A Mechanism Distinct from Highwire for the *Drosophila* Ubiquitin Conjugase *Bendless* in Synaptic Growth and Maturation

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The signaling mechanisms that allow the conversion of a growth cone into a mature and stable synapse are yet to be completely understood. Ubiquitination plays key regulatory roles in synaptic development and may be involved in this process. Previous studies identified the *Drosophila* ubiquitin conjugase *bendless* (*ben*) to be important for central synapse formation, but the precise role it plays has not been elucidated. Our studies indicate that Ben plays a pivotal role in synaptic growth and maturation. We have determined that an incipient synapse is present with a high penetrance in *ben* mutants, suggesting that Ben is required for a developmental step after target recognition. We used cell-autonomous rescue experiments to show that Ben has a presynaptic role in synapse growth. We then harnessed the TARGET system to transiently express UAS (upstream activating sequence)–*ben* in a *ben* mutant background and identified a well defined critical period for Ben function in establishing a full-grown, mature synaptic terminal. We demonstrate that the protein must be present at a time point before but not during the actual growth process. We also provide phenotypic evidence demonstrating that Ben is not a part of the signal transduction pathway involving the well characterized ubiquitin ligase *highwire*. We conclude that *Bendless* functions as a novel developmental switch that permits the transition from axonal growth and incipient synapse formation to synaptic growth and maturation in the CNS.

Key words: giant fiber; *Drosophila*; synapse formation; ubiquitin; growth; maturation

Introduction

The formation, growth, and stabilization of synapses are crucial phenomena in the establishment of functional neural circuits. An important regulatory mechanism in synapse formation and function is ubiquitination, which refers to the posttranslational modification of a target protein by the addition of one (monoubiquitination) or more (multiubiquitination and polyubiquitination) adducts of the 76 amino acid ubiquitin polypeptide (Murphey and Godenschwege, 2002; DiAntonio and Hicke, 2004; Haas and Broadie, 2008). The transfer of ubiquitin to a substrate takes place through an enzymatic cascade involving a ubiquitin activating enzyme E1, a ubiquitin conjugating enzyme E2, and a ubiquitin ligase E3 (Hershko and Ciechanover, 1998). With regard to regulation of nervous system development, ubiquitin has been implicated in the regulation of axon guidance (Campbell and Holt, 2001), axonal pruning (Watts et al., 2003), synapse development (DiAntonio et al., 2001), neurotransmitter

release (Wilson et al., 2002; Aravamudan and Broadie, 2003), number of postsynaptic receptors (Bedford et al., 2001; Buttner et al., 2001; Burbea et al., 2002; Patrick et al., 2003), and components of the postsynaptic density (Ehlers, 2003).

One of the first results that pointed to the ubiquitin system playing a role in the regulation of neuronal connectivity came from the analysis of the *Drosophila* mutant *bendless* (*ben*). *ben* was identified more than two decades ago in a behavioral screen for defects in the giant fiber system (GFS) (see Fig. 1*A,B*) that resulted in an altered escape response (Thomas and Wyman, 1982). Cloning of *ben* led to its identification as an E2 ubiquitin conjugase (Muralidhar and Thomas, 1993; Oh et al., 1994). The mutation was found to particularly affect the synapse between the giant fiber (GF) and the tergotrochanteral motoneuron (TTMn) as the GF axon, after making normal contact with the peripherally synapsing interneuron (PSI), fails to make the lateral “bend,” which represents its large presynaptic terminal on the TTMn (Thomas and Wyman, 1984). Additional phenotypes were also reported in thoracic musculature (Edgecomb et al., 1993) and in the visual system (Muralidhar and Thomas, 1993; Oh et al., 1994).

Previous studies have suggested that Ben plays an important role in axon guidance or target recognition (Muralidhar and Thomas, 1993; Oh et al., 1994). However, the underlying mechanism by which Ben regulates the formation of a mature synapse is yet to be clarified. Our analyses of the *ben* mutant in the GF

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system using anatomical studies as well as specific approaches to determine the spatial and temporal aspects of gene function have given us novel evidence indicating that Ben plays an important role in synapse growth. Our studies suggest that Ben functions as an acute developmental switch that allows axonal growth to transition into synaptic growth, allowing for the establishment of a mature synaptic connection. To our knowledge, this is the first time that a component of the ubiquitin system has been shown to play the role of a key permissive factor that allows synaptic growth and maturation to take place, in contrast to, say, the ubiquitin ligase *highwire* (*hiw*) which can be characterized to actively regulate the growth process.

Materials and Methods

Drosophila stocks. All stocks were grown on standard medium at either 22°C or 25°C unless otherwise indicated. The following fly stocks were used: *ben*¹, hereafter referred to as *ben* (Thomas and Wyman, 1984), *ben* deficiency (Bloomington stock 968, Df(1)HA92/FM7c), upstream activating sequence (UAS)-*ben* (second chromosome), *ben*; UAS-*ben*, UAS-green fluorescent protein (GFP)-*ben* (second chromosome), *fat facets* (*faf*) EP(3)0381 (Rorth et al., 1998), *hiw*^{ND8} (Wan et al., 2000), and UAS-*bsk*^{DN} (Weber et al., 2000). Three P[GAL]4 drivers that express in the GF system were used: the A307 driver has strong presynaptic expression in the GF along with weaker expression in postsynaptic targets (Allen et al., 1998), *c17* drives expression in the GF but not in its postsynaptic targets (Godenschwege et al., 2002), and ShalB-Gal4 drives expression only postsynaptically (Jacobs et al., 2000).

Generation of UAS-GFP-*ben* transgenic line. *ben* cDNA was cloned into the eGFP-C1 vector with *Kpn*I and *Sal*I. The GFP-*ben* fragment was excised with *Nhe*I and *Spe*I and subcloned into the pUAS vector cut with *Xba*I. Construct fidelity was verified by sequencing (Davis Sequencing, Davis, CA), and embryos were transformed at the Transgenic Drosophila Fly Core (Massachusetts General Hospital, Boston, MA).

Electrophysiology. Sharp electrode intracellular recordings from the TTM muscles were obtained from intact adult flies in a method similar to that described previously (Tanouye and Wyman, 1980). The physiological assay was modified and data were analyzed as described previously (Godenschwege et al., 2002).

Dye injections, imaging, and analysis. For anatomical analysis, the dissected CNS was mounted dorsal side up on a poly-lysine-coated slide. The preparation was immersed in saline and viewed with a 40× water immersion lens. The axons of the GFs are identified in the connective using differential interference contrast optics. A glass electrode (20–80 MΩ) containing 1% aqueous Lucifer yellow and backfilled with 3 M LiCl was used to impale the GF in the connective. The dye was injected into the GF by the passage of 3–5 nA of hyperpolarizing current using a Getting 5A Amplifier (Getting Instruments). Images in Figures 2 and 6 were obtained by taking maximum projections of Z-stack images taken using an advanced SPOT camera and MetaVue software. Deconvolution was performed on images using Autovisualize and Autoblur software version 9.3 (Autoquant). For UAS-GFP-*ben* localization in Figure 5, the image in **A** was taken using a coolSNAP HQ2 camera on a Nikon Eclipse FN1 microscope with 10× objective using NIS elements. Image was deconvolved with AutoquantX2 software (Media Cybernetics). Images in **B–D** were taken on a Nikon Eclipse C1si confocal system with a 40× objective on a 90i microscope.

Ultrastructure. The adult CNS was dissected from *ben* flies that were electrophysiologically tested and wild-type (wt) controls. The CNS underwent prefixation in 2.5% glutaraldehyde for 24 h and was osmicated (1% osmium tetroxide) for 1 h. Subsequent to dehydration, the CNS was embedded in Epon-Araldite. Ultrathin serial sections (50–60 nm) were taken and counterstained with 1% aqueous uranyl acetate and lead citrate. The sections were examined with a Jeol 100s electron microscope. The GF-TTMn synaptic region was photographed, and the negatives were scanned using a high-resolution flatbed scanner.

Temporal expression of UAS-*ben* using the TARGET system. The TARGET system (McGuire et al., 2003) was used to temporally express UAS-*ben* in a *ben* mutant background. We crossed *ben*; UAS-*ben* flies to A307/

Cyo; tub-Gal80ts/TM6. The cross was set up at the permissive temperature (we found 22°C sufficient for the tub-Gal80 to suppress A307 and restrict UAS-*ben* expression). Pupal day 0 (P0) pupae of *ben*/Y; A307/UAS-*ben*; tub-Gal80ts/+ were collected, and UAS-*ben* expression was induced for 24 h (~16% of pupal development (PD)) by temperature shifts to 30°C at P0, P0 plus 6 h, P0 plus 12 h, P0 plus 18 h, P0 plus 24 h, P0 plus 48 h, P0 plus 72 h, P0 plus 96 h, and P0 plus 120 h. The TTM response was determined after eclosion in 2- to 3-d-old adults and compared with control specimens: *ben*/Y; A307/UAS-*ben*; TM6/+.

Results

A residual synaptic connection is present in *ben* mutants

We examined the *ben* phenotype by performing electrophysiological recordings from the TTM and found 88% of the responses to be mutant. A wild-type response from the muscle is defined as one with a response latency below 1 ms and which follows 100% at 100 Hz stimulation. In accordance with previous studies, we found *ben* specimens to exhibit long, variable latencies and not follow 1:1 at high-frequency stimulation (Fig. 1B). To ascertain the penetrance of the mutant phenotype, we further crossed *ben* to a deficiency that spanned the entire *ben* gene and physiologically assayed offspring that were transheterozygous for *ben* and the deficiency. The physiology of the resultant transheterozygotes was 93% mutant (Table 1), which is not significantly different from the physiology of homozygous *ben* specimens as determined by a Fisher's exact test (two-tailed *p* value = 1.000).

For our anatomical analysis, we dissected the CNS out of the same *ben* specimens that had been physiologically tested and dye injected the GFs with Lucifer yellow, an anionic fluorescent dye that is small enough to pass through gap junctional contacts. In wild-type specimens, Lucifer yellow passes through the GF terminal into the TTMn 90–95% of the time depending on duration of the fill and the health of the specimen (Fig. 2A). We found transsynaptic fills from the truncated GF terminal to the TTMn in 55% (*n* = 22) of *ben* specimens (Fig. 2B). This indicated that there was a direct functional connection containing gap junctions between the GF and its target motor neuron. Because the *ben* mutation is an ethyl methane sulfonate-induced point mutant, it was possible that this synaptic contact pertains to a residual function of the mutant protein rather than being indicative of a null phenotype. To address this issue, we also performed Lucifer yellow dye injections in specimens that were transheterozygous for *ben* and the deficiency. We still saw transsynaptic fills from the GF to the TTMn with a similar penetrance of 54% (*n* = 16). To ensure that this residual synaptic connection seen in *ben* mutants was not just a case of delayed synaptogenesis or an initial stage of synaptic degeneration, we also analyzed *ben* mutants that were aged at 25°C for 2 weeks. Even in this case, the truncated GF terminal remained dye coupled with the TTMn.

Because the GF-TTMn synapse is a mixed electrical–chemical synapse (Blagburn et al., 1999; Allen and Murphey, 2007), we also wanted to determine whether components of the chemical synapse might be present in the terminals of *ben* mutants. We determined the localization of two synaptic vesicle markers, synaptotagmin and synaptobrevin, in the *ben* mutant by expressing GFP-tagged constructs in the GFs. An example of synaptotagmin–GFP localization in a wild-type and a *ben* specimen is seen in Figure 2, C and D, respectively. We could see fluorescent puncta of both proteins accumulate at the tips of the truncated terminal, suggesting the presence of synaptic vesicles (*n* = 18). We furthered this observation by performing ultrastructural analyses on the *ben* mutant and comparing the synaptic phenotype with what is seen in a wild-type specimen (Fig. 3A,B). We traced the GF to the synaptic region in three *ben* specimens and were able to isolate

Figure 1. The giant fiber system. **A**, Schematic of the GFS in the *Drosophila* CNS (modified from Godenschwege et al., 2006). The GFS is a well characterized neuronal circuit, and the mixed electrical–chemical synapse formed between the GF and the TTMn is an ideal setting to study the stages of synaptogenesis at a single-cell resolution both functionally and anatomically (King and Wyman, 1980; Blagburn et al., 1999). The image has been simplified to show the right half of the GF circuitry in color. The GF (orange) has its soma and dendrites in the brain, and its axons enter the second thoracic neuromere to make synaptic connections with the PSI (yellow) and the TTMn (blue). The PSI synapses onto the DLMn (green). The TTMn and the DLMn innervate the jump (TTM) and flight (DLM) muscles, respectively. Also indicated in the schematic are the stimulus and recording arrangement to obtain responses from the muscle. Although the recording from the right half of the circuitry is depicted, we obtain recordings from the muscle on both sides. Primary recordings are taken from the TTM, with recordings from the DLM taken to verify that the GF had indeed reached the target area. **B**, Representative recordings from wild-type and *bendless* mutant adult flies. In wild-type specimens (top trace), the TTM response latency is 0.8 ms and the pathway is able to follow 1:1 at 100 Hz stimuli. Responses from *bendless* specimens (bottom trace) have characteristic long latencies (2 ms+) and fail to follow 100% at 100 Hz stimulation.

Table 1. Spatial rescue of the physiological *ben* phenotype

Expression locus ^a	Genotype	n	Wild type in % ^b
	+ / y or + / +	40	100
	<i>ben/ben</i>	84	12
	<i>ben/Def</i>	16	7
Pre + post	<i>ben;UAS-ben/A307</i>	22	100
Pre	<i>ben;UAS-ben/C17</i>	24	83
Pre	<i>ben;UAS-bsk^{DN}/C17</i>	16	6
Pre	<i>ben;UAS-ben/C17;UAS-ben</i>	32	91
Pre	<i>ben;UAS-ben/C42.2</i>	14	20
Post	<i>ben;UAS-ben/Shak-B</i>	24	13
Pre + post	+ / +; <i>UAS-ben/A307</i>	24	100
Pre + post	<i>ben;A307/+;UAS-GFP-ben/+</i>	16	93

^aExpression of UAS constructs presynaptically (pre) in the GF, postsynaptically (post) in the TTMn, or both.

^bA wild-type synaptic connection is defined as a response latency ≤ 1 ms and the ability to follow stimuli one-to-one at 100 Hz when stimulated in the brain.

n, Number of GFs analyzed.

sections that show T-bars indicative of presynaptic release sites as well as close juxtaposition of the presynaptic and postsynaptic membrane with a single layer of vesicles indicative of gap junctional contact as described previously (Blagburn et al., 1999; Godenschwege et al., 2006) (Fig. 3C–F). The ultrastructural results are dramatic, and although the truncated GF tapers down to a much smaller axonal diameter, the essential synaptic features are still seen in the reduced contact region with the TTMn. These results suggest that an incipient chemical and gap junctional synapse is formed in a *ben* mutant background. Hence, Bendless does not seem to be required for nascent synapse formation but rather plays a role in synaptic growth and maturation.

Bendless has a cell-autonomous presynaptic function

We generated *UAS-ben* transgenic flies and conducted a series of rescue experiments to determine a spatial role for Bendless in the GFS. By driving *UAS-ben* expression in a *ben* mutant background under the control of A307, which is a strong driver that expresses in the GF and its postsynaptic target, we were able to completely rescue the Bendless phenotype physiologically and anatomically (100% wt; $n = 22$) (Table 1 and data not shown). Using the weaker presynaptic driver c17, we were still able to get a significant rescue (83% wt; $n = 24$) (Table 1), which improves with double the dosage of the *UAS-ben* transcript (91% wt; $n = 32$) (Table 1). In contrast, expression with the postsynaptic driver *ShakB-Gal4* was unable to rescue the mutant phenotype (13% wt; $n = 24$) (Table 1). Our results confirm the previous observation that Bendless does indeed have a presynaptic role in synapse function (Oh et al., 1994) and additionally proves that it functions cell autonomously in the GFS.

Our anatomical studies led us to hypothesize that Bendless was involved in synaptic growth and maturation. Interestingly, driving expression of *UAS-ben* with A307 in a wild-type background did not disrupt the synapse physiologically (100% wt; $n = 24$) (Table 1) or cause anatomical changes (data not shown). The absence of a gain-of-function phenotype, like an overgrown terminal, for Ben overexpression suggests that Bendless is not an effector molecule that is sufficient to induce synaptic growth on its own. We also expressed *UAS-ben* in a *ben* mutant background with the presynaptic c42.2 driver, which turns on expression in the latter half of PD when the giant fiber would be undergoing additional growth after having made its synapse in wild-type specimens. We did not see any significant rescue of the *ben* phe-

Figure 2. Analyses of the *bendless* incipient synapse. **A**, Dye injection of the right GF in a wild-type specimen with Lucifer yellow. Solid line box in wild-type schematic indicates region of focus in the thoracic ganglion. The presynaptic terminal is indicated with a white bracket. A transynaptic fill from the GF to the TTMn is seen (vertical white arrows). **B**, Dye injection of the right GF in a *bendless* specimen. Solid line box in *ben* schematic indicates region of focus. The presynaptic terminal is missing (white bracket), but a transynaptic fill from the truncated GF to the TTMn is still seen (vertical white arrows). **C**, Synaptotagmin-GFP localization in the GFs in the second thoracic neuromere of a wild-type specimen. Broken line box in wild-type schematic indicates region of focus in the thoracic ganglion. Strong fluorescence is seen at the contact regions between the GF and PSI (white arrow) and the GF and TTMn (white arrowheads). **D**, Synaptotagmin-GFP localization in a *bendless* specimen. Broken line box in *ben* schematic indicates region of focus. Fluorescent puncta are seen where the GF contacts the PSI (white arrow) and at the tips of the truncated GFs as well (white arrowheads). Scale bars, 10 μ m (in the respective top and panels). The horizontal white arrow in all panels serves as a point of reference indicating the region of contact between the GF and the PSI.

notype under these conditions (20% wt; $n = 13$) (Table 1). This was intriguing and contrary to what might have been expected; Ben function was not required during the actual growth of the GF presynaptic terminal.

Critical period for *bendless* before synaptic growth

Because the expression of UAS-*ben* during later stages of PD by using the c42.2 driver did not rescue the *ben* mutant phenotype, we proceeded to determine more precisely the time point when protein function was necessary to make a normal synapse. Previous anatomical studies have delineated the various developmental stages of the GFS (Phelan et al., 1996; Allen et al., 1998) (Fig. 4A) and have also identified critical periods for synapse forma-

tion (Murphey et al., 2003). We used the TARGET system (McGuire et al., 2004a,b) to regulate UAS-*ben* expression during different stages of GF development. Briefly, in the TARGET system, a temperature-sensitive variant of Gal80 (Gal80ts) is cloned behind a tubulin promoter and regulates Gal4 expression in a temperature-dependent manner. At a permissive temperature (22°C), Gal80ts binds Gal4 and inhibits gene expression, whereas at a nonpermissive temperature (30°C), the Gal80ts protein misfolds and does not bind Gal4 and gene expression is allowed to turn on.

In a *ben* mutant background, we turned on UAS-*ben* expression under the control of Gal80ts and the A307 driver by giving 24 h temperature shifts to nonpermissive temperature (30°C) at a late larval stage (L3) and various stages of PD (Fig. 4B). The timeline of GF development that corresponds to these stages is indicated in the schematic (Fig. 4A,B). Although the duration of the temperature shift is indicated (Fig. 4B, red lines), it should be noted that transgene presence is likely to be seen with an ~ 3 h delay and can persist for up to ~ 12 h after the temperature shift as detailed in the original study (McGuire et al., 2004a). Comparing these results with internal controls, we found the period of maximum rescue to be when UAS-*ben* expression is turned on in the first 24 h of PD (80% wt; $n = 132$) (Fig. 4B). The rescue ability decreases significantly for temperature shifts after P0 plus 12 h (62% wt; $n = 34$), and no rescue was seen after P0 plus 48 h (11% wt; $n = 54$). The time interval at which Ben is required to obtain any rescue of the mutant phenotype is indicated by the yellow highlight. We also performed temperature shifts of 48 h duration starting at P0, P0 plus 24 h, P0 plus 48 h, P0 plus 72 h, P0 plus 96 h, and P0 plus 120 h and still found a significant reduction in the rescue potential after P0 plus 24 h (data not shown). Hence, the critical period of UAS-*ben* expression is the first 24 h of PD, a time interval earlier than the occurrence of synaptic growth (Fig. 4, green highlight). This is rather interesting because the Ben protein is required for synaptic growth but need not be present during the growth event itself.

Bendless localization is cytoplasmic and nuclear

Ubiquitination is known to regulate synaptic function at both a protein level, by directly affecting signaling such as locally at the synapse, and a gene level, through the control of transcription factors (Murphey and Godenschwege, 2002; DiAntonio and Hicke, 2004). Hence, components of the ubiquitin system have been found to be expressed in both the nucleus and the cytosol. Sequence analysis points to Bendless being a cytoplasmic protein attributable to the lack of characterized nuclear localization motifs. To further characterize Bendless, we proceeded to determine its intracellular distribution. We generated a transgenic line containing UAS-*ben* with an N-terminal GFP fusion tag (UAS-GFP-*ben*). To verify that the GFP tag did not disrupt protein function, we expressed the construct under the control of the A307 driver in a *ben* mutant background and found that it was able to effect a robust rescue of the mutant phenotype (93% wt; $n = 16$) (Table 1). Given the functional rescue, we studied Ben protein localization by expressing UAS-GFP-*ben* in a wild-type background. We found that GFP-Ben is uniformly cytosolic throughout the entire neuron, inclusive of the GF axons, dendrites, and synaptic region (Fig. 5A,D). In addition to its ubiquitous cytosolic localization, we also find strong nuclear accumulation of GFP-Ben in cell bodies of the GF (Fig. 5B). Nuclear localization of GFP-Ben was seen in all subsets of neurons in which A307 Gal4 drives expression; an example of the dorsal longitudinal motoneuron (DLMn) cell body is seen in Figure 5C. These data highlight an

Figure 3. Ultrastructure of the presynaptic terminal in a *bendless* specimen. **A**, Electron micrograph showing the right GF in the target area of a wild-type specimen. Broken line box in the wt schematic indicates the region of focus. The GF has a pronounced 5 μ m diameter and is seen to contact the TTMn as indicated by the darker electron-dense region abutting the lighter GF tip. Scale bar: **A**, **C**, 2 μ m. **B**, Another section cut in the region marked by the solid box in the wt schematic showing a magnified view of the GF–TTMn contact region in a wild-type specimen. Note the expanded cross-sectional area of the presynaptic terminal. **C**, Electron micrograph showing the right GF in the target area of a *ben* specimen. Broken line box in schematic indicates the region of focus. The GF tapers in diameter and is seen to contact the TTMn. Scale bar: **B**, **D**, 1 μ m. **D**, Another section showing a magnified view of the GF–TTMn contact region as pointed out by the solid box in the *ben* schematic. The TTMn interdigitates with the GF, and a vesicle release site with a T-bar (black arrowhead) is seen. An area with several vesicles is also indicated (white arrowhead). **E**, Magnified image of a gap junctional contact with a characteristic single row of vesicles lining the membrane in *ben*. **F**, Magnified image of a T-bar, the release site of a chemical synapse in *ben*.

important aspect of regulation whereby a molecule can have a precise temporal role affecting one particular synaptic connection despite having a ubiquitous spatial distribution.

Bendless is in a distinct signaling pathway from Highwire

In the established ubiquitination pathway, ubiquitin is transferred from an activating enzyme to a conjugating enzyme and

finally onto a target substrate via a ubiquitin ligase that confers substrate specificity (Hershko and Ciechanover, 1998). Ben is a conjugating enzyme, and a possible candidate to be the downstream ligase for Ben in *Drosophila* is the ubiquitin ligase *highwire*. Hiw has been well characterized for regulating synaptic morphology at the *Drosophila* larval neuromuscular junction (NMJ) (Wan et al., 2000; DiAntonio et al., 2001; Wu et al., 2005, 2007; Collins et al., 2006). *hiw* mutants exhibit a dramatic increase in the number of synaptic branches and boutons along with an increase in synaptic span. NMJ synaptic development was found to be dependent on a balance of positive and negative regulators of ubiquitination. This balance is exemplified by the fact that *hiw* interacts with the deubiquitinating protease *faf*, and overexpression of *Faf* results in a phenotype similar to *hiw* mutants (DiAntonio et al., 2001).

We wanted to determine whether *ben* and *hiw* acted through the same signaling cascade in establishing a central synapse by analyzing the mutants for phenotypic similarities. If Ben were the upstream conjugase partner of the ligase Hiw, then it follows that loss of Ben function should phenocopy loss of Hiw. We characterized the GFS, both physiologically and anatomically, in specimens carrying the *hiw*^{ND8} loss-of-function allele. Loss of *hiw* function did affect the GF–TTMn synapse physiologically as well as anatomically. Anatomically, we found ectopic axonal branches off the presynaptic terminal extending outside the target region in many specimens, but we did not find truncated GF terminals as seen in *ben* specimens ($n = 30$) (Table 2, Fig. 6B). Interestingly, we ascertained a similar anatomical result of axonal overgrowth with the overexpression of *Faf* in the GFS as well ($n = 16$) (Table 2), indicating that both loss-of-function *hiw* and gain-of-function *Faf* have similar effects on growth at a central synapse. Clearly, the *ben* mutant phenotype is distinctly different from the *hiw* and *Faf* phenotypes, with the former exhibiting synaptic undergrowth and the latter two exhibiting synaptic overgrowth.

To further confirm the distinct roles played by Ben and Hiw at a central synapse, we also looked at components of the *hiw* downstream signaling pathway. The downstream pathway of *Drosophila hiw* includes the mitogen-activated protein kinase kinase kinase *wallenda* (*wnd*), the Jun kinase (JNK) *basket* (*bsk*), and the *D-fos* transcription factor. At the NMJ, the inhibition of JNK signaling by the expression of a UAS-*bsk*^{DN} construct was found to suppress the *hiw* phenotype (Collins et al., 2006). We expressed UAS-*bsk*^{DN} in a *ben* mutant background and did not find the *ben* phenotype to be suppressed

Figure 4. Critical period for Bendless function. **A**, Schematic showing the various phases of giant synapse assembly during PD (modified from Godenschwege et al., 2006). The TTMn dendrites grow into the target area of the second thoracic neuromere before the arrival of GF axons. The GF initiates growth in the late larval stage and reaches the target area to make initial contact with the TTMn dendrite by 20% of PD. This is followed by a phase of synaptogenesis (25–50% of PD) during which the GF dye couples with the TTMn at ~40% of PD and elaborates a lateral “bend” establishing a presynaptic terminal (Phelan et al., 1996; Allen et al., 1998; Jacobs et al., 2000). After 50% of PD, the synapse continues to grow and stabilize to become a fully mature synapse by the time of eclosion. The green highlight indicates the period of synaptic growth, and the yellow highlight indicates the time period when Ben function is required to affect a rescue of the mutant phenotype. **B**, Representation of temperature shifts (TS) given to specimens of genotype *ben*^Y; A307/UAS-*ben*; Gal80ts/+ at various stages of PD using the TARGET system. Specimens were raised at the permissive temperature of 22°C (blue line indicating that gene expression is “off”) and then placed at a nonpermissive temperature of 30°C for 24 h (red line indicating that gene expression is “on”). A 24 h, temperature shift represents ~16% of PD. The percentages over the red lines indicate the percentage of wild-type responses (response latency ≤1 ms and the ability to follow stimuli one-to-one at 100 Hz when stimulated in the brain) from specimens tested as adults. The number of specimens tested (*n*) are as follows: no TS (68), L3 (8), P0 (132), P0 plus 6 h (31), P0 plus 12 h (34), P0 plus 18 h (22), P0 plus 24 h (64), P0 plus 48 h (54), P0 plus 72 h (36), P0 plus 96 h (74), and P0 plus 120 h (13). Control specimens of the genotype *ben*^Y; A307/UAS-*ben*; TM6/+ (*n* = 210) were also tested under the same paradigm at different stages of PD, and responses were found to be 82–100% wild type.

(Table 1). Overall, our experiments strongly suggest that Ben and Hiw function in distinct pathways to regulate central synapse growth. A detailed description of the *hiw* phenotype, additional analysis of its function, and subsequent characterization of its downstream signaling cascade at a central synapse will be performed in a separate study.

Discussion

Bendless and Highwire

The results from this study have given us new insights into how ubiquitin system components establish functional synaptic connections. The temporal analysis of Bendless has been critical in illustrating its role as a developmental “switch” in converting a growth cone

into a mature synapse. As mentioned previously, the *ben* mutation is the result of a single amino acid change in the conserved catalytic core of the conjugase domain of the protein. This highlights the fact that the conjugase activity of the protein is necessary for the observed synaptic phenotype. Analysis of synaptic growth in *Drosophila* has primarily been done at the peripheral synapse of the NMJ (Laviolette et al., 2005; Goold and Davis, 2007; Fulga and Van Vactor, 2008; Liebl et al., 2008; Schwenkert et al., 2008; Seabrooke and Stewart, 2008). Components of the ubiquitin system, such as the ubiquitin ligase *hiw*, the deubiquitinating protease *faf*, and the synapse-associated E3 ligase PDZRN3, are known to play important roles in the growth and function of the fly NMJ (DiAntonio et al., 2001;

Figure 5. Localization of Bendless. **A**, Deconvolved image of a specimen expressing UAS-GFP-*ben* under the control of the A307 driver at 10 $\times$. The specified region is indicated by the solid black box in the schematic. **B**, Confocal image of GF cell body in the brain showing diffuse localization of UAS-GFP-*ben* in the cytoplasm as well as intense nuclear staining. Solid top red line box in schematic indicates GF cell body position in brain. **C**, Confocal image of DLMn cell body in the thoracic ganglion showing cytoplasmic and nuclear localization of GFP-Ben. Solid middle red line box in schematic indicates DLMn cell body position in thoracic ganglion. **D**, Confocal image showing the uniform localization of UAS-GFP-*ben* in the presynaptic terminal. Solid bottom red line box in schematic indicates the specified location in the thoracic ganglion. Scale bars: **A**, 25 μ m; **B**, **C**, 5 μ m; **D**, 10 μ m.

Table 2. Anatomical analysis of Hiw and Faf in the GFS

Genotype	n	% of specimens with		
		Synaptic terminal present	<i>bendless</i> -like terminal	Extra axonal branches
<i>hiw/hiw</i>	30	100	0	47
A307-ULZ; <i>faf</i> EP(3)381	16	100	0	38

n, Number of GFs analyzed.

McCabe et al., 2004; Wu et al., 2005; Collins et al., 2006; Lu et al., 2007). Significant studies have been performed with particular regard to the conserved family of *hiw* ubiquitin ligases. In *Drosophila*, *hiw* functions as a negative regulator of synapse development as mutants exhibit dramatic synaptic overgrowth at the larval NMJ (Wan et al., 2000). In *Caenorhabditis elegans*, loss of function of the *hiw* homolog *rpm-1* results in multiple phenotypes at the NMJ as well as in the CNS (Schaefer et al., 2000; Zhen et al., 2000). At the NMJ, some NMJs exhibit enlarged presynaptic terminals containing multiple active zones, whereas others contain underdeveloped or absent presynaptic terminals (Zhen et al., 2000). In the worm mechanosensory circuit, the sensory neurons were found to retract synaptic branches, extend ectopic axons, and fail to accumulate synaptic vesicles, whereas some of the motor neurons exhibited phenotypes such as altered synaptic organization, branching, and overgrowth (Schaefer et al., 2000). Downstream signaling components have been isolated for both *hiw* and *rpm-1* in *Drosophila* and *C. elegans*, respectively, and a number of conserved elements have been identified (Schaefer et al., 2000; Nakata et al., 2005; Collins et al., 2006; Grill et al., 2007; Abrams et al., 2008; Li et al., 2008). Mutations in homologs of *hiw* in zebrafish and mice are also known to cause a variety of synaptic disruptions (Burgess et al., 2004; D'Souza et al., 2005).

Ben and Hiw play distinct roles in synapse growth. In our study, we have been able to analyze the novel roles played by these ubiquitin system components at the GFS central synapse. We have shown that *ben* and *hiw* loss of function result in very different phenotypes, with *ben* specimens exhibiting synaptic un-

dergrowth and *hiw* specimens exhibiting synaptic overgrowth. We also show that Ben function does not involve JNK, a well characterized downstream signaling partner identified for Hiw in *Drosophila*. It is also interesting to compare and contrast the role Hiw plays at a peripheral synapse with the role Ben plays at a central synapse. *hiw* mutants exhibit a presynaptic overgrowth phenotype at the NMJ, whereas *ben* mutants exhibit a reduction in presynaptic growth in the CNS. Also, Hiw does not localize to the nucleus and was found to regulate synaptic growth throughout development, whereas Ben has nuclear as well as cytosolic localization and only functions in a critical time period. Finally, Hiw activity is associated with the bone morphogenetic protein (BMP) retrograde signaling pathway that is known to be dependent on the retrograde motor (McCabe et al., 2004). We found no evidence that Bendless function is dependent on the retrograde motor (our unpublished data). All these data underline the fact that there are distinct targets for the ubiquitination cascades involving Ben and Hiw.

Bendless is required for synaptic growth and maturation

Functional neuronal circuits are established through a series of events: neurite outgrowth, axon guidance, target recognition, synapse formation, and synaptic growth and maturation. When the *bendless* mutant was originally characterized,

Ben was thought to play an important role in either axon guidance or target recognition (Muralidhar and Thomas, 1993; Oh et al., 1994). Our analysis of the *ben* mutant clearly shows that Ben has an important role in synaptic growth. A number of specimens exhibit dye coupling between the GF and the motorneuron dendrite demonstrating that an incipient synapse is still formed (Fig. 2B) and that the mutant phenotype arises from a failure of this immature connection to grow into a mature synapse. In addition, we also shown that both gap junctional and chemical components are present at *ben* mutant terminals with synaptic vesicle marker localization as well as ultrastructural analyses (Figs. 2D, 3C–F).

The current view of synapse formation is that a nascent synapse can be rapidly assembled from material present in a growth cone in prepackaged vesicles and packets (Roos and Kelly, 2000; Ziv and Garner, 2004). After this primary rapid assembly of a nascent synapse, a secondary slower growth and maturation process takes place to result in a stable mature synapse. An insightful study on the *Drosophila* kinesin *immaculate connections* (*imac*) has shown it to be a permissive regulator of presynaptic maturation at the larval NMJ (Pack-Chung et al., 2007). *Imac* was found to be involved in the anterograde transport of synaptic vesicle precursors to the tip of the growth cone, an initial stage of synaptogenesis. In *ben* specimens, synaptic vesicles are still transported all the way down to the tip of the truncated terminal as evidenced by the localization of GFP-tagged synaptotagmin and synaptobrevin (Fig. 2D). Our data strongly suggest that the *ben* mutant

phenotype is resultant at a point after synaptic vesicular transport. Hence, we conclude that the *bendless* terminal is an incipient synapse that fails to grow and mature and that Ben is a permissive regulator whose function is required for the initiation of a secondary process in presynaptic growth.

Bendless as a developmental switch

It is counterintuitive that, although Bendless is required for synaptic growth and maturation, our data show that it is not required during the growth process. This highlights the important role Ben plays as a developmental switch. Transient expression of UAS-*ben* before the growth of the presynaptic terminal was sufficient to rescue the *ben* phenotype anatomically and physiologically, but expression during the growth period had no effect (Fig. 4). This suggests that Bendless is not involved in the actual growth process but rather has to be present in advance to alter signaling and initiate changes that allow growth to take place. Here it is essential to differentiate between axonal and synaptic growth, because it has been determined previously that axonal growth is unaffected in *ben* mutants (Muralidhar and Thomas, 1993). Hence, Ben function is required to permit axonal growth to switch to synaptic growth.

The molecules in the signaling pathway of this novel mechanism remain to be further investigated. In conclusion, tight spatial and temporal control of synaptic connectivity in the nervous system is undoubtedly crucial to normal function. Determining how exactly Bendless regulates the formation of a mature synapse will give us future novel insights into this phenomenon.

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Figure 6. Anatomical characterization of Hiw in the GFS. **A**, Dye injection of the left GF in a wild-type specimen with Lucifer yellow showing a normal presynaptic terminal. **B**, Overgrown GF terminal in a *hiw* mutant with extra branching in the target area and long ectopic extensions marked by white arrowheads. Scale bar, 10 μ m. The schematic shows the T2 segment of the thoracic neuromere. The solid box indicates the region of interest in which the giant fiber makes its synapse with the TTMn and is the area depicted in **B**. The black arrow in the schematic and the white arrows in **A** and **B** point to the site in which the GF makes synaptic contact with the PSI and serve as reference points.

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