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The gut reaction to traumatic brain injury

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Traumatic brain injury (TBI) is a complex disorder that affects millions of people worldwide. The complexity of TBI partly stems from the fact that injuries to the brain instigate non-neurological injuries to other organs such as the intestine. Additionally, genetic variation is thought to play a large role in determining the nature and severity of non-neurological injuries. We recently reported that TBI in flies, as in humans, increases permeability of the intestinal epithelial barrier resulting in hyperglycemia and a higher risk of death. Furthermore, we demonstrated that genetic variation in flies is also pertinent to the complexity of non-neurological injuries following TBI. The goals of this review are to place our findings in the context of what is known about TBI-induced intestinal permeability from studies of TBI patients and rodent TBI models and to draw attention to how studies of the fly TBI model can provide unique insights that may facilitate diagnosis and treatment of TBI.

Keywords: bacteria, Drosophila, glucose, innate immune response, intestine, mortality, septate junction, TBI, tight junction, traumatic brain injury

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Traumatic brain injury (TBI) is a major health issue.[1,2] According to the US. Centers for Disease Control and Prevention, TBIs result in more than 2 million emergency room visits, 280,000 hospitalizations, and 50,000 deaths each year.[3] In addition, 2.5–6.5 million people are living with TBI-caused disabilities. Despite the name, injuries that are characteristic of TBI are not limited to the brain.[4-7] Non-neurological injuries such as systemic inflammation as well as organ dysfunction involving cardiovascular, respiratory, and gastrointestinal systems often occur minutes to months after initial mechanical injury to the brain and substantially contribute to morbidity and mortality. Therefore, with the eventual goal of developing therapeutic interventions for TBI, it is necessary to understand the metabolic, molecular, and cellular events that underlie non-neurological injuries. Here, we focus on gastrointestinal injuries and highlight how studies using a *Drosophila melanogaster* TBI model can provide unique insights into causal mechanisms and therapies.

TBI Increases Intestinal Permeability in Mammals

Dysfunction of the gastrointestinal tract is a common occurrence in TBI.[4-7] In the first few weeks after injury, most patients with moderate to severe TBI have reduced intestinal contractile activity and absorption, which is manifested by vomiting and abdominal distension. In addition, evidence from humans, rodents, and now from our work in flies indicates that TBI can disrupt the intestinal barrier that normally functions to block the flow of certain ions, solutes, proteins, bacteria, and bacterial products between the inside and outside of the intestine. Severe consequences can result from disruption of the intestinal barrier. For instance, increased intestinal permeability plays a key role in the pathogenesis of Crohn's disease, Celiac disease, and diabetes,[8] and death may result from translocated bacteria that induce a systemic inflammatory response and sepsis with subsequent multiple organ failure.[9,10]

Defective Tight Junctions Underlie Increased Intestinal Permeability Following TBI in Mammals

A primary determinant of intestinal permeability is the extent of opening of

intercellular tight junctions (**Fig. 1**). The intestine is lined with a single layer of epithelial cells that separates the intestinal lumen from extra-intestinal sites.[11] Close contacts formed by tight junctions between adjacent epithelial cells restrict passive paracellular permeability to small molecules such as solvents and solutes with radii up to $\sim 3.5 \times 10^{-4}$ μm.[12] Restricted permeability is mediated by transmembrane proteins Claudins and Occludin that form charge- and size-selective paracellular channels controlled by intracellular scaffolding proteins such as PDZ (PSD-95, Discs-large, ZO-1) domain proteins as well as by myosin light chain kinase (MLCK) regulation of actomyosin contraction and Occludin endocytosis.[12,13] In contrast, active transcellular mechanisms typically regulate permeability to macromolecules such as nutrients (*e.g.*, glucose), proteins, and bacteria and their products (also known as Pathogen-associated molecular patterns (PAMPs)).[14] However, tight junctions are not static structures. Altered expression, post-translational modification, localization, or activity of tight junction proteins or their regulators can change the degree of permeability to macromolecules. For example, increased Occludin endocytosis, which occurs in response to PAMP-triggered expression of the pro-inflammatory cytokine tumor-necrosis factor-α (TNF-α), can increase permeability of tight junctions to macromolecules.[15]

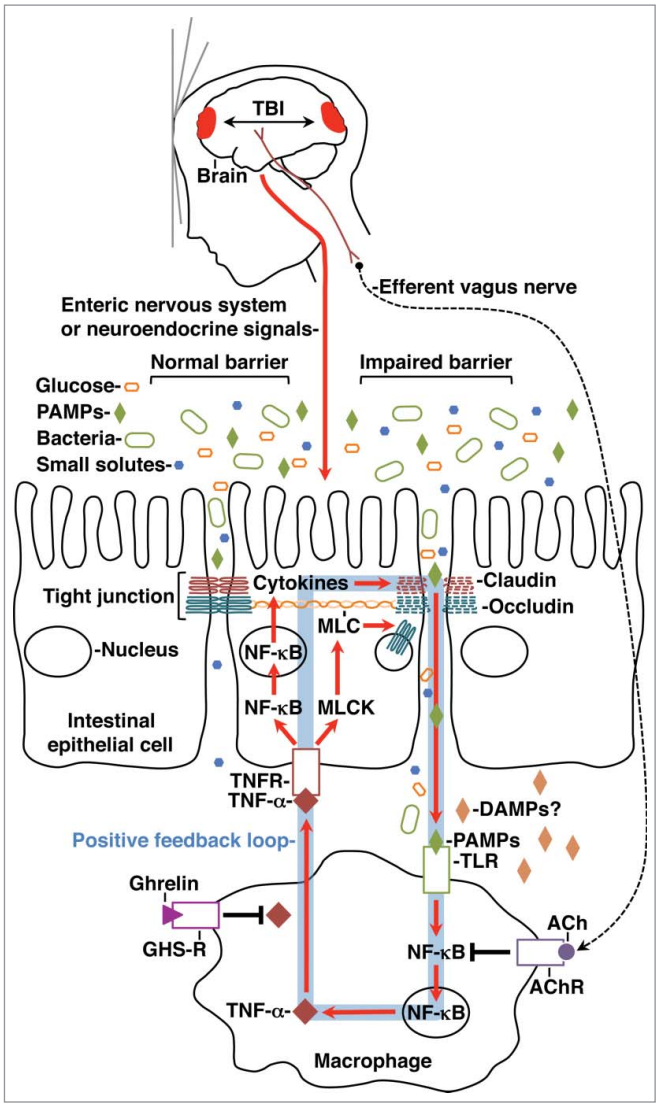

Figure 1. A model describing how TBI might increase intestinal permeability in humans. A detailed description of the model is presented in the text. Discontinuous lines for Claudin and Occludin indicate a molecular change that results in increased tight junction permeability. An Occludin-containing endosome is indicated by the cytoplasmic circle. Red spots in the brain indicate injuries, and the associated double-headed arrow indicates movement of the brain due to impact of the head with a solid object. The question mark after "DAMPs" indicates that the relative roles of PAMPs and DAMPs in this mechanism is not known.

In mammals, increased paracellular permeability appears to be the basis of increased intestinal permeability following TBI. Studies of TBI patients show an increase in the ratio of orally ingested lactulose (a marker of paracellular permeability) to mannitol (a marker of transcellular permeability) in urine.[16,17] The lactulose-mannitol test as well as a dye permeability test also shows that injury inflicted exclusively to the brain of rodents by methods such as controlled cortical impact (CCI) is sufficient to increase paracellular permeability.[18-20] Furthermore, disruption of the intestinal epithelial barrier following TBI in rodents is accompanied by decreased expression of tight junction proteins Occludin and ZO-1 as well increased expression of MLCK in the intestine, providing additional support for a paracellular mechanism.[20,21] TBI-induced gaps in intestinal tight junctions must be at least 0.25 μm (*i.e.*, the minimum dimension of *E. coli*, which is ~700 times larger than the normal gap in tight junctions) to account for the observed translocation of commensal bacteria to blood plasma and organs such as lungs.[22] Thus, rodent studies establish a direct cause-effect relationship between brain injury and increased intestinal permeability. The intermediary between brain injury and intestinal injury is yet to be resolved, but current evidence points to the enteric nervous system and neuroendocrine signals, both of which control the function of the gastrointestinal system.[23,24]

TBI Activates a Feedback Loop that Enhances Intestinal Permeability in Mammals

TNF-α is a central player in the tight junction-mediated mechanism that increases intestinal permeability following TBI in mammals (**Fig. 1**). Treatment of

mice with the hormone ghrelin following TBI blocks the increase in TNF-α and MLCK expression along with the increase in intestinal permeability.[21] Similarly, electrical stimulation of the vagus nerve before TBI in mice,[25,26] increasing glutamine levels in the diet following TBI in rats,[27,28] or injection of the antioxidant-sedative propofol following TBI in rats[29] blocks the increase in TNF-α expression as well as the increase in intestinal permeability following TBI. Conversely, mice deficient for nuclear factor erythroid 2-related factor 2 (Nrf2) have higher TNF-α expression and higher intestinal permeability than wild-type mice following TBI.[30] Ghrelin, vagus nerve stimulation, glutamine, propofol, and Nrf2 use different mechanisms to regulate TNF-α. For example, ghrelin binds the growth hormone secretagogue receptor (GHS-R) on immune cells and inhibits PAMP-induced release of TNF-α,[31] and vagus nerve stimulation promotes the release of acetylcholine (ACh) by efferent vagus nerves to inhibit the production of TNF-α by acetylcholine receptor (AChR)-expressing immune cells.[32]

TNF-α expression in mammals is induced by Toll-like receptor (TLR) signaling pathways in macrophages and other cytokine-producing cells that are part of the defense mechanism provided by the innate immune response.[33] Activation of TLRs by PAMPs turns on the NF-κB transcription factor, which promotes TNF-α transcription. Secreted TNF-α then binds TNF receptors (TNFRs) on intestinal epithelial cells and activates several pathways, including an NF-κB pathway that upregulates genes encoding pro-inflammatory cytokines such as TNF-α, interleukin 1β (IL-1β), and IL-6, which is sufficient to enhance tight junction permeability in the intestine.[33,34] So, a positive feedback loop, anchored by PAMPs and TNF-α, disrupts intestinal barrier function following TBI (**Fig. 1**). Alternatively, the positive feedback loop might be anchored by damage-associated molecular patterns (DAMPs), molecules such as nucleic acids that are released from apoptotic and necrotic cells following TBI.[35-37] DAMPs function similarly to PAMPs. They are recognized by TLRs, turn on NF-κB transcription factors, and promote TNF-α transcription. However, the relative contribution of PAMPs and DAMPs to disruption of the intestinal barrier following TBI in mammals is yet to be determined.

TBI Increases Intestinal Permeability in Flies

Our recent publication implicates increased intestinal permeability in the non-neurological injury cascade that causes flies to die from TBI.[38] This work builds upon our prior publications that describe the development and initial characterization of a fly TBI model that uses a spring-loaded device to inflict closed-head TBI in adult flies.[39,40] We found that one of the consequences of TBI is death within 24 hrs of the initial injury and that genotype plays a major role in determining the percentage of flies that die within 24 hrs, which we call the mortality index at 24 hrs (MI_{24}).[38] Through Genome-wide association study (GWAS) analysis of many wild-type fly lines, we identified single-nucleotide polymorphisms (SNPs) in three epithelial barrier-related genes, *big bang* (*bbg*), *scribble* (*scrib*), and *grainyhead* (*grh*), as being significantly associated with the MI_{24}. *bbg* encodes a PDZ domain protein that regulates the barrier function of septate junctions,[41] structures in invertebrates that are functional analogs of tight junctions in vertebrates.[42-44] Intestines of *bbg* mutants have wider paracellular gaps and greater permeability to pathogenic bacteria than wild-type flies.[41] *scrib* also encodes a PDZ domain protein that functions in the formation of septate junctions.[45,46] Lastly, *grh* and its mammalian orthologs encode transcription factors that regulate expression of septate junction and tight junction genes, respectively.[47-49] Our data suggest that flies carrying SNPs in *bbg, scrib*, or *grh*, produce septate junctions with altered sensitivity to disruption by mechanisms induced by TBI.

Moreover, we found that TBI disrupts the intestinal barrier. Our data show that, following TBI, previously ingested blue dye leaks from the intestine into the hemolymph (the circulatory fluid of flies) and travels throughout the fly producing a 'Smurf' phenotype.[38,50] In all fly lines tested, including those with extremely low or extremely high MI_{24}s, there is an almost perfect correlation between flies that Smurf and flies that die within 24 hrs following TBI, indicating that genetic variation plays a critical role in determining the magnitude of intestinal permeability following TBI. Additionally, these data suggest that death within 24 hrs is caused by factors that leak from the intestine. This conclusion is consistent with the finding that increased intestinal permeability, as assayed by Smurfing, serves as a marker of impending death caused by normal aging processes.[51]

Bacteria Do Not Affect Death-Causing Intestinal Permeability Following TBI in Flies

Surprisingly, we found that while commensal bacteria translocate from the intestine to the hemolymph within 1 hr following TBI, they do not appear to contribute to mortality within 24 hrs.[38] Bacteria-free flies that we generated by feeding flies a cocktail of antibiotics have the same MI_{24} and SI_{24} (Smurfing index at 24 hrs) as bacteria-containing flies. These findings appear to contradict the findings from rodent TBI models, which indicate that PAMPs are integral to the positive feedback mechanism that enhances intestinal permeability (**Fig. 1**). However, the apparently different role for PAMPs in fly and rodent TBI models may be explained by differences in the nature of the initial injury: in the fly TBI model, TBI occurs in the context of polytrauma and is severe enough to cause death, whereas in rodent TBI models, the initial injury is restricted to the brain and is not severe enough to cause death. So, DAMPs may play a larger role in the fly TBI model than the rodent TBI models. Alternatively, in both fly and rodent TBI models, PAMPs play a negligible role relative to DAMPs in enhancing intestinal permeability immediately following TBI, and the relative contributions of these mechanisms is only noticeable when PAMPs are eliminated (**Fig. 2**).

A negligible role for PAMPs in death following TBI does not mean that they are irrelevant to the pathophysiology of

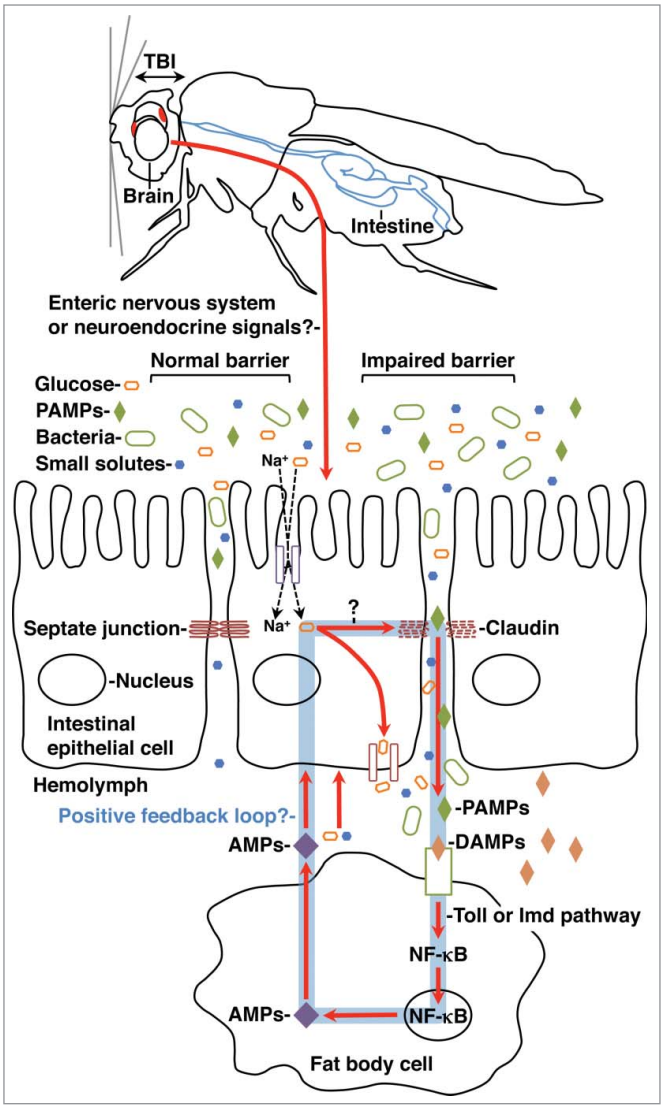

Figure 2. A model describing how TBI might increase intestinal permeability and cause death in flies. A detailed description of the model is presented in the text. "Claudin" refers to Claudin-like Drosophila proteins Kune-kune, Megatrachea, and Sinuous[75] Red spots in the brain indicate injuries, and the associated double-headed arrow indicates movement of the brain due to impact of the head with a solid object. The foregut is not included in the diagram of the fly. AMPs are not only expressed by fat body cells but also by other cells, including intestinal epithelial cells. Question marks indicate events that are presumed to occur but the mechanisms for which are unknown. Major differences between the fly model and the human model (**Fig. 1**) are roles for glucose and DAMPs (rather than PAMPs) in the positive feedback loop that increases intestinal permeability following TBI.

TBI. In fact, we found that following TBI in some fly lines, commensal bacteria significantly contribute to activation of the innate immune response,[38] most likely through the Toll and Immune deficiency (Imd) pathways, which are analogous to the TLR and TNF-α pathways, respectively, in mammals.[52] The Toll and Imd pathways use NF-κB proteins to activate the expression of antimicrobial peptide (AMP) genes,[53] which encode proteins that are cytotoxic to bacteria as well as host cells through their ability to cause membrane permeabilization.[54] Work by us and others has shown that AMP expression is associated with progressive neurodegeneration in fly models of human neurodegenerative diseases[55-58] and that brain-specific expression of AMPs is sufficient to cause neurodegeneration in flies.[59] Excessive expression of AMPs as well as TNF-α in mammals is also associated with neurodegenerative diseases.[60,61] Thus, following TBI, PAMPs that leak from the intestine activate similar molecular pathways in flies and mammals and induce the expression of proteins that may produce long-term deleterious consequences.

Glucose Ingested After TBI Increases Death-Causing Intestinal Permeability in Flies

Remarkably, we found that feeding flies water rather than standard molasses food immediately after TBI significantly reduces the percent death within 24 hrs, with some fly lines having a greater than 50% reduction in percent death within 24 hrs.[38] Thus, indigestion of a component of molasses food immediately after TBI substantially enhances the death-causing mechanism. Our data indicate that glucose is the component. Glucose levels in the hemolymph are significantly increased during the 24 hr period following TBI, indicating that TBI causes glucose to leak from the intestine (**Fig. 2**). Furthermore, feeding flies glucose rather than water immediately after TBI is sufficient to significantly increase the percent death within 24 hrs, indicating that glucose stimulates the mechanism that increases intestinal permeability and death following TBI. Thus, glucose may be a key component of an uncharacterized positive feedback loop that increases intestinal permeability following TBI.

Studies of mammalian systems shed some light on the mechanism by which glucose may increase intestinal permeability. Under high glucose (*i.e.*, hyperglycemic) conditions, transcellular Na^+-glucose cotransport causes an increase in paracellular permeability of tight junctions through phosphorylation of MLC by MLCK and reduced expression of Occludin and ZO-1.[62,63] A transcellular mechanism may also be involved in flies, since we found that a SNP in a gene *CG7882* predicted to carry out transcellular glucose transport is significantly associated with the MI_{24}.[38] *CG7882* encodes a fly ortholog of human solute carrier transporter 2

(SLC2) family proteins that are required for glucose transport in the intestine.[64] Furthermore, hyperglycemia is characteristic of TBI in humans. Patients with severe TBI have higher blood glucose levels than patients with moderate or mild TBI.[65-69] Moreover, hyperglycemia is predictive of death following TBI,[70-72] and patients with diabetes are at increased risk of death following TBI.[73,74] It remains to be determined whether hyperglycemia causes death following TBI and if so how. Glucose could directly feed into a toxic metabolic pathway or glucose could indirectly cause toxicity by facilitating paracellular leakage of a toxic molecule from the intestine (**Fig. 2**).

Future Directions

Our study indicates that cellular and molecular mechanisms underlying TBI-induced disruption of the intestinal epithelial barrier are conserved from humans to flies. This conserved mechanism provides the opportunity to use the fly experimental toolbox to make discoveries that could improve the diagnosis and treatment of TBI in humans. Our GWAS analysis has identified several potential players in the mechanisms, but much remains to be learned. By screening for mutations that modify the percentage of flies that die within 24 hrs following TBI, we should be able to address key questions such as what are the signals from the injured brain that trigger increased intestinal permeability, what role does glucose play in the TBI-induced intestinal permeability mechanism, why does increased intestinal permeability following TBI cause death, and does bacteria-dependent activation of the innate immune response following TBI cause neurodegeneration.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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