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Regulation of blood flow in diabetic retinopathy

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Review Article

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Abstract

Blood flow in the retina increases in response to light-evoked neuronal activity, ensuring that retinal neurons receive an adequate supply of oxygen and nutrients as metabolic demands vary. This response, termed "functional hyperemia," is disrupted in diabetic retinopathy. The reduction in functional hyperemia may result in retinal hypoxia and contribute to the development of retinopathy. This review will discuss the neurovascular coupling signaling mechanisms that generate the functional hyperemia response in the retina, the changes to neurovascular coupling that occur in diabetic retinopathy, possible treatments for restoring functional hyperemia and retinal oxygen levels, and changes to functional hyperemia that occur in the diabetic brain.

Blood flow within the retinal vasculature increases in response to light stimulation, ensuring that retinal neurons receive an adequate supply of oxygen and nutrients as metabolic demands vary. This response is termed functional hyperemia and is generated by neurovascular coupling mechanisms which mediate signaling from active neurons to blood vessels. The functional hyperemia response is reduced at a very early stage of diabetic retinopathy, suggesting that the reduction may contribute to the onset and development of retinopathy. This review will focus on the cellular and molecular mechanisms which generate functional hyperemia, the changes that occur in functional hyperemia during diabetic retinopathy, the mechanisms responsible for these changes, their possible contribution to retinopathy, and changes in functional hyperemia that occur in the diabetic brain.

Regulation of blood flow in the retina

The retina has one of the highest metabolic rates of any tissue in the body, and it must maintain an adequate supply of nutrients and oxygen to remain functional and healthy (Country, 2017). Many mechanisms have evolved to ensure that the retina maintains an adequate blood supply at all times. For example, strong autoregulatory mechanisms operate to maintain ample retinal blood flow in the face of variations in systemic blood pressure and variations in O_2 levels and CO_2 levels (Riva et al., 1981; Fallon et al., 1985; Movaffaghy et al., 1998).

An essential component of blood flow regulation in the retina is the functional hyperemia response (Attwell et al., 2010). When neurons are activated by light stimulation, there is a substantial increase in blood flow in retinal vessels (Fig. 1A), with basal blood flow increasing by over 59% during stimulation (Garhofer et al., 2004*a*). The blood flow increase is generated by active dilation of retinal arteries, arterioles, and capillaries. In humans, both primary arteries and veins dilate 3–7% in response to a flickering light stimulus (Fig. 1B). In rodents, arterioles and some capillaries, but not venules, actively dilate (Kornfield & Newman, 2014) (Fig. 1C and 1D).

Cellular and molecular mechanisms of neurovascular coupling

The term "neurovascular coupling" describes the cellular and molecular mechanisms that generate the functional hyperemia response, the pathways by which active neurons signal to blood vessels and cause them to dilate. These mechanisms act on the contractile cells which cover the vessels (Fig. Fig. 2A). Arteries and arterioles are surrounded by one or more layers of smooth muscle cells, which contain contractile proteins (Kur et al., 2012). Capillaries lack a continuous covering of smooth muscle cells but are partially enveloped by pericytes, which are also contractile (Hall et al., 2014). Relaxation of smooth muscle cells or pericytes results in vessel dilation and increased blood flow.

It is useful to first consider the mechanisms responsible for neurovascular coupling in the brain, where much of the research on neurovascular coupling has been conducted (Attwell et al., 2010) (Fig. Fig. 2B). These mechanisms include the release of nitric oxide (NO) and prostaglandins from active neurons onto the smooth muscle cells covering blood vessels. Both

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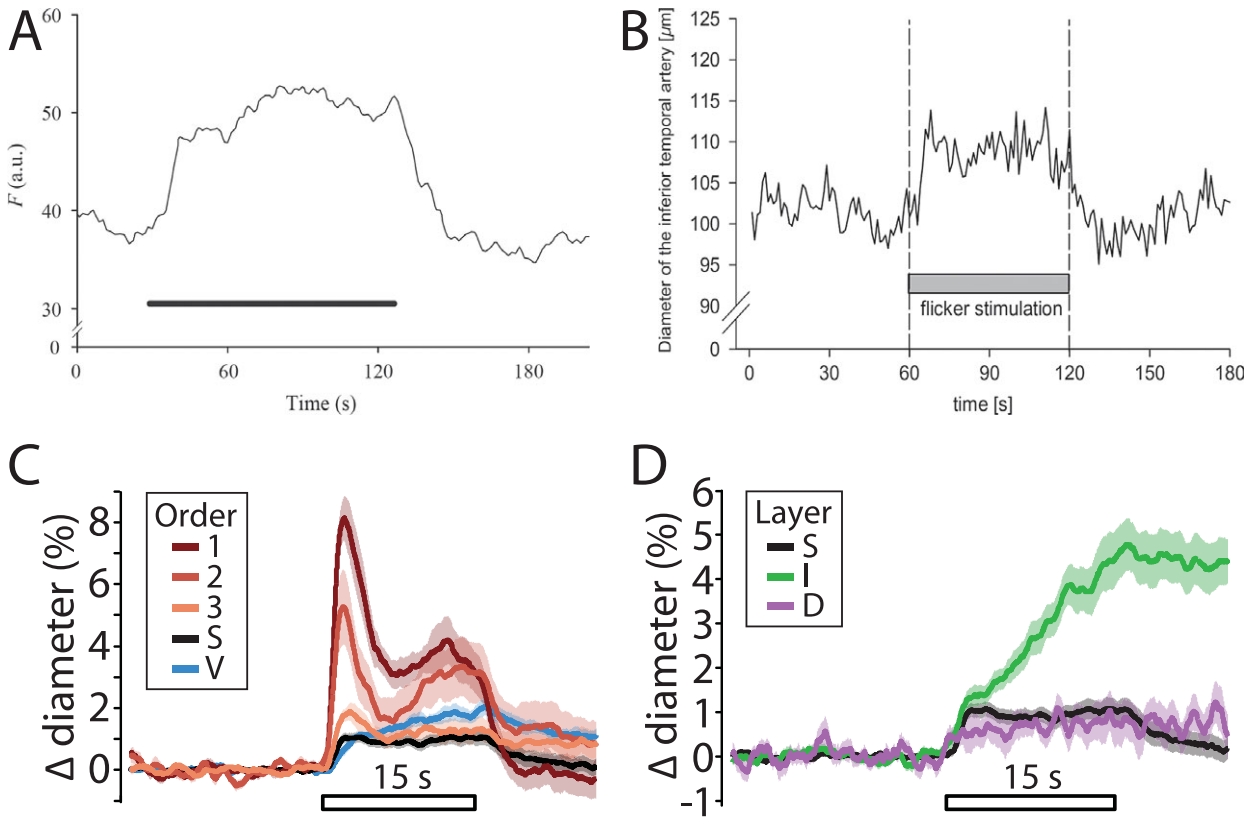

Fig. 1. Functional hyperemia in the retina. Horizontal bars in all panels indicate time course of flicker light stimuli. **(A)** Light-evoked increase in blood velocity measured from the rim of the cat optic disc with laser Doppler flowmetry. From Riva et al. (2005). **(B)** Light-evoked dilation of retinal artery from a human subject measured with the retinal vessel analyzer. From Garhofer et al. (2004*b*). **(C** and **D)** Light-evoked dilation of retinal vessels in the rat. From Kornfield and Newman (2014). **(C)** Time course of flicker-evoked dilation of vessels on the vitreal surface of the retina. First (1), second (2), and third (3) order arterioles, superficial capillaries (S), and veins (V). The largest arterioles dilate the most. **(D)** Time course of flicker-evoked capillary dilations in the superficial (S), intermediate (I), and deep (D) capillary layers. Only the intermediate layer capillaries actively dilate. The small dilations in the other capillaries are due to passive stretch.

compounds dilate vessels, leading to increased blood flow. In addition to its vasodilatory effect, NO can also modulate other neurovascular coupling pathways (Lindauer et al., 1999; Attwell et al., 2010). As we shall see, the modulatory role of NO becomes important when considering the changes to neurovascular coupling that occur in diabetic retinopathy.

In addition to direct signaling from neurons to vessels, active neurons also signal to vessels indirectly by stimulating astroglial cells that in turn release vasodilating agents (Attwell et al., 2010). Astrocytes are ideally situated to act as intermediaries in neurovascular signaling. Their fine processes surround synapses, allowing them to sense and respond to neuronal activity, while their endfeet envelop blood vessels, permitting them to release vasoactive agents directly onto vascular smooth muscle cells. Neurotransmitters released from neurons at synapses stimulate astrocytes, generating intracellular Ca^{2+} increases. The neurotransmitters glutamate and ATP play major roles in generating these astrocytic Ca^{2+} signals by activating metabotropic receptors on astrocytes. The astrocytic Ca^{2+} increases activate phospholipase A2 and other enzymes (Mishra et al., 2016), leading to the production of arachidonic acid (AA) and to the synthesis and release of AA metabolites, including prostaglandin E2 (PGE_2) and epoxyeicosatrienoic acids (EETs), resulting in vasodilation. The vasoconstrictor, 20-hydroxy-eicosatetraenoic acid (20-HETE) is also produced from AA, either by astrocytes or by vascular smooth muscle cells.

Other signaling mechanisms may also contribute to neurovascular coupling. Vascular endothelial cells may evoke vessel dilation by releasing vasoactive agents themselves (Chen et al., 2014). They contain endothelial nitric oxide synthase and can release NO, which relaxes smooth muscles cells. It is believed that signals from neurons or astrocytes stimulate vascular endothelial cells to release vasoactive agents. However, the nature of these signaling pathways is not well understood. The release of K^+ from astrocytes may also contribute to vasodilation, acting directly on smooth muscle cells or indirectly *via* the endothelial cells (Newman et al., 1984; Dunn & Nelson, 2010).

Neurovascular coupling in the retina

Neurovascular coupling mechanisms in the retina share similarities to those in the brain, with important differences. Blood flow regulation is mediated largely by Müller cells, the principal macroglial cells of the retina (Newman, 2015) (Fig. Fig. 2C). Müller cells have many of the same properties as astrocytes in the brain and assume their functions in the retina (Newman & Reichenbach, 1996). Their fine processes surround synapses, and their endfeet envelop blood vessels within the retina.

Experimental stimulation of Müller cells evokes arteriole dilation, demonstrating that these glial cells can directly release vasodilating agents (Metea & Newman, 2006; Mishra & Newman, 2010). Müller cell intracellular Ca^{2+} increases evoked by photolysis of caged Ca^{2+} or caged IP_3 result in robust dilation of arterioles. The essential role of Müller cells in mediating retinal neurovascular coupling is demonstrated by the observation that light-evoked vasodilations are abolished when neuron to Müller cell signaling

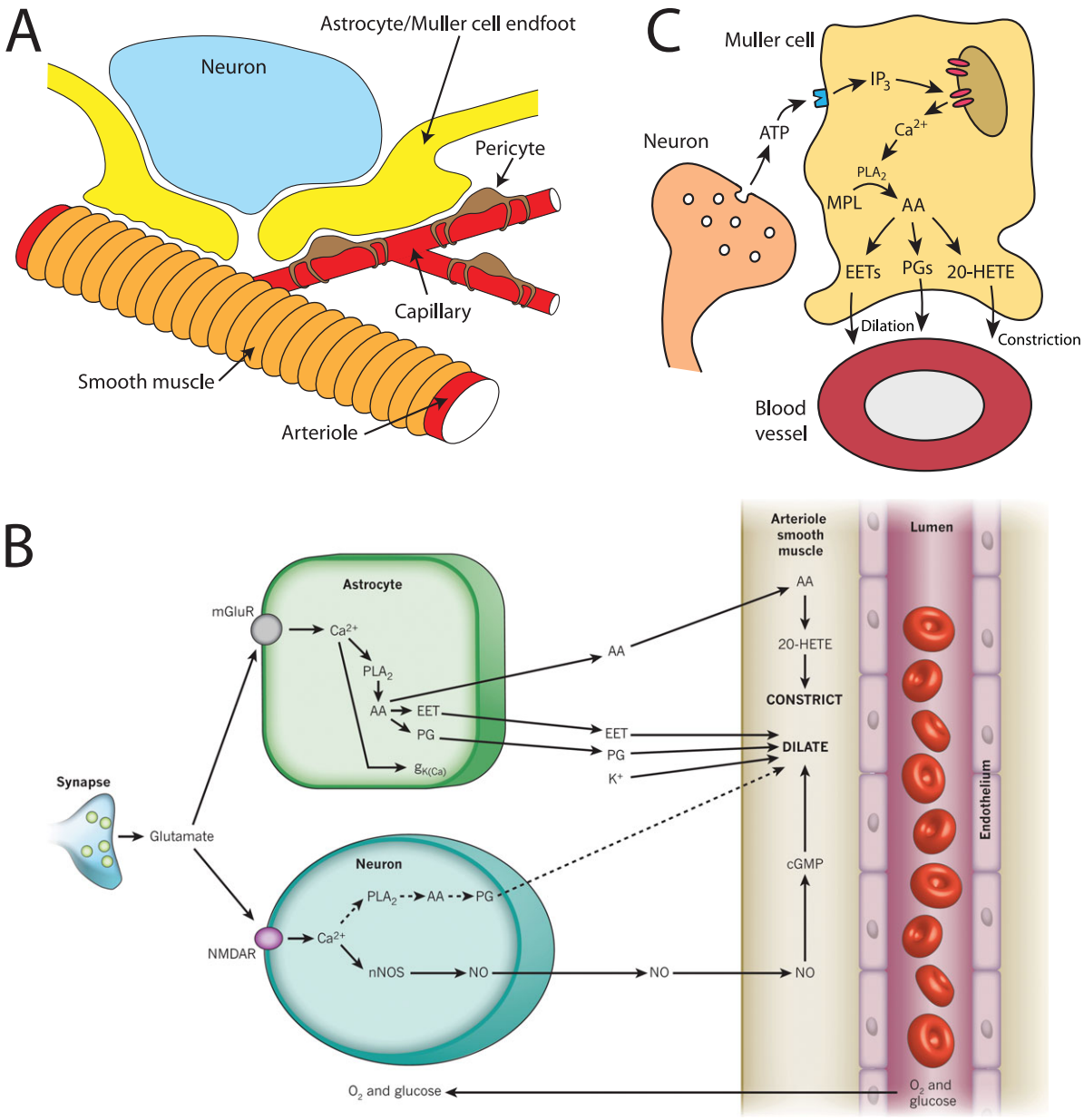

Fig. 2. Cellular and molecular mechanisms of neurovascular coupling. **(A)** Cells of the neurovascular unit regulate blood flow in the brain and retina. The diameters of arteries and arterioles are controlled by smooth muscle cells, while capillary diameter is controlled by pericytes. Astroglial cells in the brain and Müller glial cells in the retina surround arterioles and capillaries and their contractile cells. Signaling molecules released from neurons, and glial cell endfeet control the contractile state of smooth muscle cells and pericytes. From Nippert et al. (2018). **(B)** Signaling pathways that mediate neurovascular coupling in the brain. Glutamate released from neurons acts on NMDA receptors in neurons (NMDAR) to raise intracellular Ca^{2+} levels, causing neuronal nitric oxide synthase (nNOS) to release NO, dilating smooth muscle cells. Raised Ca^{2+} may also (dashed line) generate arachidonic acid (AA) from phospholipase A2 (PLA_2), which is converted to prostaglandins (PG) that dilate vessels. Glutamate also raises Ca^{2+} levels in astrocytes by activating metabotropic glutamate receptors (mGluR), generating AA and three types of AA metabolites: prostaglandins and EETs, which dilate vessels, and 20-HETE, which constricts vessels. From Attwell et al. (2010). (C) Neurovascular coupling in the retina. ATP released from neurons stimulates purinergic receptors on Müller cells, leading to the production of IP_3 and the release of Ca^{2+} from internal stores. Ca^{2+} activates PLA_2, which converts membrane phospholipids (MPL) to AA which is subsequently metabolized to the vasodilators PGs and EETs, and to the vasoconstrictor 20-HETE. From Newman (2015).

is blocked. Addition of purinergic antagonists, which block ATP receptors on Müller cells, eliminates light-evoked Ca^{2+} increases in Müller cells and prevents light-evoked arteriole dilation without reducing neuronal activity (Metea & Newman, 2006). Müller cells are responsible for dilating capillaries as well as arterioles in the retina. Ca^{2+} increases in Müller cells are both necessary and sufficient for capillary dilation (Biesecker et al., 2016).

Neurovascular coupling in the retina is mediated primarily by the release of vasodilating metabolites of AA from Müller cells (Mishra et al., 2011). Light-evoked vasodilation is reduced by 82% when PGE_2 synthesis is blocked. Similarly, vasodilation is reduced by 82% when EET synthesis is blocked. Light-evoked vasodilation is reduced by 88% when both PGE_2 and EET syntheses are blocked, indicating that the bulk of neurovascular coupling in the retina is mediated by the production and release of these two AA metabolites. A third AA metabolite, 20-HETE, mediates vasoconstriction (Metea & Newman, 2006; Mishra et al., 2011). Under physiological conditions, the vasodilating effects of PGE_2 and EETs outweigh the

vasoconstricting effects of 20-HETE, and light stimulation produces a vasodilation and an increase in blood flow. As described below, the balance between vasodilating and vasoconstricting influences is altered under pathological conditions such as diabetic retinopathy.

NO does not mediate neurovascular coupling directly in the retina. Rather, as in the cerebral cortex (Lindauer et al., 1999), NO has a modulatory role. When NO levels are raised by addition of an NO donor to the bath, light-evoked vasodilation is suppressed, revealing a light-evoked vasoconstriction (Metea & Newman, 2006). This vasoconstriction is blocked by inhibiting 20-HETE synthesis. NO may mediate its modulatory influence by inhibiting the synthesis of AA metabolites (Attwell et al., 2010). As described below, the modulatory effect of NO plays an essential role in functional hyperemia deficits in the diabetic retina.

The release of K^+ from Müller cells has been proposed to contribute to neurovascular coupling in the retina (Newman et al., 1984). However, K^+ siphoning, the process by which Müller cell depolarization generates an efflux of K^+ from Müller cell endfeet, does not contribute to vessel dilation (Metea et al., 2007). The role of vascular endothelial cells in releasing vasoactive agents and dilating vessels in response to neuronal activity has not been explored in the retina.

Neurovascular coupling in the retinas of diabetic patients

The functional hyperemia response is altered dramatically in diabetic patients. In nondiabetic subjects, light stimulation produces pronounced vasodilation. This can be measured easily with the retinal vessel analyzer and the dynamic vessel analyzer. These instruments are modified fundus cameras that present flickering light stimuli and record videos of fundus images (Blum et al., 1999; Polak et al., 2002). The vessel analyzers automatically measure the diameters of the primary arteries and veins of subjects. A flickering light typically evokes a 4–7% dilation in arteries and a 2–4% dilation in veins, leading to increased blood flow (Polak et al., 2002; Garhofer et al., 2004*a*; Riva et al., 2005).

These light-evoked vasodilations are reduced by ~60% in arteries and ~30% in veins of patients with both type 1 (Garhofer et al., 2004*a*; Mandecka et al., 2007; Nguyen et al., 2009; Pemp et al., 2009) and type 2 diabetes (Mandecka et al., 2007; Bek et al., 2008; Nguyen et al., 2009) (Fig. 3A). Importantly, these reductions in functional hyperemia occur at a very early stage of disease progression, in patients with no overt signs of retinopathy or in patients with only mild nonproliferative retinopathy, although reductions are more pronounced in mild nonproliferative retinopathy patients (Mandecka et al., 2007; Bek et al., 2008). The early deficits in functional hyperemia loss have prompted suggestions that it may be a useful diagnostic indicator for the onset of diabetic retinopathy and the probability that retinopathy will advance (Mandecka et al., 2007; Lim et al., 2017; Mozolewska-Piotrowska et al., 2019).

In addition to changes in functional hyperemia, alterations in basal retinal blood flow have been observed in diabetic patients. Retinal hypoperfusion is seen in early stages of diabetes with a shift to hyperperfusion as diabetic retinopathy progresses. However, there are quantitative and qualitative inconsistencies in the data which may be explained by several factors, including the stage of diabetes, the techniques used, the site of measurement in the retina, and demographic parameters (Grunwald et al., 1992; Pemp & Schmetterer, 2008; Curtis et al., 2009).

Neurovascular coupling in animal models of diabetes

Diabetes-induced changes in functional hyperemia have also been explored in animal models of diabetic retinopathy using both *in vivo* and isolated retina preparations. In the rat streptozotocin (STZ) model of type 1 diabetes, light-evoked dilations in retinal arterioles are reduced in early stages of diabetic retinopathy (Mishra & Newman, 2010; Mishra & Newman, 2012). *In vivo*, flickering light evokes a 10.8% dilation in arterioles of control animals. These dilations are reduced to 4.2% at 7 months after diabetes induction (Fig. 3B and 3C). This 61% reduction in vasodilation is similar to that seen in diabetic patients. The reduction occurs before major signs of retinopathy appear; at this time point, there is no loss of retinal thickness nor increase in neuronal cell death. There are, however, early signs of reactive gliosis in Müller cells (Mishra & Newman, 2010).

The diabetes-induced loss of functional hyperemia is due to an upregulation of inducible nitric oxide synthase (iNOS) in retinal neurons and glial cells, leading to increased NO levels in the retina (Mishra & Newman, 2010). This NO increase reduces neurovascular coupling, resulting in diminished light-evoked arteriole dilation (Metea & Newman, 2006). The mechanism for the NO-induced reduction in neurovascular coupling is believed to

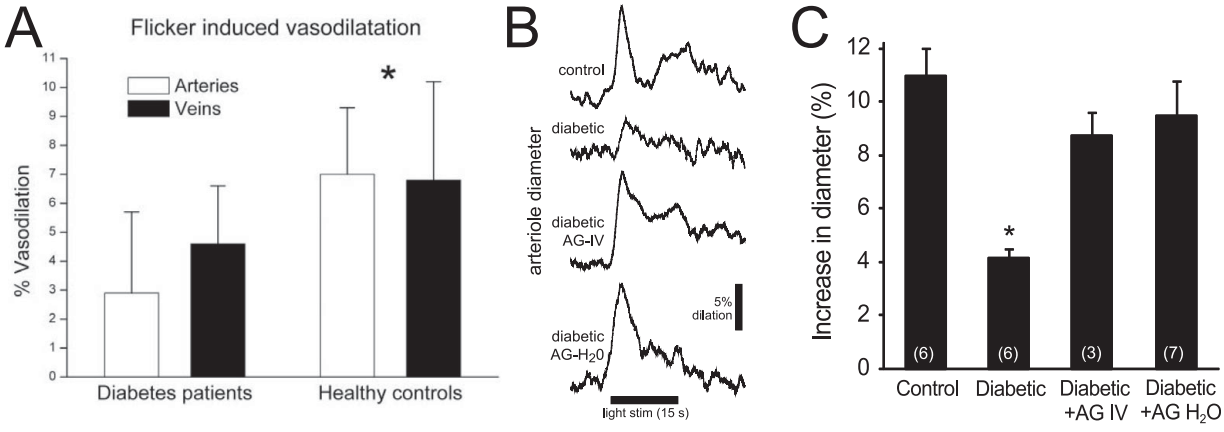

Fig. 3. Reduced functional hyperemia in the diabetic retina. **(A)** Light-evoked dilations of retinal arteries and veins are reduced in patients with type 1 diabetes. From Pemp et al. (2009). (B and C) Light-evoked dilations of retinal arterioles in the diabetic rat retina. From Mishra and Newman (2012). **(B)** Arteriole dilation is reduced in the diabetic retina compared to age-matched controls. Full dilation is restored by an acute IV injection of the iNOS inhibitor aminoguanidine (AG-IV) or by chronic administration of aminoguanidine in the drinking water (AG-H_2O). **(C)** Summary of results from B.

be due to an NO block of the synthesis of vasodilatory agents released from Müller cells (Attwell et al., 2010). Inhibition of iNOS, which leads to a reduction in retinal NO levels, results in a restoration of neurovascular coupling in diabetic retinas. The iNOS inhibitors aminoguanidine (AMG) and 1400W both restore light-evoked vasodilations to control levels in isolated diabetic retinas (Mishra & Newman, 2010). *In vivo*, AMG restores light-evoked vasodilations to control levels when administered chronically though drinking water or acutely by intravenous injection (Fig. 3B and 3C) (Mishra & Newman, 2012).

iNOS inhibition in diabetic retinopathy

The loss of light-evoked increases in blood flow in both diabetic patients and animals may reduce the supply of oxygen and nutrients to the retina and lead to retinal hypoxia. It has been suggested that hypoxia could exacerbate or even be a root cause of diabetic retinopathy. In that case, inhibiting iNOS, which restores functional hyperemia in animal models of diabetes, should slow the progression of diabetic retinopathy. Indeed, the iNOS inhibitor AMG has been shown to be extremely effective in preventing the development of diabetic retinopathy in animal models (Kern et al., 2000; Kowluru et al., 2000). In a notable 5-year study on diabetic dogs, AMG prevented the development of retinopathy entirely (Kern & Engerman, 2001).

The above results must be interpreted cautiously, however, as AMG has many effects besides the inhibition of iNOS. AMG inhibits the nonenzymatic formation of advanced glycation end-products (AGEs), and its effects on diabetic retinopathy have been attributed to this action (Brownlee et al., 1986). However, findings from several studies suggest that AMG could also be acting by mechanisms other than inhibition of AGE formation, including inhibition of iNOS (Kern et al., 2000; Kowluru et al., 2000; Kern & Engerman, 2001; Mishra & Newman, 2010).

There has been one clinical trial on the effects of AMG (pimagedine) in diabetic patients (Bolton et al., 2004). The trial was primarily a study of the effects of AMG on diabetic nephropathy in type 1 patients. However, the study also evaluated the progression of retinopathy and found that AMG reduced the number of patients whose retinopathy progressed three steps or more in the Early Treatment Diabetic Retinopathy Study retinopathy score in a dose-dependent manner. However, the study did not demonstrate statistically significant beneficial effects on the slowing of retinopathy, and some patients receiving high-dose AMG experienced glomerulonephritis. No trials on the efficacy of AMG or other iNOS inhibitors in diabetic retinopathy have subsequently been conducted.

Hypoxia hypothesis of diabetic retinopathy

As described above, the development of diabetic retinopathy may be triggered, at least in part, by the loss of functional hyperemia and the development of retinal hypoxia. More generally, retinal hypoxia has been proposed as a root cause of retinopathy (Sivaprasad & Arden, 2016). It is noteworthy that in psychophysical experiments, reduced contrast sensitivity and reduced color vision perception, both seen in diabetic patients, are restored when patients breathe 100% O_2 (Harris et al., 1996; Dean et al., 1997). O_2 saturation measurements in diabetic patients (Fondi et al., 2017) and measurements of retinal O_2 levels in diabetic animal models (Linsenmeier et al., 1998; de Gooyer et al., 2006) are consistent with a hypoxic origin of diabetic retinopathy. However, other studies indicate that basal retinal blood flow is raised and that retinal oxygen levels are increased during diabetic retinopathy, casting doubt on the hypoxia hypothesis (Wright et al., 2011; Lau & Linsenmeier, 2014; Wanek et al., 2014).

G.B. Arden has suggested that the high metabolic demand of photoreceptors in the dark, which lower O_2 levels in the proximal retina, will exacerbate retinal hypoxia and may be a primary cause of diabetic retinopathy (Arden et al., 1998). He proposed that preventing the rod photoreceptors from dark adapting, which will significantly reduce their metabolic demand, may lessen hypoxia and slow the progression of retinopathy. However, a multicenter, phase 3 trial showed that wearing a light mask to light adapt the eyes while sleeping was ineffective in slowing the progression of retinopathy, as assayed by changes in retinal thickness (Sivaprasad et al., 2018). A study on diabetic rats reached the same conclusion. Maintaining animals in dim light during the night to light adapt the retina did not slow the progression of retinopathy (Kur et al., 2016).

Vascular dysfunction in the diabetic brain

Diabetes is associated with cognitive deficits as well as retinopathy (Moheet et al., 2015). This cognitive impairment is likely due to a variety of factors, including vascular dysfunction and a reduction in cortical functional hyperemia. Indeed, many signaling pathways involved in neurovascular coupling in the brain are altered in diabetes, including changes in iNOS and caveolin expression as well as astrocyte signaling (Song et al., 2008; Nagayach et al., 2014; Bonds et al., 2019). It is unclear, however, whether and how these changes in signaling pathways contribute to neurovascular coupling deficits or cognitive decline.

Measuring functional hyperemia in the brain in human studies is more difficult than in the retina as brain vessels cannot be directly visualized. Instead, researchers have used blood oxygenation level–dependent functional magnetic resonance imaging (BOLD fMRI) to measure changes in functional hyperemia. A recent study using BOLD fMRI found no global deficits in functional hyperemia in type 2 diabetes, although there were region-specific deficits (Hu et al., 2019). Other studies examining task performance and BOLD fMRI in patients with type 2 diabetes found decreases in the BOLD signal, indicating decreased functional hyperemia (Chung et al., 2015; Duarte et al., 2015; Wong et al., 2016).

Diabetes-induced deficits in brain functional hyperemia have also been noted in animal studies. Impairments in functional hyperemia have been observed in a lean type 2 model of diabetes, where researchers found a decrease in functional hyperemia in the barrel cortex in response to whisker stimulation (Fig. 4A and 4B) (Kelly-Cobbs et al., 2012). A second study found that dilation of pial arteries on the surface of the brain evoked by sciatic nerve stimulation was diminished in STZ-induced diabetic rats (Fig. 4C and 4D) (Vetri et al., 2012). This decrease in functional hyperemia was restored with topical application of a PKC-α/β/γ inhibitor on the surface of the brain. Impairment in functional hyperemia was also seen in a zebrafish model of prolonged hyperglycemia. Zebrafish maintained in high glucose water developed impairments in functional hyperemia in the optic tectum in response to visual stimulation (Chhabria et al., 2018). Functional hyperemia was restored in the zebrafish with application of sodium nitroprusside, an NO donor. This is an intriguing finding, as it is at odds with the situation in the retina of diabetic rats, where neurovascular coupling is restored by lowering NO levels (Mishra & Newman, 2010).

Interestingly, cognitive decline in diabetic patients may be associated with the development of diabetic retinopathy (Ding et al.,

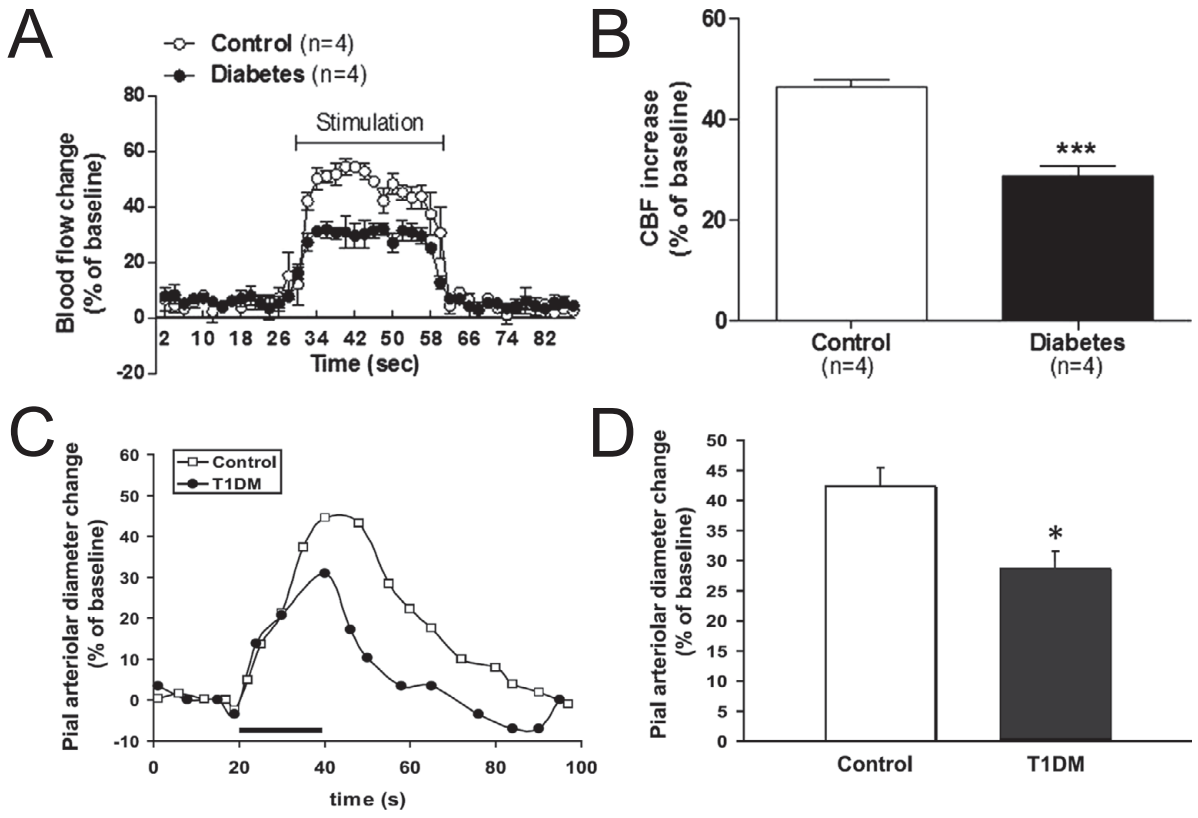

Fig. 4. Reduced functional hyperemia in the diabetic rodent brain. **(A)** Increase in cerebral blood flow in the somatosensory cortex in response to whisker stimulation is reduced in a genetic rat model of type 2 diabetes. **(B)** Summary of results from A in control and diabetic rats. **(A)** and **(B)** are from Kelly-Cobbs et al. (2012). **(C)** Pial artery dilation in response to sciatic nerve stimulation is reduced in a rat model of type 1 diabetes. Sciatic nerve stimulation is indicated by the black bar. **(D)** Summary of results from C in control and diabetic rats. **(C)** and **(D)** are from Vetri et al. (2012).

2011). One longitudinal study found that patients with retinopathy in a baseline visit were more likely to have cognitive decline and decreased gray matter mass at a 40-month follow-up visit (Hugenschmidt et al., 2014). Other studies failed to find a correlation between retinopathy and cognitive decline, perhaps owing to differences in subjects and methodologies (Crosby-Nwaobi et al., 2012).

Conclusions

Functional hyperemia regulates blood flow in the retinal vasculature, ensuring that retinal neurons receive an adequate supply of oxygen and nutrients as metabolic demands vary. The functional hyperemia response is compromised in patients with both type 1 and type 2 diabetes as well as in animal models of diabetes. The cellular mechanisms responsible for diminished neurovascular coupling have been elucidated in an animal model of diabetes, indicating that an upregulation of iNOS and an increase in retinal NO are responsible for the loss of functional hyperemia. Inhibition of iNOS reverses the loss of functional hyperemia in an animal model of diabetes, but additional studies are needed to determine the efficacy of iNOS inhibition in diabetic patients. While minor impairments in functional hyperemia are seen in the brain, the retina appears to be particularly vulnerable to damage from diabetes. Future research examining differences in neurovascular coupling between the brain and the retina may provide important insights into retina-specific vulnerabilities in diabetes.

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