Intra-carotid angiotensin II activates tyrosine hydroxylase expressing rostral ventrolateral medulla neurons following blood-brain barrier disruption in rats

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Running Title: BBB disruption facilitates Ang II mediated RVLM activation

Key Words: sympathetic nervous system, brain stem, blood-brain barrier, Ang II, blood pressure, hypertension
Abstract

Angiotensin II (Ang II) in the periphery and within the brain plays important roles in blood pressure control. Circulating angiotensin is normally excluded from the brain by the blood-brain-barrier (BBB), but there is evidence that in some diseases there is disruption of the BBB that could allow circulating Ang II to access nuclei from which it is normally excluded, such as the rostral ventrolateral medulla (RVLM). We therefore investigated whether disruption of the BBB leads to increased activation by circulating Ang II of tyrosine hydroxylase (TH) containing neurons in the RVLM. In anaesthetised rats, in which the BBB was disrupted with intracarotid hypertonic mannitol (1.6M, 2mL/kg), subsequent intracarotid infusion of a subpressor dose of Ang II (5ng/kg) activated ~24% of TH containing RVLM neurons whereas saline only activated ~8% of TH neurons. Intracarotid pretreatment with the Ang II receptor type 1 (AT1R) blocker, losartan (20µg/kg), significantly reduced the number of TH cells activated to ~11% (P<0.05, one-way ANOVA, n=5). In summary, disruption of the BBB resulted in increased activation by circulating Ang II of TH containing cells in the RVLM. The activation was reduced by losartan indicating a specific action on AT1Rs. These results suggest that disruption of the BBB allows entry of circulating Ang II into the RVLM, which could increase sympathetic outflow. This potential source of Ang II within the RVLM might be an important consideration when assessing sympathetic nerve activity in disease states associated with a compromised BBB.
Introduction

Angiotensin II (Ang II) plays a central role in the control of cardiovascular function (Allen et al., 2009; Saavedra, 2012; Verdecchia et al., 2012) and in the control of fluid and electrolyte balance (McKinley et al., 2008). Circulating Ang II has multiple peripheral actions that act to increase arterial pressure, including vasoconstriction (Hofbauer et al., 1983), aldosterone release (Campbell et al., 1979), and stimulation of sympathetic nerve activity (SNA; Jackson and Campbell, 1980). Circulating Ang II also acts on the circumventricular organs (CVOs), such as the area postrema and lamina terminalis, and via actions on angiotensin type 1 receptors (AT1R) causes drinking (Simpson & Routtenberg, 1973; McKinley et al., 2008) and desensitisation of the arterial baroreflex (Casto and Phillips, 1986; Paton and Kasparov, 1999). In the rostral ventrolateral medulla (RVLM), a key region for the control of sympathetic outflow, microinjection of Ang II is sympathoexcitatory and increases blood pressure (Muratani et al., 1991). At the cellular level, patch clamp experiments reveal that Ang II depolarises bulbospinal RVLM neurons in both normotensive and hypertensive rats (Kumagai et al., 2012), suggesting that endogenously produced Ang II, presumably from astrocytes, where angiotensinogen is synthesised (Stornetta et al., 1988), tonically activates bulbospinal AT1R expressing RVLM neurons in hypertensive rats (Kumagai et al., 2012). Whether circulating Ang II can gain access to brain areas, apart from those areas that lack a blood-brain barrier (BBB) viz the CVOs, is still not clear.

The BBB forms a physiological barrier between the peripheral circulation and the brain parenchyma. It is of vital importance for normal brain function as it regulates the delivery of nutrients and removal of metabolites from the central nervous system (Bernacki et al., 2008). The BBB is thought be affected by a number of diseases and conditions including hypertension. Disruption of the BBB in hypertensive animals has
been attributed partly to the effects of mechanical shear stress on the vascular walls with increased pressure (Kaya et al., 2003). Whether this disruption allows the entry of substances such as Ang II into the brain is unclear. The question of whether Ang II crosses the BBB is important particularly in relation to autonomic cardiovascular regulation.

Therefore, in this study, we investigated whether hypertonic mannitol-induced disruption of the BBB (Kozler & Pokorny, 2003) allows peripherally administered Ang II to enter the brain and activate tyrosine hydroxylase (TH) expressing bulbospinal RVLM neurons. To achieve this we used c-Fos protein immunohistochemistry as a marker of neuronal activation and quantified the number of c-Fos protein expressing neurons that were also positive for TH in response to carotid infusions of subpressor doses of Ang II following mannitol-induced BBB disruption.

**Experimental Procedures**

Male Sprague-Dawley rats (10-12 weeks of age) were purchased from the Animal Resource Centre (ARC, Western Australia). All experimental protocols used in this study were performed in accordance with the Prevention of Cruelty to Animals Act, Australia 1986 and conformed with guidelines set out by the National Health and Medical Research Council of Australia (2007). The protocol was approved by the Florey Neuroscience Institutes Animal Ethics Committee. Upon arrival the rats were given free access to food (standard laboratory rat chow, Barastoc, Australia) and tap water. The animals were allowed at least 5 days to acclimatise to the animal housing facilities before being used.
**Blood-brain barrier disruption**

We used a mannitol-mediated BBB disruption protocol (Kozler & Pokorny, 2003). To confirm that hypertonic mannitol (1.4M) disrupts the BBB we infused Evans Blue (2% at 2 mL/kg) immediately after intracarotid infusion of mannitol. For this initial set of experiments, rats were anaesthetised with sodium pentobarbitone (60mg/kg i.p.) and the right carotid artery was cannulated with PE-40 tubing. After cannulation, either mannitol (1.4M; 2 mL) or saline (2 mL; control) was infused at a rate of 2 mL over 10 seconds immediately followed by Evans Blue infusion. After 90 minutes rats were decapitated with a small animal guillotine (Harvard Apparatus), the brain rapidly removed, snap-frozen over liquid nitrogen and stored at -80°C until cut on a cryostat (14µm) and thaw-mounted on gelatin-chrome-alum coated slides. Sections were then washed in PBS (0.1M, pH 7.4), incubated in mouse anti-rat endothelial cell antigen (RECA; AbD Serotec; 1:1000 dilution) overnight at 4°C. Sections were then washed in PBS (3 x 5 mins), incubated in an AlexaFluor-488 conjugated horse anti-mouse secondary antibody (Molecular Probes, Invitrogen, 1:500 dilution) for 1 hour at room temperature and washed prior to being coverslipped with anti-fade mountant.

To test whether a subpressor dose of Ang II (5 ng/kg) can activate RVLM neurons when the BBB is compromised, rats were randomly assigned to one of four groups; i), Mannitol/AngII (Man-Ang II; n=5), ii) Mannitol/Saline (Man-Sal; n=5), iii) Mannitol/Losartan/Ang II (Man-Los-Ang II; n=5) and iv) saline/AngII (Sal-AngII, n=5).

The dose of Ang II we chose was based on that reported by Xue and colleagues (2012). Rats were anaesthetised with sodium pentobarbitone (60 mg/kg i.p.) and the right carotid artery was cannulated for mannitol and drug infusions. The right femoral artery and vein were cannulated for blood pressure measurement and supplemental anaesthetic administration (sodium pentobarbitone, 20 mg/kg i.v. as required) respectively. After establishing a stable baseline blood pressure, mannitol (2 mL;
1.4M) was injected into the right carotid artery. Immediately after the mannitol injection, either Ang II (5 ng/kg in 0.1 mL bolus) or saline (0.9 %, 0.1 mL) was administered into same carotid artery. In order to see if we could block the effects of Ang II, in a third group of rats we used the angiotensin receptor blocker, losartan. This group of animals were prepared as described above, however, losartan (20 µg/kg; 0.1 ml bolus) was administered into the carotid artery just prior to Ang II (5 ng/kg in 0.1 mL) immediately following the mannitol infusion. A fourth, control group, was also included where saline was administered (instead of hypertonic mannitol) prior to Ang II (5 ng/kg in 0.1 mL). Following the treatment protocol rats were then left for 60 minutes to allow for the c-Fos protein to be expressed before transcardial perfusion.

**Transcardial perfusion and tissue collection**

Sixty minutes after administering the Ang II, the rats were transcardially perfused with 200 mL of 0.1M phosphate buffered saline (PBS, pH 7.4) and 300 mL of 4% (w/v) paraformaldehyde (PFA) made up in PBS. The brains were removed and stored in 4% PFA for 4 hours at 4ºC prior to being transferred to PBS containing 20% (w/v) sucrose to cryoprotect the tissue. After being stored for 2-3 days in 20% sucrose/PBS the brain tissue was blocked, rapidly frozen over liquid nitrogen and stored at -80ºC until further processed.

**Immunohistochemistry**

Serial coronal sections (30 µm) of the medulla oblongata were cut using a cryostat (Leica Cryocut 1850, Leica Microsystems, Germany) and collected in 24-well tissue culture plates containing 0.1M PBS. For immunohistochemical detection of c-Fos, a commercial rabbit polyclonal c-Fos antisera directed against a recombinant protein corresponding to amino acids 4-17(SGFnADYEAssSRC) of c-Fos of human origin
was used (Calbiochem, catalogue number: PC38). For the detection of tyrosine hydroxylase (TH), we used a commercially available mouse monoclonal antibody (clone: LNC 1) of the TH of human-origin (Millipore, catalogue number: MAB318).

Immunohistochemistry was performed as described previously (Yao et al., 2005). Free-floating rat brain stem sections were first incubated for 15 minutes in 0.3% (v/v) \( \text{H}_2\text{O}_2 \) in PBS to eliminate endogenous peroxidase activity. Sections were then incubated in a blocking solution comprising 10% normal goat serum (NGS, Sigma) and 0.3% Triton X-100 (Sigma) in 0.1 M PBS followed by rinses in PBS (3 x 10 minutes). Sections were then incubated in a polyclonal rabbit anti-c-Fos primary antiserum (1:20,000 dilution) in PBS containing 1% NGS and 0.3% Triton X-100 for 4 hours at room temperature then overnight at 4°C. After the primary antibody incubation, the sections were rinsed in PBS (3 x 10 minutes) prior to a 1 hour incubation in PBS containing biotinylated goat anti-rabbit IgG (1:500 dilution, Vector Laboratories), 1% NGS and 0.3% Triton X-100 at room temperature. Following rinses in PBS (3 x 10 minutes), the sections were incubated for 1 hour in streptavidin-conjugated horseradish peroxidase (1:500 dilution, Vector Laboratories, UK) in PBS containing 1% NGS and 0.3% Triton X-100. Subsequent to further washes (3 x 10 minutes) c-Fos immunopositive neurons were visualised using nickel intensified 3,3'-diaminobenzidine substrate (Sigma, MO) to yield an intense black chromogenic product. The colour reaction was terminated at 30 minutes by transferring the sections into PBS (3 x 10 minutes). The sections were then incubated in the TH primary antibody (1:5000 dilution) for 4 hours at room temperature then overnight at 4°C. After washing in PBS (3 x 5 mins) sections were incubated in a horseradish peroxidase conjugate goat anti-mouse secondary antibody (1: 500 dilution, Vector Laboratories) for 1 hour at room temperature followed by 3 x 5 min washes in PBS. Sections were then incubated in 3,3'-diaminobenzidine substrate (Roche, Australia)
producing a brown product. After further washes, the sections were then mounted onto glass microscope slides with 0.5% gelatin and allowed to air-dry overnight. Once dry, the slides were dehydrated in ethanol (70%, 90%, 3 x 100%), cleared in Histoclear (RA Lamb, UK), and coverslipped in DPX mountant (VWR, UK).

**Immunohistochemical controls**

In order to control for non-specific staining, sections were also processed in the absence of either the primary or secondary antisera. No staining was observed after the omission of either the primary or secondary antibodies (data not shown).

**Photomicroscopy and preparation of figures**

The processed sections were examined on a Nikon E800 microscope equipped with Nikon CFI plan optics. Photomicrographs were taken with an Olympus digital camera (DP70, Olympus, Japan) using DP Controller software (V2.1.1, Olympus, Japan), saved as JPEG images at 300 dpi resolution and arranged in their respective sequences in Adobe Illustrator (version CS2, Adobe Corp., USA) for figure production. The schematic drawings were prepared from representative sections of the RVLM at the respective levels. Digital images of the sections were taken as described above and imported into Adobe Illustrator for figure production.

Line drawings of medullary sections were made using a microfilm histological slide projector (Allen Microfilm Products, Bournemouth, England), digitally scanned, imported into Adobe Illustrator CS2 and superimposed over the digital image and circles were placed over single c-Fos positive, TH positive and c-Fos and TH double-labelled immunoreactive neurons within the RVLM. Anatomical landmarks including the nucleus ambiguus, mammillary bodies, and the border of the ventral surface of the brain stem were used to identify the RVLM. c-Fos and TH like immunoreactivity
were quantified and mapped at four different levels corresponding to sections which were -12.0, -12.12, -12.24, -12.36 mm posterior of bregma.

**Quantification and Statistical analysis**

To assess Evans Blue leakage across the BBB pictures of the RVLM were taken at X20 magnification using a fluorescent microscope (Fluorescent Stereology Microscope Olympus BX51, Olympus, Japan). Evans Blue fluoresces in the red spectrum (610 nm) when excited at 550 nm. The photomicrographs were captured at maximum resolution, saved as JPG and the spread of Evans Blue analysed by tracing the extent of Evans Blue diffusion from the outer edge of the blood vessel walls (clearly defined by the RECA staining) using Image J (NIH, USA) and expressed in number of pixels.

To quantitatively analyse the labelling observed we counted the number of double and single labelled immunopositive neurons located within the defined borders of the RVLM as delineated in the atlas of Paxinos and Watson (2004). Using the guidelines previously proposed (Saper, 1996), each section at the appropriate level caudal of bregma was viewed and photographed under sufficient magnification (20X objective) to visualise all immunopositive cells at two different focal planes and the number of immunopositive neurons counted using Image J (NIH, USA) software. As the relative sizes of TH-like and c-Fos- like immunoreactive neurons remained constant throughout the rostro-caudal extent of the RVLM, we were confident that any error in our counts would be much smaller than those arising from biological variation. All values are given as mean ± SEM. The data were statistically analysed with a one-way ANOVA followed by a Tukey’s *post-hoc* test using GraphPad Prism 6.0 (GraphPad Software Inc. CA). Statistical significance was assumed when $P<0.05$. 
Results

**Disruption of the blood-brain barrier with mannitol**

Our first objective was to confirm that we could successfully disrupt the BBB with hypertonic mannitol. Therefore, we infused Evans Blue (2%; 2ml/kg) into the carotid artery immediately following mannitol (1.4M) infusion. We observed significantly greater amounts of Evans Blue extravasation into the brain parenchyma following BBB disruption with mannitol compared with the control group (14567±2756 vs 1274±978 pixels, respectively, P<0.05, Student's t-test, n=5) where physiological saline, instead of hypertonic mannitol, was used (Figure 1).

**Effect of mannitol, Ang II and losartan on blood pressure and heart rate**

Infusion of hypertonic mannitol (1.4M) into the carotid artery of pentobarbitone-anaesthetised rats induced a transient decrease in blood pressure (-16±11 mmHg) and heart rate (-10±12 bpm) lasting approximately 1 minute. Infusion of Ang II (5 ng/kg) into the carotid artery after the mannitol infusion did not elicit any significant changes to blood pressure (1.3±3 mmHg) or heart rate (2±3 bpm). Likewise, losartan (20 µg/kg) administered just before Ang II infusion did not elicit any significant changes to blood pressure (-1.6±4 mmHg) or heart rate (3±4 bpm).

**Distribution of TH and c-Fos immunoreactive cells in the RVLM**

The distribution and number of TH-positive cells were virtually identical in all of the brain stems examined. These cells showed brown cytoplasmic staining with a ramified morphology and were scattered throughout the RVLM (Figures 2 and 3). The morphology and distribution of these cells have been described previously.
(Hökfelt et al., 1984; Phillips et al., 2001). In addition, c-Fos positive cells, indicated by intense black nuclear staining, were observed scattered throughout the rostro-caudal extent of the RVLM (Figures 2 and 3). The distribution of c-Fos, TH and c-Fos and TH double-labelled neurons within the RVLM in 4 representative rats (one from each of the 4 treatment groups; Man-Ang II, Man-Sal, Man-Los-Ang II, Sal-AngII) is shown in Figure 3.

**Effect of Mannitol, Ang II and Losartan on c-Fos expression within the RVLM**

In mannitol-Ang II treated rats, we observed a significantly ($P<0.05$, one-way ANOVA, n=5) higher number of both c-Fos labelled and double-labelled TH and c-Fos immunoreactive cells compared with the mannitol-saline treated group; where only a small number of c-Fos positive and a smaller number of TH/c-Fos double labelled cells were observed (Figure 2 and Figure 4A,B). Control experiments where we administered saline (instead of mannitol) prior to Ang II administration revealed low numbers of c-Fos and TH/c-Fos double-labelled cells (Figure 2). Prior treatment with the AT1R blocker, losartan, significantly ($P<0.05$, one-way ANOVA) reduced the number of c-Fos positive TH expressing RVLM neurons (see Figure 2 and Figure 4).

In the mannitol-Ang II group ~24% of TH positive cells were also c-Fos positive whereas only ~8% and ~11% of TH positive cells were also c-Fos positive in the mannitol-saline and mannitol-losartan-Ang II groups, respectively. Similarly, only ~7% of TH positive cells were also c-Fos positive in the saline-Ang II group.

Examination and mapping of the distribution of TH and c-Fos double-labelled cells showed tight clustering of TH and c-Fos positive cells along the RVLM in the mannitol-saline and mannitol-losartan-ang II groups with only one or two double-labelled cells observed in the saline treated group (Figure 2 and Figure 3).
Discussion

In this study we demonstrate that intracarotid administration of hypertonic mannitol effectively disrupts the BBB such that we see greater Evans Blue leakage into the brain parenchyma. Furthermore, mannitol-mediated disruption of the BBB allows peripherally administered subpressor amounts of Ang II to gain access to and activate TH and non-TH expressing RVLM neurons within the brain stem. We also show that the acute effects of Ang II can be partially attenuated by prior treatment with the AT1R blocker, losartan, suggesting that activation of TH-containing RVLM neurons was indeed mediated by an action of peripherally administered Ang II on AT1Rs, and not by the transient blood pressure changes induced by mannitol. This observation is supported by the fact that significantly fewer TH positive RVLM neurons were activated in the saline-treated compared with the Ang II-treated group.

The RVLM located in the medulla oblongata contains adrenergic (TH-containing) neurons that are thought to be of critical importance for maintaining sympathetic vasomotor tone and blood pressure (Cochrane and Nathan, 1993; Guyenet et al., 1989; Lipski et al., 1995). RVLM neurons project to the sympathetic pre-ganglionic neurons located in the intermediolateral cell column of the spinal cord that control the sympathetic nerves to the heart, blood vessel and kidneys (Lipski et al., 1995; Dampney 1994). Hence, activation of these RVLM cells results in increased sympathetic outflow and increased blood pressure. There is strong evidence to suggest that Ang II depolarises RVLM neurons (Kumagai et al., 2012) and direct injection of Ang II within the RVLM induces increases in sympathetic nerve activity and blood pressure (Kishi et al., 2012). While the evidence that Ang II has a pressor and sympathoexcitatory role within the RVLM appears to be unequivocal, the source of Ang II within this nucleus and indeed in other areas of the brain is still debateable.
Contrary to an earlier report of Ang II immunoreactive terminal fields within the RVLM (Lind et al., 1985), Ang II is unlikely to be released from nerve terminals in the same manner as a conventional neurotransmitter. Using the same antibody employed by Lind and colleagues (1985), Allen and others (2009) found that the antibody produced similar staining patterns in Ang II knockout mice suggesting that this antibody might be not specific for Ang II. The current evidence suggests that Ang II might be locally produced in the extracellular space (Lavoie et al., 2004; Allen et al., 2009). The Ang II precursor, angiotensinogen, is produced and presumably released by astrocytes into the extracellular space. Similarly, renin, the enzyme that converts angiotensinogen to Angiotensin I, is also released into the extracellular space by neurons. Angiotensinogen and renin are not expressed in the same cells within the RVLM (Lavoie et al., 2004). Hence, angiotensinogen might be converted to Ang II by renin and angiotensin converting enzyme in the extracellular space and directly activate RVLM neurons via this manner. The findings of the present study are significant because they highlight another mechanism by which Ang II can activate RVLM neurons. Our data suggests that circulating Ang II can enter the brain, via a disrupted BBB, to activate RVLM neurons.

Ang II can act at other sites in addition to the RVLM. As such infusion of Ang II could activate the subfornical organ, which lacks a BBB and via projections to the paraventricular nucleus of the hypothalamus activate RVLM neurons (Braga et al., 2011). The fact that we did not see increased c-Fos expression after Ang II administration when the BBB was not disrupted suggested that in our paradigm, Ang II appears to have had direct effects on RVLM neurons. However, we cannot rule out the possibility that disruption of the BBB also allows the entry of Ang II into the paraventricular nucleus of the hypothalamus, which is known to send excitatory projections to the RVLM (Stocker et al., 2005).
The question arises as to why we did not see increases in blood pressure in response to intra-carotid Ang II administration following BBB disruption despite activation of RVLM neurons. We propose that only a proportion of RVLM neurons were activated following Ang II administration. Hence, either an insufficient number of RVLM neurons were activated to elicit a haemodynamic response or, more likely, some degree of compensation took place whereby the vasoconstriction of some vascular beds was compensated for by vasodilation of others. It would be interesting to ascertain where the activated TH-expressing RVLM cells project, along the spinal cord, to get a better idea of which sympathetic nerves might be affected.

A previous study by Potts and colleagues (1999) show that intravenous infusion of Ang II (1.4 g/kg/hr) in the rabbit mainly activates forebrain areas such as the organum vasculosum of the lamina terminalis and the SFO while activation of the medullary regions is primarily a secondary effect resulting from the stimulation of arterial baroreceptors (Potts et al., 1999). Hence, in the current study, we chose to administer a subpressor dose of Ang II (5 ng/kg) in order not to activate the arterial baroreceptors, which stimulates c-Fos expression in the RLVM (Potts et al., 1999). The fact that we did not see a significant amount of c-Fos staining in the RVLM following Ang II administration except after BBB disruption suggests that Ang II-induced activation of RVLM neurons was likely to be a direct and not a secondary effect.

Increased BBB permeability occurs not only as a result of neural trauma and cerebral ischemia (Persidsky et al., 2006), but there is good evidence to suggest that it might also be compromised in other disease states such as hypertension (Pelisch et al., 2011), multiple sclerosis (Floris et al., 2004) and in normal aging (Popescu et al.,
Our results suggest that under these conditions an impaired BBB might allow the entry of Ang II into the RVLM leading to increased sympathetic drive which might contribute to disease progression (i.e. in hypertension and heart failure).

Interestingly, patients with multiple sclerosis also show signs of autonomic dysfunction (Monge-Argiles et al., 2003). There is also evidence that Ang II itself can change BBB permeability via activation of AT1R located on brain endothelial cells. Fleegal-DeMotta and colleagues (2009) have shown that Ang II increases brain microvascular endothelial cell transcytosis of $^{125\text{I}}$-albumin in vitro, an effect they could inhibit with the AT1R blocker, telmisartan but not AT2R blocker, PD123,319.

Furthermore, they also presented evidence that the Ang II-induced increase in paracellular and transcellular permeability is mediated by the activation of protein kinase C downstream of the AT1R. These findings are supported by earlier studies showing that intravenous injections of Ang II into rats caused a reversible increase in Evans Blue permeability across the BBB (Oztas and Sandalci, 1984).

One of the limitations of the present study is the method we used to disrupt the BBB. Hypertonic mannitol has been extensively used in the past and has been shown to be an uncomplicated and robust technique to reversibly disrupt the BBB (Hawkins and Egleton, 2006; Kaya and Ahishali, 2011). However, it might be argued that the resulting changes in blood pressure during the hypertonic mannitol administration might activate cells in the RVLM. While this may be the case, we observed significantly fewer c-Fos activated cells in the RVLM of saline treated versus Ang II treated rats, suggesting that intracarotid Ang II did significantly increase c-Fos protein expression in the RVLM. The fact that we could attenuate the effects of Ang II with an AT1R blocker gives us confidence that the effects we observed were specifically Ang II mediated. There is functional evidence suggesting that losartan crosses the BBB in rats (Li et al., 1993). In the present study we found that prior administration of
losartan significantly attenuated the actions of Ang II within the RVLM following BBB disruption. While the precise site of action was not investigated, the literature suggests the presence of AT1R in the subfornical organ (Lippoldt et al., 1992), paraventricular nucleus of the hypothalamus (Li et al., 1993) and RVLM (Hirooka et al., 1996). Hence, while it is highly likely (given that losartan crosses the BBB) that losartan was acting directly on AT1R expressing RVLM neurons, there is the distinct possibility that it might also have acted at other sites such as the subfornical organ and paraventricular nucleus to attenuate the actions of Ang II. In this study we also used Evans Blue as a marker of BBB permeability as it strongly binds to plasma albumin, which does not normally cross the BBB. Some have argued that Evans Blue might not be the most robust method to assess BBB permeability since unbound Evans Blue might cross the BBB. However, dyes with a molecular weight greater than 180 Da preclude passage across an intact BBB (Kozler and Pokorny, 2003), as such Evans Blue with a molecular weight of 961 Da is unlikely to easily cross a normally functioning BBB. Moreover, we saw very little Evans Blue leakage in our control saline-treated animals suggesting that in our paradigm, Evans Blue was a robust assay for the assessment of BBB integrity.

In conclusion, this study shows that disruption of the BBB facilitates the entry of Ang II into the RVLM to activate TH-containing neurons, a majority of which are thought to play a role in central cardiovascular regulation (Guyenet et al., 1989). Furthermore, activation of the TH-containing neurons appears to be specific to Ang II, since the effects were attenuated by prior treatment with the AT1R blocker, losartan, supporting the notion that the effects were specific to Ang II. While the functional consequences of increased Ang II permeability into the RVLM was not investigated in the present study, but given that exogenous Ang II directly injected into the RVLM causes sympathoexcitation and increased blood pressure, it is likely that this
additional source of Ang II in the RVLM may be important in pathological conditions where the BBB is disrupted.

**Acknowledgements**

We thank Professor Michael J McKinley for reading the manuscript and offering helpful advice. This work was supported by the NH&MRC (Australia) and the Victorian Government through the Operational Infrastructure Scheme. C. N. May is supported by a National Health and Medical Research Council Research Fellowship (566819).

**References:**


AT1 receptors protects the blood-brain barrier and improves cognition in Dahl salt-sensitive hypertensive rats. Am J Hyperten, 24: 362-368.


Figure Legends

**Figure 1.** Representative photomicrographs showing Evans Blue extravasation (red staining) from rostral ventrolateral medulla (RVLM) blood vessels stained for rat endothelial cell antigen (RECA; green) following; A) control saline infusion and B) mannitol (1.4M) intracarotid infusion. There was significantly ($P<0.05$, $n=5$; Student’s t-test) more Evans Blue seen outside of the blood vessels (in the RVLM) after mannitol treatment compared to saline treated controls (C). Data expressed as mean±SEM. Scale bar = 10µm.

**Figure 2.** Representative photomicrographs of c-Fos (black nuclear) and tyrosine hydroxylase (brown cytoplasmic) immunohistochemistry in the RVLM of A) mannitol-angiotensin II (5 µg/kg ica), B) mannitol-saline, C) mannitol-losartan-angiotensin II and D) saline-angiotensin II treated rats. A greater number of double-labelled c-Fos and tyrosine hydroxylase (TH) labelled cells (red arrows) were observed in the mannitol-angiotensin II treated rats (A) compared with the mannitol-saline treated rats (B). Pre-treatment with losartan (20 µg/kg ica) reduced the number of double-labelled cells (C). Angiotensin II administration after saline control injections did not induce much c-Fos activation in TH-labelled cells (D). Panels A’, B’, C’ and D’ are pictures taken at higher power of the area indicated by the black rectangular boxes in panels A, B, C and D respectively. The blue arrow heads point to cells that are TH positive only, while the black arrows point to cells that were c-Fos positive only. Scale bar = 200µm in A, B, C and D; 100µm in A’, B’, C’ and D’.

**Figure 3.** Line drawings of representative brainstem sections showing the distribution of c-Fos positive (black circles), tyrosine hydroxylase (TH) positive (open circles) and c-Fos and TH double-positive cells (red circles) at four different levels within the
rostral ventrolateral medulla (RVLM) of mannitol-angiotensin II, mannitol-saline, mannitol-losartan-angiotensin II and saline-angiotensin II treated rats. A larger number of TH and c-Fos double-positive cells were observed in the Ang II group compared with the saline and losartan treated groups.

**Figure 4.** Column graphs showing the number of c-Fos and tyrosine hydroxylase (TH) positive, c-Fos positive only and TH positive only cells in each of the four groups of rats: mannitol-saline (white bars), mannitol-angiotensin II (black bars), mannitol-losartan-angiotensin II (dark grey bars) and saline-angiotensin II (light grey bars). Following mannitol-induced disruption of the blood-brain barrier, angiotensin II (5 ng/kg i.c.a.) treatment induced a significantly greater amount of c-Fos expression in TH positive cells compared with saline treatment. Losartan (20 µg/kg i.c.a) significantly attenuated the effects of Ang II. *P<0.05 compared to mannitol-saline group, one-way ANOVA followed by a Tukey’s post-hoc test.
Figure 1

(A) RECA/EB

(B) RECA/EB

(C) Area of EB leakage ('000s of pixels)

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* Indicates significant difference.
Figure 3
Highlights

Angiotensin II plays an important signalling role in the brain.

Disruption of the blood-brain barrier allows its entry into the RVLM.

Peripherally administered Angiotensin II activates RVLM neurons.

Losartan can block Angiotensin II-mediated RVLM activation.
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**Title:**
INTRA-CAROTID ANGIOTENSIN II ACTIVATES TYROSINE HYDROXYLASE-EXPRESSING ROSTRAL VENTROLATERAL MEDULLA NEURONS FOLLOWING BLOOD-BRAIN BARRIER DISRUPTION IN RATS

**Date:**
2013-08-15

**Citation:**

**Publication Status:**
Accepted manuscript

**Persistent Link:**
http://hdl.handle.net/11343/41846