Regulation of neuroblast proliferation by surface glia in the Drosophila larval brain

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Despite the importance of precisely regulating stem cell division, the molecular basis for this control is still elusive. Here, we show that surface glia in the developing Drosophila brain play essential roles in regulating the proliferation of neural stem cells, neuroblasts (NBs). We found that two classes of extracellular factors, Dally-like (Dlp), a heparan sulfate proteoglycan, and Glass bottom boat (Gbb), a BMP homologue, are required for proper NB proliferation. Interestingly, Dlp expressed in perineural glia (PG), the most outer layer of the surface glia, is responsible for NB proliferation. Consistent with this finding, functional ablation of PG using a dominant-negative form of dynamin showed that PG has an instructive role in regulating NB proliferation. Gbb acts not only as an autocrine proliferation factor in NBs but also as a paracrine survival signal in the PG. We propose that bidirectional communication between NBs and glia through TGF-β signaling influences mutual development of these two cell types. We also discuss the possibility that PG and NBs communicate via direct membrane contact or transcytotic transport of membrane components. Thus, our study shows that the surface glia acts not only as a simple structural insulator but also a dynamic regulator of brain development.

The Drosophila CNS develops from neural stem cells called neuroblasts (NBs). The self-renewal and differentiation of NBs are thought to be controlled by both intrinsic programs in the NBs as well as extrinsic cues. Despite a wealth of knowledge about intrinsic factors that regulate NB development, very little is known regarding non-autonomous factors that affect this process. In the embryonic CNS, it has been shown that NBs receive an extrinsic signal from the overlying epithelium to properly orient the mitotic spindle axis. Several lines of evidence suggest that post-embryonic NBs are also controlled by extrinsic factors. One previous study showed that the activin receptor, Baboon (Babo), and its transcriptional mediator, Smad2 (Smox), regulate NB proliferation in the larval CNS, indicating that activin-like ligands may play a role in this process. Furthermore, expression of a dominant-negative form of E-cadherin in glia reduced the number of proliferating NBs. This observation indicated that physical contact of larval NBs with glia is critical to NB proliferation. Larval glia are also known to secrete Anachronism, which maintains NBs in a mitotically inactive state (the quiescent state) during early larval stages. Thus, although there is no direct evidence that Drosophila larval glia have a niche function, it is assumed that division and differentiation of larval brain NBs may be controlled by surrounding glial cells.

Recent studies demonstrated vital roles of heparan sulfate proteoglycans (HSPGs) as important regulators of stem cell behaviors. HSPGs are a class of carbohydrate-modified proteins involved in a variety of biological processes such as growth factor signaling, cell adhesion, and enzymatic catalysis. Importantly, these molecules serve as co-receptors for growth factor signaling, regulating reception and tissue distribution of secreted signaling proteins such as FGFs, BMPs, Wnts, and Hedgehog on the cell surface. Although HSPGs are generally thought to regulate growth factor signaling on the surface of signal-receiving cells, they can also activate signaling in trans from adjacent cells to play non-autonomous roles.

The TGF-β pathway is an evolutionarily conserved signal transduction module that mediates diverse biological processes in animals, including stem cell control. Like in mammals, both the BMP and Activin branches are required for Drosophila development and homeostasis. BMPs are involved in numerous processes throughout all developmental stages, from patterning of the embryo and imaginal discs to stem cell maintenance.
Activin branch ligands also play diverse roles in growth and patterning in *Drosophila*, including control of imaginal cell proliferation, neuroblast proliferation, neuron remodeling, axon guidance, and sugar homeostasis. Glial cells in the *Drosophila* larval brain have been classified into a few different types based on their positions, morphology, and gene expression profiles, including perineurial glia (PG), subperineurial glia (SPG), cortex glia, and astrocyte-like glia, which send processes into the neuropil. Two surface glia cell types, PG and SPG, provide a structural basis for an insulation barrier between the CNS and hemolymph known as the blood–brain barrier (BBB). The most outer layer is formed by PG, which is characterized by the formation of extensive, thin cell protrusions. The SPG are located immediately below the PG. The SPG forms septate junctions (SJs) and are central for the *Drosophila* BBB function. The BBB restricts molecular movement at the interface between blood and nervous tissues, and it plays critical roles in maintaining a regulated microenvironment for reliable neural signaling. On the other hand, the BBB is a major obstacle for pharmacologic treatments to the brain. Past studies have focused on the functions of surface glia as an effective shield, which separates the nervous system from the open circulatory system, to maintain brain homeostasis. However, the biological role of surface glia in regulating neural development remains to be elucidated.

In this study, we identified bidirectional interactions of NBs and surface glial cells in the developing larval brain. We found that Dally-like (Dlp), a HSPG of the glypican family, is expressed in PG and is critical for proper NB proliferation. Selective ablation of glial cells supported the idea that PG play an instructive role in regulating NB proliferation. We also found that Glass bottom boat (Gbb), a BMP homologue, is expressed in NBs and functions with Dlp to regulate normal proliferation of NBs. In addition to serving as an autocrine signal in NBs, Gbb also acts as a paracrine survival signal in the PG. Thus, the development of NBs and glia is controlled by reciprocal communication between these cells through TGF-β signaling pathways in the NSC niche. Finally, we discuss the possibility that PG make physical membrane contact with NBs.

**Results**

Dlp is required for normal NB proliferation. HS-deficient mutants, *tutelula* (*ttv*) and *sulfateless*, show a small brain phenotype mainly because the sizes of brain lobes and ventral ganglion do not properly increase during third larval instar stage. A previous study showed that mutations in activin signaling components cause a similar small brain phenotype, which is associated with altered innervation of photoreceptor (PR) axons (Fig. 1B). The PR axon innervation abnormality is due to a reduction in the size of PR target field in the CNS, the lamina and medulla, rather than defects of PR cells. Thus, PR axon projection is a sensitive readout of brain growth. Similar to a mutant for *baboon* (*babo*), the *Drosophila* activin type I receptor, *ttv* mutant brains show PR axon targeting defect revealed by staining with anti-Chaoptin antibody (22B10) (Fig. 1C). This observation suggests that one or more HSPG molecules are required for normal NB proliferation. No obvious small brain phenotype is caused by mutations in *daily*, one of two integral-membrane HSPGs of the glypican type (Fig. 1D), or *dsyndecan*, the only *Drosophila* Syndecan homologue (data not shown). We found that brains mutant for *dlp*, the second glypican homologue, exhibit a very severe small brain phenotype with abnormalities of PR axon projection (Fig. 1E), suggesting that *dlp* is required for normal brain development.

To determine if the small brain phenotype of *dlp* mutants is caused by reduced proliferation of NBs, we analyzed NB proliferation in control and mutant brains by BrdU labeling (Fig. 1G–I). In control (*yw* third instar larval brains, where NBs are actively dividing, BrdU incorporation showed a unique and robust pattern in NBs and their progeny (Fig. 1G). Although *dlp* null mutants have been described as embryonic lethal, trans allelic heterozygotes of certain *dlp* loss-of-function mutations (*dlp*/*dlp^A187*) survive to late third larval instar stage. We found that *dlp*/*dlp^A187* mutant brains show significantly impaired NB proliferation (Fig. 1H and N; Proliferation Index (PI) = 0.74).

Interestingly, Dlp is predominantly, although not exclusively, expressed at the surface of larval brains (Fig. 1J and K). To further define the localization of Dlp on the brain surface, we used well-established surface glia Gal4 drivers, NP6293-Gal4 and NP2276-Gal4. We carefully assessed the expression patterns of these Gal4 drivers and confirmed that NP6293-Gal4 and NP2276-Gal4 are specifically expressed in PG and SPG, respectively (Fig. S1, data not shown), consistent with previous studies. Therefore, hereafter, we will call these drivers *PG-Gal4* and *SPG-Gal4*, respectively, in this paper. To define Dlp-expressing cells, fluorescent UAS transgene reporters were induced to mark glia membranes using *PG-Gal4* and *SPG-Gal4*. Anti-Dlp antibody staining of brains from *PG > mCD8ChRFP* (animals bearing *PG-Gal4* and UAS-mCD8ChRFP) and *SPG > mCD8ChRFP* showed that the area of Dlp expression is largely overlapping with PG (Fig. 1L and M).

To determine if Dlp on the surface glia non-autonomously regulates NB development, we specifically expressed *dlp* in *PG* or *SPG* mutants using *PG-Gal4*. We found that the reduced NB proliferation of *dlp* mutants was completely restored by PG-specific expression of *dlp* (Fig. 11 and N; PI = 1.07). Since no leaky expression of *PG-Gal4* is detectable in NBs or any parts of the brain inside the surface glia (Fig. S1), this rescue experiment demonstrated that Dlp expressed in PG can sufficiently function to maintain normal NB proliferation.

**Perineural glia are required for normal development of NBs.** To examine the role of PG in NB proliferation, we tested the effects of functionally ablating glial cells *in vivo* using a temperature-sensitive dominant-negative form of *shibire* (*shi*), the *Drosophila* dynamin. Since dynamin is required for the formation of membrane vesicles, expression of this dominant negative form of *shi* (*shi^ns*) can block various types of membrane trafficking events, including endocytosis and protein secretion, and has been effectively used to disrupt glial cell function. We expressed *UAS-shi^ns* specifically in PG using *PG-Gal4* (*PG > shi^ns*) and assessed its effect on NB proliferation. We found that the functional ablation of PG by expression of *shi^ns* severely disrupted NB cell proliferation (Fig. 2D and K; PI = 1.01 and 0.43 at permissive (18 °C) and restrictive (32 °C) temperatures, respectively), which resulted in a small brain phenotype (data not shown). Normal NB proliferation was observed.
in control (yw) brains for both temperature conditions (Fig. 2A and B). PG > shi<sup>64</sup> reared at 32 °C also showed a decreased number of M-phase cells labeled with anti-phosphorylated histone H3 (pH3) (Fig. 2E and F). Brains from various control animals (PG > shi<sup>64</sup> reared at the permissive temperature, UAS > shi<sup>64</sup> without Gal4 driver) were indistinguishable from wild-type (Fig. 2C and E, data not shown). These observations show that functional ablation of PG disrupts normal proliferation of neighboring NBs.

To further explore the cellular and molecular basis for the role of PG in NB proliferation, we examined the effect of shi<sup>64</sup> on membrane structures in PG. We observed a gross abnormality in patterns of a membrane-bound...
form of GFP (mCD8GFP) in PG > shi⁰ at the restrictive temperature (Fig. 2G and H). This cell membrane marker accumulated in restricted areas, forming patches, and failed to uniformly distribute on the PG plasma membrane. Expression of shi⁰ in PG also affected a marker for the Golgi (ManII-eGFP) (Fig. 2I and J). Altered patterns of these membrane markers suggested that normal membrane trafficking is required for proper control of NB development.

We next asked if Dlp localization is affected by shi⁰ expression. In a section near the brain surface, the PG focal plane, anti-Dlp staining showed abnormal aggregations of Dlp-containing membrane in PG > shi⁰ brain at 32°C.
(Fig. 2L, compare to Fig. 1J). This observation suggests that the mislocalization of Dlp in the PG cell membrane may contribute to the NB proliferation defect of shi" brains. Altogether, these results support the idea that Dlp on the PG surface regulates NB proliferation.

**Gbb signaling regulates NB proliferation.** The requirement for Dlp in NB proliferation suggests that a signaling molecule controls NB development in a HSPG-dependent manner. Previous studies have shown that most HS-dependent ligands are dispensable for NB development at the third instar larval stage23,34,35, or they are not expressed in the corresponding regions34–36. An exception is Gbb, a BMP-like ligand, which has been found to be expressed in the developing larval CNS by RNA in situ hybridization37. We monitored gbb expression at higher resolution, using gbb-lacZ and gbb-LexA > LexAop-rCD2GFP, in which gbb enhancers drive expression of a membrane-bound form of GFP, to determine the specific cell types that express gbb. As shown in Fig. 3A, a reporter assay revealed that gbb expression overlapped with a NB-marker, Mira. We also asked if gbb is expressed in PG. In this and subsequent experiments, we used an antibody against a nuclear receptor Seven-up (Svp) as a specific marker for PG (Fig. S2). Thus, gbb is specifically expressed in the NBs but not in PG.

We next examined whether gbb is required for NB proliferation. Blocking gap activity at the third instar using a temperature-sensitive allelic combination (gbb"/gbb") severely disrupted NB proliferation (Fig. 3C and H; PII = 0.72 at permissive temperature, 0.26 at restrictive temperature). An even more severe NB division defect was observed in the early third instar survivors of gbb null (gbb"/gbb") mutants (Fig. 3D and I; PII = 0.07). This gbb"/gbb" phenotype was fully rescued by induction of UAS-gbb using either gbb-Gal4 or pros-Gal4, another NB-specific driver (Fig. 3E and I; PII = 1.21 and 1.05, respectively). These results confirmed that Gbb produced in NBs is responsible for normal cell proliferation of NBs (Fig. 3E and F).

To determine if gbb and dlp function in the same pathway to regulate NB development, we examined genetic interactions between the two genes. The NB proliferation defect of dlp mutants was significantly aggravated by heterozygosity for gbb in gbb"+/gbb"/dlp"/dlp" brains (Fig. 3G and I, compare to Fig. 1B; PII = 0.29), while gbb heterozygotes (gbb"+/gbb") show normal NB proliferation (data not shown). This synergistic effect is consistent with the notion that the gbb and dlp genes function in the same pathway to regulate NB proliferation.

**Role of Gbb autocrine signaling in NB proliferation.** We next asked which cells in the developing brain respond to the Gbb signal using dad-lacZ11, a widely-used reporter of BMP signaling activity. Interestingly, we detected specific dad-lacZ signals in both Dpn-positive NBs and Svp-positive PG (Fig. 4A–B"), showing that Gbb signaling is received by both NBs and PG. Most dad-lacZ expression was lost in gbb mutant brains (Fig. 4C–C"). This result indicated that gbb is responsible for most, if not all, BMP signaling in this region of the brain at third instar larval stage. When we specifically blocked Gbb signaling in NBs by using gbb-Gal4 to express UAS-dad, an inhibitor of BMP signaling, NB proliferation was strongly inhibited (Fig. 4D and K; PII = 0.17). Since surrounding glia are wild-type, this result demonstrated an autonomous role for Gbb signaling in NB proliferation.

If PG support NB proliferation through activation and/or maintenance of Gbb signaling, one could expect that shi" expression in PG would impair Gbb signaling in NBs. We found that this was the case. Gbb signaling in NBs can be monitored by co-expression of dad-lacZ and Dpn (arrows in Fig. 4E and F). Expression of dad-lacZ in Dpn-positive cells was eliminated by PG-specific expression of shi" (Fig. 4G). In addition, we also observed that dad-lacZ signal was diminished in dlp mutants (Fig. 4H–I"). Together with the genetic interactions between gbb and dlp shown above, these results indicated that Dlp in the PG potentiates autocrine Gbb signaling in NB to promote its proliferation. We also evaluated the effects of gbb or dlp overexpression on the NB phenotype, but neither caused any significant change in NB proliferation (Fig. 4K; PII = 0.91 and 1.16, respectively).

We have previously shown that Dlp acts as a co-receptor for Gbb in vitro using a trans signaling assay34. This assay measures the activity of a co-receptor expressed in a contacting neighboring cell to regulate signaling in trans in the signal-receiving cell. This in vitro data supports our model that Dlp (on PG surface) can act as a trans co-receptor for Gbb (received by NB) in the brain. Since no previous work has shown a direct binding between Dlp and Gbb, we attempted to show Dlp-Gbb complex formation by co-immunoprecipitation of the proteins expressed in S2 cells. However, the Gbb-Dlp complex was not detectable, probably because the level of HS modification onto the Dlp core-protein in S2 cells is too low. As shown in Fig. S3A and a previous work37, Gbb binds to heparin-sepharose beads. Thus, similar to many other BMP members, Gbb is a heparin-binding protein39, characteristic of HS-dependent factors.

We also analyzed the cellular localization of Gbb and Dlp by expressing Gbb-HA and Dlp-Myc in S2 cells. Immunostaining with anti-HA and anti-Myc antibodies using an extracellular staining protocol revealed that Gbb and Dlp extensively co-localize on the S2 cell surface (Fig. S3). Together, our results and previous reports suggest that Dlp serves as a co-receptor for Gbb signaling to regulate NB proliferation in the larval brain.

**Role of Gbb signaling in perineural glia.** The observation that both NBs and PG receive Gbb signaling (monitored by dad-lacZ, Fig. 4A–B") raised the question of what role this pathway plays in PG. Remarkably, we found that the number of PG cells, monitored by anti-Svp antibody staining, was substantially reduced in gbb null mutant brains (Figs 5B and 6B; PG Index (PGI) = 0.44). This phenotype is unique to the gbb null condition since gbb hypomorphic mutations did not affect PG cell numbers (Fig. 5C and G; PGI = 0.94). The loss of PG cells in gbb null mutants was rescued by UAS-gbb expression in NBs using gbb-Gal4 (Fig. 5D and G; PGI = 0.89). Thus, in addition to NB proliferation, Gbb signaling regulates PG maintenance. The lack of this phenotype in gbb"/gbb" and dlp mutants (Fig. 5G; PGI = 0.95) suggests that the critical threshold required for PG maintenance is lower than that for NB proliferation. Consistent with the phenotype of gbb null mutants, blocking BMP signaling in PG by UAS-dad expression using PG-Gal4 also reduced the PG cell number (Fig. 5E and G; PGI = 0.35). This
**Figure 3.** *gbb* controls NB proliferation. (A–A’)* gbb* is expressed in NBs. Expression of *gbb-LexAVP16* was monitored by *LexAop-rCD2GFP* (A) and NBs are labeled with anti-Mira antibody (A’). (A’) Merged image of A and A’. IOA: inner optic anlage. (B–G) BrdU incorporation of control (yw reared at 32°C; B), *gbb1/gbb4* (reared at 32°C; C), *gbb1/gbb2* (D), *gbb1 gbb-Gal4/gbb2 UAS-gbb* (E), *gbb1 pros-Gal4/gbb2 UAS-gbb* (F), and *gbb1/+; dlp1/dlpA187* (G) brains. NB proliferation was severely disrupted in *gbb* mutants. This phenotype is rescued by *gbb* expression in NBs. Heterozygosity for *gbb* enhanced the NB proliferation defect of *dlp* mutants, suggesting that these two genes function in the same pathway. (H and I) Summary of PI for each genotype. Numerical figures depict the mean ± SE. n.s.: not significant; **p < 0.01; ***p < 0.001.
treatment also resulted in impaired NB proliferation (Figs 5F and 4K; PI = 0.32), consistent with the essential role of PG in the normal proliferation of NBs.

In order to determine if PG cells are lost by cell death in gbb null mutants, we expressed p35, an apoptosis inhibitor, in PG. In this experiment, we counted the PG cell number by marking PG with nlsGFP driven by...
PG-Gal4. We found that p35 expression significantly restored the PG in gbb null mutant brains (Figs 6A–C and 5H). Altogether, these results indicate that Gbb acts not only as an autocrine factor in NB but as a paracrine survival signal to inhibit PG apoptosis.

Interestingly, in addition to rescuing PG, p35 expression in gbb null mutants also partially restored NB proliferation (Fig. 6D–F and H; PI = 0.50). Given that this restoration occurs in the absence of Gbb function, this observation implies that additional factor(s) derived from PG promote NB division, independent of Gbb signaling. Possible candidates for such molecules are activin-like ligands since, as mentioned above, a previous study showed that mutations in baboon, the activin type I receptor, or dSmad2, the downstream activin signal transducer, strongly disrupt NB proliferation. We examined the expression pattern of one of the Drosophila activin-like ligands, daw, in the CNS. Extensive co-localization of Svp and daw-Gal4 expression indicates that this ligand is exclusively expressed in PG (Fig. 6G–G”). This result is consistent with the idea that Daw, and possibly other activin-like ligands, serve as PG factors to control NB proliferation.

Since both dlp and activin signaling components show NB proliferation defect (Figs 6D–F and H; PI = 0.50). This result is consistent with the idea that Daw, and possibly other activin-like ligands, serve as PG factors to control NB proliferation.

Since both dlp and activin signaling components show NB proliferation defect, we asked if they function in the same pathway (i.e. Dlp acts as a co-receptor for activins). We examined the genetic interactions between baboon and dlp for brain growth using the PR projection assay. No synergistic effect was observed between baboon and dlp mutations (data not shown), suggesting that activins regulate NB proliferation, independent of Gbb-Dlp signaling.

**Possible mechanisms for communications between perineural glia and NBs.** Our results showed that PG and NBs communicate to regulate each other’s development. Specifically, our findings support the idea that Dlp serves as a co-receptor for Gbb signaling during larval brain development. However, the mechanism by which these cells communicate across SPG, the major barrier component of the BBB, is unknown. One obvious possibility is that “transcytosis” in SPG may carry specific molecules, either alone or in complexes with lipid particles, to the other side of the BBB. Alternatively, SPG may have unknown openings, which allow PG to directly...
contact NBs. Although we do not know the definite answer to this question, several observations described below are consistent with these possibilities.

When a membrane-bound form of GFP (mCD8GFP) was expressed in SPG by the SPG-Gal4 driver, GFP signal was detected non-uniformly, showing openings within the SPG layer (Fig. 7A). These numerous holes suggest either the presence of a zone where mCD8GFP is excluded from the membrane or a discontinuity of the plasma membrane. Cross-sections of brains doubly stained for PG (PG-Gal4 > UAS-mCD8ChRFP) and SPG (Mdr65-LexA > mCD8GFP) often revealed regions in which the PG and SPG signals are found in the same layer (Fig. 7B). They do not appear to be co-localized since we do not see yellow signals indicative of co-localization. Instead, in these domains, the SPG layer is undetectable and the only PG signal can be detected (Fig. 7B). Such discontinuity of the SPG signal is consistently observed in virtually all brain samples analyzed. To further confirm that this staining pattern is not due to leaky expression of PG-Gal4 in SPG, we used eGFP-tagged Dlg, which is localized to septate junctions formed between neighboring SPGs. When this construct was expressed in SPG using Mdr65-Gal4, eGFP-Dlg highlighted the septate junction structures (Fig. S1E). On the other hand, eGFP-Dlg did not show this pattern when expressed by PG-Gal4 or daw-Gal4. Thus, these two Gal4 drivers are specific to PG and do not drive expressions in SPG. Together, our observations suggest that the PG layer may directly communicate with more interior NBs through the openings in the SPG.

To examine whether there is close physical proximity of NBs and PG cell membranes, we employed the split-GFP (spGFP) system. This method is based on the fact that complementation of two split-GFP proteins, spGFP1-10 and spGFP11, reconstitutes green fluorescence while each spGFP fragment alone is not fluorescent61. Membrane-bound forms of spGFP proteins have been successfully used to label areas of close membrane apposition between two cells62. We developed a system to express each spGFP fragment separately in different set of cells in vivo using the Gal4/UAS63 and the LexA/LexAop64 systems. We generated and used gbb-LexA as a NB driver to express a membrane-tethered form of spGFP1-10 in NBs (Fig. 7E). Independent of spGFP1-10, expression of spGFP11 was induced in several different types of glial cells using the Gal4/UAS system. In addition to PG-specific PG-Gal4, we used NP2222-Gal4 and NP3233-Gal4 to induce expression of a membrane bound form of spGFP11 in cortex glia and astrocyte-like glia, respectively65.

Using PG-driven spGFP11, we detected complementation of the two spGFP fragments, suggesting that PG cell membranes are in close proximity to NB membranes (Fig. 7C and D). Control brains lacking one of the
Figure 7. Possible membrane contact between NBs and PG. (A) Distribution of a membrane-bound form of GFP in SPG (SPG > mCD8GFP). (B) Cross-sections of brains doubly stained for PG (PG-Gal4 > UAS-mCD8ChRFP) and SPG (Mdr65-LexA > mCD8GFp). (C–D') Reconstitution of spGFP fragments. spGFP signal was detected in PG-Gal4/gbb-LexAVP16; UAS-spGFP11/LexAop-spGFP1-10 brains in the glial cell focal plane (C and C') and NB focal plane (D and D'). Anti-Mira signal is shown in C' and D' (magenta). (E) Schematic representation of spGFP reconstitution. spGFP1-10 was expressed in NBs under the control of the gbb promoter through the LexA/LexAop system. spGFP11 expression was driven in the PG by NP Gal4 drivers. (F–G') shi' expression disrupts the PG-NB contact. spGFP signal (green) in PG-ablated brains (PG-Gal4/gbb-LexAVP16; UAS-spGFP11/LexAop-spGFP1-10, UAS-shi') at permissive (F–F') and restrictive (G–G') temperatures. Anti-Mira staining marks NBs (magenta). Images for X-Z sections (F' and G') and Y-Z sections (F'' and G'') at white bars are shown. Arrows show the lack of GFP signal, showing the elimination of physical contact between PG and NBs (G). An example is shown of a large gap in the spGFP signal on the brain surface (H).
transgenes showed no GFP signal, proving the specificity of the GFP reconstitution (Fig. S4A–D). Similar experiments with other Gal4 drivers showed that cortex glia have large area of direct contact with NBs (Fig. S4F), while astrocyte-like glia show very limited contact with NB neuropil (Fig. S4H). These are expected results based on the anatomy of the larval CNS, further validating the spGFP experiments to detect the membrane contact. Notably, PG and cortex glia showed distinct patterns of spGFP signals: cortex glia contact the entire surface of a NB, while PG contact only the apical membrane of NBs (Figs 7C, D and S4), suggesting that each glial cell population may have a unique contribution to NB development.

We also examined NB–PG membrane contact by the spGFP system in PG > shi0 brain, which shows severely reduced NB proliferation (Fig. 2). At 18 °C, GFP complementation was observed uniformly and continuously on the surface of the brain lobe although the GFP signal is weak due to the low level of Gal4-induced expression at low temperature (Fig. 7F–F”). In contrast, inactivation of shi at the restrictive temperature caused gaps in the spGFP signal, indicating loss of the PG–NB contact on the brain lobe surface (arrowheads in Fig. 7G–G”). Figure 7H shows an example of a large gap in the spGFP signal on the surface of a shi0 brain lobe at 32 °C. These observations suggest that the NB proliferation defect in shi0 brain may be caused by loss of contact-dependent communication between NB and PG due to disrupted membrane trafficking in PG.

Taken together, our observations using the spGFP complementation system suggest that PG–NB communication may be mediated through direct membrane contact or transcytotic transport of membrane components across the BBB.

Discussion
Glia cells play essential roles in the Drosophila neural stem cell niche66,67. Our study demonstrated that cellular communication between NBs and surface glia is critical for development of both cell types. Gbb signaling is required autonomously for the proliferation of NBs as well as non-autonomously for the survival of PG. PG is essential for Gbb autocrine signaling. Given that the NB phenotype in PG > shi0 is associated with aberrant subcellular localization of Dlp in PG (Fig. 2L), one contribution of PG appears to be presenting Dlp on their surface, which likely potentiates Gbb autocrine signaling.

Alignment of amino acid sequences of Drosophila BMPs identified an HS-binding domain in Dpp and Gbb, but not Screw64. A truncated form of Dpp lacking this HS-binding fails to bind to its co-receptor Dally and was quickly degraded by receptor-mediated endocytosis69. This suggested that glypicans can enhance BMP signaling by disrupting receptor-mediated internalization and degradation of the Dpp-receptor complex. We have also shown that both Dally and Dlp can act as a trans co-receptor to enhance Gbb signaling in vitro41.

Expression of p35 by PG-Gal4 in gbb null mutants not only rescued PG but also partially restored NB proliferation. This observation suggests that additional PG-derived factor(s) contribute to NB proliferation. The most likely candidates for these PG factor(s) are activin-like ligands, whose role in NB division has been previously reported3. We identified specific expression of Daw in PG, supporting the idea that activin signaling together with Gbb regulates NB proliferation. Altogether, glia play a “niche-like” role, contributing to the microenvironment required for normal development of contacting NBs. Glia provide Dlp to support Gbb autocrine signaling, and express activin-like ligands as additional promoters of NB proliferation. Thus, we propose a model that bidirectional communication between NBs and glia through TGF-β signaling pathways influences each other’s development in the NSC niche.

Our study raises a fundamental question: how can PG and NBs communicate across the BBB and affect each other’s development? Our experiments using spGFP complementation favor a model in which NBs make direct contact with PG. Thus, it is possible that Dlp acts as a trans co-receptor for Gbb at the contacting sites. However, our study does not exclude other possibilities. For example, one can expect that transcytosis in SPG would allow molecular communications across this layer. Thus, the spGFP complementation signals we observed may result from the transport of membrane components of PG and NBs. It has been shown that lipoprotein particles can cross the Drosophila BBB40. The major Drosophila lipoprotein, lipophorin (Lpp), forms large particles with lipid molecules and transports them between different organs. In addition to lipid, Lpp particles carry sterol-linked and GPI-linked proteins. Dlp is a GPI-linked HSPG and has been shown to be incorporated into the Lpp particles30. This raises an interesting possibility that Dlp is transcytosed by the SPG through a Lpp-mediated mechanism.

Recent studies have shown that the surface glia are required to maintain the structural integrity of the neural lamella and to control the shape of the CNS1. A transcriptome study demonstrated that the surface glia express metabolic enzymes and signaling molecules at high levels in addition to various transporters, cell adhesion molecules, and ECM components25. Our study also supports the idea that the surface glia play a role not only as a simple structural insulator but a dynamic regulator of brain physiology development. Further study will be required, to fully elucidate the cellular and molecular mechanisms by which surface glia regulate the microenvironment internal to the BBB.

Methods
Fly stocks. Detailed information for the fly strains used is described in Flybase (http://flybase.bio.indiana.edu/) except where noted. Fly strains used were: dlp15, dlp41873, gbb28, gbb3, gbb4, dally germ21, tta224, bab3243, pros-Gal4 (a gift from F. Matsuzaki), four Gal4 drivers for glial cells: NP6293-Gal4 (PG-Gal4), NP2276-Gal4 (SPG-Gal4), NP3233-Gal4, and NP2222-Gal4865, daw-Gal4, gbb-Gal4, Mdr65-LexA (Bloomington Stock Center, BL61562), UAS-lacZ, UAS-dlp39.215, UAS-p35 (Bloomington Stock Center), UAS-daf34, UAS-gbb35, UAS-mCD8GFP36, UAS-ManII-GFP77, UAS-shi46, UAS-nlsGFP, UAS-eGFP-Dlg (a gift from V. Budnik), LexAop-rCD2::GFP84, LexAop2-mCD8GFP (Bloomington Stock Center, BL32203), vkg (viking;GFP)78, gbb-lacZ, dad-lacZ91, and UAS-mCD8ChRFP. Plasmid DNAs for gbb-LexA VP16, LexAop-spGFP PI-10, and UAS-spGFP11 were constructed as described below and transgenic strains bearing these constructs were generated by Rainbow Transgenic Flies, CA.
For overexpression experiments using the Gal4/UAS system, flies were raised at 25 °C for 24 hours AEL and then incubated at 29 °C until the mid-third instar larval stage. To compromise temperature sensitive alleles, flies bearing either UAS-shi5 or gbb4 were exposed to temperature shift from 18 °C to 32 °C with water-bath until the stage we examined. Larvae were staged by counting the number of teeth on the mouth hook. Homozygotes and trans-heterozygotes were selected based on GFP expression from the CyO-GFP balancer or the Tb dominant marker of TM6B.

**Immunostaining and imaging.** The following antibodies were used: Guinea pig anti-β-galactosidase (1:500, a gift from Y. Hiromi), rat anti-β-galactosidase (1:500, a gift from Y. Hiromi), rabbit anti-β-galactosidase (1:500, Cappel), mouse anti-Repo (1:200, Developmental Studies Hybridoma Bank (DSHB) 8D12), mouse anti-Chaoptin (1:100, DSHB, 22B10), rat anti-Miranda (Mira) (1:100), guinea pig anti-Deadpan (Dpn) (1:1000), mouse anti-BrdU (1:250, Becton, Dickinson and Company), mouse anti-Dlp (1:4, DSHB, 13G8), rabbit anti-active caspase 3 (1:500, Abcam), mouse anti-Svp (1:4, a gift from Y. Hiromi), rabbit anti-pH3(1:1000, Upstate Biotechnology), and Alexa Fluor 488, 546, 633 conjugated secondary antibodies (1:500, Invitrogen).

Larval brains were dissected in PBS and fixed with 3.7% paraformaldehyde in PBS for 30 min at room temperature. After washing with PBST (0.1% triton X-100 in PBS), tissues were blocked with 5% normal goat serum in PBST for 30 min. The samples were incubated with primary antibodies at 4 °C overnight. After washing with PBS, the samples were incubated with secondary antibodies for 2 hours at room temperature. The tissues were mounted in Vectashield (Vector Labs) and fluorescent signals were observed by confocal laser scanning microscope (Zeiss LSM710) with a 40X water immersion objective.

**BrdU incorporation.** Larval brains were dissected in PBS and incubated with Schneider’s Drosophila medium (Invitrogen) containing 40 ng/ml of BrdU (Roche) for 30 min at room temperature, and subsequently with Schneider’s Drosophila medium without BrdU for 24 hours. Tissues were washed in PBST and fixed with 3.7% paraformaldehyde in PBT for 30 min at room temperature. After tissues were treated with 1 N HCl for 30 min at 37 °C, BrdU incorporation. was eluted by 0.25 M NaCl and analyzed by immunoblotting using anti-HA antibody.

For overexpression experiments using the Gal4/UAS system, flies were raised at 25 °C for 24 hours AEL and then incubated at 29 °C until the mid-third instar larval stage. To compromise temperature sensitive alleles, flies bearing either UAS-shi5 or gbb4 were exposed to temperature shift from 18 °C to 32 °C with water-bath until the stage we examined. Larvae were staged by counting the number of teeth on the mouth hook. Homozygotes and trans-heterozygotes were selected based on GFP expression from the CyO-GFP balancer or the Tb dominant marker of TM6B.

**Plasmid construction.** The original plasmids of spGFP fragments, ace-4p CD4-2 spGFP1-10 and rig-3p CD4-2 spGFP11, were provided by C. Bargmann62. To optimize the spGFP constructs for Drosophila, the original signal peptide (Par3) was replaced with the Drosophila myospheroid signal peptide (1-28). The spGFP11 and spGFP1-10 fragments were amplified by PCR using primers 1 and 3, and primers 2 and 3, respectively (see below), and subcloned into the pENTR/D-TOPO Gateway entry vector (Invitrogen). PCR fragments were verified by DNA sequencing. The destination vectors used for germline transformation were pTW from the Gateway Vector Collection and pLot W from S. Diegelmann80.

**gbb-4** and gbb-lacZ constructs were made by ligating gbb4 and lacZ sequences downstream of the 5′ genomic sequences of the gbb gene, respectively, and subcloned into pCaSpeR3. gbb-LexAVP16 was constructed in the same manner after the LexAVP16 fragment was amplified by PCR using primers 4 and 5.

**Data availability.** All data generated or analyzed during this study are included in this published article and its Supplementary Information files.
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Author Contributions
H.N. and M.O. designed the project. M.I.K., and M.K. conducted the experiments. M.T. performed in vitro assays. M.I.K., M.K., and H.N. analyzed the results. T.A. and K.W. provided critical unpublished materials. M.I.K. and H.N. wrote and all authors reviewed the manuscript.

Additional Information
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