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Integrins regulate DLG/FAS2 via a CaM kinase II-dependent pathway to mediate synapse elaboration and stabilization during postembryonic development

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SUMMARY

Calcium/calmodulin dependent kinase II (CaMKII), PDZ-domain scaffolding protein Discs-large (DLG), immunoglobin superfamily cell adhesion molecule Fasciclin 2 (FAS2) and the position specific (PS) integrin receptors, including βPS and its alpha partners (αPS1, αPS2, αPS3/αVolado), are all known to regulate the postembryonic development of synaptic terminal arborization at the *Drosophila* neuromuscular junction (NMJ). Recent work has shown that DLG and FAS2 function together to modulate activity-dependent synaptic development and that this role is regulated by activation of CaMKII. We show that PS integrins function upstream of CaMKII in the development of synaptic architecture at the NMJ. βPS integrin physically associates with the synaptic complex anchored by the DLG scaffolding protein, which contains CaMKII and FAS2. We demonstrate an alteration of the FAS2 molecular cascade in integrin regulatory mutants, as a result of CaMKII/integrin interactions. Regulatory βPS integrin mutations increase the expression and synaptic localization of FAS2. Synaptic structural defects in βPS integrin mutants are rescued by transgenic overexpression of CaMKII (proximal in pathway) or genetic reduction of FAS2 (distal in pathway). These studies demonstrate that βPS integrins act through CaMKII activation to control the localization of synaptic proteins involved in the development of NMJ synaptic morphology.

Key words: *Drosophila*, CaMKII, FAS2, Neuromuscular junction, Synaptic proteins

INTRODUCTION

Molecular mechanisms mediating the initial establishment and elaboration of neuronal synapses during embryogenesis are likely to be retained for postembryonic, experience-dependent development of synaptic architecture. Indeed, several molecules that mediate embryonic pathfinding, target recognition and synaptogenesis are known to play roles in later synaptic modulation events (reviewed by Zhang et al., 2001). One class of cell adhesion molecules (CAM) important in both synaptic development and later modulation is the immunoglobin (Ig) superfamily, including NCAM and L1 in vertebrates (Dahme et al., 1997; Luthl et al., 1994), apCAM in *Aplysia* (Bailey and Chen, 1989; Mayford et al., 1992) and Fasciclin 2 (FAS2) in *Drosophila*/grasshopper. FAS2 is involved in axon sorting, fasciculation/ defasciculation and target selection in the embryo (Bastiani et al., 1987; Davis et al., 1997; Grenningloh et al., 1991; Lin et al., 1994), and is later necessary for synaptic stabilization and growth modulation in the postembryonic neuromuscular junction (NMJ) (Schuster et al., 1996a; Schuster et al., 1996b; Stewart et al., 1996; Thomas et al., 1997).

Synaptic localization of FAS2 is regulated by Discs Large (DLG) through a C-terminal PDZ consensus binding site (Thomas et al., 1997; Zito et al., 1997). *Drosophila* DLG, like its mammalian homologue PSD95, is a PDZ-domain scaffolding protein with multiple binding sites that are capable of mediating the formation of large molecular complexes. PSD95 binds NMDA receptors, K^+ channels and semaphorins at central glutamatergic synapses (Chung et al., 2000; Inagaki et al., 2001; Kim et al., 1996; Kim et al., 1995; Kornau et al., 1995; Niethammer et al., 1996; Xia et al., 2000). Similarly, DLG binds both FAS2 and Shaker K^+ channels, and is responsible for their synaptic localization at the *Drosophila* glutamatergic NMJ (Tejedor et al., 1997; Thomas et al., 1997; Zito et al., 1997).

DLG-dependent localization of FAS2 to the *Drosophila* NMJ is negatively regulated by Ca^{2+}/calmodulin dependent kinase II (CaMKII). Phosphorylated DLG disassociates from the synaptic protein complex, leading to the synaptic loss of FAS2 (Koh et al., 1999b). NMJs in animals expressing a CaMKII inhibiting peptide (ala) display impaired synaptic modulation and altered synaptic morphology (Griffith et al., 1994; Wang et al., 1994). Thus, the current model suggests that synaptic activity leads to CaMKII activation via Ca^{2+}-dependent auto-phosphorylation. CaMKII, in turn,

phosphorylates DLG to release FAS2 from the synaptic complex, allowing developmental growth in response to increased synaptic activity (Koh et al., 1999b).

An independent line of investigation has shown that integrins also regulate activity-dependent development of *Drosophila* NMJ architecture (Beumer et al., 1999; Rohrbough et al., 2000). Integrins are heterodimeric transmembrane receptors for the extracellular matrix with both adhesion and bi-directional transmembrane signaling functions. During embryogenesis, integrins play roles in neuronal pathfinding in vertebrates, *C. elegans* and *Drosophila* (Baum and Garriga, 1997; Hoang and Chiba, 1998; Ivins et al., 2000). Moreover, at least ten different integrin receptors are found in postembryonic vertebrate synapses, and at least three in larval *Drosophila* NMJs (Beumer et al., 1999; Burkin et al., 1998; Cohen et al., 2000; Fernandes et al., 1996; Martin et al., 1996; Pinkstaff et al., 1999; Rodriguez et al., 2000; Rohrbough et al., 2000), suggesting a persistent synaptic function. *Drosophila* integrin mutants display disrupted short-term memory and a loss of functional synaptic plasticity (Grotewiel et al., 1998; Rohrbough et al., 2000). Mutations of the *Drosophila* β integrin (βPS) subunit, or one α integrin partner (αVolado), cause multiple alterations in synaptic architecture, indicating a role for integrins in synaptic morphological development (Beumer et al., 1999; Rohrbough et al., 2000).

Do integrins affect synaptic morphological development via CaMKII or via an independent, parallel pathway? We propose the hypothesis that integrins may act through CaMKII at the *Drosophila* NMJ to modulate synaptic structure. Integrins regulate CaMKII activation in cultured cells (Bilato et al., 1997; Blystone et al., 1999), and this regulation has been proposed to mediate communication between heterologous integrin receptors expressed together in single tissues, a condition seen at the *Drosophila* NMJ (Beumer et al., 1999; Rohrbough et al., 2000). We show here that transgenic overexpression of CaMKII completely rescues synaptic structural defects in integrin mutants, indicating that integrins act through alteration of CaMKII expression or activity. We also show that synaptic FAS2, whose regulation is downstream of CaMKII activity, is misregulated in integrin mutants and that genetic compensation for this misregulation rescues synaptic structural defects in integrin mutants. We therefore propose that synaptic integrin receptors act upstream of CaMKII to regulate NMJ morphological development, largely via regulation of FAS2 expression, and that this integrin function is required for synaptic structural alterations.

MATERIALS AND METHODS

Fly stocks and genetic manipulations

Drosophila melanogaster stocks were reared in well-yeasted, uncrowded vials on standard medium at 25°C (Ashburner, 1989) unless otherwise noted. Oregon-R or Canton S flies were used as wild-type control animals. An extensive allelic series of *myospheroid* (*mys*; encodes βPS integrin) mutant stocks were used: mys^{xg43} (Bunch et al., 1992), an embryonic lethal genetic null mutant; *cm* ct^{6} mys^{ts1} (Bunch et al., 1992), a regulatory mutant with greatly reduced pre- and postsynaptic βPS expression at the NMJ (Beumer et al., 1999); *sn* mys^{b9} *v* (D. Brower) (FlyBase, 1998), a hypomorphic mutant with moderately reduced βPS expression at the NMJ due primarily to postsynaptic mislocalization (Beumer et al., 1999); and a series of mys^{olfC} alleles, $olfC^{X5}$, $olfC^{X10}$, $olfC^{X14}$ and $olfC^{X17}$ (C. Ayyub) (Ayyub et al., 2000), which have been less thoroughly characterized. For some experiments, *sn* mys^{b9} *v* and *cm* ct^{6} mys^{ts1} were backcrossed a minimum of eight times to a Canton S (CS) stock, removing all marker mutations and producing a uniform genetic background that is conservatively 96% CS. To generate double mutants, *yw* was crossed onto *cm* ct^{6} mys^{ts1}, and *sn* mys^{b9} *v*.

CaMKII protein levels were manipulated by crossing P[w^{+}, *UASCAMKII*$^{+}$] R3, located on chromosome 3 (V. Budnik) (Koh et al., 1999b) into *mys* mutant backgrounds. To drive the inducible UAS constructs in muscle and nerve, we used previously described GAL4 constructs (FlyBase, 1998). One GAL4, *elavGAL4* (Lin and Goodman, 1994) is located on the tip of the *X* chromosome, and was crossed onto each of the *mys* mutant chromosomes and the stock tested for proper genotype by testing for viability and wing blister phenotypes over mys^{xg43}. To inhibit CaMKII, we used the heat-shock inducible construct P[*hsp70-ala*]2, which expresses the CaMKII inhibitor peptide (ala) at a low level without heat shock induction (Griffith et al., 1993; Wang et al., 1994). For descriptions of the marker mutations used in this study see Lindsley and Zimm (Lindsley and Zimm, 1992).

To genetically reduce FAS2 levels in integrin mutant backgrounds, we recombined *mys* chromosomes with the P-element alleles $fas2^{e76}$, which reduces wild-type FAS2 to ~10%, and $fas2^{e86}$, which reduces FAS2 protein ~50% (C. Goodman) (Grenningloh et al., 1991). Recombinants were tested for proper expression of the FAS2 protein by western blot analysis of flies putatively homozygous for the appropriate *fas2* mutant allele but heterozygous for the integrin allele (i.e., $fas2^{e76}$ *sn* mys^{b9} *v* / $fas2^{e76}$). The continued presence of the *mys* allele was reconfirmed in each new stock by testing for viability and wing blister phenotypes over mys^{xg43}.

Immunocytochemistry and imaging

Wandering third instar larvae were dissected and immunologically stained as previously reported (Beumer et al., 1999; Broadie and Bate, 1993). To examine NMJ morphology, preparations were probed with a mouse monoclonal anti-cysteine string protein (CSP) antibody (1:200) (Zinsmaier et al., 1990). Staining was visualized using a Vectastain ABC Elite kit with $NiCl_2$ and $CoCl_2$ enhancement (Broadie and Bate, 1993). Images were captured digitally. For clarity, different focal planes were occasionally combined in one picture using Adobe PhotoShop. In confocal preparations, either rabbit anti-DLG (1:1000) (Woods and Bryant, 1991), rabbit anti-synaptotagmin (1:500) (Littleton et al., 1993), mouse anti-CSP (1:500) (Zinsmaier et al., 1990), rabbit anti-HRP (1:500; Cappel/Oreon Teknica Corp.) or Texas-Red-conjugated goat anti-HRP (1:500; Jackson Laboratories) was used to mark synaptic arbors.

Integrin expression was examined with the following antibodies: mouse monoclonal anti-βPS integrin antibody CF6G11 (1:500) or CF6G11 ascites (1:300) (Brower et al., 1984); rabbit polyclonal 185E (1:1000; R. Hynes) (Zusman et al., 1993), rat polyclonal anti-αPS2, PS2hc2 (1:5) (Bogaert et al., 1987) and rabbit rabPS21 (1:1000) (Bloor and Brown, 1998). All anti-integrin antibodies were independently tested for their well-characterized embryonic staining patterns prior to probing the larval preparations (Bloor and Brown, 1998; Brower et al., 1984). FAS2 was visualized using mouse monoclonal anti-FAS2 1D4G9 (1:10; C. Goodman) (Zito et al., 1999). CaMKII was visualized with rabbit anti-CaMKII (1:4,000; V. Budnik) (Koh et al., 1999b). Fluorescent secondaries used were Alexa-red 594- and Alexa-green 488-conjugated goat anti-mouse and goat anti-rabbit (1:500; Molecular Probes). Confocal microscopy was performed on either a BioRad MRC-600 or a Zeiss LSM 510. ImageQuant software from Molecular Dynamics was used to quantify staining intensity. Statistical significance of all measurements was determined with the ANOVA test using InStat software.

Western blot analysis

To prepare proteins for western blot quantification, 10 third instar larvae were homogenized in 100 μl NP40 buffer (150 mM NaCl, 1% IGEPAL CA630, 50 mM Tris (pH 8), 1× Complete™ Protease inhibitor (Boehringer Mannheim), incubated on ice for 45 minutes and centrifuged at high speed for 20 minutes at 4°C. The supernatant was removed to a fresh tube, and the pellet was resuspended in an additional 100 μl NP40 and spun again for 20 minutes. The supernatants were combined and 25 μl of non-reducing sample buffer was added to 100 μl of the supernatant. The sample was incubated at 85°C for 10 minutes and separated on a 10% SDS-PAGE gel, then transferred onto PVDF membrane. Immunoblotting was performed with anti-FAS2 monoclonal 34B3C2 (1:100; C. Goodman) (G. Helt and C. Goodman, unpublished) and visualized with chemiluminescence reagents (Amersham). Anti-actin was used as a loading control (developed by J. J.-C. Lin and obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, Iowa City, IA 52242 under contract NO1-HD-7-3263 from the NICHD). Bands were quantified using NIH Image.

Immunoprecipitation

Immunoprecipitation assays were performed essentially as described by Thomas et al. (Thomas et al., 1997); however, antibodies were coupled as described by Zhang et al. (Zhang et al., 2001) to recombinant Protein A sepharose beads. Briefly, beads were washed in phosphate-buffered saline (PBS) 0.2% NP40, 5% bovine serum albumin (BSA) and incubated for 2 hours with rabbit anti-DLG (Woods and Bryant, 1991). Beads were washed, coupled with disuccinimidyl suberate, washed again and finally blocked with PBS 0.2% NP40 and 5% BSA. These beads were gently rocked with *Drosophila* head extract prepared in the following manner. OR flies were frozen in liquid N_2, agitated and heads isolated by sieving. Heads were homogenized in 50 mM Tris (pH 7.2) 150 mM NaCl 2 mM EGTA 0.5% Triton X-100 and 2× complete protease inhibitors (Roche). The supernatants were pre-cleared for 45 minutes with protein A Sepharose at 4°C. The cleared supernatant was incubated with Protein A beads coupled to either anti-kinesin (SUK4), anti-DLG (Woods and Bryant, 1991) or naïve beads for 1 hour at 4°C. The beads were washed six times in 800 μl PBS-NP40 buffer once with 5 ml PBS and resuspended in 2× SDS-sample buffer. The proteins were separated on a 4-20% SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked (5% powdered milk), and probed with either rabbit anti-DLG (1:1000; D. Woods) (Woods and Bryant, 1991) or rabbit anti-βPS (1:1500; R. Hynes) (Zusman et al., 1993). Bands were visualized with alkaline phosphate using NBT/BCIP as a substrate.

RESULTS

Differential NMJ structural growth defects caused by postsynaptic versus pre/postsynaptic βPS misregulation

A single β integrin receptor family is present on both sides of the *Drosophila* NMJ, comprising the βPS subunit encoded by *myospheroid* (*mys*) and its α-integrin partners α*PS1 (multiple edematous wings; mew*), αPS2 (*inflated; if*) and αPS3 (*Volado*; *Vol*) (Beumer et al., 1999; Rohrbough et al., 2000). Mutations in *mys* alter the synaptic level of the heterodimeric integrin receptors in parallel (Beumer et al., 1999), allowing the assay of total PS integrin synaptic function. Null *mys* alleles are embryonic lethal because of catastrophic failure of development (i.e. dorsal closure failure, gut defects and muscle detachment), precluding analysis of mutant phenotypes at the postembryonic NMJ. Therefore, we used two well-characterized regulatory hypomorphic *mys* mutant alleles in these analyses; mys^{b9}, which causes loss of ~50% of synaptic integrin, and mys^{ts1}, which causes a more severe ~80% integrin loss (Beumer et al., 1999). Phenotypically, mys^{b9} NMJ synapses display profound undergrowth with a reduced number of small, tightly spaced type I boutons, and an increased number of satellite boutons (Fig. 1A, Fig. 3A), which probably represent developmentally arrested type I boutons (Beumer et al., 1999; Zito et al., 1999). The mys^{ts1} synapses display the opposite phenotype of structural overgrowth, with an increased number of boutons in a highly branched, over-elaborated

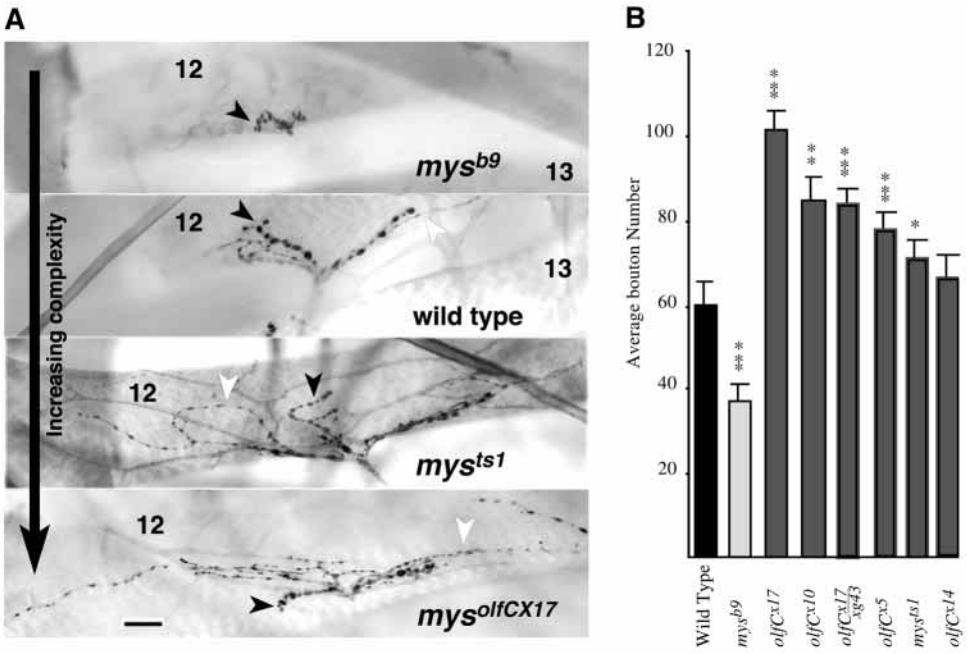

Fig. 1. Synaptic bouton quantification in mys^{olfC} alleles. (A) Representative muscle 12 NMJ morphology in mature third instars of wild-type, mys^{b9}, mys^{ts1} and $mys^{olfCX17}$ alleles. Black arrowheads indicate type I boutons. White arrowheads indicate type II boutons. Scale bar indicates 20 μm. (B) Quantification of boutons for each allele and for $mys^{olfCX17}/mys^{XG43}$, a null allele. Note that mys^{ts1} is among the least severe of the *mys* overgrowth alleles, whereas the mys^{b9} undergrowth allele is clearly very different. Error bars indicate s.e.m. (*P<0.05; **, P<0.01; ***, P<0.001).

synaptic arbor (Fig. 1A, Fig. 3A) (Beumer et al., 1999). Thus, two integrin mutant alleles, which differentially alter integrin expression at the NMJ, cause opposite morphological growth phenotypes.

Our results suggest that the mys^{b9} mutation causes specific mislocalization of postsynaptic (muscle) integrin, whereas the mys^{ts1} mutation results in globally decreased synaptic expression in both the pre- and postsynaptic membranes (Beumer et al., 1999). In support of this conclusion, many mys^{b9} phenotypes, including NMJ undergrowth, reduced bouton number and reduced physiological function, improve over a *mys* deficiency, while mys^{ts1} morphological and functional phenotypes are either unchanged or more severe over the same deficiency (Beumer et al., 1999). For further support, we analyzed the *olfC* locus, which has recently been shown to be allelic to *myospheroid* (Rodrigues and Siddiqi, 1978; Ayyub et al., 2000). Mutants in *olfC* are recessive, with nervous system deficiencies in responding to a variety of odorants (Ayyub et al., 2000). Two alleles, $olfc^{x3}$ and $olfc^{x14}$, have been assayed for βPS protein expression and, like mys^{ts1} and mys^{b9}, express normal protein when assayed by western blot, and are therefore proposed to be regulatory mutants. We assayed *olfC* mutants for changes in NMJ morphology. Of five mutant combinations assayed, four ($olfc^{x17}$, $olfc^{x17xg43}$, $olfc^{x10}$ and $olfc^{x5}$) showed NMJ overgrowth phenotypes that were more severe than mys^{ts1} (Fig. 1), and the fifth ($olfc^{x14}$) showed less overgrowth than mys^{ts1}. No allelic combinations showed undergrowth. These results suggest that synaptic overgrowth represents a simpler genetic lesion that represents loss of function. Thus, the mys^{b9} phenotype is probably attributable to a more complex gain-of-function alteration of the normal βPS integrin expression pattern, which reduces postsynaptic integrin while increasing extrasynaptic integrin (Beumer et al., 1999).

To test this idea further, we used tissue-specific GAL4 drivers (Brand and Dormand, 1995) to overexpress βPS integrin specifically either in muscle [*myosin heavy chain* (*MHC*)*-GAL4*] or neurons [*embryonic lethal, abnormal vision* (*elav)-GAL4*], and assayed NMJ structure in *mys* mutants. The undergrowth phenotype of the mys^{b9} NMJ can be completely rescued by postsynaptic muscle expression, which increases the amount of integrin localized to the synapse, of either βPS alone or with its alpha partners (Table 1). This observation provides good evidence that the mys^{b9} synaptic undergrowth is due to postsynaptic misregulation of βPS. By contrast, expression of βPS integrin in muscle alone provided no rescue of the mys^{ts1} phenotype (Table 1). We also attempted to rescue the mys^{ts1} phenotype by overexpressing βPS integrin in the presynaptic neuron, but were unable to do so because overexpression of either βPS alone or with its α partners in the nervous system caused extensive NMJ overgrowth, even in wild-type animals (data not shown). The observation that both presynaptic overexpression (*elavGAL4*; UAS-βPS) and underexpression (mys^{ts1}) causes structural overgrowth suggests that βPS integrins are involved in a regulatory mechanism sensitive to both over- and underactivation and pre- and postsynaptic balance.

Thus, we suggest that mys^{ts1} most probably represents the integrin loss-of-function phenotype, whereas the mys^{b9} synaptic undergrowth defect is due to alteration in the βPS integrin expression pattern in postsynaptic muscle.

Table 1. Rescue by Gal4 expression

Genotype	*n*
Wild type	57.9
mys^{b9}	37.45***
mys^{b9}*;; UASβPS UASPS2*	40.71***
mys^{b9}*; mhcGAL4; UASβPS; UASPS2*	61.7°°°
mys^{ts1}	71.75*
mys^{ts1}*; UASβPS; UASPS2*	67.64
mys^{ts1}*; mhcGAL4; UASβPS; UASPS2*	68.75

n, average bouton number, muscle 12. A minimum of 20 A2 NMJ synapses per genotype were assayed. Data are for only one set of rescue combinations. Data for rescue with *mhcGAL4; UASβPS; UASPS1* or *mhcGAL4; UASβPS* are essentially identical. Asterisks indicate significance relative to wild type. Circles indicate significance relative to each *mys* mutant without manipulated integrin levels. Asterisks indicate significance (*P<0.05; *** or °°°, P<0.0001).

Integrins and DLG are associated in a neuromuscular junction complex

βPS integrin is a transmembrane receptor present in both pre- and postsynaptic membranes at the larval NMJ (Beumer et al., 1999). DLG is a synaptic scaffolding protein associated with both pre-and postsynaptic membranes and is also involved in the regulation of synaptic morphology, through the localization of diverse transmembrane proteins (including FAS2) (Guan et al., 1996; Lahey et al., 1994). We wished to determine whether βPS integrin associates with the DLG complex to tie together these disparate receptor components in a common molecular machine.

In confocal analyses, βPS integrin and DLG co-localize at the larval NMJ (Fig. 2A). DLG clearly has less extensive expression, more tightly localized at NMJ boutons, whereas βPS is more extensive through the subsynaptic reticulum (SSR) and also localized at extrasynaptic sites in the muscle, including the muscle sarcomere (Volk et al., 1990) and attachment sites (Leptin et al., 1989). However, both proteins are most intensively expressed at the NMJ pre-/postsynaptic interface, where they co-localize (Fig. 2A). Therefore, we tested to determine if βPS integrins form part of the synaptic complex linked by DLG. We performed protein immunoprecipitation assays using rabbit anti-DLG to probe Oregon R head extracts. DLG antibodies clearly co-immunoprecipitate DLG and βPS integrin protein (Fig. 2C), consistent with co-localization observed in confocal analysis. Inspection of the ratio of both bound proteins and proteins not bound to beads (immunoprecipitation versus flow through lanes, Fig. 2C) indicates that a large portion of the βPS integrin protein associates with a complex containing DLG. Control experiments with either naïve protein beads or beads coupled to anti-kinesin antibodies failed to immunoprecipitate sufficient levels of βPS integrin protein to be detected (Fig. 2B), demonstrating the specificity of the co-immunoprecipitation. The fact that βPS was found to co-immunoprecipitate with the complex mediated by DLG provides support for integrins existing in a synaptic complex with FAS2 and CaMKII at the synapse.

Genetically increasing CaMKII levels rescues *mys* phenotypes at the NMJ

Animals with directly reduced CaMKII activity show altered

synaptic structure at the larval NMJ (Griffith et al., 1993; Wang et al., 1994), comparable with *mys*^{ts1} mutant phenotypes. Our hypothesis is that integrins may mediate their regulation of NMJ architecture through the modulation of CaMKII, both pre- and postsynaptically. If integrins modulate CaMKII in regulating synaptic structure, then transgenically manipulating CaMKII levels in integrin mutants should ameliorate *mys* synaptic structural defects.

We first inhibited synaptic CaMKII activity in *mys* mutants with constructs that constitutively expressed the inhibitory peptide ala (Griffith et al., 1993; Wang et al., 1994). In wild-type animals, expression of the ala peptide results in a ~40% increase in NMJ bouton number (Wang et al., 1994) (data not shown). Expression of ala in integrin mutants resulted in similar, though smaller, increases in bouton number, with 20% and 16% increases in *mys*^{b9} and *mys*^{ts1} mutants animals, respectively (data not shown). Thus, integrin mutant phenotypes cannot be rescued by inhibition of CaMKII activity. Indeed, inhibition of CaMKII has less effect in altering NMJ structure in *mys* mutants than in controls.

We next overexpressed CaMKII^{+} through introduction of an inducible *UAS-CaMKII*$^{+}$ construct (Koh et al., 1999b). We again used a muscle driver (*MHC-GAL4*) and neuronal driver (*elav-GAL4*), and also expressed CaMKII ubiquitously (*GAL4-e22c*) (Koh et al., 1999a). Overexpression of CaMKII ubiquitously, or either pre- or postsynaptically, in wild-type animals produced no significant alteration in synaptic architecture (Fig. 3). Similarly, presynaptic CaMKII over-expression in *mys*^{b9} mutants did not detectably alter the NMJ undergrowth phenotype. By contrast, transgenic expression of CaMKII in postsynaptic muscle completely rescued the *mys*^{b9} undergrowth phenotype (Fig. 3A). The *mys*^{b9} NMJ is spatially contracted and contains 35% fewer type 1 boutons than normal, but CaMKII expression in muscle rescued both the abnormal growth and the reduced number of type I boutons to normal levels (Fig. 3B). This finding reinforces our conclusion that the *mys*^{b9} defect of NMJ undergrowth is due entirely to a postsynaptic impairment.

In the *mys*^{ts1} mutant, expressing CaMKII in the presynaptic terminal caused no detectable alteration in the overgrowth phenotype (Fig. 3A). Expressing CaMKII in postsynaptic muscle caused a slight, but insignificant, decrease in the overgrowth phenotype, to partially rescue the *mys*^{ts1} mutant overproduction of type I boutons (Fig. 3A,B). By contrast, transgenically expressing CaMKII ubiquitously completely rescued the *mys*^{ts1} overgrowth phenotype (Fig. 3A). The *mys*^{ts1} NMJ is overly expansive and contains 25% more type 1 boutons than normal, but combined pre/postsynaptic CaMKII expression rescued the abnormal growth and reduced the number of type I boutons to normal (Fig. 3B). Ubiquitous CaMKII overexpression in wild-type animals had no effect on synapse morphology, but also rescued *mys*^{b9}, as expected (Koh et al., 1999a) (Fig. 3B).

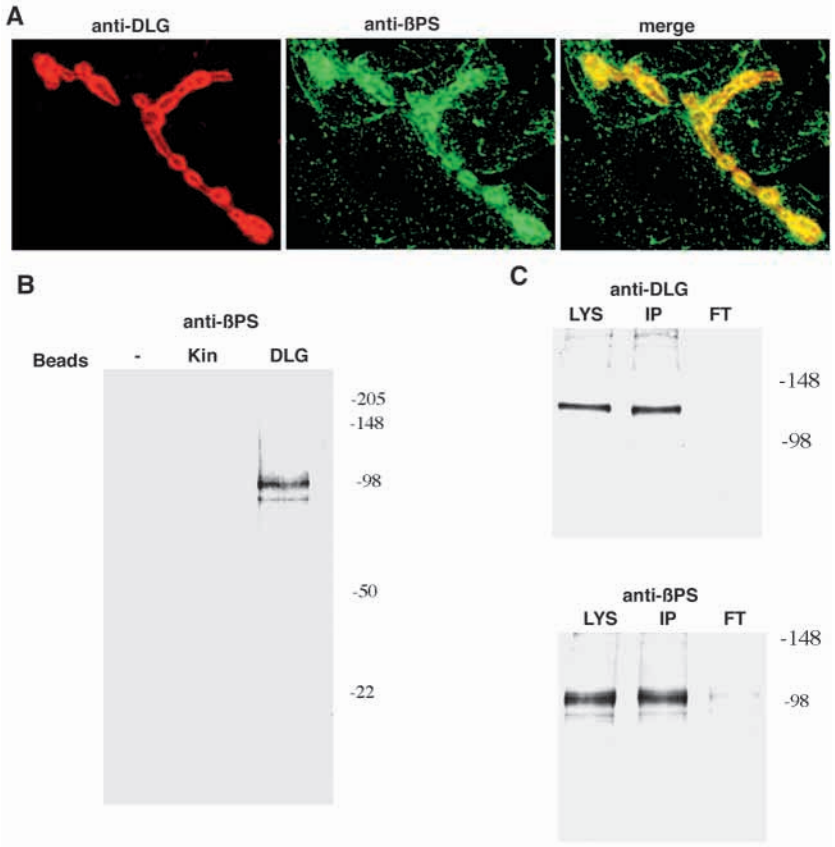

Fig. 2. βPS integrin interacts with DLG. (A) Co-localization of DLG (red) and βPS integrin (green) in the third instar NMJ. βPS staining overlaps, but is not restricted to, the domain of DLG staining. (B) Immunoprecipitations of fly head extracts using protein A beads or beads coupled to either anti-kinesin or DLG were examined by western analysis. Note detectable βPS is found only in the IP with DLG beads validating the DLG IP assay. (C) Anti-DLG immunoprecipitation (IP) experiments from adult heads were performed and western blots of total lysate (LYS), material on beads (IP-resuspended to same volume as lysate) and unbound proteins or flow through (FT) were probed with either anti-DLG or anti-βPS antibodies. These results indicate that quantitative immunodepletion of DLG leads to a substantial reduction of βPS from the lysate.

We conclude that *mys*^{b9} synaptic undergrowth can be rescued by genetically increasing CaMKII only postsynaptically, whereas CaMKII must be increased on both sides of the synapse to completely rescue *mys*^{ts1} synaptic overgrowth. These results are consistent with integrins regulating synaptic growth through a CaMKII-dependent pathway, and with *mys*^{ts1} having defects in both pre- and postsynaptic CaMKII regulation, whereas *mys*^{b9} is specifically defective in postsynaptic CaMKII regulation.

Fasciclin 2 expression is increased in *myospheroid* integrin mutants

Although CaMKII is known to have multiple synaptic targets, one of the most clearly understood is DLG. The ultimate result of CaMKII phosphorylation of DLG is to limit the localization of the homophilic adhesion receptor FAS2, which dictates the directionality and degree of synaptic elaboration. As we have demonstrated that βPS integrin lies upstream of CaMKII, we

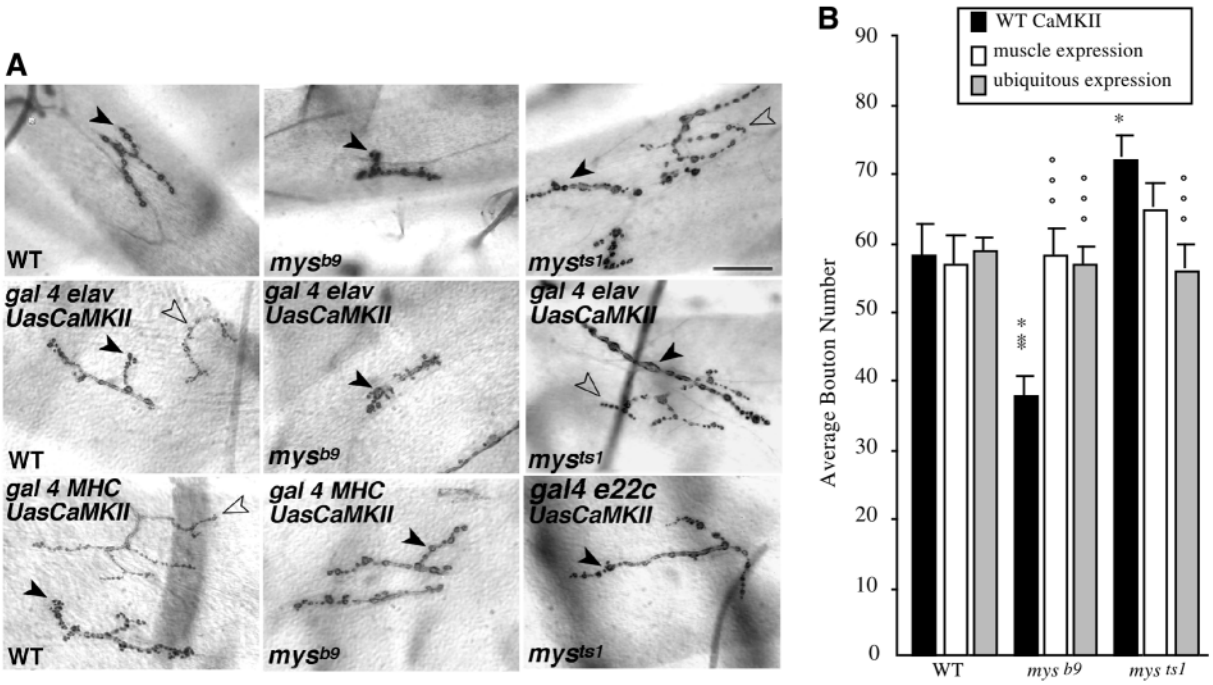

Fig. 3. Rescue of *mys* mutant phenotypes by genetic manipulation of CaMKII expression. (A) Phenotypic rescue of structural defects at the third instar NMJ on muscle 4. Black arrowheads indicate type Ib boutons; open arrowheads indicate type 1s boutons. Scale bar: 20 μm. (B) Quantification of synaptic bouton number in *mys* and CaMKII overexpression mutant animals. In all cases, type I boutons on a minimum of 20 muscle 12 NMJs in hemisegment A2 were quantified. Error bars indicate s.e.m. Asterisks indicate significance relative to wild type. Circles indicate significance relative to each *mys* mutant without manipulated CaMKII levels. (* or °P<0.05; *** or °°°, P<0.001).

hypothesized an alteration in FAS2 localization. We therefore assayed FAS2 expression in mys^{b9} and mys^{ts1} alleles in two ways: (1) quantitative confocal immunocytochemistry to assay FAS2 localization at the NMJ (Fig. 4A,B), and (2) western blot analysis to assay FAS2 levels in whole animal protein extracts (Fig. 4C,D).

FAS2 levels at the larval NMJ were clearly elevated in both mys^{b9} and mys^{ts1} mutants (Fig. 4A). To quantify this elevated expression, we measured the average anti-FAS2 fluorescent intensity (green) relative to the internal control of anti-HRP (red), a neuronal membrane marker. In both *mys* alleles, FAS2 synaptic localization was very significantly increased relative to the internal control (Fig. 4B). The increase was most marked for mys^{ts1} mutants, with a 64% (P<0.0001) increase in FAS2 synaptic expression. In mys^{b9} mutants, FAS2 expression was also greatly increased (48%; P<0.0001). The two *mys* alleles were also significantly different from each other (P<0.002). In the reverse experiment, βPS integrin expression and localization levels were unaltered in *fas2* mutant animals (data not shown). When we measured FAS2 levels in the ubiquitous CaMKII transgenic line, which rescues both mys^{b9} and mys^{ts1} structural defects (Fig. 3), we found that FAS2 expression was reduced in both mys^{b9} and mys^{ts1} to levels comparable with controls (Fig. 4C). Indeed, overexpression of CaMKII in wild-type, mys^{b9} and mys^{ts1} reduced FAS2 levels in all three genotypes, to a uniform level that represented ~80% of wild-type levels (Fig. 4C) (Koh et al., 1999b).

We next assayed total FAS2 protein levels using quantified western blot analysis of staged third instar larva (Fig. 4D,E). As a control and verification test for subsequent experiments (see below), we quantified FAS2 levels in two *fas2* mutants, $fas2^{e76}$ and $fas2^{e86}$. We observed a ~90% and ~50% reduction of FAS2, respectively, in these alleles which is consistent with published levels of expression (Grenningloh et al., 1991). In both mys^{b9} and mys^{ts1}, quantification of the FAS2 band relative to an actin internal control band supported the increased FAS2 expression observed at the synapse in confocal immunocytochemistry experiments (Fig. 4E). We measured a 35% increase in FAS2 in mys^{ts1} animals and a 17% increase in mys^{b9} animals. Note that the relative change of FAS2 levels in mys^{b9} and mys^{ts1} mutants is the same using both techniques. Thus, at least part of the increased synaptic FAS2 levels could be explained by more abundant FAS2 protein in the *mys* mutants.

Taken together, these data are consistent with the hypothesis that the βPS integrin receptor, together with its α partners, functions via CaMKII to regulate both FAS2 expression and its localization at the NMJ synapse. By contrast, FAS2 has no detectable effect on the expression or localization of βPS integrin.

Genetically reducing FAS2 expression in *mys* mutants rescues synaptic structural defects

As the synaptic structural defects of mys^{b9} and mys^{ts1} are accompanied by a striking increase in FAS2 expression at the synapse, we next determined if genetically reducing FAS2 expression would rescue these developmental phenotypes. We recombined two *fas2* regulatory alleles, $fas2^{e76}$ (~90% loss of FAS2) and $fas2^{e86}$ (~50% loss of FAS2) (Fig. 4E) (Grenningloh et al., 1991) onto chromosomes carrying the βPS integrin mutations, generating four stocks: $fas2^{e76}$ mys^{b9}, $fas2^{e76}$ mys^{ts1},

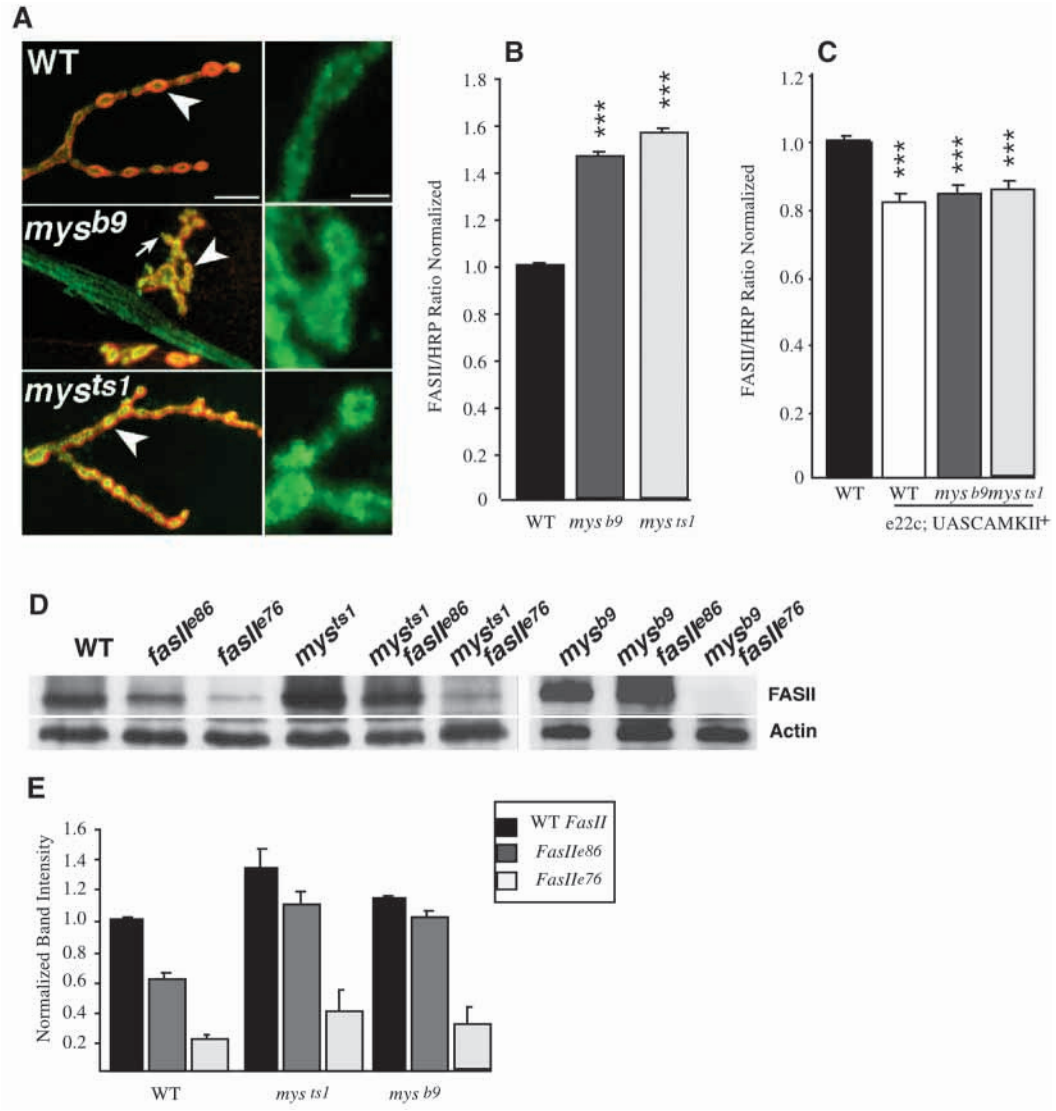

Fig. 4. FAS2 is increased in *mys* mutant NMJ synapses. (A) Anti-FAS2 staining (green) and anti-DLG staining (red) at the third instar NMJ. FAS2 staining is significantly increased in both *mys* alleles. Arrowhead marks a type Ib bouton; arrow indicates a satellite bouton. Scale bar: 12 μm. High magnification views of FAS2 in NMJ boutons. Scale bar: 3 μm. (B) Quantification of the average intensity of FAS2 staining normalized to HRP (neuronal marker). Each bar represents the average of 80-100 boutons for each genotype. Error bars indicate s.e.m. Asterisks indicate significance. (C) Quantification of FAS2 in animals overexpressing wild type CaMKII. (***, $P<0.001$) (D) Sample western blots of FAS2 in the same *mys* mutants and the FAS2 mutants, fas^{e76} and fas^{e86}. (E) Graph quantifying band intensities for western blot experiments. Staining intensities are normalized to wild type.

$fas2^{e86}$ mys^{b9} and $fas2^{e86}$ mys^{ts1}. Each genotype was then tested for alterations in the synaptic architecture phenotype at the third instar NMJ.

Animals doubly mutant for $fas2^{e76}$ and either *mys* allele reduced FAS2 expression to very low levels, below 20% of wild type (Fig. 4E), and showed reduced NMJ growth phenotypes, consistent with those published for $fas2^{e76}$ mutants alone (data not shown) (Schuster et al., 1996a; Schuster et al., 1996b; Stewart et al., 1996). By contrast, $fas2^{e86}$ in conjunction with either mys^{b9} or mys^{ts1}, produced FAS2 protein at near normal levels, as measured by western blot analysis (Fig. 4E). Most strikingly, NMJ synaptic architecture, including bouton size, neurite branch length and number, and synaptic area, is altered towards normal by genetically reducing FAS2 levels in both mys^{b9} and mys^{ts1} animals (Fig. 5A). In mys^{ts1} mutants, the reduced FAS2 level resulted in a highly significant (P=0.0002) decrease in bouton number to near normal numbers (Fig. 5B), resulting in no significant difference between wild-type and mys^{ts1} $fas2^{e86}$ bouton numbers. This result is particularly striking because either mys^{ts1} or $fas2^{e86}$ (Stewart et al., 1996) alone causes excessive NMJ overgrowth. By contrast, in mys^{b9} mutants, the reduced FAS2 level resulted in an increase in bouton number towards normal, although the rescue was not complete (Fig. 5B).

Taken together, these data are consistent with regulation of FAS2 being a primary mechanism by which postsynaptic integrins regulate synaptic growth at the NMJ. However, the overgrowth of mys^{ts1} in the presence of high expression of FAS2, and the incomplete rescue of mys^{b9} by restoring FAS2 levels, indicates that other mechanisms must be involved.

DISCUSSION

Integrins regulate CaMKII activity and FAS2 expression during postembryonic synaptic development of the NMJ

Several studies in the laboratories of Budnik, Goodman, Griffith and Wu have shown that activity-dependent development at the *Drosophila* NMJ is mediated by the Ca^{2+}-dependent kinase CaMKII phosphorylating the scaffolding

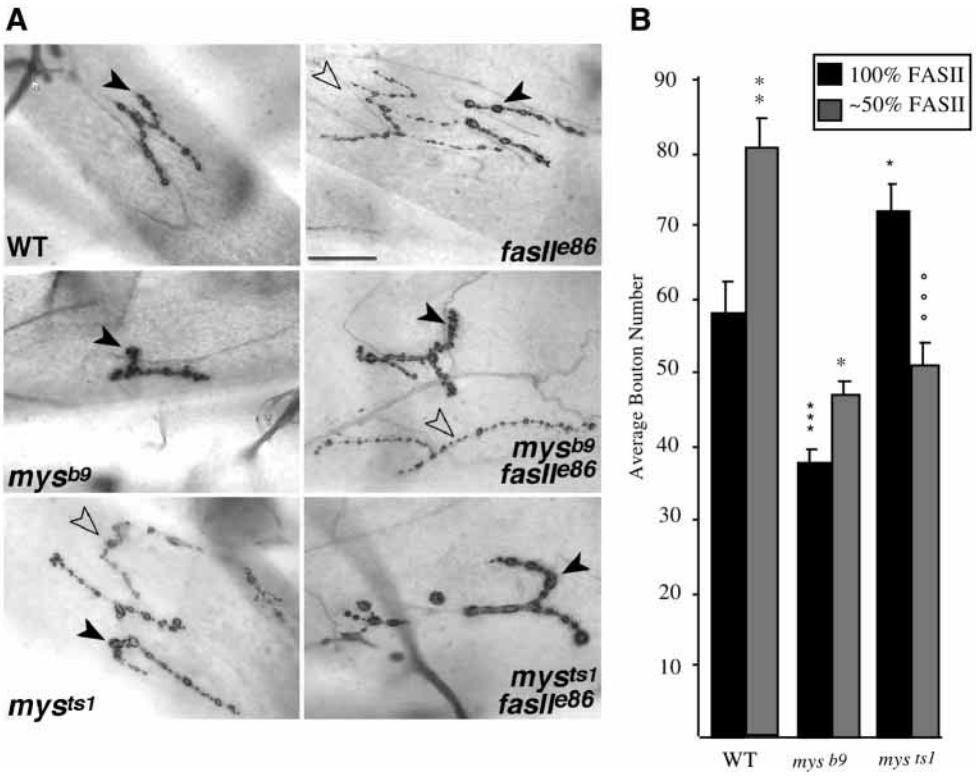

Fig. 5. Rescue of *mys* synaptic structure defects by genetic manipulation of FAS2 expression. (A) Phenotypic rescue at the third instar muscle 4 NMJ. mys^{b9} causes undergrowth, whereas mys^{ts1} causes overgrowth. Other phenotypic characteristics include increased satellite boutons and irregular bouton size. Black arrowheads indicate type Ib boutons. Open arrowheads indicate type 1s boutons. Scale bar: 20μm. (B) Quantification of NMJ synaptic growth in *mys* and *fas* mutant animals. In all genotypes, type I boutons on a minimum of 20 muscle-12 NMJs in hemisegment A2 were quantified. Error bars indicate s.e.m. Asterisks indicate significance relative to wild type. Circles indicate significance relative to each genotype without manipulated FAS2 levels. (* or °, $P<0.05$; ** or °°, $P<0.01$; *** or °°°, $P<0.001$).

protein DLG, which, in turn, tethers the homophilic adhesion molecule FAS2 to control its synaptic localization (Budnik et al., 1996; Koh et al., 1999b; Tejedor et al., 1997; Thomas et al., 1997; Zito et al., 1997). Independently, integrins have been implicated in activity-dependent synaptic development following the isolation of integrin mutants that affect short-term memory in *Drosophila* and in our subsequent demonstration that synaptic integrins modulate NMJ architecture in a manner reminiscent of alterations in the CaMKII-DLG-FAS2 pathway (Beumer et al., 1999; Grotewiel et al., 1998; Rohrbough et al., 2000). The purpose of this study was to determine whether integrins function to regulate FAS2 synaptic expression by the CaMKII pathway, or rather affect morphology via an independent, parallel mechanism. If the former were true, our aims were to determine where in the CaMKII-DLG-FAS2 pathway the integrins function and to assess whether disruption of CaMKII-mediated regulation of FAS2 is sufficient to account for the structural defects observed in integrin mutant synapses.

To assay the *mys* requirement in synaptic development comprehensively, we compared and contrasted two regulatory alleles with opposing structural phenotypes: mys^{b9}, which causes NMJ undergrowth, and mys^{ts1}, which causes NMJ overgrowth (Beumer et al., 1999). In both mutants, we have observed a correlation between the morphological alterations and synaptic function (Beumer et al., 1999), in contrast to the homeostasis seen in *fas2* mutant synapses (Davis et al., 1997; Stewart et al., 1996) but consistent with the parallel alterations seen in CaMKII-inhibited synapses (Wang et al., 1994). However, the correlation between synaptic structural and functional alterations in *mys* mutants is not striking, and it is clear that integrins primarily mediate architectural regulation. Therefore, our focus in this study was exclusively on the mechanism(s) by which integrins modulate NMJ structural development. This article presents molecular and genetic evidence that strongly support the hypothesis that synaptic integrin receptors containing βPS modulate CaMKII activation on both sides of the synaptic cleft and, through CaMKII, control both the expression and the synaptic localization of FAS2 at the synapse. The primary experimental results supporting these conclusions are detailed below.

First, transgenic expression of CaMKII is sufficient to completely rescue all synaptic structure defects in *mys* mutants. Genetically increasing CaMKII in postsynaptic muscle, but not presynaptic neuron, completely rescues the structural undergrowth of the mys^{b9} integrin mutant, whereas it is necessary to elevate CaMKII both pre- and postsynaptically to rescue structural overgrowth in the mys^{ts1} mutant. These results are consistent with the postsynaptic mislocalization of βPS integrin in the mys^{b9} mutant, as opposed to the global loss of synaptic integrin in the mys^{ts1} mutant (Beumer et al., 1999). These data indicate that coordinate regulation of CaMKII in the muscle and motoneuron is necessary for proper development of synaptic architecture.

Second, at the distal end of the cascade, both the expression and synaptic localization of FAS2 are increased in *mys* mutants, although the extent of FAS2 misregulation was significantly different between the two regulatory mutants. Importantly, both the NMJ overgrowth (mys^{ts1}) and undergrowth (mys^{b9}) phenotypes are rescued towards wild-type structure by genetically reducing the amount of FAS2 available at the synapse to near normal levels. In mys^{ts1} mutants, correcting for FAS2 level suppresses the synaptic overgrowth, while in mys^{b9} mutants, correcting the FAS2 level suppresses the synaptic undergrowth. These results support the conclusion that FAS2 is centrally involved in mediating synaptic growth,

but suggest that the FAS2-mediated mechanism is more complex than previously thought.

Do integrins do more than regulate FAS2 at the synapse?

Our results demonstrate that integrins regulate morphological growth at the postembryonic NMJ through activation of CaMKII in both pre- and postsynaptic compartments. One important target of CaMKII is FAS2 and it is clear that regulation of FAS2 expression and localization is an important component of integrin regulation. However, the modulation of FAS2 levels alone is unlikely to account fully for the alterations in synaptic architecture in integrin mutants. In particular, both mys^{ts1} and mys^{b9} upregulate synaptic FAS2 levels, albeit to different degrees, yet show opposite alteration in synaptic growth. Moreover, reducing FAS2 levels rescues both under- and overgrowth defects, but the rescue is not perfect.

Precise control of FAS2 levels finely tunes morphological development at the NMJ. Different degrees of reduced FAS2 expression can either facilitate or inhibit the growth/maintenance of the NMJ, and reduced FAS2 expression has been demonstrated in other overgrowth mutants (Schuster et al., 1996b). However, overexpression of FAS2 in specific muscles drives increased NMJ elaboration/bouton differentiation and selective preference for a high-expressing muscle over a low-expressing muscle (Davis and Goodman, 1998). How can this complexity be interpreted? One likely explanation is interaction between FAS2 and other developmental pathways regulated by CaMKII. To date, the only known FAS2-independent regulation of synaptic morphology involves a deubiquitinating protease encoded by *fat facets* (*faf*), and a putative ubiquitin ligase encoded by *highwire* (*hiw*), which have been shown to work together to modulate the degree of NMJ growth (DiAntonio et al., 2001; Wan et al., 2000). Loss-of-function *hiw* mutants display NMJ structural overgrowth, importantly without a concomitant decrease in FAS2. Indeed, overexpression of FAS2 cannot suppress the overgrowth seen in *hiw* mutants (Wan et al., 2000). These observations are consistent with the overgrowth combined with overexpression of FAS2 observed in mys^{ts1} mutants in this study. However, any putative interaction between FAS2-dependent and -independent mechanisms of morphological growth regulation at the *Drosophila* NMJ synapse remain to be elucidated. We expect that further study will reveal an additional structural control mechanism regulated by CaMKII, acting in parallel to and interacting with FAS2. The ubiquination mechanism discussed here is one candidate mechanism, but as CaMKII is known to have many synaptic targets, it is clearly not the only candidate.

Presynaptic CaMKII has previously been shown to be important for behavioral change, including learning in mice, and habituation in *Drosophila* (Griffith et al., 1993; Soderling, 2000). In mice, animals null for αCaMKII are unable to learn, and do not manifest experience-dependent plasticity (Frankland et al., 2001). Animals missing just one copy of αCaMKII learn normally, but are unable to form long-term memories, which is accompanied by an inability to maintain LTP in the cortex. In *Drosophila*, presynaptic CaMKII has been shown to bidirectionally regulate habituation of a simple motor response (Jin et al., 1998). Inhibition of CaMKII activation by expression of the ala peptide reduces the initial response, which is then followed by facilitation instead of habituation. Moreover, presynaptically targeted expression of a constitutively active form of CaMKII eliminated habituation. Thus, appropriate regulation of CaMKII appears necessary for many aspects of developmental plasticity.

In summary, we conclude that architectural developmental defects observed in NMJ synapses mutant for βPS integrin are due to the loss of the ability to regulate synaptic CaMKII properly. One function of CaMKII is to phosphorylate the scaffolding protein DLG, and thus regulate the proteins synaptically localized by this scaffold. FAS2 is the central known output of this regulatory cascade. Loss of this regulation is central to the *mys* mutant defects in the postembryonic elaboration of NMJ structure. It is clear from this study, however, that regulation of FAS2 localization via CaMKII is only one facet of how integrins function at the synapse.

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