An original infection model identifies host lipoprotein import as a route for blood-brain barrier crossing

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Pathogens able to cross the blood-brain barrier (BBB) induce long-term neurological sequelae and death. Understanding how neurotropic pathogens bypass this strong physiological barrier is a prerequisite to devise therapeutic strategies. Here we propose an innovative model of infection in the developing Drosophila brain, combining whole brain explants with in vivo systemic infection. We find that several mammalian pathogens are able to cross the Drosophila BBB, including Group B Streptococcus (GBS). Amongst GBS surface components, lipoproteins, and in particular the B leucine-rich Blr, are important for BBB crossing and virulence in Drosophila. Further, we identify (V)LDL receptor LpR2, expressed in the BBB, as a host receptor for Blr, allowing GBS translocation through endocytosis. Finally, we show that Blr is required for BBB crossing and pathogenicity in a murine model of infection. Our results demonstrate the potential of Drosophila for studying BBB crossing by pathogens and identify a new mechanism by which pathogens exploit the machinery of host barriers to generate brain infection.
Central nervous system (CNS) infections are rare, yet extremely damaging. They lead to fatal outcomes and long-term neurological disabilities in surviving infants and adults, including cognitive deficit and motor impairment. A major route for CNS infection is the bloodstream, which pathogens enter after crossing the epithelial barriers of the skin and gut, and in which they circulate as free particles or carried by blood cells. To infect the brain, pathogens must ultimately bypass an additional guardian: the blood–brain barrier (BBB). The BBB is both a selective physical and chemical filter controlling molecular import into the CNS, thus enabling neuroprotective functions. In higher vertebrates, brain microvascular endothelial cells form the core structure of the BBB. These cells are equipped to provide selective insulation, harbouring intercellular tight junctions, absence of fenestrae, and asymmetrically localised transport systems. The BBB also includes perivascular pericytes, astrocytes and a basal membrane made of the extracellular matrix, which regulate BBB integrity and functions. This complex set of interlinked layers behaves as a double-edge sword for the organism: it restricts the entry of pathogens as well as therapeutic molecules, such as antibiotics.

Pathogens that manage to cross the BBB thus secure their access to the CNS, where they tend to be immunologically protected. Accordingly, neuro-invasive, neurotropic pathogens have developed intricate mechanisms that allow them to cross this layer and invade the CNS. Three main strategies have been proposed so far: transcellular, paracellular and Trojan horse. The transcellular entry occurs through a receptor-mediated mechanism or pinocytosis, while the paracellular mechanism follows the increase of BBB permeability due to tight junction disruption. The Trojan horse mechanism uses infected blood cells which transmigrate from the periphery to the CNS, where they tend to be immunologically protected. Despite induced pluriotent stem cell–related advances and new set-ups like microfluidic organ-on-chips, these models struggle to recapitulate complex parameters crucial to BBB properties, including 3D architecture and dynamic cellular interactions. Animal models, mostly mice and rats, but also zebrafish, exist and have provided essential contributions to mechanistic explorations, including revisiting results from in vitro models. Manipulating these organisms to reach a cellular resolution and causal relationships is nevertheless still challenging. Cost and ethical issues also hinder their extensive use.

Drosophila is a powerful and tractable model system, with unrivalled genetics. It has been very successful in identifying conserved molecular mechanisms in innate immunity, such as the Toll pathway, with a focus on systemic and epithelial immunity (skin and gut). Strikingly, many aspects of mammalian neurogenesis are conserved in the Drosophila larva CNS, a postembryonic, juvenile stage which also harbours a BBB (Fig. 1a, b). The open circulatory system of the fly carries the haemolymph, which is in direct contact with all the organs including the CNS. The BBB represents its outermost structure and is composed of two gial layers. The subperineurial glia (SPG) are large polarised cells forming an epithelium-like structure with septate junctions (Fig. 1c), the equivalent of tight junctions in vertebrates. These represent a physical barrier to paracellular diffusion, similarly to the mammalian brain vascular endothelium. The perineurial glia (PG) cover the SPG and are proposed to be a haemolymph sensor. Several studies have now uncovered a striking conservation of molecules and import mechanisms between fly and mouse BBB cells. Thus, the Drosophila BBB represents a physical and chemical barrier that retains conserved chemoprotective strategies with the mammalian BBB, ensuring brain homeostasis.

Here we show that the Drosophila larval brain is a relevant and valuable system to model brain infection and discover cellular mechanisms of BBB crossing by mammalian pathogens. Taking advantage of this innovative model, we identified the lipoprotein Blr as a new virulence factor contributing to GBS neurotropism in the fly and mouse. We further identified the Drosophila lipoprotein receptor LpR2 as a host receptor for Blr in the BBB, mediating GBS internalisation through endocytosis.

Results

Group B Streptococcus actively invades the Drosophila larval brain in an explant set-up. Establishing a model of brain infection in the fly larva required experimental set-ups in which whole, intact living brains would be in contact with pathogens. We devised an ex vivo protocol, as a straightforward platform for screening pathogens and conditions (Fig. 1d). Whole third instar larvae were opened posteriorly to expose all tissues while preventing damages to the brain and minimising injuries of the peripheral nerves (see Methods section). These brain explants were transferred to culture conditions that preserve cell viability, cell proliferation and BBB permeability, and that do not induce oxidative stress (Supplementary Fig. 1a–d). The culture medium was then inoculated with the chosen pathogens at selected doses at 30 °C, close to mammalian body temperature yet tolerated by Drosophila. Brain explants were left in contact with pathogens for a given time (usually 3 h) to allow binding, washed to remove unattached microorganisms and kept in culture until the desired time of analysis. Whole fixed brains were analysed under confocal microscopy in order to distinguish brain entry from adhesion and precisely localise and quantify individual pathogens (Fig. 1d and see Methods section).

We used this set-up to screen for prokaryotic or eukaryotic pathogens known to trigger encephalitis and/or meningitis in mammals. We found that several were able to cross the Drosophila BBB and generate brain infection 24 h after inoculation (Streptococcus agalactiae, Streptococcus pneumoniae, Neisseria meningitidis, Listeria monocytogenes, Candida glabrata and non-lymphal Candida albicans; Fig. 1e). In contrast, non-pathogenic strains (Lactobacillus plantarum, non-pathogenic Escherichia coli, and Saccharomyces cerevisiae) were not able to enter the Drosophila brain, pointing to specific entry mechanisms under these conditions.

Amongst the various pathogens tested, S. agalactiae (Group B Streptococcus, GBS) proved to be the most efficient to cross the fly BBB and was detected both inside the brain and attached to its surface (Fig. 2a). We thus focused on GBS, an opportunistic gram-positive bacterium responsible for severe invasive infections in neonates leading to pneumonia, septicemia and meningitis. Despite available antibiotic treatments and intrapartum prophylaxis, these cases still represent 10% of mortality and neurological sequelae in 25 to 50% of survivors. Among the various clinical isolates tested, NEM316, being the most efficient to infect Drosophila larval brain explants, was chosen as our reference GBS strain (Fig. 2b, from now on called GBS). Interestingly, dead, formaldehyde-fixed GBS were unable to enter Drosophila brain explants, underlining the fact that GBS needs to be alive to cross the BBB.

Dissecting GBS strategies to cross the multiple layers of the BBB. Using confocal and specific markers of the extracellular matrix (ECM), PG and SPG layers, as well as transmission...
electron microscopy, we noticed several morphological disruptions under GBS infection. First, using protein trap lines (vkg::GFP\textsuperscript{27} and trol::GFP\textsuperscript{28}) to visualise conserved components of the ECM (respectively collagen IV and heparan sulfate proteoglycan (HSPG) Perlecan), we revealed: (i) that GBS was laying on or embedded in the ECM (Fig. 2c) and (ii) that both the overall Collagen IV and Perlecan networks were disrupted (Supplementary Fig. 2a, b) and appeared locally clumped around the embedded bacteria (Fig. 2c and Supplementary Fig. 2c). In addition, we observed a decrease in signal intensity of Trol::GFP (Supplementary Fig. 2b, c). Second, analysis of the cellular layers showed alterations in membrane morphology under GBS infection. Indeed, staining for a PG membrane reporter revealed a partial and fainter signal under infection (Fig. 2d). Furthermore, we noticed in some cases an altered morphology of SPG membrane and septate junctions (Fig. 2e and Supplementary Fig. 2d, e).

These observations led us to investigate GBS transport means across the PG and SPG. The first cellular layer to cross, the PG,
The B Streptococcal surface lipoprotein Blr is required for BBB crossing. To identify GBS surface component(s) involved in this process, we first tested known virulence and colonisation factors (Fig. 3a), such as the polysaccharide capsule (capsular mutant ΔepsE), the haemolytic lipid toxin (non-haemolytic strain ΔcylE) and hyper-haemolytic strain cyl+), or cell-wall anchored proteins (ΔAstrA). None of these mutants strongly affected BBB crossing (Supplementary Fig. 3a). We thus tested the contribution of surface lipoproteins which are tethered to the cell membrane by an N-terminal lipid moiety. In Gram+ bacteria, lipoprotein biosynthesis involves two specific enzymes, Lgt (prolipoprotein diacylglycerol transferase) and Lsp (lipoprotein signal peptidase). In our model, removing either Lgt or Lsp decreased bacterial count within the brain at 24 h post-infection compared to wild-type (WT) GBS, and the double mutant (Δlgt/Δlsp) displayed an additive drop (Fig. 3b), leading to a strong impairment in GBS brain entry. A significant decrease in GBS translocation into the brain was also demonstrated at 6 h post-infection for Δlgt/Δlsp mutant (Fig. 3c).

Next, we sought to identify specific GBS lipoprotein(s) involved in BBB crossing. The GBS repertoire consists of 39 putative lipoproteins,32 most of them being substrate-binding proteins of ATP-binding cassette (ABC) transporters. We selected Blr (group B leucine-rich), a His-triad/Leucine-Rich Repeat (LRR) protein,33 as an interesting candidate (Fig. 3a’). LRR domains are classically associated with protein-protein interaction and ligand recognition.34 Similar LRRs are actually found within the internalin A (InLA) of *Listeria monocytogenes*, a surface protein crucial for the bacterial crossing of the gut barrier,36 albeit seemingly not of the *Drosophila* BBB37,38. To test the role of blr (annotated as gbs0918), we deleted the gene in GBS NEM316. We first checked that GBS and its isogenic mutants grew similarly in various rich laboratory media as well as in *Drosophila* culture medium (Supplementary Fig. 3b). Moreover, using SEM, we observed no obvious morphological difference between WT GBS and Δblr, that we found attached to the brain surface and in chains, and displaying biofilm-type matrix on their surface (Fig. 3d, matrix colourised in yellow). However, Δblr displayed a significant decrease in bacterial count in the *Drosophila* larval brain at 24 h post-infection compared to the control WT and complemented (Δblr+/+ blr) strains (Fig. 3b). GBS translocation into the brain was also significantly decreased at 6 h post-infection for Δblr mutant (Fig. 3c). Altogether, these results showed that GBS lipoproteins, and in particular Blr, are key contributors to cross the *Drosophila* larval BBB and enter the brain ex vivo.

Surprisingly, we noticed that infection by Δblr mutant resulted in significant damages to SPG membranes, altered septate junction architecture and increased BBB permeability compared to WT GBS (Fig. 3e and Supplementary Figs. 2d and 3c). These differences were decreased but still remained when culture medium pH was maintained (Fig. 3e and Supplementary Figs. 2d and 3c), and acidification was similar regardless of the bacterial
strain (Supplementary Fig. 2f). Of note, none of these features 
were observed with the double Δlgt/lsp mutant (Supplementary 
Figs. 2d and 3c). In addition, using SEM, we did not notice a 
detectable difference in the morphology of Δblr mutants with or 
without medium acidification (Fig. 3d). Interestingly, we also 
detected on Δblr-infected brains large film-like structures 
embedding bacteria and reminiscent of the polysaccharidic coat 
produced during biofilm formation (Supplementary Fig. 3d, 
colourised in yellow). Using a marker for polysaccharides (the 
lectin Concanavalin A, see Methods section), we con 
firmed that 
both WT GBS and Δblr mutants were actually able to form 
biomembranes on the 
Drosophila 
larval brain (Supplementary Fig. 3e).

Altogether, these data suggest that, in the absence of Blr, GBS 
turns on more destructive, yet much less ef 
icient alternative 
mechanisms. They also point to speci 
fic Blr-dependent mechan-
isms for GBS crossing of the BBB.
The Drosophila lipoprotein receptor LpR2 is essential in the BBB for brain invasion by GBS. We then asked how the lipoprotein Blr overcomes the physical barrier of the SPG (Fig. 1a–c).

GBS surface lipoprotein Blr binds to the Drosophila LpR2, allowing the endocytosis-dependent transcellular crossing of the BBB. Interestingly, LpR2 has been shown to be an endocytic receptor, able to mediate the uptake of lipoprotein particles. We hypothesised that binding of LpR2 to Blr could first help GBS adheres to the SPG, and ultimately lead to its internalisation through endocytosis.

We further assessed the role of the endocytic pathway in GBS entry. We blocked endocytosis specifically in the SPG by preventing dynamin function (shibire<sup>DN</sup> and dominant-negative shibire<sup>ON</sup>). This led to a strong decrease in bacterial counts within the brain at 6 h post-infection (Fig. 4f). In addition, we were able to detect GBS in vesicles co-staining for a marker of early endosomes (Rab5-GFP) and SPG membrane (Fig. 4g). Expressing another early endocytic marker (FYVE-GFP) specifically in the SPG gave similar results (Supplementary Fig. 4f). In addition, we detected GBS in lysosomal vesicles, coming from the SPG layer, through the specific expression of Lamp1-GFP (Supplementary Fig. 4f) or of Spinster-RFP (Supplementary Fig. 4f, which also marks late endosomes). Finally, we found that GBS and LpR2::GFP colocalised in vesicles staining for SPG membranes (Fig. 4h).

Taken together, these results strongly indicate that SPG crossing by GBS occurs via endocytosis, likely through binding of Blr to LpR2 and internalisation of the resulting complexes.

Blr is a virulence factor essential for BBB crossing in the Drosophila larva. To confirm the relevance of these findings in an...
**Fig. 3** Screening for surface factors identifies the lipoprotein Blr as essential for BBB crossing in *Drosophila*. **a** Schematic representation of GBS surface structures and tested virulence factors with corresponding mutants. **a’** Schematic structure of Blr lipoprotein. **b, c** Screening of GBS surface structures and virulence factors at **b** 24 h and **c** 6 h post-infection identified GBS surface lipoproteins, and in particular Blr, as crucial for BBB crossing. A Kruskal–Wallis test followed by Dunn’s multiple comparisons test generated adjusted *p*-values. **b** WT GBS (*n* = 31) is compared to **Δlgp** (*p* = 0.0124, *n* = 12), **Δlsp** (*p* = 0.0022, *n* = 8), **Δlgp/Δlsp** (*p* < 10^-10, *n* = 43), **Δblr** (*p* = 9.77 * 10^-7, *n* = 45), **Δblr + Δlsp** (*p* > 0.9999, *n* = 13), and **Δblr + Δlsp** (*p* > 0.9999, *n* = 15). **c** WT GBS (*n* = 19) is compared to **Δlgp/Δlsp** (*p* = 1.27 * 10^-8, *n* = 22), **Δblr** (*p* = 0.0029, *n* = 16). Results are presented as box and whisker plots: whiskers mark the minimum and maximum, the box includes the 25th–75th percentile, and the line in the box is the median. *n* represents the number of larvae analysed. *p* ≤ 0.05; **p** ≤ 0.01; ****p** ≤ 0.0001; ns, not significant. **d** SEM pictures of WT GBS and **Δblr** GBS attached to the brain surface, without or with HEPES. Colourisations show biofilm-type matrix (yellow) present at the surface of the bacteria. **e** Close-up of confocal images of brain infected with **Δblr**, with and without acidosis, showing the SPG membrane (*mdr65-mtd-tomato*, red) and septate junctions (*Lachesin::GFP*, green) at 6 h post-infection. Septate junctions are strongly affected under **Δblr** infection without HEPES and partially rescued with HEPES (compare to Fig. 2e). SPG membranes are still damaged under **Δblr** infection with HEPES (6 h post-infection). Dashed lines outline SPG damages. Source data are provided as a Source Data file for **b, c**.
in vivo set-up, we developed a protocol of brain infection through pathogen microinjection into the Drosophila circulatory system (Fig. 5a). It was preferred to feeding in order to control the dose and bypass the variability in gut crossing efficiency.

Bacterial counts in the brain of surviving larvae at 4 h post-injection revealed that GBS was able to access and enter the Drosophila brain via the systemic route (Fig. 5b). We were also able to observe an altered SPG layer in brains with high bacterial counts (Supplementary Fig. 5a). Survival curves showed that all infected animals died between 4 and 5 h post-injection while mock-injected animals could pass developmental stages and reach adulthood (Supplementary Fig. 5b). These results demonstrated
that GBS is able to infect the *Drosophila* brain from a circulating, systemic route, causing animal mortality.

Next, we tested the virulence of Δ*lgt/lsp* and Δ*blr* mutants in this set-up. First, bacterial counts in the brains of surviving larvae injected with Δ*lgt/lsp* or Δ*abl* were significantly reduced compared to WT or complemented (Δ*blr + blr*) GBS strains at 4 h post-injection (Fig. 5b). To discard differences in fitness or survival between these isogenic GBS strains, we determined through cfu (colony-forming units) counts the exact quantity of bacteria per animal: in or attached to the brain, in the haemolymph, and in all other solid tissues (Supplementary Fig. 5c). We then calculated three ratios: brain to haemolymph, brain to tissues, brain to haemolymph and tissues (Supplementary Fig. 5d). In all cases, we found a significant decrease in Δ*abl* ratios vs wild-type ratios. This shows that the loss of Blr specifically affects the neurotropic ability of GBS to adhere and/or enter the brain. In agreement with these results, survival scores (0–4 h post-infection) were significantly higher in larvae injected with Δ*lgt/lsp* or Δ*abl* mutants compared to the two control strains, with a lethality level similar to non-infected animals (Fig. 5c).

We then assessed the role of LpR2 in the BBB during systemic infection. Infection by WT GBS larvae in which LpR2 was specifically depleted in the SPG resulted in a dramatic reduction of bacterial count in the brain (Fig. 5d), showing that LpR2 is also crucial for GBS entry into the brain in vivo. Survival curves showed that depleting LpR2 in the SPG did not significantly alter lethality compared to wild-type animals (compare black and orange curves in Fig. 5e). This suggests that, although lethality might result from a brain infection, it mainly depends on a systemic effect and the infection of other organs and compartments.

**Blr is a virulence factor essential for BBB crossing in mice.** To determine whether Blr-dependent virulence and CNS invasion mechanism are conserved in mammals, we used the mouse model of GBS hematogenous brain infection and compared wild-type GBS strain with the isogenic Δ*abl* mutant.

Time-course infection analysis showed that GBS could be detected in the brain as early as 3 h post-infection, was maintained at similar levels at 6 and 24 h, and reduced at 48 h (Fig. 6a). In parallel, bacterial counts in the blood were measurable at 3 and 6 h post-infection and dropped sharply at 24 h (Fig. 6b). Using a fluorescent GFP-tagged GBS, we observed bacteria attached to and in the capillaries of the brain parenchyma at 4 h post-infection (Fig. 6c) and Supplementary Fig. 6a) suggesting that the primary entry point for GBS is through the endothelial barrier. Interestingly, we were able to detect LDLR on mouse brain capillaries (stained with CD31), underlying the availability of this receptor at GBS putative point of entry (Supplementary Fig. 6b). Then, at 24 h after infection, we detected bacteria at the choroid plexuses and walls of the ventricles, including the lateral ventricle (Fig. 6d), that also play a barrier role in the mammalian brain. Very few cells were detected in the brain parenchyma, in regions far from the ventricles, except for some small clusters in which typical streptococcal chains were identified (Fig. 6d).

Survival curves showed that infection with wild-type GBS led to more than 50% of lethality over 7 days (Fig. 6e). The mice that survived up to 7 days exhibited aberrant behaviour indicative of neurological deficits, including unilateral palsy, immobility, and imbalance. Mood aberrations, such as isolation and lack of explorative behaviour, were also observed. Moreover, the brains of these mice revealed meningitis hallmarks including meningeal thickening and leukocyte accumulation in the meninges compared with saline-injected control mice (Supplementary Fig. 6c), as identified by co-staining for macrophages (CD68, pan-macrophage marker) and microglia (Iba-1, microglia/macrophage marker).

In contrast, no deaths were recorded in mice infected with Δ*blr* mutant and their survival curve was significantly different compared to mice inoculated with WT GBS (Fig. 6e). We then analysed bacterial levels in the brain and in the blood over the course of infection. The levels of the Δ*blr* mutant in the blood were not significantly different from WT GBS neither at 3 h nor at 6 h post-infection and we observed a similar clearance at 24 h (Supplementary Fig. 6d). Importantly, the brain levels of the Δ*abl* mutant at 3 h and at 6 h were lower, yet not significantly (Supplementary Fig. 6e). A significant reduction was then observed at 24 h post-infection when compared with the WT strain. Normalising brain-to-blood levels confirmed that the Δ*abl* strain was significantly altered in its capacity to invade the mouse brain at 3 and 6 h post-infection, as compared to the WT (Fig. 6f).

Interestingly, none of the mice infected with the Δ*lgt/lsp* mutant died (Supplementary Fig. 6f). Bacterial levels of Δ*lgt/lsp* mutant were reduced both in the blood and brain at 6 h post-infection as compared to WT GBS (Supplementary Fig. 6g). Yet, the brain-to-blood ratios were not significantly different between these two strains (Supplementary Fig. 6h) suggesting that Δ*lgt/lsp* mutants are generally less fit in vivo.

Altogether, these results identify Blr as a new, conserved virulence factor endowing GBS the ability to cross the BBB in *Drosophila* and mouse.

**Discussion**

Here we propose an original model of brain infection, using the *Drosophila* larval brain, as a mean to investigate molecular and
cellular mechanisms contributing to the crossing of the BBB. Our model combines an ex vivo approach with brain explants for the straightforward, versatile and scalable screening of putative virulence factors and associated mechanisms, with a full in vivo approach to assessing virulence and impact on the whole organism. Even though the ex vivo protocol does not allow to assess the contribution of circulating immune cells in BBB crossing, bypassing it can unveil BBB-specific mechanisms that could be masked either by an earlier, systemic effect (e.g. general inflammation) or by the difficulty to detect or assess it (e.g. acidosis). Interestingly, for example, Cryptococcus neoformans cannot enter the Drosophila larval brain in the ex vivo conditions (Fig. 1e), a finding congruent with the contribution of the Trojan horse mechanism proposed to explain C. neoformans barrier crossing31. It is worth noting that fly experiments were performed at 30 °C, and not at 37 °C, the usual environment of mammalian pathogens, to allow Drosophila development. This constitutes a limitation of our model since the expression of some virulence factors can be temperature-dependent.

Using our model, we aimed to identify novel factors crucial for BBB crossing by GBS. Our approach demonstrated for the first time the contribution of surface-exposed lipoproteins in mediating GBS entry into the Drosophila larval brain, and in particular the role of a specific lipoprotein known as Blr. Blr was
shown to be expressed in vivo but no role in virulence has been demonstrated yet. Interestingly, Blr was shown to bind to the pathogen recognition receptor SR-A (scavenger receptor A), expressed on most macrophages and known to endocytose modified low-density lipoproteins. This finding strongly supports our results that Blr interacts with a specific lipoprotein receptor LpR2 and is then internalised through endocytosis in the SPG. The physiological role of LpR2 in the BBB is not known and the

Fig. 6 Blr is a streptococcal virulence factor in mice involved in BBB crossing by GBS. a, b GBS counts in a the brain (including bacteria found in the parenchyma and inside the capillaries) [log10(cfu/g)] and b the blood [log10(cfu/ml)] of mice inoculated with WT GBS at 3 h (n = 10), 6 h (n = 10), 24 h (n = 18), and 48 h (n = 10). One-way ANOVA followed by Sidak’s multiple comparisons tests: p(Brain 24 h vs 48 h) = 0.0003, and Kruskal-Wallis followed by Dunn’s multiple comparisons test: p(Blood 6 h vs 24 h) = 8.5 * 10^-6. c Confocal images showing GBS-GFP (green) attached to and in the capillaries (CD31, red) of the brain parenchyma at 4 h post-injection. d Confocal images of sagittal brain sections of mice injected with a fluorescent GBS WT-GFP strain showing GFP-positive bacteria (green) at the choroid plexus (CP) inside the lateral ventricle (LV; outlined; left image) as well as at the walls of the LV and in the brain parenchyma adjacent to the LV (upper right image), at 24 h post-infection. In the lower right image, a representative cluster of GFP-positive bacteria (also positive for anti-GBS; red) detected in the brain parenchyma. Typical streptococcal chains found in the clusters are presented in the inset. DNA is stained with DAPI (blue). e Kaplan-Meier survival curves of mice intravenously injected with WT GBS (n = 22) or Δblr (n = 10). Log-rank test p = 0.0055. f The ratio of bacterial counts in the brain vs blood [log10(cfu/g brain]/[cfu/ml blood])] in mice inoculated with Δblr was significantly lower than in mice inoculated with WT GBS, at 3 and 6 h post-inoculation (n = 10 for each condition). Two-tailed Student’s t-test, 3 h: p = 0.0351; 6 h: p = 0.0404. For results presented as box and whisker plots: whiskers mark the minimum and maximum, the box includes the 25th–75th percentile, and the line in the box is the median. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001. Source data are provided as a Source Data file for a, b and e, f.
other lipoproteins could substitute Blr on the bacterial surface of expressed in brain endothelial cells, where they are linked to the VLDLR proteins. Both LDLR and VLDLR were shown to be as a potential interactor and interesting pharmacological target.

domain of Blr (Supplementary Fig. 4j), a surprising result protein moiety. This interaction does not seem to require the LRR modules. LpR2, which bears between 7 and 9 LA motifs through clusters of cysteine-rich LDL receptor type-A (LA) motifs depending on the isoform, could thus bind Blr through its protein moiety. This interaction does not seem to require the LRR domain of Blr (Supplementary Fig. 4j), a surprising result entailng that histidine-triad domain of Blr should be considered as a potential interactor and interesting pharmacological target. Blr is also a virulence factor critical for BBB crossing in mice. Drosophila LpR2 is orthologous to mammalian LDLR and VLDLR proteins. Both LDLR and VLDLR were shown to be expressed in brain endothelial cells, where they are linked to the uptake of molecular complexes across the BBB. Here we confirmed LDLR localisation in blood vessels of the mouse brain in situ (Supplementary Fig. 6b).

Of note, we found that GBS acidifies the extracellular environment, a known parameter during meningitis. Production of lactic acid by GBS contributes to the weakening of SPG and/or of upstream layers, especially the PG. It has been proposed as a virulence factor in rat fetal lung explants, where it is also linked to tissue destruction. Interestingly, we noticed destroyed blood capillaries in the brain of mice infected with WT GBS (Supplementary Fig. 6a), as well as brains with highly altered PG during infection by WT GBS in our in vivo Drosophila model (Supplementary Fig. 5a). This suggests that acidosis-linked alterations of the BBB might be a conserved mechanism taking place during GBS infection, likely localised around concentrations of bacteria releasing lactic acid. In addition, other events could also account for BBB destruction in vivo.

Surprisingly, Blr-deficient bacteria caused higher damages of the SPG, suggesting that, in the absence of Blr, GBS turn to an alternative pathway, less efficient and more destructive. Such damages were not seen with the lipoprotein-deficient mutant, in which GBS brain entry is extremely low. We hypothesise that other lipoproteins could substitute Blr on the bacterial surface of Δblr mutant, leading to entry into the brain through alternative pathways and thus explaining why Δblr still enters better than Δglt/lsp. The presence of biofilm is intriguing and could be a way Δblr causes additional damage to the BBB. Altogether, these different results underline the ability of GBS to shapeshift and use different mechanisms independently or together, depending on the conditions.

How GBS adheres to the *Drosophila* brain is a crucial step that remains to be determined in our model. The ECM is a layer rich in glycosaminoglycans recognised by many pathogens, and the fly ECM indeed contains HSPGs, including Perlecan (Supplementary Fig. 2b, c). Moreover, several HSPGs, such as the PG-secreted Dally-like, were shown to be important for GBS adhesion to *Drosophila* S2 cells, as well as for virulence using an infection model in which adult flies were pricked with GBS serotype Ia (A909 strain).

In conclusion, we propose the following model for GBS entry into the fly developing brain: adhesion, the crossing of the ECM through localised rearrangement, and then traversal of the PG layer, through paracellular and/or destructive mechanisms. Then Blr comes at play, binds to LpR2 on the surface of the SPG allowing GBS endocytosis and brain invasion (Fig. 7). Our work, using an original model of brain infection in *Drosophila*, thus proposes a detailed mechanism behind pathogen crossing of the complex BBB structure and identifies the specific lipoprotein Blr as a new, conserved virulence factor for GBS.

**Methods**

**Animal models.** *Drosophila* strains and larval culture conditions

The following fly stocks were used: *w*1118 (from F. Schweiguth), *mdr65-md-tomato* (this study), *mdr65-GalD* (BDSC 504726), *Uas-mCD8-RFP* (BDSC 27399 and 27400), *NP6293-Gal4* (Kyoto DGGR 105188); *ub-Gal80* UAS-shg RNAi (BDSC stock 34831), UAS-LpR2 RNAi (BDSC stock 106364), UAS-LpR2 RNAi (BDSC stock 107597), UAS-arr RNAi (BDSC stock 4818), UAS-mgl RNAi (BDSC stock 105071), yw; *Mi(PT-GFRSTF.1)LpR2MI04745-GFRSTF.1* (BDSC stock 58121), UAS-shp* (BDSC stock 44222), UAS-shp* (BDSC stock 5811), yw; *E GFP-Rub59*, UAS-GFP-myc-2xFYVE; UAS-spin.myc-mRFP (BDSC stock 42716). *vkg:: GFPT*, *frw::GFP*.

Embyros were collected for 2–3 h on grape juice egg-laying plates. Equivalent numbers (100) of hatching first instar larvae were transferred to standard food plates at 25 °C or 29 °C (for RNAi knockdown) until mid-third instar larval stage. For the *mdr65-GalD, UAS-RFP x UAS-shp*, hatching first instar larvae were transferred to standard food plates at 18 °C until early-third instar larval stage and transferred then to 30 °C.

**Microorganisms used and culture conditions.** The microorganisms that were tested in our experimental set-up are shown in Table 1. All strains were grown overnight at 37 °C in BHI (Brain Heart Infusion) broth for bacteria or in YPD (Yeast extract Peptone Dextrose) medium for fungi. They were stored at −80 °C in BHI broth containing 20% glycerol for bacteria or in YPD broth containing 30% glycerol for yeast. The only exception was *L. plantarum*, which was grown in de Man, Rogosa and Sharpe (MRS) broth and stored at −80 °C in MRS broth containing 20% glycerol.

![Fig. 7 Proposed model for the mechanisms used by GBS during BBB crossing.](https://example.com/image)
Table 1 Microorganisms and PCR primers used in this study.

| Strains or primers | Relevant characteristics | Reference |
|--------------------|--------------------------|-----------|
| Bacteria           |                          |           |
| *Escherichia coli* | DH5α™-pEGFP-C1           | 71        |
| *Lactobacillus plantarum* | Lp°/EGFP   |           |
|                     |                         |           |
| *Neisseria meningitidis* | 2C4.3-GFP |           |
| *Listeria monocytogenes* | GEODe   |           |
| Yeast              | Saccharomyces cerevisiae | MAT a his3Δ1 leu 2Δ10 ura3Δ10 TPI-GFP-HIS3Δx | 78        |
|                    | Candida albicans         | CEC4061 ura3Δ17 Δmm434 ura3Δ17 Δmm434   |           |
|                    | Cryptococcus neoformans  | H990-E2-Crimson |           |

**Mouse ethics statement.** All animal experiments in this study were carried out in the Department of Animal Models for Biomedical Research of the Hellenic Pasteur Institute in strict compliance with the European and National Law for Laboratory Animals Use (Directive 2010/63/EU and Presidential Decree 156/2013), with the FELASA recommendations for euthanasia and Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal work was conducted according to protocols approved by the Institutional Protocols Evaluation Committee of the Hellenic Pasteur Institute (Animal House Establishment Code: EL 25 BIO 013). License No 6317/27-11-2017 for experimentation was issued by the Greek authorities, i.e. the Veterinary Department of the Athens Prefecture. The preparation of this manuscript was done in compliance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

**Protocols**

Construction of NEM316Δblr mutant and complemented strain. In-frame deletion mutant of *blr* in NEM316 was constructed by splicing-by-overlap-extension PCR64. The primers used were the following:

- **blr-1Eco** 5′-TTCCTgaattCTGCGGTTGCTGTAATGGAGT-3′/blr-2 5′-TAGTCCTGTAAGAAGTAGGATGCTCCTACATAGT-3′ and
- **blr-3** 5′-AACATTTATGGAGGACTCTAATTCTTGGAGCTA-3′/blr-4 5′-TTCCTgtacccACCCCATGTAGTACACT-3′.

The chromosomal gene inactivation was carried out by cloning *blr*-1/blr-4 fragment into the plasmid pPS3Δ1. The resulting plasmid was transformed into the harbouring the *gfp* gene cloned under the control of the P*da* promoter66.

Construction of GFP expressing NEM316. pMV158GFP is a mobilisable plasmid harbouring the *gfp* gene cloned under the control of the P*da* promoter66.

DNA cloning and *Drosophila* transgenics. A portion of the *mdr65* enhancer (GMR54C07, Flybase ID F81000165529), which drives in the eye, was amplified from genomic DNA extracted from *Drosophila* adults, with a minimal *Drosophila* synthetic core promoter [DSCP68] fused in C-terminal. The *mdr*-Tomato DNA codes for a Tomato fluorescent protein tagged at the N-terminal end with Tag-MycPalm (MGCOSFT, directing myristoylation and palmitoylation) and at the C-terminal with 3 Tag-HA epitope. It was amplified from genomic DNA extracted from *Drosophila* adults (BDSC30005, Chris Potter lab). The two amplicons were joined using the MultiSite gateway system63 to generate a *mdr65DSCP-mtd-Tomato* construct. The construct was integrated in the fly genome at an *attP* docking site through PhiC31 transgenesis.
Culture of Drosophila brain explants. Staged larvae were washed successively in PBS and ethanol 70% v/v in water then transferred in cold Drosophila Schneider’s Medium in a dissection well. Larvae were cut at around a quarter from the posterior spiracle to minimise damages to motor nerves. The posterior part was discarded and the anterior part was turned inside-out to expose the brain. All larval tissues were kept except for the gut, which is removed to avoid contamination with intestinal symbiotic pathogens. Eight larvae were transferred to one well (24-well carded and the anterior part was turned inside-out to expose the brain. All larval Medium in a dissection well. Larvae were cut at around a quarter from the poss-
with a Leica Ultramicrotome Ultracut UC7 sections (60 nm), stained with uranyl acetate and lead citrate. Images were taken with a Tecnai SPIRIT (FEI-ThermoFisher Company) using TIA software V4.

**Mouse infection.** Eight to 10-week-old male CD-1 mice (body weight, 40.99 ± 3.62 g [mean ± standard deviation]) were randomly grouped and injected intravenously (i.v.), via the tail vein, with 10^8 CFU of bacterial suspensions in sterile 3.62 g [mean ± standard deviation] were randomly grouped and injected intravenously (i.v.), via the tail vein, with 10^8 CFU of bacterial suspensions in sterile normal saline. A priori sample size estimation was performed using GPower version 3.1. For the determination of bacterial levels in blood and brain, mice were anesthetised by intraperitoneal (i.p.) injection of a mixture containing ketamine (Imalgene 1000, MERAL, Lyon, France; 100 mg/kg of body weight) and xylazine (Rompun, Bayer, Leverkusen, Germany; 10 mg/kg of body weight). Blood samples were collected by cardiac puncture. Immediately after, each mouse was killed by cervical dislocation and its brain was aseptically removed. One brain hemisphere from each mouse was homogenised in sterile normal saline. Bacterial levels in blood samples and brain homogenates were determined by plating serial tenfold dilutions on Columbia Agar with Sheep Blood plates (ThermoFisher Scientific, Waltham, MA, USA) and counting of bacterial colonies 16 h later. The numbers of mice in each group of analysis are shown in Table 2. The bacterial loads per animal were then represented in a Log10 scale, and the brain/blood ratios were calculated as follows: ratio brain/ blood = \frac{10^{log(CFU/g brain)}}{10^{log(CFU/ml blood)}}

**Mouse immunohistology.** Mice were euthanized by (i.p.) injection of a ketamine/xylazine mix. After transcardial perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS), the brains of the infected mice were dissected out, post-fixed in the same fixative, cryoprotected in 30% w/v sucrose solution in PBS for 2 d at 4 °C, embedded in O.C.T. compound (VWR Chemicals) and frozen at −80 °C. Sections of coronal or sagittal 20-µm-thick sections were collected on Superfrost Plus microscope slides and stored at −20 °C until further processing. The cryosections were thawed and subjected to antigen retrieval in 10 mM sodium citrate solution, pH 6, followed by 1 h blocking of non-specific sites with 5% v/v normal donkey serum (NDS), simultaneously with permeabilization using 0.1% v/v Triton X-100 in PBS. Primary antibodies diluted in 2.5% NDS in PBS were applied overnight at 4 °C, followed by incubation with the appropriate secondary antibodies for 2 h at room temperature. The following primary antibodies were used: rat anti-Cluster of Differentiation 68 (CD68; 1:100; Bio-Rad Antibodies, Oxford, UK; MCA1957GA), rabbit polyclonal anti-ionised calcium-binding adapter molecule 1 (Iba-1; 1:400; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan; 019-7941), rabbit anti-CD31 (1:50; Abcam, Cambridge, UK; ab28364), goat anti-LDLR (1:100, R&D Systems, MN, USA; AF2255), rabbit anti-GHS (1:300); homemade). Secondary antibodies (all from Thermofisher Scientific) used for immunofluorescence were conjugated with Alexa Fluor 488 or 546 and cell nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI; 1:1000; Thermofisher Scientific). Prolong Gold antifade curing mountant (Cell Signaling Technology, Danvers, MA, USA) was used for mounting. Images were acquired using Leica TCS SP8 confocal microscope with Leica Application Suite X software version 3.5.5. Images were processed using Fiji (ImageJ version 2020 2.1.0/1,53c and version 1.52p), Icy (2.0.3.0) or Volocity (6.3) were used to process confocal data. Adobe Photoshop and Illustrator were used to assemble Figs.

**Statistics and reproducibility.** GraphPad Prism software (version 7 and version 2020 8.4.2 (464)) was used for all analyses.

**Bacterial quantifications in infected *Drosophila* brain.** The same region of the CNS (Ventral Nerve Cord, VNC) was scanned at an optimised number of slices (distance between each slice of 0.38 µm) using a Zeiss LSM 880 microscope with Zen software (2012 S4). The exact number of bacteria for each brain was then determined manually by counting each individual bacterium contained within the boundary of the BBB (mib65-mtd-Tomato).

**CFU counts following in vivo larval GBS-injection.** Each injected larva was washed on a paper with ethanol 70% then bled in 10 µl PBS. The brain is then dissected, transferred and homogenised in 10 µl PBS. The rest of the larval carcass (other tissues except the gut) is also transferred and homogenised in 10 µl PBS. This protocol was done for 5 larvae by the condition. Haemolymph, brain and carcass bacterial levels were determined by plating 7 serial tenfold dilutions two times on

### Table 2 Sample size per time point per bacterial strain.

| Blood and brain levels | Survival |
|------------------------|----------|
|                         | 3 h | 6 h | 24 h | 48 h | 7 d |
| WT GBS                  | 10  | 10  | 18   | 10   | 22  |
| Δft/Δls                  | 12  | 17  | 10   | 10   | 10  |
| Δlps                   | 10  | 10  | 9    | 10   | 10  |

### Table 3 Experimental reproducibility.

| Figure | Total number of samples | Number of experiments | Overall penetrance (%) |
|--------|-------------------------|-----------------------|------------------------|
| 1b     | 16 CNS                  | 2                     | 100                    |
| 1d     | 20 CNS                  | 2                     | 100                    |
| 2a     | 80 CNS                  | 12                    | 100                    |
| 2c     | ≥14 CFU per condition   | 2                     | 100                    |
| 2d     | ≥12 CFU per condition   | 2                     | 100                    |
| 2e     | ≥12 CFU per condition   | 3                     | 100                    |
| 3d     | ≥4 CFU per condition    | ≥1                    | 100                    |
| 3e     | ≥12 CFU per condition   | 3                     | 100                    |
| 4c     | 18 CNS                  | 4                     | 100                    |
| 4d     | 10 adult CNS            | 2                     | 100                    |
| 4e     | NA                      | 3                     | 100                    |
| 4g     | 8 CNS                   | 1                     | 100                    |
| 4h     | 12 CNS                  | 1                     | 100                    |
| 5a     | 13 larvae               | 3                     | 100                    |
| 6c, d  | ≥3 mice per condition   | 1 (based on the 3R principle) | 100                    |
| Supp. 1d | ≥5 CNS per condition  | 1                     | 100                    |
| Supp. 2a | ≥14 CNS per condition  | 2                     | 100                    |
| Supp. 2b, c | 8 CNS per condition | 2                     | 100                    |
| Supp. 2d | ≥12 CNS per condition  | 3                     | 100                    |
| Supp. 2e | ≥5 CNS per condition   | 1                     | 100                    |
| Supp. 3d | 5 CNS                   | 2                     | 40 (2 CNS)             |
| Supp. 3e | ≥7 CNS per condition   | 2                     | 15–20% (1–2 CNS)       |
| Supp. 4b | ≥14 larval VNCs; ≥3 wing discs; ≥3 egg chambers | 4; 2; 2 | 100 for each tissue |
| Supp. 4d | ≥7 CNS per condition   | 2                     | 100                    |
| Supp. 4e | 13 CNS                  | 2                     | 45 (6 CNS)             |
| Supp. 4f | 17 CNS                  | 3                     | 40 (7 CNS)             |
| Supp. 4g, h | NA                    | 2                     | 100                    |
| Supp. 4i | NA                      | 1                     | 100                    |
| Supp. 4k, l | 8 CNS per condition | 1                     | 100                    |
| Supp. 5a | 8 control CNS/infected CNS | ≥2                  | Control: 100           |
| Supp. 6a-c | ≥3 mice per condition | 1 (based on the 3R principle) | 100                    |
Columbia Agar with Sheep Blood plates (Biomerieux 43041) and counting of bacterial colonies after 16 h at 37 °C. The average CFU/ml was calculated as an average from all the different dilutions. The bacterial loads per animal were then represented in a log10 scale, and ratios were calculated from raw counting then represented on a log10 scale:

- Ratio brain/haemolymph = log10 (cfu per brain/cfu per haemolymph)
- Ratio brain/other tissues = log10 (cfu per brain/cfu per Other tissues)
- Ratio brain/haemolymph + other tissues = log10 (cfu per brain/cfu per haemolymph + cfu per other tissues).

**Drosophila statistical analysis.** All p-values are exact.

In order to perform statistical tests on several experimental replicates, each value (corresponding to one brain) was normalised to the mean of the control condition within one replicate. Statistical tests were then run on all the normalised values from all replicates, which were considered as biological replicates.

Comparisons between BBB permeability, GBS entry into the brain, the ratio of bacterial levels for brain/haemolymph, the ratio of bacterial levels for brain/other tissues and ratio of bacterial levels for brain/other tissues + haemolymph were performed by Student’s t-test (two conditions) or one-way ANOVA test followed by Tukey’s post-hoc analysis (more than two conditions) when values followed a normal distribution (assessed by Shapiro–Wilk normality test). Otherwise, non-parametric Mann–Whitney tests (two conditions) or Kruskal–Wallis tests (more than two conditions) were performed. The data were represented with Box and whiskers plots. All Box and whiskers plots display minimal value (bottom whisker), first quartile (25th percentile, lower limit of the box), a median of the interquartile range (middle horizontal line), third quartile (75th percentile, the upper limit of the box) and maximal value (top whisker). All individual points are plotted.

Comparison of survival curves was performed using the log-rank test. The log-rank test is based on a chi-square distribution and tests for the difference between individual points are plotted.

Comparisons between BBB permeability, GBS entry into the brain, cell viability, Ratio brain/(haemolymph + other tissues) and the brain, as well as between ratios of bacterial levels for brain/blood were performed using a Kruskal–Wallis test followed by Dunn’s multiple comparisons test was performed. The data were represented as Kaplan–Meier curves with error bars corresponding to standard errors (SE). p-values lower than 0.05 were considered significant.

**Mouse statistical analysis.** Comparisons between bacterial levels in the blood and the brain, as well as between ratios of bacterial levels for brain/blood were performed by unpaired Student’s t-test or one-way ANOVA followed by Sidak’s multiple comparisons test when values followed a normal distribution (assessed by D’Agostino–Pearson normality test). Otherwise, non-parametric Kruskal–Wallis followed by Dunn’s multiple comparisons test was performed. The data were represented with Box and whiskers plots. All Box and whiskers plots display minimal value (bottom whisker), first quartile (25th percentile, lower limit of the box), a median of the interquartile range (middle horizontal line), third quartile (75th percentile, the upper limit of the box) and maximal value (top whisker). All individual points are plotted.

Comparison of survival curves was performed using the log-rank test. p-values lower than 0.05 were considered significant.

**Representative pictures.** For representative pictures of phenotypes and experiments, the total number of biological samples and independent experiments, as well as the percentage of samples showing the representative phenotype are displayed in Table 3.

**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. Source data are provided with this paper.

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Author contributions
B.B. performed most Drosophila experiments. N.R. generated Figs. 3c, 4c, e-f as well as parts of Fig. 2c and of Supplementary Fig. 2a, d under the supervision of B.B. P.S. devised the brain explant protocol and started the pathogen screen. B.B. and P.S. designed and analysed all Drosophila experiments. B.P. and S.D. generated GBS strains and constructs as well as performed growth curves. S.D. designed and advised on all GBS experiments. F.P. designed, performed and analysed, together with K.S., the mouse experiments and generated Fig. 6 and Supplementary Fig. 6. V.M. provided consultation on the infection model and P.S. R.M. supervised and advised on mouse experiments and analysis. C.S. generated the EM and SEM data. B.B., S.D. and P.S. wrote the article with input from F.P. and R.M.
