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Selective C1 Lesioning Slightly Decreases Angiotensin II type I Receptor Expression in the Rat Rostral Ventrolateral Medulla (RVLM)

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Abstract

Cardiovascular homeostasis is regulated in large part by the rostral ventrolateral medulla (RVLM) in mammals. Projections from the RVLM to the intermediolateral column of the thoracolumbar spinal cord innervate preganglionic neurons of the sympathetic nervous system causing elevation of blood pressure and heart rate. A large proportion, but not all, of the neurons in the RVLM contain the enzymes necessary for the production of epinephrine and are identified as the C1 cell group. Angiotensin II (Ang II) activates the RVLM acting upon AT_1 receptors. To assess the proportion of AT_1 receptors that are located on C1 neurons in the rat RVLM this study employed an antibody to dopamine-beta-hydroxylase conjugated to saporin, to selectively destroy C1 neurons in the RVLM. Expression of tyrosine hydroxylase immunoreactive neurons in the RVLM was reduced by 57 % in the toxin injected RVLM compared to the contralateral RVLM. In contrast, densitometric analysis of autoradiographic images of ^{125}I-sarcosine^1, isoleucine^8 Ang II binding to AT_1 receptors of the injected side RVLM revealed a small (10%) reduction in AT_1 receptor expression compared to the contralateral RVLM. These results suggest that the majority of AT_1 receptors in the rat RVLM are located on non-C1 neurons or glia.

The rostral ventrolateral medulla (RVLM) contains bulbospinal neurons that are critical mediators of sympathetic tone and are essential for many neurally-mediated cardiovascular (1) reflexes stimulated by the peripheral baroreceptors, chemoreceptors, and cardiopulmonary receptors (2). In addition, excitatory inputs from the forebrain, primarily the paraventricular nucleus of the hypothalamus (PVN), also activate the bulbospinal neurons of the RVLM (3-6).

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The renin-angiotensin system (RAS) through its active hormone angiotensin II (Ang II) has long been known to be a critical regulator of peripheral blood pressure. It is now known that all components of the renin-angiotensin system exist in the brain, and a brain-specific angiotensin system exists there (7). In the RVLM, microinjection of Ang II produces a significant pressor response that can be attenuated by an angiotensin II type 1 receptor (AT1R) antagonist such as losartan or valsartan (8-10). Alone, microinjection of losartan or valsartan into the RVLM produces no response in normotensive animals (3, 9, 11), but causes a depressor response in hypertensive animals such as the spontaneously hypertensive rat (1, 12). This suggests that elevated activity of the brain-specific renin-angiotensin system contributes to hypertension. In support of these physiological studies, it had been reported that AT1Rs are present in the RVLM of many species including rat, rabbit, cat, dog, sheep, and human (13-19). Our laboratory further characterized the binding characteristics of AT1Rs in the RVLM of the rat (20, 21) and showed that AT1R expression in the RVLM is increased in the spontaneously hypertensive rat compared to the Wistar-Kyoto rat (21). This is consistent with a previous report indicating an increase in AT_1 receptor gene expression in the RVLM of the SHR (22).

Within the RVLM, there are two phenotypically distinct types of neurons: those that contain the enzymes required to synthesize epinephrine (C1 neurons), and those that lack these enzymes (non-C1 neurons). Approximately two thirds of the spinally projecting neurons in the RVLM have been shown to co-express tyrosine hydroxylase and phenylethanolamine N-methyltransferase (PNMT) and hence are part of the C1 cell group, whereas the other one third of spinally projecting neurons present in the RVLM do not contain PNMT, but do express vGlu2, suggesting that they are glutamatergic (2).

Early attempts to determine the relative importance of the C1 and non-C1 neurons in cardiovascular responses were largely unsuccessful as the C1 neurons, despite containing all of the enzymes required for the synthesis of epinephrine, do not use catecholamines as their primary neurotransmitter and in fact use glutamate like the non-C1 neurons that project to the preganglionic sympathetic neurons (23-30). Later studies have consistently shown that both the C1 and non-C1 neurons are involved in cardiovascular responses. For example, both electrophysiological and c-fos expression studies have confirmed that C1 as well as non-C1 neurons are responsive to decreases in mean arterial pressure (31-34) and found that both C1 and non-C1 neurons increase sympathetic nerve activity in response to sciatic nerve stimulation.

It is currently unknown whether the effects of Ang II in the RVLM are due to activation of the C1 neurons, non-C1 neurons, or both. There have been recent reports that suggest that the majority of the AT1Rs in the RVLM are found specifically on the C1 neurons. For example, studies in AT1a receptor knockout mice have found that AT1a receptor expression occurs mostly on C1-neurons following either lentiviral vector transgene expression or bacterial artificial chromosome expression (35, 36). However, these studies were limited to the mouse brain, which differs from the rat brain in its distribution of AT_1 receptors on catecholaminergic neurons; e.g., in the mouse brain the locus coeruleus contains AT_1 receptors, while in the locus coeruleus of the rat brain, the noradrenergic neurons contain AT_2 receptors (37); in the mouse brain the dopamine rich striatum contains abundant AT_1

receptors (38), while in the striatum of the rat brain, AT_1 receptors are barely discernable (39)

Since most studies of the physiological significance of the brain renin-angiotensin system have been carried out in rats, the purpose of this study was to determine the relative abundance of AT1Rs on C1 and non-C1 neurons of the rat brain using quantitative densitometric analysis after unilaterally lesioning the C1 cells using an anti-dopamine-beta-hydroxylase antibody conjugated to saporin.

Materials & Methods

Animals and Experimental Treatment

Male Sprague-Dawley (Charles River) rats weighing 250-375g were individually housed with ad libitum access to standard rat chow and tap water. Room temperature was maintained at 22-23°C and a 12:12 hour cycle was maintained with lights on at 07:00. Unilateral RVLM injections of 10 or 15 ng/200 nL, n= of DSAP (Advanced Targeting Systems Inc., San Diego, CA) were made from a dorsal approach via a glass micropipette angled at 20 degrees. Target stereotaxic coordinates, taken from the calamus scriptorius, were: 1.8 mm rostral, 1.8 mm lateral, and 3.2 mm ventral. Control animals (n=4) received 20 ng/200 nL, unilateral injections of Mouse IgG-saporin (MZAB) (Advanced Targeting Systems, Inc.). Three to four weeks after injection, rats were decapitated and brains were rapidly removed and frozen in liquid isopentane over dry ice.

Tissue Sectioning and Staining

In a cryostat, brainstem tissue from each animal was cut onto glass microscope slides at 20 μm in a 1:10 series and stored at -80°C. A set of brainstem sections from each rat was brought to room temperature and immersed in 4% paraformaldehyde and rinsed in sodium phosphate buffer (SPB) before being incubated for 24 hours at room temperature in a rabbit anti-tyrosine hydroxylase (TH) polyclonal antibody that is specified for use with fresh tissue (Chemicon; 1:10,000) and diluted in sodium phosphate buffer (SPB) containing 0.3% Triton-X and 1% donkey serum. Sections were rinsed in SPB and then incubated in biotinylated donkey anti-rabbit IgG diluted in SPB containing 0.3% Triton-X (Jackson ImmunoResearch Laboratories; 1:500). Sections were rinsed in SPB and then processed according to the avidin-biotin immunoperoxidase method using Elite Vectastain reagent (Vector Laboratories). To determine the absence of non-specific tissue damage or microglia infiltration, a second set of slides from each rat were stained with neutral red. After immunohistochemical staining, tissue was dehydrated in a graded ethanol series, defatted in xylene, and coverslipped with Histomount (VWR).

To determine the extent of the C1 cell depletion in each rat, cell counts of TH-positive neurons were performed using brightfield illumination microscopy. Cell counts were performed in every tenth brain stem section through the entire rostral-caudal extent of the RVLM. The RVLM was anatomically defined as the area extending 600 μm caudally from the caudal pole of the facial nucleus and cells were considered TH-positive when they displayed characteristic brown cytoplasmic staining. A third set of Nissl-stained tissue

sections was used to assess nonspecific tissue damage such as necrosis and microglia infiltration. The four rats injected with 15 ng/200 nL of DSAP showed nonspecific damage to the RVLM were not included in the analysis.

Quantitative Angiotensin Receptor Autoradiography

Quantitative angiotensin receptor autoradiography was performed essentially as described previously (40). Two of the ten slides from tissue sectioning were used for angiotensin receptor autoradiography, one for non-specific binding and one for AT1 receptor binding. Slides were pre-incubated in AM5 buffer (150 mM NaCl, 5 mM EDTA, 0.1 mM bacitracin, 50 mM NaPO4, pH 7.2) for 30 minutes at room temperature. Slides were then incubated in AM5 buffer containing 500 pM ^{125}I-sarcosine^{1},isoleucine^{8}-Angiotensin II (^{125}I-SI Ang II) in the presence of either 3 μM Ang II (non-specific binding) or 10 μM PD123,319 (AT1 binding) for 60 minutes at room temperature. Slides were then rinsed in two changes of distilled water, five changes of AM5 buffer for one minute each, and then two additional changes of distilled water. Slides were dried under a stream of cool air, taped to cardboard, and exposed to autoradiographic film (Kodak Biomax MR-1) for approximately 3 days at -20°C. A set of ^{125}I calibration standards (Microscales, RPA 522, General Electric Healthcare) were included with each film for densitometric quantitation.

Image Analysis & Densitometry

Specific binding of ^{125}I-SI Ang II was quantitated essentially as previously described (41). Briefly, images of the autoradiograms were analyzed using AIS 6.0 software. The ^{125}I calibration standards included on the autoradiograms were used to make a standard curve, allowing quantitation of brain regions of interest. Specific AT1 receptor binding was calculated by subtracting non-specific binding of the corresponding section from the total ^{125}I Ang II binding in the presence of PD123,319.

Statistics

AT1 receptor and TH immunostaining data from three levels of the RVLM (approximately -12.2, -12.4, and -12.6 mm relative to Bregma) were averaged. Two-way Analyses of Variance (ANOVA) of a 2 × 2 design (Type of Injection × Side) with repeated measures were used for both AT1 receptor and TH immunostaining data followed by Bonferroni post-hoc analysis where appropriate. *A priori* contrasts to assess TH immunostaining and AT1R density following DSAP injection were considered one-tailed analyses and stated p values reflect this fact. $p < 0.05$ was considered statistically significant.

Results

TH Immunostaining

As expected, DSAP produced a substantial 57% depletion of C1 neurons in the RVLM compared to the control MZAB injected RVLM, $F(1,6) = 6.42$, $p = 0.022$. The main effect of injection side was significant, $F(1,6) = 5.31$, $p = 0.03$, which was primarily attributed to the significant loss of TH immunostaining following DSAP injection into the injected side. As expected, there was a highly significant interaction, $F(1,6) = 17.66$, $p = 0.003$ as DSAP injection reduced TH immunostaining on injected side relative to the contralateral side while

MZAB injection did not differentially affect TH immunostaining between sides. Figure 1 shows the effect of MZAB and DSAP injection on TH immunostaining on both the injected side and contralateral side. Figure 3 panels C and D show a demonstrative example of TH immunostaining on both sides of the RVLM in a brain section from a rat given a unilateral DSAP injection. Figure 3 panels E and F convey the Nissl staining of the ipsilateral and contralateral RVLM of a DSAP injected brain.

AT1 Receptor Density

As shown in Figure 2, the AT1 receptor binding was 19% lower in the RVLM of the DSAP injected brains compared to the MZAB injected brains, $F(1,6) = 3.95$, $p = 0.047$. The main effect of side was not significant, $F(1,6) = 0.01$, $p = 0.45$. Despite the slightly greater reduction in AT_1 receptor binding on the DSAP injected side relative to the uninjected side, or the ipsilateral MZAB injected side, there was no interaction between side and the different conjugates, $F(1,6) = 0.99$, $p = 0.18$. Figure 3 panels A and B depict a demonstrative AT1 receptor autoradiogram in a brain receiving DSAP injection.

Discussion

Our results provide further validation that DSAP injection into the RVLM produces a significant, selective C1-cell lesion. In this experiment, DSAP injection (10 ng/200 nL) decreased TH immunostaining (a marker for C1-cells) by 57% compared to the contralateral side, whereas MZAB injection produced no significant change in TH immunostaining compared to the contralateral side. Overall, brains receiving DSAP injection or MZAB injection showed the same number of TH immunopositive cells on the side not receiving the injection. While it has been reported that there is some contralateral innervation of the C1 it is reported to be sparse (42) and was not observable in our analyses.

It should be noted that we used a higher dose of DSAP in some animals (15 ng/200 nL). However, this dose proved to be too high as Nissl staining showed profound gliosis and a complete loss of AT1 receptor binding on the side of the lesion. While the data from those animals was not included in the final analysis, it is noteworthy that the infiltrating microglia did not express AT1 receptors. Previously, higher doses of up to 21 ng/200 nL had been used, and these doses provided selective C1 lesioning without causing gliosis (43, 44). It is possible that over time, preparation of this toxin-conjugate has become more efficient, and hence the final product more potent.

Our AT1 receptor data provide intriguing results. It has previously been assumed that the AT1 receptors present in the RVLM are found on the C1 cells as these make up the majority of neurons in the region (45) and by extension, must provide the majority of the input into the preganglionic sympathetic neurons. However, it has been shown that non C1-cells within the RVLM are also responsive to changes in blood pressure and provide input to the pre-ganglionic sympathetic neurons (31-33), but the overall contribution to sympathetic tone by each cell phenotype has not been determined. Our results show that the majority of the AT1 receptors within the RVLM are not found on C1 cells. A 57% loss of TH immunopositive cells following DSAP injection compared to the contralateral side only produced a 10% decrease in AT1 receptor density that was not statistically significant using the same

comparison. These results contrast with those observed in the mouse RVLM, in which nearly all AT1 receptor expressing cells displayed tyrosine hydroxylase immunoreactivity (35, 36, 46). It should be noted that there are distinct differences in the expression of angiotensin receptors in the rat and mouse brains, e.g. in the rat brain the neurons of the locus coeruleus express AT2 receptors (37), while in the mouse locus coeruleus the receptors are primarily the AT1 subtype (47); in the basal ganglia (a dopamine rich region of the brain) of the mouse there is high expression of AT1 receptors (38), while in the rat AT1 receptors are barely discernable in the basal ganglia (14, 39, 48). Additionally, studies measuring AT1 receptor in the mouse RVLM have used AT1aR transgenic mice or have specifically measured AT1a receptor or its promoter expression (35, 36, 46, 49, 50),. Both rats and mice express two subtypes of the AT1 receptor – the AT1a receptor and AT1b receptor. The autoradiography method used in this study to measure angiotensin receptor expression will detect both the AT1a and AT1b receptors. It has previously been shown (51) that the rat RVLM expresses both the AT1a and AT1b receptor – the AT1a predominating on cell bodies and the AT1b being evenly expressed on neuronal fibers. However, that study did not determine the relative distribution of AT1a and AT1b receptors specifically on the C1 and non-C1 neurons, so it is entirely possible that the C1 neurons express primarily AT1aRs whereas the non-C1 neurons express primarily AT1bRs.

Interestingly, the DSAP injection led to a significant decrease in AT1 receptor expression (19%) when both the left and right RVLM of the DSAP-injected brains was compared to the left and right RVLM of the MZAP-injected brains. The bilateral reduction in AT1 receptor binding was not anticipated and requires explanation. As noted above, C1-cells of the RVLM send projections not only to the pre-ganglionic sympathetic neurons of the spinal cord, but also send a sparse projection to the contralateral RVLM (42). Thus it is expected that there will be a loss of axonal projections of the C1 neurons to the RVLM contralateral to the DSAP injected RVLM. This suggests that there may be AT1 receptors on contralaterally projecting axon terminals of C1 neurons, moreso than on the neuronal cell bodies. Another possibility is that the destruction of the C1 neurons in the RVLM by DSAP may have had secondary effects on the functionality of the contralateral RVLM resulting in a reduction of AT1 receptor expression on non-C1 neurons or glia.

While this study clearly shows that the majority of the AT1 receptors found in the RVLM are not present on the C1 neuronal cell bodies, it does not definitively identify the phenotype of the neurons that express AT1 receptors in the RVLM. However, regardless of phenotype, these results provide evidence that the non-C1 neurons of the RVLM make significant contributions to the maintenance of blood pressure during periods of dehydration, as well as the maintenance of a hypertensive state in hypertensive animals in response to AT1 receptor stimulation. It has been previously shown that angiotensinergic activity in the RVLM supports blood pressure during these states, but is not an important modulator of blood pressure in water-replete, normotensive animals (1, 4, 52). Moreover, the possibility exists that it only requires a small number of C1 neurons for Ang II to generate a strong stimulatory response to presynaptic sympathetic neurons in the intermediolateral column of the spinal cord.

Another possibility is that a significant proportion of AT1 receptors in the RVLM are found on glial cells or the microvasculature. It is known that the brain microvasculature of dogs (53) and rats (54) express angiotensin receptors, and it is likely that at least some proportion of the measured angiotensin receptors in this study were found on the microvasculature. Studies using a bacterial artificial chromosome indicator of the AT1 receptor demonstrate the occurrence, albeit scarce, of AT1 receptors on astrocytes in the mouse RVLM (35). It has also been shown that injection of an adenoviral vector containing either the native form of the rat AT1a receptor or a constitutively active form of the receptor (termed [N111G]AT1a) which was made by site-directed mutagenesis of asparagine 111 to glycine into the RVLM, leads to an increased expression of AT1 receptors exclusively on glial cells (55) Interestingly, overexpression of the (N111G)AT1a receptor in glial cells lead to a sustained increase in arterial blood pressure, whereas overexpression of the native form of the receptor did not cause a change in blood pressure. It is currently not understood how an increase in AT1 receptor expression and activation in glial cells of the RVLM leads to an increase in arterial pressure. However, it is known that angiotensinogen synthesis in the brain occurs primarily in glial cells (56, 57), and in the periphery angiotensinogen synthesis is stimulated by activation of AT1 receptors (58). It has been speculated that AT1 receptor activation on glial cells stimulates angiotensinogen synthesis from the cell, thus increasing the concentration of Ang II in the RVLM, which could then activate AT1 receptors on nearby neurons to increase their firing rate (55).

Previously we have characterized the AT1 receptor population in the RVLM compared to those found in the nucleus of the solitary tract, dorsal motor nucleus of the vagus, and area postrema (NTS area) (20). We found that while the AT1 receptor density was much lower in the RVLM compared to the NTS area (as expected), the affinity of the receptors was higher in the RVLM compared to the NTS area. This led us to conclude that lower concentrations of circulating Ang II could produce relatively profound effects on arterial blood pressure and sympathetic tone. However, that study did not assess whether the AT1 receptors were on neurons or glia. If indeed a large proportion of the AT1 receptors are on glia, our recent conclusion would still be valid, but for different reasons. In light of this recent data, it is entirely possible that the higher affinity of the AT1 receptors in the RVLM is due to the cell type expressing the receptors (neurons versus astrocytes), and a higher affinity of the AT1 receptors found on astrocytes has direct implications for the regulation of angiotensinogen synthesis, indirectly affecting blood pressure and sympathetic tone.

We also observed that AT1 receptor expression in the RVLM is significantly higher in spontaneously hypertensive rats (SHR) compared to normotensive Wistar-Kyoto (WKY) rats (21). Again, in light of the observation that the majority of these receptors are not found on the C1-cells and are potentially more involved with astrocyte function than direct neuronal function, it is possible that the centrally derived component of hypertension associated with the SHR model is more directly attributable to an increased local concentration of angiotensinogen (and hence Ang II) instead of an increased neuronal sensitivity for the normal presence of Ang II. On the other hand, the AT1 receptor density in the RVLM of the SHR was only 10% higher than that of the WKY rats. As we did not attempt to identify the cell types responsible for the increased AT1 receptor expression, it is entirely possible that the increase in AT1 receptor expression in the RVLM of SHR is

restricted to an increase in neuronal AT1 receptors as opposed to glial AT1 receptors, or possibly a combination of both.

There are similar (albeit smaller) increases in the responsiveness of the RVLM to Ang II in rats that are water-deprived compared to water-replete as is seen in hypertensive rats compared to normotensive rats (4). We have recently studied both AT1 receptor and angiotensin converting enzyme (ACE) expression in the RVLM of normotensive rats that were water-deprived compared to those given free access to water (59), and interestingly we did not find an increased AT1 receptor density in the RVLM of animals that were water-deprived. However, there was a 34% higher ^{125}I-351A binding to ACE in the RVLM of water-deprived rat brains compared to water-replete control brains. This led us to conclude that water-deprivation leads to an increased local concentration of Ang II in the RLVM via an increased expression of ACE.

Our recent observations of increased AT1 or ACE expression in the RVLM of rats that are hypertensive or water-deprived, respectively, led us to suggest that the brain renin-angiotensin system is differentially altered based on the physiological circumstances. However, in light of our current results along with the observations of others, it is entirely possible that the renin-angiotensin system within the RVLM is only altered by changing the local concentration of Ang II, either by increasing angiotensinogen production or by increasing the conversion of Ang I to Ang II. However, this suggestion has not been directly studied and thus a true conclusion as to the local modulation of the renin-angiotensin system during hypertension or water-deprivation cannot be made at this time.

In conclusion, we have demonstrated that unilateral injection of DSAP into the RVLM, despite producing a large decrease in the number of C1 neurons limited to the injected side, produced only a small and bilateral reduction in AT1 receptor binding. This suggests that only a small proportion of the AT1 receptors are found on the C1-cells within the RVLM of the rat, but this small population of AT1 receptors is found at both the dendritic and axonal ends of the C1 neurons. Taken together with the observations of others, this suggests that it is likely that the majority of the AT1 receptor population within the rat RVLM is found on glial cells or non-C1 neurons, and these glial receptors affect the bulbospinal neurons of the RVLM only indirectly.

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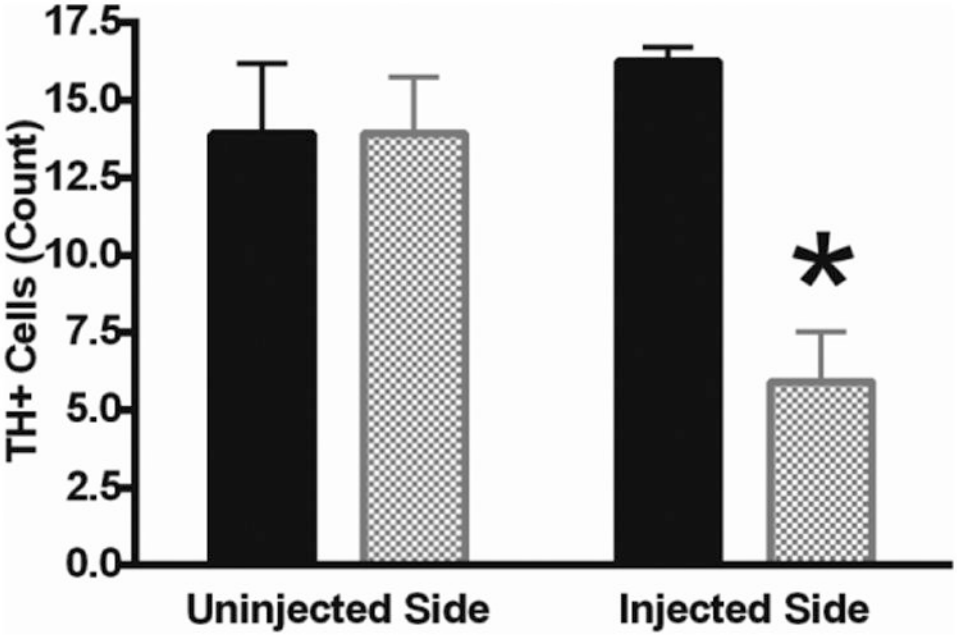

Fig 1. Effect of Mouse IgG-saporin (MZAB) or Anti-Dopamine-Beta-Hydroxylase-Saporin (DSAP) Injection on TH Immunostaining in RVLM

Effect of MZAB (solid black bars) or DSAP injection (hatched gray bars) on TH immunostaining in the RVLM on both the injected side and the contralateral side. MZAB injection had no effect on TH immunostaining on either side, whereas DSAP injection markedly decreased TH immunostaining in the RVLM on the injected side, but not the contralateral side. * $p < 0.05$ v MZAB and uninjected side.

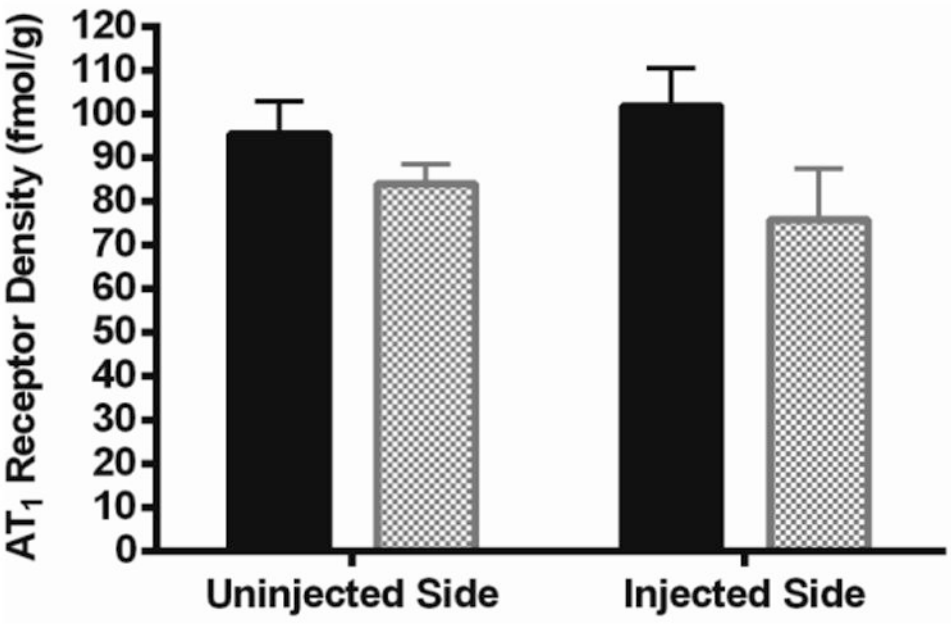

Fig 2. Effect of MZAB or DSAP Injection on AT1 Receptor Density in RVLM

Effect of MZAB (solid black bars) or DSAP injection (hatched gray bars) on AT1 receptor density in the RVLM on both the injected side and the contralateral side. MZAB injection had no effect on AT1 receptor density on either side, whereas DSAP injection slightly (but significantly, $p < 0.05$) decreased AT1 receptor density in the RVLM on both sides compared to MZAB.

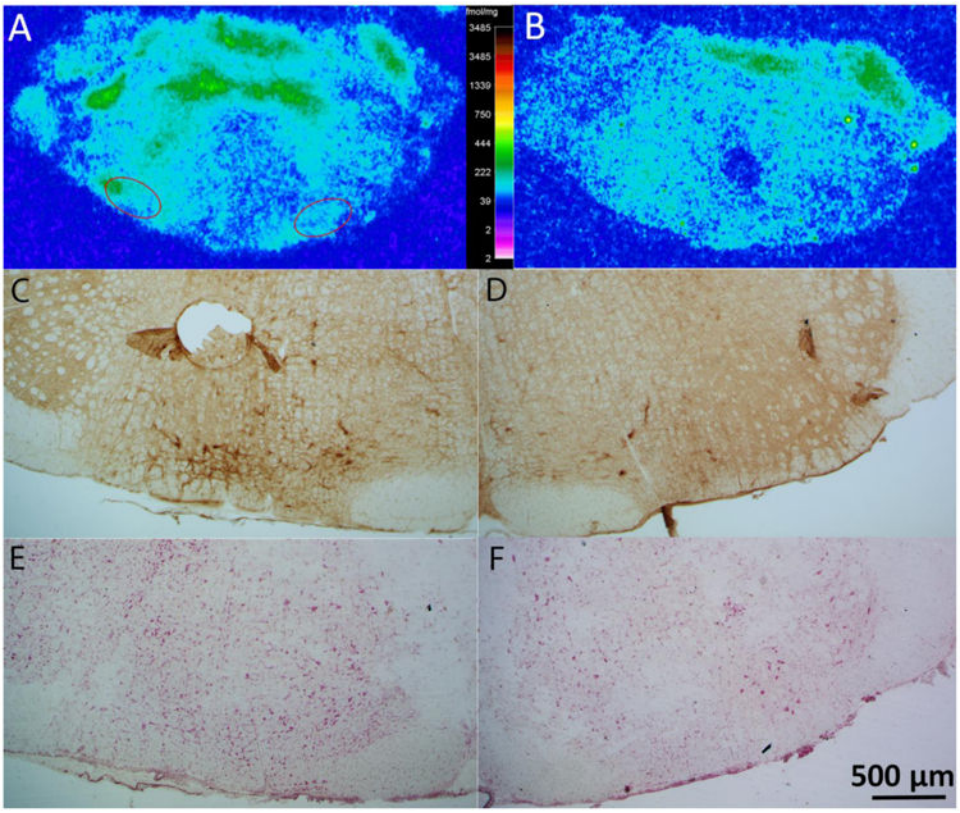

Fig 3. Effect of DSAP Injection on AT1R Density, TH, and Nissl Staining

Photomicrographs of a brainstem that received a unilateral (right sided) DSAP injection. All photomicrographs are taken at approximately -12.0 mm relative to Bregma. Panel A) AT1 receptor binding in the brainstem. Circles indicate the area sampled as the RVLM. Note the small reduction in AT1 receptor binding on the right side of section within the red circle compared to the area within the red circle on the left side of the section. Panel B) Non-specific 125I-SI Ang II binding in the brainstem. Panels C and D) TH immunostaining in the left and right RVLM and surrounding areas, respectively. Panel E and F) Nissl staining in the left and right RVLM and surrounding areas, respectively