

Chapter 10

Roles of Drug Transporters in Blood-Retinal Barrier



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Abstract Blood-retinal barrier (BRB) includes inner BRB (iBRB) and outer BRB (oBRB), which are formed by retinal capillary endothelial (RCEC) cells and by retinal pigment epithelial (RPE) cells in collaboration with Bruch's membrane and the choriocapillaris, respectively. Functions of the BRB are to regulate fluids and molecular movement between the ocular vascular beds and retinal tissues and to prevent leakage of macromolecules and other potentially harmful agents into the retina, keeping the microenvironment of the retina and retinal neurons. These functions are mainly attributed to absent fenestrations of RCECs, tight junctions, expression of a great diversity of transporters, and coverage of pericytes and glial cells. BRB existence also becomes a reason that systemic administration for some drugs is not suitable for the treatment of retinal diseases. Some diseases (such as diabetes and ischemia-reperfusion) impair BRB function via altering tight junctions, RCEC death, and transporter expression. This chapter will illustrate function of BRB, expressions and functions of these transporters, and their clinical significances.

Keywords Blood-retinal barrier · Transporters · Retinal capillary endothelium · Retinal pigment epithelium · Diabetic retinopathy

10.1 General Introduction

The retina has a unique position in that blood-retinal barrier (BRB) separates the retina from the circulating blood. The BRB regulates fluids and molecular movement between the ocular vascular beds and retinal tissues, prevents leakage of macromolecules and other potentially harmful agents into the retina, and keeps the microenvironment of the retina and retinal neurons. BRB existence is also a reason that

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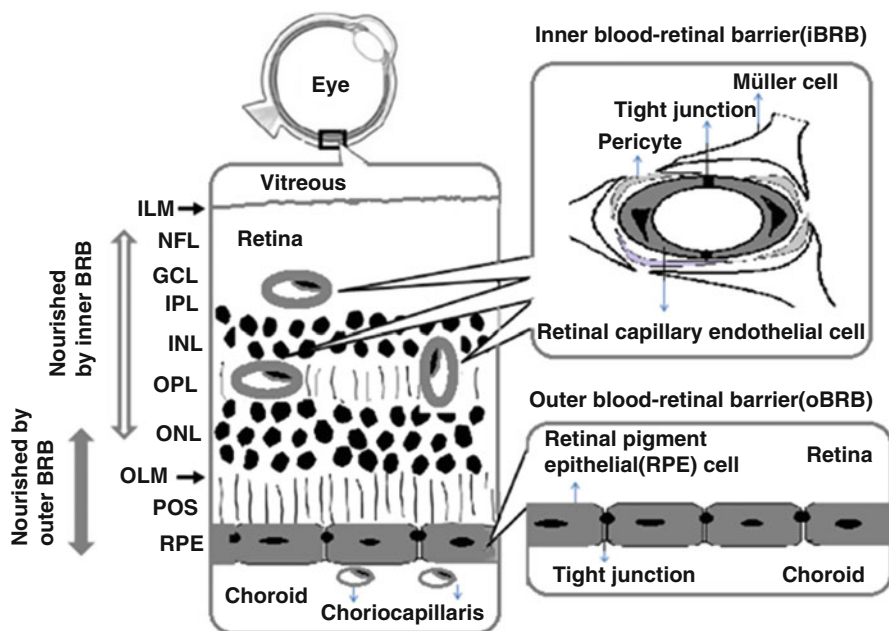


Fig. 10.1 Schematic diagram of blood-retinal barrier (BRB). Symbol: *ILM* inner limiting “membrane,” *NFL* nerve fiber layer, *GCL* ganglion layer, *IPL* inner plexiform, *INL* inner nuclear layer, *OPL* outer plexiform, *ONL* outer nuclear layer, *OLM* outer limiting “membrane,” *POS* photoreceptor outer segments

systemic drug administration is not suitable for the treatment of retinal diseases. The BRB includes inner BRB (iBRB) and outer BRB (oBRB), which are formed by retinal capillary endothelial cells (RCECs) and by retinal pigment epithelial (RPE) cells in collaboration with Bruch’s membrane and the choriocapillaris, respectively (Fig. 10.1). It is well known that two third of the human retina is nourished by retinal capillaries via the iBRB and the remainder is attributed to choriocapillaris via the oBRB (Hosoya and Tomi 2005; Kur et al. 2012).

The paracellular and transcellular transport across BRB are generally involved in the following five different mechanisms (Fig. 10.2) (Rizzolo et al. 2011):

1. Paracellular diffusion: Paracellular diffusion is mainly regulated by the tight junction. Tight junctions, boundaries between the apical and basolateral plasma membrane domains, are considered to be essential for the integrity of tissue barrier and the maintenance of cell polarity, which restrict paracellular movement of fluids and molecules between the blood and retina.
2. Facilitated diffusion: Transporters expressed in the plasma membrane allow the passage of preferred solutes across the monolayer along with a concentration gradient. An example is glucose transport via glucose transporter 1 (GLUT1).

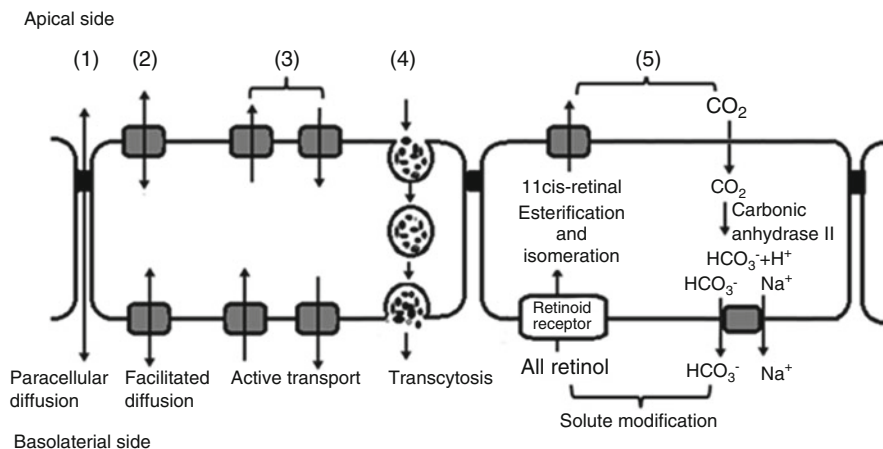


Fig. 10.2 Mechanisms for the transepithelial transport of solutes in the BRB

- Active transport: Transporters expressed in the plasma membrane consume ATP to move solutes against a concentration gradient or establish electrochemical gradients that drive vectorial transport through antiporters and cotransporters.
- Transcytosis: Vesicles can invaginate and bud from the apical or basal membrane, traverse the cell, and fuse with the opposite membrane to release their contents on the opposite side of the cell. Normal BRB lacks transcytosis, which become a reason limiting transcellular passage (Chow and Gu 2017).
- Solute modification: During transport, solutes can be degraded or transformed into something else. For example, in RPE, retinol enters the basal side of the RPE by receptor-mediated endocytosis and is delivered to microsomes, where retinol is transformed into cis-retinal. The cis-retinal transports across the monolayer and is endocytosed by photoreceptors and bound to opsin. Another example is CO_2 . CO_2 is converted to $\text{HCO}_3^- + \text{H}^+$ as it is transported from the apical to the basal side of the monolayer.

10.1.1 The Inner Blood-Retinal Barrier (iBRB) and Outer Blood-Retinal Barrier (oBRB)

The iBRB is structurally similar to the blood-brain barrier (BBB). The RCECs connected by tight junctions are covered with pericytes and glial cells (Muller cells or astrocytes) (Cunha-Vaz et al. 2011). The iBRB is formed by the inner or outer capillary beds. The inner capillary bed lies in the ganglion nerve cell layer, and the iBRB function is induced by astrocytes. The outer capillary bed lies in the inner and outer plexiform layers, where function of BRB is regulated by Müller cells (Rizzolo et al. 2011).

The oBRB is established by RPE cells connected by tight junctions. RPE is a monolayer of pigmented cells situated between the neuroretina and the choroids. The apical membrane of RPE exhibiting long microvilli faces the light-sensitive outer segments of the photoreceptors cells, while its basolateral membrane faces the Bruch's membrane, which separates the neural retina from the fenestrated endothelium of the choriocapillaris. It is different from the epithelium of the choroid plexus and other transporting epithelia that the apical membrane of RPE cells abuts a solid tissue rather than a lumen. Moreover, the transepithelial electrical resistance of RPE shows large species differences ranging from 135 to 600 $\Omega \times \text{cm}^2$ (Rizzolo et al. 2011).

The main functions of the RPE (Kay et al. 2013; Simó et al. 2010; Willermain et al. 2014a) are to (1) transport nutrients, ions, and water or waste products; (2) absorb light and protect against photooxidation; (3) reisomerize all-*trans*-retinal into 11-*cis*-retinal, which is a key element of the visual cycle; (4) phagocyte shed photoreceptor membranes; (5) release K^+ into the subretinal space to maintain constant excitability of the photoreceptors; (6) secrete growth factors such as pigmented epithelium-derived factor (PEDF) at the apical side and vascular endothelial growth factor (VEGF) at the basolateral side for the structural integrity of the retina; and (7) maintain the immune privilege of the eye due to its outer BRB function but also by interfering with signaling pathways coordinating the immune system.

10.1.2 Tight Junctions in the BRB

Tight junctions consist of specialized proteins such as occludins, claudins, and zonula occludens, which play an important role in maintaining the barrier function via regulating the transport of solutes and molecules across RCEC or RPE cell layers. Both the iBRB and the oBRB cells have tight junctions, but they differ in their organization and composition. Tight junctions of the oBRB are more concentrated at the apical side of the cell, whereas tight junctions of the iBRB are more dispersed between adherens junctions and gap junctions. The adherens junctions consist of vascular endothelial-cadherin and its associated proteins, such as catenins and plakoglobin, both of which are linked to the cytoskeleton (Dejana et al. 2008). The cell-cell contacts also contain additional adhesion molecules, such as platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule 2 (ICAM2), endoglin, and other clusters (Dejana et al. 2008).

10.1.3 Astrocytes, Müller Cells, and Pericytes

Astrocytes, Müller cells, and pericytes, closely connected to blood vessels in the retina, are considered to influence activities of the BRB via secreting regulatory signal molecules such as glial cell line-derived neurotrophic factor (GDNF), transforming growth factor beta 1 (TGF- β 1), and VEGF (Igarashi et al. 2000;

Abukawa et al. 2009; Le 2017; Wisniewska-Kruk et al. 2012). In immortalized rat retinal capillary endothelial (TR-iBRB2), an *in vitro* model cell line of the iBRB, it was found that incubation with conditioned medium of Müller (TR-MUL5) cells increased alkaline phosphatase activity, induced expression of plasminogen activator inhibitor 1 (PAI-1) gene, and suppressed expression of an inhibitor of DNA binding 2 gene. TGF- β 1, secreted from TR-MUL5 cells, showed similar effects, indicating that paracrine interactions occur between TR-iBRB2 and TR-MUL5 cells via secreting TGF- β 1 (Abukawa et al. 2009). Müller cells are also involved in the regulation of retinal iron homeostasis. Loss of Müller cells was reported to break down the BRB and increase iron levels throughout the neurosensory retina of mice (Baumann et al. 2017). Müller cell loss or dysfunction often occurs in patients with macular telangiectasia type 2 (MacTel2) and diabetic retinopathy, which may explain the clinical findings that a patient with MacTel2 and a patient with diabetic retinopathy had the increased iron levels in RPE or neurosensory retina (Baumann et al. 2017). TGF- β was reported to impair function of BRB via stimulating metalloproteinases (MMPs) from Müller cells, later degrading tight junction protein occludin (Behzadian et al. 2001). Moreover, interactions between endothelial cells and pericytes or astrocytes also increase the BRB properties through enhancing expression of tight junctions (Kim et al. 2009).

The pericytes play important roles in regulating vascular tone, secreting extracellular material, and being phagocytic. The frequency of pericyte coverage on human retinal capillaries was reported to be high up to 94.5%, substantially greater than that of human choriocapillaris (11%) (Chan-Ling et al. 2011), demonstrating that retinal microvasculature characterizes a uniquely high density of pericytes. The communication between pericytes and endothelial cells is mediated by diverse molecules such as angiopoietin, TGF- β 1, platelet-derived growth factor- β (PDGF- β), and sphingosine-1-phosphate. In endothelial cells, genetic deletion of PDGF- β (Park et al. 2017) or blocking PDGF receptor beta using antibody (Ogura et al. 2017) could lead to severe vascular impairments such as vascular engorgement, leakage, severe hemorrhage, retinal detachment, and severely impaired pericyte coverage of the vessels. Moreover, pericyte loss also promotes pathologic angiogenesis. In consistence, pericyte dropout or loss in the retina is considered to be one of the earliest pathological changes in diabetic retinopathy. Moreover, hyperglycemia, advanced glycation end products, basement membrane thickening, and hypertension trigger pericyte apoptosis and dropout, all of which become reasons leading to diabetic retinopathy (Eshaq et al. 2017).

10.2 Major Drug Transporters in the Retina

Several transporters, including the solute carrier (SLC) family and the ATP-binding cassette (ABC) family have been identified in the retina (Fig. 10.3). The SLC transporters utilize facilitated diffusion, or they couple an ion or electrochemical

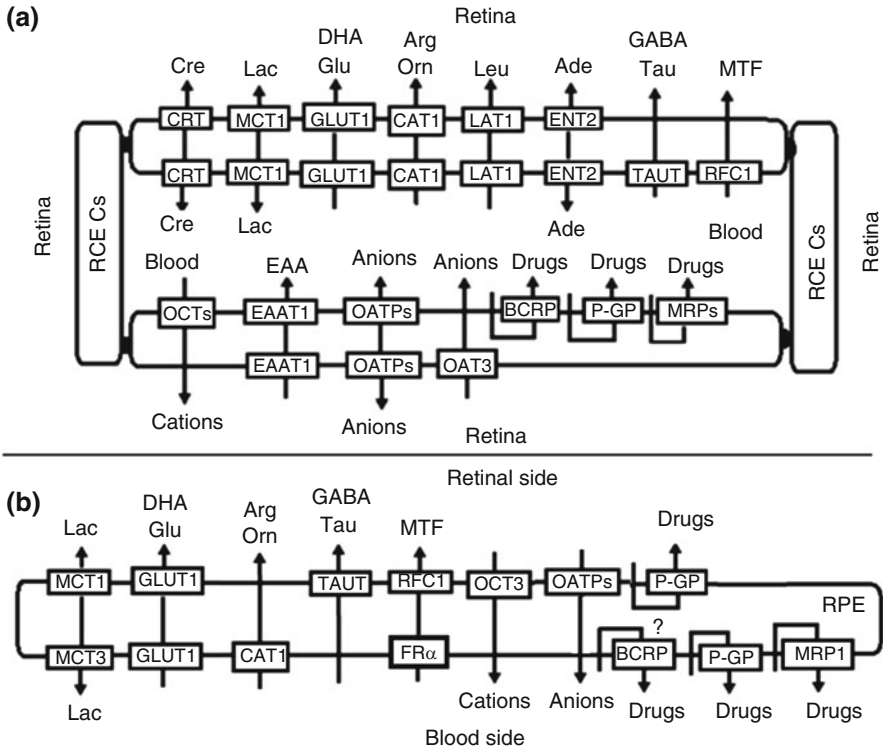


Fig. 10.3 Hypothetical localization and physiological function of several transporters in the iBRB (a) and the oBRB (b). *Ade* adenosine, *Arg* L-arginine, *Cre* creatine, *DHA* dehydroascorbic acid, *EAA* excitatory amino acid, *GABA* gamma-aminobutyric acid, *Glu* glucose, *Lac* lactate, *Leu* L-leucine, *MTF* methyltetrahydrofolate, *Orn* L-ornithine, *RCECs* retinal capillary endothelial cells, *RPE* retinal pigment epithelial (RPE) cells, *Tau* taurine

gradient to transfer their substrates across the cell membrane. ABC transporters, on the other hand, use ATP as the energy source to drive the transport.

In the retina, neuronal cells, including photoreceptor cells, require a large amount of metabolic energy for phototransduction and neurotransduction metabolic substrates, such as D-glucose, amino acids, vitamins, and nucleosides. These compounds are hydrophilic, and their transport is often mediated by influx transporters, belonging to SLC family. The identified influx transporters in the retina include glucose transporter 1 (GLUT1), Na⁺-dependent multivitamin transporter (SMVT), taurine transporter (TAUT), cationic amino acid transporter 1 (CAT1), excitatory amino acid transporter 1 (EAAT1), L-type amino acid transporter 1 (LAT1), creatine transporter (CRT), nucleoside transporters, and monocarboxylate transporters (MCTs). A series of influx transporters for drugs such as organic cation transporters (OCTs), organic anion transporting polypeptides (OATPs), and organic anion transporters (OATs) have been also identified in the retina.

10.2.1 *Influx Transporters*

10.2.1.1 Glucose Transporter 1 (GLUT1/SLC2A1)

D-glucose is the main energy source for the retina, whose transport from the blood to the retina is mainly mediated by GLUT1 (Tomi and Hosoya 2004). GLUT1 is mainly localized at both luminal and abluminal membranes of the iBRB and the oBRB, although the expression of GLUT1 at abluminal membrane of the iBRB is approximately two- to threefold greater than that at its luminal membrane (Fernandes et al. 2003). The asymmetrical distribution of GLUT1 at the iBRB suggests that D-glucose transport is limited at the blood-to-luminal rather than the abluminal-to-interstitial interface. GLUT1 is also expressed in Müller cells (Hosoya et al. 2008a). In addition to hexoses, GLUT1 also transports dehydroascorbic acid from blood to the retina, where dehydroascorbic acid is converted to ascorbic acid. The uptake of dehydroascorbic acid by GLUT1 may be completely inhibited under diabetic conditions due to high glucose concentration (Minamizono et al. 2006).

10.2.1.2 Taurine Transporter (TAUT/SLC6A6)

Taurine, functioning as osmolyte and antioxidant, is the most abundant free amino acid in the retina, accounting for more than 50% of the free amino acid content in the rat retina, which is considered to be essential for maintenance of retinal structure. Taurine transport across BRB is mainly mediated by TAUT (Tomi et al. 2007). TAUT, an Na^+ - and Cl^- -dependent transporter, is mainly expressed in apical membrane of RPE, ganglion cells, Müller cells (El-Sherbeny et al. 2004), and RCECs (Tomi et al. 2007, 2008). Some TAUT inhibitors such as β -alanine and hypotaurine may inhibit taurine transport across BRB. TAUT also mediates transport of β -alanine and gamma-aminobutyric acid (GABA) (Tomi et al. 2008; Usui et al. 2013). Hyperosmolar conditions could stimulate activity of TAUT, leading to increase in V_{\max} of taurine without affecting K_m (El-Sherbeny et al. 2004; Yahara et al. 2010). Both in vivo and in vitro data demonstrated that taurine itself enhanced expression of retinal TAUT (Zeng et al. 2010). High glucose was reported to downregulate expression of retinal TAUT (Lee and Kang 2013; Stevens et al. 1999), which was attenuated by taurine treatment (Zeng et al. 2010). In consistence with this issue, 8-week and 12-week diabetic rats demonstrated significantly lower levels of retinal TAUT, and taurine treatment completely reversed the decreased expression of retinal TAUT by diabetes (Zeng et al. 2010). Similarly, TAUT deficiency was reported to lead to severe retinal degeneration in mice (Heller-Stilb et al. 2002). All these results demonstrated important roles of retinal TAUT in normal retinal development.

10.2.1.3 Cationic Amino Acid Transporter 1 (CAT1/SLC7A1)

L-arginine is the precursor molecule for the synthesis of nitric oxide (NO) by nitric oxide synthase (NOs). L-arginine transport from the blood to the retina across BRB is mediated by CATs. CAT1 is highly expressed at both luminal and abluminal membrane of RCECs, TR-iBRB2 cells, the basal membrane of PRE (Kubo et al. 2015; Tomi et al. 2009), and immortalized rat retinal pericyte cell line (TR-rPCT1 cells) (Zakoji et al. 2015). QT-PCR analysis showed that levels of CAT1 mRNA in TR-iBRB2 and the isolated rat RCECs were 25.9- and 796-fold greater than that of CAT3, respectively. CAT1-specific small interfering RNA, L-arginine, and L-lysine inhibited [³H]L-arginine uptake in TR-iBRB2 cells (Tomi et al. 2009). Moreover, high glucose exposure significantly inhibited L-arginine transport in TR-iBRB, and simvastatin might reverse high glucose-induced-alterations via increasing expression of endothelial NOS mRNA and NO production (Tun and Kang 2017).

L-ornithine is a cationic amino acid produced in the urea cycle and ingested from the diet. Clinical trials have suggested the beneficial effects of L-ornithine in the body, but long-term treatment or high concentrations of L-ornithine in the blood induce retinal toxicity, forming gyrate atrophy of the choroid and retina (Hayasaka et al. 2011), an autosomal recessive disease due to the genetic defect of ornithine aminotransferase. Both in vivo and in vitro data demonstrated that CAT1 mediates transport of [³H]L-ornithine across the BRB (Kubo et al. 2015). In RPE cells, it was reported that the basal-to-cell uptake of [³H]L-ornithine was greater than that of the apical-to-cell uptake, and the basal-to-cell transport was inhibited by L-ornithine, suggesting the involvement of CAT1 in the blood-to-cell transport of L-ornithine across the basal membrane of the oBRB (Kubo et al. 2015). Moreover, in human telomerase reverse transcriptase-RPE cells, CAT1 siRNA decreased both L-[¹⁴C] ornithine uptake and L-ornithine cytotoxicity (Kaneko et al. 2007). All these results indicate that reduction of the ornithine transport via inhibiting CAT1 may be a new target for treatment of gyrate atrophy.

10.2.1.4 L-Type Amino Acid Transporter 1 (LAT1/SLC7A5)

L-type amino acid transporters (LATs) prefer branched-chain and aromatic amino acids. LAT1 (SLC7A5) and LAT2 (SLC7A8) mRNAs were reported to be expressed in cultured human RPE cell line (ARPE-19 cells), but the level of LAT1 mRNA was 42-fold higher than that of LAT2 (Yamamoto et al. 2010). LAT1, an Na⁺-independent transporter, was also identified in TR-iBRB2, isolated rat RCECs, and primary cultured human RCECs (Tomi et al. 2005; Usui et al. 2013; Yamamoto et al. 2010), mediating blood-to-retina transport of large neutral amino acids including L-leucine (Tomi et al. 2005), L-histidine (Usui et al. 2013), and L-phenylalanine (Atluri et al. 2008). Some drugs such as L-dopa, melphalan, alpha-methyl-dopa, and gabapentin are substrates of LAT1 and LAT2 (del Amo et al. 2008), indicating the roles of LAT1 in retinal disposition of these drugs.

10.2.1.5 Creatine Transporter (CRT/SLC6As)

Creatine and phosphocreatine are required to maintain ATP needed for normal retinal function and development. Creatine is transported from the blood to the retina against the creatine concentration gradient via creatine transporter (CRT), an Na^+ - and Cl^- -dependent transporter. The creatine transport from the blood to the retina is considered to be a major pathway for supplying creatine to the retina although local creatine is preferentially synthesized in the glial cells (Tachikawa et al. 2007; Nakashima et al. 2005a). In the inner retina, CRT is expressed in cells of intense metabolic activity, such as photoreceptors and selected cells, but not in glial cells (de Souza et al. 2012). CRT is also expressed at both the luminal and abluminal membranes of the iBRB (Nakashima et al. 2004). CRT expressed in the luminal membrane would mediate creatine supply to the retina, and CRT in the abluminal membrane may be involved in the metabolite uptake of creatine (Nakashima et al. 2004).

10.2.1.6 Monocarboxylate Transporters (MCTs/SLC16As)

The retina produces more L-lactic acid aerobically than any other tissues. In addition to D-glucose, L-lactic acid appears to be required as an energy source in photoreceptors. Transports of L-lactic acid and other monocarboxylates (such as pyruvate and the ketone bodies across cellular membranes) are facilitated by specific monocarboxylate transporters (MCTs). Four MCTs (including MCT1, MCT2, MCT3, and MCT4, encoded by SLC16A1, SLC16A7, SLC16A8, and SLC16A3, respectively) have been identified in the retina (Bergersen et al. 1999; Gerhart et al. 1999; Philp et al. 1998). MCT1 is detected on four retinal cell types: RPE, photoreceptor cells, Müller cells, and endothelial cells (Gerhart et al. 1999). MCT1 is mainly located in the apical membrane of RPE and in both the luminal and abluminal plasma membranes of RCECs (Bergersen et al. 1999; Gerhart et al. 1999). MCT2 is only found to be abundantly expressed on the inner (basal) plasma membrane of Müller cells and by glial cell processes surrounding retinal microvessels (Gerhart et al. 1999). High expression of MCT4 is only detected in the RPE of younger animals, but RPE of adult animals only show very weak expression (Bergersen et al. 1999). MCT3 is preferentially expressed in the basolateral membrane of the RPE (Philp et al. 1998, 2001), forming a heteromeric complex with the accessory protein CD147 (Philp et al. 2001). The absence of *Mct3* was reported to impair expression of CD147 from the basolateral but not apical RPE of mice. Moreover, the amount of L-lactate in retinal of *Mct3*^{-/-} mice was approximately four times higher than that from the wild-type retinas, accompanied by decreases in the magnitude of the light suppressible photoreceptor current (Daniele et al. 2008). These results demonstrate the pivotal roles of MCT3 in regulating the ionic composition of the outer retina.

MCTs also accept monocarboxylic acid drugs such as moxifloxacin (Barot et al. 2014) and nicotinate (Tachikawa et al. 2011) as their substrates, indicating that

MCT-mediated transport at BRB is a possible route for delivery of monocarboxylic acid drugs to the retina (Hosoya et al. 2001).

10.2.1.7 Nucleoside Transporters

Adenosine is an important intercellular signaling molecule, showing a number of roles in retinal neurotransmission. Most of the adenosine in the retinal interstitial fluid originates from the catabolism of adenosine monophosphate, which is localized in the innermost process of Müller cells. Thus, almost all of the retinal adenosine is distributed in the neighborhood of the innermost process of Müller cells in the ganglion cell layer, inner plexiform layer, and inner nuclear layer. Adenosine in the blood may penetrate the iBRB via adenosine transport systems. Four transporters including two equilibrative nucleoside transporters (ENT1/ENT2, SLC29A1/SLC29A2) and two concentrative nucleoside transporters (CNT1/CNT2, SLC28A1/SLC28A2) have been identified in the rat retina, although the expression of ENT2 mRNA was reported to be 5.5-fold greater than that of ENT1 mRNA. Among the four transporters, only CNT1 was not detected in TR-iBRB. Adenosine uptake in TR-iBRB2 cells is predominantly mediated by ENT2, which is strongly inhibited by adenosine, inosine, uridine, and thymidine, but neither nitrobenzylmercaptapurine riboside (NBMRP) nor dipyrindamole, characterizing NBMRP- and dipyrindamole-insensitive transport of adenosine. An *in vivo* study suggested that [³H]adenosine transport from the blood to the retina was also significantly inhibited by adenosine and thymidine, demonstrating that ENT2 most likely mediates adenosine transport at the iBRB (Nagase et al. 2006). ENT2 is also expressed in Müller cells, contributing to transport of adenosine and its metabolite hypoxanthine. ENT2 also mediates elimination of hypoxanthine from the retina (Akanuma et al. 2013a). In addition, ENT2 accepts some antiviral or anticancer nucleoside drugs, such as 3'-azido-3'-deoxythymidine, 2'3'-dideoxycytidine, 2'3'-dideoxyinosine, cladribine, cytarabine, fludarabine, gemcitabine, and capecitabine, as preferred substrates (Baldwin et al. 2004; Yao et al. 2001), indicating that ENT2 at the BRB could be a potential route for delivering nucleoside drugs from the circulating blood to the retina.

10.2.1.8 Folate Transport Proteins

Folates, water-soluble vitamins, play an essential role as cofactors for one-carbon metabolism in cells. Most of the folate in the plasma is in the reduced form, methyltetrahydrofolate. Folate transport from blood to the retina across BRB is mediated by some specific transport process. Three transport proteins, folate receptor- α (FR α), reduced folate carrier 1 (RFC1/SLC19A1), and proton-coupled folate transporter (PCFT/SLC46A1), have been described for folate uptake. FR α , a receptor for folate, is expressed in basolateral membrane of PRE and in retinal Müller cells, mediating the influx of its ligand into cells via receptor-mediated endocytosis

(Bozard et al. 2010; Chancy et al. 2000). RFC1, a pH-sensitive transporter, is involved in folate⁻/OH⁻ exchange. RFC1 is highly expressed in TR-iBRB2 cells, isolated rat RCECs, normal mouse RPE, and in cultured human RPE cells (Chancy et al. 2000; Hosoya et al. 2008b). Although both RFC1 and PCFT mRNA are expressed in TR-iBRB2 cells and isolated rat RCECs, the expression level of RFC1 mRNA was reported to be 83- and 49-fold greater than that of PCFT, respectively, indicating that RFC1 probably predominates at the iBRB. In TR-iBRB2 cells, transports of methotrexate, formyltetrahydrofolate and methyltetrahydrofolate are mediated by RFC1 (Hosoya et al. 2008b). RFC1 is also expressed in RPE cells. Importantly, FR α and RFC1 are localized in the basolateral and apical membrane of the RPE, respectively, demonstrating that the two proteins work in a concerted manner to operate the vectorial transfer of folate across RPE from choroidal blood to the retina. Diabetes might downregulate expression and activity of RFC1 in mouse PRE. In ARPE-19 cells, it was reported that 6 h exposure of high glucose (45 mM) led to decreases in methyltetrahydrofolate uptake by 35%, which was consistent with decreases in levels of RFC1 mRNA and protein (Naggar et al. 2002). PCFT mRNA was detected in all tested retinal cells including primary cultures of ganglion, Müller, and RPE cells of the mouse retina and their cell lines (Umapathy et al. 2007), mediating H⁺-coupled transport of folate. In retinal Müller cells, PCFT and FR α are expressed and colocalized in the endosomal compartment, where the two proteins may work coordinately to mediate folate uptake (Bozard et al. 2010).

10.2.1.9 Organic Anion-Transporting Polypeptides (OATPs)

Several OATPs (OATPs for human and Oatps for animal), such as Oatp1a4, Oatp1c1, Oatp4a1, and Oatp1a5, have been detected in rat retina. Oatp1a4 and Oatp1c1 mRNA are predominantly expressed in isolated rat RCECs (Tomi and Hosoya 2004). Oatp1a4 proteins are detected on both the abluminal and luminal membrane of rat RCECs and RPE, but Oatp1a4 protein is preferentially localized on the abluminal membrane of the RCECs and the apical membrane of rat RPE (Akanuma et al. 2013b; Ito et al. 2002). Oatp1c1 protein is expressed in both the abluminal and luminal membrane of the RCECs and is preferentially expressed in the basolateral membrane of rat RPE (Akanuma et al. 2013b; Ito et al. 2002). Oatp1a5 protein is predominantly localized in optic nerve fibers, not in RPE (Gao et al. 2002; Ito et al. 2002). Expressions of Oatp1a4 and Oatp1c1 on two side membranes of BRB indicate their contributions to the transcellular transport of amphipathic organic anions including digoxin and [³H]estradiol 17- β glucuronide (E17 β G) across the BRB in both the blood-to-retina and retina-to-blood directions. It was found that elimination rate constant of [³H]- E17 β G from the vitreous humor of rats was 1.9-fold greater than that of [¹⁴C]D-mannitol. The efflux transport of E17 β G from rat retina was significantly inhibited by organic anions probenecid, sulfobromophthalein, digoxin, and dehydroepiandrosterone sulfate (Katayama et al. 2006). Oatp4a1 is also expressed in the RPE, inner and outer nuclear layers,

ganglion cell layer, and nerve fiber layer of rat eyes. In the cultured rat RPE cells, it was found that uptake of triiodothyronine, a known substrate of Oatp4a1, was significantly inhibited by OATP inhibitor sulfobromophthalein, indicating roles of OATPs in the transport of thyroid hormone in the retina (Ito et al. 2003).

Human OATP1A2 is expressed in photoreceptor bodies, somas of amacrine cells and RPE, and mediates the cellular uptake of all-trans-retinol (Chan et al. 2015; Gao et al. 2015), inferring roles of OATP1A2 in canonical visual cycle. The OATP1A2-mediated uptake of all-trans-retinol may be inhibited by chloroquine and hydroxychloroquine (Xu et al. 2016), which may provide novel insights into retinal dysfunction induced by certain drugs. For instance, digoxin and antimalarial drugs (chloroquine and hydroxychloroquine) have induced retinopathy (Kinoshita et al. 2014; Weleber and Shults 1981; Yaylali et al. 2013), which may be partly contributed to decrease in all-trans-retinol uptake into the retinal cells via inhibiting retinal OATP1A2 function, resulting in dysfunction of the canonical visual cycle and toxic accumulation of retinoids. Moreover, human OATP1A2 and OATP2B1 are abundantly expressed in retina amacrine neurons containing substance P and vasoactive intestinal peptide. The two peptides are also substrates of OATP1A2 and OATP2B1, demonstrating roles of OATP1A2 and OATP2B1 in reuptake of these neuropeptides released from retinal neurons and in the homeostasis of neuropeptides (Gao et al. 2015).

10.2.1.10 Organic Cation Transporters

Organic cation transporter 3 (Oct3) is detected in mouse RPE and in several cell types of the neural retina, including photoreceptor, ganglion, amacrine, and horizontal cells, where Oct3 participates in the clearance of dopamine, histamine, and neurotoxin 1-methyl-4-phenyl pyridinium (MPP^+) from the subretinal space (Rajan et al. 2000). In cultured ARPE-19 cells, it was found that uptake of typical OCT3 substrate MPP^+ was completely inhibited by several cationic drugs and monoamine neurotransmitters (dopamine and histamine) (Rajan et al. 2000), although an organic cation transporter, functionally similar to plasma membrane monoamine transporter (SLC29A4), was reported to mediate retina-to-blood transport of MPP^+ at BRB (Kubo et al. 2017a).

L-carnitine is essential for the translocation of acylcarnitine esters into mitochondria for β -oxidation of long-chain fatty acids and ATP generation. L-carnitine transport in the retina is mainly mediated by organic cation/carnitine transporter 1/organic cation/carnitine transporter 2 (OCTN1/OCTN2 for human and Octn1/Octn2 for animal). QT-PCR analysis showed that the expressions of Octn2 mRNA in TR-iBRB2 and isolated rat RCECs were 27.3- and 45.9-fold greater than Octn1 mRNA, respectively, indicating that Octn2 predominantly accounts for the transport of acetyl-L-carnitine from the blood to the retina across the iBRB (Tachikawa et al. 2010). In consistence, acetyl-L- $[^3H]$ carnitine uptake in rat retina was significantly suppressed by L-carnitine and acetyl-L-carnitine. In TR-iBRB2 cells, OCTN substrates and inhibitors (as L-carnitine, acetyl-L-carnitine, tetraethylammonium,

quinidine, and betaine) remarkably decreased uptake of L- ^3H carnitine and acetyl-L- ^3H carnitine (Tachikawa et al. 2010), further confirming roles of retina OCTNs in transport of L-carnitine.

Several novel organic cation transporters have been identified. Transport of the cationic drugs clonidine and diphenhydramine at the mouse BRB is mainly attributed to a carrier-mediated system. The transporter for transporting clonidine is an Na^+ -independent proton-antiporter and insensitive to the *trans*membrane potential. The transporter also transports other cationic drugs such as nicotine, tramadol, diphenhydramine, cocaine, verapamil, methadone, and oxycodone (Chapy et al. 2015). Transport of ^3H verapamil across the iBRB is also mediated by a novel organic cation transporter. In vivo data demonstrated that influx transport of verapamil across the BRB was about fivefold higher than that across the BBB. Verapamil (3 mM) and quinidine (10 mM), not pyrilamine (3 mM), slightly increased the retinal uptake of ^3H verapamil. However, these compounds markedly increased brain uptake of ^3H verapamil. Moreover, pyrilamine (40 mM) significantly reduced the retinal uptake index to 72.9% but not for the brain uptake index. These in vivo results clearly demonstrate that differently from the BBB, transport of verapamil across the BRB is mediated by both influx transporters and efflux transporters (Kubo et al. 2013a). Importantly, in P-GP-deficient rats, P-GP inhibitors (vinblastine and verapamil) inhibited verapamil uptake by the retina but not the brain, which conformed that ^3H verapamil is permeated across the BRB via influx transporters (Fujii et al. 2014). In human RPE cell lines (RPE/Hu and ARPE-19), it was found that verapamil uptake is active, pH-dependent, and independent of the membrane potential (Han et al. 2001), but in TR-iBRB2 cells, verapamil uptake was independent of pH (Kubo et al. 2013a), indicating that it has characteristics of influx transporter for verapamil in the iBRB was different from that in the oBRB. The verapamil uptake was inhibited by metabolic inhibitors, quinidine, pyrilamine, diphenhydramine, diltiazem, timolol, propranolol, and L-carnitine, but not by other known OCT/OCTN2 substrates nor inhibitors (such as tetraethylammonium, cimetidine, decynium-22, and MPP^+) (Han et al. 2001; Kubo et al. 2013a). Similarly, ^3H pyrilamine uptake in TR-iBRB2 cells was also inhibited by verapamil and some cation compounds (such as desipramine, imipramine, propranolol, memantine, quinidine, and nipradilol), not tetraethylammonium, serotonin, choline, or choline. However, the transporter mediating pyrilamine uptake seemed not to be identical to verapamil transport system, because they showed different sensitivity to pH and L-carnitine. Moreover, kinetic analysis indicated that verapamil had no competitive effect on the pyrilamine uptake although verapamil uptake exhibited a competitive-like inhibition (Kubo et al. 2013a).

Transport of propranolol across iBRB is mediated by an influx carrier and was reduced by several organic cations (Kubo et al. 2013b). Propranolol uptake in TR-iBRB2 cells was also inhibited by some organic cations (such as pyrilamine, verapamil, imipramine) but not substrates nor inhibitors of OCTs (MPP^+ , tetraethylammonium, and cimetidine). The propranolol transport is pH dependent, Na^+ independent, and L-carnitine insensitive, which is different from verapamil transport system (Kubo et al. 2013a, b). Blood-to-retina transport of nicotine across the iBRB is driven by an outwardly directed H^+ gradient, which was stimulated by

an outwardly directed H^+ gradient and significantly inhibited by organic cations (pyrilamine and verapamil) but not OCT inhibitors (Kubo et al. 2014; Tega et al. 2015). These findings that substrate specificity of the cationic drug transport at the BRB is different from those of well-characterized organic cation transporters OCTs, OCTNs, and MATEs, suggesting the involvement of novel organic cation transporters in the influx transport of these cationic drugs across the BRB (Chapy et al. 2015; Kubo et al. 2013a, b, 2014).

10.2.1.11 Other Transporters

Organic anion transporter 3 (OAT3) is expressed in RCECs and TR-iBRB cells. Rat Oat3 is possibly located at the abluminal membrane of the RCECs, where it effluxes its substrates such as *p*-aminohippuric acid, benzylpenicillin, and 6-mercaptopurine from the vitreous humor/retina to the blood across the iBRB, limiting the retinal distribution of these substrates (Hosoya et al. 2009). Unlikely BBB, androgen receptor ligand dihydrotestosterone did not affect expression of Oat3 mRNA in TR-iBRB cells (Ohtsuki et al. 2005). Anion transporter inhibitor probenecid also inhibited transport of digoxin across BRB of rats, leading to significant increases in uptake retina index by 1.6-fold of control, indicating roles of OATs in efflux of digoxin across BRB (Toda et al. 2011).

Excitatory acid transporter 1 (EAAT1), an Na^+ -dependent high-affinity L-glutamate transporter, is localized on the abluminal membrane of the RCECs and mediates elimination of L-glutamate, a neuroexcitatory neurotransmitter, from the retina across the iBRB (Sakurai et al. 2015). Multivitamin transporter (SMVT/SLC5A6), an Na^+ -dependent transporter, was detected in TR-iBRB2 cells and isolated rat RCECs, mediating the uptake of vitamins and some essential cofactors such as biotin, pantothenic acid, and lipoic acid (Quick and Shi 2015). In vivo, [3H] biotin uptake by the rat retina was significantly inhibited by biotin and pantothenic acid. [3H]biotin uptake in TR-iBRB2 cells was also significantly inhibited by biotin, pantothenic acid, lipoic acid, and desthiobiotin (Ohkura et al. 2010). SMVT mRNA and protein were also detected in human RPE cells. Hypoxia induced expression and function of SMVT in human RPE cells, indicating that hypoxia may alter disposition of ophthalmic drugs (Vadlapatla et al. 2013). Riboflavin transport across blood-to-retina is mediated by riboflavin transporter (SLC52A/RFVT), an Na^+ - and Cl^- -independent transporter. In TR-iBRB2 cells, two RFVTs RFVT2 (SLC52A2) and RFVT3 (SLC52A3) were detected (Kubo et al. 2017b).

10.2.2 Efflux Transporters

Efflux transporters mainly belong to ABC family transporters. Several ABC transporters, such as P-glycoprotein (P-GP/*ABCB1*), multidrug resistance proteins

(MRPs/ABCCs for human and Mrps/ABCCs for animal) (Asashima et al. 2006), and breast cancer resistance protein (BCRP/ABCG2), were identified in the BRB.

10.2.2.1 P-Glycoprotein (P-GP/ABCB1)

P-GP is mainly expressed at the iBRB. However, reports on P-GP in the oBRB are often contradictory, which may result from different tissues or cells from several species or different detection methods. In mice, it was found that the oBRB showed low or lack expression of P-GP (Chapy et al. 2016). In human fetal RPE and ARPE-19, no P-GP protein was detected by LC-MS/MS (Pelkonen et al. 2017). But, in the oBRB tissue of porcine, P-GP protein was measured to be 2.01 fmol/ μ g protein by LC-MS/MS, although it was less than that of the iBRB (8.70 fmol/ μ g protein) (Zhang et al. 2017a). Similarly, among three human RPE cell lines (ARPE-19, D407 and h1RPE), only ARPE-19 cells do not express P-GP. D407 and h1RPE cells express P-GP, but functional activity is demonstrable only in D407 cells (Constable et al. 2006). Expression and function of P-GP were also detected in both cultured human RPE and in D407 cells. Furthermore, P-GP immunoreactivity is predominantly associated with localization to both apical and basolateral cell membranes of human RPE (Kennedy and Mangini 2002). Importantly, expression and function of P-GP are also demonstrated in the mitochondria of D407 cells and upregulated by H₂O₂ (Zhang et al. 2017b). Expression and function of P-GP were also clearly demonstrated in the oBRB tissue of porcine. Permeabilities of verapamil and rhodamine in the retina-to-choroid direction were reported to be higher 5.5- and 2.6-fold than those in the opposite direction. Moreover, cellular calcein accumulation in the presence of verapamil was twice as strong as control (Steuer et al. 2005). PET data showed that P-GP inhibitor tariquidar significantly increased influx rate constant k_1 across the BRB and total retinal distribution volume of (R)-[¹¹C]-verapamil in human subjects, by 1.4-fold and 1.5-fold of baseline. In accordance with this, retinal efflux rate constant k_2 was significantly decreased by 2.8 in the presences of P-GP inhibitor (Bauer et al. 2017).

It is worth noting that the impact of P-GP on BRB permeability to its substrates is greatly lower than that on BBB permeability (Chapy et al. 2016; Fujii et al. 2014). For example, uptake indexes of verapamil, quinidine, and digoxin in the retina of *Abcb1a*^{-/-} rats were 1.6-, 1.07-, and 3.7-fold of wild-type rats, respectively. But, the brain uptake parameters for verapamil, quinidine, and digoxin were high up to 8.3-, 12.3-, and 14.0-fold of wild-type rats, respectively. Quinidine, verapamil, and digoxin are substrates of P-GP, but P-GP inhibitors only inhibited transport of digoxin in the retina, which was different from the brain. These results indicate that P-GP may play a substantial role in the retinal distribution of digoxin, but not verapamil nor quinidine (Toda et al. 2011), which may partly be attributed to influx transporters for these drugs. Similarly, transport of [³H]-verapamil at BRB of mice was significantly increased by ~1.5-fold following P-GP inhibition using elacridar (5 μ M) or valsopodar (5 μ M) and by 1.3-fold in triple knockout (*Abcb1a/Abcb1b*^{-/-} and *Abcg2*^{-/-}) mice compared with control wild-type mice, but extents of these

alterations at the BRB were remarkably lower than those (5.6-fold for elacridar, 8.4-fold for valsopodar, and 10.3-fold for triple knockout) at the BBB (Chapy et al. 2016).

10.2.2.2 Breast Cancer Resistance Protein (BCRP/ABCG2)

Likely to P-GP, BCRP is mainly located at the luminal membrane of RCECs (Asashima et al. 2006; Chapy et al. 2016), acting as the efflux transporter for photosensitive toxins and drugs in retinal tissue. In TR-iBRB2 cells, BCRP mediates cellular efflux of phototoxic compounds pheophorbide and protoporphyrin IX, which is inhibited by ABCG2 inhibitor Ko143 (Asashima et al. 2006). BCRP is often co-located with P-GP in the retina. In mice, P-GP and BCRP are uniformly expressed in the physiologically developing retinal vasculature of the neonatal mouse (Tagami et al. 2009). Expressions of P-GP and BCRP mRNA and proteins were also detected in the retinal vascular endothelial cells from the adult mouse retina (Tachikawa et al. 2008; Chapy et al. 2016).

Similarly to P-GP, the importance of BCRP efflux at the retina is less than that at the BBB. Triple knockout (*Abcb1a/Abcb1b*^{-/-}:*Abcg2*^{-/-}) and coadministration of BCRP inhibitor elacridar significantly increased the mitoxantrone entry rate to the mouse brain, with 3.3-fold increases of control mice, but these increases did not occur at the retina (Chapy et al. 2016).

Several reports have also demonstrated expression of BCRP in the oBRB, although contradictory may be often contradictory. LC-MS/MS analysis demonstrated no expression of BCRP in human fetal RPE and ARPE19 (Pelkonen et al. 2017), but high levels of BCRP protein were still detected in both the iBRB (22.8 fmol/ μ g protein) and the oBRB (2.76 fmol/ μ g protein) of pig (Zhang et al. 2017a). Among the tested three human RPE cell lines (ARPE-19, D407, and HRPEpiC) and bovine primary cells, BCRP was only detected in D407 cells (Mannermaa et al. 2009). However, BCRP mRNA was also detected in the neural retina, RPE eyecup, and primary mouse RPE cells. Immunoreactivity showed that the expression of BCRP is almost exclusively restricted to the RPE cell layer, mainly at the basolateral membrane of PRE (Gnana-Prakasam et al. 2011), indicating that the function of BCRP in the oBRB is to efflux of heme from the retina to choroidal blood, showing roles in retinal hemochromatosis. Iron overload downregulated BCRP expression in PRE, whose defective function in RPE may lead to increases in the cellular levels of phototoxin, thus contributing to oxidative stress and enhancing the progression of retinal diseases such as age-related macular degeneration (Gnana-Prakasam et al. 2011).

10.2.2.3 Multidrug Resistance-Associated Proteins (MRPs/ABCCs)

MRPs seem to be mainly expressed in the oBRB, but different tissues or cells from several species or detected methods often show different patterns of MRP expression. Mannermaa et al. (2009) investigated expressions of several MRPs in three

human RPE cell lines (ARPE-19, D407 and HRPEpiC) and bovine primary RPE cells. The results showed that expressions of MRP1, MRP4, and MRP5 proteins were detected in the tested three human RPE cell lines. Unlike MRP1 and MRP4, Mrp5 protein was not detected in bovine primary RPE cells. MRP2 protein was only detected in the D407 cells (Mannermaa et al. 2009). Activities of MRP1 and MRP5 in ARPE-19 cells were also confirmed by the calcein-AM and CDCF efflux tests (Mannermaa et al. 2009), respectively. In PRE of porcine, permeability of known MRP substrate fluorescein in the retina-to-choroid direction across was reported to be higher 11.3-folds than that in opposite direction. The transport of fluorescein in the retina-to-choroid direction was blocked by probenecid, with the result that permeability was equalized in both directions, which was in line with expression of Mrps (Steuer et al. 2005).

LC-MS/LC-MS analysis demonstrated high expression of MRP1 in primary human PER cells, ARPE-19 cells, and oBRB tissue of porcine. High levels of MRP5 protein were only detected in primary human PER cells but not ARPE-19 cells nor oBRB tissue of porcine (Pelkonen et al. 2017; Zhang et al. 2017a). Mrp1 and Mrp4 were also detected in the basal membrane of the mouse RPE; in accordance, the efflux of [³H]-zidovudine from the retina might be inhibited by MRP inhibitor MK571 (Chapy et al. 2016). Moreover, Mrp4 was reported to be also uniformly expressed in the physiologically developing retinal vasculature of the neonatal mouse including the capillaries and large vessels (Tagami et al. 2009). RCECs of the adult mouse also showed expression of Mrp3, Mrp4, and Mrp6 mRNA (Tachikawa et al. 2008).

Generally, MRPs efflux a wide variety of endogenous compounds and therapeutic drugs. For example, MRP4 mediates cellular efflux of both cAMP and prostaglandin E₂ (PGE₂), indicating involvement of MRP4 in angiogenesis. In human RCECs, VEGF was reported to dose-dependently decrease expression of MRP4 mRNA and protein. *Abcc4* knockdown using RNAi enhanced cell migration and attenuated serum starvation-induced cell apoptosis, assembled and aggregated into a massive tube-like structure (Tagami et al. 2010). Similarly, *Abcc4* deficiency did not cause overt abnormalities in the development of the retinal vasculature of mice, but retinal vascular development was suppressed in response to forskolin administration. The forskolin-treated *Abcc4*^{-/-} mice showed an increased number of Ki67-positive and cleaved caspase 3-positive RCECs and significant decreases in the amount of pericyte coverage and number of empty sleeves. Moreover, following exposure of hyperoxia, the *Abcc4*^{-/-} mice showed a significant increase in the unvascularized retinal area. These results indicate that MRP4 may have its protective roles in the retinal vascular development by regulating the intracellular cAMP level (Matsumiya et al. 2012).

Many kinds of drug transporters, such as OATPs, OATs, P-GP, BCRP, and MRPs, are expressed at the BRB, mediating transport of therapeutic drugs across the BRB. These transporters often overlap substrate specificity. Thus net effect is attributed to their interplay. For example, the elimination of p-aminohippuric acid, benzylpenicillin, and 6-Mercaptopurine from vitreous humor is mediated by several transporters. P-aminohippuric acid, benzylpenicillin, and 6-Mercaptopurine are

substrates of MRP4 (Uchida et al. 2007) and OAT3 (Hosoya et al. 2009), indicating that OAT3 and MRP4 in common contribute to the efflux transport of PAH, PCG, and 6-MP from the retina across the BRB. Another example is verapamil. Although P-GP is also highly expressed at the iBRB, P-GP has little involvement in the retinal uptake of verapamil, which is partly attributed to the existence of influx transporters (Chapy et al. 2015; Fujii et al. 2014). Transport of E17 β G and dehydroepiandrosterone sulfate across BRB may be attributed to the combined effects of OATP1A4, OAT3, and MRP4 (Hosoya et al. 2011).

10.3 Alterations in BRB Function Under Disease Status and Clinic Significances

10.3.1 *The BRB and Diabetic Retinopathy*

Diabetic retinopathy, a complication of diabetes, is the leading cause of acquired blindness, which is involved in functional and structural changes of the BRB. Microvascular disorders are often diabetic retinopathy although other cells such as RPE cells are affected by diabetes. The vascular changes are clearly linked to the loss of visual acuity and clinical alterations in the retinal vasculature. Early vascular changes include leukostasis, aggregation of platelets, alteration in blood flow, degeneration of pericytes, and basement membrane thickening. The increased retinal vascular permeability associated with diabetic retinopathy may result from alterations in the tight junction and adherens junction complexes or from endothelial cell death. Macular edema is closely associated with the loss of visual acuity in diabetic retinopathy, which is attributed to the increased BRB permeability (Frey and Antonetti 2011; Arden and Sivaprasad 2011).

10.3.1.1 Inflammation and Diabetic Retinopathy

Retinal inflammation plays a major role in the pathogenesis of diabetic retinopathy. Hyperglycemia is considered as a pro-inflammatory environment via releasing some inflammatory cytokines [such as tumor necrosis factor- α (TNF- α), interleukin -1 β (IL-1 β), and interleukin-6 (IL-6)] and chemokine ligands (such as CCL2, CCL5, and CCL12)]. These cytokines and chemokines induce the disorganization and redistribution of junctional proteins in microvasculature, leukocyte activation, release of intercellular adhesion molecule-1 (ICAM-1), and other cell adhesion molecules, in turn, increasing vascular permeability and further exacerbating the inflammatory milieu of the retina. For example, TNF- α downregulated expression of tight junction proteins (such as claudin-5 and ZO-1). Hyperglycemia upregulated levels of ICAM-1 and high mobility group box-1 (HMGB-1), which was in line with increases in leaky of Evans blue in rat retina (Ran et al. 2016). Streptozotocin (STZ)-induced

diabetes significantly upregulated expression of retinal CCL2 and increased monocyte trafficking in rats. In accordance with this, intraocular injection of the CCL2 into nondiabetic rats also increased retinal monocyte trafficking, indicating the ability of the chemokine to attract monocytes/macrophages into retinal tissue. High glucose exposure was also reported to upregulate CCL2 expression in human RCECs. Contrarily, CCL2 deficiency prevented the increase in vascular permeability and monocyte trafficking in the retinas of diabetic rats (Rangasamy et al. 2014). Moreover, diabetes also significantly increased levels of retinal cathepsin D and CCL2, accompanied by increases in vascular permeability to albumin in the retinas of mice. Patients with diabetic macular edema also showed increases in levels of serum cathepsin D protein. In human RCECs, cathepsin D was reported to disrupt endothelial junctional barrier via increasing RhoA/ROCK cell contractility (Monickaraj et al. 2016). Significant increases in expression of CXC chemokine platelet factor-4 (PF-4/CXCL4) were also found in both vitreous fluid from patients with proliferative diabetic retinopathy and the retinas of diabetic rats. In human RCECs, it was found that PF-4/CXCL4, an angiostatic chemokine, inhibited VEGF-induced signal transduction and inhibited cell migration (Nawaz et al. 2013). Platelet factor-4 variant (PF-4var/CXCL4L1) was also reported to inhibit VEGF-mediated hyperpermeability. In accordance, intravitreal PF-4var/CXCL4L1 or bevacizumab attenuated diabetes-induced BRB breakdown in rats, leading to decreases in vascular leakage by approximately 70% and 73%, respectively, compared with phosphate buffer saline injection. These effects were also associated with upregulation of occludin and vascular endothelial-cadherin and downregulation of hypoxia-inducible factor (HIF)-1 α , VEGF, TNF- α , receptor for advanced glycation end products (RAGE), and caspase-3 (Abu El-Asrar et al. 2016). Nuclear factor- κ B (NF- κ B) pathway is also involved in the pathogenesis of diabetic retinopathy. It was reported that administration of NF- κ B inhibitor dehydroxymethylepoxyquinomicin suppressed retinal adherent leukocytes, expression of inflammatory molecules (ICAM-1 and VEGF), and renin angiotensin system (RAS)-related molecules such as angiotensinogen and angiotensin-II receptor 1 (AT1-R) induced by diabetes (Nagai et al. 2007).

10.3.1.2 Vascular Endothelial Growth Factor (VEGF) and Diabetic Retinopathy

VEGF, a pro-angiogenic growth factor secreted preferentially from the basal surface of the RPE and Müller cells, modulates and maintains the extracellular space in and around the Bruch's membrane and modulates the growth/density of endothelial cells in the choriocapillaris (Kay et al. 2013; Le 2017). Diabetic retinopathy is often associated with the increases in retinal VEGF levels (Kay et al. 2013; Lin et al. 2011; Nawaz et al. 2013). Animal and clinical trials have demonstrated that diabetes-induced increases in retinal VEGF levels are coincided with BRB breakdown (Le 2017). VEGF is involved in the pathogenesis of diabetic retinopathy via inducing retinal ICAM-1 expression, vascular permeability, leukostasis, BRB breakdown, or vascular lesions (Le 2017). In normal rats, it was found that VEGF₁₆₄/VEGF-A was

at least twice as potent as VEGF₁₂₀ at inducing ICAM-1-mediated retinal leukostasis and BRB breakdown following intravitreal injections (Ishida et al. 2003). Moreover, blockade of endogenous VEGF-A with EYE001 significantly suppressed diabetes-induced retinal leukostasis and BRB breakdown, indicating that VEGF-A is an important isoform in the pathogenesis of diabetic retinopathy. Roles of VEGF in pathogenesis of diabetic retinopathy were confirmed by conditional VEGF knockout mice. It was found that conditional VEGF knockout significantly reduced leukostasis, expression of inflammatory biomarkers, depletion of tight junction proteins, numbers of acellular capillaries, and vascular leakage in the retina of diabetic mice (Wang et al. 2010). In general, VEGF shows its pathophysiological effects in diabetic retinopathy in two ways (Deissler et al. 2014). One, VEGF increases vascular permeability and causes fluid extravasation and retinal edema via affecting endothelial tight junctions. Second, VEGF causes leukocyte aggregation in the retinal microvasculature, resulting in local cytokine production and inflammatory cell migration through the endothelium, both of which contribute to BRB breakdown. Anti-VEGF therapy with aflibercept, bevacizumab, or ranibizumab has been a hallmark strategy to prevent diabetic retinopathy in the clinic (Virgili et al. 2017), demonstrating efficiency of anti-VEGF drugs on the improvement of vision in people with diabetic macular edema. Some drugs such as brimonidine, memantine (Kusari et al. 2007, 2010), and corticosteroids (Edelman et al. 2005; Wang et al. 2008) also attenuate diabetic macular edema and retinopathy partly via affecting expression of retinal VEGF expression or downstream signal proteins of the VEGF receptor.

10.3.1.3 Hyperosmolar Stress and Diabetic Retinopathy

Diabetic retinopathy is associated with osmotic stress resulting from hyperglycemia and intracellular sorbitol accumulation. *In vitro*, it was reported that high glucose increased expression of the water channel aquaporin-1 (AQP1) and cyclooxygenase (COX)-2, increased activity of the osmolarity-sensitive transcription factor tonicity enhancer-binding protein (TonEBP), and enhanced endothelial migration and tubulization. These alterations by high glucose were reversed by AQP1 and TonEBP siRNA, indicating that high glucose-induced hyperosmolarity promotes angiogenesis and retinopathy via activating TonEBP (Madonna et al. 2016). It is generally accepted that glucose is reduced to sorbitol by aldose reductase, and sorbitol is eventually metabolized to fructose by sorbitol dehydrogenase. Intracellular sorbitol accumulation induces osmotic damage of the retinal vascular cells and RPE cells, loss of pericytes, basement membrane thickness, and oxidative stress, all of which contribute to iBRB rupture during diabetic retinopathy (Lorenzi 2007). RPE cells subjected to hyperosmolar stress also underwent osmoadaptive responses such as shrinkage of RPE cells, alterations in AQP expression, increases in VEGF expression, placental growth factor, monocyte chemoattractant protein-1, and basic fibroblast growth factor (Willermain et al. 2018), further impairing RPE function. Hyperosmolar conditions also increases activity and expression of TAUT in RPE

cells, leading to increases in the uptake of taurine (El-Sherbeny et al. 2004). Moreover, hyperosmolar condition also increased expression of aldose reductase (Winges et al. 2016), in turn, further enhancing sorbitol accumulation.

10.3.1.4 Plasma Kallikrein-Kinin System (PKKs) and Diabetic Retinopathy

Accumulating evidences have demonstrated contribution of plasma kallikrein-kinin system (PKKs) to diabetic retinopathy. It was reported that levels of plasma prekallikrein and plasma kallikrein in vitreous from subjects with diabetic macular edema were increased to 2.0-fold and 11.0-fold, respectively, of those with a macular hole (Kita et al. 2015). In normal rats, intraocular injection of bradykinin dose-dependently might increase plasma extravasation, which was inhibited by bradykinin receptor 2 antagonist Hoe140 (Abdouh et al. 2008; Phipps et al. 2009). Similarly, the bradykinin receptor 2 agonist bradykinin might vasodilate retinal vessels in a concentration-dependent manner, which was completely blocked by Hoe140. But bradykinin receptor 1 agonist des-Arg9-bradykinin had no this effect. However, in diabetic rats, des-Arg9-bradykinin could also produce a concentration-dependent vasodilatation and was also inhibited by the bradykinin receptor 1 receptor antagonist des-Arg10-Hoe140 (Abdouh et al. 2003), which may be explained by these findings that the bradykinin receptor 1 in the retina is only minimally expressed under physiological conditions, but diabetes significantly upregulated expression of bradykinin receptor 1 without affecting bradykinin receptor 2 (Abdouh et al. 2008; Kita et al. 2015; Pouliot et al. 2012). Animal experiments have been demonstrated that diabetes-induced retinal vascular permeability is attenuated by plasma kallikrein inhibitor or bradykinin receptor antagonists (Abdouh et al. 2008; Catanzaro et al. 2012; Clermont et al. 2011; Pouliot et al. 2012) or plasma prekallikrein-gene deficiency (Kita et al. 2015). FOV-2304, a non-peptide selective bradykinin receptor 1 antagonist, also blocked retinal vascular permeability, inhibited leukocyte adhesion, and abolished the retinal mRNA expression of several inflammatory mediators in diabetic rats (Pruneau et al. 2010). These results indicate that the blockade of the PKKs is a promising therapeutic strategy for diabetic retinopathy.

Interestingly, the response of plasma kallikrein following injection into vitreous of diabetic rats was blocked by bradykinin receptor antagonist but not by bevacizumab. In mice, administration of VEGF receptor 2 antibody DC101 did not affect bradykinin-induced retinal thickening. Moreover, although increased VEGF levels were also observed in diabetic macular edema vitreous, no correlation of plasma kallikrein level and VEGF level was found. These results indicate that diabetic macular edema induced by PKKs is VEGF-independent (Kita et al. 2015). However, a report showed that plasma prekallikrein-gene deficiency partly decreased the VEGF-induced retinal vascular permeability and retinal thickening or TNF α -induced retinal thickening in mice. Systemic administration of plasma kallikrein inhibitor VA999272 also reduced VEGF-induced retinal thickening in both mice and rats, indicating that plasma kallikrein is required for the full effects of VEGF on retinal

vascular permeability and retinal thickening (Clermont et al. 2016). Retinal hemorrhages also occur under diabetic condition. Intravitreal injection of autologous blood was reported to induce retinal vascular permeability and retinal leukostasis, which were ameliorated by plasma kallikrein inhibition. Intravitreal injections of exogenous plasma kallikrein also induced retinal vascular permeability, leukostasis, and retinal hemorrhage, indicating that retinal hemorrhage increases retinal vascular permeability and leukostasis partly via plasma kallikrein (Liu et al. 2013).

Real mechanisms that PKKs induce retinal vascular permeability and retinal thickening are not fully understood. Bradykinin was reported to evoke intracellular Ca^{2+} transients in primary human RPE, which was enhanced by pretreatment with TNF- α and/or IL-1 β but inhibited by fasitibant chloride, a selective bradykinin receptor 2 antagonist. TNF- α and/or IL-1 β enhanced bradykinin-induced Ca^{2+} response via increasing expression of both bradykinin receptor 2 and COX-2, as well as secretion of prostaglandin E₁ and E₂ into the extracellular medium (Catalioto et al. 2015). Intravitreal injections of either plasma kallikrein or collagenase, but not bradykinin, also induced retinal hemorrhage in rats. Proteomic analysis showed that plasma kallikrein increased collagen degradation in pericyte-conditioned medium and purified type IV collagen, indicating that plasma kallikrein leads to breakdown of BRB due to its collagenase-like activity (Liu et al. 2013). However, intravitreal injection of bradykinin also increased retinal vascular permeability, which might be prevented by both vaso-inhibins and Hoe-140. In ARPE-19, bradykinin also increased permeability of the BRB and decreased endothelial monolayer resistance, which was attributed to redistribution of actin cytoskeleton, subsequently reorganization of tight and adherens junctions. These effects were reversed by NO synthase inhibitor L-NAME, vaso-inhibins, and N-acetyl cysteine (Arredondo Zamarripa et al. 2014). Bradykinin could also inhibit TGF- β 1-stimulated ARPE-19 cell proliferation, collagen I, fibronectin, and MMP-2 secretion as well as Akt phosphorylation via activating bradykinin receptor 2 (Cai et al. 2016). In normal rats, bradykinin-induced vasodilatation was involved in intracellular Ca^{2+} mobilization and products of the cyclooxygenase-2 (COX-2) pathway, but in STZ-diabetic rats, the vasodilatation in response to des-Arg⁹-bradykinin was involved in both calcium influx and intracellular calcium mobilization, which was blocked by GdCl₃, 2,5-di-*t*-butylhydroquinone, and cADP ribose (Abdoun et al. 2003).

10.3.1.5 Renin Angiotensin System (RAS) and Diabetic Retinopathy

Clinical and experimental studies have demonstrated that abnormalities of the renin angiotensin system (RAS) may play a significant role in the progression of the diabetic retinopathy, presumably through local changes in the blood flow and the production of angiotensin II (Ang II) (Ola et al. 2017; Phipps et al. 2012). Besides circulating RAS, RAS locally exists in the retina. Animal experiments have demonstrated that diabetes increases components of RAS including prorenin, angiotensinogen, Ang II, angiotensin-converting enzyme (ACE), angiotensin-converting enzyme 2 (ACE2), and Ang II receptor 1 (AT1R) in the retina (Ola et al. 2017; Nagai et al. 2007; Verma et al. 2012). Several reports have shown that

vitreous pool of proliferative diabetic retinopathy patients also exhibited an increased level of Ang II and other RAS components compared to nondiabetic subjects (Ola et al. 2017; Phipps et al. 2012). Rodent experiments have also revealed that ACE inhibitors and AT1R blockers could reduce diabetes-induced retinal microvascular damage with reductions in vascular leakage, decreased formation of acellular capillaries, and decreased expression of angiogenic factors such as VEGF (Mori et al. 2002; Wilkinson-Berka et al. 2007; Zhang et al. 2007). ACE inhibition or AT1R blockade also decreased diabetes-induced retinal leukostasis and upregulation of adhesion molecules (Mori et al. 2002; Chen et al. 2006; Silva et al. 2007), but AT2R blockade did not show this effect (Nagai et al. 2007).

AT1R antagonist candesartan could decrease diabetes-induced or Ang II-stimulated retinal vascular permeability without affecting retinal vascular permeability in normal rats although candesartan decreased hypertension both in diabetic and normal rats (Phipps et al. 2009). Ang II infusion also significantly increased expression of plasma kallikrein in rat retina. Bradykinin receptor 2 antagonist Hoe140 and plasma kallikrein inhibitor (ASP-440) might attenuate Ang II-induced retinal vascular permeability, indicating that activation of AT1R increases retinal vascular permeability partly via PKCs (Phipps et al. 2009). Clinical trial (Mauer et al. 2009) demonstrated effects of ACE inhibitor enalapril and the AT1R inhibitor losartan on progression of diabetic retinopathy to a similar extent, whose odds of retinopathy progression were reduced by 65% with enalapril treatment and 70% with treatment, respectively. Data from 5321 diabetic patients demonstrated that treatment with candesartan significantly reduced the incidence of development of diabetic retinopathy in type 1 diabetics without affecting its progression (Chaturvedi et al. 2008). But in type 2 diabetes, treatment with candesartan reduced the development of retinopathy and even resulted in a 34% regression of retinopathy compared with the control group (Sjølie et al. 2008). Taken together, these results suggest that RAS blockade may be useful for slowing progression and decreasing the severity of diabetic retinopathy.

ACE2 is expressed in the retina, which converts the vasoconstrictive and pro-inflammatory peptide Ang II into the vasodilatory and anti-inflammatory peptide angiotensin-(1-7) [Ang(1-7)], which exhibits its effects primarily through the receptor Mas. A report showed that STZ-induced diabetes significantly increased mRNA levels of the vasodeleterious axis of the RAS (angiotensinogen, renin, pro/renin receptor, ACE, and AT1R) in the retina of *eNOS*^{-/-} mice, leading to increases in ratios of ACE/ACE2 and AT1R/Mas mRNA levels by approximately tenfolds and threefolds, respectively (Verma et al. 2012). The increases in vascular permeability, infiltrating CD45-positive macrophages, and activation of CD11b-positive microglial cells as well as formation of acellular capillaries in the retina of diabetic mice were attenuated by intraocular administration of adeno-associated virus-ACE2 or Ang-(1-7) vector (Verma et al. 2012). Intraocular administration of adeno-associated virus-ACE2 vector was also reported to attenuate the increase of acellular capillaries and leaky of macrophages/microglia in the retina of STZ-induced diabetic mice (Dominguez et al. 2016). Similarly, in STZ-induced diabetic rat retinas, the

increased numbers of acellular capillaries were almost completely prevented by gene delivery of either ACE2 or Ang-(1-7) (Verma et al. 2012).

Diabetes is often associated with hypertension, and patients with hypertension are at a greater risk of developing diabetic complications including retinopathy, inferring that therapeutic effects of RAS blockade on diabetic retinopathy are also attributed to the reduction in blood pressure. Clinical trials have demonstrated no different effects on progression of retinopathy between captopril and atenolol treatments or between enalapril and nisoldipine in type 2 diabetes (Phipps et al. 2012). In consistence, diabetic spontaneously hypertensive rats (SHR) showed significantly higher number of ED1/microglial-positive cells and the expression of ICAM-1 in the retina than in control SHR. The SHR also possessed higher NF- κ B p65 levels than Wistar Kyoto (WKY) rats. These abnormalities in diabetic SHR rats were completely prevented by losartan or complex of hydralazine+ reserpine +hydrochlorothiazide) (Silva et al. 2007). Similarly, SHR rats showed higher number of BrdU-positive retinal cells than WKY rats. A significant reduction in cell replication was found only in diabetic SHR, and this reduction was associated with enhanced p27^{Kip1}, fibronectin, and VEGF retinal expressions and greater blood-retinal barrier breakdown (Lopes et al. 2008). These results indicate contribution of concomitant diabetes and hypertension to diabetic retinopathy. However, a rat experiment showed that ramipril, losartan, and nifedipine showed similar antihypertensive efficiencies, but ramipril and losartan showed stronger decreases in retinal leukostasis and expression of ICAM-1 induced by diabetes than nifedipine, indicating that their effects seem to be partly independent of blood pressure and to be associated with a decrease in ICAM-1 gene expression (Chen et al. 2006), which need further investigation.

It is worth noting that systemic Ang II reduces RPE renin production via stimulating AT1R and that systematic application of ACE inhibitors strongly activates local RAS in the retina, indicating that the systemic treatment with RAS blockade for retinal degeneration and systemic disease may cause side effects detrimental, or perhaps beneficial, to retinal disease (Strauß 2016) via affecting retinal RAS.

10.3.1.6 Alterations in Transport under Diabetic Status

10.3.1.6.1 Alterations in Glucose Transport

The retina and RPE are highly metabolically active tissues with substantial demands for glucose. In STZ-induced diabetic rats, Glut1 expression in retinal microvessels but not in RPE was decreased by approximately 50%, without altering the retina microvascular density, indicating that the fraction of the glucose entering the retina of diabetes is likely to be greater across the RPE than across the retinal vasculature (Badr et al. 2000). Decreases in expressions of Glut1 proteins not mRNA were also found in the retinas of diabetic GK rats and alloxan-treated rabbits (Fernandes et al. 2004). In TR-iBRB cells, high glucose exposure also decreased expression of Glut1 protein. Moreover, higher content of high molecular weight ubiquitin conjugates

was found in both membrane fractions of diabetic retinas and endothelial cells treated with high glucose exposure, indicating that the decreased expression of Glut1 protein may be associated with its increased degradation by a ubiquitin-dependent mechanism (Fernandes et al. 2004). Importantly, although expressions of Glut1 on the luminal plasma membrane of the RCECs and in homogenates of the whole retina in diabetic rats were significantly decreased (about 55% and 36% of control rats, respectively) (Tang et al. 2000), retinal glucose levels were significantly elevated by fourfold to sixfold compared with the nondiabetic rats. It was also found that glucose influx increased with increasing plasma glucose in both diabetic and normal rats (Puchowicz et al. 2004). Dehydroascorbic acid transport across BRB is mediated by GLUT1; it was consistent with the decreases in Glut1 expression that [^{14}C] dehydroascorbic acid transport across the BRB in STZ-induced diabetic rats was less than 35% of normal rats, inferring that hyperglycemia reduces the supply of vitamin C to the retina (Minamizono et al. 2006).

10.3.1.6.2 Alterations in H₂O Transport

BRB maintains fluid homeostasis in the retina by removing fluid out of the retina via the retinal vasculature. Besides the ionic and osmotic gradients, transport H₂O across BRB is mainly mediated by aquaporins (AQPs). Thirteen AQPs have been identified in mammals, most of which are expressed in the retina. Diabetes significantly altered the expression and distribution of retinal AQPs in the retina (Xia and Rizzolo 2017) in a species-dependent manner. For example, diabetes upregulated AQP5, 9, 11, and 12 but downregulated AQP0 in the RPE of rats (Hollborn et al. 2011). Diabetic retinopathy is often associated with cellular stressors (such as hypoxia and oxidative stress). In primary cultures of human RPE, it was found that exposure of stressors (chemical hypoxia, oxidative stress, VEGF, and high glucose) upregulated expression of AQP9 (Hollborn et al. 2012). AQP9, an aquaglyceroporin, is not only permeable to water but also to non-charged solutes, such as lactate. AQP9 expression is considered to be required for L-lactate to maintain retinal neuronal survival (Akashi et al. 2015), indicating that the upregulation of AQP9 in RPE cells may prevent lactic acidosis and subretinal edema under ischemic and oxidative stress conditions (Akashi et al. 2015; Hollborn et al. 2012). Hyperosmolarity could alter AQP expression. It was reported that 10 min exposure to an osmolar stress (400 mM sucrose or 200 mM NaCl) significantly decreased AQP4 expression in ARPE-19 cells (Willermain et al. 2014b). Under normal condition, expression of AQP1 in the rat retina is at minimal levels, diabetes increased expression of AQP1, and hypertension also enhanced expression of AQP4 under diabetic conditions, which were reversed by valsartan and metoprolol (Qin et al. 2012). Furthermore, intravitreal injection of VEGF also increased AQP4 expression in the retina of normal and diabetic rats. TGN-020, a selective AQP4 inhibitor, suppressed VEGF-induced enlargement of Müller cells and increases in intracellular levels of NO. Thus, the authors gave a conclusion that VEGF induced Müller cell swelling through the formation of NO and AQP4 channels (Kida et al. 2017). Importantly, although

diabetes or administration of VEGF increased AQP4 expression in the retina of rats (Cui et al. 2012; Kida et al. 2017), AQP4 downregulation exacerbated diabetic retinopathy and aggravated inflammatory response (Cui et al. 2012). Thus, the cellular mechanisms mediating expression of AQPs by diabetes and their clinic significances need further investigation.

10.3.1.6.3 Alterations in Active Transport

Tight junctions maintain the ion gradients essential for transcellular transport mechanisms to function. Diabetes can directly decrease the ion gradients via decreasing Na^+/K^+ -ATPase activity, contributing to retinal edema (Xia and Rizzolo 2017). Transport of L-lactic acid across the RPE is dependent on a pH gradient. Intracellular pH is regulated by $\text{Na}^+/\text{HCO}_3^-$ cotransporters. The Na^+ -dependent movement of HCO_3^- is actively driven by the physiologic Na^+ gradient established by the Na^+/K^+ -ATPase. Thus, inadequate activity of the Na^+/K^+ -ATPase impaired transport of L-lactic acid. Taurine is the most abundant free amino acid in the retina, which functions as an antioxidant and may attenuate the spread of cell death in RPE cells. Diabetes and high glucose downregulate expression and activity of retinal TAUT (Zeng et al. 2010; Lee and Kang 2013) partly due to overexpression of aldose reductase (Nakashima et al. 2005b; Stevens et al. 1999). Taurine deficiency can lead to severe damage to photoreceptors (Ripps and Shen 2012). Thus, decreases in transport of retinal taurine by diabetes and subsequent taurine depletion from the retina could contribute to visual impairment.

10.3.1.6.4 Alterations in ABC Transporters

Some diseases may affect expression of retinal P-GP and BCRP. A report showed that 24-week STZ-induced diabetic mice demonstrated lower expression of retinal P-GP and BCRP, with the breakdown of the iBRB, which might be linked to the pathogenesis of early diabetic retinopathy (Li et al. 2017). In D407 cells, it was reported that high glucose exposure significantly decreased P-GP expression of both mRNA and protein levels, attenuated P-GP activity, and increased expressions of both mRNA and protein of inducible nitrate oxide synthase (iNOS). High glucose exposure also decreased expression of pregnane X receptor (PXR) mRNA. These alterations by high glucose were partially blocked by a selective iNOS inhibitor, whose effects were antagonized with the addition of L-arginine, a substrate for NO synthesis. These results demonstrate roles of iNOS induction in decreased P-GP expression and function at the human oBRB under hyperglycemic conditions (Zhang et al. 2012).

Diabetes is often associated with oxidative stress, which is a contributing factor to RPE cell dysfunction in diabetic retinopathy and age-related macular degeneration. In general, cellular antioxidants in RPE also play a critical role in combating oxidative stress; among the cellular antioxidant constituents, reduced glutathione

(GSH) plays a significant role in cellular defense against pro-oxidants. MRP1, expressed in PRE, regulates levels of cellular GSH via efflux GSH. In ARPE-19 cells, it was found that MRP inhibitors (MK571 and sulfinpyrazone) significantly decreased GSH efflux by 50%. MRP1 silencing using MRP1-specific siRNA also caused a significant 60% reduction in GSH efflux. Moreover, the downregulation of MRP1 developed to resistance to H₂O₂-induced cell death. Contrarily, overexpression of MRP1 was susceptible to H₂O₂-induced cell death, which was consistent with lower cellular GSH levels in MRP1 overexpressed cells (Sreekumar et al. 2012). These results indicate that MRP1 shows its roles in oxidant-induced cell death via efflux GSH and that MRP1 may be a potential therapeutic target in pathological retinal degenerative disorders linked to oxidative stress.

10.3.2 Ischemia-Reperfusion/Hypoxia and BRB Breakdown

Retinal ischemia-reperfusion injury is associated with many ocular diseases such as acute retinal vein occlusion, diabetic retinopathy, and glaucoma. Oxidative injury is one of the complications after retinal ischemia-reperfusion injuries, accompanied by retinal swelling, neuronal cell death, and glial cell activation, due to oxidation stress and release of inflammatory cytokines and chemokines (Kaur et al. 2008). Ischemia-reperfusion was reported to significantly increase retinal mRNA expression of several pro-inflammatory cytokines such as IL-1 β (3.2-fold), IL-6 (4.2-folds), TNF- α (5.6-fold), and CCL2 (116.9-folds) in the retinas, accompanied by significant increases in leaky of Evans Blue (3.8-fold) and number of CD45-positive cells (Gonçaves et al. 2016). The roles of TNF- α in cytokine-induced BRB breakdown were demonstrated in TNF- α -deficient mice. It was found that compared with wild-type mice, TNF- α deficiency significantly reduced leukocyte accumulation in retinal vessels by 80%, 100%, and 100% after intravitreal injection of VEGF, IL-1 β , and platelet-activating factor (PAF), respectively. The absence of TNF- α significantly reduced retinal vascular permeability induced by injection of PAF, but not VEGF nor IL-1 β . Moreover, TNF- α deficiency significantly reduced leukostasis and mild reduction in vascular leakage, but did not affect hypoxia-induced retinal neovascularization (Vinores et al. 2007). Ischemia-reperfusion was also reported to induce occludin Ser490 phosphorylation and ubiquitination, distribution of specific tight junction proteins, and activation and phosphorylation of VEGF receptor-2 (VEGFR-2) at tyrosine 1175, all of which contribute to the increased vascular permeability. Intravitreal injection of bevacizumab prevented VEGFR-2 activation, occludin phosphorylation, and vascular permeability induced by ischemia-reperfusion (Muthusamy et al. 2014). Furthermore, ischemia-reperfusion also altered distribution of glial fibrillary acidic protein (GFAP) and AQP4 in the retina. In normal rats, expression of GFAP was confined to astrocytes in ganglion layer; ischemia-reperfusion markedly increased expression of GFAP, whose expression was not just confined to the ganglion layer but also found in the Muller cell processes. AQP4 staining is discontinuous along the blood vessels and appeared weak in normal rats. AQP4

immunoreactivity surrounding retinal vessels became more intense in the ischemia-reperfusion retina (Li et al. 2011). The increased expression of AQP4 and alterations in its distribution led to increases in transport of water from blood vessels to the retinal tissues, contributing to edema formation in hypoxic conditions (Kaur et al. 2008; Li et al. 2011). Moreover, under normal conditions, Müller cells do not express GFAP, but many insults including hypoxia to the retina lead to a rapid upregulation of GFAP in these cells. Upregulation of GFAP in stressed Müller cells is often associated with an upregulation of heat shock proteins and alterations in cytoskeletal protein synthesis, swelling of Müller/astrocytes cells, and vascular leakage in hypoxic condition (Kaur et al. 2008). Caveolae-mediated vesicular transport is a system of transcellular transport of macromolecules in the BRB. Caveolin-1 is a principal structural component of caveolae. Under normal condition, expression of caveolin-1 in the RCECs contributes to the integrity of the BRB. Hypoxia increased expressions of caveolin-1 mRNA and protein and caveolin-1 siRNA significantly reduced hypoxia-induced albumin leakage in the retinas and neovascularization, which was in line with reduction of caveolin-1 mRNA and protein, indicating that overexpression of caveolin-1 in the retina is associated with BRB breakdown and neovascularization formation (Tian et al. 2012).

BRB breakdown by hypoxia is linked to induction of hypoxia-inducible factor (HIF)-1 in Müller cells. In vitro, it was found that unlikely to wild-type mice, hypoxia did not induce expression of abundance of VEGF in Müller cells from conditional Hif-1 α knockout mice, which was consistent with decreases in expression of HIF-1, indicating that HIF-1 α in Müller cells is a major mediator of ischemia-/hypoxia-induced VEGF overproduction in the retina. In vivo, hypoxia induces expression of HIF-1, VEGF, ICAM-1, vascular leakage, and retinal neovascularization in mice, which may be attenuated by conditional HIF-1 α deficiency. Moreover, HIF-1 α deficiency also reverses the diabetes-induced inflammation, vascular leakage, and leucostasis (Lin et al. 2011), indicating that Müller cell-derived HIF-1 α is therefore a promising therapeutic target for diabetic retinopathy.

Acknowledgments The project was in part supported by the National Natural Science Foundation of China (No. 81872930; 81573490) and “Double First-Class” University project (No. CPU2018GY22).

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