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SYMPOSIUM REVIEW

Compromised blood–brain barrier permeability: novel mechanism by which circulating angiotensin II signals to sympathoexcitatory centres during hypertension

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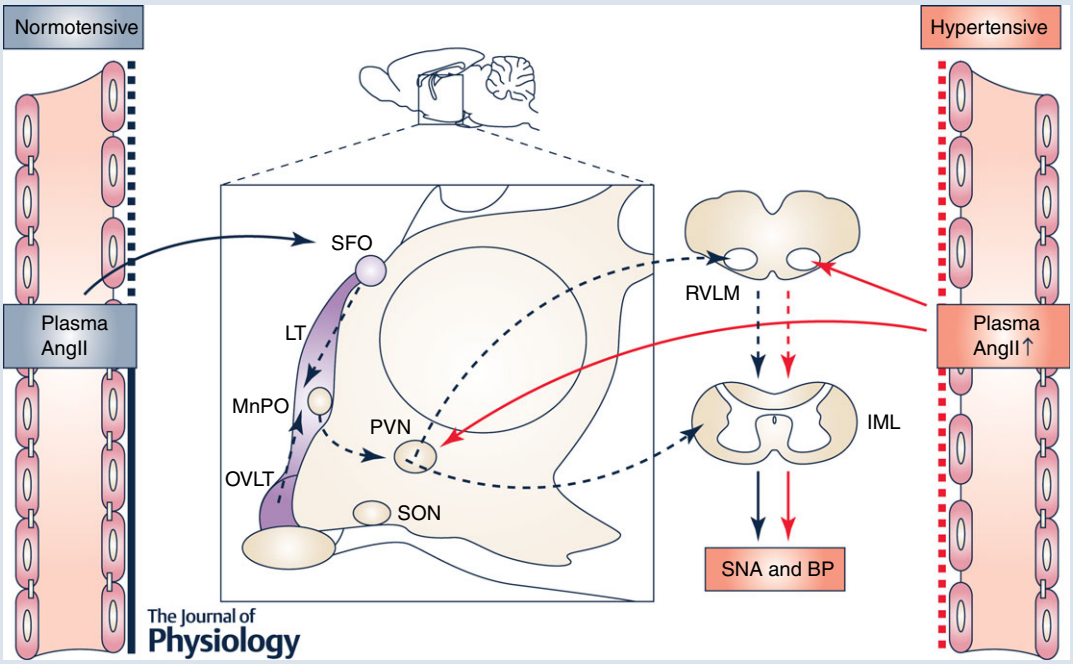

Abstract Angiotensin II (AngII) is a pivotal peptide implicated in the regulation of blood pressure. In addition to its systemic vascular and renal effects, AngII acts centrally to modulate the activities of neuroendocrine and sympathetic neuronal networks, influencing in turn sympatho-humoral outflows to the circulation. Moreover, a large body of evidence supports AngII signalling

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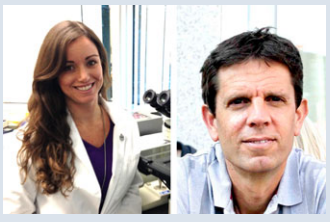

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dysregulation as a key mechanism contributing to exacerbated sympathoexcitation during hypertension. Due to its hydrophilic actions, circulating AngII does not cross the blood–brain barrier (BBB), signalling to the brain via the circumventricular organs which lack a tight BBB. In this review, we present and discuss recent studies from our laboratory showing that elevated circulating levels of AngII during hypertension result in disruption of the BBB integrity, allowing access of circulating AngII to critical sympathoexcitatory brain centres such as the paraventricular nucleus of the hypothalamus and the rostral ventrolateral medulla. We propose the novel hypothesis that AngII-driven BBB breakdown constitutes a complementary mechanism by which circulating AngII, working in tandem with the central renin–angiotensin system, further exacerbates sympatho-humoral activation during hypertension. These results are discussed within the context of a growing body of evidence in the literature supporting AngII as a pro-inflammatory signal, and brain microglia as key cell targets mediating central AngII actions during hypertension.

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Abstract figure legend Proposed model of circulating angiotensin II (AngII) signalling pathways in the brain under normotensive and hypertensive conditions. Under normal conditions (left, blue), and due to its hydrophilic nature, circulating AngII does not cross the blood–brain barrier (BBB) and signals to the brain through the circumventricular organs (mainly the SFO), which lack a tight BBB. The SFO then engages the PVN–RVLM–IML pathway, increasing sympathetic nerve activity to the circulation. During hypertension (right, red), circulating AngII leads to BBB breakdown, allowing its own leakage into the PVN and RVLM parenchyma, resulting in overactivation of the PVN–RVLM–IML pathway and exacerbated sympathoexcitatory outflow to the circulation. BP, blood pressure; IML, intermediolateral cell column; LT, lamina terminalis; MnPO, median preoptic nucleus; OVLT, organum vasculosum of the lamina terminalis; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla; SFO, subfornical organ; SNA, sympathetic nerve activity; SON, supraoptic nucleus.

Abbreviations 2K1C, Goldblatt hypertensive model; two-kidneys, one-clip; AngII, angiotensin II; AT1R, angiotensin type 1 receptor; BBB, blood–brain barrier; BP, blood pressure; CVOs, circumventricular organs; NTS, nucleus of the tractus solitarius; PVN, paraventricular nucleus of the hypothalamus; ROS, reactive oxygen species; RVLM, rostral ventrolateral medulla; SFO, subfornical organ; SHR, spontaneous hypertensive rats; WKY, Wistar Kyoto rats.

Introduction

Hypertension remains a global public health challenge. It affects one-third of US adults, being a key risk factor for stroke, myocardial infarction, vascular disease and chronic kidney disease. A large proportion of the hypertensive population is categorized as 'neurogenic', displaying an increased activation of the sympathetic nervous system during the initiation and maintenance phases of the disease (Parati & Esler, 2012). Importantly, sympathoexcitation is a major determinant of morbidity and mortality in hypertensive patients (Mancia *et al.* 1999; Parati & Esler, 2012). Thus, understanding the precise neuroanatomical pathways and neurobiological mechanisms underlying increased sympathetic outflow in hypertension is of critical physiological and clinical importance.

Regulation of blood pressure (BP) by the central nervous system involves coordinated activities among highly interconnected neuronal networks distributed throughout the spinal cord, brainstem and forebrain, including, among others, the nucleus of the tractus solitarius (NTS), the rostral ventrolateral medulla (RVLM) and the paraventricular nucleus of the hypothalamus (PVN) (Swanson & Sawchenko, 1983; Guyenet, 2006). Within these centres, numerous chemical signals have been identified to play critical roles in regulating sympathoexcitatory outflow to the cardiovascular system (Gabor & Leenen, 2012). Among them, the neuropeptide angiotensin II (AngII), which also acts as a circulating hormone, constitutes a pivotal signal modulating the activities of both central neuroendocrine and sympathetic neuronal networks. Moreover, a large body of evidence supports AngII signalling dysregulation as one of the key mechanisms involved in stimulating the sympathetic nervous system within the brain (Fink, 1997; Leenen, 2014). There are excellent reviews in the literature covering the contribution of central and systemic AngII to hypertension (Ferrario, 1983; Osborn *et al.* 2007; Paton *et al.* 2008; Coble *et al.* 2015).

Recent findings from our and other laboratories, however, provide support for a novel mechanism by which the peripheral and central renin–angiotensin systems, via disruption of the blood–brain barrier (BBB), may act

in tandem to promote exacerbated sympathoexcitatory activity, contributing in turn to neurogenic hypertension (Biancardi *et al.* 2014). We summarize and review here these novel findings, discussing them in the context of previous literature in the field.

AngII brain signalling and the regulation of blood pressure

AngII is the major effector peptide of the renin–angiotensin systems. It is produced by cleavage of angiotensinogen by the proteolytic enzyme renin into angiotensin I, which is in turn converted to AngII by the angiotensin-converting enzyme. Besides its classical peripheral actions, including vasoconstriction and kidney sodium reabsorption (Cogan, 1990), AngII has been implicated in the regulation of the cardiovascular system via actions within the brain. Central AngII actions include stimulation of fluid intake, sodium appetite, secretion of hormones (adrenocorticotropic hormone, oxytocin and vasopressin), modulation of baroreflex and activation of the sympathetic nervous system (Lang *et al.* 1981; Iovino & Steardo, 1984; Ganong & Murakami, 1987; McKinley *et al.* 1996, 2001, 2003; Paton *et al.* 2001; Grippo *et al.* 2002; Ito *et al.* 2002; Sanderford & Bishop, 2002; Guyenet, 2006; Tan *et al.* 2007; Coble *et al.* 2015). Because AngII is highly hydrophilic, the general consensus is that circulating AngII triggers changes in the brain through actions on the circumventricular organs (CVOs), which have an incomplete BBB, consisting of fenestrated capillaries and an incomplete basal membrane and astrocyte ensheathment (Broadwell & Brightman, 1976; Shaver *et al.* 1992; Daneman, 2012). This particular topographical arrangement creates a large perivascular space that allows passage of large molecules from the circulation into the brain parenchyma. The CVOs are divided into secretory and sensory groups. The secretory group includes the median eminence, the neurohypophysis, the intermediate lobe of the pituitary gland and the pineal gland, while the sensory group includes the subfornical organ (SFO), the organum vasculosum lamina terminalis and the area postrema (Fry & Ferguson, 2007). Circulating AngII signalling through the sensory CVOs is then integrated and conveyed to major brain autonomic and neurosecretory centres, including the PVN, which through descending projections to the RVLM and the NTS mediate the sympathoexcitatory, neurosecretory and modulation-of-baroreflex effects of the circulating peptide (Bains *et al.* 1992; Ferguson & Bains, 1997; Anderson *et al.* 2001; Dampney *et al.* 2007; Tan *et al.* 2007; Ferguson, 2009). Indeed, elevated circulating levels of AngII, resulting in over-activation of the PVN and RVLM, is recognized as a critical factor contributing to excessive sympathetic activation and vasopressin outflow in neurogenic hypertension in different experimental animal models (Ferguson & Bains, 1997; Bergamaschi *et al.* 2002; de Oliveira-Sales *et al.* 2010; Chen *et al.* 2011; Huber & Schreihofer, 2011; Qi *et al.* 2013).

Most of the central AngII actions are mediated by activation of the AngII type 1 receptor (AT1R), a G protein-coupled receptor (McKinley *et al.* 1996). AT1Rs have been shown to be present within the sensory CVOs, the hypothalamus and the brainstem in several mammals, including the human (Mendelsohn *et al.* 1984; Song *et al.* 1992; MacGregor *et al.* 1995; McKinley *et al.* 1996; Lenkei *et al.* 1997). In fact, AngII microinjected into those nuclei causes dose-dependent pressor responses (Casto & Phillips, 1984; Muratani *et al.* 1991; Jensen *et al.* 1992; Toney & Porter, 1993*b*), while AT1R blockade with losartan prevents the increase in BP and vasopressin secretion following intracerebroventricular injections of AngII (Toney & Porter, 1993*a*).

In addition to circulating AngII, all the components of the renin–angiotensin system are also present within the brain, including the newly discovered (pro)renin receptor, allowing local formation of AngII and its use as a central neurotransmitter (Ganten & Speck, 1978; McKinley *et al.* 2003; Li *et al.* 2012). For example, AngII has been identified as a key neurotransmitter in the SFO–PVN pathway (Osborn *et al.* 2007; Ferguson, 2009; Coble *et al.* 2015). Ferguson and collaborators have shown that either electrical stimulation of the SFO, or AngII directly applied within the PVN, increased the firing activity of PVN neurons, responses that were attenuated by the AT1R blocker losartan (Li & Ferguson, 1993; Bains & Ferguson, 1995). Brain AngII has also been shown to be functionally relevant within the RVLM, where an injection of AT1R antagonist decreased mean arterial pressure (∼14 mmHg) in animal models with either high or low plasma renin activity, such as transgenic rats with over-expression of a mouse renin gene (Fontes *et al.* 2000), the Dahl salt-sensitive hypertensive rat (∼35 mmHg) (Ito *et al.* 2003) and the spontaneous hypertensive rat (SHR) (∼35 mmHg) (Ito *et al.* 2002), with no effect in normotensive animals.

Although local formation has been implicated as the main source of central AngII contributing to sympathoexcitation and hypertension (Fink, 1997), we have obtained recent evidence, as summarized and discussed further below, that circulating AngII, under certain conditions, may gain access to cardiovascular centres within the central nervous system, exerting its own direct actions on sympathoexcitatory-related nuclei.

AngII and brain inflammation during hypertension

Vascular brain inflammation has emerged as a novel pathophysiological mechanism contributing to neurogenic hypertension (Paton & Waki, 2009; Lazartigues, 2010; Winklewski *et al.* 2015). A growing body of

evidence supports AngII as a pro-inflammatory molecule, particularly in hypertension. For example, AngII has been shown to be critical for T-cell activation and development of vascular inflammation during hypertension, via both central and peripheral mechanisms (Marvar *et al.* 2010, 2011). Moreover, Francis and collaborators have shown that in an AngII-induced hypertensive model, AngII stimulated production and release of the transcription factor NFκB along with various pro-inflammatory cytokines, including TNFα, IL-1β and IL-6 within the PVN, all factors that contributed to neurohumoral excitation via oxidative stress (Kang *et al.* 2009; Cardinale *et al.* 2012; Sriramula *et al.* 2013). Similar results have been recently reported in renovascular hypertensive rats (Goldblatt model, two-kidneys, one-clip (2K1C)), in which mRNA expression of AT1 receptors and NAD(P)H oxidase subunits were shown to be increased within the RVLM and PVN of hypertensive animals, contributing to elevated BP and sympathetic activity (Oliveira-Sales *et al.* 2009), effects that were attenuated by AT1R blockade within the RVLM (Nishi *et al.* 2013). In addition, higher levels of cytokines (TNFα, IL-1β and IL-6, and chemokine MCP-1) have also been reported within the RVLM in this hypertensive model (Li *et al.* 2014). Likewise, pro-inflammatory factors such as the junctional adhesion molecule-1 were found to be upregulated within the NTS of SHR (Waki *et al.* 2007, 2011).

Microglia cells are the primary resident immune cells of the brain (Saijo & Glass, 2011), and thus likely candidates mediating central AngII pro-inflammatory actions. In fact, several recent papers reported that AngII can turn microglia from a resting state into an activated, inflammatory state (Miyoshi *et al.* 2008; Rodriguez-Pallares *et al.* 2008; Benicky *et al.* 2009). Moreover, AngII-mediated microglia cell activation within the PVN has been shown to contribute to high BP in an angiotensin II-induced hypertensive rat model, via generation of pro-inflammatory cytokines (IL-6, IL1β and TNFα) (Shi *et al.* 2010).

Disruption of the blood–brain barrier (BBB) integrity in neuro-inflammatory disorders and hypertension

The BBB acts as a dynamic physical barrier at the brain–blood interface, which effectively excludes substances that are lipid insoluble, as well as those of high molecular weight (Abbott *et al.* 2006, 2010; Obermeier *et al.* 2013; Wong *et al.* 2013). The BBB is mainly composed of tight junctions formed between endothelial cells and the enwrapping astrocyte endfeet, which limit the paracellular diffusion of hydrophilic molecules (Stamatovic *et al.* 2008; Abbott *et al.* 2010). In addition, BBB endothelial cells, under normal conditions, have minimal vesicle transport activity, which limits transcellular transport (Stamatovic *et al.* 2008). This process is essential to maintain homeostasis of the brain environment. In general, astrocytes, pericytes and neurons that are in direct physical contact with the capillary endothelium play an important role in the regulation of BBB integrity (Abbott *et al.* 2010).

Breakdown of the BBB, resulting in increased permeability and access to the brain of circulating substances normally excluded, is a common feature in neuro-inflammatory disorders, including ischaemia and multiple sclerosis (Waubant, 2006; Yang & Rosenberg, 2011). An altered BBB state has also been shown in hypertension (Mayhan *et al.* 1989; Ueno *et al.* 2004; Vital *et al.* 2010; Pelisch *et al.* 2011, 2013). Still, the precise underlying mechanisms contributing to BBB disruption in hypertension, and the potential consequences in terms of brain access and actions of circulating AngII within central nervous system pathways involved in BP regulation have not yet been thoroughly assessed.

To further study changes in BBB integrity and function, and to determine whether circulating AngII gains access to the brain during hypertension, we performed a study in which we used a combination of fluorescent dyes of different sizes that were injected into the systemic circulation of Wistar Kyoto (WKY) and SHR rats. This approach enabled us to perform a quantitative assessment of the degree of intravascular and extravascular dyes within the parenchyma of specific brain nuclei, specifically within hypothalamus and brainstem regions, crucial to BP regulation and baroreceptor function (Biancardi *et al.* 2014). As previously reported (Mayhan *et al.* 1989), the presence of extravasated dye within the brain parenchyma was considered indicative of BBB increased permeability. To validate this approach, we compared first in control rats the presence or absence of extravasated dyes in brain areas known to reside within and outside the BBB. As shown in Fig. 1*A–D*, we found large amounts of the intravascularly injected dye dextran-fluorescein isothiocyanate (FITC) 10 kDa (FITC10) extravasated in the parenchyma of the SFO (Fig. 1*A*) and the area postrema (Fig. 1*B*), both brain regions known to lack a tight BBB. Conversely, minimal extravasation was observed in areas known to possess a tight BBB, such as the PVN (Fig. 1*C*) and the supraoptic nucleus of the hypothalamus (Fig. 1*D*). When we applied this approach to SHR, we observed a large degree of FITC10 extravasation within the PVN (~85% increase), which was significantly more abundant compared to aged-matched WKYs (Fig. 1*E–G*). Importantly, similar results were observed in a renovascular hypertensive model (the 2K1C model of hypertension) (Fig. 1*H–J*), indicating that BBB disruption and increased permeability is not a unique feature of the SHR model. Similar to the PVN, the brainstem (RVLM and the NTS) also displayed disrupted BBB in both hypertensive models (SHR and 2K1C) (Biancardi *et al.* 2014).

To determine whether in addition to the inert dextran dyes, physiologically relevant molecules could also leak through the disrupted BBB during hypertension, we

repeated the experiments using systemic infusions of a fluorescently labelled form of AngII (AngII-FITC). Similar to FITC10, we found large levels of extravasated AngII-FITC within the PVN of hypertensive animals (~44%) (Fig. 2*A–C*). These results indicate that the increased BBB permeability observed during hypertension allows the leakage and access of circulating factors, namely AngII, directly into key sympathoexcitatory brain areas, such as the PVN and the RVLM. These results are in line with a recent study by Yao & May, who showed that when the BBB was disrupted with hypertonic mannitol, systemically applied AngII activated tyrosine hydroxylase-expressing RVLM neurons, indicative of leakage of AngII into the RVLM (Yao & May, 2013).

We found the extravasated AngII-FITC in the PVN of hypertensive animals to be associated both with neurons and microglia cells (Fig. 2*D* and *E*), supporting these two cell types as likely targets for circulating AngII actions within the brain during hypertension. These results are thus in agreement with several other reports, as discussed above, supporting microglia as likely mediators of pro-inflammatory actions of AngII within the CNS.

Mechanisms contributing to BBB disruption during hypertension

Endothelial dysfunction associated with high BP conditions is a likely underlying factor contributing to vascular inflammation and downstream BBB disruption during hypertension (Ueno *et al.* 2004; Pires *et al.* 2013). Alternatively, direct AT1R-mediated signalling could also contribute to this phenomenon. For example, AT1R activation has been shown to affect BBB permeability in cultured microvessels (Fleegal-DeMotta *et al.* 2009). Likewise, chronic infusion of AngII, in an AT1R-dependent manner, has been implicated in increased BBB permeability, when measured in whole mouse brain homogenates (Vital *et al.* 2010). Moreover, the AT1R blocker olmesartan was shown to prevent altered BBB permeability within the hippocampus of AngII-induced and Dahl salt-sensitive hypertensive rats (Pelisch *et al.* 2011, 2013).

Thus, to gain more insights into the specific mechanisms contributing to altered BBB permeability during hypertension, we experimentally assessed the relative contribution of high blood pressure itself *vs.* AngII-AT1R signalling. To this end, we evaluated the effect of treating SHR with either the vasodilator hydralazine (which lowered BP independently of AngII signalling) or the AT1R blocker losartan, which also lowered BP by blocking AngII actions. We found that the disrupted BBB in SHR was prevented by the treatment with losartan (decrease of ~76% in dye extravasation), but not with hydralazine (Fig. 1*G*), despite similar decreases in BP obtained with the two treatments (Biancardi *et al.* 2014). These results support a major contribution of

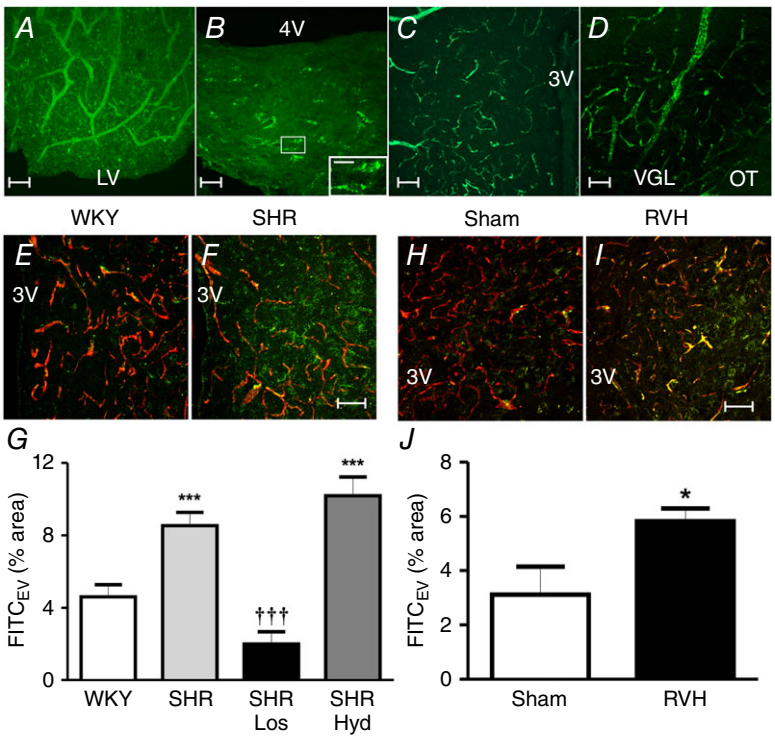

Figure 1. Extravasation of FITC10 within the brain in different conditions
A–D, representative images showing a comparison of extravasated (EV) FITC10 in control rats in brain areas lacking (*A*, subfornical organ; *B*, area postrema) or possessing (*C*, PVN; *D*, supraoptic hypothalamic nuclei) an intact BBB. The inset in *B* shows a higher magnification of the squared area. Note the presence of extravascular FITC10 in *A* and *B*, but not *C* or *D*. *E–J* shows increased extravasated small size FITC10 (green dye) but not large size dextran-rhodamine 70 kDA (RHO70) (red dye), indicative of increased BBB permeability, within the PVN of spontaneous (SHR, *E–G*) and renovascular hypertensive rats (RVH, *H–J*) as indicated in the respective summary. *G*, summary data showing that leakage of FITC10 is blunted in SHR treated with losartan (Los) but not hydralazine (Hyd). Scale bars: 25 μm for *A*, *C* and *D* and inset in *B*; 50 μm for *B*, *E*, *F*, *H* and *I*. $^{***}P < 0.001$ and $^{*}P < 0.05$ *vs.* WKY or Sham; $^{†††}P < 0.001$ *vs.* SHR. $n = 8$ SHR/WKY in *G*; $n = 4$ SHR-Los/SHR-Hyd in *G*; $n = 3$ Sham/RVH in *J*. 3V and 4V, third and fourth ventricle; LV, lateral ventricle; VGL, ventral glial lamina; OT, optic tract (modified from Biancardi *et al.* 2014).

AT1R-mediated signalling to altered BBB permeability during hypertension, rather than high BP itself.

One technical aspect of these studies that needs to be taken into consideration is that any residual free fluorescein-isothiocyanate present in our FITC-dextran preparations could potentially be transported across the BBB via organic anion transporters (Sun *et al.* 2001), resulting in false positive signals. However, the facts that (a) we observed a significantly larger amount of leaked FITC10 in hypertensive rats, (b) that only the FITC-dextran of smaller molecular size leaked, and (c) that these differences were largely prevented in rats treated with AT1 receptor blockers, would argue against a positive signal due to transport of residual free FITC10. Still, the precise route by which the FITC-dextran influx accessed these areas of the brain, and particularly whether it occurs via the paracellular space, remains unknown.

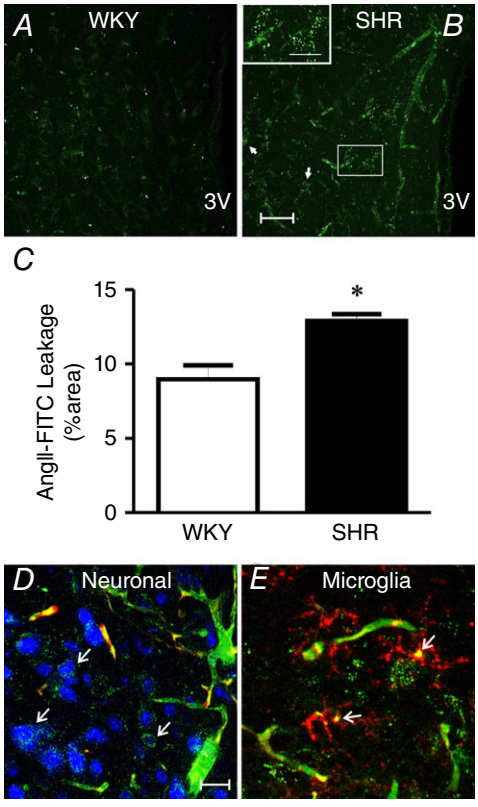

Figure 2. Circulating angiotensin II leaks into the PVN parenchyma in SHR, targeting neurons and microglia
A–C, images showing increased extravasated levels of AngII-FITC (intravascularly delivered) within the PVN of hypertensive (SHR, *B*) compared with normotensive (WKY, *A*) rats, as indicated in the summary data (*C*). *D* and *E*, extravasated AngII-FITC (green) is co-localized with neurons (*D*, neuronal marker NeuN (blue) and the RHO70 dye contained in the vasculature, red) and with microglia (*E*, microglia marker CD11b, red). Scale bars: 50 μm. $^*P < 0.05$ *vs.* WKY. $n = 4$ WKY/SHR. 3V: third ventricle (modified from Biancardi *et al.* 2014).

Do active microglia contribute to AngII-driven reactive oxygen species production?

As summarized above, it is now generally recognized that AngII-mediated pro-inflammatory actions constitute a critical mechanism contributing to sympathoexcitation in hypertension, and a growing body of evidence supports microglia as key cellular targets mediating central AngII pro-inflammatory effects. Moreover, our recent studies also support this mechanism to contribute to BBB disruption during hypertension (Biancardi *et al.* 2014).

AngII-mediated generation of reactive oxygen species (ROS) within the SFO–PVN–RVLM pathway has also been implicated as an important contributor to sympathoexcitation in hypertension (Zimmerman *et al.* 2002,2004; Braga *et al.* 2011; Capone *et al.* 2012). Despite the robust evidence supporting this mechanism, the precise cellular targets and sources of ROS remain largely unknown. In this sense, activated microglia, besides releasing a variety of pro-inflammatory cytokines, also generate and release ROS (Saijo & Glass, 2011). However, whether AngII-dependent, microglia-derived ROS contribute to altered BBB permeability during hypertension remains to be determined.

Final remarks

AngII and its AT1Rs is one of the most important and most widely studied signalling pathways contributing to the central regulation of blood pressure, both in health and disease conditions. We believe that our recent studies summarized above further our current understanding of the mechanisms by which circulating AngII exerts its central effects. While the general consensus in the field is that circulating AngII accesses the central nervous system through the CVOs that reside outside of the BBB, our recent data suggest, that under pathological conditions such as hypertension, an additional route for AngII signalling in the brain is gated. Thus, we propose that a compromised BBB facilitates the direct access of circulating AngII to critical sympathoexcitatory brain centres that are normally protected, constituting a complementary mechanism that, working in tandem with the local central renin–angiotensin system, further exacerbates AngII-driven neurohumoral activation during hypertension. The facts that elevated circulating levels of AngII at the onset of hypertension (a) contribute to BBB integrity, (b) facilitate their own access to brain sympathoexcitatory centres, and (c) contribute to further increasing BP thus support a highly deleterious AngII-mediated feedforward mechanism during hypertension.

AT1R blockade, as well as angiotensin-converting enzyme inhibitors, are widely used therapeutic agents for the treatment of cardiovascular diseases

(Perret-Guillaume *et al.* 2009). Moreover, AT1R blockade has been shown to have neuroprotective effects when used for inflammatory brain disorders that accompany BBB disruption such as traumatic brain injury, stroke, dementia, Alzheimer's and Parkinson's diseases (Villapol & Saavedra, 2015). Thus, AngII and its central AT1 receptors stand as novel therapeutic targets that may help prevent and/or rescue an altered BBB status in numerous inflammatory diseases, including hypertension.

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Additional information

Competing interests

None declared.

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