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## Angiotensin modulation of rostral ventrolateral medulla (RVLM) in cardiovascular regulation

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### Abstract

The rostral ventrolateral medulla (RVLM) and the presympathetic bulbospinal neurons in this region play a critical role in cardiovascular regulation. However, there is ambiguity regarding the precise anatomical coordinates of the RVLM and much still needs to be learned regarding the regulation and neurochemistry of this region. This brief review discusses some of these issues and focuses on the role of angiotensin-mediated signaling in the RVLM in blood pressure regulation.

### Keywords

Neural control of blood pressure; Angiotensin II; angiotensin receptors; AT1 receptors; C1 neurons

## 1. Introduction

The rostral ventrolateral medulla (RVLM) is a brainstem region that contains bulbospinal neurons providing a major input to the preganglionic neurons of the sympathetic nervous system. Indeed, complete inhibition of the RVLM outflow reduces sympathetic nerve activity and arterial blood pressure (AP) to the same extent as cervical spinal cord transection, suggesting that it is the brain region responsible for all descending tonic sympathetic vasomotor tone (Guyenet, 2006; Sved et al., 2003). A wealth of data supports an important role of the RVLM in the tonic and reflexive maintenance and control of AP. This brief review will discuss some of this evidence for a role of the RVLM in

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cardiovascular regulation and will then examine the role of angiotensin acting in this region. This review focuses primarily on data generated in rat, the species in which this area has been most thoroughly studied.

## 2. RVLM Location, Neuron Phenotypes, and Function

The RVLM has been extensively studied with regards to the regulation of sympathetic tone and the baroreceptor reflex. However, upon critical review of the relatively large body of literature, a problem associated with studying the RVLM becomes clear - there is no consensus on exactly where the RVLM is located or what it entails.

The specific location of the RVLM has historically been difficult to identify because it is not a defined anatomical nucleus with clear boundaries. Rather, the RVLM lies within the ventrolateral aspect of the medullary reticular formation. Although it is now generally agreed that the correct descriptor of this region is the rostral ventrolateral medulla (RVLM), previous researchers have referred to it as the C1 region (Reis et al., 1984b; Atkinson et al., 1986; Morrison et al., 1988), the subretrofacial nucleus (Dampney et al., 1987; Head and Howe, 1987; Allen et al., 1988), the nucleus paragigantocellularis (Guyenet and Young, 1987), the ventral nucleus ambiguus (Swanson, 2004), the rostroventrolateral reticular nucleus (RVL) (Paxinos and Watson, 1986; Paxinos and Watson, 2005), a rostral longitudinal cell column of the nucleus reticularis rostroventrolateralis (Ruggiero et al., 1994) and the vasopressor area (Willette et al., 1983; Sapru, 1996), while at the same time other researchers were already using the term RVLM (Pilowsky et al., 1986; Morris et al., 1987; Blessing et al., 1987). Also complicating the identification and function of this region is the fact that there are at least two types of bulbospinal neurons located in the RVLM. Somewhat more than half of these bulbospinal neurons in rat contain the enzymes necessary for catecholamine biosynthesis and are part of the C1 catecholaminergic neuron group. However, other bulbospinal RVLM neurons are non-catecholaminergic (Chan and Sawchenko, 1994; Sved et al., 1994; Schreihofner and Guyenet, 1997; Madden and Sved, 2003a; Madden and Sved, 2003b), and hence not part of the C1 population. In addition, the C1 cell group extends well beyond the boundaries defined for the RVLM such that the C1 cell group overlaps with the caudal ventrolateral medulla (CVLM) as well as the RVLM (Schreihofner and Sved, 2008). Furthermore, only a small fraction of the neurons in the RVLM region are bulbospinal, though often it seems that the term RVLM is used to refer specifically to the bulbospinal neurons in this region. (See below for listing of other RVLM efferents.) Regardless of the multiple descriptors used to identify this region and the phenotypes of the neurons present there, it is now agreed that this region is found in the ventrolateral region of the medulla and in rat (the species in which it is most studied) it spans approximately 1 mm in the rostrocaudal plane beginning posterior to the caudal pole of the facial nucleus (12.0 mm caudal to Bregma) and extending caudally (Paxinos and Watson, 2005; Card et al., 2006; Schreihofner and Guyenet, 2002; Schreihofner and Sved, 2008). The mediolateral and dorsoventral extent of the RVLM is less well defined; however it is generally agreed upon that a triangle drawn between the nucleus ambiguus, ~1.5 mm lateral to the midline at the ventral surface of the medulla, and 3 mm lateral to the midline at the ventral surface of the medulla will encompass the majority of the RVLM. (See Figure 1 for details.)

It is well established that the RVLM receives inhibitory GABAergic input from the CVLM. While this inhibitory CVLM input plays a critical role in the baroreceptor reflex (Guyenet, 2006), it is not solely a baroreceptor relay (Sved et al., 2000). The RVLM receives modulatory input from a number of other brain structures (see figure 2) including the paraventricular nucleus of the hypothalamus (PVH) (Badoer, 2001; Tagawa and Dampney, 1999), the paratrigeminal nucleus (Pa5) (de Sousa et al., 2001), the rostral parvicellular reticular formation (rPCRt) (Oskutyte et al., 2006), the NTS (Ross et al., 1985; Dampney et al., 2003; Milner and Pickel, 2003), the ambiguous complex, Kolliker-Fuse nucleus, lateral hypothalamic area (Dampney et al., 1987) and the central nucleus of the amygdala (cNAmyg) (Saha, 2005; Saha et al., 2005).

It is also well established that the RVLM sends projections to the spinal cord intermediolateral cell column (IML), providing a major excitatory input to the sympathetic preganglionic neurons. While the RVLM is often considered to be a bulbospinal nucleus, the RVLM also contains interneurons and neurons that project to a variety of other brain areas, including the CVLM, the raphe pallidus (RPa) and obscurus (ROb), N.Amb, DMV, the locus coeruleus (LC), the nucleus subcoeruleus (SubCA), and the area postrema (AP) (Dampney, 1994; Sved et al., 2001; Card et al., 2006; Frugiere et al., 1998) (Figure 3).

Examination of physiological studies that have evaluated the role of the RVLM in the baroreceptor reflex shows that most researchers localize the RVLM by noting a pressor response to glutamate injection in the region they target to be the RVLM. However, there is substantial variance in the locations reported. For example, (Willette et al., 1983) described a pressor response to glutamate injection between 12.1 mm and 13.7 mm caudal to Bregma, which is in relatively good agreement with the 2<sup>nd</sup> Edition of the Paxinos & Watson Rat Brain Atlas (Paxinos and Watson, 1986). At the other end of the spectrum the pressor region of the RVLM was determined to extend only 450 microns starting at the caudal extent of the facial nucleus which can be approximated as 12 mm caudal to Bregma to 12.45 mm caudal to Bregma (Ruggiero et al., 1994). Others described the pressor responses to glutamate between 11.3 mm and 12.3 mm caudal to Bregma (Muratani et al., 1991), which only incorporates the most rostral portion of the RVLM. There have been numerous reports defining the RVLM as the most rostral aspect of the RVLM and possibly incorporating other closely localized brainstem regions such as the facial nucleus, the inferior olive, the nucleus ambiguus, pre-Botzinger complex, Botzinger complex, linear nucleus of the medulla, the lateral paragigantocellular nucleus, and the gigantocellular reticular nucleus (Fontes et al., 1994; Averill et al., 1994; Reja et al., 2002a; Reja et al., 2002b; Ito and Sved, 1996; Ito et al., 2000). Further complicating the picture is a large range in the magnitude of the pressor response evoked by injection of glutamate into this region, in part resulting from different rat strains or suppliers (Madden and Sved, 2003a) or different anesthetics.

As mentioned previously, it is inappropriate to consider the region of the C1 neuron group as synonymous with the RVLM as the C1-region is known to extend beyond the boundaries typically ascribed to the RVLM and extends into what is generally considered to be the CVLM. Nonetheless, the spinally projecting C1 cells are localized to the rostral portion of C1 and map quite well to the region of the rostral RVLM. Paxinos & Watson describe the C1 cell group (associated with the RVLM) to be between 11.30 mm and 13.68 mm

caudal to Bregma in the 2nd edition of their rat brain atlas (Paxinos and Watson, 1986) (see table 1). However, in the 5th edition, they describe the C1 cell group between 12.00 mm and 14.16 mm caudal to Bregma (Paxinos and Watson, 2005). Clearly there has been a wealth of information gathered about the anatomy and localization of the RVLM and while it may seem as if there is still some ambiguity over the anatomical location of the RVLM, the general location of this region is becoming increasingly clear. The use of anatomical landmarks such as Bregma to define the RVLM (or any other brain structure) is inherently difficult as there are potential differences in brain size depending on strain, sex, age, and weight. Nonetheless, it is useful to use the atlas descriptions as a way to allow ready comparison among studies. It seems that using the rostrocaudal coordinate of 12.0 mm caudal to Bregma provides a good reference for the caudal pole of the facial nucleus. While there is an anterior tip of the RVLM that exists ventral to the caudal few hundred microns of the facial nucleus, it is generally agreed upon that the RVLM begins at the caudal pole of the facial nucleus. Based largely on physiological studies of the pressor regions of the ventrolateral medulla, it would appear that the caudal limit of the RVLM is no further than 12.6 mm caudal to Bregma which is consistent with the fifth edition of the rat atlas (Paxinos and Watson, 2005). Thus the entire rostrocaudal extent of the pressor region of the brain defined as the RVLM extends less than 1 mm.

As noted previously, there are at least two types of neurons located in the RVLM, and this has also complicated the identification and assessment of the functionality of the region. Initially it was assumed that the C1 cells were responsible for the cardiovascular effects of the RVLM based on the observation that the majority of the spinally projecting neurons in the RVLM were of the C1 population and that the characteristics of these neurons seemed to fit with a role in cardiovascular regulation (Reis et al., 1984a). Later, electrophysiology studies showed that both the C1 and non-C1 RVLM spinally projecting neurons were responsive to changes in arterial pressure (Haselton and Guyenet, 1989; Schreihofer and Guyenet, 1997), and Fos expression studies showed that both C1 and non-C1 cells are activated by decreases in arterial blood pressure (Chan and Sawchenko, 1994; Sved et al., 1994)

Other studies investigating the role of C1 and non-C1 cells in the cardiovascular responses elicited by RVLM failed to produce evidence that the C1 cells were involved in those responses at all. For example, intrathecal phentolamine and pindolol (alpha- and beta-receptor antagonists) did not significantly alter blood pressure responses to RVLM stimulation (Mills et al., 1988a). On one hand, this would suggest that epinephrine and/or norepinephrine (and hence the C1 neurons) are not important in the cardiovascular responses elicited by the RVLM. On the other hand, however, the validity of that conclusion is in question as it is now believed that the spinally projecting C1 cells of the RVLM do not normally utilize epinephrine as their primary neurotransmitter (Sved, 1989; Madden and Sved, 2003a), but instead use glutamate (Stornetta et al., 2002a; Stornetta et al., 2002b; Morrison, 2003), which is also believed to be true of the non-C1 neurons (Stornetta et al., 2002a; Stornetta et al., 2002b). Indeed, pharmacological studies are also consistent with this notion, as the effects of RVLM stimulation on blood pressure and sympathetic vasomotor outflow can be blocked by antagonists of excitatory amino acid receptors administered

into the spinal cord (Mills et al., 1988b; Morrison et al., 1989; Bazil and Gordon, 1991; Sundaram and Sapru, 1991).

One way of assessing the relative contributions of C1 and non-C1 neurons in autonomic regulation is through cell-specific lesioning. The classic pharmacological neurotoxins that have been utilized in the past to lesion catecholaminergic neurons include 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4). However, these toxins have not been useful in studying C1 neurons. Although a few studies using 6-OHDA suggested a potential role of C1 neurons (Head and Howe, 1987; Howe et al., 1988), these studies are problematic for a couple of reasons. First, the lesions that were caused by 6-OHDA administration in these studies were extensive and not localized to the C1 neurons; indeed, they may not even have been selective for catecholamine neurons. Secondly, it is now known that C1 neurons do not have a catecholamine uptake mechanism (Comer et al., 1998; Lorang et al., 1994), and thus the lesions induced by 6-OHDA administration were most likely not affecting the C1 neurons selectively, if at all (Madden and Sved, 2003a). More recently, an antibody to dopamine-beta-hydroxylase (DBH) linked to the ribosome-inactivating toxin saporin has been developed as a selective toxin for neurons expressing DBH (Wrenn et al., 1996). This neurotoxin (anti-DBH-sap) is selectively taken up into DBH containing neurons (Madden and Sved, 2003a) and the saporin then kills the cell by blocking protein synthesis (Picklo et al., 1994). One of the major benefits of this neurotoxin over 6-OHDA or MPTP is its selectivity for adrenergic/noradrenergic neurons. Immunohistochemical evidence showed that anti-DBH-sap selectively lesioned noradrenergic neurons while sparing cholinergic, dopaminergic, and serotonergic neurons (Wrenn et al., 1996). Although these authors initially described the relative resistance of the medullary DBH-containing cell groups, including C1, to the anti-DBH-sap neurotoxin administered into the lateral cerebral ventricle, subsequent studies demonstrated that direct microinjection of the neurotoxin into the cell-group of interest or its terminal field does lead to significant, selective lesioning. Thus, the C1 neurons of the RVLM have been selectively destroyed either by injection of the toxin directly into RVLM (Madden et al., 1999; Madden and Sved, 2003b) or into the thoracic spinal cord, e.g., (Schreihofer and Guyenet, 2000) .

Studies utilizing anti-DBH-sap to define the physiological contribution of the C1 neurons in the RVLM in rats have revealed that the C1 neurons do most likely make a contribution to the tonic and reflexive control of AP exerted by the RVLM. In the first studies of this sort, anti-DBH-Sap was injected unilaterally into the RVLM and showed that large, selective destruction of the C1 neurons of the RVLM reduced by approximately 50% the magnitude of the pressor response evoked by glutamate injected into the RVLM (Madden et al., 1999). Interestingly, the response to stimulating the RVLM contralateral to the lesion was increased, supporting the notion of cross talk between the RVLM on the two sides of the brain. In subsequent studies with bilateral injection of the toxin into the RVLM, destruction of greater than 80% of C1 neurons in the RVLM reduced resting AP in unanesthetized rats by approximately 10 mm Hg and significantly impaired the baroreceptor reflex and other sympathoexcitatory reflexes (Madden and Sved, 2003a; Madden and Sved, 2003b; Madden et al., 2006). Using spinal injections of anti-DBH-Sap to destroy spinally-projecting C1 neurons in RVLM, it was also shown that sympathoexcitatory reflexes, including the

baroreceptor reflex were decreased (Schreihofner and Guyenet, 2000). Note that in these studies, some aspect of sympathoexcitation was attenuated but RVLM function remained largely intact. Clearly, the exact participation of the C1 and non-C1 neurons on sympathetic nervous system activity has yet to be defined, although both are most likely contributing to cardiovascular reflexes mediated via the RVLM in some way.

### 3. Angiotensin and the RVLM

#### 3.1 The brain angiotensin system

Angiotensin II (Ang II), long known for its peripheral effects (Kaschina and Unger, 2003), is now recognized as having significant central effects including thirst and sodium appetite (Booth, 1968; Simpson and Routtenberg, 1973; Morris et al., 2002; McKinley et al., 2004), as well as modulating centrally controlled cardiovascular responses - for review see (Severs and Daniels-Severs, 1973). Originally, these central effects of Ang II were believed to be mediated by peripherally derived Ang II acting on angiotensin receptors found on neurons in the brain regions lacking a blood-brain barrier such as the area postrema and subfornical organ (Bickerton and Buckley, 1961; Joy and Lowe, 1970; Dampney et al., 2002b). However, this view changed when angiotensin receptors were discovered in brain regions located within the blood-brain barrier (Bennett and Snyder, 1976; Mendelsohn et al., 1984; Gehlert et al., 1984), and all of the components necessary for local production of Ang II (angiotensinogen, renin, angiotensin-converting enzyme) were found to exist within the brain (Ganten et al., 1971; Yang and Neff, 1972; Lewicki et al., 1978; Stornetta et al., 1988). This indicated that a tissue-specific renin-angiotensin system exists in the brain, and many studies have reinforced this idea (reviewed in (Ganten and Speck, 1978; Moffett et al., 1987; Unger, 2001; Morimoto and Sigmund, 2002; Speth et al., 2003; Phillips and de Oliveira, 2008). The highest density of angiotensin receptors in the brain stem is found in the NTS and many studies have shown that stimulation of AT1 receptors in the NTS can alter AP and the baroreceptor reflex, although the effects on the baroreflex vary (Casto and Phillips, 1984; Casto and Phillips, 1986; Matsumura et al., 1989; Boscan et al., 2001; Wong et al., 2002).

#### 3.2 Angiotensin AT-1 receptor localization to and activation of the RVLM

Angiotensin receptors, primarily of the AT1 subtype, are also present in the RVLM (Allen et al., 1987; Yang et al., 1997; Bourassa and Speth, 2008) and impact the activity of RVLM neurons (Li and Guyenet, 1995; Li and Guyenet, 1996; Seyedabadi et al., 2001). Although angiotensin receptors in the RVLM appear to contribute to cardiovascular regulation – for reviews see (Tagawa et al., 2000; Sved et al., 2003), the physiological role played by angiotensin in modulating the activity of the RVLM has been controversial. This is largely because of issues relating to the pharmacological actions of certain drugs that impact the renin-angiotensin system, uncertainty regarding the nature of brain angiotensin signaling, and minimal effects of selective AT1 receptor antagonists on sympathetic nervous system activity when administered directly into the RVLM of normotensive animals. It was initially reported in 1988 that injection of Ang II into the RVLM of the cat produced a pressor response (Allen et al., 1988; Andreatta et al., 1988). Subsequent studies showed that pharmacological blockade of AT1 receptors, the predominant angiotensin subtype present in RVLM, attenuated the pressor response to Ang II microinjection in the RVLM in rats

(Hirooka et al., 1997; Averill et al., 1994). After the initial observation that stimulation of the RVLM by Ang II caused a pressor response, it was observed that injection into the RVLM of the peptide antagonists of Ang II receptors, Sar<sup>1</sup>Thr<sup>8</sup>-Ang II (sarthran), or Sar<sup>1</sup>Ile<sup>8</sup>-Ang II (sarile) caused a large depressor response, similar in magnitude to the effect to ganglionic blockade (Ito and Sved, 1996). Administration of Sar<sup>1</sup>Thr<sup>8</sup> Ang II, but not losartan (a nonpeptidic AT1 receptor selective antagonist), into the RVLM of the rabbit also reduced sympathetic nervous activity (Hirooka et al., 1997). This led to the hypothesis that Ang II is tonically active in the RVLM and provides the majority of the sympathoexcitatory drive attributable to the RVLM, but that it might involve a novel, non-AT1, non-AT2 angiotensin receptor subtype (Hirooka et al., 1997). Sarthran, but not losartan, reduced blood pressure in rats made hypertensive by oral administration of the nitric oxide synthase inhibitor L-NAME for one week, also leading to the suggestion that a non-AT1 receptor in the RVLM must be mediating this pressor response (Bergamaschi et al., 2002). Other investigators also observed an inability of non-peptidic AT1 receptor-selective antagonists (as well as AT2 antagonists) to reproduce this effect of the sarcosine-containing Ang II receptor antagonists (Averill et al., 1994; Fontes et al., 1997; Tagawa et al., 1999; Potts et al., 2000; Ito and Sved, 2000). Furthermore, it was shown that fragments of angiotensin such as Ang-(3–8) that by themselves have no effect on AP could attenuate the effects of Sar<sup>1</sup>Thr<sup>8</sup>-Ang II or Sar<sup>1</sup>Ile<sup>8</sup>-Ang II (Ito and Sved, 2000). The mechanism by which these sarcosine-containing angiotensin antagonists injected into RVLM produce such a large decrease in AP has yet to be explained. However, recently we have observed the presence of a novel non-AT1, non-AT2 binding site for angiotensins in the rat brain (Karamyan and Speth, 2007; Karamyan and Speth, 2008) and this site is present in the RVLM (Bourassa, Fang, Li, Sved and Speth, unpublished observations). It binds Ang II and Sar<sup>1</sup>Ile<sup>8</sup> Ang II with high affinity but it does not bind losartan or telmisartan or PD123319 (IC<sub>50</sub> > 10 μM) (Karamyan et al., 2008). Thus it is possible that there is tonic angiotensinergic activity in the RVLM that is mediated by this novel, non-AT1, non-AT2 binding site. However, much more needs to be learned about this site before any conclusions can be drawn about its physiological role.

### 3.3 Angiotensin in the RVLM in animal models of hypertension

The role of Ang II in the RVLM has been studied in animal models of hypertension, and the results are rather different from what is observed in normotensive rats. In particular, in hypertensive animals the actions of angiotensin seem to be enhanced and AT1 receptors seem to be tonically stimulated. In the spontaneously hypertensive rat (SHR), for example, it was shown that Ang II injected into the RVLM increased AP in SHR significantly more than in normotensive control rats of the Wistar-Kyoto (WKY) strain, (Ito et al., 2002; Muratani et al., 1991) (see Table 1). In contrast to this increased pressor response to Ang II in the RVLM of the SHR, excitation of this region with glutamate produced a similar increase in AP in SHR and WKY (Ito et al., 2002) suggesting that the effects of AngII in RVLM were selectively enhanced. Importantly, injection into the RVLM of a selective non-peptide AT1 receptor antagonist (either candesartan or valsartan) decreased AP in SHR to near normotensive levels but had no effect in normotensive rats (Ito et al., 2002; Allen, 2001) (Table 1). Interestingly, blockade of glutamate receptors in RVLM also decreases AP in SHR

but not normotensive rats (Ito et al., 2000) (Table 1) and this seems to be distinct from the effect of the AT1 receptor antagonist as the two responses are additive (Ito et al., 2002).

Data from the Dahl salt-sensitive (DS) rat model of hypertension are in agreement with the data from SHR, and also reveal the importance of sodium and/or circulating angiotensin in the ability of central Ang II to modulate RVLM activity. It was reported that hypertensive DS rats fed a high salt diet (8% NaCl) had a significant decrease in AP (~35 mm Hg) following injection of valsartan into the RVLM whereas the DS rats fed a low salt diet (0.3% NaCl) were normotensive and had no response to valsartan (Ito et al., 2003) (see table 2). Also of interest, these investigators found that while Dahl salt-resistant (DR) rats fed either a high or low salt diet both were normotensive, the DR rats fed a low salt diet but not a high salt diet had a significant decrease in AP (~10 mm Hg) following valsartan injection into the RVLM. Also, Ang II injection into the RVLM of both DR and DS rats fed either diet produced an increase in AP, but the increase in AP was significantly higher in DS rats fed a high salt diet compared to the other groups. These results suggest that in DS hypertensive rats, like in SHR, Ang II in the RVLM helps support hypertension, but the response in DR rats fed a low salt diet was unexpected and suggests that in normotensive DR rats fed a low salt diet, Ang II in the RVLM also plays a tonic role in maintaining blood pressure. It is noteworthy that a low sodium diet is associated with increased plasma renin activity and elevated plasma levels of Ang II. Dietary salt intake may alter RVLM responsiveness to Ang II (Adams et al., 2008), which correlates well with data showing that high dietary salt intake increases RVLM responsiveness to glutamate (Pawloski-Dahm and Gordon, 1993; Ito et al., 1999; Ito et al., 2001; Adams et al., 2007) but raises the issue of what it is about dietary salt intake that alters RVLM responsiveness to Ang II and glutamate, even in normotensive animals. While much is still unclear regarding these interactions of dietary salt and responsiveness of RVLM, the available data provide support of the hypothesis that central Ang II is an important mediator of sympathetic tone in the RVLM, at least under certain circumstances.

Other models of hypertension also mimic the response seen in DS and SHR models. One particularly interesting model is the transgenic rat that overexpresses the mouse Ren-2 renin gene, the TGR(mREN2)27 rat; this rat has elevated brain AngII levels but circulating levels are normal (Moriguchi et al., 1995; Campbell et al., 1995; Fontes et al., 2000). It has been shown that an AT1 antagonist (candesartan) injected into the RVLM produces a significant fall in AP (~15 mmHg) in TGR(mREN2)27 rats (Fontes et al., 2000). In other models of hypertension the effect of injection of AT1 receptor antagonists into the RVLM has not been tested, though in the Goldblatt model (two-kidney one-clip) there is indirect evidence for the involvement of angiotensinergic activity in the RVLM (Carvalho et al., 2003). However, in at least one model of hypertension in rats, that produced by long term infusion of L-NAME, injection into the RVLM of an AT1R antagonist had no effect (Bergamaschi et al., 2002).

#### 4. Source of stimulation of RVLM AT1 receptors

Given the potential importance of AT1 receptors in the RVLM in cardiovascular regulation, the question of the source of endogenous ligand for these receptors naturally arises. It is unlikely that circulating Ang II accesses these receptors that are inside the blood brain



barrier; more likely, Ang II is generated within the RVLM. (This makes the assumption that Ang II is the agonist acting on these receptors, and at present there is still some controversy as to whether Ang III rather than Ang II is the angiotensin peptide that acts on brain AT1 receptors (Reaux-Le Goazigo et al., 2005; Kokje et al., 2007). Based on immunocytochemical staining with a rabbit polyclonal antiserum to Ang II showing Ang II-like immunoreactive neurons in PVN and terminals in RVLM (Lind et al., 1985), it has been suggested that angiotensinergic neurons in the PVN innervate the RVLM (e.g., (Dampney et al., 2002a) although the parabrachial nucleus also stained for Ang II (Lind et al., 1985) and projects to the RVLM (Herbert, 1996). The notion that the PVN is responsible for the Ang II input to the RVLM is further supported by the observation that the increase in AP produced by disinhibition of the PVN with local injection of the GABA receptor antagonist bicuculline can be attenuated by blockade of AT1 receptors in the RVLM (Tagawa et al., 1999). Furthermore, the decrease in AP in SHR rats caused by blockade of AT1 receptors in RVLM and that caused by inhibition of the PVN are of similar magnitude and not additive (Ito et al., 2002). While there have been arguments that angiotensin is synthesized within neurons and behaves as a true neurotransmitter (Grobe et al., 2008), the data is not conclusive and this remains as a hypothetical mechanism. Rather, by analogy to the peripheral renin-angiotensin system one might expect Ang II to be generated in the extracellular fluid through enzymatic cleavage of angiotensinogen, and the evidence favors that arrangement (Moffett et al., 1987; Diz, 2006; Speth and Karamyan, 2008).

## 5. Is the AT2 receptor functionally significant in the RVLM?

Recent studies (Li et al., 2007; Gao et al., 2008a; Gao et al., 2008b) suggest that the AT2 receptor may mediate the actions of Ang II in the rat RVLM, at least under some conditions. Viral vector-induced overexpression of the AT2 receptor in the RVLM is associated with a reduction in blood pressure, diuresis and reduced urinary excretion of norepinephrine (Li et al., 2007; Gao et al., 2008a).

In a rat model of chronic heart failure, Ang II administered into the RVLM caused a greater increase in renal sympathetic nerve activity (RSNA) in the presence of the AT2 receptor antagonist PD123319 than in its absence (Gao et al., 2008b). This suggests that the sympathoactivation mediated by Ang II at AT1 receptors in the RVLM is partially antagonized by its actions at AT2 receptors in the RVLM. In addition, these authors showed that administration of an AT2 selective agonist peptide, CGP42112 into the RVLM, caused a reduction in RSNA that could be blocked by prior administration of PD123319 in sham, but not in the chronic heart failure rats. However, based on the stereotaxic coordinates reported, 2.5 to 3 mm rostral to obex, these injections may have been well rostral to RVLM (Gao et al., 2008b) (shown in the online data supplement).

Additional support for an AT2 receptor-mediated sympathoinhibitory effect of Ang II in the RVLM comes from studies of AT1A receptor knockout mice (Matsuura et al., 2005); in a brainstem slice preparation from these knockout mice, they observed hyperpolarization of RVLM neurons in response to Ang II and this effect was antagonized by PD123319. This contrasts with the predominant depolarization response elicited from wild-type neurons by Ang II. However, the high concentrations of Ang II (6  $\mu$ M) and PD123319 (60 – 120

$\mu\text{M}$ ) used in these experiments call into question the specificity of the responses for AT-2 receptors.

In receptor autoradiography studies in control rats, there is negligible binding of  $^{125}\text{I}$ -Ang II or the radiolabeled antagonist  $^{125}\text{I}$ -Sar<sup>1</sup>,Ile<sup>8</sup> Ang II in the presence of the AT-1 receptor antagonist losartan; thus the level of AT-2 receptor expression in the RVLM of the rat is below our reliable detection limit. However, sulfhydryl reducing agents (e.g., dithiothreitol, beta-mercaptoethanol) can enhance radioligand binding to the AT-2 receptor (Speth et al., 1991) and such modification of assay conditions might reveal measurable levels of AT-2 receptors in the RVLM.

## 6. Conclusions

It has been known for the past twenty years that Ang II can modulate the activity of the RVLM, and therefore the activity of the sympathetic nervous system. Since the discovery that Ang II injected into the RVLM causes an increase in blood pressure (Allen et al., 1988; Andreatta et al., 1988), many studies have been performed to further elucidate the role that Ang II plays in modulating the RVLM. One thing is clear: Ang II does influence the activity of the RVLM, and its role is more pronounced in animal models of hypertension as well as some pathological conditions in which compensatory mechanisms are needed to maintain AP. The sympathoinhibitory actions of Ang II mediated by the AT-2 receptor in the RVLM of sham, but not chronic heart failure rats, a pathological state associated with increased sympathetic drive (Gao et al., 2008b), and the presence of a novel non-AT1, non-AT2 binding site for Ang II in the RVLM (Bourassa, Fang, Li, Sved and Speth, unpublished observations), suggests that Ang II may have a much more complex role than could be anticipated based solely on its AT-1 receptor-mediated effects. Thus, despite the progress that has been made in this area, there are many questions yet to be answered, many of which are very basic to our understanding of RVLM function and angiotensin signaling in the brain.

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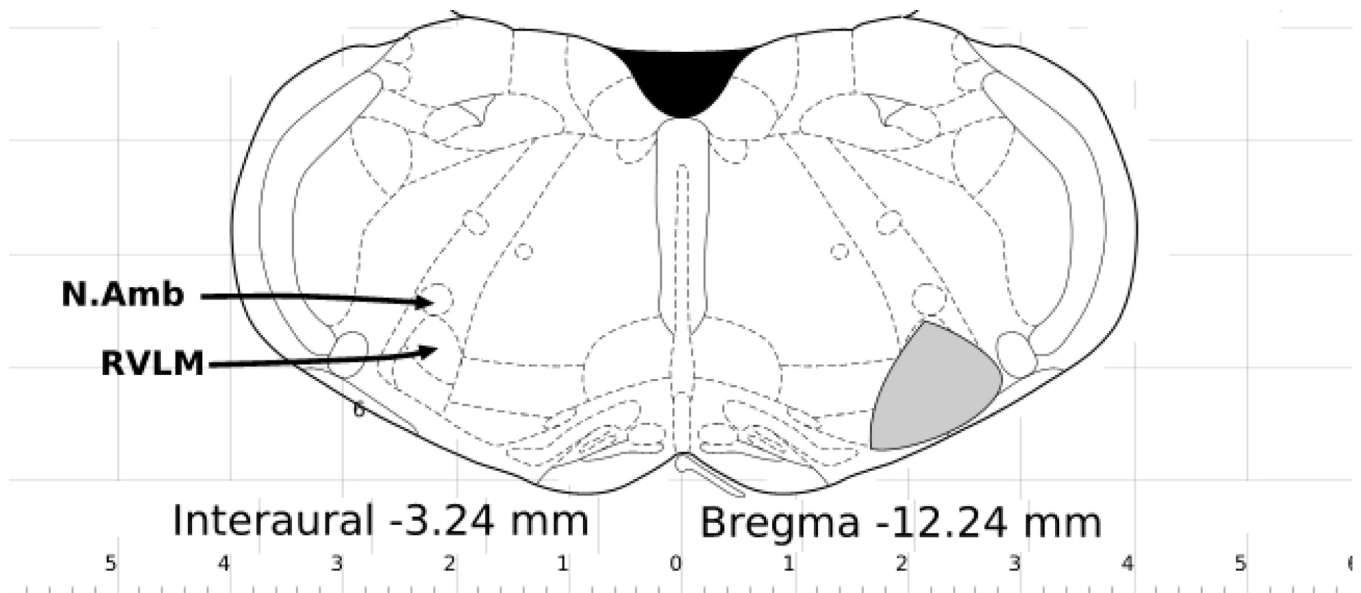
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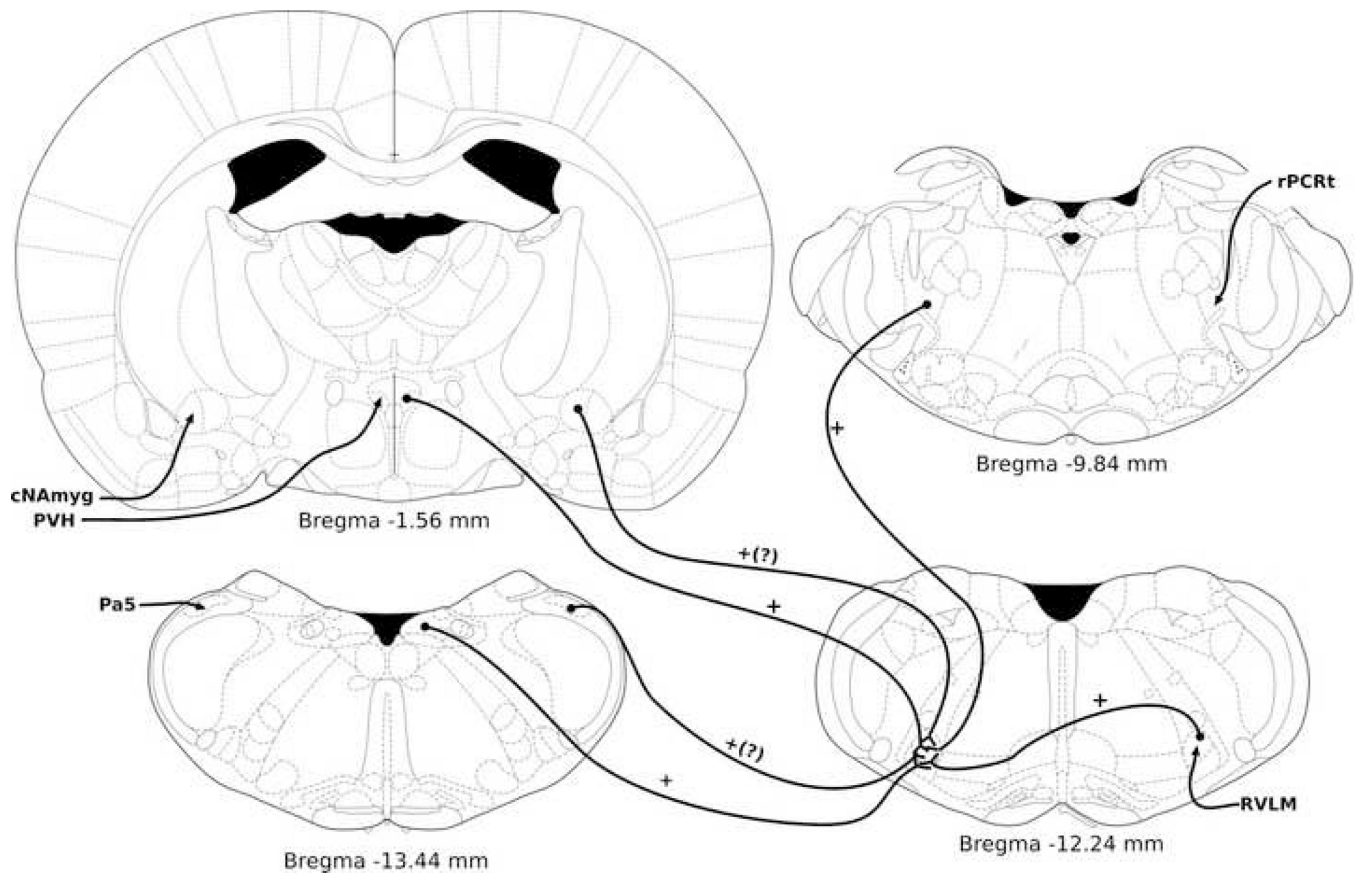
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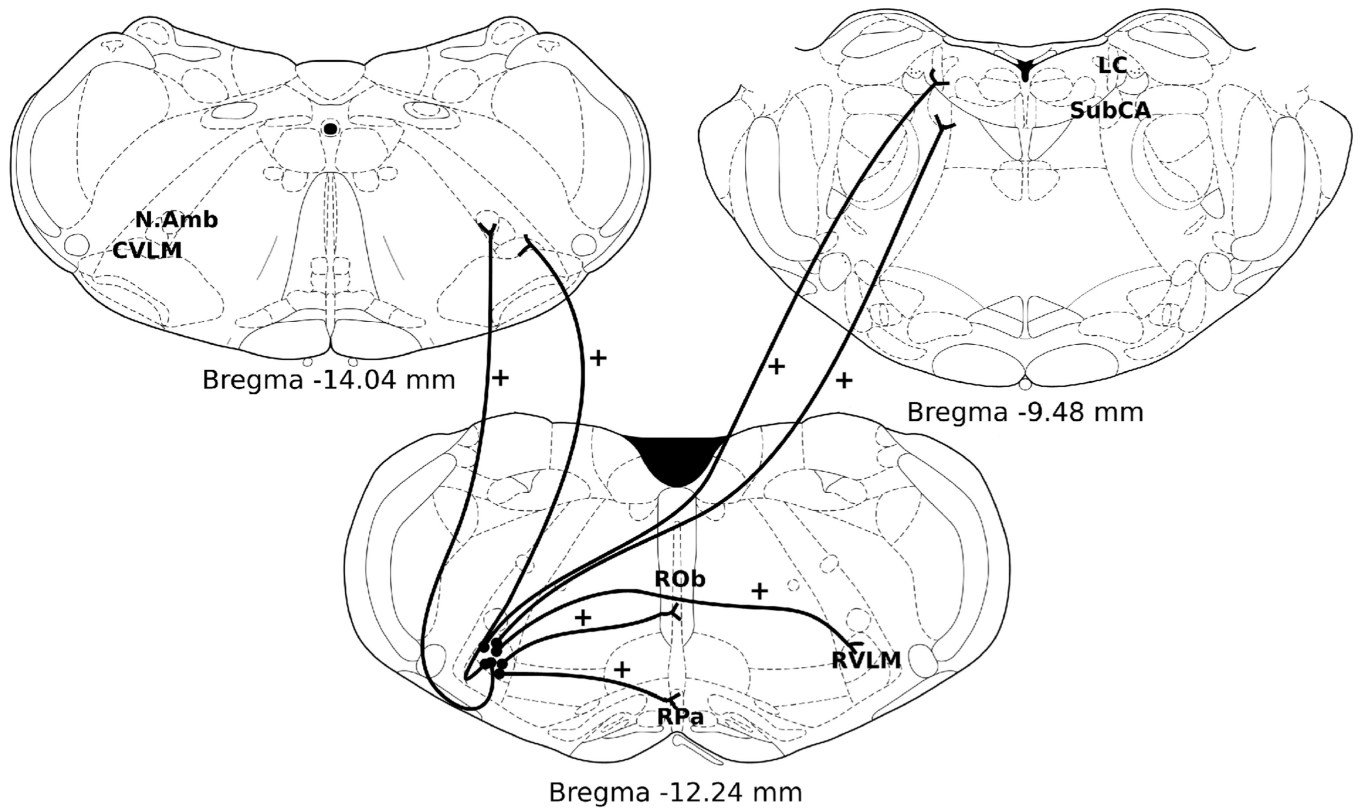
**Figure 1- Dorsoventral and Mediolateral Extent of the RVLM**

Figure 1 illustrates the triangular region between the N.Amb, 2 mm lateral to the midline at the ventral surface of the medulla, and 3 mm lateral to the midline at the ventral surface of the medulla that defines the mediolateral and dorsoventral extent of the RVLM (shaded in grey). The coronal section taken 12.24 mm caudal to Bregma and was adapted from vector graphics data supplied with the CD included in the Paxinos & Watson's "The Rat Brain in Stereotaxic Coordinates - The New Coronal Set 5th Edition." (Paxinos and Watson, 2005) All adapted and produced vector graphics data were processed using Xara Xtreme (v 0.7) and GIMP Image Editor (v 2.2) on the Linux (Ubuntu 6.10) platform.



### Figure 2. Afferent Projections to the RVLM

Figure 2 illustrates the afferent projections to the RVLM (other than the CVLM). The cNAmyg and PVH are found on the coronal section taken at Bregma  $-1.56$  mm, the rPCRt is found at the coronal section taken at  $9.84$  mm caudal to Bregma, the Pa5 is found at the coronal section taken  $13.44$  mm caudal to Bregma, and the RVLM is found at the coronal section taken  $12.24$  mm caudal to Bregma. A (+) indicates that the connection is excitatory, whereas a (+?) indicates that the connection appears to be excitatory, however it has not been definitively determined. Coronal diagrams were adapted from vector graphics data supplied with the CD included in the Paxinos & Watson's "The Rat Brain in Stereotaxic Coordinates - The New Coronal Set 5th Edition." (Paxinos and Watson, 2005) All adapted and produced vector graphics data were processed using Xara Xtreme (v 0.7) and GIMP Image Editor (v 2.2) on the Linux (Ubuntu 6.10) platform.



### Figure 3- Efferent Projections from the RVLM

Figure 3 illustrates the efferent projections from the RVLM (other than the IML of the thoracic spinal cord). The N.Amb and CVLM are found on the coronal section taken 14.04 mm caudal to Bregma, the LC and SubCA are found on the coronal section taken 9.48 mm caudal to Bregma, and the RPa, ROb, and RVLM are found on the coronal section taken 12.24 mm caudal to Bregma. A (+) indicates that the connection is excitatory. Coronal diagrams were adapted from vector graphics data supplied with the CD included in the Paxinos & Watson's "The Rat Brain in Stereotaxic Coordinates - The New Coronal Set 5th Edition." (Paxinos and Watson, 2005) All adapted and produced vector graphics data were processed using Xara Xtreme (v 0.7) and GIMP Image Editor (v 2.2) on the Linux (Ubuntu 6.10) platform.

**Table 1-****Blood Pressure Responses in WKY and SHR**

<b>Treatment</b>	<b>WKY</b>	<b>SHR</b>
Ang II (1)	~ 20 mmHg Increase	~35 mmHg Increase
AT1 Antagonist (1,3)	No Change	~30 mmHg Decrease
Glutamate (1)	~45 mmHg Increase	~50 mmHg Increase
Kynurenic Acid (2,4)	~5 mmHg Decrease	~40 mmHg Decrease

Data summary of blood pressure responses to RVLM injection of different agents in WKY and SHR. Data from (1) are adapted from (Ito et al., 2002). Data from (2) are adapted from Ito et al 2000. Antagonist used (3) in this study was the non-peptide AT1 antagonist valsartan. Kynurenic acid (4) is a “broad-spectrum” excitatory amino acid antagonist.

**Table 2-****Blood Pressure Responses in DS and DR Rats**

<b>Treatment</b>	<b>DR Low Salt</b>	<b>DR High Salt</b>	<b>DS Low Salt</b>	<b>DS High Salt</b>
Ang II (1)	~25 mmHg ↑	~20 mmHg ↑	~20 mmHg ↑	~40 mmHg ↑
ATI Antagonist (1, 3)	~10 mmHg ↓	No change	No Change	~35 mmHg ↓

Data summary of blood pressure responses to RVLM injection of different agents in DS and DR fed either a low or high salt diet. Data from (1) are adapted from (Ito et al., 2003). Antagonist used (3) in this study was the non-peptide AT1 antagonist valsartan.