Immunofluorescence localization of the receptor Mas in cardiovascular-related areas of the rat brain

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Becker I.K., Etelvino GM, Walther T, Santos RAS, Campagnole-Santos MJ. Immunofluorescence localization of the receptor Mas in cardiovascular-related areas of the rat brain. Am J Physiol Heart Circ Physiol 293: H1416–H1424, 2007. First published May 11, 2007; doi:10.1152/ajpheart.00141.2007.—The G protein-coupled receptor Mas was recently described as an angiotensin-(1–7) [ANG-(1–7)] receptor. In the present study we evaluated the anatomical localization of Mas using immunofluorescence in the central nervous system of adult male Wistar rats. An abundant labeling was found in the hippocampus, amygdala, anterodorsal thalamic nucleus, cortex, and hypoglossal nucleus. More importantly, a dense ANG-(1–7) receptor Mas immunoreactivity was observed in cardiovascular-related areas of the medulla and forebrain, shown in several previous studies as sites for the action of ANG-(1–7) in the brain. A strong staining was found in the nucleus of the solitary tract, caudal and rostral ventrolateral medulla, inferior olivary, parvo and magnocellular portions of the paraventricular hypothalamic nucleus, supraoptic nucleus, and lateral preoptic area. Furthermore, Mas staining was predominantly present in neurons. At the medullary sites, a specific and high-intensity binding for rhodamine-ANG-(1–7) was also shown. The specific ANG-(1–7) binding was completely displaced by the anti-Mas antibody or by the ANG-(1–7) antagonist, A-779. The data presented provide the first anatomical basis for the physiological role of ANG-(1–7)/Mas axis in the modulation of different cardiovascular functions and give new insights for clarifying the role of ANG-(1–7) in the central nervous system.

angiotensin-(1–7), medulla; hypothalamus

EMERGING EVIDENCE SUGGESTS that several angiotensin (ANG) II actions can be counterbalanced by its congener, ANG-(1–7), which may provide a more precise regulation of the physiological functions of the renin-angiotensin system (29, 30). The physiological role of ANG-(1–7) was more firmly established by two recent discoveries: 1) the identification of angiotensin-converting enzyme-2, an enzyme that generates ANG(1–7) from ANG I or ANG II (11, 12, 36, 37) and 2) the characterization of the G protein-coupled receptor (GPCR) Mas as a receptor that is associated to several ANG-(1–7) actions (32). Mas was identified as an ANG-(1–7) receptor in experiments showing that ANG-(1–7) does not bind to the kidney of Mas-knockout mice (32). Moreover, Mas-deficient mice completely lack the antidiuretic action of ANG-(1–7) after an acute water overload, and ANG-(1–7) does not induce a relaxation response in the aorta of these animals (32). In contrast, Mas-knockout mice preserve the antidiuretic response to vasopressin and the binding of ANG II to ANG II type 1 (AT1) and type 2 (AT2) receptors and of ANG IV to ANG II type 4 (AT4) receptors in the kidney. In addition, ANG-(1–7) binds to Mas-transfected Chinese hamster ovary (CHO) cells, and in Mas-transfected COS or CHO cells, ANG-(1–7) elicits arachidonic acid release that was completely abolished by the Mas receptor antagonist D-Ala7-ANG-(1–7), A-779, and not affected by AT1 or AT2 receptor antagonists (32). In these and subsequent studies, Mas was shown to mediate most of the known peripheral actions of ANG-(1–7), including antidiuresis (26, 32) vasodilatation (22) improvement of endothelial function (9, 13) and its antifibrotic effect (14).

In the central nervous system, ANG-(1–7) acts as an important neuromodulator, especially in those areas related to tonic and reflex control of arterial pressure, in the hypothalamus and in the dorsomedial and ventrolateral medulla (30). At these sites, the cardiovascular effects induced by ANG-(1–7) are blocked by A-779 (30, 31), suggesting that in the brain, ANG-(1–7) actions may be mediated by the interaction with the GPCR Mas. Previous studies have described high levels of Mas mRNA in the forebrain areas, including hippocampus, cortex, and olfactory bulb of the rat and mouse (6, 24). Low amounts of Mas mRNA were also detected in the medulla oblongata using RNase protection assay (24). The characterization of Mas as an ANG-(1–7) receptor prompted us to evaluate whether Mas is present in cardiovascular-related areas in which biological effects of ANG-(1–7) have been described. Therefore, we determined the distribution of the GPCR Mas protein in the rat brain using immunofluorescence.

METHODS

Animals. Wistar rats (300–350 g) were obtained from the animal facilities (Centro de Bioterismo) of the Biological Sciences Institute of the Federal University of Minas Gerais (UFMG, Minas Gerais, Brazil). All experiments were approved by and performed in accordance with the guidelines established by our Institutional Animal Welfare Committee (Comite de Ética em Experimentação Animal, UFMG). The animals were kept in a temperature-controlled room on a 14-h:10-h light-dark cycle (lights on at 6:00 AM) with free access to water and food.

Antisera. Polyclonal antisera directed against Mas was produced in Mas-knockout mice (40) using as antigen, a 12 amino acids peptide (LAEEKAMNTSSR) corresponding to the NH2-terminal domain of the mouse Mas protein. This sequence has 100% homology with......
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mouse and 91.6% homology with rat Mas, and it is not present in any other known protein (see Fasta protein database, www.ebi.ac.uk/ fasta33). Mas-knockout mice were submitted to three injections of the peptide conjugated to keyhole limpet hemocyanin by glutaraldehyde at 15-day intervals each. Just after the third injection, the antibody titer was determined by ELISA. The mice were next inoculated intraperitoneally with Ehrlich tumor cells. After 3–5 days, the peritoneal liquid was removed and centrifuged and the titer of the antibody was determined in the supernatant by ELISA. This antibody was used in a recent study in which an anti-human Mas commercially available antibody was also used for comparison (28a). To further document the specificity of this antibody, Western blot analyses of brain samples were performed.

Western blot analysis was performed using a modification of a previously described protocol (28a). Briefly, four rats were euthanized, the brains were quickly removed, and the medulla and hypothalamus were dissected and homogenized in 2 ml of Tris-acetate buffer (50 mM, pH 7.4) containing (in μM) 1 leupeptin, 1 aprotinin, and 1 pepstatin and 1 mM phenylmethylsulphonyl fluoride (PMSF). For a positive control, rat testis, which was previously shown to express Mas (24), was processed in parallel. The solubilized protein (50 μg for the brain and testis) was separated by electrophoresis in 10% SDS-PAGE and transferred to nitrocellulose membranes. Non-specific binding was blocked by the incubation of the membrane with 5% skimmed milk in Tris-base buffer (0.2 M, pH 7.4) containing 0.1% Tween 20. Membranes were incubated with the Mas antibody (1:500) overnight at 4°C, followed by incubation with horseradish peroxidase conjugated with secondary antibody (1:2,000) during 1 h at room temperature. Immunoactivity bands were visualized by chemiluminescence. As can be seen in the Fig. 1, brain tissue (medulla and hypothalamus) and testis gave a single protein band of 33 kDa, equivalent to the Mas protein molecular mass. Actually, the protein band appears as a doublet, which may represent different degrees of glycosylation since Mas possess putative glycosylation sites (see Fasta protein database).

Immunofluorescence staining procedure. The immunostaining protocol used was modified from Block et al. (4). Briefly, rats (n = 11) were euthanized with an overdose of tribromoethanol and were perfused transcardially with PBS (0.02 M, pH 7.4) for 2 min followed by 10% paraformaldehyde in PBS (0.02 M, pH 7.4) for 2 h and then placed in 30% sucrose solution overnight. Serial coronal sections (30 μm) of the brain were then made in a cryostat. Free-floating sections were hydrated with PBS (0.02 M, pH 7.4) and permeabilized with 0.2% Tween 20, and the nonspecific staining was blocked with 5% bovine serum albumin (BSA), each one for 15 min. Sequentially, the tissues were incubated with a mouse anti-Mas primary antibody (1:500) at 4°C during 24 h, for the medulla, and 48 h for the rest of the brain. The sections were then incubated with an Alexa 594-labeled anti-mouse secondary antibody during 1 h at room temperature. After incubation, sections were washed with PBS (0.02 M, pH 7.4) and were mounted onto chromo-alum-coated slides, air dried, and coverslipped with glycerol-PBS mounting media (1:3).

The immunostaining specificity was characterized by preincubating the antiserum with 30 or 50 μg of the immunogenic peptide and staining the brain tissue as described above.

To identify whether Mas was present in neurons, in additional experiments brain sections were simultaneously stained with Mas (1:500); a neuronal marker, anti-Neu-N-Alexa fluor 488 conjugated (1:1,000); and the nuclear marker, Draq-5 (1:200) in the same conditions as described above.

ANG-(1–7) binding. ANG-(1–7) fluorescent binding in the rat medulla was obtained using rhodamine (Rho)-ANG-(1–7). Rats (n = 8) were euthanized by decapitation, and the brain was removed and frozen on dry ice (or liquid nitrogen) and kept in a –80°C freezer. Serial coronal sections (30 μm) of the medulla were then made in a cryostat, and adjacent sections were mounted onto separated 1%-gelatinized slides and dried at 4°C. All the slides were incubated in phosphate-buffered saline (PBS, 0.02 M, pH 7.4) containing 5% BSA (5%) and 0.005% bacitracin for 15 min at 4°C. The total binding sections were next incubated in the same assay buffer described above with the addition of 0.001% PMSF, 0.005% orthophenandalinone, and 10–5 M enalaprilat. The nonspecific binding sections were incubated for 15 min at 4°C in the same assay buffer in the presence of ANG-(1–7). Other sections were incubated with Mas antibody (1:500) or the ANG-(1–7) antagonist A-779 at a concentration of 10–5 M. Subsequently, all sections were incubated with Rho-ANG-(1–7) in assay buffer for 1 h at 4°C. The nonspecific binding sections were then incubated with Rho-ANG-(1–7) in the presence of ANG-(1–7) (10–5 M). The sections were then rinsed (3 times for 1 min in assay buffer), dried under a stream of air at room temperature, and coverslipped with glycerol-PBS mounting media (1:3).

ANG-(1–7) assay stability. To evaluate the stability of ANG-(1–7) in the presence of the anti-Mas antibody assay condition, 125I-labeled ANG-(1–7) (750 nCi) (26) was incubated for 1 h alone or in the presence of Mas antibody (1:500) and subsequently extracted using phenyl bond-elut cartridges (Analytichem International). The samples were dried at 45°C until residue and resuspended in 150 μl of aqueous solution containing 0.13% heptafluorobuturic acid (HFBA, mobile phase A) and 0.13% HFBA-80% acetonitrile in water (mobile phase B), corresponding to 31% of mobile phase B. The solution was filtered (0.45 μm, Costar) and injected into a high-performance liquid chromatography system (Shimadzu), equipped with SLC-6B gradient system and with a Nova Pack C18 column (3.9 × 150 mm, particle size 4 μm). The experimental condition was as follows: mobile phase A, 0.13% HFBA; mobile phase B, 0.13% HFBA-80% acetonitrile; and flow rate, 1.0 ml/min. ANG-(1–7) was separated with the following gradient: 31% to 41% mobile phase B during 30 min, 41.1% to 60% mobile phase B during 30.1 to 40 min, and 60% to 31% mobile phase B during 40.1 to 50 min. Fractions of 1 ml were collected and counted in a gamma counter. Incubation of 125I-labeled ANG-(1–7) with the anti-Mas mouse serum in the assay conditions used did not result in any apparent hydrolysis, only one peak corresponding to tubes 18–21 was observed before or after incubation with Mas antibody in the binding assay condition (data not shown).

Fig. 1. Western immunoblot analysis of Mas receptor expression in the medulla and hypothalamus. Testis was used as positive control. A protein band of 33 kDa, equivalent to the Mas protein molecular mass, can be seen.
Fig. 2. Illustrative images (×10) show receptor Mas immunofluorescence in different areas of the medulla: nucleus tractus solitarius and dorsal motor nucleus of the vagus complex (NTS/nX; A1), caudal ventrolateral medulla (CVLM; B1), inferior olive (IO; C1), and rostral ventrolateral medulla (RVLM; D1). Staining disappeared in adjacent coronal sections preincubated with 30–50 μg of the synthetic immunogen (A2–D2, D3) or incubated without secondary antibody (D4). Scale bar = 100 μm.

Fig. 3. Higher magnification (×40) images show receptor Mas immunofluorescence in medullary areas: RVLM (left), IO (middle), and CVLM (right). Scale bar = 50 μm.
Image analyses. Fluorescent images were obtained using confocal microscopy (Zeiss Confocal LSM 510). Specific laser and channels for excitation and emission of the fluorescence were used, as appropriate for each fluorescent compound. The control and stained images were performed in adjacent slices, and the configuration of the scan was kept the same for both slices. The images were exported to Microsoft PowerPoint software (Windows XP platform) and edited. Some figures were built from 2–8 superimposed captures of ×10 magnification of one section. Adjacent slices were collected for conventional histology and stained with neutral red (1%) for the identification of the different brain areas.

Drugs and reagents. ANG-(1–7) and α-Ala²-ANG-(1–7), A779, were from Bachem Holding; horseradish peroxidase conjugated with secondary antibody was from Pierce Biotechnology; the fluorescent secondary antibody, Alexa 594, was from Invitrogen-Molecular Probes; Rho-ANG-(1–7) was from Phoenix Peptides (USA); neuronal specific nuclear protein antibody conjugated to Alexa 488, anti-Neu-N, was from Chemicon; and the nuclear marker, Draq5, was from Bio-Status. All other reagents were commercial products of the highest available grade of purity.

RESULTS

The immunofluorescence for the Mas receptor was found in different areas of the rat brain, mainly in those areas of the medulla and forebrain related to the cardiovascular and hydro-electrolyte controls. In addition, the presence and characteristics of the staining in the different areas of the brain were very similar in all animals studied. In the medulla, the staining pattern was documented in relation to the rostrocaudal subdivisions of the nucleus tractus solitarius (NTS). An intense Mas-positive immunofluorescence was found in the cardiovascular-related areas, such as the caudal ventrolateral medulla (CVLM), the rostral ventrolateral medulla (RVLM), the NTS, the dorsal motor nucleus of the vagus (nX), and the inferior olive (IO), as illustrated in Fig. 2. The images in Fig. 2 represent the more intense staining of each area; however, the staining pattern was not substantially different throughout the rostrocaudal axis of all these areas. It is important to emphasize that the staining was completely eliminated by preabsorption of the antiserum with 30–50 μg of the synthetic immunogen (A2–D2). In addition, no labeling could be seen when the tissue was incubated only with the primary antibody (antibody autofluorescence) as illustrated in Fig. 2, D4.

Figure 3 presents, in higher magnification (×40), the Mas-positive cells in the IO and in the RVLM and CVLM. As can be seen in these higher magnification images, the staining extends for the whole cell body and, in some cells, also to the...
dendrites. It appears that the cell nucleus remained immunonegative.

In the forebrain, Mas-positive cells were also more strongly concentrated in the areas related to cardiovascular control and hydroelectrolyte balance control. Figure 4 presents the more intense labeling for the paraventricular hypothalamic nucleus (PVN), the supraoptic nucleus (SO), and the preoptic area (PO). An intense staining for Mas was observed in the SO and PO in all rostrocaudal axes. Figure 5 presents the Mas-positive cells in the PVN and in the SO in higher magnification (×40). Mas immunoreactivity in the PVN was observed in both subdivision, magnocellular, and parvocellular nucleus. The cell nucleus in the forebrain areas also appears to remain immunonegative and clear.

Fig. 6. Illustrative images show receptor Mas immunofluorescence in areas not related to cardiovascular control: anterodorsal thalamic nucleus (Ad; top, left), basomedial and basolateral amygdaloid nucleus (Amg; top, right), hippocampal nucleus (HC; bottom, left), in hindlimb area of the frontal cortex (Co; bottom, middle), and in hypoglossal nucleus (nXII; bottom, right). Images are a composite of 2–6 captures of adjacent regions of 1 section in ×10 magnification and were edited with Microsoft PowerPoint software. Scale bar = 100 µm.

Fig. 7. Colocalization of receptor Mas in neuronal cells of the ventrolateral medulla. The neuron marker, anti-Neu-N, is shown in green (A), the anti-Mas is shown in red (B), and the nuclear marker, Draq-5, in blue (C). Image shown in C represents the overlay, and the image in D is an enlargement of a neuron shown in C. Scale bar = 50 µm.
Mas immunofluorescence was also found in other areas not directly related with cardiovascular control or hydroelectrolite balance (Fig. 6), including the hippocampal nucleus, different subregions of the frontal cortex, anterodorsal thalamic nucleus, basomedial and basolateral amygdaloid nucleus, and hypoglossal nucleus (nXII).

To verify in which cellular type the Mas immunostaining was present, we have incubated medullary sections of the brain with anti-Neu-N, a neuronal marker, and the fluorescent compound Draq5, a nuclear marker, simultaneously with the Mas antibody. As illustrated in Fig. 7, the Mas immunoreactivity was mainly observed in neuronal cells.

Figure 8 summarizes in diagrams, based on the atlas of Paxinos and Watson (25), the localization of the immunoreactivity for the receptor Mas in different areas of the rat brain.

Because only low expression of Mas mRNA in the medulla has been reported in a previous study (24), we next tested whether the anti-Mas antibody could displace the Rho-ANG-(1–7) binding in this region. As shown in Fig. 9, binding of Rho-ANG-(1–7) was found in different areas of the medulla, especially in the CVLM, RVLM, and NTS/nX complex. In addition, the total Rho-ANG-(1–7) binding was greatly displaced by unlabeled ANG-(1–7) and by the ANG-(1–7) receptor antagonist, A-779, and was totally displaced by the anti-Mas antibody, suggesting that ANG-(1–7) binds to Mas receptor in the medulla.

DISCUSSION

In the present study, using a specific polyclonal antibody combined with immunofluorescence, we showed, for the first time, the presence of the GPCR Mas in specific areas of the brain, including many important cardiovascular-related sites of the forebrain and medulla. In addition, our data suggest that the Mas receptor is mainly expressed in neurons. Our results extended previous observations that showed Mas mRNA, using in situ hybridization with radio-

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Fig. 8. Diagrams of frontal sections of the brain from the atlas of Paxinos and Watson (Ref. 25) at different levels (number on diagrams) showing the localization (shaded area) of Mas immunoreactivity into different areas of the rat brain as observed in all animals studied. 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus; AD, anterodorsal thalamic nucleus; Amb, ambiguous nucleus; Amb, amygdala; AP, area postrema; CVL, caudal ventrolateral reticular nucleus; LPO, lateral PO; 4V, 4th ventricle; LV, lateral ventricle; py, pyramidal tract; RVL, rostro ventrolateral reticular nucleus; LPO, lateral preoptic area.
labeled Mas cRNA probes, in forebrain areas, such as the hippocampus and limbic system (6, 24).

The presence of the ANG-(1–7) receptor Mas in cardiovascular-related areas of the rat medulla is in keeping with several studies describing the biological effects of this heptapeptide in the same regions (29, 30). The observation that the specific binding of Rho-labeled ANG-(1–7) to these brain nuclei was completely displaced by the anti-Mas antibody strongly supports the specificity of the immunoreactive labeling. Mas mRNA was also detected in a previous study, in the medulla, although low levels were reported (24).

ANG-(1–7) induces changes in blood pressure when microinjected in many brain areas, including the NTS (hypotension and bradycardia) (7), RVLM (pressor effect) (15), and the CVLM (hypotension) (1). The cardiovascular effects elicited by ANG-(1–7) at these brain regions were abolished by its selective antagonist, A-779 (1, 15, 31), and not affected by AT1 or AT2 receptor antagonists, which suggested that these effects were elicited by a selective angiotensin receptor. In addition to its effect on baseline pressure, it has been shown that, in opposition to the inhibitory effect of ANG II, ANG-(1–7) facilitates the baroreflex control of heart rate, either after lateral ventricle infusion (8, 19) or microinjection into the NTS (10). The selective effect of ANG-(1–7) on the baroreflex modulation gave a further support for the evidence that ANG-(1–7) actions also in the brain were mediated by a distinct receptor, different from AT1 or AT2. The identification of Mas immunoreactivity in medullary areas involved in cardiovascular control, in which ANG-(1–7) produces biological effects, provides substantial evidence that the mechanism of the action of ANG-(1–7), in brain areas involved in cardiovascular regulation, involves its newly described receptor.

It should be pointed out that it has been reported in some instances the central and peripheral effects of ANG-(1–7) can be blocked or attenuated by AT1 or AT2 receptor antagonists (2, 16, 17, 27). Whether these results represent interference with Mas-mediated intracellular mechanisms or a true physical competition at these binding sites or, yet, are due to a distinct binding site, as recently proposed by us (35), remains to be clarified.

Indeed, in the present study we described for the first time the binding of Rho-ANG-(1–7) to medullary areas, such as the CVLM, RVLM, and NTS, which correspond to the areas where immunofluorescent Mas receptor and ANG-(1–7) actions were identified. The blockade of the Rho-ANG-(1–7) binding by the Mas antibody and A-779 are further evidence for Mas as a mediator of the actions of ANG-(1–7) in the brain.

Our data showing Mas staining in the hypothalamic areas are in agreement with those reported by Block et al. (4), which showed intense immunoreactivity staining for ANG-(1–7) in the paraventricular, supraoptic, and suprachiasmatic nuclei. The presence of the peptide and its receptor in close proximity is consistent with the role of ANG-(1–7) on the modulation of cardiovascular and neuroendocrine function. ANG-(1–7) was found to be a potent stimulator of vasopressin release from rat hypothalamic-neurohypophysial explants (33). In addition, it has been shown that ANG-(1–7) at the paraventricular hypothalamic nucleus can increase the fire rate of neuronal cells (3) and increases renal sympathetic nerve activity (34). These effects are blocked by the selective receptor Mas antagonist, A-779 (3, 34).

We have also detected the presence of the GPCR Mas in areas apparently not directly related to cardiovascular function, such as in the amygdala, hypopcampal nucleus, and different areas of the cortex. These results are in keeping with previous studies showing Mas mRNA expression in limbic, thalamic, and cortical structures (6, 24). It has been shown that the deletion of Mas leads to an increased durability of long-term potentiation in the dentate gyrus, without affecting hippocampal morphology, basal synaptic transmission, and presynaptic
function (40). In addition, ANG II administration into the amygdala leads to an AT₁ receptor-mediated decrease in the amplitude of the field potentials in the Mas-knockout mice, whereas, in normal mice, there was an increase in the amplitude of the field potentials (38). The identification of Mas in these forebrain areas is consistent with a role of this receptor in synaptic plasticity and behavior and with the observation that, centrally, ANG-(1–7) increases learning, memory, and motility behavior (20). Furthermore, Hellner et al. (18) have recently reported that ANG-(1–7) enhances long-term potentiation in the CA1 region of the hippocampus and that this effect was abolished in Mas-deficient mice, suggesting a role for ANG-(1–7)/Mas axis on learning and memory mechanisms.

Although in the present study immunolocalization of ANG-(1–7) in the brain was not performed, many studies have shown the presence of the angiotensin peptides precursor, angiotensigen (5, 23, 28), in different brain areas where Mas mRNA (6, 24) or protein (present study) are present. In a recent and elegant study by Lavoie et al. (21), expressions of renin and angiotensinogen were observed in adjacent cells of brain areas where we now report the presence of Mas, including cardiovascular-related areas, such as NTS and the RVLM, and noncardiovascular-related areas, such as hypocampus, cortex, and amigdala. Thus it is very likely that ANG-(1–7) can be formed and/or released in close proximity to Mas receptor expression. Future studies will be necessary to evaluate this possibility.

In summary, the present study shows for the first time the presence of the ANG-(1–7) receptor Mas in cardiovascular and hydroelectrolyte control areas of the rat brain, providing a clearer morphological basis for the ANG-(1–7) effects in these regions. In addition, this study provides new insights for other possible physiological roles of the ANG-(1–7)/Mas axis in other brain regions.

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