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The IgLON protein Lachesin is required for the blood–brain barrier in *Drosophila*

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In the mammalian peripheral nervous system, nerve insulation depends on the integrity of paranodal junctions between axons and their ensheathing glia. Ultrastructurally, these junctions are similar to the septate junctions (SJ) of invertebrates. In *Drosophila*, SJ are found in epithelia and in the glia that form the blood–brain barrier (BBB). *Drosophila* NeurexinIV and Gliotactin, two components of SJ, play an important role in nerve ensheathment and insulation. Here, we report that *Drosophila* Lachesin (Lac), another SJ component, is also required for a functional BBB. In the developing nervous system, Lac is expressed in a dynamic pattern by surface glia and a subset of neurons. Ultrastructural analysis of Lac mutant embryos shows poorly developed SJ in surface glia and epithelia where Lac is expressed. Mutant embryos undergo a phase of hyperactivity, with unpatterned muscle contractions, and subsequently become paralyzed and fail to hatch. We propose that this phenotype reflects a failure in BBB function.

Keywords: Nervous system; Septate junctions; GPI-linked IgCAM; Peristalsis

Introduction

Proper function of the nervous system requires insulation from the surrounding environment, a feature also referred to as the blood–brain barrier (BBB). The BBB is essentially a diffusion barrier that compartmentalizes certain ions and molecules on each side of this interface to meet the particular requirements for the chemical composition of the neuronal bathing fluid. In both vertebrates and invertebrates, the barrier is thought to depend on occluding junctions formed by specialized cells that surround the nervous system (reviewed in Bellen et al., 1996; Ballabh et al., 2004).

Occluding junctions are operationally defined as subcellular specializations at points of tight adhesion among cells that are required to limit free diffusion of solutes across a sheath of cells. Ultrastructurally distinct occluding junctions have been described on the basis of electron microscopy studies, most notably septate junctions (SJ) and tight junctions (TJ) (Tepass et al., 2001; Van Itallie and Anderson, 2004). At TJ, the outer leaflets of the membranes of the two adjacent cells appear to contact each other at multiple points (Farquhar and Palade, 1963; Tsukita et al., 2001). At SJ instead, the membranes of the adjacent cells are not in direct contact. SJ are characterized by regularly spaced electron-dense material (septa) connecting adjacent membranes (Locke, 1965; Tepass and Hartenstein, 1994).

In insects, a sheath of glial cells enwraps and insulates the nervous system (reviewed in Carlson et al., 2000; see also Schwabe et al., 2005 and references therein). Ultrastructural analysis of the contacts between these cells demonstrated that they form occluding junctions of the SJ type (Tepass and Hartenstein, 1994; Auld et al., 1995; Baumgartner et al., 1996; Carlson et al., 2000; Schwabe et al., 2005). While SJ appear to form only at points of glia-to-glia contacts in the embryonic fly nervous system (Carlson et al., 2000; Pereanu et

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al., 2005), the existence of SJ also between glia and neurons has been recently reported for the *Drosophila* larval CNS (Pereanu et al., 2005).

In mammals, both types of occluding junctions are employed for the insulation of the nervous system. In the central nervous system (CNS), the BBB is sustained by TJ between endothelial cells of blood vessels. In addition, TJ run along myelin sheaths that enwrap central and peripheral axons, and their integrity is crucial to nerve conduction (Morita et al., 1999; Gow et al., 1999; Miyamoto et al., 2005). In the peripheral nervous system (PNS), axonal insulation also depends on so-called paranodal junctions. Paranodal junctions are formed in nerves at sites of close apposition between the axon and its myelinating glia next to the nodes of Ranvier and contain regularly spaced septa similar to those found in insect SJ (reviewed in Spiegel and Peles, 2002).

From a molecular standpoint, it should be pointed out that several homologous proteins were found to localize to occluding junctions in invertebrate and vertebrate organisms. Unexpectedly, homologues of insect SJ proteins have been found not only at paranodal junctions, but also at TJ, despite their different ultrastructure. For example, the *Drosophila* SJ proteins NeurexinIV (Nrx), Neuroglian (Nrg), Coracle (Cor), and Contactin (DCont) are homologous to Caspr/Paranodin, Neurofascin155, Protein4.1, and Contactin, respectively, all paranodal components (Fehon et al., 1994; Baumgartner et al., 1996; Bieber et al., 1989; Hortsch, 2000; Faivre-Sarrailh et al., 2004; Spiegel and Peles, 2002). On the other hand, *Drosophila* Megatrachea and Sinuous are homologous to mammalian Claudins, found at TJ; similarly, Discs Large (Dlg) is homologous to the TJ component ZO-1 (Behr et al., 2003; Wu et al., 2004; Woods and Bryant, 1991; Woods et al., 1996; Willott et al., 1993; Van Itallie and Anderson, 2004).

Genetic analysis in flies and mice demonstrated that mutations in genes that encode components of occluding junctions lead to ultrastructural alterations in the junctions and disrupted barrier function in epithelia and/or nervous tissue (e.g., Auld et al., 1995; Baumgartner et al., 1996; Lamb et al., 1998; Bhat et al., 2001; Boyle et al., 2001; for reviews, see Bhat, 2003; Spiegel and Peles, 2002). Because of the molecular and cellular parallels between insect and vertebrate occluding junctions and diffusion barriers, *Drosophila* has become an attractive model system to study BBB development and function. The involvement of SJ proteins in the fly BBB has nonetheless been investigated at the ultrastructural level only for Nrx and Gliotactin (Gli).

The cellular components of the fly BBB are thought to be the surface glia in the CNS and the exit and peripheral glia in the PNS (Ito et al., 1995; Auld et al., 1995; Baumgartner et al., 1996 and references therein). The surface glia wrap the entire CNS. The exit glia lie at the exit point of peripheral nerves, while the peripheral glia lie outside the CNS (Klambt and Goodman, 1991; Ito et al., 1995).

We had previously identified Lachesin (Lac) as a surface protein of the immunoglobulin superfamily (IgSF) that is expressed in epithelial and nervous tissues in *Drosophila* (Llimargas et al., 2004). Lac bears structural similarity to members of the IgLON subclass that includes vertebrate LAMP, ObCAM, and Neurotrimin (Pimenta et al., 1995; Schofield et al., 1988; Struyk et al., 1995; reviewed in Karagogeos, 2003). We showed that in epithelial tissues Lac is necessary for morphogenesis as well as for the formation/maintenance of the trans-epithelial barrier. We found that in epithelia Lac localizes to SJ, and that it is required for the correct subcellular localization of other SJ components, including Nrx and Cor. Conversely, Lac localization to SJ is dependent on that of other SJ components.

In the present study, we investigate the functional relevance of Lac in the nervous system. We show that Lac is expressed in the glial cells that form the barrier around both the CNS and PNS, and that the ultrastructure of SJ in these glia is disrupted in homozygous *Lac* mutant embryos. This is accompanied by swollen neuronal fibers and a behavioral phenotype: mutant embryos display an early phase of uncoordinated hyperactivity followed by paralysis and inability to hatch, which we also observe in *nrx* and *Gli* mutants. We propose that Lac contributes to the formation and/or maintenance of a functional BBB.

Results

Lachesin, a cell surface protein of the Ig superfamily, is expressed in subsets of glia and neurons in the developing Drosophila nervous system

Expression of Lac mRNA and protein in epithelial tissues has been described in Llimargas et al. (2004). Here, we describe the expression in nervous tissue. Lac mRNA is expressed in the embryonic nervous system in a dynamic pattern starting from stage 11. It is first detected in a small group of two-three cells per hemineuromere in the ventral nerve cord (VNC) (Figs. 1A–B). The number of *Lac*-expressing cells increases, and by stage 14/15, three groups of expressing cells are detected per hemineuromere. These cells are most likely neurons (Figs. 1C–F). In addition, staining can be detected in the nerves and nerve roots, most likely reflecting expression in exit and peripheral glia (Figs. 1D–F).

We analyzed expression of Lac protein either in a 'wild-type' reference stock (genotype: *yw*) using a polyclonal mouse anti-Lac antibody (Llimargas et al., 2004), or in the *LacGFP* stock, where a chimera between Lac and GFP is expressed from the endogenous Lac promoter (described in Morin et al., 2001). The expression of LacGFP reflects the tissue and subcellular distribution of the endogenous protein (Llimargas et al., 2004). The use of neuronal and glial markers confirmed that Lac is first expressed in neurons and later also in glia, starting from stages 13 to 14. We concentrate here on Lac expression in stages 15–16 embryos and first instar larvae (Fig. 2).

Firstly, we monitored Lac expression in the CNS dissected from first instar larvae, where the BBB is fully matured, and compared it to that of Gli (Auld et al., 1995). Lac and Gli are coexpressed in the surface glia wrapping the CNS (Fig. 2A). We then looked at Lac expression in the embryo. Double labeling with antibodies to Lac and either Nrg (Figs. 2B–C) or Nrx antibodies shows colocalization of Lac with these antigens in exit and peripheral glial cells (Baumgartner et al., 1996; Bieber et al., 1989). Lac is also expressed in two cells at the midline starting from stage 16. These cells do not express the midline glia markers Nrx and Wrapper (Wra) (Fig. 2D and data not shown) (Noordermeer et al., 1998), but express Repo and Engrailed (En), as well as the Mz820 GAL-4 driver (Figs. 2H–I and data not shown), indicating that they are channel glia, a subtype of surface glia located at the midline and lining the dorsoventral channels (Ito et al., 1995). Lac also colocalizes with membrane-targeted GFP expressed in glial cells with the UAS/GAL4 system (not shown). We then visualized sensory or motorneuron axons in the periphery, with 22c10 (anti-Futsch) or anti-FasciclinII (FasII) monoclonal antibodies respec-

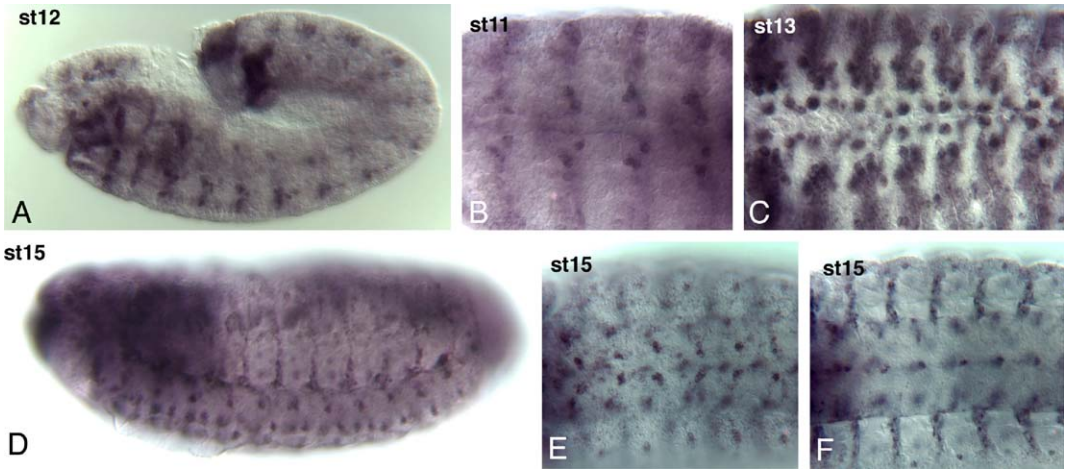

Fig. 1. *Lac* mRNA expression in the embryonic nervous system. RNA in situ hybridization with a *Lac* anti-sense probe. (A, D) Lateral views, stages 12 and 15 respectively. (B, C, E, F) Ventral views, stages 11, 13, 15, and 15, respectively. *Lac* expression starts in a small subset of cells in the CNS at around stage 11 (B) and evolves to include 3 small cell clusters per hemisegment, as well as putative exit and peripheral glia. Panels E and F are images of the same stage 15 embryo, with F a slightly more dorsal focal plane to visualize the nerves.

tively, while monitoring LacGFP expression (Fujita et al., 1982; Grenningloh et al., 1991; Lin and Goodman, 1994). The LacGFP staining appears to surround that of the axonal fibers, especially at the level of the nerve roots, as expected for a marker of the ensheathing glia (Figs. 2E–F, J; compare to glial Nrg and Gli expression in Figs. 6A and 7A of Sepp and Auld, 2003). Lac is also expressed in the glia of chordotonal organs (not shown).

We also tried to relate the expression of Lac to that of known neuronal markers. Lac-positive cell bodies are preferentially detected in the ventral half of the VNC (Figs. 2J–K), suggesting that they may be born at relatively late stages; this is consistent with the earliest detectable expression of Lac mRNA in the VNC at around stage 11. Neurons that stain for FasII, FasciclinIII (FasIII), 22c10, or Connectin (Conn) do not seem to express Lac (Figs. 2E–G and data not shown) (Grenningloh et al., 1991; Lin and Goodman, 1994; Patel et al., 1987; Nose et al., 1992; Gould and White, 1992).

The anti-Lac antibody clearly marks axons in both the anterior and posterior commissures. 'Confocal sagittal sections' reveal bundles of Lac-positive axons in the anterior and posterior commissures. The bundles are surrounded by Nrx-positive midline glial extensions (Fig. 2K) and appear as distinct from Conn- or FasIII-positive 'bundles' (not shown).

In conclusion, Lac is expressed in the glia that surround the nervous system and form the cellular basis for the BBB (surface glia, including channel glia; exit and peripheral glia), as well as in a subset of neurons.

Lachesin functions as a homophilic adhesion molecule in a cell aggregation assay

We previously showed that Lac works as a homophilic adhesion molecule in a bead aggregation assay (Llimargas et al., 2004). To further test the ability of Lac to mediate adhesion in a more physiological context, we used the S2 cell aggregation assay (Fehon et al., 1990; Bieber, 1994). *Drosophila* S2 cells normally grow in suspension as single cells. In this assay, transfected S2 cells are induced to express a candidate cell adhesion molecule; cells are kept in suspension by placing them on a shaker, and the formation of cell aggregates is then monitored by microscopy at different time intervals. Formation of aggregates is taken as an indication that the candidate adhesion molecule can indeed mediate homophilic adhesion. We transiently cotransfected S2 cells with an inducible Lac expression vector and a constitutive GFP expression vector, induced expression, and a day later performed the assay (see Experimental methods for details). Lac-expressing cells form aggregates within 4–6 h. Nrg-expressing cells also form aggregates as previously reported (Hortsch et al., 1995), while uninduced or untransfected cells do not (Figs. 3A–C and data not shown). Further, the appearance of the anti-Lac staining at the sites of cell–cell contact within the aggregates is similar to that of other adhesion molecules in the same assay (e.g., Tsiotra et al., 1996; Dhar-Malhotra et al., 1998) (Fig. 3D). Therefore, Lac also functions as a homophilic adhesion molecule in the S2 cell aggregation assay.

Next, we tested whether Lac and Gli could be engaged in heterophilic adhesion in the S2 assay. Gli localizes to SJ, is expressed in subsets of glial cells where it colocalizes with Lac, and is required for the BBB in *Drosophila* (see also Discussion). Gli-expressing cells cannot be recruited to Lac-expressing cells when the two cell populations are mixed (not shown; see Experimental methods). Furthermore, clusters of cells expressing both Lac and Gli do not appear to be larger than the ones expressing Lac alone (not shown). Therefore neither trans nor cis interactions between Lac and Gli appear to sustain adhesion in this assay.

Abnormal ultrastructure of the blood–brain barrier and neurons in Lachesin mutants

To examine whether Lac has a role in the establishment/maintenance of SJ in the fly nervous system, we undertook an ultrastructural analysis of mutant embryos with transmission electron microscopy. This revealed a serious disruption of the SJ in the glial cover of both the central and peripheral nervous system. At stage 17, when these special glial junctions are formed and the corresponding diffusion barrier is operating (Tepass and Hartenstein, 1994; Carlson et al., 2000; Schwabe et al., 2005),

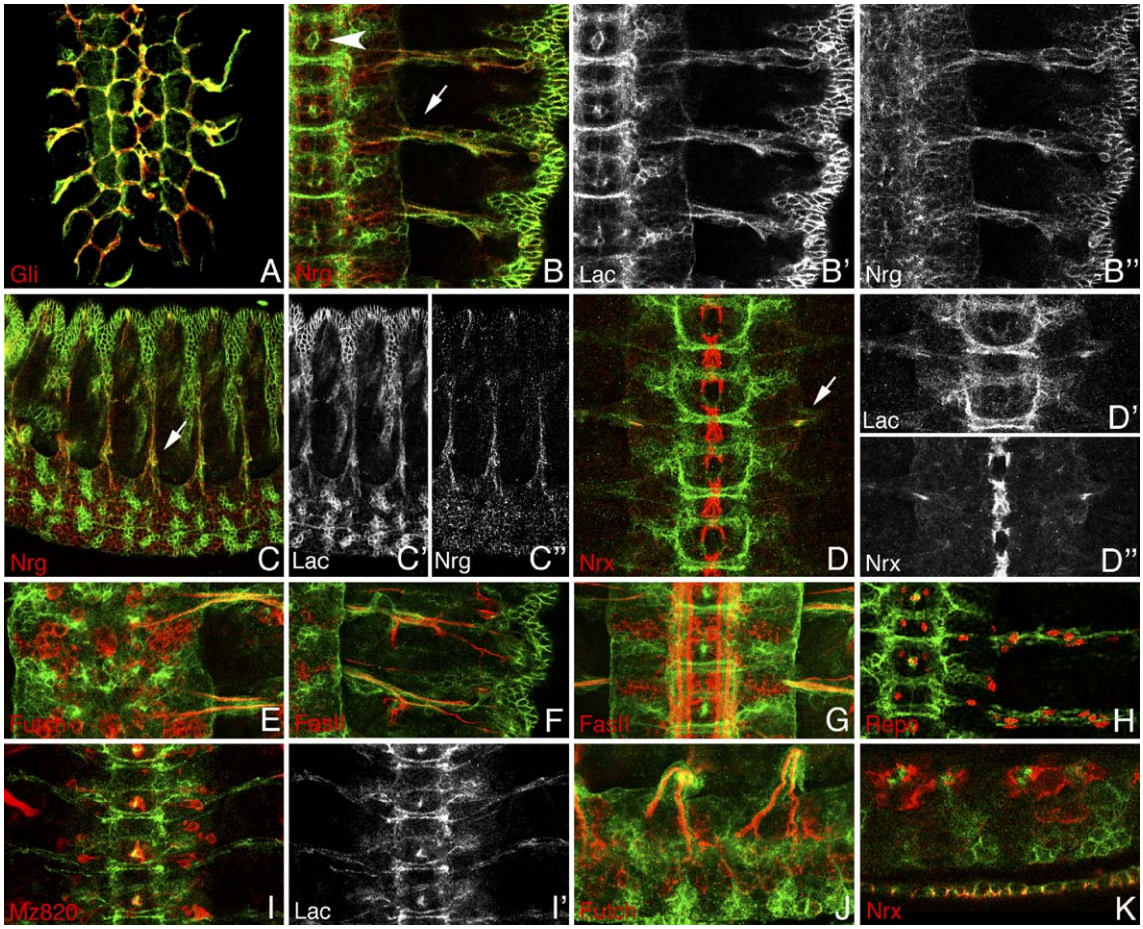

Fig. 2. Lac protein expression in the nervous system: Lac is expressed in the surface glia and subsets of neurons. Anterior is to the top in panels A–B, D–I, and to the left in panels C, J–K. Lac expression (green in all panels) is detected with an anti-Lac antibody or with an anti-GFP antibody in *LacGFP* embryos, where a fusion protein between Lac and GFP is expressed from the endogenous *Lac* promoter (see text). Samples were simultaneously labeled with another antibody (red in all panels), as follows: (A) Gli. Dissected 1st instar larval CNS. Lac and Gli are coexpressed in the large surface glia of the larval CNS. (B, C) Nrg (monoclonal antibody 1B7, against all Nrg isoforms, i.e., both neuronal and non-neuronal). Note that Lac is expressed in subsets of neurons in the VNC, as well as in the exit and peripheral glia surrounding nerves (arrow). Panel B is a ventral view of a stage 16 embryo (the arrowhead points to the channel glia); panel C is a lateral view of a stage 15 embryo. Panels B, C show double labeling, panels B′, C′ show the LacGFP channel, panels B′′, C′′ show the Nrg channel. (D) Nrx. Ventral view, stage 15 embryo. Note that midline expression of Lac and Nrx do not overlap: Lac is expressed in commissures, while Nrx is expressed in the midline glia wrapping the commissures. Also note coexpression of Lac and Nrx in the exit glia (in focus where arrow points); panel D′ shows the Lac channel, panel D′′ shows the Nrx channel. (E) Futsch (22c10). Lac and Futch neuronal staining do not overlap in the VNC. Lac-positive peripheral glia surround Futsch-positive axons in the periphery. (F, G) FasII (1D4). Lac and FasII neuronal staining do not overlap in the VNC. Lac-positive peripheral glia surround FasII-positive axons in the periphery. (H) Repo. The Lac antibody stains peripheral glia as well a pair of Repo-positive glia at the midline in the VNC. (I) Beta-galactosidase (Mz820-GAL4, UAS-tau-lacZ). At the midline Mz820-GAL4 is expressed in the channel glia, where it is coexpressed with Lac; panel I′ shows the Lac channel. (J) Futsch (22c10). Lateral view. Lac-positive peripheral glia surround Futsch-positive axons in the periphery. Note that Lac-positive neurons are located ventrally. (K) Nrx. Lateral view (confocal sagittal section at the level of the midline). Nrx-positive midline glia surround the commissures. Note the section of Lac-positive axon bundles crossing the commissures. A monolayer of epidermal cells is visible ventral to the VNC.

their characteristic electron-dense septa are severely reduced in *Lac* mutants compared to heterozygous embryos (Figs. 4A–B, showing the PNS) and in some cases not detectable. This abnormality is also observed in other epithelial tissues where Lac is expressed, including epidermis, fore-gut and tracheae. In the largest tracheal trunks, the taenidia, characteristic spiral folds that reinforce the cuticular lining of the respiratory tube, are also disorganized (not shown). Neuronal fibers in the neuropil (the synaptic region in the CNS) are significantly enlarged (compare heterozygous and homozygous mutant embryos in Figs. 4C–D and 5). On the other hand, axons in the PNS do not appear to be enlarged (Figs. 4A–B and 5).

Behavioral phenotypes: Lachesin mutants display unpatterned peristalsis and become paralyzed by the end of embryogenesis

Lac mutant embryos do not hatch. We wondered whether this could be due to a failure in the peristaltic movements with which the embryo emerges through the egg envelopes. We imaged live embryos for a period of about 3 h before hatching. 18–21 h after egg laying (AEL) corresponds to the time window within which wild-type and heterozygous control embryos start displaying patterned muscle contractions characteristic of peristaltic larval crawling (Movie3, Supplementary material). It is known that these movements are neurally

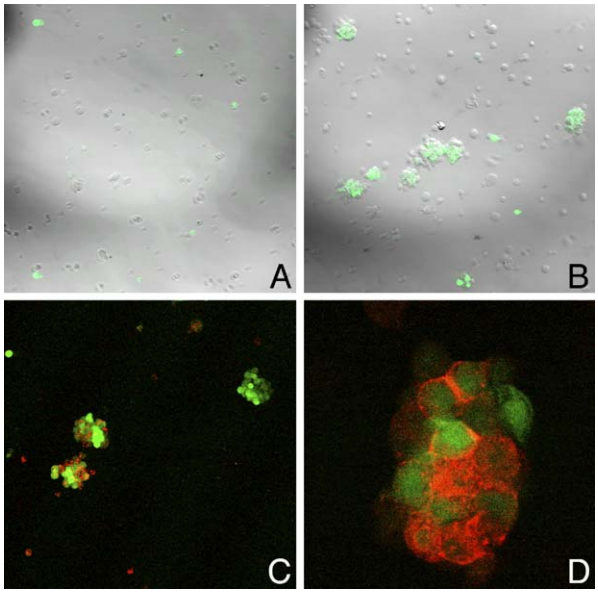

Fig. 3. Lac functions as a homophilic cell adhesion molecule in the S2 aggregation assay. (A–B) S2 aggregation assay, 6 h from start; merge of the GFP channel and transmission light. Cells transfected only with the GFP vector do not form aggregates (A), while cells transfected with the GFP and the Lac expression vector do (B) (see Experimental methods for details). (C) S2 aggregation assay with mixed populations of Lac and Nrg expressing cells. Cells were transiently transfected with a constitutive GFP expression vector and either a Lac- or Nrg inducible expression vector. The latter were labeled with the vital dye DiI (red) 1 h before the aggregation assay, and the two populations were mixed for the assay. Thus, Lac-expressing cells appear green only, while Nrg-expressing cells are green and red. Both Lac-expressing cells (green only) and Nrg-expressing cells (red and green) form aggregates but do not form mixed aggregates. (D) An aggregate of Lac-expressing S2 cells, similar to those shown in panel B, stained with anti-Lac (red).

controlled (Sarah J. Crisp and M.B., in preparation). Before this neurally controlled phase of movement there is an extended period of myogenic twitching. The transition between myogenically and neurogenically controlled movements occurs at 17–18 h of embryogenesis. We concentrate on this transition phase. Before 17 h *Lac* mutant embryos resemble wild-type embryos (see movies 1 and 2 in Supplementary material). After 18 h *Lac* mutant embryos twitch in a random and uncontrolled fashion, moving far more frequently than heterozygous controls, but show no patterned contraction (see movies 3 and 4 in Supplementary material). We conclude that this is a defect associated with the onset of movement controlled by the nervous system. The hyperactivity observed in the mutant embryos gradually disappears and the embryos become immobile by the end of embryogenesis.

This phenotype may be associated with a failure in the BBB. We checked whether it could be observed in embryos with mutations in other components of SJ known to contribute to BBB function, namely *Gli* and *nrx* (Auld et al., 1995; Baumgartner et al., 1996). We found that *Gli* and *nrx* homozygous mutant embryos display essentially the same phenotype as *Lac* mutants, consisting of an early hyperactive phase followed by paralysis.

Discussion

We have shown here that the IgLON protein Lachesin is expressed in subsets of glial and neuronal cells in the developing *Drosophila* nervous system, including the glia that insulate the CNS and PNS. With transmission electron microscopy, we documented that loss of *Lac* leads to defective ultrastructural organization of SJ in the glial cells that function as the fly BBB. We also observed an enlargement of neuronal fibers in the CNS. Time-lapse video recording of mutant *Lac* embryos during late embryogenesis revealed abnormal muscle activity followed by

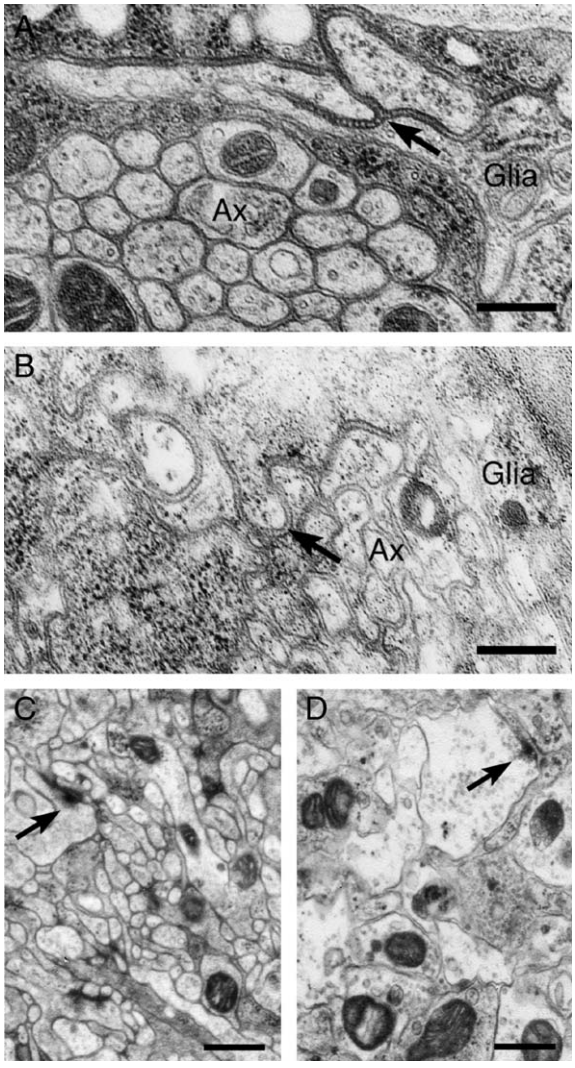

Fig. 4. Ultrastructural analysis of nervous tissue in *Lac* mutant embryos at stage 17. (A–B) PNS. In the nerves of heterozygous embryos (A), axons (Ax) are insulated by peripheral glia (Glia) forming convoluted wrappings with septate junctions (arrow) as in wild-type embryos. In homozygous mutant embryos (B), the septa in these glial junctions are fewer, thinner, and less regularly spaced. Scale bar in panels A, B = 2 μm. (C–D) CNS. The two bottom micrographs show the neuropil (synaptic region) of the central nervous system in either heterozygous (C) or homozygous (D) *Lac* mutant embryos. Mutant homozygotes have larger fibers than heterozygotes (see also Fig. 5 for quantification). Both images are from transverse sections across thoracic nerve cord, and the scale bar represents 0.5 μm. The arrows in panels C and D point to synapses.

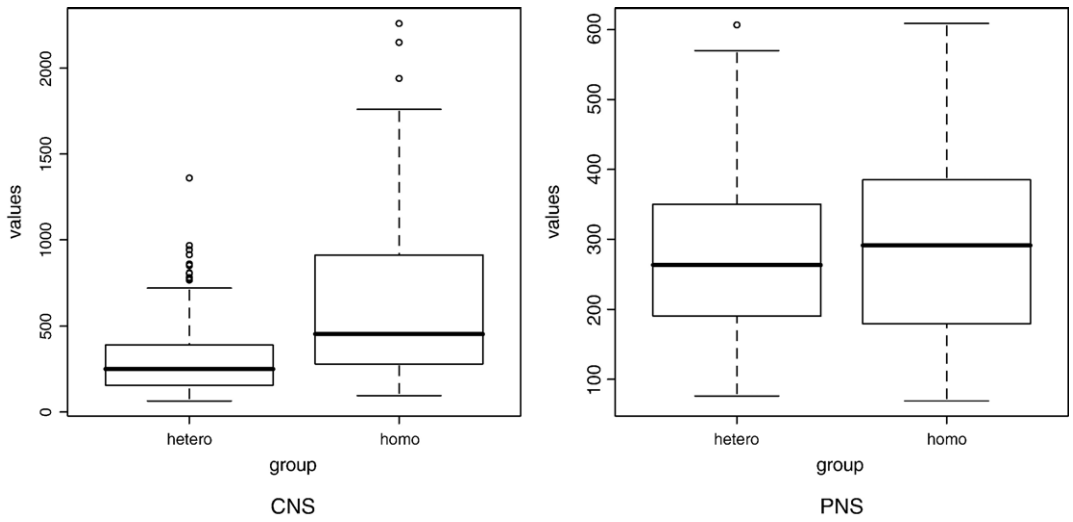

Fig. 5. Box and whisker plots of fiber diameters. The lower edge of the rectangle is at the 1st quartile, the upmost edge at the 3rd quartile. The median is plotted as a horizontal line within the box. The whiskers extend to the 1.5× range between median and 1st quartile, and median and the 3rd quartile, if data are within this range. Extreme values outside this interval are plotted as circles. The plots show the fiber diameters in the CNS of control heterozygous embryos versus *Lac* homozygous embryos (left) and in the PNS of control heterozygous embryos versus *Lac* homozygous embryos (right). The *y* axis shows fiber diameter, in nanometer. Note that the scale is different in the two panels. CNS: the mean diameter of neuronal fibers in the neuropil is 310.48 nm (SD 209.24 nm) in heterozygous embryos and 631.58 nm (SD 507.11 nm) in homozygous *Lac* mutant embryos. The difference in the mean between the two data sets is statistically highly significant ($P < 0.01$, *t* test). PNS: the mean diameter of axons in the PNS is 274.04 nm (SD 116.77 nm) and 292.75 nm (SD 129.66 nm) for heterozygous and homozygous embryos, respectively. The difference is not statistically significant.

paralysis, which may be a consequence of the impairment of the BBB. We found similar behavioral defects in embryos with mutations in Nrx and Gli, two other components of SJ that are essential for BBB function. These data support the hypothesis that Lac is an essential component of the SJ formed by surface glia and necessary for the establishment of the BBB.

Ultrastructural and behavioral defects in Lac mutants

Lac is a SJ component, it is expressed in surface glia and *Lac* mutants fail to hatch (Llimargas et al., 2004; this study). Nrx and Gli are also SJ components, they are expressed in glial cells, and the corresponding mutants are essentially paralytic by the end of embryogenesis (Auld et al., 1995; Baumgartner et al., 1996). Ultrastructural and electrophysiological analyses had shown that the BBB is disrupted in *nrx* and *Gli* mutants. We observed a similar ultrastructural defect in the SJ made by glial cells surrounding the PNS (nerves) and CNS (nerve cord) in *Lac* mutants (Fig. 4). We have not undertaken an electrophysiological analysis of nerve conduction in *Lac* mutants, but we have characterized the behavioral phenotypes and found them to be essentially indistinguishable from those of *nrx* and *Gli* mutants: late embryos display unpatterned movements followed by paralysis. Thus, we propose that Lac, similarly to Nrx and Gli, contributes to the formation and/or maintenance of the BBB.

Our TEM study also revealed an extraordinary enlargement of neuronal fibers in the CNS (Fig. 4). A simple interpretation for this phenotype could be swelling caused by the osmotic pressure that will result from uncontrolled ion flow if the BBB does not operate properly. However, we did not detect axonal swelling in the PNS. This difference between CNS and PNS may reflect a specific requirement for *Lac* activity in the CNS. Alternatively, it could reflect the operation of compensatory mechanisms specific for the PNS. In this respect, it should be pointed out that the BBB becomes functional at earlier stages in the PNS than the CNS (Carlson et al., 2000). In theory, this temporal difference could make possible an amelioration of the BBB in nerves through upregulation of other SJ genes or other mechanisms.

SJ components and the BBB

Lac, Gli, and Nrx are three SJ proteins expressed in surface glia with a documented function in the formation of the BBB (this study; Auld et al., 1995; Baumgartner et al., 1996). We might then expect the involvement of many if not all SJ proteins in the formation of SJ by surface glia and in BBB function. The analysis of phenotypes in loss of function mutants for other known SJ components has primarily focussed on epithelial structures and has not directly addressed their role in the BBB. Yet, the availability of mutants for most of these proteins makes it possible to test such predictions. The Na/K ATPase pump has been shown to be a component of SJ in epithelia (Hemphala et al., 2003; Genova and Fehon, 2003; Paul et al., 2003). Previous studies had suggested that the Na/K pump could possibly contribute to the maintenance of the correct ion concentrations inside and outside neurons (Palladino et al., 2003; Trotta et al., 2004). It is not clear yet whether such function is to be ascribed solely to the activity of the Na/K ATPase in neurons or instead (also) in the glia, and it may thus well be that this protein too contributes to the fly BBB. Indeed, recent work by Schwabe, Gaul, and colleagues strongly suggests that Nervana2 (encoding a component of the Na/K ATPase) and Nrg also contribute to the BBB (Schwabe et al., 2005).

Interestingly, the regulation of SJ components in glial cells may be under direct coordinated control of the glial cell-specific transcription factors Glial Cells Missing (Gcm) and Gcm2. Two

recent studies (Egger et al., 2002; Freeman et al., 2003) have identified several SJ components among the putative target genes of Gcm/Gcm2, including *Lac*, *Nrg*, *nervana 1* and *2* (the beta-subunits of the Na/K ATPase; Genova and Fehon, 2003; Paul et al., 2003). The level of *Lac* mRNA was indeed shown to be dependent on *gcm* activity by Freeman et al. (2003).

We had previously shown that Lac works as a homophilic adhesion molecule in a bead aggregation assay (Llimargas et al., 2004), and now, we show that Lac also mediates homophilic adhesion in a cellular context, in the S2 cell aggregation assay (Bieber, 1994; Fehon et al., 1990). We have also asked whether Lac can be engaged in heterophilic adhesive interactions with Gli. Gli localizes to SJ, is expressed in subsets of glial cells where it colocalizes with Lac, and is required for the BBB in *Drosophila*. Gli does not function as a homophilic cell adhesion molecule in the S2 assay and its ligands are currently unknown (Fig. 2; Auld et al., 1995). Gli is an electrotactin. Electrotactins are membrane proteins characterized by a structurally similar extracellular domain, carrying charged residues at comparable positions, and include cell adhesion molecules such as *Drosophila* Neurotactin (Nrt) and vertebrate Neuroligins (Botti et al., 1998). Interestingly, Nrt is known to mediate heterophilic cell adhesion with Amalgam (Ama), that in turn shows very high sequence similarity to Lac at the amino acid level (38% identity, 60% similarity) (Seeger et al., 1988; Karlstrom et al., 1993; Fremion et al., 2000; Liebl et al., 2003). We therefore tested whether Lac can be the Gli ligand and be engaged in heterophilic binding to Gli in the S2 aggregation assay but could not find support for this hypothesis.

Other functions for Lac in the nervous system?

Lac is expressed both in glia and neurons. The neuronal function of Lac remains unclear, but its expression appears to be conserved within the insects. In particular, the expression in the fly is highly reminiscent of that in the grasshopper, including expression in commissural neurons, midline cells and subperineural glia. *Drosophila* Lac lacks the widespread early expression in neuroblasts found for the grasshopper homologue (Karlstrom et al., 1993). Lac expression in axons has also been reported in the cricket and locust (Malaterre et al., 2002; Schaffer and Lakes-Harlan, 2001). Given the homology to vertebrate IgLON proteins with a known function in axon guidance (reviewed in Karagogeos, 2003), Lac could work as an axon guidance molecule in insects.

At least two other *Drosophila* genes predicted to encode IgLON like proteins (CG11320 and CG14521) are expressed in subsets of neurons in the embryonic CNS (X.M., unpublished observations). One could then speculate on the existence of an IgLON combinatorial code used for axon guidance in the fly, reminiscent of the one proposed for vertebrates.

We could not detect any obvious axon guidance phenotype in *Lac* mutants using a variety of markers including HRP, BP102, FasII, 22c10, nor when looking at Lac-expressing axons in *LacGFP* homozygous embryos (not shown). We could not detect fasciculation defects in Lac-expressing axons in the commissures of *LacGFP* embryos either. Although *LacGFP* behaves as a strong loss of function allele with respect to the tracheal phenotype (Llimargas et al., 2004), we cannot exclude that its protein product may retain sufficient activity for neuronal functions.

Finally, it is also possible that neuronal Lac expression is required in later stages for the formation of SJ between neurons and glia described in the larval CNS (Pereanu et al., 2005).

It should be noted that while all SJ components for which data are available appear to be expressed in the surface glia, they do not share the same expression pattern throughout the nervous system. For example, some are expressed in the midline glia that surround the commissures, while others in the channel glia, and neuronal expression has been reported only for some SJ proteins (Fig. 2; Auld et al., 1995; Baumgartner et al., 1996; Noordermeer et al., 1998; Behr et al., 2003). The significance of these differences is unclear.

IgLON and occluding junctions

Lac consists of 3 Ig domains and is linked to the membrane by a GPI-tail, a domain arrangement and membrane association also characteristic of vertebrate LAMP, OBCAM and Neurotrimin, the founding members of the small IgLON subfamily (Struyk et al., 1995; reviewed in Karagogeos, 2003). Vertebrate IgLONs have long been implicated in axon outgrowth and guidance, on the basis of their expression pattern and of functional in vitro assays (e.g., Pimenta et al., 1995; Marg et al., 1999). Recently, IgLONs have also been detected at synapses: mainly at postsynaptic sites of dendritic and somatic synapses, where they appear to colocalize with vesicle-associated membrane protein 2 (Miyata et al., 2003a,b). To our knowledge, the only function for vertebrate IgLONs in glial cells relates to a possible role of Neurotrimin/Kilon as a growth-promoting factor for outgrowing axons following hippocampal denervation (Schafer et al., 2005). Nonetheless, given that homologues of insect SJ components are found in vertebrate occluding junctions (paranodal and tight junctions), we speculate that IgLON proteins may also contribute to the function of occluding junctions and the BBB in particular. Interestingly, human diseases that could be associated with a defect in paranodal or tight junctions have been mapped to chromosomal regions where the three known human IgLONs map, including several types of non-syndromic sensorineural deafness.

Experimental methods

Fly strains

Lac^1 and Lac^2 are described in Llimargas et al. (2004). The *Lac*GFP line is described in Morin et al. (2001). The following fly lines were also used: nrx^{4304} (Baumgartner et al., 1996), Gli^b (Auld et al., 1995), Mz-820-GAL4 (Ito et al., 1995), UAS-tau-lacZ (Hidalgo et al., 1995). Most lines are also described in FlyBase (http://www.flybase.bio.indiana.edu).

in situ hybridization and antibody stainings

In situ hybridizations were performed according to the protocol kindly provided by Nipam Patel (U.C. Berkeley) (based on Patel, 1996). *Lac* RNA probes were generated using the whole cDNA as template and produced with the Megascript kit (Ambion). Immunostainings were performed on embryos or dissected larval CNSs fixed in 4% formaldehyde for 15′–29′ according to standard protocols (Patel, 1994). The following antibodies were used: rabbit anti-βGal (Cappel) 1:10,000,

mouse anti-FasII (1D4, Grenningloh et al., 1991) 1:10, mouse anti-FasIII (7G10, Patel et al., 1987) 1:10, mouse anti-Futsch (22c10, Fujita et al., 1982) 1:50, rabbit anti-GFP (Molecular Probes) 1:1000, mouse anti-Gli (Auld et al., 1995) 1:75, mouse anti-Lac (Llimargas et al., 2004) 1:1000, mouse anti-Nrg (1B7, Bieber et al., 1989) 1:10, rabbit anti-Nrx (Baumgartner et al., 1996) 1:1000, rabbit anti-Repo (Halter et al., 1995, 1:1000), mouse anti-Wrapper (Noordermeer et al., 1998) 1:5. Fluorescently labeled secondary antibodies (Molecular Probes) were used for fluorescent labeling following the manufacturers' instructions. Samples were mounted in 80% glycerol with 0.5% *n*-Propylgallate (n-PG) as anti-fading reagent or in Vectashield mounting medium. Confocal images were obtained at a Biorad Radiance 2100 confocal microscope or at a Leica confocal microscope.

Expression plasmids

Expression plasmids used for transient transfection experiments of S2 cells were pRmHa (Bunch et al., 1988), pRmHa(puro)-Lac, pRmHa-Nrg180 (a kind gift of M. Hortsch, Dhar-Malhotra et al., 1998), and pMet(6)-7.41 (a kind gift of V. Auld, Auld et al., 1995). pRmHa(puro)-Lac was made by cloning the whole Lac cDNA into the pRmHa modified vector pRmHa(puro) generated by Lidia Perez in Stephen Cohen's laboratory (EMBL, Heidelberg), which also carries a gene for resistance to puromycine. pRmHa(puro)-Lac allows Cu+ inducible expression of Lachesin, pRmHa-Nrg180 allows Cu+ inducible expression of Neuroglian, and pMet-7.41 allows Cu+ inducible expression of Gliotactin. pHS-GFP (provided by Christos Delidakis) confers constitutive GFP expression to transfected cells.

Transfection of S2 cells

1.0 × 10^6 cells/ml were plated in 2.5-ml culture dishes and transiently transfected after about 24 h with 5 μm total DNA (see below) according to the Ca/Phosphate protocol. Cells were induced after another 24 h with 0.7 mM CuSo4 (final concentration) and collected for the S2 cell aggregation assay after about 14–16 h. Control cells were either mock transfected or were transfected with 4.5 μm of empty pRmHa vector plasmid and 0.5 μm of pHS-GFP. Experimental samples were transfected with 0.5 μm of pHS-GFP, 2.0 μm of pRmHa(puro)-Lac plasmid, and/or 2.0 μm of pMet(6)7.41 (pMet-Gliotactin), or 2.0 μm of pRmHa-Nrg180, and the necessary amount of empty pRmHa vector plasmid to add up to a total of 5 μm of DNA. Uninduced cells were similarly treated, except that CuSO4 solution was not added before the aggregation assay. Expressions of Lac, Nrg, and Gli were measured by Western blots on cell extracts from aliquots of induced, uninduced, and untransfected cells used for the aggregation assays, and normalized against Tubulin. Lac, Nrg, or Gli expression was indeed induced only in transfected cells upon Cu+ induction. Up to 5% of cells are transfected with this protocol (Fehon et al., 1990), as judged by constitutive GFP expression, as well as by antibody staining against the surface molecules (in the induced samples only). Some cells may stochastically express higher levels of the induced protein and/or GFP.

S2 cell aggregation assay

We followed Fehon et al. (1990) and Bieber (1994). In brief, cells were collected about 14–16 h after induction, gently pipetted up and down a couple of times and 0.6 ml were plated into wells of a 24-well plate. The 24-well plate was then placed on a rotating platform at 110 rpm, at room temperature. At different time intervals cells were treated in one of the following ways. (a) The whole sample (0.6 ml) was fixed by adding 0.6 ml of 4% FA-PBS for 10′ always on the rotating platform, transferred to 1.5-ml Eppenforf tubes, centrifuged at 800 rpm, 3 min, in a table-top centrifuge, placing the tubes on top of 15-ml falcon tubes. Pellets were washed twice with PBS, 3% fetal bovine serum and centrifuged as above. Cells were then resuspended in 80% glycerol, 0.5% nPG, and mounted on microscope slides. (b) Alternatively, 30-μl samples were collected with 200-μl pipette tips whose tip had been cut off, and placed on a Lysine coated 8-well microscope slide. Cells were allowed to settle in a humidified chamber for 1–2.5 h. The liquid was then gently sucked, and cells were fixed with 30 μl 2% FA-PBS for 15 min. The fixative was removed, and cells were washed twice with PBS. The samples were then either processed for Ab staining (see below) or 80% glycerol was added to the wells. When antibody staining was performed, cells were incubated with the appropriate dilution of primary antibody in PBS/0.1%TritonX, 3% NGS, 0.1% BSA for 45–60 min, rinsed twice with PBS, incubated similarly with secondary antibodies, rinsed twice again with PBS and finally 80% glycerol, 0.5% nPG was added. All samples were then observed at the transmission and confocal microscope (Biorad Radiance 2100) and scored for the formation of aggregates. For labeling of S2 cells with the vital dye DiI, we followed Hortsch et al. (1995). In short, control, Nrg-transfected or Gli-transfected S2 cells were incubated in culture medium containing the vital dye DiI for 1 h before the aggregation assay. Thus, cells emitted in the red channel when illuminated with UV light. Aliquots of DiI-labeled cells were then assayed for the formation of aggregates, either alone or mixed with Lac-transfected cells. DiI-labeled Nrg-transfected cells still formed aggregates among themselves. Control or Gli-transfected DiI-labeled cells did not form aggregates on their own nor were they recruited to Lac-expressing cell aggregates.

Transmission electron microscopy

Transverse sections of three heterozygous and three homozygous embryos were processed as described elsewhere (Cantera et al., 2002) and analyzed with a Jeol JEM 1010 microscope operated at 80 kV. Images were taken with a digital camera (Hamamatsu C4742-95) and processed with the software AMT Advantage CCD.

Statistical analysis

Statistical comparisons of neuronal fiber size were performed with two-sided *t* tests. In the CNS, we measured the diameter of all fibers (axons, dendrites, axonal branches) within a given image, making sure that all the images were selected from approximately the same area of the CNS, to avoid differences specific for particular CNS regions. We measured 204 fibers from 3 heterozygous embryos and 75 fibers from 3 homozygous embryos. In the PNS, we measured the diameter of all the fibers, i.e., all the axons, in the cross sections of a nerve (orthogonal to the long axis of the nerve). We measured 128 fibers from 3 heterozygous embryos and 49 fibers from 2 homozygous embryos.

Video recording of prehatching embryos

Embryos were dechorionated in bleach and selected for the right stage at the dissecting scope. Homozygous mutant embryos were identified by selecting against 'GFP balancers'. Stage 16 embryos were placed ventral side up in a drop of halocarbon oil on double sticky tape. Continuous video recording at room temperature was made (Sony Digital Video cassette Recorder DSR-309) at 25 frames per second, on the stage of a Leica M420 microscope using a JVC TK-C1380 video camera. Wild-type embryos develop coordinated peristaltic movements shortly before trachea was filled with air (late stage 17). Clips of these stages were downloaded to a Macintosh G5 computer and stored as Quicktime movies (see Supplementary material).

Note added in proof

Data documenting the role of DCont, Nrx and Nrg in axonal ensheathment of the fly PNS have been reported when this paper was in press by Banerjee et al., in J. Neurosci. 26: 3319–29.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mcn.2006.03.001.

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