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Erick A. Bourassa
Northwest Missouri State University

Xiefan Fang
University of Mississippi

Xia Li
University of Pittsburgh

Alan F. Sved
University of Pittsburgh

Robert C. Speth
Nova Southeastern University, rs1251@nova.edu

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AT_1 angiotensin II receptor and novel non-AT_1, non-AT_2 angiotensin II/III binding site in brainstem cardiovascular regulatory centers of the spontaneously hypertensive rat

Erick A. Bourassa[1,2,*], **Xiefan Fang**[1,*], **Xia Li**[3], **Alan F. Sved**[3], and **Robert C. Speth**[4]

[1]Biological Sciences, Northwest Missouri State University, Maryville, MO 64468

[2]Department of Pharmacology, School of Pharmacy, University of Mississippi, University, MS 38677

[3]Department of Neuroscience, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

[4]Department of Pharmaceutical Sciences, College of Pharmacy, Nova Southeastern University, Fort Lauderdale, FL 33328

Abstract

Spontaneously hypertensive rats (SHR) have an activated brain angiotensin system that contributes to the elevation of blood pressure in this animal model. Physiological and pharmacological studies suggest that hyperactivation of brain AT_1 angiotensin receptors is a major pathophysiological factor. Consistent with these observations, radioligand binding studies indicate widespread up-regulation of brain angiotensin receptors in SHR. One key brainstem site in which AT1 receptor stimulation appears to contribute to the elevated blood pressure in SHR is the rostral ventrolateral medulla (RVLM). However, no quantitative comparison of AT_1 receptor binding in the RVLM has been made in SHR versus normotensive rats. A novel, non-AT_1, non-AT_2 binding site, specific for angiotensins II and III has recently been discovered in the brain. To determine if radioligand binding to either AT_1 receptors or this novel angiotensin binding site are altered in the RVLM and other caudal brainstem regions of SHR, a quantitative densitometric autoradiographic comparsion of radioligand binding in SHR versus normotensive Wistar-Kyoto rats was made. In both the RVLM and caudal ventrolateral medulla (CVLM) as well as dorsomedial medulla (DMM), there was increased expression of AT_1 receptor binding in SHR (13, 9 and 23%, respectively). Conversely, expression of the novel, non-AT_1, non-AT_2, angiotensin II and III binding site was decreased in the RVLM and DMM of SHR (37 and 13%, respectively). This increased AT1 receptor binding in the RVLM may contribute to the hypertension of SHR. Reduced radioligand binding to the novel, non-AT_1, non-AT_2, angiotensin binding site in the RVLM of SHR may indicate a role for this binding site to reduce blood pressure via its interactions with angiotensins II and III.

Correspondence: Robert C. Speth, Ph.D., Dept. Pharmaceutical Sciences, College of Pharmacy, Nova Southeastern University, Fort Lauderdale, FL 33328, Robert.speth@nova.edu, 954-262-1330, 954-262-2278 (Fax).
*Joint first authors based upon equivalent contributions to this manuscript.

Keywords

Rostral ventrolateral medulla (RVLM); Caudal ventrolateral medulla (CVLM); Dorsomedial medulla (DMM); Novel, non-AT_1, non-AT_2 Angiotensin II and III binding site; Receptor autoradiography; ^{125}I-sarcosine^1, isoleucine^8 angiotensin II

Introduction

Spontaneously hypertensive rats (SHR) (Okamoto and Aoki, 1963) have many features of human essential hypertension and therefore are one of the most widely used animal models of hypertension. Among their most commonly noted features are an increased peripheral vascular resistance that is mostly under neurogenic control (Yamori, 1984), an increased plasma concentration of norepinephrine (Grobecker et al., 1975), and an upregulation of the renin-angiotensin system (Gehlert et al., 1986; Haddad and Garcia, 1996; Ito et al., 2002; Veerasingham and Raizada, 2003). Also, the hypertension in SHR is affected by dietary salt consumption (Adams and Blizard, 1991) and is treatable using antihypertensive agents (Barone et al., 2007; Okamoto, 1969; Xie et al., 2007).

A substantial body of evidence suggests that an increased action of angiotensin II (Ang II) within the brain contributes to the elevated blood pressure in SHR. For example, blockade of angiotensin receptors (Phillips et al., 1977; Yang et al., 1992) and pharmacological interference with angiotensin formation (Hutchinson et al., 1980) in SHR brain decreases arterial blood pressure (ABP) to normal level; see reviews (Bourassa et al., 2009; Phillips and de Oliveira, 2008; Veerasingham and Raizada, 2003).

The rostral ventrolateral medulla (RVLM) is critically involved in the sympathetic control of ABP. Activation of the RVLM increases sympathetic vasomotor tone and ABP, whereas inhibition of the RVLM reduces ABP to the same extent as cervical transaction (Guyenet, 2006; Sved et al., 2003). Overactivity of the RVLM contributes to hypertension in SHR, as well as other animal models of hypertension. Angiotensin II can act in the RVLM to increase sympathetic vasomotor tone and ABP (Allen et al., 1998; Ito et al., 2002; Saigusa and Head, 1993). The caudal ventrolateral medulla (CVLM) and nucleus of the solitary tract (NTS), two other brainstem areas critical to the control of blood pressure, are also sites of action of Ang II. In contrast to the RVLM where Ang II acts to increase ABP, Ang II acts in the CVLM to decrease ABP, at least in part by enhancing CVLM inhibition of RVLM activity (Alzamora et al., 2006; Fow et al., 1994; Guyenet, 2006; Tan et al., 2005). A critical function for the NTS is alteration of sympathetic nervous system (SNS) output in response to changed baroreceptor input. The action of Ang II in the NTS is generally pressor by directly stimulating SNS output (Casto and Phillips, 1984) and by blunting baroreceptor-mediated reduction of heart rate with increasing ABP (Casto and Phillips, 1986); see also review (Veerasingham and Raizada, 2003).

The RVLM, CVLM, and NTS of SHR are more sensitive to the effects of microinjected Ang II than normotensive Wistar-Kyoto rats (WKY) (Ito et al., 2002; Katsunuma et al., 2003; Muratani et al., 1991). Importantly, inhibition of AT_1 receptors in the RVLM by local injection of an AT_1 receptor-selective antagonist decreases ABP in SHR but not WKY (Allen, 2001; Ito et al., 2002). Furthermore, expression in the RVLM of a constitutively active form of the AT_1 receptor increases ABP (Allen *et al.*, 2006). Taken together, these data support the notion that increased stimulation of AT_1 receptors in the RVLM contributes to the elevated ABP in SHR. This appears to generalize to other models of experimental hypertension (Sved et al 2003; Bourassa et al 2009).

We recently measured angiotensin AT_1 receptor binding in the RVLM and other medullary regions in normotensive rats (Bourassa et al., 2010). One goal of the present studies was to compare AT_1 receptor binding in the RVLM and other medullary cardiovascular control regions between SHR and WKY to address the hypothesis that AT_1 receptors are more abundant in the RVLM of SHR.

We recently reported the presence of a novel non-AT_1, non-AT_2 Ang II and angiotensin III (Ang II/Ang III) specific binding site present in brain-derived membranes of multiple species that is unmasked by parachloromercuribenzoate (PCMB) (Karamyan et al., 2008b; Karamyan et al., 2008a; Karamyan and Speth, 2007). This binding site is not inhibited by selective AT_1 or AT_2 agonists or antagonists. It is present in brain membranes of mice lacking AT_{1A}, AT_{1B} and AT_2 receptors establishing it as different from these classical angiotensin receptors (Karamyan et al., 2008a). This binding site is also present in brain membranes of mice lacking the Mas oncogene, which encodes the receptor for Ang1–7, and the endopeptidase neprilysin (EP 24.11, neutral endopeptidase, NEP) (Karamyan et al., 2008a).

The molecular structure and physiological function of this novel Ang II/Ang III binding site is presently unknown. It is widely distributed in the rat brain, including the brainstem, and is present in many brain regions devoid of AT_1 and AT_2 receptors (Karamyan and Speth, 2008). It is also present in the testis and several other structures in the mouse (Rabey et al., 2010). Thus, a second goal of these studies was to compare the relative expression of this binding site between SHR and WKY in the DMM, RVLM and CVLM, to test the hypothesis that this binding site is differentially expressed in hypertensive rats.

Results

AT_1 Receptor Density

Demonstrative autoradiograms of AT_1 receptor binding in the DMM and CVLM of SHR and WKY brainstems are shown in Figure 1. Demonstrative autoradiograms of AT_1 receptor binding in the RVLM are shown in Figure 2. AT_1 receptor densities were significantly higher in SHR compared to WKY in all three brainstem regions studied. There was a 23% increase in AT_1 receptor binding in the DMM, a 13% increase in AT_1 receptor binding in the RVLM and a 9% increase in AT_1 receptor binding in the CVLM (Figure 3).

Novel non-AT_1, non-AT_2 Ang II/Ang III binding site density

The binding of ^{125}I SI Ang II to the novel non-AT_1, non-AT_2 Ang II/Ang III binding site in the DMM and CVLM is shown in Figure 4. Binding in the RVLM is shown in Figure 5. Specific binding of ^{125}I SI Ang II to the novel Ang II/Ang III binding site was 37% lower in the RVLM and 13% lower in the DMM of SHR compared to WKY, however there was not a significant difference in the binding density of the CVLM between SHR and WKY (Figure 6).

Discussion

The present studies compared radioligand binding to the AT_1 receptor and a novel non-AT_1, non-AT_2 binding site in medullary cardiovascular regulatory areas in SHR and WKY. Differences were observed in the regions surveyed. Previous studies of Ang II receptor binding in SHR demonstrated increased concentrations of Ang II receptors in brain nuclei associated with the regulation of blood pressure, e.g., organum vasculosum of the lamina terminalis (OVLT), subfornical organ, median preoptic nucleus, paraventricular nucleus of the hypothalamus, NTS and dorsal motor nucleus of the vagus (DMV) (Gehlert et al., 1986; Gutkind et al., 1988; Song et al., 1994). This study replicates previously reported increases

in Ang II receptor binding in the NTS/DMV/DMM showing that this increase in Ang II receptor binding is to the AT_1 receptor. The highest level and largest increase of AT_1 receptor binding in SHR occurred in the caudal DMM (Figure 2), which contains the portion of the NTS most heavily innervated by the ninth and tenth nerve afferents that convey cardiovascular signals (Potts, 2002). However, due to the low abundance of Ang II receptor binding in the ventrolateral medulla of the rat and ambiguity regarding the precise locations of the RVLM and CVLM, few Ang II receptor binding studies have focused on these brain regions in rats (Bourassa et al., 2009; Bourassa et al., 2010).

The higher level of AT_1 receptor binding in the RVLM of SHR seen herein is consistent with studies suggesting that sensitivity to Ang II in the RVLM of SHR is increased. Ang II directly administered into the RVLM of SHR causes a larger increase in ABP in SHR than in WKY (Ito et al., 2002; Muratani et al., 1991). AT_1 antagonists administered into the RVLM of SHR cause a larger depressor response than in WKY (Allen, 2001; Ito et al., 2002). The mRNA for the AT_1 receptor in the RVLM of SHR (assessed by real-time PCR) is 2.7 times that of WKY (Reja *et al.*, 2006). AT_1 immunoreactive material in the RVLM of SHR is reported to be increased 50% over WKY in selected neurons displaying AT_1 immunoreactivity using a semiquantitative assay (Hu *et al.*, 2002), although documentation of the specificity of the AT_1 staining is lacking. The convergence of data from microinjection, mRNA, immunorective protein, and binding studies strongly suggests that an increase in functional AT_1 receptors in the RVLM of SHR contributes to the increased angiotensin-mediated stimulation of RVLM neurons causing increased ABP in SHR.

The increase in AT_1 receptors in the brainstem of SHR is not unique to the RVLM and NTS/DMV/DMM. In the CVLM, where Ang II acts to decrease ABP, AT_1 receptor binding is also increased. In the spinal trigeminal tract (SpV), a brain region considered to have little cardiovascular influence, an increase in AT_1 receptor binding in SHR has also been reported (Song et al., 1994). The functional significance of these changes and their relevance to SHR hypertension is not apparent.

In contrast to the change in AT_1 receptors, binding of radioligand to the novel non-AT_1, non-AT_2, Ang II/Ang III binding site was lower in the DMM and RVLM of SHR compared to WKY. Because DMM and RVLM regulate blood pressure, differences in the expression of this novel Ang II/Ang III binding site between WKY and SHR in these two regions could indicate a physiological role of this binding site in the regulation of blood pressure.

The function and identity of this novel non-AT_1, non-AT_2 Ang II/Ang III binding site is not yet known (Speth and Karamyan, 2008), but such investigation is in progress (Karamyan et al., 2010). Binding of Ang II and Ang III to this protein is unmasked by the organomercurial protease inhibitor PCMB, which may mimic alterations in redox state that could regulate functionality of the protein. Its presence in mouse (Karamyan et al., 2008a) and human brains (Karamyan et al., 2008b) indicates an evolutionary conservation, consistent with a functional role for this binding site. Three possible functions of this protein are: 1) it is an enzyme that degrades angiotensins, 2) a binding protein that serves as a clearance receptor that internalizes Ang II and Ang III, or, 3) a novel Ang II/Ang III receptor. Thus the non-AT_1, non-AT_2 binding site may serve to limit the access of Ang II to the AT_1 receptor or possibly function in a counter-regulatory role as does the AT_2 receptor (Jones et al., 2008) and the Ang 1–7 receptor (Ferreira et al., 2010). In such scenarios, a reduction in the novel non-AT_1, non-AT_2 binding site in the RVLM of SHR might contribute to the hypertension by increasing the stimulation of AT_1 receptors by Ang II and Ang III.

An additional indicator of possible functionality of this novel Ang II/Ang III binding site is the observation that Sar^1,Ile^8 Ang II and Sar^1,Thr^8 Ang II (sarthran), but not AT_1 receptor-

selective antagonists can lower blood pressure when administered directly into the RVLM of the normotensive WKY (Hirooka et al., 1997; Ito and Sved, 1996; Ito and Sved, 2000). This observation led Hirooka et al., (1997) and Ito and Sved (2000) to suggest that there may be a novel, non-AT_1, non-AT_2 angiotensin receptor subtype mediating this effect. Since this novel, non-AT_1, non-AT_2, Ang II/Ang III binding site has high affinity for Sar^1,Ile^8 Ang II (Karamyan and Speth, 2007), but not for non-peptidic AT_1 receptor-selective antagonists, it is possible that this binding site could be a novel Ang II receptor mediating responses to Sar^1,Ile^8 Ang II and Sar^1,Thr^8 Ang II in the RVLM that counteract the effects of AT_1 receptors.

In summary, this study found an increased AT_1 receptor binding in DMM, CVLM, and RVLM of SHR compared to WKY, whereas there was decreased binding to a novel non-AT_1, non-AT_2 binding site in DMM and RVLM of SHR. These results suggest that the increased ABP in SHR may be due to increased AT_1 receptor density and decreased novel non-AT_1, non-AT_2, Ang II/Ang III binding site density in cardiovascular regulatory regions of the brainstem.

Materials & Methods

Chemicals

Angiotensin and other peptides were obtained from Phoenix Pharmaceuticals, Bachem, or American Peptides. Losartan was a gift of Dr. Ron Smith of Dupont Merck. PD123319 (1-[[4-(Dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7- tetrahydro-1H-imidazo[4,5-c]pyridine-6 -carboxylic acid ditrifluoroacetate), was purchased from Tocris Bioscience. P-chloromercuribenzoic acid (PCMB) sodium salt was purchased from MP Biomedicals. The ^{125}I-Sarcosine^1, Isoleucine^8-Angiotensin II (^{125}I-SI Ang II) was prepared by the Peptide Radioiodination Service Center at the University of Mississippi as described elsewhere (Speth and Harding, 2001).

Animals & Tissue Preparation

Age-matched 16–17 week old male SHR (n = 6) and WKY (n = 6) rats (Charles River, Boston, MA), housed individually, and fed Purina 5001 diet ad lib with tap water on a 12 hour light-dark cycle were used for these experiments. The animals were decapitated and the brains were quickly removed and frozen in isopentane on dry ice. The brains were stored at −80°C. Brainstems were serially sectioned (sets of ten) using a cryostat at a thickness of 20 microns and thaw-mounted onto polylysine-coated slides (Superfrost, Thermo Fisher Scientific). The slides were air dried and kept frozen at −80°C until used for autoradiography, within six weeks of sacrifice. All animal procedures were approved and followed the guidelines set by the IACUC of the University of Pittsburgh and the University of Mississippi.

Quantitative Autoradiography

Quantitative AT_1 receptor autoradiography was performed essentially as previously described (Falcon *et al.*, 2004). Briefly, slides were pre-incubated in 150 mM NaCl, 5 mM EDTA, 0.1 mM bacitracin, and 50 mM NaPO4 buffer at pH 7.1–7.2 (AM5 buffer) for 30 minutes at room temperature. Slides were then incubated in Coplin jars containing 35 ml of AM5 buffer containing 500 pM ^{125}I-SI Ang II in either the presence of 3 μM Ang II (non-specific binding), or 10 μM PD123,319 (Total plus AT_1 binding) for 60 minutes at room temperature. Specific, AT_1 binding was determined by subtracting non-specific binding from total plus AT_1 binding. Under these conditions, the fractional metabolism of radioligand is small due to the large amount of radioligand present. Slides were 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 then dried under a stream of cool air, taped to cardboard, and exposed to autoradiographic film (Kodak Biomax MR-1) for 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.

Quantitative non-AT_1, non-AT_2 receptor autoradiography was performed as described above, except 0.3 mM PCMB, 10 uM losartan and 10 uM PD 123319 were also present in the assay buffers. Specific non-AT_1, non-AT_2 binding was determined by subtracting non-specific binding (in the presence of 3 μM Ang II) from total binding determined in the absence of Ang II.

Image analysis and Densitometry

Specific binding of ^{125}I-SI Ang II was quantitated essentially as previously described (Speth *et al*., 1999). Briefly, images of the autoradiograms were analyzed using AIS 6.0 software (Interfocus Imaging). The ^{125}I calibration standards included on the autoradiograms were used to construct a standard curve relating optometric density to ^{125}I, allowing quantitation of ^{125}I-SI Ang II binding to brain regions of interest. Specific binding was calculated by subtracting non-specific binding of the corresponding section from total AT_1 or non-AT_1, non-AT_2 binding. A thresholding technique was used to measure the DMM, enabling more accurate quantitation of this irregularly shaped brain region compared to the low level of binding surrounding it. The threshold was 500 fmol/g for the DMM and 0 fmol/g for CVLM and RVLM (as there is not a large amount of binding within these brainstem areas relative to the areas surrounding them). Measurement of binding density in the brain sections were made without knowledge of the group (SHR or WKY) of animals from which the sections were obtained. Values are expressed as density (fmoles/g wet weight).

Autoradiograms representing 2.4 mm rostral through 0.2 mm caudal to the calamus scriptorius were obtained. For AT_1 receptor binding, the DMM was measured between 0.2 mm caudal and 1.0 mm rostral to the calamus scriptorius. Rostral to 1.0 mm rostral to the calamus scriptorius the density of AT_1 receptor binding was considerably diminished and was not measured in this study. Densitometric values were obtained for the ventrolateral medulla between 0.2 mm and 2.4 mm rostral to the calamus scriptorius. The CVLM was measured between 0.2 mm and 1.0 mm rostral to the calamus scriptorius, whereas the RVLM was measured between 1.0 mm and 2.4 mm rostral to the calamus scriptorius. For the final analysis, five measurements corresponding to the peak of AT_1 receptor binding in each brainstem region were averaged.

For non-AT_1, non-AT_2 receptor binding, the DMM was measured between 0 mm caudal and 0.8 mm rostral to the calamus scriptorius. Densitometric values were obtained for the ventrolateral medulla between 0.2 mm and 2.2 mm rostral to the calamus scriptorius. The CVLM was measured between 0.2 mm and 0.8 mm rostral to the calamus scriptorius, and the RVLM was measured between 1.0 mm and 2.2 mm rostral to the calamus scriptorius. The basis for the size of the sampling area is described previously (Bourassa et al., 2010).

Statistics

AT_1 receptor and non-AT_1, non-AT_2 receptor densities for each brainstem region between WKY and SHR were compared using Student's t-test. $p < 0.05$ was considered statistically significant.

Research Highlights

- AT_1 angiotensin receptor binding in the RVLM and CVLM is increased in the SHR

- Expression of a novel non-AT_1, non-AT_2 Ang II binding site is lower in the NTS of SHR
- Expression of a novel non-AT_1, non-AT_2 Ang II binding site is lower in RVLM of SHR

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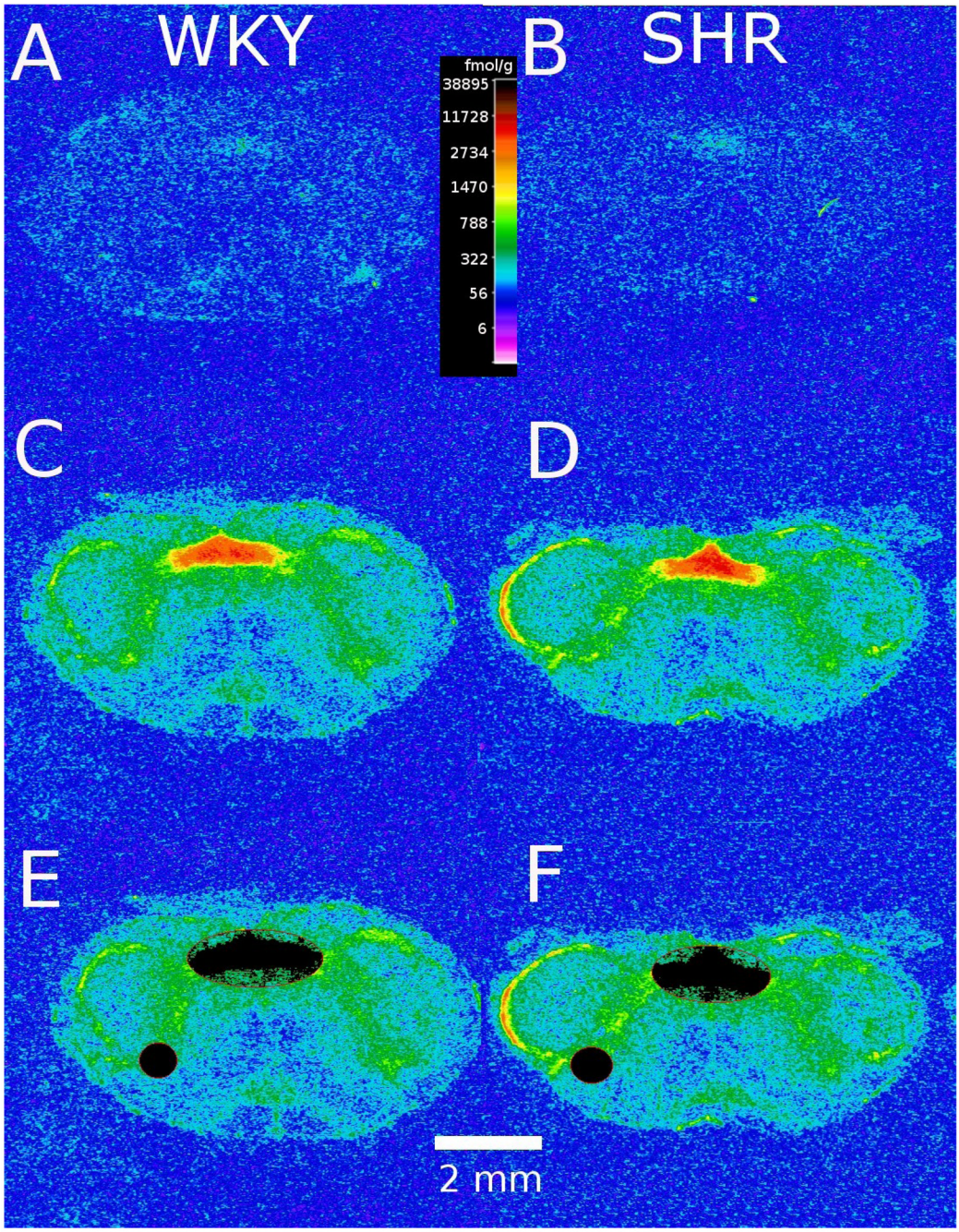

Figure 1.

Representative AT_1 receptor autoradiograms depicting DMM and CVLM (approximately 0.2 mm rostral to calamus scriptorius). Panels A and B depict non-specific binding in WKY and SHR, respectively. Panels C and D depict total binding in WKY and SHR, respectively. Panels E and F are the same as C and D except the areas surveyed as DMM (upper oval) and CVLM (lower circle) using the thresholding technique are shown. (Note: Total binding data was obtained by sampling bilaterally for all regions.)

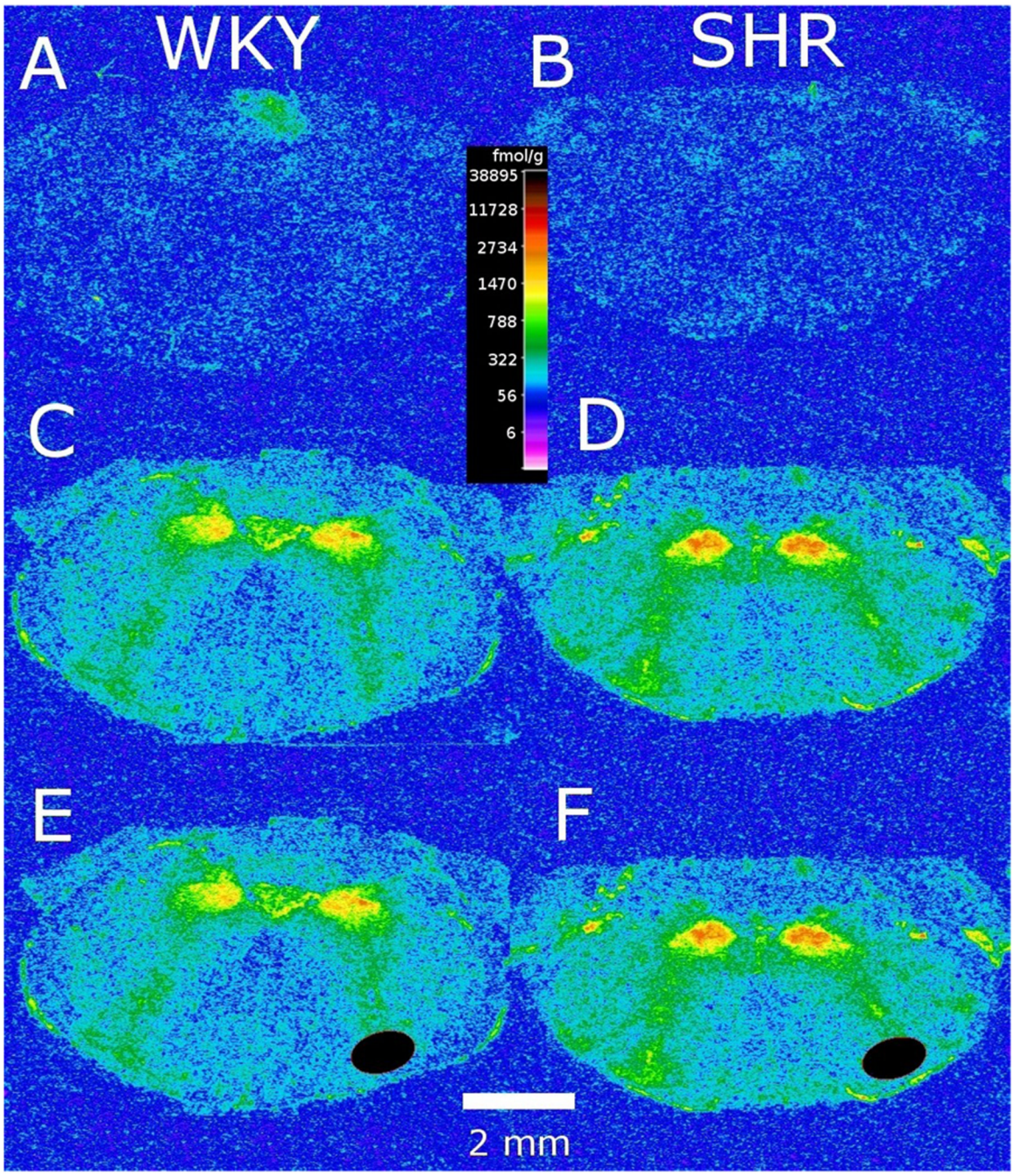

Figure 2.

Representative AT_1 receptor autoradiograms depicting RVLM (approximately 1.4 mm rostral to the calamus scriptorius). Panels A and B depict non-specific binding in WKY and SHR, respectively. Panels C and D depict total binding in WKY and SHR, respectively. Panels E and F are the same as C and D except the area surveyed as RVLM (lower circle) using the thresholding technique is shown.

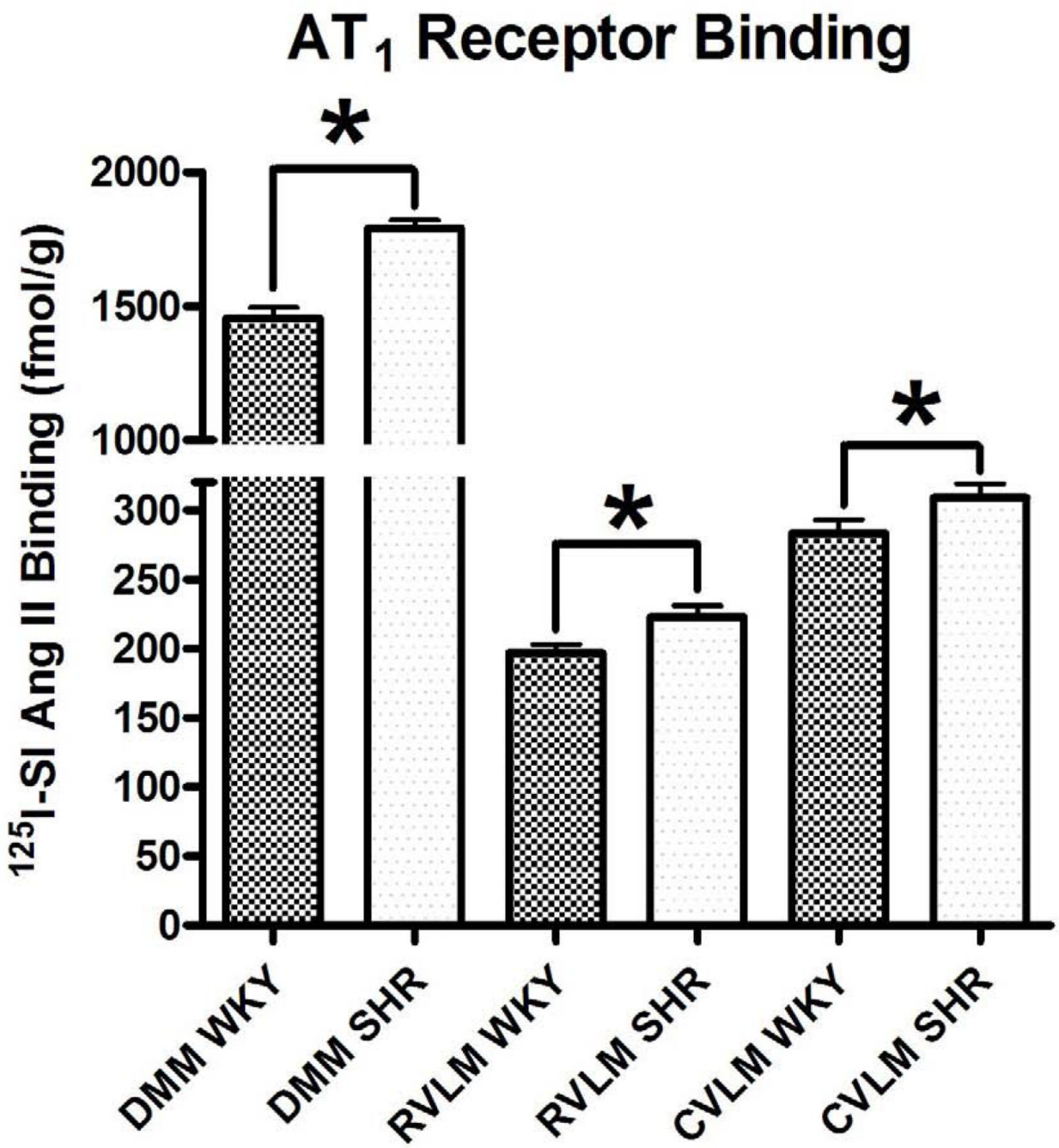

Figure 3.

Average (+/− SEM) specific AT_1 receptor binding in DMM, CVLM, and RVLM between WKY and SHR strains are shown. * indicates $p < 0.05$ by Student's t test.

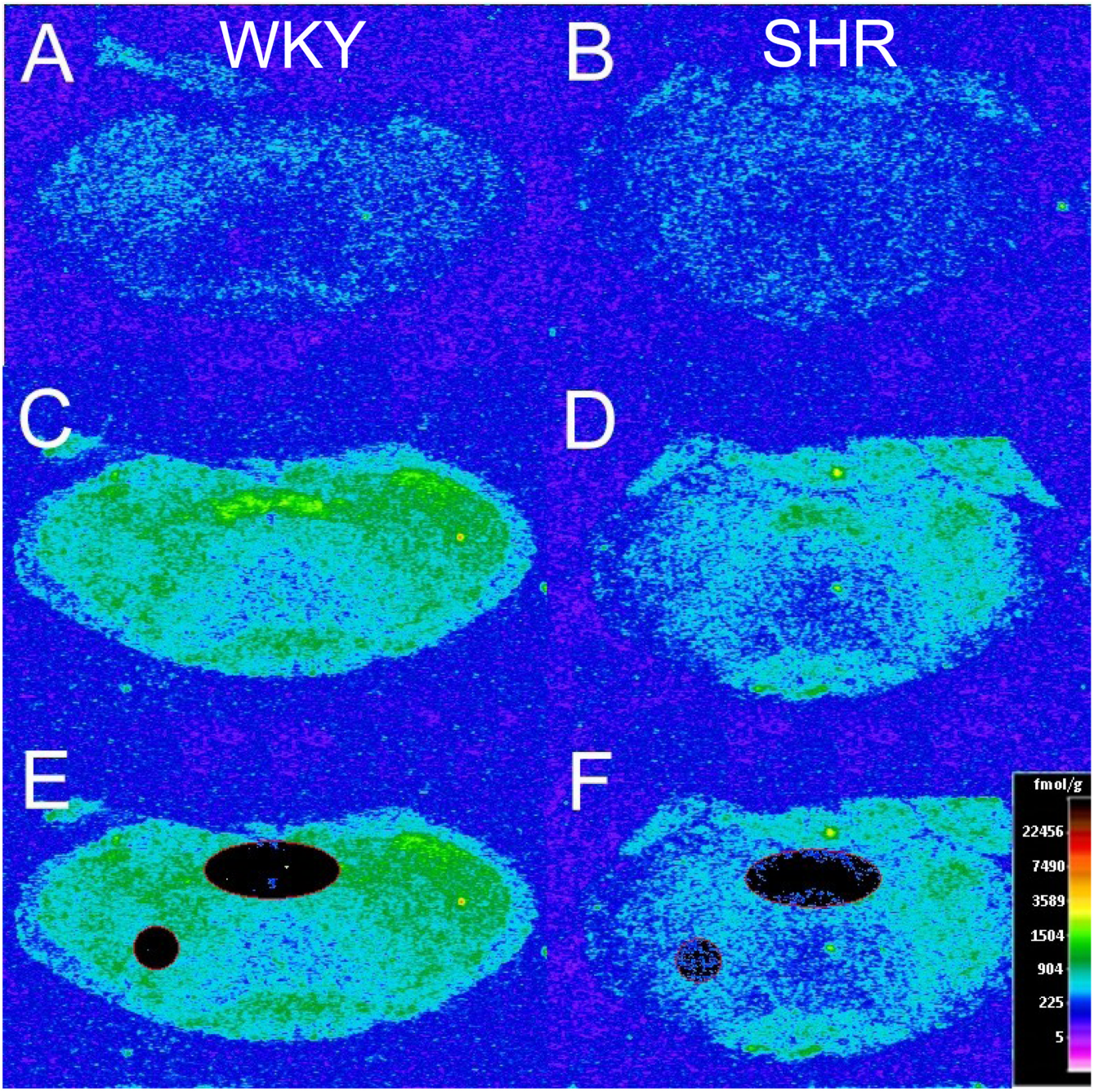

Figure 4.

Representative non-AT_1, non-AT_2 binding site autoradiograms depicting DMM and CVLM (approximately 0.4 mm rostral to the calamus scriptorius). Panels A and B depict non-specific binding in WKY and SHR, respectively. Panels C and D depict total binding in WKY and SHR, respectively. Panels E and F are the same as C and D except the areas surveyed as DMM (upper oval) and CVLM (lower circle) using the thresholding technique are shown.

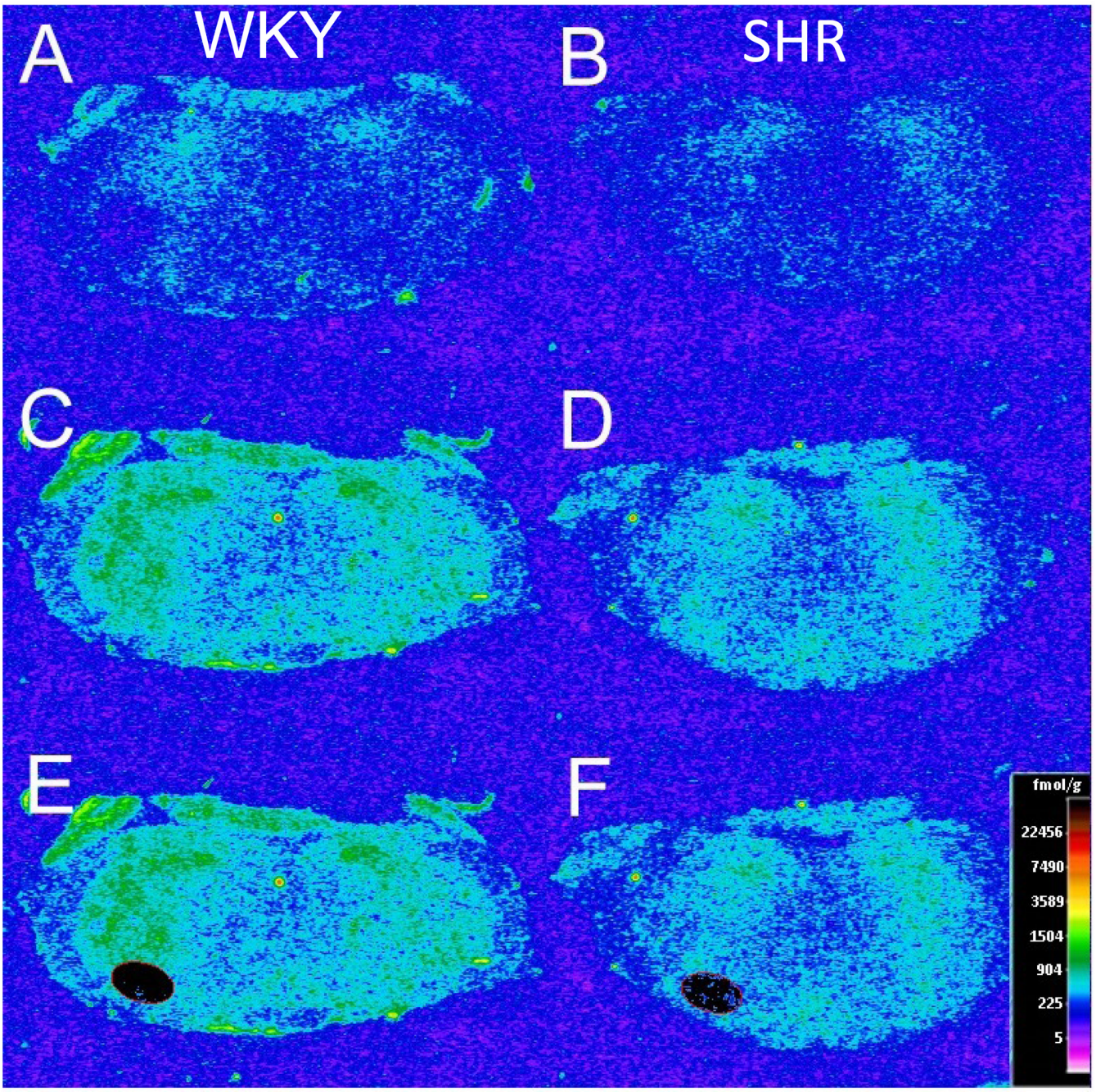

Figure 5.

Representative non-AT_1, non-AT_2 binding site autoradiograms depicting RVLM (approximately 1.6 mm rostral to the calamus scriptorius). Panels A and B depict non-specific binding in WKY and SHR, respectively. Panels C and D depict total binding in WKY and SHR, respectively. Panels E and F are the same as C and D except the area surveyed as RVLM using the thresholding technique is shown.

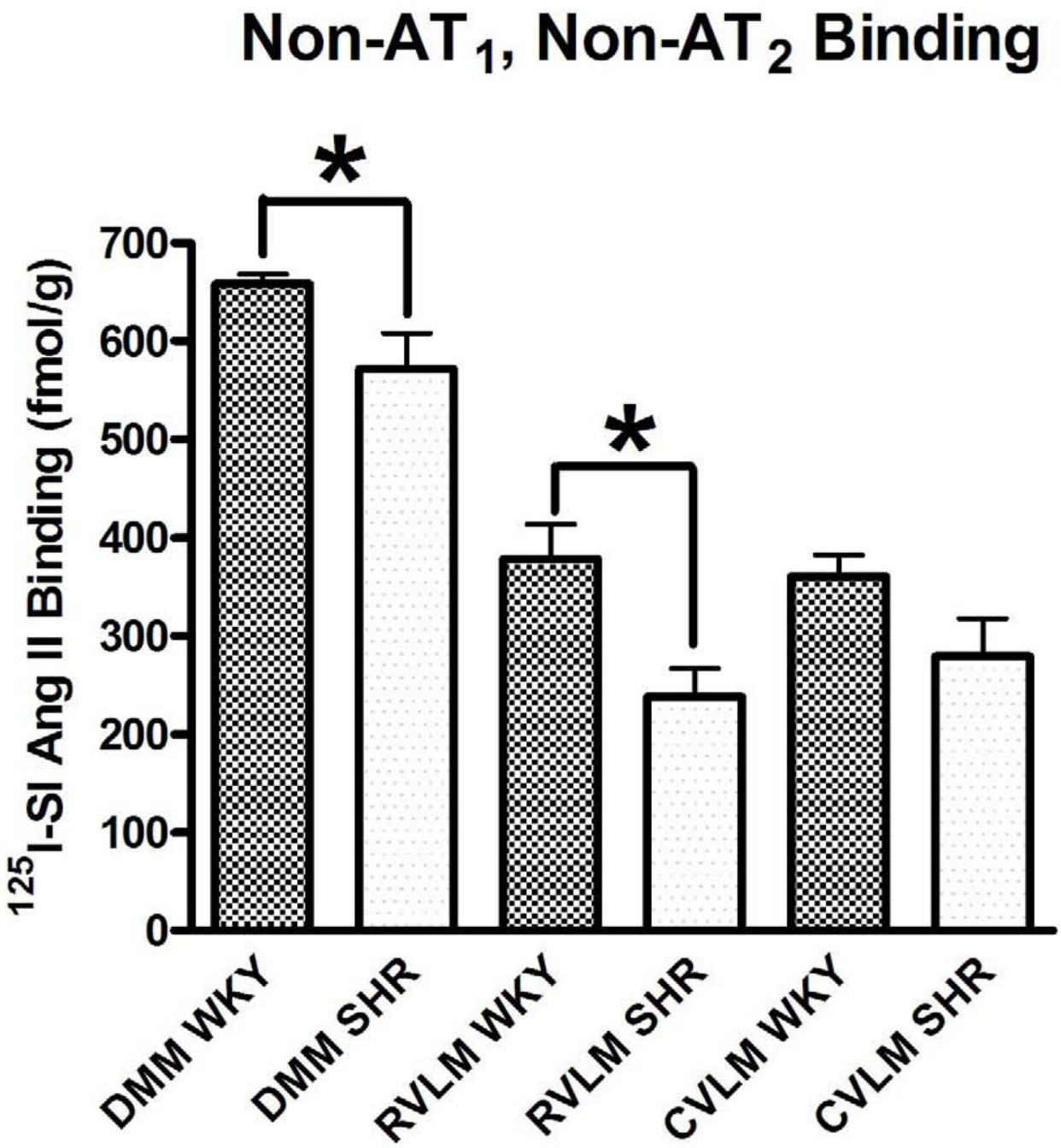

Figure 6.

Average (+/− SEM) specific non-AT_1, non-AT_2 binding in DMM, CVLM, and RVLM between WKY and SHR are shown. * indicates $p < 0.05$ by Student's t test.