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Minireview

highwire, *rpm-1*, and *futsch*: Balancing Synaptic Growth and Stability

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Nervous system function depends on the construction of complex, ordered synaptic connections among neurons during development. Presynaptic machinery specialized to release neurotransmitter, and postsynaptic machinery specialized for neurotransmitter detection and signal transduction, are assembled in a highly regulated fashion. Presynaptic terminal boutons contain active zones that support neurotransmitter release, consisting of several hundred vesicles clustered around an electron-dense array of voltage-gated Ca^{2+} channels and protein complexes involved in vesicle docking, fusion, and recycling, surrounded by a filamentous cytomatrix. Precisely apposed to presynaptic active zones are postsynaptic specializations that contain, among other machinery, neurotransmitter receptor clusters and protein complexes involved in receptor membrane insertion, clustering, anchoring, and retrieval. Once these structures are assembled, pre- and postsynaptic specializations must remain structurally intact and aligned for synaptic transmission to occur, yet paradoxically retain the ability to respond to signals that modulate synaptic form and function. Five papers in this issue of *Neuron* (Hummel et al., 2000; Roos et al., 2000; Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000) begin to explain at a molecular level why this paradox is more apparent than real. The Goodman, Jin, and Nonet labs report the identification of two homologous members of a new family of proteins, Highwire (HIW) in *Drosophila* and Regulator of presynaptic morphology (RPM-1) in *C. elegans*, that modulate synaptic growth. HIW/RPM-1 are localized to presynaptic boutons, in periactive zones, first described in fly by Sone and Hama (Sone et al., personal communication). Periactive zones appear to be molecularly distinct from active zones and may contain the machinery that modulates synaptic growth. The Davis and Klämbt labs report the identification of a *Drosophila* microtubule binding protein, Futsch, that modulates dendritic, axonal, and synaptic growth via interactions with the neuronal cytoskeleton. Together, these five papers provide substantial new insight into the modulation of the dynamic balance between synaptic growth and stability.

hiw *and* rpm-1 *Mutants Affect Presynaptic Growth*

Over the last decade, forward genetics in fly and worm (reviewed by Broadie, 1998) and reverse genetics in mice (reviewed by Sanes and Lichtman, 1999) have identified a number of proteins that mediate and modulate synapse formation and neurotransmitter release. For the most part, these studies have focused on neuromuscular junctions, the synapses between motor neurons and muscle fibers, because of their large size, relative simplicity, and experimental accessibility compared to CNS synapses. At fly neuromuscular junctions, presynaptic terminals consist of a series of branches, decorated with boutons containing active zones, whereas in worm, presynaptic terminals are arrayed like beads on a string. In fly, presynaptic terminals are highly dynamic throughout development (Zito et al., 1997), much like their vertebrate counterparts in the CNS and periphery. Despite powerful technical and biological tools, dissecting the molecular and cellular mechanisms regulating presynaptic differentiation and growth has proven to be difficult. The identification of HIW/RPM-1 and Futsch and their functions has begun to change that.

Using a primary behavioral screen of the X chromosome designed to detect viable mutants in walking behavior, coupled to a secondary screen designed to detect alterations in presynaptic motor terminal morphology, Wan et al. (2000) identified *highwire* (*hiw*) as a gene that modulates synaptic growth of larval neuromuscular junctions. From the onset of synaptogenesis, presynaptic terminals in *hiw* mutants have a larger number of branches, and more complex branches, than wild-type terminals (Figure 1B). The number of presynaptic boutons was observed to be increased, while the size of boutons was decreased; however, the localization of several pre- and postsynaptic proteins, and the number, ultrastructure, and spacing of active zones, was essentially normal. Because synapse formation per se, as well as preceding axon guidance and target selection, were normal, *hiw* appears to selectively affect the mechanisms underlying synaptic growth.

The synaptic overgrowth observed in *hiw* mutants suggested that synaptic strength might be correspondingly increased. However, electrophysiological analyses of synaptic transmission revealed that *hiw* synapses were weaker than their wild-type counterparts. Postsynaptic sensitivity to neurotransmitter was preserved in *hiw* mutants, but quantal content, a measure of the number of neurotransmitter quanta released per presynaptic action potential, was reduced to a third of that measured at wild-type junctions. If a decrease in quantal content were the primary defect in *hiw* mutants, the observed synaptic overgrowth might be compensatory, to maintain synaptic strength. Wan et al. argue that this is unlikely, since synaptotagmin mutants, which have an even greater reduction in quantal content, do not have a corresponding increase in terminal branch or bouton number. Thus, while the decrease in quantal content may be secondary to the defect in synaptic growth, the mechanism linking synaptic overgrowth in *hiw* mutants with a reduction in synaptic strength is presently unclear.

Zhen et al. (2000) and Schaefer et al. (2000) report the independent identification of a gene in *C. elegans*, *rpm-1*, that is homologous to *hiw*. These workers used a clever strategy, devised by Nonet and colleagues (Nonet, 1999), of screening mutant worms for alterations in the expression of a synaptobrevin-GFP fusion construct, that when driven by neuron-specific promoters reveals the location of vesicle clusters in presynaptic boutons. Zhen et al. report that in *rpm-1* mutants, synap-

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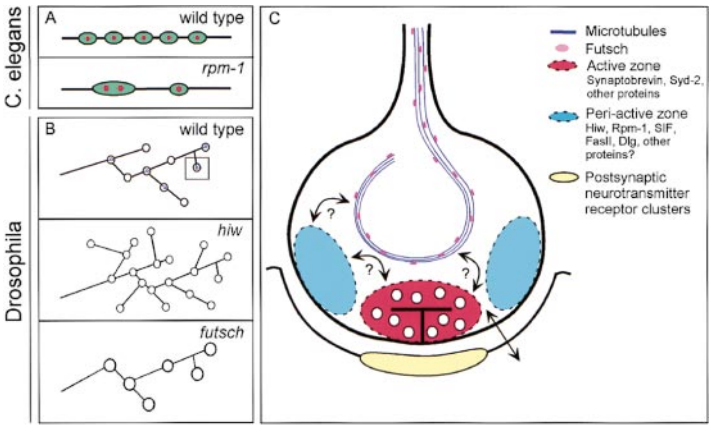

Figure 1. Phenotype of *rpm-1*, *hiw*, and *futsch* Mutants and Machinery Regulating Synaptic Growth

(A) In *C. elegans*, wild-type neuromuscular junctions are formed en passant with postsynaptic targets (top). Presynaptic boutons can be marked by a synaptobrevin-GFP fusion protein (green), and each bouton typically contains one active zone (red) that can be marked by expression of SYD-2, a liprin homolog that modulates active zone formation (Zhen and Jin, 1999). In GABAergic neuromuscular junctions in *rpm-1* mutants, bouton number is reduced and bouton size is increased; some boutons contained more than one active zone (bottom).

(B) In *Drosophila*, wild-type neuromuscular junctions with relatively stereotyped presynaptic branching and bouton number are formed with muscle fiber targets (top). Many boutons contain loops of microtubules that may play a role in synaptic stabilization (blue loops within circular boutons). In *hiw* mutants, presynaptic branching and bouton number are increased roughly 2-fold (middle). *futsch* mutants have reduced numbers of boutons and abnormal branching, and microtubule loops are absent (bottom).

(C) Enlargement of a wild-type bouton in box in (B) (top). HIW/RPM-1 are localized to regions surrounding active zones, called periactive zones by Hama and colleagues. Active zone domains (red) contain the machinery for neurotransmitter release, including electron-dense T bars, synaptic vesicles, synaptobrevin, SYD-2, and other proteins too numerous to name here. Active zones are precisely apposed to postsynaptic regions containing neurotransmitter receptor clusters (yellow). While active zone assembly appears to be cell autonomous, in that it does not appear to require the presence of a differentiated target cell, active zone localization is affected by retrograde signals from target cells (straight arrow) (reviewed by Broadie, 1998). Other proteins that play a role in the modulation of synaptic growth and differentiation, such as Fas II, Discs large (DLG), SIF, DAP160, and likely other proteins, are also localized to periactive zones. While direct evidence is presently lacking, it seems likely that there are interactions between active and periactive zone domains that modulate bouton size and number and active zone size and number (curved arrows with question marks).

Presynaptic boutons contain a filamentous cytomatrix, filled with actin filaments and microtubules (blue). The *Drosophila* protein Futsch (pink) is a microtubule binding protein. It is presently unclear whether Futsch modulates microtubule organization via direct binding, as diagramed here, or via more indirect interactions. Loops of microtubules and associated Futsch are found in some boutons in wild-type neuromuscular junctions ([B], top). The aberrant presynaptic branching and bouton number in *futsch* mutants suggests that modulation of the presynaptic cytoskeleton is one of several mechanisms underlying synaptic growth. Whether there are interactions between the presynaptic cytoskeleton, active zones, and periactive zones (curved arrows with question marks) seems likely but remains to be determined.

tobrevin-GFP in GABAergic motor terminals is abnormally distributed, in that the number of GFP^+ boutons was reduced, and the remaining boutons were enlarged. Electron microscopic analyses showed that while wild-type boutons contain a single active zone, in *rpm-1* mutants some boutons contained more than two active zones, some were filled with debris and contained less prominent active zones, and other boutons appeared normal. Moreover, the localization of another presynaptic molecule, SYD-2, that modulates active zone formation (Zhen and Jin, 1999) was also disrupted. SYD-2 is normally localized in a single spot in the center of synaptobrevin-GFP^+ boutons, but in *rpm-1* mutants, multiple SYD-2 spots were observed within boutons, and these likely correspond to multiple active zones (Figure 1A). Using a functional GFP-tagged GABA receptor subunit, Zhen et al. found that receptor clustering was also disrupted in *rpm-1* mutants, although rescue and genetic mosaic analyses suggested that this was secondary to the presynaptic defect. Behavioral and pharmacological observations suggested that synaptic transmission was probably intact, although this was not examined directly. Thus, as in fly, assembly of presynaptic specializations by GABAergic and other motor neurons appears normal, but synaptic growth is disrupted.

Schaefer et al. describe the phenotype of neuron–neuron glutamatergic terminals of posterior lateral mechanosensory and other neurons in *rpm-1* mutants. While overall mechanosensory neuron differentiation appeared normal, their terminals failed to accumulate synaptobrevin-GFP-tagged vesicles, retracted some terminal branches, and ectopically extended others. As a possible consequence of the defect in synapse formation, mechanosensory neurons in *rpm-1* mutants extend branches to ectopic locations and retract others. The inability of mechanosensory neurons to form synapses may be due to defects in the targeting of presynaptic machinery to appropriate locations along the axon, or to defects in the detection of the appropriate retrograde signals from potential postsynaptic targets. The aberrant branch growth and retraction observed in motor neurons innervating head muscle in *rpm-1* mutants is similar to that of neurons in mutants with reduced synaptic activity (Zhao and Nonet, 2000). Thus, defects in synapse formation may result in reduced activity, in turn leading to aberrant branch formation. Together with results from Zhen et al., these observations suggest that *rpm-1* function may differ among GABAergic and other motor neurons, where it appears to modulate the spatial arrangement or restrict the formation of synapses, and glutamatergic mechanosensory and other neurons, where it appears to positively modulate synapse formation and growth.

HIW/RPM-1 Are Localized to Presynaptic Periactive Zones that Contain Machinery Regulating Synaptic Growth and Plasticity

The observations of Wan et al., Zhen et al., and Schaefer et al. show that the functions of *hiw* and *rpm-1* as regulators of synapse formation and growth appear to be conserved between fly and worm, although their function may differ across neurons. Immunostaining showed that in *Drosophila* the localization of HIW, and in *C. elegans*

of RPM-1, surrounds active zones containing synaptic vesicles that are precisely apposed to neurotransmitter receptor clusters in the postsynaptic membrane (Figure 1C). This region has been called the periactive zone by Hamas and colleagues (Sone et al., personal communication).

Several lines of evidence have implicated this region as containing machinery involved in the regulation of presynaptic growth and plasticity. In *Drosophila*, several proteins that function to regulate presynaptic architecture are localized to periactive zones, including the product of *still life*, a guanine nucleotide exchange factor (GEF), SIF, that may modulate synaptic growth and other processes (Sone et al., 1997); the cell adhesion molecule Fasciclin II (Fas II); and DAP160, a membrane-associated protein that complexes with dynamin and is associated with the filamentous cytomatrix surrounding active zones. Thus, the molecular architecture of periactive zones appears to be distinct from that of active zones. Active zones contain the machinery required for neurotransmitter release, while periactive zones may contain the machinery involved in the regulation of terminal growth and plasticity, and may also directly or indirectly modulate active zone assembly.

The localization of Fas II to periactive zones is particularly interesting, as genetic analyses have demonstrated that the downregulation of Fas II is both necessary and sufficient for structural plasticity at neuromuscular junctions. *FasII* mutants that have a 50% reduction of Fas II expression have increased branch and bouton number, while transgenes that maintain synaptic Fas II levels suppress synaptic overgrowth in other mutants such as *ether-a-go-go*/*Shaker* and *dunce*. These mutants exhibit synaptic overgrowth due to an activity- and cAMP-dependent signaling cascade that in part decreases Fas II localization at synapses (Schuster et al., 1996a, 1996b). These and other data suggest Fas II–mediated cell–cell adhesion is modulated by activity and is required for synaptic stabilization: adhesion stabilizes synapses and inhibits growth, while loss of adhesion destabilizes synapses and facilitates growth. Wan et al. showed that Fas II overexpression pre- or postsynaptically failed to rescue the *hiw* mutant phenotype, suggesting that *FasII* is not downstream of *hiw* genetically. Thus, while both HIW and Fas II are localized to periactive zones, these mutants may define parallel pathways in the control of synaptic growth.

HIW/RPM-1 Are Members of a New Protein Family

Cloning of the proteins encoded by *highwire* in *Drosophila* by Wan et al., and *rpm-1* in *C. elegans* by Zhen et al. and Schaefer et al., showed that these genes encode enormous proteins (5233 amino acids in HIW, 3766 in RPM-1), with significant homology distributed throughout the entire protein. HIW and RPM-1 are homologous to a human protein associated with Myc, known as hPam, that was originally identified in an expression screen for proteins that bind to the product of the c-*myc* protooncogene. The function of hPam is presently unknown.

The several protein motifs present in HIW/RPM-1 hint at possible protein–protein interactions and function. The N terminus contains a region of seven tandem repeats, moderately similar to those in a protein known as Regulator of chromosome condensation (RCC1). RCC1 functions in part as a GEF for the nuclear small GTP binding protein RAN. Thus, this domain in HIW/RPM-1 may function as a GEF for GTPases found in presynaptic terminals. The *Drosophila* protein SIF is a putative GEF localized to periactive zones that activates RAC in presynaptic terminals and may modulate synaptic growth (Sone et al., 1997). Another recently identified GEF, *Drosophila* Trio (cf. Bateman et al., 2000), has been shown to play a role in axon guidance and may also affect synapse formation and growth at neuromuscular junctions. This raises the possibility that synapse formation and growth may be controlled by several GEFs in periactive zones that differentially modulate GTPases in response to intrinsic or extrinsic cues.

The C terminus is highly conserved among HIW/RPM-1 and hPam, and contains a cysteine-rich domain that appears to have multiple zinc finger motifs, one of the RING-H2 type and one of the B-box type, that mediate protein–protein interactions in other molecules. In other proteins, RING-H2-type zinc finger domains function as ubiquitin ligases, and thus may play a role in protein degradation. This may be related to a possible function of HIW/RPM-1 in modulating the formation of presynaptic structures by targeting proteins for degradation. The middle of the HIW/RPM-1 proteins contains a coiled-coil domain and two repeats of $\sim$90 amino acids, conserved in hPam, HIW, and RPM-1. Wan et al., Zhen et al., and Schaefer et al. designate these PHR repeats, and this domain will be useful to identify other members of this protein family.

Regulation of the Neuronal Cytoskeleton by Futsch, a Microtubule Binding Protein that Modulates Synaptic Growth

In *Drosophila*, presynaptic nerve terminal growth occurs, in part, by division of existing synaptic boutons (Zito et al., 1997) that may involve regulated, but largely unknown, modifications to the presynaptic cytoskeleton similar to those that exist within neuronal growth cones of many organisms. This involves the assembly and disassembly of microtubule hairpin loop structures that are present in a large proportion of growth cone microtubules. The assembly of microtubule loops is correlated with an arrest of growth cone motility, while the disassembly of loops occurs prior to the onset of motility (Dent et al., 1999). Thus, regulation of microtubule organization may also play an important role in synaptic growth and stability.

Work by Hummel et al. (2000) and Roos et al. (2000) implicates a novel *Drosophila* protein, Futsch, in the regulation of the neuronal microtubular cytoskeleton and in dendritic, axonal, and synaptic growth. Futsch protein turns out to be the antigen recognized by the monoclonal antibody 22C10, generated by S. Benzer's lab, that has been widely used to visualize the morphology and axonal projections of many *Drosophila* neurons. Futsch is a large ($>$500 kDa) protein that shares homology at its N and C termini with vertebrate microtubule-associated proteins (MAPs), in particular with MAP1B and 1A, and likely represents a novel member of the MAP family. In its central domain, repetitive sequences share some homology with vertebrate neurofilament proteins. Immunostaining analyses suggest that Futsch is localized to the presynaptic cytoskeleton, in close association with microtubules.

Hummel et al. show that in wild-type embryos, Futsch is expressed by all postmitotic neurons, and is localized

in cell bodies, axons, and dendrites. In *futsch* amorphic mutants, dendrites of lateral chordotonal organs fail to grow; sensory axons fail to grow into the CNS, longitudinal connectives are reduced in the ventral nerve cord; and motor axons fail to reach muscle fibers 12 and 13. Axonal pathfinding appears unaffected, because axons are often observed to select their normal path but subsequently stop growing. These data suggest that Futsch is necessary for the normal growth of axons and dendrites.

Roos et al. show that at neuromuscular junctions, Futsch modulates the microtubule loops normally present within some presynaptic boutons (Figure 1B, top). Microtubule loops form throughout development and may be associated with stable synaptic boutons, while the disassembly of microtubule loops may be associated with boutons undergoing division or with sites of sprouting. In *futsch* hypomorphic mutants, microtubule organization is disrupted and loops are absent (Figure 1B, bottom). While axon outgrowth appears normal, synaptic morphology is disrupted, with a halving of presynaptic bouton number and a doubling in bouton size. These phenotypes are partially rescued by overexpression of the N-terminal portion containing the predicted microtubule binding domain in mutant backgrounds.

These data suggest that Futsch stabilizes microtubule organization within presynaptic nerve terminals (Figures 1B and 1C). Similar loops are observed in the neurofilament cytoskeleton of motor nerve terminals at developing and adult rodent neuromuscular junctions, although it is unclear whether the presence of loops is associated with stable presynaptic terminal areas. Because the disassembly of microtubule loops in *futsch* mutants is associated with aberrant synaptic growth, much as loop disassembly is associated with motility in neuronal growth cones, regulation of the cytoskeleton may also have a profound impact on the balance between synaptic growth and stability.

Synaptic Machinery Regulating Growth: Future Directions

HIW/RPM-1 and Futsch can thus be added to the growing repertoire of proteins involved in the modulation of synaptic growth. The available evidence suggests that these processes are under both positive and negative regulation, and HIW/RPM-1 and Futsch play roles in this regulation. One issue of interest will be to determine what genes are upstream and downstream of these proteins in fly and worm and thus define a signaling cascade that modulates synaptic growth and plasticity. The large size and multiple domains of HIW/RPM-1 and Futsch may allow these proteins to complex with several others in presynaptic terminals. A second issue of interest, then, will be to determine how these protein complexes are assembled in periactive zones. The different phenotypes observed in motor versus sensory and other neurons in *rpm-1* mutants raises the possibility that these protein complexes in periactive zones may differ among different neurons, perhaps reflecting the wide variety of signaling interactions with potential postsynaptic targets.

A third issue is whether HIW/RPM-1 homologs in addition to hPam exist in vertebrates, whether periactive zone domains exist in vertebrate presynaptic terminals, and whether these have similar functions as those in fly and worm. Two proteins shown to be localized to presynaptic terminals in rodents, Bassoon and Piccolo/Aczonin, also contain two zinc finger domains, as do HIW/RPM-1 (cf. Fenster et al., 2000), but the in vivo functions of these proteins are presently unknown.

Finally, it will be interesting to explore how activity-dependent regulation of synaptic growth may be linked to HIW/RPM-1 and Futsch function. While studies in worm, fly, and mouse show that presynaptic activity is not required for synapse formation (cf. Verhage et al., 2000), synaptic growth and stabilization are modulated by neural activity. Combining genetics with structural and functional analyses at the molecular and cellular levels will expand our understanding of how neural activity, and other signals, tip the balance between synaptic growth and stability during development as well as in adulthood.

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