The Ig cell adhesion molecule Basigin controls compartmentalization and vesicle release at Drosophila melanogaster synapses

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Abbreviations used in this paper: BRP, Bruchpilot; CAM, cell adhesion molecule; CSP, cysteine string protein; eEJC, nerve-evoked excitatory junctional current; NMJ, neuromuscular junction; PSD, postsynaptic density; SSR, subsynaptic reticulum.

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Synapses can undergo rapid changes in size as well as in their vesicle release function during both plasticity processes and development. This fundamental property of neuronal cells requires the coordinated rearrangement of synaptic membranes and their associated cytoskeleton, yet remarkably little is known of how this coupling is achieved. In a GFP exon-trap screen, we identified Drosophila melanogaster Basigin (Bsg) as an immunoglobulin domain-containing transmembrane protein accumulating at periactive zones of neuromuscular junctions. Bsg is required pre- and postsynaptically to restrict synaptic bouton size, its juxtamembrane cytoplasmic residues being important for that function. Bsg controls different aspects of synaptic structure, including distribution of synaptic vesicles and organization of the presynaptic cortical actin cytoskeleton. Strikingly, bsg function is also required specifically within the presynaptic terminal to inhibit nonsynchronized evoked vesicle release. We thus propose that Bsg is part of a transsynaptic complex regulating synaptic compartmentalization and strength, and coordinating plasma membrane and cortical organization.

Introduction

Synapses are highly specialized and asymmetric intercellular junctions organized into morphologically, biochemically, and physiologically distinct subdomains. At the presynaptic terminal membrane, active zones mediate Ca2+-dependent synaptic vesicle fusion, whereas the surrounding periactive zones are essential for synaptic vesicle endocytosis and the control of synaptic terminal growth (Sone et al., 2000; Zhai and Bellen, 2004). Definition of distinct synaptic subdomains is not restricted to the plasma membrane but is also clearly visible within the presynaptic terminal cytoplasm. Notably, synaptic vesicles are clustered at the cell cortex, in the vicinity of active zones. In addition, they seem organized into functional subpools displaying distinct release and recycling properties (Rizzoli and Betz, 2005). Such an organization requires the precise trafficking and targeting of vesicles to their appropriate location and the specific recruitment and release of subsets of vesicles, depending on the stimulation conditions. One of the main challenges synapses have to face is maintaining such a highly organized structure while constantly adapting their morphology and strength in response to developmental programs and/or external stimuli. Indeed, synaptic terminals can adjust their size; the number, size, and composition of their pre- and postsynaptic membrane specializations; and the availability and release competence of cytoplasmic synaptic vesicles. These dynamic changes require the maintenance of precise physical and functional connections between pre- and postsynaptic compartments, as well as between cytoplasmic and plasma membrane subdomains.

To date, the mechanisms allowing such a dynamic reorganization are still poorly understood. However, using the Drosophila melanogaster neuromuscular junction (NMJ) as a genetic model, different components of periactive zones, including transmembrane proteins and adaptor molecules, have been implicated in the control of terminal outgrowth (Schuster et al., 1996b; Beumer et al., 1999; Sone et al., 2000; Koh et al., 2004; Marie et al., 2004). Cell adhesion molecules (CAMs) of the Ig superfamily seem particularly important in maintaining
the integrity of synaptic terminals but also in transmitting signals to the cell interior, thereby promoting differentiation of pre- and postsynaptic specializations and regulating synaptic structure and function (Schuster et al., 1996a; Stewart et al., 1996; Sone et al., 2000; Polo-Parada et al., 2001; Rougon and Hobert, 2003; Yamagata et al., 2003). Moreover, the actin-rich presynaptic cytoskeleton is important for rearranging synaptic domains and for controlling synaptic vesicle distribution and release ability (Dillon and Goda, 2005). How the linkage between cortical cytoskeleton, cytoplasmic vesicle pools, and specialized membrane domains is mediated and, more generally, how plasma membrane and cytoplasmic membranes are spatially and functionally connected largely remain to be elucidated.

Here, we identify the transmembrane Ig CAM Basigin (Bsg) as a new component of periactive zones at D. melanogaster NMJ synapses. Bsg is the only D. melanogaster member of the Basigin/Embigin/Neuroplastin family of glycoproteins, of which mammalian Bsg has been shown to have multiple functions, including in tumor progression (Nabeshima et al., 2006). It seems to regulate cell architecture and cell–cell recognition (Fadool and Linser, 1993; Curtin et al., 2005), act in signaling (Guo et al., 1997; Tang et al., 2006), and act as a chaperone for transmembrane proteins (Kirk et al., 2000; Zhou et al., 2005). By analogy to other mammalian cell surface glycoproteins, and in particular to the CD44 transmembrane protein family (Ponta et al., 2003), Bsg may be essential for establishment of transmembrane complexes and for organization of cell structure and signal transduction cascades. Interestingly, mammalian Bsg and Neuroplastin have been suggested to play a role in memory functions and long-term potentiation, respectively, although their precise function has not been determined (Naruhashi et al., 1997; Smalla et al., 2000).

Our in vivo study shows that D. melanogaster Bsg is required in both pre- and postsynaptic compartments to control formation and growth of synaptic varicosities (or boutons) at larval NMJs. We also show that Bsg is a bona fide Ig CAM because (1) it can promote cell–cell adhesion and (2) its transmembrane and/or juxtamembrane cytoplasmic domains are critical for its function in vivo. Furthermore, down-regulation of bsg affects the size of postsynaptic receptor fields, as well as the distribution of synaptic vesicles within neuronal terminals. These defects are associated with alterations of the actin/Spectrin network, suggesting that Bsg accumulation at the plasma membrane regulates synaptic compartmentalization and architecture. Strikingly, we found that Bsg function is also essential within the presynaptic compartment for the restriction of neurotransmitter release. Based on our in vivo data, we propose that Bsg may be part of a transsynaptic complex surrounding active zones and involved in the coordinated development of pre- and postsynaptic membranes, as well as in the functional coupling of plasma membrane and cortical subdomains.

Results

Basigin accumulates at D. melanogaster NMJs

To identify new proteins controlling synapse development, we searched for proteins specifically accumulating at developing NMJs of D. melanogaster larvae. We performed a protein-trap screen in which GFP fusion proteins expressed from their endogenous promoters are randomly generated (Morin et al., 2001) and screened the expression pattern of ~350 GFP+ lines (see Materials and methods). Thereby, we identified 10 lines exhibiting GFP expression at the larval NMJ and focused on three independent lines showing strong GFP accumulation at larval NMJs, but only low GFP levels along the motoneuron axons, and at the surface of muscle fibers (Fig. 1, A and A′). In these lines, a strong GFP signal is also observed in different neuropil structures of the larval brain (Fig. 1 B).

Using inverse PCR, we found that in each of these three lines the protein-trap cassette was inserted in the gene CG31605, encoding the D. melanogaster homologue of the mammalian protein CD147/EMMPRIN/Basigin, Basigin (Bsg; Curtin et al., 2005). According to predictions, the artificial GFP exon should be incorporated upon splicing into mature transcripts whose transcription starts upstream of the insertion, resulting in the in-frame incorporation of GFP. We confirmed this by RT-PCR

Figure 1. GFP expression pattern in bsg protein-trap lines. (A and A′) Third instar larvae heterozygous for the protein-trap insertion, stained with anti-GFP antibodies (A and A′, green), anti-HRP antibodies (A′, red), and phalloidin (A′, blue). (B) GFP-Bsg accumulation in heterozygous larval brain. (C) Western blot of total extracts from w larvae (left) or larvae heterozygous [protein-trap/+; middle] or homozygous [protein-trap; right] for a protein-trap insertion, probed with anti-Bsg and anti-GFP antibodies. (D) Alignment of D. melanogaster and human Bsg proteins. The green triangle indicates the location of GFP insertion.
Basigin accumulates at periaactive zones

To check if the distribution of tagged Bsg reflects that of the endogenous protein, we stained wild-type larvae with anti-Bsg antibodies. Endogenous Bsg shows a localization pattern identical to that of the GFP fusion (Fig. 2 A), and both precisely colocalize with Discs large (Dlg), a transmembrane protein present both pre- and postsynaptically, but mainly accumulating in stacks of postsynaptic membranes named subsynaptic reticulum (SSR; Fig. 2, A″ and B″; Lahey et al., 1994). Like Dlg, Bsg accumulates to higher levels at type I than at smaller type I boutons (Fig. 2, A and B; and not depicted). To exclusively visualize the presynaptic expression of Bsg, we next expressed a GFP-tagged Bsg fusion specifically in the presynaptic compartment (Fig. 2 C) and observed a robust GFP signal at NMJs. Consistent with an accumulation of Bsg at the presynaptic membrane, the inner aspect of both endogenous Bsg and GFP-Bsg protein-trap fusion labels partially overlap with the presynaptic membrane marker HRP (Fig. 2, D and E).

Further analysis revealed that Bsg is not homogeneously distributed at the membrane but is excluded from active zones labeled with anti-Bruchpilot (BRP) NC82 antibodies (Fig. 2 F; Wagh et al., 2006). Therefore, similar to other transmembrane proteins involved in the structural control of synaptic terminals, such as Dlg or Fasciclin II (Fas II), Bsg localizes to periaactive zones (Sone et al., 2000).

Identification of basigin mutants

According to data from the *D. melanogaster* genome project, bsg encodes nine distinct putative transcripts, of which eight encode the same protein (Fig. 3 A). One of the predicted transcripts, RG, encodes a slightly different protein. However, this transcript is barely detected after quantitative RT-PCR on larval fillet extracts (unpublished data).

To address the in vivo requirement for Bsg at the larval NMJ, we searched for *P* element insertions near the transcription starts of the longer transcripts and found five belonging to the same lethal complementation group. Three of these ([l2]k13638, [l2]k14308, and NP3198), when placed in trans over a deficiency covering the locus (*Df* [2L] Exel7034, hereafter referred to as *Df*), cause an embryonic/early larval lethality that can be rescued by ubiquitous expression of a *bsg* transgene (Fig. 3 B), and represent strong hypomorphic alleles (Curtin et al., 2005; unpublished data). Two other insertions, NP6293 and l(2)SH1217, behave genetically as weaker hypomorphic alleles, as, respectively, 30 and 50% of the corresponding hemizygotes
reach third larval instar (Fig. 3 B). This semilethality is reverted after precise excision of NP6293 (Fig. 3 B). Consistent with these data, Western blot analysis shows that Bsg expression levels are greatly reduced in Df/NP6293 and Df/l(2)SH1217 mutant larvae but restored to normal levels after precise excision of NP6293 (Fig. 3 C). The amount of Bsg specifically accumulating at the NMJ is also significantly reduced in Df/NP6293 larval fillets, compared with wild type (Fig. 3 D). Together, these results show that NP6293 and l(2)SH1217 are bsg mutant alleles suitable for analysis of larval NMJ development and maturation. We therefore renamed them bsg6293 and bsg1217 and used them for subsequent studies.

Basigin is required in both pre- and postsynaptic compartments to restrict bouton size and allow efficient NMJ expansion

To determine whether bsg mutants exhibit defects in their motoneuron connection pattern and/or NMJ morphology, we examined synaptic boutons and motoneuron membranes of both Df/bsg6293 and Df/bsg1217 third instar larvae. Axonal targeting is not altered to a visible degree in these animals. However, the growth of synaptic boutons is strongly altered, as revealed by the considerable increase in their size (Fig. 4, C–G; and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200701111/DC1). In particular, the proportion of very large boutons (>12 μm²) is greatly increased in bsg mutants compared with controls (Fig. 4 G and Table S1; P < 0.001). The observed increase in bouton size is associated with a concomitant reduction of both NMJ branching and bouton number (Fig. 4, C–F and H; and Table S1), keeping the overall NMJ size close to normal (muscle 4 NMJ area: 165.33 ± 9 μm² [n = 18] and 163.97 ± 15 μm² [n = 12] for Df/bsg6293 and w larvae, respectively; P > 0.05). Moreover, defects in bouton size and number are already observed in second instar animals (Fig. S1) and revert after precise excision of NP6293 (Fig. 4 H and Table S1).

To explicitly determine whether these growth defects could be rescued and whether they reflected pre- and/or postsynaptic function of bsg at the NMJ, we expressed a wild-type copy of bsg in specific compartments of Df/bsg6293 larvae. Expression of wild-type Bsg solely in muscles (using mhc-Gal4 or 24B-Gal4) or in neurons (using elav-Gal4), partially, but significantly, rescued both the increase in bouton size and the reduction of bouton number observed in mutant larvae (Fig. 4, I, J, L, and M; and not depicted). Near-complete rescue of bouton size and junction growth was obtained only when expressing wild-type Bsg both pre- and postsynaptically (Fig. 4, K, L, and M; and Table S1).

Collectively, we conclude that Bsg is needed for efficient outgrowth of larval NMJs and that its function is required in both pre- and postsynaptic compartments to define boutons of proper size. Such a dual requirement is documented for the Ig CAM Fas II and is thought to reflect the establishment of transsynaptic homophilic interactions (Schuster et al., 1996a). We thus tested whether Bsg might also promote cell–cell adhesion. As shown in Fig. 4 N, S2 cells transfected with a GFP-Bsg construct strongly adhere to each other, whereas S2 cells transfected with a control GFP construct do not. Thus, Bsg promotes cell aggregation, consistent with the idea that Bsg could regulate the addition and growth of synaptic boutons through modulation of cell adhesion.
A conserved juxtamembrane basic motif is crucial for Basigin function in vivo

Depending on the cell type and/or the protein partners, different domains of mammalian Bsg are required for its activity (Guo et al., 1997; Kirk et al., 2000; Sun and Hemler, 2001). Thus, to determine which domains of D. melanogaster Bsg are required for its function at the larval NMJ, we generated GFP-tagged truncated variants (Fig. 5 A) and assayed their capacity to rescue Df/bsg6293 morphological defects.

Bsg lacking the most C-terminal part of the intracellular domain (∆intra) rescues defects in bouton size and number similarly to the full-length tagged form (∆fl  ) when expressed presynaptically (Fig. 5, D and E). In contrast, forms composed of the two Ig domains only (Extra) or of the two Ig domains of Bsg fused to the transmembrane and intracellular domains of rat CD2 (Bsg-CD2) do not significantly rescue the decrease in bouton number observed in bsg mutants and only poorly rescue bouton growth defects (Fig. 5, D and E). Thus, Bsg transmembrane and/or juxtamembrane cytoplasmic domains appear crucial for regulation of NMJ bouton growth and budding by Bsg.

The cytoplasmic juxtamembrane region of Bsg contains a conserved cluster of positively charged residues (KRR; Fig. 5 B). We found that when KRR is substituted with NGG, the mutated protein only poorly rescues the reduced bouton number and only to a low extent the increased bouton size of bsg mutants and only poorly rescue bouton growth defects (Fig. 5, D and E). Moreover, ubiquitous expression of the KRR→NGG mutated protein does not significantly rescue the early lethality of the strong mutant combination Df/l(2)k13638, whereas full-length Bsg does (Fig. 5 C), further indicating a crucial and previously unknown role of this motif for Bsg function.
Figure 5. Differential rescue capacity of various mutated Bsg proteins. (A) Scheme of Bsg variants. Δintra corresponds to a form lacking the last 14 amino acids but including the juxta-membrane KRR stretch, Extra to a form lacking both the transmembrane and the cytoplasmic domains, KRR→NGG to a full-length protein where the KRR residues have been substituted to NGG, and Bsg-CD2 to a chimeric protein composed of the two Ig domains of Bsg fused to the transmembrane and cytoplasmic domains of rat CD2. (B) Alignment of Bsg transmembrane and intracellular domains. H.s., Homo sapiens; M.m., Mus musculus; D.r., Danio rerio; Dr. m., D. melanogaster; A.g., Anopheles gambiae. The black box indicates the amino acids deleted in the Δintra construct. (C) Percentages of non-CyO-GFP third instar larvae recovered among the non-TM6 progeny of a cross between Df/CyO-GFP; UAS-GFP; Bsg+/TM6 females and (1;2)13;638/CyO-GFP; tubGal4/TM6 males. 33% of non-CyO-GFP animals are expected in case of complete rescue (left bar). Numbers correspond to the total numbers of non-TM6 larvae scored in the entire progeny of each cross. Statistical comparison to GFP-Bsg Δintra #28 animals: **, P < 0.001 (χ² test). (D) Quantification of muscle 6/7 type I bouton numbers in Df/bsg6293 larvae and different rescue contexts (driver used for rescue: elav-Gal4). Statistical comparisons to Df/bsg6293 animals: **, P < 0.001 (t test). The number of junctions scored for each genotype is represented in white. Error bars indicate SEM. #7 and #28 represent two independent insertions of the GFP-tagged full-length transgene. (E) Percentage of synaptic boutons >12 μm² in Df/bsg6293 larvae and different rescue contexts. Statistical comparison to w animals: **, P < 0.001 (χ² test).

The specification of active zones and periactive zones is normal in basigin mutants

Given that bsg mutants exhibit defective NMJ morphology, we next tested whether the assembly and/or maintenance of pre- and postsynaptic specializations might also be altered. As shown in Fig. S2 (A and B; available at http://www.jcb.org/cgi/content/full/jcb.200701111/DC1), the overall distribution and complementary accumulation of markers specific to perisynaptic zones and PSDs seems to be normal at bsg junctions. Moreover, no alteration of SSR integrity could be detected at the light microscopy level (Fig. S2, C and D) or at the ultrastructural level (Fig. 6 E).

Next, we assayed the distribution of receptor fields and active zones, using antibodies recognizing the glutamate receptor subunit GluRIID (Qin et al., 2005) in combination with anti-BRP NC82 antibodies (Wagh et al., 2006). As illustrated in Fig. 6 (A and B), the distribution of these two markers is normal at bsg junctions: BRP and GluRIID remain concentrated in individual puncta of normal intensity and distribution (density of BRP⁺ puncta: 1.35 ± 0.3/μm² and 1.19 ± 0.2/μm² for w and Df/bsg6293 third instar larvae, respectively; P > 0.05). Moreover, as described for wild-type animals, BRP⁺ release sites are reproducibly found in direct apposition to postsynaptic glutamate receptor clusters in bsg larvae (Fig. 6, A and B, insets; and not depicted). Consistent with these observations, transmission EM showed that active zones are found at normal frequency and that their characteristic electron-dense specializations (T-bars) are of normal morphology (Fig. 6, D–I). Quantification, however, indicated a slight increase in the electron-dense PSD diameter (Fig. 6 I), which correlates with a slight, but significant, increase in the mean size of GluRIID clusters observed using light microscopy (w = 0.76 ± 0.01 μm, n = 525; Df/bsg6293 = 0.84 ± 0.01 μm, n = 501; P < 0.001; Fig. 6 C). Thus, these results suggest that, although Bsg is involved in definition of receptor field size, its function is not essential for specifying active and periactive zone domains.

Presynaptic actin cytoskeleton organization is altered in basigin boutons

Given that D. melanogaster Bsg has been suggested to regulate cell architecture, possibly by modulating the cell cytoskeleton (Curtin et al., 2005), we checked the integrity of the actin-based cytoskeleton at bsg NMJs. α-Spectrin (α-Spec) closely associates with the NMJ juxtamembrane actin-rich cytoskeleton (Ruiz-Canada et al., 2004). Although it is mainly enriched in the postsynaptic peribouton area, α-Spec is also found at the inner presynaptic bouton cortex (Fig. 7, A and C1; Pielage et al., 2005). In bsg larvae, even though no major alterations of the postsynaptic Spectrin cytoskeleton are observed, α-Spec aggregates are detected within the bouton lumen (Fig. 7, B, D1, and E1) in ~38% of NMJ branches (Fig. 7 L). These aggregates are ~0.5 μm large and contain other α-Spec–associated proteins, such as β- and βH-Spectrin (not depicted), as well as the actin-associated protein Wasp (Fig. 7, G and G′). In contrast, no enrichment of microtubule-associated proteins was observed in these aggregates (Fig. 7, I′ and I″). To more directly and specifically visualize the presynaptic F-actin network, we expressed the F-actin–binding domain of Moesin fused to GFP (GFP-GMA) exclusively in neurons (Dutta et al., 2002). As shown in Fig. 7 J, GFP-GMA accumulates at the cortex of wild-type synaptic boutons. In bsg mutants, although a cortical actin network is still clearly detected at the periphery of boutons, clusters of F-actin filaments are also
frequently present within them (Fig. 7, K and L). Altogether, these observations indicate that the organization of the presynaptic F-actin network is altered at bsg NMJs.

Diffuse distribution of synaptic vesicles at basigin NMJs

In the course of our ultrastructural analysis, we observed that abnormally large vesicles (diameter of up to $\sim300$ nm) are present in $Df/bsg^{6293}$ boutons (Fig. 6, E and H) but are only rarely observed after presynaptic reexpression of Bsg in this background (not depicted). The exact nature of these vesicles remains unclear, as we have not observed any concomitant alteration in the distribution and/or size of the FYVE-GFP$^+$ endosomal compartment (Wucherpfennig et al., 2003) at the light microscopy level (unpublished data).

To determine whether these defects could be associated with an alteration of the synaptic vesicle compartment, we analyzed synaptic vesicle distribution using specific markers. In wild-type boutons, synaptic vesicles are clearly enriched at the cortex but are largely excluded from their central core (Fig. 8, A and A$'$). In contrast, in bsg larvae, preferential association of vesicles with the bouton cortex is lost in $\sim60\%$ of NMJ branches (Fig. 8 D), and CSP$^+$ (cysteine string protein) vesicles fill parts of (Fig. 8, B–B$''$) or even the entire lumen (Fig. 8, C–C$''$) of the bouton. CSP staining is also abnormally strong in axonal tracts connecting boutons and appears more granular than in control animals (Fig. 8, B and C). An essentially identical mislocalization was observed using two other independent markers of synaptic vesicles, Synaptotagmin and Synapsin (Fig. 8, E–H). These defects do not indirectly result from the increase in bouton size observed in bsg mutants, as synaptic vesicle localization appears normal in fas$^{50E}$ hemizygous larvae, which also form abnormally large boutons (Schuster et al., 1996a; Stewart et al., 1996; unpublished data). Together with the fact that such a diffuse distribution can be observed upon tracking of freshly endocytosed synaptic vesicles (FM 1–43 loading assay; Fig. S3, C and D, available at http://www.jcb.org/cgi/content/full/jcb.200701111/DC1), our data suggest that Bsg specifically regulates the spatial distribution of synaptic vesicles and, in particular, their proper anchoring to the cortex of synaptic boutons.

Presynaptic down-regulation of Basigin causes excessive, atypically delayed vesicle release

To address whether the observed changes in the distribution of synaptic vesicles might be linked to functional changes in transmitter release, we recorded postsynaptic currents at larval NMJs. As shown in Fig. 9 A, the amplitude of the postsynaptic response to the fusion of single vesicles (minis) is increased above wild-type levels in bsg mutants (Fig. 9 A, bottom right; Fig. S3 B; and Table S2, available at http://www.jcb.org/cgi/content/full/jcb.200701111/DC1). This effect is most likely related to the observed enlargement of the postsynaptic glutamate receptor clusters (Fig. 6 C), given that no increase in the size of
which largely correlates with the observed enlargement of minis
tional currents (eEJCs) is also increased at
often occur clustered in “exocytotic bursts” (Fig. 9 A, asterisk).
Notably, the frequency of spontaneous release events is strongly
elevated in
controls (Df/bsg
d, 34.4 ± 6.6 nm; w, 34.8 ± 7.2 nm; P > 0.5).
Notably, the frequency of spontaneous release events is strongly
highly dynamic during lar-
ed by bsg mutant minis is only moderately increased (1.5-fold
increase; Table S2), a near eightfold elevation of the charge transferred
to the postsynapse after exocytosis occurs upon initial nerve stimulation (Fig. 9 C and Table S2). Notably, this value decreases progressively after further low-frequency stimulation, which may result from the exhaustion of the abnormally recurited pool of vesicles responsible for the atypically delayed release component. Averaging the charge transferred over 15 consecutive sweeps nonetheless reveals a near fivefold increase in bsg mutants (Table S2); therefore, a more than threefold elevation of the number of vesicles released per action potential (quantal content) is estimated to occur.

These defects reflect a requirement for Bsg within the pre-
synaptic terminal, as sole presynaptic expression of wild-type
Bsg in the mutant background rescues both the asynchronous evoked release (Fig. 9 B) and the high frequency of spontaneous release (Fig. 9 A, bottom left), whereas its sole postsynaptic re-expression does not (Fig. 9, A–C). Interestingly, the presynaptic reexpression of Bsg even decreases the amplitude of eEJCs and the frequency of minis below control levels, indicating a dose-dependent role of presynaptic Bsg in restricting vesicle release.

Discussion
In this study, we have identified the small transmembrane Ig
CAM Bsg as a new component of perisynaptic zones of D. melanogaster NMJs. We have shown that Bsg function is required in pre- and postsynaptic compartments for the formation and growth of synaptic boutons and that Bsg controls different aspects of synapse structure, including distribution of synaptic vesicles and organization of the presynaptic terminal cortical actin network. Bsg behaves as a canonical Ig CAM, as it promotes cell–cell adhesion and has a conserved motif in its cytoplasmic tail essential for its function in vivo. We propose that
Bsg is part of a transsynaptic complex regulating synaptic growth and structural organization. Moreover, and very originally for an Ig CAM, we found that Bsg is essential for inhibiting transmitter release and that this function is restricted to the presynaptic compartment.

Basigin controls synaptic terminal growth and synaptic organization
In D. melanogaster, the final pattern of larval motoneuron connections and the establishment of synapses are complete by the end of embryogenesis, yet NMJs are highly dynamic during larval development, expanding through sprouting of new branches and addition of new synaptic boutons (Schuster et al., 1996a). Here, we have shown that down-regulation of Bsg levels at the D. melanogaster NMJ strongly affects bouton growth and budding, resulting in a decrease in bouton number. This effect is probably independent of the increased transmitter release observed in bsg larvae because (1) it is already observed in early second instar larvae (2), in contrast to the increased neurotransmission phenotype, it corresponds to a requirement for bsg

synaptic vesicles was found in bsg mutants compared with w
controls (Df/bsg
d, 34.4 ± 6.6 nm; w, 34.8 ± 7.2 nm; P > 0.5).
Notably, the frequency of spontaneous release events is strongly elevated in bsg mutants (Fig. 9 A, bottom left), and these events often occur clustered in “exocytotic bursts” (Fig. 9 A, asterisk).

The mean amplitude of nerve-evoked excitatory junc-
tional currents (eEJCs) is also increased at bsg NMJs (Fig. 9 B), which largely correlates with the observed enlargement of minis

![Figure 7. Distribution of actin cytoskeleton markers is altered in bsg larvae.](image)

(A and B) Wild-type (A) and Df/bsg
d muscle 4 NMJs stained with anti–α-Spec antibodies. Bar, 10 µm. (C–E) Heterozygous control (C) and Df/bsg
d (D and E) boutons stained with anti–α-Spec [C1, D1, and E1], anti-HRP [C2, D2, and E2], and anti-Dlg [C4, D4, and E4] antibodies. Bar, 5 µm. [F and G] w [F] and Df/bsg
d [G] bouton stained with anti-Wasp [F' and G] and anti–α-Spec [F" and G", red] antibodies. (H and I) w [H] and Df/bsg
d [I] boutons stained with anti–α-Spec [H' and I'] and anti-Futsch [H" and I"] antibodies. Bar, 5 µm. (j and K) Synaptic boutons of wild-
type [j] and Df/bsg
d [K] larvae expressing a fusion of GFP with the F-actin binding domain of Moesin [GMA], under the control of elavGα4. GFP-GMA expression is shown in J and K, and is in green in J' and K'. HRP staining is in red in J' and K'. Bar, 5 µm. Images A and B corre-
spond to z projections of serial confocal sections throughout entire boutons (step size: 0.3 µm), and images C–K correspond to single optical slices taken through bouton centers. (l) Graph showing the percentage of NMJ
6/7 branches containing presynaptic Spec" or GMA" aggregates. ***, P < 0.001 (χ² test).
function in both pre- and postsynaptic compartments. This may reflect a role of Bsg in regulating adhesion between pre- and postsynaptic membranes, as described for the Ig CAM Fas II (Schuster et al., 1996a). Bsg function is, however, not restricted to modulation of synaptic membrane adhesiveness, as mutant forms lacking the transmembrane and/or juxtamembrane cytoplasmic domains can promote cell–cell aggregation (Fig. S1 B) but function poorly in vivo. Bsg may thus also signal toward the cell cytoplasm and/or regulate the actin cytoskeleton (see the following section).

In addition, Bsg controls synaptic architecture: it modulates the size of postsynaptic glutamate receptor fields and, more strikingly, is required for the anchoring of synaptic vesicles to the presynaptic terminal cortex. This suggests that Bsg could be a key component coupling organization of the plasma membrane and cytoplasmic vesicular compartments. Consistent with such a role, we have observed defects in “plasma membrane versus internal membrane” sorting of presynaptic transmembrane components (for internal accumulation of HRP epitopes, see Fig. 7, D and E; not depicted). We thus propose that Bsg may be part of a transsynaptic complex surrounding active zones and involved in the coordinated development of pre- and postsynaptic membranes, as well as in the functional coupling of plasma membrane and cytoplasmic vesicles. Notably, Bsg recently has been identified within synaptic vesicle preparations (Takamori et al., 2006).

Bsg might act directly, or through interaction with transmembrane and cytoplasmic partners. Consistent with this latter hypothesis, we have shown that conserved amino acids found in the cytoplasmic tail of Bsg are crucial for the function of the protein in vivo and that they may thus mediate transduction of a signal toward the cell cytoplasm and/or interaction with the cortical cytoskeleton (see the following section).

Basigin and the actin cytoskeleton

The F-actin/Spectrin cytoskeleton underlying pre- and postsynaptic membranes seems essential for different aspects of synaptic terminal growth and plasticity, including terminal expansion, organization of presynaptic vesicle pools, and postsynaptic receptor clustering (Dillon and Goda, 2005; Pielage et al., 2006; Ruiz-Canada and Budnik, 2006). Here, we have shown that Bsg modulates the organization of the presynaptic actin cytoskeleton, as revealed by the presence of ectopic aggregates of F-actin and actin-associated proteins at cell–cell contacts and that expression of Bsg in cultured cells results in reorganization of the F-actin network and consequent formation of lamellipodia (Schlosshauer et al., 1995; Curtin et al., 2005).

At the NMJ, Bsg may modulate actin cytoskeleton organization indirectly, by interacting with integrin subunits at the plasma membrane. Indeed, mammalian Bsg has been found in...
of the FERM domain protein family have been shown to organize a cortical actin network. Interestingly, different membrane-associated proteins and thereby directly participates in or juxtamembrane cytoplasmic motif, recruits Spectrin or other data). Another attractive hypothesis is that Bsg, through its similar to that of larval NMJ development, we identified several encodes the main unpublished data). Furthermore, although we have not detected a genetic interaction between bsg and myspheroid (mys, which encodes the main D. melanogaster β-integrin subunit) during larval NMJ development, we identified several mys missense mutations displaying a junction undergrowth phenotype very similar to that of bsg mutants (Jannuzzi et al., 2004; unpublished data). Another attractive hypothesis is that Bsg, through its juxtamembrane cytoplasmic motif, recruits Spectrin or other actin-associated proteins and thereby directly participates in organizing a cortical actin network. Interestingly, different members of the FERM domain protein family have been shown to link cell surface glycoproteins and the actin cytoskeleton by directly binding to both the intracellular region of transmembrane proteins and to actin or Spectrin (Tsukita and Yonemura, 1999; Bretscher et al., 2002). In particular, Moesin directly interacts with the intracytoplasmic domains of mammalian CD43, CD44, and intercellular adhesion molecule 2, through a positively charged amino acid cluster found in the juxtamembrane region of these proteins (Legg and Isacke, 1998; Yonemura et al., 1998). The striking conservation and functional importance of the KRR juxtamembrane motif of Bsg suggests that such cytoplasmic proteins may physically link cell-surface Bsg to the underlying F-actin network and mediate organization of cortical domains at the NMJ.

**Basigin controls synaptic vesicle release**

Down-regulation of bsg at D. melanogaster NMJ terminals causes a dramatic increase in transmitter release, which, to our knowledge, is unique among Ig CAM mutants. This phenotype corresponds to a specific presynaptic function of Bsg and may be explained by (1) an alteration of the excitability of the synaptic terminal or (2) an altered definition of the different functional synaptic vesicle pools.

Mammalian Bsg has been shown to promote translocation of transporter proteins to the plasma membrane, as well as regulate the activity of multiprotein transmembrane complexes (Kirk et al., 2000; Zhou et al., 2005). At the D. melanogaster NMJ, Bsg may thus be required for the proper distribution and/or clustering of ion channels regulating Ca^{2+} dynamics. In this context, it was recently demonstrated that the presynaptic scaffolding protein BRP is required for the clustering of Ca^{2+} channels and for their spatial coupling with synaptic vesicles at the D. melanogaster active zone. This process appears to be required for the rapid evoked component of synaptic vesicle release but not for spontaneous release (Kittel et al., 2006).

Therefore, both the additional spontaneous and delayed evoked component of transmitter release in bsg mutants might correspond to the fusion of vesicles lacking a tight association with Ca^{2+} channels. An elevated contribution of asynchronous release has also been reported to occur naturally at particular synapses of the mammalian central nervous system and is thought to reflect long-lasting Ca^{2+} transients and a loose coupling between Ca^{2+} sources and vesicles (Hefft and Jonas, 2005). It is thus conceivable that down-regulation of Bsg alters Ca^{2+} dynamics, leading to an abnormal recruitment of vesicles distant from Ca^{2+} sources.

Alternatively, the observed transmitter release phenotype may not be associated with an alteration of Ca^{2+} signals, but rather reflects a role of Bsg in organizing synaptic vesicle populations. It has been suggested that synaptic vesicles are organized into functionally distinct pools (readily releasable pool, recycling pool, and reserve pool) with specific recycling and mobilization properties (Rizzoli and Betz, 2004, 2005). Here, we have shown that down-regulation of bsg leads to an abnormal distribution of vesicles in resting terminals (Fig. 8), as well as an aberrant trafficking of vesicles to the center of boutons (where reserve pool vesicles are thought to reside) under conditions where synaptic vesicle recycling is normally restricted.
to the periphery (and to the recycling pool; Fig. S3, C and D). These data suggest that the definition of different synaptic vesicle populations may be altered in bsg mutants, which in turn may explain the observed defects in precise recruitment and release of vesicles. This is of particular interest given that mammalian Bsg has been suggested to physically associate with synaptotagmin (Takamori et al., 2006). Additionally, presynaptic actin filaments have been proposed to provide a physical barrier impeding vesicle dispersion and, in particular, to regulate the availability of the reserve pool (Dillon and Goda, 2005). They have also been suggested to participate in a mechanism restraining fusion of synaptic vesicles in cultured hippocampal neurons (Morales et al., 2000). An attractive possibility is therefore that Bsg controls synaptic vesicle organization and retention through regulating fusion of synaptic vesicles in cultured hippocampal neurons (Morales et al., 2000). An attractive possibility is therefore that Bsg controls synaptic vesicle organization and retention through regulating fusion of synaptic vesicles in cultured hippocampal neurons (Morales et al., 2000).

**Materials and methods**

**Screening procedure**

In brief, + / Y, pBac[3xP3-DsRed; GFPexon]/ + ; pHer[3xP3-ECFP; a-tub-piggyBac1K10] / + ; jumpstarter males, carrying the protein-trap transposon and a source of piggyBac transposase (Horn et al., 2003), but not expressing any detectable GFP, were used to mobilize the GFP cassette and create new insertions. Jumpstarter males were crossed en masse with w virgins, and embryos were collected at 25°C on apple juice plates for either 5 h (for late embryo sorts) or overnight (for L1 sorts). They were then aged to stage 16–17 embryos and L1 larvae, respectively. Dechorionated embryos or L1 larvae were sorted using an embryo sorter (COPAS Select 500; Union Biometrica), following a procedure adapted from Furlong et al. (2001). Sorted larvae were then manually rescreened at later stages using a stereomicroscope (MZFLIII; Leica) equipped with standard GFP filters. About 50 GFP-positive individuals from each insertion were sorted (1 million sorted as young larvae and 0.5 million sorted as late embryos) were selected, which roughly corresponds to a frequency of GFP+ event recovery of 1/2,000–2,500 (estimated after taking into account the percentage of lethality).

Emerging GFP+ individuals were then crossed individually to w flies to establish independent lines. Third instar larvae from each line were dissected, briefly fixed (5 min in 4% PFA), and washed several times in PBT. The exact positions (in the genomic scaffold AE003619) of the three insertions were selected, which roughly corresponds to a frequency of GFP+ event recovery of 1/2,000–2,500 (estimated after taking into account the percentage of lethality).

**Fly stocks and transgenes**
The NP6293, I(2)SH1217, and I(2)k13638 insertions, and the deficiency covering bsg locus [Df(2L)Ejel7034], were obtained from the Kyoto, Szeged, and Bloomington stock centers, respectively. NP6293 and I(2)1217 lie at position 267161 and 267380 of 2L, respectively. The NP6293 chromosome contains a single P element insertion (as verified by inverse PCR), but contains an additional mutation outside of the region covered by Df(2L)Ejel7034, as indicated by the fact that both NP6293 and NP6293/+ homozygous animals never reach larval stages, whereas a good number of NP6293/Df(2L)Ejel7034 larvae survive until larval instar 4 for precise excision of NP6293. NP6293/CyO; A2-3, Sb/+ males were individually crossed to CyO females. A single v- CyO male per vial was then selected among the progeny and crossed to CyO virgin females to establish a stock. Exclusions were then evaluated at the molecular level, by PCR amplification and sequencing of genomic DNA of hemizygous larvae. mhc-Gal4, elav-Gal4, tub-Gal4, UAS-synEFPP, UAS-GMA stocks were obtained from the Bloomington stock center. All crosses were reared at 25°C.

The UAS-bsg transgene was generated by insertion of a 2.7-kb EcoRI-Xhol fragment obtained by double digestion of the DGC clone LD19437 into an EcoRI-Xhol digested pCasper3 vector. The following procedure was used to construct GFP-tagged full-length and mutated Bsg variants in which GFP insertion mimics that generated upon insertion of the protein-trap transposon. Total RNA was isolated from bsg protein-trap larval body wall preparations by standard Trizol extraction and used for reverse transcription with Superscript I RT (Invitrogen) and oligo dT primers. Reverse transcription products were then subjected to further PCR and cloned into a pENTR/DTOPO vector (Gateway technology; Invitrogen). One of the full-length GFP-tagged Bsg clones obtained was used as a template to construct the other Bsg variants. Insertion of the different Bsg-tagged proteins into pUAS vector was achieved through a LR recombination reaction (Gateway technology; Invitrogen) using pYW piggyBac transposase (Drosophila Gateway Vector Collection; http://www.ciwemb.edu/ labs/murphy/Gateway%20vectors.html). Lines expressing similar levels of GFP-tagged mutant proteins were used for the rescue experiments described in Fig. 5 (not depicted).

**Immunocytochemistry**

Dissections and immunostainings were performed as described by Qin et al. (2005), using the following antibodies: rabbit anti-GFP (1:1,000; Invitrogen), mouse anti-Dig (1:1,000; Developmental Study Hybridoma Bank [DSHB]), mouse anti-CSP (1:40; a gift from E. Buchner), mouse anti-Synapsin (1:20; a gift from E. Buchner), rabbit anti–α-Spectrin (1:100; a gift from R. Dubreuil, University of Illinois at Chicago, Chicago, IL), mouse anti–α-Spectrin (1:50; clone 3A9; DSHB), mouse anti–Futsch (1:50; clone 22C10; DSHB), guinea pig anti-Wasp (1:400; Bogdan et al., 2005), FITC- and Cy3-conjugated anti-HP (Cappel and Jackson ImmuneResearch Laboratories, respectively). Alexa Fluor 488 (Invitrogen), Cy3 or Cy5 (Jackson ImmuneResearch Laboratories) conjugated secondary antibodies were used at a 1:500 dilution. Unless specified, confocal pictures are those of muscles 6/7 NMJs (segments A2–A4) and were taken with a microscope (DMRE; Leica) equipped with a scan head (TCS SP2 AOBs; Leica) and an oil-immersion 63× 1.4 NA objective.

For bouton number quantifications, type I, boutons of muscles 6/7 on segment A2 were counted using anti-CSP and anti-HP double stainings. Muscles were photographed at 20× magnification and then traced and measured using ImageJ. The normalized bouton number was calculated by dividing the bouton number by the muscle surface area (data are expressed as a percentage of the w controls in each experiment). Although Df/bsg1217 larvae exhibited muscles of a rather normal size, Df/ bsg1217 larvae have smaller muscles. Larvae with extremely thin muscles were excluded from the quantification of normalized bouton numbers. For bouton area quantification, confocal pictures of muscle 4 NMJs (segment A2) stained with anti-HP antibodies were taken with a 63× magnification. Sections along the z axis were projected, and individual type I boutons were then manually traced and measured using ImageJ.

For measurement of BRP puncta density, sections of NMJ 6/7 branch were photographed along the z axis, and the density was calculated as the ratio between the total number of puncta and the projected surface of the branch (using Image J).

**Generation of antibody and Western blotting**

Rat polyclonal anti-Bsg antibodies were raised against an N-terminal synthetic peptide (QSLDKLYPVDY) obtained from Thermo Scientific. Crude serum was used at a 1:200 dilution for immunostainings and at a 1:1,500 dilution for Western blot analysis. Insertion of GFP within the epitope used to generate anti-Bsg antibodies probably explains the lower signal observed for tagged versus untagged Bsg proteins (Fig. 1 C). The highest molecular weight isoform detected in total larval extracts is not detected in body wall extracts (Fig. 3 C) and might correspond to a glycosylated form, as described for mammalian Bsg. The following antibodies were used for Western blot analysis: rabbit anti-GFP (1:500 [Santa Cruz Biotechnology, Inc.] or 1:2,500 [Torey Pines]) and HRP-conjugated anti–mouse, –rat, or –rabbit (1:2,000; GE Healthcare) antibodies.

**Cell aggregation assay**

3 ml of a 10⁶ cells/ml culture of metGal4–expressing S2 cells were plated and transfected the next day with 3.5 μg of either UAS-Gal3-GFP or UAS-GFP-Bsg. After overnight recovery, cells were induced for 30 h with 0.7 mM CuSO₄, shortly centrifuged, and resuspended at 2 × 10⁶ cells/ml in D. melanogaster SFM medium (Invitrogen) containing 18 mM L-Glutamine, 50 μM/ml penicillin, and 50 μg/ml streptomycin and puromycin. 1 ml of each suspension was placed in a 2-ml tube and shaken for 1 h at room temperature.
Kidokoro (1998). For quantification of FM1-43 distribution upon loading FM1-43 labeling assays by single minis (see Tables S2 for raw values).

**Electrophysiology**

Two-electrode voltage clamp recordings were obtained at 22°C from VM6 in segments A2 and A3, of late third instar larvae, essentially as previously described (Kittel et al., 2006). The composition of the extracellular haemolymph-like saline (HL-3) was as follows: 70 mM NaCl, 5 mM KCl, 20 mM MgCl₂, 10 mM NaHCO₃, 5 mM tretalose, 115 mM sucrose, 5 mM Heps, and 1 mM CaCl₂, pH adjusted to 7.2. Minis (voltage clamp at −80 mV) and eEJs (voltage clamp at −60 mV) were recorded with intracellular microelectrodes filled with 3 M KCl to give final resistances of 8–21 MΩ. The data are reported as mean ± SEM. n indicates the number of cells examined, and where included, p-values denote the significance according to the Mann-Whitney Rank Sum test. In the figures, the level of significance is marked with asterisks: *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. The quantal content was roughly estimated as the ratio between the mean charge transferred per action potential and the mean charge carried by single minis [see Tables S2 for raw values].

**FM1-43 labeling assays**

The procedure used for FM1-43 labeling was adapted from Kuromi and Kidokoro (1998). For quantification of FM1-43 distribution upon loading (Fig. S3 C), fillets of third instar larvae were dissected in Ca²⁺-free saline (30 mM NaCl, 36 mM sucrose, 5 mM KCl, 4 mM MgCl₂, and 5 mM Heps) and incubated for 6 min with high-K⁺ saline (45 mM NaCl, 36 mM sucrose, 90 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 5 mM Heps, and 0.5 mM EGTA containing 7 μl/ml (−10⁻⁴ mol/l) of 1 μg/μl FM1-43X solution (Inviron). Fillets were then briefly washed two times in Ca²⁺-free saline, fixed for 5 min in 4% formaldehyde, and washed quickly before mounting on a slide. FM1-43 fluorescence was imaged right after using a confocal microscope [SP2; Leica]. For analysis of FM1-43 loading and unloading, fillets of third instar larvae were dissected in Ca²⁺-free saline, transferred into a microscope chamber, and incubated for 5 min with high-K⁺ saline containing 6 μl/ml (−10⁻⁴ mol/l) of FM1-43 dye (Inviron). Preparations were then briefly rinsed three times with Ca²⁺-free saline, further washed (once for 5 min and once for 10 min) with Ca²⁺-free saline, and imaged using confocal microscopy and a 40× immersion objective (“after loading” pictures). We could not quantify the amount of FM1-43 dye loaded after the first part of the procedure, as bsq muscles contract longer than wild-type muscles upon transfer to Ca²⁺-free saline, and thus start unloading part of the dye. We also observed that contractions provoked by the high-K⁺ solution are weaker in muscle 4 than in muscles 6/7, and therefore imaged exclusively muscle 4 NMJs. For unloading, two consecutive rounds of high-K⁺ saline stimulation (1 min each), separated by washes with Ca²⁺-free saline (three times for 5 min), were then applied in the absence of any dye. Junctions were imaged after further washing (“after unloading” pictures), using the same confocal settings as for the “after loading” pictures.

**Online supplemental material**

Fig. S1 shows second instar larvae NMJ morphology and cell–cell aggregation assays performed with Bsg variants. Fig. S2 shows the distribution of pre- and postsynaptic markers in bsg larvae. Fig. S3 shows the localization of synaptic vesicle–associated proteins (varial confocal sections), the distribution of mini amplitudes, and FM1-43 labeling assays. Table S1 shows raw bouton number and size in mutant and rescued controls. Table S2 shows recorded electrophysiological properties of mutant and rescued larvae. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200701111/D1C1.

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Temperature. Aliquots were spotted on slides and examined using Nomarski optics and epifluorescence microscopy.

Transmission EM

Transmission micrographs were obtained from dissected preparations of third instar larvae (NM) 6/7 and 12/13, segment A2/A3, as described by Wagh et al. (2006). All measurements were made using Image. The data are reported as mean ± SEM, and where included, p-values denote the significance according to the Mann-Whitney Rank Sum test. Measurement of synaptic vesicle diameter was performed on populations of vesicles found within a radius of 300 nm around active zone Tbars.

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