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14. ABSTRACT This research assayed the role of three Drosophila gene products in the synaptic mechanisms of short-term memory: 14-3-3zeta, origin of replication 3 (ORC3) and alpha-integrin proteins. Null mutation of all three genes result in developmental arrest and lethality, whereas partial loss-of-function mutations cause defects in short-term memory retention in the adult. All three proteins were found to be localized in presynaptic terminals, specifically associated with presynaptic boutons. Electrophysiological analyses of both null and hypomorphic mutations revealed defects in synaptic transmission. Each class of mutants was

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ABSTRACT(con't)

defective in presynaptic calcium-dependent neurotransmitter release mechanisms
and multiple forms of calcium- and activity-dependent functional presynaptic
modulation properties. In particular, all three classes of mutants displayed
defective synaptic potentiation following tetanic stimulation (post-tetanic
potentiation; tpt). These results suggest that these three gene products play
roles in the presynaptic terminal necessary for the synaptic potentiation
underlying memory formation.

Final Technical Report

"Genetic and Electrophysiological Investigations of Learning and Memory Mechanisms"
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Technical Summary:

Genetic mutations have been generated in the fruitfly, *Drosophila melanogaster*, which both decrease and potentiate the ability to learn and remember. These mutations affect specific aspects of the learning to memory pathway including initial acquisition (learning), immediate retention (short-term memory (STM); < 5 minutes), intermediate of amnesia-resistant memory (ARM), and long term memory (LTM) representing consolidated retention of > 1 day. This class of mutations identifies genes which encode proteins with essential functions in learning and memory. This research program focussed on mutations in three gene products with a known role in STM: 1) 14-3-3ζ protein implicated in protein kinase signal transduction, 2) original of replication (ORC) protein implicated in nuclear replication and transcriptional control, and 3) integrin protein implicated as a transmembrane receptor for extracellular matrix. Our goal was to assay putative functions for each of these proteins in neuronal synaptic plasticity mechanisms believed to mediate memory retention. Investigations with specific antibodies against each protein showed that they localize to synaptic contacts and specifically reside in presynaptic boutons. Furthermore, electrophysiological assays of loss-of-function mutations in each gene showed defects in functional neurotransmission. In particular, calcium-dependent forms of synaptic plasticity were impaired in these genetic mutants and all three showed a severely compromised ability to manifest post-tetanic potentiation (PTP). This type of potentiated synaptic transmission has long been postulated to provide the cellular basis for memory formation. Therefore, the results of this study support the hypothesis the synaptic plasticity, specifically maintained functional potentiation, provides a cellular mechanism of memory retention. Moreover, this study has shown that 14-3-3ζ, ORC, and integrin proteins are molecular components of the synapses required to mediate both synaptic potentiation and STM formation.

Research Production:

This research program gave rise to three publications during the funding period of 1997-2000. Each manuscript focuses on one of the three genes targeted in the research program. Two studies were published in Neuron and the third in the Journal of Neuroscience:

1. Broadie, K., Rushton, E., Skoulakis, E.C.M. and Davis, R.L. (1997). Leonardo, a 14-3-3 protein involved in learning, regulates presynaptic function. *Neuron* 19: 391-402.

2. Rohrbough, J., Pinto, S., Mihalek, R., Tully, T. and Broadie, K. (1999). Latheo, a Drosophila gene involved in learning, regulates functional synaptic plasticity. *Neuron* 23: 55-70.

3. Rohrbough, J., Grotewiel, M.S., Davis, R.L. and Broadie, K. (2000). Integrin-mediated regulation of synaptic morphology, transmission, and plasticity. *Journal of Neuroscience* 20: 6868-6878.

Results of Specific Aims:

I. Leonardo, a 14-3-3ζ protein regulating presynaptic plasticity and STM

Viable *leonardo* (*leo*) mutants are deficient in short-term memory (3 and 15 mins following training; 1). Null mutants are embryonic lethality, indicating that *leo* is also an essential gene. The *leonardo* gene encodes a member of the 14-3-3 protein family (14-3-3ζ), which is prominently expressed in neural tissues throughout development (1). There are multiple isoforms of the 14-3-3 proteins and at least six distinct genes in mammals. 14-3-3 proteins are abundant acidic cytosolic proteins of approximately 30 kDa which function as dimers (2-5). All family members bind to a consensus sequence (RSXSXP) present in a wide range of target molecules in a number of intracellular signaling mechanisms, and quite probably act as integrators of information between different signaling cascades (6-8). Two pathways seem primarily significant: 1) 14-3-3 proteins act as negative regulators of protein kinase C (PKC) and directly effect kinase activity upon phorbol ester stimulation (9-10); 2) 14-3-3 proteins regulate the activity of the small GTP-binding protein Ras through its effector kinase Raf-1 in the mitogen-activated protein kinase (MAPK) pathway (11-16). 14-3-3 proteins complex with Raf-1 during recruitment to the plasma membrane and activation by Ras (17-18). Genetic studies in yeast show that 14-3-3 proteins play a role in Ras/Raf-dependent vesicular trafficking (19). Likewise, catecholamine release from bovine adrenal chromaffin cells is enhanced by 14-3-3 proteins, which regulate a Ca^{2+}-independent, PKC-dependent step of the vesicular trafficking pathway (20-21). 14-3-3 proteins function in this neurosecretory role by mediating the reorganization of the cortical actin cytoskeleton (21), which normally forms a barrier between the vesicles and the plasma membrane. The 14-3-3 protein family is particularly enriched in the mammalian brain (>1% total protein), where these proteins are also thought to be involved in Raf/PKC regulation and vesicular trafficking (2,8). 14-3-3 proteins bind the membranes of synaptic vesicles (SVs) in central synapses (22). Thus, 14-3-3 proteins occupy a key interface between synaptic vesicles, the cytoskeleton and intracellular regulatory kinase cascades and play a crucial role in early associative learning.

Leonardo/14-3-3ζ is highly expressed at presynaptic contacts in both the neuromuscular junction (NMJ) and central nervous system (CNS) neuropil in *Drosophila* larvae. Leonardo is expressed in all classes of synapses independent of the nature of the neurotransmitter, both in the periphery (type I, II and III NMJs) and CNS. The protein is specifically enriched in presynaptic boutons where it co-localizes with synaptic vesicle (SV) pools. Electrophysiological assays in null *leo* alleles show that basal synaptic function is not strongly impaired, indicating that Leonardo does not play an essential role in synaptogenesis or neurotransmission. However, null mutants show a 30% decrease in basal transmission amplitude, suggesting that Leonardo plays a facilitory role in neurotransmitter secretion. This defect is dramatically heightened under conditions that challenge function; 1) decreasing extracellular $[Ca^{2+}]$ causes transmission failure during high frequency stimulation, and 2) >10 Hz stimulation causes progressive loss of transmission amplitudes and fidelity. While short-term plasticity, including paired pulse and short-term facilitation, are not impaired in *leonardo* mutants, longer term plasticity, including both augmentation and PTP, are lost. Indeed, the strong potentiation that normally follows a tetanic stimulation is replaced with a period of depression in the mutant synapse. Thus, Leonardo plays a role in maintaining transmission under conditions of high usage and long-term plasticity. Two models of Leonardo function have been suggested: 1) The specific *leo* functional defects, coupled to earlier work of 14-3-3 proteins outlined above, suggest that Leonardo may function to regulate SV dynamics through the direct or indirect modulation of the presynaptic actin cytoskeleton. Leonardo may function through interactions with Raf-1 kinase and PKC, as these

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kinases have been independently implicated in synaptic modulation (23-26). 2) Leonardo may function in K^+ channel regulation (27). Leonardo biochemically interacts with the slow-poke (slo)-binding protein (slob). Slowpoke is a Ca^{2+}-dependent voltage-gated K^+ channel which may potentially be a site of integration of electrical and biochemical signals. Leonardo may act to regulate the activity of the slo channel, thereby modulating neurotransmission (27).

II. Latheo, an ORC3 protein regulating presynaptic plasticity and STM

Viable *latheo* mutants, like *leo*, show a specific defect in short-term memory (3 and 15 mins following training; 28,29). Null alleles are lethal in the late larval/early pupal stages, showing that *latheo*, like *leo*, also encodes an essential protein. The *latheo* gene encodes a protein of ~70 KD with homology (~30% identical) to yeast origin of replication 3 (ORC3; 29). Consistent with this homology, *latheo* plays an essential role in cell division and lethality in null alleles results from the absence of post-embryonic cell division (29). Latheo biochemically interacts with other members of the ORC complex, including ORC2. ORCs have at least two roles in the nucleus; 1) initiation of DNA replication during cell division, and 2) the regulation of transcription, including transcriptional silencing. Latheo is present in the nucleus of mitotic neuronal precursors and in the cytoplasm of post-mitotic mature neurons, including particularly adult neurons of the brain mushroom bodies (4). These studies suggest that *latheo* plays distinctive functions during development and learning. One intriguing possibility is that Latheo acts as a liaison between the nucleus and synapse mediating the synaptic modulation underlying memory formation.

Latheo/ORC3 is expressed in the cytoplasm and synaptic regions of post-mitotic neurons in the adult brain MBs (29) and in NMJ boutons in *Drosophila* larvae. Like Leonardo, Latheo is localized to SV-dense boutons at all classes of NMJ terminals (type I, II and III) independent of the neurotransmitter type. Null *latheo* mutants display a weak reduction in synaptic growth, although this phenotype isn't striking and may not be physiologically relevant. The striking functional defect is a Ca^{2+}-dependent increase in synaptic transmission amplitude; null mutants show a 3-4 fold increase in basal transmission amplitude in 0.2 mM Ca^{2+}. The disparity between wild-type and mutants is less severe at higher $[Ca^{2+}]$ so that the Ca^{2+}-dependency curve for transmission is effectively shifted; a slope of 3.5-4 in wild-type and 2-2.5 in null mutants. All forms of synaptic modulation properties are strongly perturbed in *latheo* mutants. Both short-term forms of facilitation and longer-term potentiation are severely depressed. One possibility to explain these results is that the heightened basal transmission in *latheo* mutants is effectively saturating the SV release machinery and thereby preventing any form of functional plasticity. In experiments in lower $[Ca^{2+}]$ to equalize transmission amplitude, we found that some modulation properties were weakly rescued but others were not, showing that Latheo independently affects basal transmission and modulation properties. Two models may account of Latheo's synaptic function: 1) Synaptic and nuclear functions may be related. Latheo may act as a shuttle to relay activity-dependent information from the synapse to the nucleus where it acts via the ORC complex to regulate transcription of synaptic genes. 2) Synaptic and nuclear roles may be unrelated. Latheo may play one role during development and a second, unrelated role in post-mitotic neurons during synaptic modulation. Numerous examples of such pleiotropy are known (e.g. the role of *disks large* (*dlg*) as a tumor suppressor and synaptic organizer; 30-31).

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III. Volado, an α-integrin protein regulating presynaptic plasticity and STM

The final gene in the trilogy, *Volado (Vol)*, like the other two target genes, impairs memory formation from 3-15 minutes after training (32). However, *Volado* has a uniquely dominant effect in perturbing short-term memory. Null mutations cause late larval lethality, showing once again that these learning genes play essential functions in development. *Volado* encodes two splice isoforms of an α-integrin protein, Volado-long (Vol-l) and short (Vol-s), differing only in the first 63 amino acids (32). The splice isoforms appear functionally redundant since mutation of either Vol-l or Vol-s equally impairs memory. Like, *leonardo* and *latheo*, both *Volado* isoforms are strongly expressed in adult brain MBs (32). Integrins are transmembrane α/ß heterodimers that link the cytoskeleton and extracellular matrix (ECM; 33-36). Integrins function in cell adhesion and the bidirectional flow of information across cell membranes. Intracellular signals alter integrin affinity for ECM ligands (inside-out signaling) and ECM ligands trigger intracellular events such as cytoskeletal re-organization and the modulation of gene expression (outside-in signaling) (34-37). It is also clear that integrins act by modulating other classes of receptors and that adhesive and signaling functions may be distinct (38-39). Integrins function in axonal outgrowth, target recognition, and sprouting of synaptic arbors (33,40), but are also involved in mature synaptic function (41-42). Integrins participate in the modulation and Ca^{2+}-dependent regulation of neurotransmitter release (43-44). The pharmacological blocking of integrins in rat hippocampal slices interferes with the stabilization of long term potentiation (LTP), thought to underlie certain types of learning and memory (45-46). Integrin activation/signaling occurs within minutes of LTP induction and is crucial for the maintenance and stabilization of synaptic potentiation (47). These studies show that integrins play a role in memory formation in both rats and flies.

Volado and other members of the position-specific (PS) integrin family (37) are expressed at the *Drosophila* larval NMJ: a beta subunit (ßPS), expressed in both pre- and postsynaptic membranes, two alpha subunits (αPS1, αPS2), expressed in the postsynaptic membrane and Volado/αPS3, expressed presynaptically (48). The integrin complement on both synaptic faces is analogous to the situation at vertebrate synapses. All PS-integrins are first detected in the postembryonic NMJ, coincident with the onset of terminal type differentiation and robust plasticity (48). Volado, uniquely, appears to be "dynamically expressed" with only a small subset of boutons expressing high levels at any given time both in the NMJ and CNS. Like the other synaptic integrins (48), *Volado* plays a role in morphological plasticity, although, like *latheo*, this phenotype appears relatively mild and may not be relevant to the STM function. Our focus here is on Volado's surprising function as a regulator of Ca^{2+}-dependent transmission strength and functional plasticity. The null *Volado* phenotype is essentially identical to the null *latheo* phenotype in every regard. Transmission amplitude at low external $[Ca^{2+}]$ is elevated 3-4 fold and the calcium-dependence of secretion is reduced by approximately an order of magnitude. Like *latheo*, all forms of functional plasticity are reduced or absent in *Volado* synapses; including paired-pulse and short-term facilitation, augmentation and PTP. Integrins interact with components of the ECM to mediate their function and it is known that small peptides containing the RGD sequence inhibit these interactions (49). Thus, RGD peptides are a pharmacological tool with which we can phenocopy the *Volado* mutant phenotypes. RGD incubation mimics the amplitude and plasticity defects observed in *Volado* null mutants in <30 mins. These studies demonstrate that the Volado integrin plays a <u>dynamic role</u> in functional plasticity and complement the 3 hr transgenic rescue of the adult *Volado* learning phenotype (32). Two models may explain Volado's synaptic role: 1) Volado may act as an adhesion molecule to rapidly (mins) alter synaptic architecture to modulate transmission through activation of "silent synapses" . 2) Volado may act as to rapidly (seconds) alter intracellular signaling pathways. Integrins regulate Ca^{2+} entry, Ca^{2+} sensing through the calreticulin

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protein and PKC/Raf-1 kinase activation (50-51). Integrins also structure the cytoskeleton and may regulate SV dynamics either directly or in combination with downstream signaling molecules.

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