light dark cycle. Individual 1-3 day old male flies were initially placed in a Drosophila Activity Monitor System (DAMS, Trikinetics, Waltham, MA, USA) inside 5mm diameter glass tubes (one fly/tube) containing standard fly food at one end and a cotton plug on the other. These monitors were housed inside a humidified Digitherm incubator, where humidity and a temperature of 23°C were kept constant. Each of the mutant strains was simultaneously run to wild type Canton-S (CS), with the average total activity for each strain being recorded in thirty-minute bins. The activity recorded for each bin was determined by how many times each individual fly crossed an
infrared light beam that bisected the tube. Each tube is monitored by an infrared emitter-detector that will record how many times the walking fly has interrupted the light beam during a defined period of time (bin). Each bin of the experiment (usually 1 or 2 weeks long) will be associated with an activity value and the resulting file will be analyzed to detect rhythmic patterns.
RESULTS

Morphology of synapses

The distribution and morphology of glutamatergic synapses on Drosophila body wall muscle fibers were examined at the single-synapse level using immunocytochemistry. Glutamate-immunoreactive motor endings innervate the entire larval bodywall musculature, with each muscle fiber receiving at least one glutamatergic ending. The innervation is initiated at stereotyped locations on each muscle fiber from where moderately branched varicose nerve processes project over the internally facing muscle surface. Individual muscle fibers have distinct stereotypic patterns of nerve endings that occupy characteristic regions on the cell surface. The muscle-specific branching pattern of motor endings is reiterated by segmentally homologous fibers. Two morphological types of innervating nerve processes can be distinguished by their bouton size distributions.

The full-grown larva is 3-4 mm in length, and has about 400 striated muscle fibres arranged in a constant pattern (P1. 1). A muscle is a single fibre about 400,um long, 80,um wide, and 25 /tm thick; its polytene nuclei (about 10-20 per fibre) are flattened and aligned in longitudinal rows on the innermost surface of the fibre. Sarcoplasmic reticulum and dyads are found among the filaments within each fibril; perforate Z-disks are also found which seem to be characteristic of supercontracting muscles (Osborne, 1967b). A tracheolar plexus is in close contact with each fibre to supply air. Each segmental nerve radiating from the ventral ganglion approaches the left or right segment at the ventral mid line, branches, and innervates fibres of that half segment.
Loco mutantants show enlarged but a decrease in boutons number

The locomotor assay described before was used to simultaneously assess both circadian and sleep behavior. Like many other organisms, the fruit fly Drosophila melanogaster operates on a 24-hour schedule maintained by environmental input to an internal body clock. The molecular basis of the clock relies on oscillations in the activation of particular genes at certain times of the day. The key feature of these molecular oscillations is a negative feedback loop in which the protein products of genes actually turn off production of more protein. This process is possible in all cells of Drosophila; however, the highest concentrations of the essential molecules are found in lateral neurons of the central nervous system. These lateral neurons, or pacemaker cells, are the Drosophila equivalent of mammalian neurons in the suprachiasmatic nucleus. Results showed a shift in activity for all loco mutants (Liu et al). CS flies typically anticipate the start of day by 9 AM and end of day by 9pm. During these times, they are the most active and thus record the most activity. Loco flies anticipated day around 1PM and night at 1AM. During these times, they record the most activity. (Fig. 7)

Is There an Interaction Between Rugose and Loco?

Akaps may be anchors for other signaling pathways (Shamloula et. al, 2002) and are involved in other pathways such as RAS signaling(Fishman et. al). RAS belongs to a small GTPase class and Loco regulates GTPases. Therefore, is there a direct interaction between rugose and loco? To test for this hypothesis, we made crosses between Rugose and Loco mutants. We collected their offspring at third instar larva and adult stages and tested them using immunohistochemistry and Drosophila locomoter activity assays.
Discussion

Our data show that morphology and physiological properties of the larval as well as adult nmj synapses are significantly altered. We have shown that rg mutants show slight enlargement in bouton size and less oval-shaped boutons. We found that there were significant changes in the number of boutons at the NMJ. Overall, there was a decrease in the number of boutons in more severe alleles of rg (rgγ 1, 7, 9 and 11). These results support our conclusion that disruption of AKAP function can lead to significant changes at the synapse. Taken together we have presented data that suggests AKAPs specifically Rugose (DAKAP550) facilitates key intermediate steps that govern proper synaptic transmission and synaptic plasticity.

Loco is as an RGS protein, inactivating the inhibitory α subunit of g-coupled protein receptor. This interaction may lead to changes in synaptic transmission as was previously observed with shift in locomotor activity and disorganization of boutons. Due to the shift in locomotor activity observed in loco mutants, Loco may be influence the internal biological clock of flies. Our results also showed that there is an interaction between rugose and loco. This interaction may be allele specific since phenotypes are based of certain alleles.
Fig. 1. At the neuromuscular junction, there is a precise alignment and neurotransmitters are released from the presynaptic neuron to the post synaptic muscle.
Fig. 2. The axon ends in boutons. Boutons are small button-like swellings at the end of an axon that package, store and release neurotransmitters into the synaptic cleft of the junction between neuron and muscle, overlapping the muscle site where the receptors are located.
Fig. 3. AKAPs forms multi-protein complexes that integrate cAMP signaling with other pathways (Carnegie et. al.).
Fig 4. RGS proteins regulate the α-subunit of the G-protein through GTPase activity.
Genetics and Transgenetic Lines

The following mutant rugose and loco flies were used:

<table>
<thead>
<tr>
<th>Rugose allele</th>
<th>Phenotype</th>
<th>Classification</th>
<th>Mutagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>rg^1f</td>
<td>Severe</td>
<td>Null</td>
<td>γ-rays</td>
</tr>
<tr>
<td>rg^3a</td>
<td>Mild</td>
<td>Hypomorph</td>
<td>γ-rays</td>
</tr>
<tr>
<td>rg^6n</td>
<td>Severe</td>
<td>Null</td>
<td>γ-rays</td>
</tr>
<tr>
<td>rg^7f</td>
<td>Moderate</td>
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<td>Moderate</td>
<td>Hypomorph</td>
<td>γ-rays</td>
</tr>
<tr>
<td>rg^11f</td>
<td>Moderate</td>
<td>Hypomorph</td>
<td>γ-rays</td>
</tr>
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<td>rg^p2</td>
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<td>P-element</td>
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<tr>
<td>------------</td>
<td>-----------</td>
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<td>Loco$^{t1}$</td>
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<td>Loss of function allele</td>
<td>Ethyl Methanesulfonate</td>
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<td>behavior defective</td>
<td>Loss of function allele</td>
<td>Ethyl Methanesulfonate</td>
</tr>
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<td>Loco$^{P283}$</td>
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<td>Amorphic allele-genetic evidence</td>
<td>Delta 2-3</td>
</tr>
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<td>Loco$^{P542}$</td>
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<td>Imprecise excision of locoEY04589</td>
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<tr>
<td>lococ1ΔGoLoco.Scen\UAS</td>
<td>lethal</td>
<td>Loss of function allele</td>
<td>in vitro construct - regulatory fusion, in vitro construct - deletion</td>
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Fig. 3. (A) Larva stained with phalloidin conjugated with cy5. (B) To show boutons at the presynapse, the larva is stained with goat anti-mouse-FITC. (C) Axons are shown at the neuromuscular junction. Larva stained with HRP-TRITC. (D) Merge of different staining. The musculature of Drosophila larvae is remarkably simple, consisting of up to 31 discrete unicellular muscle fibers per hemisegment. Each muscle fiber is uniquely identifiable, and many are segmentally repeated. Five segment-specific patterns exist for segments T1, T2, T3, A1-A7, and A8, respectively. Each muscle fiber may possess regional cell-surface recognition factors that are necessary for the different and specific innervation patterns to be established.

Nerves end in clusters of boutons about 3-5/µm in diameter
Figure 4. Camera lucida tracings of glutamate-immunoreactive endings on identified muscle fibers in Drosophila. Examples of the entire innervation pattern of muscles 4, 6, 7, 12, and 13 are shown. Examples of Type I and Type II endings are indicated by ~I~ow.r. Note the extensive ramifications of the pattern of muscles 4, 6, 7, 12, and 13 are shown. Examples of Type I and Type II endings are indicated by ~I~ow.r(Keshishian et al, 1989)
**Fig. 5.** *rugose* mutants exhibit altered synaptic structure. Confocal images of the Neuromuscular junction from third-instar larvae stained with antibodies, anti-Dlg (green) anti-HRP (red) and Phalloidin (blue). Canton-S (A). In rugose mutant alleles, boutons number appears decreased and boutons are slightly enlarged (B-H) with few axonal branching and vacuous spacing within the boutons are seen (c). rugose mutant allele *rgp2* (I) and *rgp5* (H) exhibit less severe phenotype than the gamma allelic series. Note all images (A-I) are from *Muscles 6,7-segment 3.*
Fig. 6. Bouton size is altered loco mutants. There is a significant change in the size of the boutons. In loco the boutons are significantly decrease (0.015027103) in comparison to wild-type.
Fig. 7. A-D. Shows activity profiles for CS and loco mutants. Loco mutants in (A-D) exhibit the greatest shift in activity while locoT1/TM6 alleles (E) show the least shift in activity.
Rugose Mutants Exhibit Hyperactivity

Activity Profile Over Four Successive Days of a 12:12 Hour L:D Cycle

- **A**

Activity Profile Over Four Successive Days of a 12:12 Hour L:D Cycle

- **B**
Fig. 8. Rugose mutants show overall hyperactivity. The average beam crossing/30 min for CS is 30. In rg1 mutants, average beam crossings/30 min is about 70 which is more than 2 times the amount of activity (A-D). Rugose P-element mutant shows the highest average beam crossing/30 min. The activity of rgP2/x\textsuperscript{xyf} is averages about 100 which is more than 3 times the activity of the wildtype CS, (E).
Loco and Rugose interact at the synapse
FIG. 9. A-D. Flies have copies of both rugose γ and loco mutations. Results show even greater activity when the fly carries copies of both rugose γ and loco mutations as compare to flies carrying single alleles of rugose γ or loco mutations. In contrast, a decrease in activity was observed in flies that carried copies of both rugose p-element and loco mutations as compared to flies with only rugose P-element or Loco(E-F).
Rugose and Loco Double Mutant Shows Improved Morphology

Fig.11. A fly carrying both copies of rugoseɤ6 and locoP452 showed improved bouton morphology as compared to single rugoseɤ6 mutant.

Fig. 12. A fly carrying both copies of rugoseɤ4 and locoP452 shows subtle change improved, in bouton morphology as compared to single rugoseɤ4 mutant.
Fig. 13. A-B. Results from test cross still shows no significant change in activity.
Fig 14. Results show no significant change rgp2/+ as compared to rgp2/x^xyf

Fig 15. A copy of the wildtype allele in the locop283 mutant showed a more organized and significant change in bouton morphology.
References


Yishi Jin, Synaptogenesis, The online review of C. Elegans Biology, Department of Molecular, Cell and Development Biology, Howard Hughes Medical Institute, University of California, Santa Cruz, Santa Cruz, CA 95064, USA


Tavalin, SJ. (2008) “AKAP79 Selectively Enhances Protein Kinase C Regulation of GluR1 at a Ca2+-Calmodulin-dependent Protein Kinase II/Protein Kinase C Site” J Biol Chem  283(17): 11445–11452


