The *Drosophila* blood brain barrier is maintained by GPCR-dependent dynamic actin structures

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The blood brain barrier (BBB) is essential for insulation of the nervous system from the surrounding environment. In *Drosophila melanogaster*, the BBB is maintained by septate junctions formed between subperineurial glia (SPG) and requires the Moody/G protein–coupled receptor (GPCR) signaling pathway. In this study, we describe novel specialized actin-rich structures (ARSs) that dynamically form along the lateral borders of the SPG cells. ARS formation and association with nonmuscle myosin is regulated by Moody/GPCR signaling and requires myosin activation. Consistently, an overlap between ARS localization, elevated Ca²⁺ levels, and myosin light chain phosphorylation is detected. Disruption of the ARS by inhibition of the actin regulator Arp2/3 complex leads to abrogation of the BBB. Our results suggest a mechanism by which the *Drosophila* BBB is maintained by Moody/GPCR-dependent formation of ARSs, which is supported by myosin activation. The localization of the ARSs close to the septate junctions enables efficient sealing of membrane gaps formed during nerve cord growth.

**Introduction**

The blood brain barrier (BBB) functions to insulate the central nervous system from its changing molecular environment. In *Drosophila melanogaster*, the BBB is maintained by septate junctions formed between subperineurial glia (SPG) and requires the Moody/G protein-coupled receptor (GPCR) signaling pathway. In this study, we describe novel specialized actin-rich structures (ARSs) that dynamically form along the lateral borders of the SPG cells. ARS formation and association with nonmuscle myosin is regulated by Moody/GPCR signaling and requires myosin activation. Consistently, an overlap between ARS localization, elevated Ca²⁺ levels, and myosin light chain phosphorylation is detected. Disruption of the ARS by inhibition of the actin regulator Arp2/3 complex leads to abrogation of the BBB. Our results suggest a mechanism by which the *Drosophila* BBB is maintained by Moody/GPCR-dependent formation of ARSs, which is supported by myosin activation. The localization of the ARSs close to the septate junctions enables efficient sealing of membrane gaps formed during nerve cord growth.

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Abbreviations used in this paper: ANOVA, analysis of variance; ARS, actin-rich structure; BBB, blood brain barrier; GPCR, G protein–coupled receptor; HPF, high-pressure freezing; MLC, myosin light chain; Nrg, Neuroglian; NrxIV, NeurexinIV; P-MLC, phospho-MLC; SPG, subperineurial glia; Sqh, Spaghetti-squash.
cells, and glia cells (Furuse and Tsukita, 2006; Banerjee and Bhat, 2007). The dynamics of septate junction formation have not been elucidated. Specifically, their ability to provide the sealing function during growth and morphogenesis of these distinct tissues is not understood at the structural or the molecular level.

In Drosophila, different types of glia cells exhibit differential functions in providing nurture, insulation, and support for the nervous system (Freeman and Doherty, 2006; Parker and Auld, 2006; Stork et al., 2008). The SPG are large cells defined during embryonic development that form a continuous cell layer located between the more external layer of perineural glia cells and the more internal cortex glia enveloping individual axons or axon bundles within the nerve cord. The SPG layer consists of uniquely large cells that maintain tight adhesion with both the external perineural glia and the more internal cortex glia. Importantly, the SPG cells form tight septate junctions in their lateral intercellular border with neighboring SPG cells to form the BBB (Schwabe et al., 2005; Stork et al., 2008). Although the SPG cells are polarized, they do not exhibit the typical epithelial belt of apical cadherin-mediated adherens junctions and therefore lack the mechanical support that these junctions provide.

To maintain insulation of the brain and nerve cord during development from embryo to adult stages, the septate junctions along the entire circumference of the SPG cells must accommodate the growth and morphological changes of the developing nervous system. SPG cells grow in size to mediate their function in the changing environment without further divisions throughout development. Thus, cross talk between the growing brain and nerve cord and the dynamic formation of septate junctions in the SPG cells would be expected to account for the maintenance of nervous system sealing during development. The precise mechanism regulating septate junction continuity along the changing borders of neighboring SPG cells has yet to be characterized.

The signaling pathway regulated by Moody, a G protein–coupled receptor (GPCR) expressed uniquely by SPG cells, might be related to this function. In moody mutant flies, BBB function is disrupted, leading to behavioral abnormalities (Bainton et al., 2005). Ultrastructural analysis revealed that in moody mutant embryos, septate junctions are nevertheless established, suggesting that all the elements required for their formation exist within the SPG cells. However, the continuity and length of these junctions along the entire circumference of the SPG cells is disrupted, leading to holes in the seals between neighboring SPG cells and consequently to aberrant BBB function (Schwabe et al., 2005). Based on glial-specific rescue experiments, it was proposed that the Moody/GPCR pathway is involved in the regulation of septate junction continuity along the SPG borders and might act autonomously within these cells. moody mutant SPG cells exhibit abnormalities in the actin cytoskeleton (Schwabe et al., 2005). However, the molecular link between Moody signaling, actin skeleton arrangement, and the establishment of elongated septate junctions along the SPG plasma membrane is not clear.

In this study, we describe novel highly dynamic actin-rich structures (ARSs) associated with convoluted membrane extensions detected in close proximity to the septate junctions.

Further analysis demonstrated that the formation of the ARSs depends on the Moody signaling pathway as well as on the activity of the actin regulatory Arp2/3 complex and myosin activation. Importantly, ARS disruption leads to discontinuities in the septate junctions along the borders of the SPG cells, abrogating BBB function, similarly to the phenotype observed in moody mutants. We suggest that the ARSs support specialized membrane convolutions of the SPG plasma membrane that help to stabilize septate junctions at the lateral borders of the SPG cells.

**Results**

**Specialized ARSs are present along the borders of the SPG cells**

To gain insight into the actin cytoskeleton organization of the SPG cells, we expressed moesin–GFP (GMA–GFP) in the SPG cells using a specific Gal4 driver, moody-gal4. To analyze the cytoskeletal organization of the SPG cells, we chose to look at nerve cords of third instar larvae (half the way to adult fly development), as at this stage the SPG cells are well defined and do not divide anymore. The moesin-GFP labeling was thus specific for the SPG cells, whereas phalloidin marked the F-actin throughout the entire nerve cord. GFP-positive rounded structures were detected along the lateral borders of the SPG cells. These structures overlapped the phalloidin-labeled regions, suggesting that they contain F-actin (Fig. 1, A–C); we therefore named them ARSs. The diameter of the GFP-positive ARSs was around 1–2 µm, which is significantly larger than typical F-actin distribution in adherens type junctions.

Expression of Lifeact-GFP, a 17-aa actin-binding peptide fused to GFP in the SPG cells, similarly decorated the ARSs. Previous experiments demonstrated that overexpression of Lifeact-GFP does not interfere with actin dynamics (Riedl et al., 2008). Thus, based on the co-distribution of moesin-GFP, Lifeact-GFP, and phalloidin, we concluded that the intercellular border of the SPG cells is characterized by novel ARSs.

To reveal the distribution of the ARSs relative to the plasma membrane of the SPG cells, we expressed a membrane form of GFP (CD8-GFP) within the SPG cells and colabeled the nerve cord with phalloidin (Fig. 1, G–I). High magnification showed that the ARSs were in close proximity to the CD8-GFP–labeled membranes, suggesting that the ARSs accumulate around specialized rounded plasma membrane structures at the intercellular SPG borders. The CD8-GFP labeled additional structures within the SPG cells, including weblike structures located at the basal surfaces of these cells.

We next performed immuno-EM experiments on nerve cords taken from larvae expressing moesin-GFP driven by moody-gal4, using anti-GFP antibodies to label the ARSs (Fig. 2). This approach enabled visualization of the actin distribution specifically in the SPG cells. The nerve cords were fixed and processed by high-pressure freezing (HPF) followed by a freeze substitution procedure, which preserved the antigenicity of the GFP. Ultrathin cross sections of the nerve cord were obtained and labeled with anti-GFP antibody, followed by secondary antibody conjugated to 10-nm gold particles. A low magnification...
of unlabeled cross section through the nerve cord fixed by chemical fixation is shown in Fig. 2 A, indicating the distribution of perineurial (PN), subperineurial (SPG), and axons (N) as well as the elongated septate junctions formed between the SPG cells. A corresponding section in similar orientation and magnification processed by HPF and labeled with anti-GFP is shown in Fig. 2 B–D. Notice that very close to the site of septate junction (Fig. 2, B and D, arrows) a convoluted rounded membrane extension was labeled with the anti-GFP antibody (see arrows in high magnification in Fig. 2 C). The location, appearance, and size of this structure were similar to that of the ARSs detected by confocal microscopy. Therefore, we suggest that this structure represents an ARS. Notice that the ARS is located next to the septate junction but does not overlap it.

The septate junction components Nrg and Scribble but not NrxIV are associated with the ARSs

Nrg and NrxIV are two membrane proteins essential for proper septate junction formation (Bieber et al., 1989; Baumgartner et al., 1996). To gain insight as to the relationships between the localization of these two proteins and the ARSs, we analyzed

Figure 1. **ARSs are distributed along the SPG intercellular borders.** (A–I) Nerve cords were dissected from third instar larvae, labeled with moesin-GFP (A–C), Lifeact-GFP (D–F), or CD8-GFP (G–I), driven to be expressed in the SPG cells, and then double labeled with phalloidin (Phall). The GFP labeling is shown in A, D, and G, phalloidin in B, E, and H, and their merged images in C, F, and I. The insets in each panel represent high magnification of the region marked by an arrow in the nerve cords. Note that the ARSs are distributed along the borders of the large SPG cells. Overlap between GFP and phalloidin is observed.
spatial overlapping pattern of Nrg and ARSs suggests their functional association. Interestingly, overexpression of Scribble-GFP, a cytoplasmic component of the septate junctions, also exhibited a distribution that overlapped with the ARSs, similarly to Nrg (Fig. 3, M–O). Collectively, these observations suggest that the membranes along the lateral borders of the SPG form unique convoluted looplike structures, which contain Nrg and Scribble but not NrxIV and are often surrounded by the ARSs.

The ARSs are included within the boundaries of a single SPG cell

The tight lateral association between the cell membranes of neighboring SPG cells did not allow us to determine whether the ARSs are formed on the membranes of two neighboring SPG cells or, alternatively, whether a given ARS originates within a single SPG cell. To address the relationships between the ARSs and the SPG cells, individual glial cells were labeled with GFP by glial-specific Flippase-dependent excision of a genomic spacer localized between a tubulin promoter and GFP (see Materials and methods). Using this method, additional types of individual glia cells were also labeled; however, the SPG cells were easily recognized because of their typical large cubical appearance (Fig. 4, arrow). We analyzed the localization of the ARSs, labeled with phalloidin, in individual GFP-labeled SPG cells that were surrounded by non-GFP-labeled SPG.
In the majority of cases, ARSs were detected within the boundaries of a single GFP-labeled SPG cell (Fig. 4, B–E). We assume that in the few cases in which the relative localization of the ARSs was not clearly visible within a single cell, it was because of the relatively weak GFP expression levels at the edges of the SPG cell, which did not permit adequate identification of the cell borders. Thus, the clonal analysis demonstrated that the ARSs are produced within a single SPG cell, suggesting that at the lateral borders of the SPG cells, the plasma membrane is highly convoluted, forming looplike structures that contain Nrg (but not NrxIV) and are surrounded by F-actin–rich structures as demonstrated in Fig. 4 (F–H).

Cross sections of third instar larvae nerve cords expressing moesin-GFP in the SPG cells and labeled with anti-NrxIV antibody (Fig. 4, F and G) were performed. High magnification of a single confocal optical section shows that the ARSs are located at both sides of the NrxIV-labeled septate junction formed between two neighboring SPG cells, supporting the notion that the ARSs are included within the borders of a single SPG cell and localized in close proximity to the septate junctions as shown in Fig. 2. Moreover, cross sections of nerve cords of larvae carrying Nrg-GFP protein trap and labeled with phalloidin showed that in contrast to NrxIV, Nrg is localized along convoluted membrane indentations that wrap the ARSs (Fig. 4, H and I). To confirm that these membrane indentations are separated from the septate junction site (as was also suggested by the immuno-EM analysis shown in Fig. 2), we labeled nerve cord cross sections from larvae carrying the Nrg-GFP with phalloidin and NrxIV. Whereas NrxIV appeared as a straight line localized between two neighboring SPG cells, corresponding to the septate junction, Nrg-GFP and the ARSs were located proximal to that straight line (Fig. 4, J–L). These results combined with the immuno-EM analysis suggest that the ARSs are located within Nrg-positive membrane convolutions next to the septate junctions at the lateral borders of the SPG cells (Fig. 4 M).

We have excluded the possibility that the ARSs associate with early endosomal compartments, as we did not detect overlapping distribution between the ARSs and Rab-5–GFP in the
moesin-GFP driven by the moody-gal4. Third instar larvae were immobilized between two cover slides, and the GFP labeling was analyzed during a 20–30-min period. This analysis revealed that the ARSs are highly dynamic structures; they continuously form and disintegrate (Fig. 5).

Interestingly, we detected elevation of ARS number in early pupal stages (1 h after pupal formation; Fig. S2 A). In contrast, in the adult brain, we did not detect typical ARSs (Fig. S2 B), which is consistent with the idea that these structures are more pronounced during brain morphogenesis.

Live larvae simultaneously labeled for F-actin and Nrg using both Lifeact-Ruby driven by moody-gal4 and Nrg-GFP SPG cells (Fig. S1, D–F). Similarly, the possible association between the ARSs and cellular lipid droplets was excluded as no overlap in the staining of fluorescent nonpolar BODIPY 493/503 (a specific marker for cellular lipid droplets) and phalloidin in the SPG cells was detected (Fig. S1, A–C).

**Live imaging of the ARSs reveals their dynamic appearance in wild-type nerve cords**

To further characterize the dynamics of the ARSs within the SPG cells, we followed nerve cords of live larvae carrying the moesin-GFP driven by the moody-gal4. Third instar larvae were immobilized between two cover slides, and the GFP labeling was analyzed during a 20–30-min period. This analysis revealed that the ARSs are highly dynamic structures; they continuously form and disintegrate (Fig. 5). Interestingly, we detected elevation of ARS number in early pupal stages (1 h after pupal formation; Fig. S2 A). In contrast, in the adult brain, we did not detect typical ARSs (Fig. S2 B), which is consistent with the idea that these structures are more pronounced during brain morphogenesis.

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constructs to compromise Arp2/3 function was demonstrated previously (Massarwa et al., 2009). These experiments demonstrate that ARS formation in the SPG cells depends on activation of the Arp2/3 complex, which is recruited to the intercellular SPG borders.

To directly assess the contribution of the ARSs to BBB function, we performed a permeability assay for larvae in which the ARSs were disrupted by SPG-specific knockdown of the Arp2/3 complex. Live larvae were submerged into a solution containing 3-kD fluorescein-dextran for 30 min. The larvae were then dissected and fixed, and dye penetration to the ventral cord was assessed by measuring the fluorescent intensity in the nerve cords using confocal microscopy. The fluorescence intensity was measured in three distinct areas in each nerve cord within a single optical section taken from the ventral cord. As expected, dye penetration was significantly higher in the moody mutant compared with wild-type larvae, as demonstrated previously (n = 12; P < 0.0066; Fig. 6 G; Bainton et al., 2005; Schwabe et al., 2005). Significantly, dye penetration of larvae carrying SPG-dependent RNAi for the Arp2/3 complex was also significantly higher when compared with controls of either larvae carrying RNAi for Arp2/3 alone (arp2/3RNAi/+; Fig. 6 H) or moody-gal4 alone (moody-gal4/+; Fig. 6 H).

Comparison of the three groups of nerve cords (Moody-Gal4>Sop2i;Arp3i, Moody-gal4/+, and Sop2i;Arp3i/) was performed by one-way analysis of variance (ANOVA) with Dunnett’s t test (using SAS program) to determine the statistical significance of the difference between the experimental group (Moody-Gal4>Sop2i;Arp3i) and the control groups (Moody-gal4/+ or Sop2i;Arp3i/+). The difference between the experimental group and each of the control groups was statistically significant (at α = 0.05).

Figure 5. ARS dynamics in live third instar larvae. [A–H] GFP images of the ARSs taken at distinct time intervals (in minutes) of the nerve cord of third instar larvae immobilized between two coverslips carrying the moesin-GFP under Moody-Gal4. The arrows and arrowheads indicate to the same ARSs at the different time points. I and J are images of live larvae carrying Nrg-GFP and Lifeact-Ruby driven by Moody-Gal4. An Nrg loop and the ARS associated with it, labeled with Lifeact-Ruby, are indicated [empty arrowheads].

Disruption of the ARSs leads to abrogated septate junctions and BBB function

To address the molecular basis for ARS formation and its functional significance, the subcellular distribution of the actin regulatory subunit Arp3 was analyzed in SPG cells. Driving Arp3-GFP to SPG cells led to its specific colocalization with the ARSs (Fig. 6, A–C), suggesting that the Arp2/3 complex is specifically recruited to the sites of ARSs and might regulate their formation. Reducing the expression of the Arp2/3 complex using RNAi for both Sop2 and Arp3 subunits of this complex in the SPG cells led to a severe reduction of their size (Fig. 6 D and Fig. S3 G) and number (Fig. 6 D). Temporal restriction of the knockdown of Sop2 and Arp3 to third instar larvae stage (using Gal80°) led to similar reduction of the ARS size (Fig. S3, A–C). These results suggest that the correct size of the ARSs is regulated by the activity of the Arp2/3 complex, which is localized along the lateral borders of the SPG cells.

ARS size reduction induced by knockdown of Sop2 and Arp3 by RNAi led to gaps in Nrg distribution along the intercellular borders of the SPG cells, and its convoluted structure disappeared (Fig. 6 E). Importantly, the overlap between the Nrg-GFP and the ARSs was eliminated, supporting the hypothesis that the ARSs are essential to maintain septate junctions along the intercellular SPG borders and for Nrg-GFP membrane convolutions. The efficiency of both Sop2 and Arp3 RNAi constructs to compromise Arp2/3 function was demonstrated previously (Massarwa et al., 2009). These experiments demonstrate that ARS formation in the SPG cells depends on activation of the Arp2/3 complex, which is recruited to the intercellular SPG borders.

To directly assess the contribution of the ARSs to BBB function, we performed a permeability assay for larvae in which the ARSs were disrupted by SPG-specific knockdown of the Arp2/3 complex. Live larvae were submerged into a solution containing 3-kD fluorescein-dextran for 30 min. The larvae were then dissected and fixed, and dye penetration to the ventral cord was assessed by measuring the fluorescent intensity in the nerve cords using confocal microscopy. The fluorescence intensity was measured in three distinct areas in each nerve cord within a single optical section taken from the ventral cord. As expected, dye penetration was significantly higher in the moody mutant compared with wild-type larvae, as demonstrated previously (n = 12; P < 0.0066; Fig. 6 G; Bainton et al., 2005; Schwabe et al., 2005). Significantly, dye penetration of larvae carrying SPG-dependent RNAi for the Arp2/3 complex was also significantly higher when compared with controls of either larvae carrying RNAi for Arp2/3 alone (arp2/3RNAi/+; Fig. 6 H) or moody-gal4 alone (moody-gal4/+; Fig. 6 H). Comparison of the three groups of nerve cords (Moody-Gal4>Sop2i;Arp3i, Moody-gal4/+, and Sop2i;Arp3i/) was performed by one-way analysis of variance (ANOVA) with Dunnett’s t test (using SAS program) to determine the statistical significance of the difference between the experimental group (Moody-Gal4>Sop2i;Arp3i) and the control groups (Moody-gal4/+ or Sop2i;Arp3i/+). The difference between the experimental group and each of the control groups was statistically significant (at α = 0.05).
Myosin activation is required for ARS formation

The specific F-actin organization of the ARSs might be promoted by myosin contractile activity. To address this possibility, we first characterized the distribution of nonmuscle myosin relative to the ARS in wild-type larval nerve cords. The subcellular distribution of two nonmuscle myosin proteins, Spaghetti-squash (Sqh; nonmuscle myosin regulatory light chain) and

These experiments demonstrate that disruption of the ARSs in the SPG promotes discontinuous appearance of Nrg-GFP along the SPG intercellular borders, leading to the opening of the BBB in a similar fashion as detected in moody mutant larvae. Therefore, we suggest that the ARSs provide support for the formation of continuous septate junctions along the entire circumference of the SPG cells, which is essential for the maintenance of the BBB.

Figure 6. The Arp2/3 complex is required for ARS formation and for maintenance of BBB function. (A–C) Nerve cord of third instar larvae expressing Arp3-GFP fusion protein driven by Moody-Gal4. The Arp3-GFP (A) colocalizes with the ARSs labeled with phalloidin (B). The merged image is shown in C. Arrowheads in A–C indicate ARSs colabeled with phalloidin and Arp3-GFP. (D–F) Nerve cord from larvae expressing RNAi for both Arp2 and Arp3 proteins labeled for phalloidin (D). The larvae also carried the Nrg-GFP protein trap. Nrg-GFP is shown in E, and the merged image is shown in F. Arrows show aberrant ARSs that no longer associate with Nrg-GFP labeling. Arrowheads indicate sites lacking Nrg-GFP continuity. (G) Dye penetration to the nerve cord was measured by the fluorescent intensity of nerve cords dissected from wild-type (WT) larvae and compared with moody mutant larvae (moody17). The difference between the averaged fluorescent intensity of the two groups was calculated by Student's t test and was found to be significant (***, P < 0.0066). (H) Dye penetration was compared between three groups: control larvae carrying RNAi to the Arp2/3 components alone (UAS-sop2i;arp3i/+), control larvae carrying moody-gal4 alone (moody-gal4/+), or an experimental group of Arp2/3 knockdown larvae (moody-gal4;UAS-sop2i;arp3i). One-way ANOVA test with Dunnett's test (using SAS program) was used to determine the statistical significance of the difference between the experimental and the control groups (Moody-gal4/+ or Sop2i;Arp3i/+). In both cases, the difference between the experimental group and each of the control groups was statistically significant (at α = 0.05; indicated by asterisks). (G and H) Error bars indicate standard deviation.
Zipper (nonmuscle myosin heavy chain), was analyzed using flies carrying Sqh-GFP under its endogenous promoter (Bertet et al., 2004) or UAS-Zipper-GFP (Franke et al., 2005) driven by moody-gal4 driver. Both myosin-GFP constructs showed specific labeling that was tightly associated with the ARSs at the intercellular SPG borders (Fig. 7, A–F). Interestingly, myosin/GFP labeling appeared to wrap the ARSs.

Importantly, inhibition of Zipper activation by expressing a dominant-negative form of Zipper led to its complete dissociation from the ARSs (Fig. 7 G) and accumulation around the nucleus. Furthermore, dissociation of myosin from the ARSs led to shrinking of the ARSs but did not affect their localization (statistical analysis of the reduction in ARS size is shown in Fig. S3 H). Thus, myosin activation is required to maintain the normal morphology of the ARSs but not their association with the intercellular SPG borders.

Labeled cross sections of nerve cords dissected from larvae expressing moesin-GFP with anti-phospho–myosin light chain (MLC [P-MLC]) showed a specific positive labeling of P-MLC at the ARSs (Fig. 7, J–L). Moreover, in larvae expressing a recently developed sensitized Ca^{2+} indicator, GCaMP3 (Tian et al., 2009), driven to the SPG cells, we detected elevated levels of Ca^{2+} at the ARSs (Fig. 7, M and N). Collectively, these results demonstrate that specific activation of myosin takes place at the ARSs presumably as a result of elevated internal Ca^{2+} levels and that this activation is essential to maintain the ARS size.
staining was closely associated with the ARSs but did not entirely overlap these structures (Fig. 8, G–I).

We have attempted to rescue the ARS phenotype in moody mutants by expressing UAS–Moody or UAS–Moody in moody mutant larvae. Only partial rescue of the ARSs was detected, presumably because of the sensitivity of the ARSs to Moody levels (Fig. S3, D–F). These results suggest an essential supportive function of the ARSs in the formation of persistent septate junctions along the entire circumference of the SPG cells.

Discussion

The nervous system of the developing fly undergoes significant morphological changes during its development from embryo to adult stages. Surprisingly, the number of the SPG cells does not change (Sepp et al., 2000; Stork et al., 2008). Because these cells maintain the sealing function of the nervous system, we assumed that a mechanism must exist to couple nerve cord growth and the maintenance of septate junctions along the changing borders of the SPG cells. Our analysis suggests that as part of this mechanism, the SPG cells produce specialized convoluted membrane structures that are associated with accumulated F-actin, the ARSs, along their intercellular lateral borders. These structures are highly dynamic and contain Nrg but not NrxIV. Significantly, they are essential for maintaining BBB

The Moody/GPCR signaling pathway regulates ARS localization and association with myosin

To address whether the Moody signaling pathway regulates the morphology of the ARSs, myosin distribution was examined in moody mutant nerve cords. In contrast to the association of the Sqh-GFP with the ARSs detected in wild-type larvae nerve cords (Fig. 7 A), it was completely eliminated from the ARSs in moody mutant nerve cords (Fig. 8 A). Significantly, in these mutants, the ARSs lost their normal morphology and shrank into small dots that remained localized along the SPG borders (Fig. 8, B and E). This result suggests that the moody pathway regulates the association of nonmuscle myosin with the ARSs. The change in the morphology of the ARSs in moody mutant nerve cords might result from their dissociation from the myosin.

As reported previously (Schwabe et al., 2005), the distribution of Nrg-GFP was discontinuous along the lateral intercellular borders of the SPG (Fig. 8 D, arrowhead). In addition, Nrg-GFP distribution lost its convoluted membrane morphology in moody mutants, and importantly, there was no overlap between the remaining ARS dots and Nrg-GFP (Fig. 8, D–F, arrows).

These experiments strongly suggest that the Moody/GPCR pathway controls the association of nonmuscle myosin with the ARSs, possibly by inducing phosphorylation of MLC as a result of localized elevation of Ca^2+ levels. Consistently, Moody-α staining was closely associated with the ARSs but did not entirely overlap these structures (Fig. 8, G–I).

We have attempted to rescue the ARS phenotype in moody mutants by expressing UAS–Moody-α or UAS–Moody-β in moody mutant larvae. Only partial rescue of the ARSs was detected, presumably because of the sensitivity of the ARSs to Moody levels (Fig. S3, D–F). These results suggest an essential supportive function of the ARSs in the formation of persistent septate junctions along the entire circumference of the SPG cells.
function in larval nerve cords, and their maintenance depends on their association with and activation of nonmuscle myosin, which is downstream of Moody/GPCR signaling. Failure to maintain this signaling or direct inhibition of myosin activation leads to dissociation of myosin from the ARSs, shrinking and detachment of the ARSs from the Nrg-positive membrane convolutions, and BBB disruption.

A functional link between the ARSs, moody/GPCR signaling, and myosin activation

A previous study (Schwabe et al., 2005) demonstrated that the BBB is disrupted in moody mutant embryos. This analysis indicated that although septate junctions are formed in moody mutants, their morphology is aberrant. Importantly, in moody mutants, the septate junctions were discontinuous along the entire circumference of the SPG cells, leading to abrogation of the BBB. Comparative analysis performed showed that the lack of moody leads to a less severe BBB disruption phenotype relative to that of an NrxIV mutant, as measured by dye injection assay (Stork et al., 2008). This is presumably because of the fact that in moody mutants, septate junctions are formed and part of the BBB function is preserved; however, persistent junction formation at the lateral SPG border is aberrant. These results are consistent with a function for the Moody pathway in the regulation of septate junction continuity.

Our results indicate that moody signaling controls the association of the ARSs with membrane convolutions formed along the lateral borders of the SPG cells. Disruption of the ARSs by expression of RNAi against the Arp2/3 components abrogated BBB function to a similar extent as that of moody mutants. We suggest a model in which Moody/GPCR signaling promotes myosin activation and association with the ARSs. GPCR signaling often leads to activation of myosin contraction in different cellular/developmental contexts because of a transient release of internal Ca\(^{2+}\) pools (Blaser et al., 2006; Turu and Hunyady, 2010). Indeed, we detected elevation of Ca\(^{2+}\) levels at the ARS sites. Activation of myosin is essential to preserve ARS morphology and may promote their dynamic behavior. How this behavior relates to the persistent appearance of septate junctions along the lateral SPG borders is yet to be determined. The correlation between the disruption of the ARSs and elimination of the Nrg-positive membrane convolutions is consistent with the idea that the ARSs provide mechanical support for these structures. We hypothesize that these Nrg-positive membrane convolutions are essential for the fast reconstitution of septate junctions during nerve cord growth and morphogenesis. Consistently, lack of the ARSs leads to discontinuity in the appearance of septate junctions as well as abrogation of BBB function. Because some of the ARSs were still detected at the SPG cellular borders in moody mutant larvae, we suggest that a Moody-independent mechanism promotes ARS formation at the SPG cellular borders. The Moody/GPCR pathway then maintains their morphology and association with septate junction components by regulation of myosin activation.

The ARSs are unique structures of the SPG cell

The ARSs are novel structures and have not been previously described. One possible explanation is that these are highly delicate structures and might disintegrate during fixation. We suspect that the association of the moesin-GFP with the ARSs led to their stabilization, enabling their visualization both in fixed and live nerve cords. Indeed, when staining wild-type larvae nerve cords only with phalloidin, fewer ARSs were detected; however, their presence was clearly observed in third instar larvae. Moreover, we also detected ARSs using Lifeact-GFP. Two additional types of F-actin structures were detected in SPG cells, apical stress fibers arranged in an anterior posterior polarity and basal weblike stress fibers forming large circles of F-actin. The function of this basal F-actin network as well as its possible contribution to BBB maintenance is yet to be elucidated.

An interesting feature of the ARSs is their association with the plasma membrane. Because the SPG cells do not exhibit a typical epithelial-like belt of apical adherens junctions, we suspect that the ARSs instead provide mechanical support for the intercellular septate junctions formed along the SPG intercellular borders. In summary, we have described novel ARSs involved in the maintenance of convoluted Nrg-positive membrane indentations in the SPG cells, which are essential for maintaining septate junctions between neighboring SPG cells.

In vertebrates’ peripheral nervous system, myelinated Schwann cells form a myelin sheath that wraps the axon in segments separated by the nodes of Ranvier. The segmented myelin sheaths enable saltatory movement of the nerve impulse from node to node. The myelin membrane of the Schwann cell forms convoluted loops at the paranodal region separating between the Na\(^+\) channels at the node of Ranvier and the juxtaparanodal K\(^+\) channels located along the paranodal loops (Spiegel and Peles, 2002). These paranodal loops form septate junctions with the axonal membrane, enabling separation between the electrical activity at the node of Ranvier and the internodal region. Whereas the precise cytoskeletal composition of the paranodal loops as well as potential signaling involved in their formation is yet to be elucidated, the convoluted looplike structures of the ARSs in Drosophila SPG cells are structurally reminiscent of these paranodal loops, which are associated with the septate junctions. It remains to be elucidated whether similar GPCR-dependent molecular signaling mediates the formation of vertebrate peripheral nervous system paranodal loops and axoglial septate junctions.

Materials and methods

Fly strains

The following fly strains were obtained from published sources: UAS-GCaMP3 (Tian et al., 2009), UAS-Moody–GFP and UAS-Moody–β-Gal (Mayer et al., 2009), UAS-GMA–GFP (Kiehart et al., 2000), UAS-Zipper–GFP and UAS-Zipper/SP (Franke et al., 2003), moody-gold and Repo-flp1/Cyo; tub >6k> gal4-UAS-GFP/TM6 (C. Klämbt, University of Münster, Münster, Germany) and moody\(^{17}\) (Bainton et al., 2005), UAS-Lifeact-GFP (F. Schnorrer), UAS-CDB–GFP (Lee and Luo, 1999), Nrg-GFP (Morin et al., 2001), Neurexin-GFP (Edendfeld et al., 2006), UAS-Scribble-GFP (Zeiller et al., 2004), Wasp\(^{11}\) (Tai et al., 2002), arp3-GFP (Hudson and Cooley, 2002), and UAS-slp2BNA;UAS-arp3BNA (E. Scheiter, Weizmann Institute of Science, Rehovot, Israel). RNAi knockdown strains
Sp2 (VDC42172) and Arp3 (VDC35260) were obtained from the Vienna Drosophila RNAi Center stock center (Vienna, Austria).

**Immunohistochemistry and imaging**

Larvae nerve cords and brains were dissected in PBSX1.66 and fixed for 10–15 min in 2% PFA, which was then washed twice for 10 min in PBS containing 0.1% Triton X-100 (PBT) and then blocked with 10% BSA for 1 h. Samples were transferred into primary antibody and incubated overnight at 4°C. After additional washes in PBS, samples were incubated with secondary antibody for 1 h, washed with PBS, and mounted in Aqua-mount. The following primary antibodies were used: rabbit anti-NrxIV (1:1000; C. Klämbt), rabbit anti–Moody-Gal4/Cyo were crossed to males homozygous for UAS–Moody-Gal4–GFP or UAS–Moody-Gal4–GFP, and the GFP-positive moody mutant larvae males were labeled with phallolidin and examined.

**Dye penetration assay**

Third instar larvae were placed in fluorescein-dextran (3 kD, 10 mg/ml; Invitrogen) diluted 1:50 for 30 min. The larvae were dissected immediately and fixed as described in Immunohistochemistry and imaging, and the nerve cords and brains were dissected and examined by an LSM 710 system for fluorescent intensity. Dye penetration was quantified using ImageJ (National Institutes of Health). A single optical section from each nerve cord was examined, three equal-sized areas were selected and averaged for their mean pixel intensity, and background intensity was subtracted. The light intensity of the areas taken from each larva was averaged. For each experiment, 3–10 larvae were analyzed. The statistics were calculated in the following manner: for comparing permeability of moody+/− with wild-type, we have used Student’s t-test, and the p-value in this assay was P < 0.0006. For comparing the three groups of nerve cords (Moody-Gal4>Sop2i;Arp3i, Moody-gal4+/−, and Sop2i;Arp3i/+), we used a one-way ANOVA test with Dunnnett’s test (using SAS program) to determine the statistical significance of the difference between the experimental group (Moody-Gal4>Sop2i;Arp3i) and the control groups (Moody-gal4+/− or Sop2i;Arp3i/+). In both cases, the difference between the experimental group and each of the control groups was statistically significant (at α = 0.05).

**Online supplemental material**

Fig. S1 shows that there is no overlap between the distribution of the ARSs and lipid droplets (labeled with BODIPY) or with Rab-5–associated endosomes. Fig. S2 shows enrichment of the ARSs in pupal but not in adult fly brains. Fig. S3 shows that temporal knockdown of Arp2/3 only during the third instar larval stage leads to aberrant ARSs and includes statistical analysis of ARS size after Apr2/3 knockdown after expression of dominant-negative Zipper. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201007095/DC1.

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