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Identification of Membrane-bound Variant of Metalloendopeptidase Neurolysin (EC 3.4.24.16) as the Non-angiotensin Type 1 (Non-AT₁), Non-AT₂ Angiotensin Binding Site^{*S}

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Background: Angiotensin II, the renin-angiotensin system effector peptide, interacts with a recently discovered binding site that is distinctly different from its classic receptors.

Results: A radioiodinated angiotensin II photoprobe bound to a ~75-kDa membrane protein, enabling its isolation and identification.

Conclusion: Membrane-bound metalloendopeptidase neurolysin (EC 3.4.24.16) is the novel angiotensin-binding protein.

Significance: This metalloendopeptidase may be a crucial component of the renin-angiotensin system.

Recently, we discovered a novel non-angiotensin type 1 (non-AT₁), non-AT₂ angiotensin binding site in rodent and human brain membranes, which is distinctly different from angiotensin receptors and key proteases processing angiotensins. It is hypothesized to be a new member of the renin-angiotensin system. This study was designed to isolate and identify this novel angiotensin binding site. An angiotensin analog, photoaffinity probe ¹²⁵I-SBpa-Ang II, was used to specifically label the non-AT₁, non-AT₂ angiotensin binding site in mouse forebrain membranes, followed by a two-step purification procedure based on the molecular size and isoelectric point of the photoradiolabeled binding protein. Purified samples were subjected to two-dimensional gel electrophoresis followed by mass spectrometry identification of proteins in the two-dimensional gel sections containing radioactivity. LC-MS/MS analysis revealed eight protein candidates, of which the four most abundant were immunoprecipitated after photoradiolabeling. Immunoprecipitation studies indicated that the angiotensin binding site might be the membrane-bound variant of metalloendopeptidase neurolysin (EC 3.4.24.16). To verify these observations, radioligand binding and photoradiolabeling experiments were conducted in membrane preparations of HEK293 cells overexpressing mouse neurolysin or thimet oligopeptidase (EC 3.4.24.15), a closely related metalloendopeptidase of the same family. These experiments also identified neurolysin as the non-AT₁, non-AT₂ ang-

iotensin binding site. Finally, brain membranes of mice lacking neurolysin were nearly devoid of the non-AT₁, non-AT₂ angiotensin binding site, further establishing membrane-bound neurolysin as the binding site. Future studies will focus on the functional significance of this highly specific, high affinity interaction between neurolysin and angiotensins.

The renin-angiotensin system (RAS),² one of the phylogenetically older hormonal systems, is most known for its essential role in regulation of hydromineral and cardiovascular homeostasis (1). The system was introduced to the scientific community by the discovery of renin, the main enzyme generating effector peptides of RAS, by Tigerstedt and Bergmann in 1898 (2). With the discovery of the existence of the two major angiotensin II (Ang II) receptor subtypes (AT₁ and AT₂) in the late 1980s, most of the questions related to the biochemical, pharmacological, and physiological features of the RAS seemed to be resolved (3). Based upon extensive studies of the RAS for over a century, three classes of drugs targeting different levels of the system (angiotensin-converting enzyme inhibitors (-prils), angiotensin receptor blockers (-sartans), and renin inhibitors (-kirens)) are in worldwide clinical use. However, new discov-

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[§] This article contains [supplemental Table 1 and Figs. 1–4](#).

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² The abbreviations and trivial names used are: RAS, renin-angiotensin system; Ang II, angiotensin II; AT₁ and AT₂, angiotensin type 1 and 2, respectively; SBpa-Ang II, sarcosine¹-*p*-benzoyl-L-phenylalanine⁸-angiotensin II; SI-Ang II, sarcosine¹-isoleucine⁸-angiotensin II; PCMB, *p*-chloromercuribenzoate; NS, nonspecific; Tot, total; P10 postnatal day 10; WT, wild type; KO, knockout; ZD7155, 5,7-diethyl-3,4-dihydro-1-[[2'-(1H-tetrazol-5-yl)](1,1'-biphenyl)-4-yl]methyl]-1,6-naphthyridin-2(1H)-one hydrochloride; PD123319, 1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate.

eries extending fundamental aspects of RAS continue to occur. Among such discoveries are agonistic autoantibodies against angiotensin type 1 receptor (4), the existence of a homolog of angiotensin-converting enzyme (ACE2) (5, 6), the novel role of (pro)renin acting at a specific receptor (7), physiologically important actions of angiotensin 1–7 and angiotensin IV through their specific receptors (8–10), the finding of angiotensin 1–12 as an alternative precursor of effector peptides of the RAS (11), and the presence of a physiologically relevant intracellular RAS (12–14).

Recently, in the course of radioligand receptor binding studies of brain angiotensin receptors, we discovered a novel, non-AT₁, non-AT₂ angiotensin binding site in rat brain membranes (15), which was also confirmed in mouse and human brain membranes (16, 17). The non-AT₁, non-AT₂ binding site has nanomolar affinity and high specificity for angiotensins I, II, and III and is pharmacologically, biochemically, and anatomically different from classical angiotensin type 1 (AT₁) and type 2 (AT₂) receptors, G-protein coupled Mas receptor, and nepri-lysin (EC 3.4.24.11, also known as neutral endopeptidase), a protease involved in the processing of angiotensins (15–19). Increased expression of the novel binding site was strongly associated with neuronal cell death following oxidative stress (20), whereas it is reduced in spontaneously hypertensive rat brains (21). However, little is known about the functional significance of these changes and molecular mechanisms of their potential effects. A unique feature of the novel angiotensin binding site is that it is unmasked (*i.e.* attains high affinity for angiotensins) in the presence of optimal concentrations of organomercurial sulfhydryl reagents *p*-chloromercuribenzoate (PCMB) or *p*-chloromercuribenzenesulfonate (15, 20). This effect is reversed by glutathione and disulfide-reducing agents, suggesting the involvement of cysteine residues (thiol groups) in unmasking and function of the binding site, with a potential role of extracellular redox state in regulation of the function of this protein (15, 20).

The purpose of this study was to identify the non-AT₁, non-AT₂ angiotensin binding site. Here we report experiments involving photoradiolabeling, stepwise electrophoretic, and mass spectroscopic methods, followed by molecular-genetic and receptor pharmacological approaches used to isolate, characterize, and establish the identity of the binding protein.

EXPERIMENTAL PROCEDURES

Animals—Postnatal day 10 (P10) mouse brains were collected from in-house-born pups of both genders from timed pregnant CD-1 female mice (Charles River Laboratories) and stored at -80°C until their use. Animals were maintained in 12-h light/dark cycle and fed *ad libitum*. All procedures were carried out according to a protocol approved by the Texas Tech University Health Sciences Center institutional animal care and use committee (IACUC). The choice of P10 mouse forebrains as a source for isolation and identification of the non-AT₁, non-AT₂ angiotensin binding site was based on our recent observation indicating ~ 5 -fold higher density of the binding site in P10 compared with adult mouse forebrain membranes (20).

Neurolysin (EC 3.4.24.16) knock-out mice were generated using the commercially available embryonic stem cell line

NPX481 (Baygenomics®). A detailed description of the generation and characterization of these and wild-type mice will be published elsewhere.

Cell Culture and Transfections—HEK293 (human embryonic kidney) cells were purchased from American Type Culture Collection and maintained according to their recommended protocol. Transfection-ready plasmids with mouse cDNA clone of neurolysin or thimet oligopeptidase (EC 3.4.24.15) and empty vector (pCMV6-Kan/Neo) were obtained from OriGene Technologies. Cell transfections were carried out with Lipofectamine™ LTX (Invitrogen) as described by the manufacturer. After 24 h, cell medium was replaced by fresh medium containing 1 mg/ml G418 for about 3 weeks to select stably transfected cells. To eliminate individual colony artifacts, colonies of cells resistant to G418 were pooled together for propagation and maintained under a selection pressure of 0.5 mg/ml G418 (22).

Photoaffinity Labeling and Radioligand Binding—A photoprobe analog of Ang II, sarcosine¹-*p*-benzoyl-L-phenylalanine⁸-angiotensin II (SBpa-Ang II), which was extensively used for molecular-structural studies of AT₁ and AT₂ receptors (23, 24), was prepared at the Department of Pharmacology, Université de Sherbrooke. Radioiodination of SBpa-Ang II and sarcosine¹-isoleucine⁸-angiotensin II (SI-Ang II) was carried out at American Radiolabeled Chemicals or the University of Florida.

Photoradiolabeling of the non-AT₁, non-AT₂ angiotensin binding site with ¹²⁵I-SBpa-Ang II was conducted using an established procedure (25) except that neurolysin knock-out and wild-type or P10 CD-1 mouse forebrains or HEK293 cells were used for preparation of crude membranes, and 2 μM final assay concentration of ZD7155 and 20 μM PD123319 (Tocris Bioscience) were used to block AT₁ and AT₂ receptors, respectively. Photoradiolabeling experiments designed for purification of the angiotensin binding site utilized a mixture of ^{127/125}I-SBpa-Ang II (95 and 5%, respectively), to have a minimal amount of radioactivity in the final samples intended for LC-MS/MS analysis.

Radioligand binding studies of the non-AT₁, non-AT₂ binding site in HEK293 cells and neurolysin knock-out and wild-type mouse forebrain membranes using ¹²⁵I-SI-Ang II were carried out according to our established procedures (15, 20). In these experiments, 2 μM ZD7155 and 20 μM PD123319 were used to block AT₁ and AT₂ receptors, respectively. All photoradiolabeling and radioligand binding experiments of the novel binding site were carried out in the presence of a 150 μM final assay concentration of *p*-chloromercuribenzoate (PCMB) to unmask the non-AT₁, non-AT₂ angiotensin binding site (15, 20).

Photoradiolabeling and radioligand binding studies of AT₁ and AT₂ receptors in neurolysin knock-out and wild-type mouse brains were carried out essentially as described previously (26) in combined membrane preparations of hypothalamus, thalamus, and brainstem using ¹²⁵I-SBpa-Ang II or ¹²⁵I-SI-Ang II, respectively. In these experiments, an additional mixture of protease inhibitors (*o*-phenanthroline, puromycin, phenylmethanesulfonyl fluoride, and glutamate phosphonate) was used to protect the ligands and receptors from proteolytic degradation (26, 27). To avoid aggregation of AT₁ and AT₂ receptors, photoradiolabeled membrane pellets were dissolved in

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modified Laemmli sample buffer (containing 5 M urea and 0.125 M dithiothreitol instead of 2-mercaptoethanol), periodically vortexed for 30 min at room temperature, and resolved on 7.5% Tris-HCl Ready Gels (Bio-Rad) without prior boiling. For all experimental procedures, protein concentration was determined by the BCA assay (Pierce) using bovine serum albumin as a standard.

In Vitro Receptor Autoradiography—*In vitro* receptor autoradiography studies in neurolysin knock-out and wild-type mouse forebrain coronal sections using ¹²⁵I-SI-Ang II were carried out essentially as described (18).

Protein Purification and Two-dimensional Gel Electrophoresis—Crude membrane preparations of P10 mouse forebrains (~4 g total wet weight starting material) were pelleted after photoradiolabeling and multiple washes, solubilized in SDS sample buffer, and separated in 10% Tris-HCl preparative Criterion gels (Bio-Rad). Gel sections corresponding to a ~75-kDa region were combined from multiple gels, and the radioactivity was extracted into Tris-glycine SDS-PAGE running buffer at 4 °C for 4 days (>90% recovery of the iodine-125). The extracted sample was concentrated using centrifugal filtering units (Amicon Ultra and Nanosep Omega), and an aliquot was saved for two-dimensional gel electrophoresis. The sample was further separated by isoelectric focusing using one-dimensional pH gradient strips (pH 3–10, 11-cm ReadyStrip™ IPG; Bio-Rad). Strip sections from the pH ~5.5–7.0 region were combined, the radioactivity was extracted, and the sample was concentrated as described above.

To conduct two-dimensional gel electrophoresis, aliquots of the final concentrate of the sample were acetone-precipitated, solubilized in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.2% pH 3–10 ampholytes, 10% glycerol) and loaded onto pH 5–8 immobilized gradient strips (11-cm ReadyStrip™ IPG; Bio-Rad). Isoelectric focusing was followed by separation in 10% Tris-HCl Criterion gels.

Representative gels after SDS-PAGE or two-dimensional gel electrophoresis were stained with Bio-Safe Coomassie Blue (Bio-Rad), dried in a vacuum gel drier, and incubated with x-ray film and intensifying screen at –80 °C for 2–5 days for autoradiographic visualization of the photoradiolabeled proteins.

Mass Spectrometry Analysis and Identification of Proteins—After two-dimensional gel electrophoresis of the final purified sample, the region of the Coomassie Blue-stained gel containing radioactive signal was cut and stored at 4 °C to decay the radioactivity to background levels. Mass spectrometry analysis was performed on trypsin-treated gel segments. In brief, gel pieces were diced into 1-mm squares, rinsed with water and 50 mM ammonium bicarbonate buffer, and dehydrated. Reduction of disulfide bonds was conducted with dithiothreitol, followed by alkylation with iodoacetamide. Proteins were digested by rehydrating the gel slices in 20 μg/ml trypsin (Promega) in ammonium bicarbonate buffer plus 10% acetonitrile for 1 h at 24 °C, followed by overnight incubation at 37 °C and a second addition of trypsin the next day for 3 h. The digested material was extracted from the gel, combined, and dried, using a vacuum concentrator. 10–20% of the digest was loaded on a Magic C18 AQ (Michrom) nanospray tip on a Thermo LTQ mass spectrometer and washed with 5% methanol, 0.1% formic acid for 10 min before peptide elution began, using a 5–60% meth-

anol gradient. The LTQ ion trap mass spectrometer was equipped with a nanoelectrospray ionization source, running a full MS survey scan every 3 s in the data-dependent mode to collect the MS/MS fragmentation spectrum. The MS and fragmentation spectrum data were used in a Mascot search of the whole mouse proteome. Mascot search parameters included precursor and fragment ion mass tolerance of 1.5 and 0.8 daltons, respectively, one ¹³C incorporation, one missed trypsin cleavage site, fixed carbamidomethyl-cysteine modification, and variable methionine oxidation, against the complete mouse proteome (NCBI 20100930). The ion score/Expect cut-off score was set for 15, and peptides with an Expect score less than 0.05 were considered positive identification if more than one peptide was identified for a given protein.

Immunoprecipitation and Western Blotting—Immunoprecipitation experiments were performed in photoradiolabeled preparations of P10 mouse forebrain membranes using specific polyclonal antibodies against neurolysin (EC 3.4.24.16), thimet oligopeptidase (EC 3.4.24.15), α-adducin (Abcam; product numbers ab59523, ab59487, and ab51130, respectively), and collapsin response mediator protein 2 (Millipore; product number AB9218). Preparations for immunoprecipitation were made by extraction of proteins from photoradiolabeled forebrain membranes by radioimmune precipitation assay buffer (Boston Bioproducts) containing protease inhibitors (Halt Protease Inhibitor Mixture, Pierce). Protein A/G plus agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or Protein G Dynabeads (Invitrogen) were used to precipitate the antibody-antigen complexes from the solution according to a protocol detailed by Marimuthu *et al.* (28).

Western blotting of these proteins was carried out essentially as described by Alfonso *et al.* (29), using β-actin as a loading control (Sigma; product number A5441). The only exception was that α-adducin and thimet oligopeptidase samples were not boiled prior to SDS-PAGE.

Statistical Analyses—Determination of B_{\max} (fmol of radioligand bound/mg of protein) and K_d (nM) values were carried out using one-site saturation binding models of Prism software (GraphPad). Values reported were significantly different from zero (95% confidence interval) and are presented as mean ± S.E. Comparisons of radioactivity in immunoprecipitates, B_{\max} and K_d values obtained from radioligand binding experiments in membrane preparations of HEK293 cells, and specific binding to AT₁ and AT₂ receptors in neurolysin knock-out and wild-type control animal brains were carried out by one-way analysis of variance followed by the Tukey-Kramer test. Values reported are mean ± S.E.

RESULTS

Photoradiolabeling of the non-AT₁, non-AT₂ angiotensin binding site with ¹²⁵I-SBpa-Ang II in mouse forebrain membranes was demonstrated by SDS-PAGE analysis of samples. Specific and reproducible photoradiolabeling of a ~75-kDa protein was observed, with minor labeling at ~50 kDa (Fig. 1). As expected, nonspecific (NS) samples, which contained saturating concentrations of Ang II to prevent ¹²⁵I-SBpa-Ang II binding to the binding site, showed negligible radioactive signal at ~75 and ~50 kDa (Fig. 1). Therefore, all experiments were carried out with parallel use

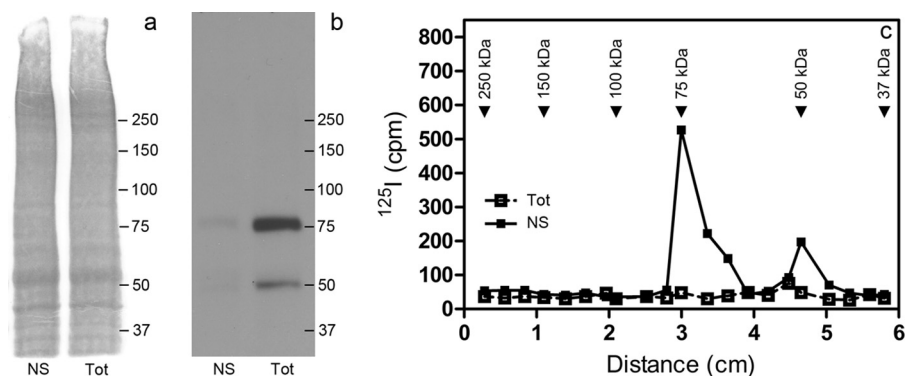


FIGURE 1. Photoradiolabeling of the non-AT₁, non-AT₂ angiotensin binding site in P10 mouse forebrain membranes. Shown are total (Tot) groups composed of membrane preparations incubated with ¹²⁵I-SBpa-Ang II (~1 nM) in the presence of 2 μM ZD7155, 20 μM PD123319, and 150 μM PCMB (1 h at 24 °C, followed by UV irradiation, pelleting, and solubilization). Nonspecific (NS) groups also contained 10 μM Ang II, to preclude radioligand binding to the binding site. *a*, representative SDS-PAGE analysis of NS and Tot samples (Coomassie Blue-stained gel). *b*, autoradiogram corresponding to *a*. *c*, representative migration of iodine-125 in NS and Tot lanes of an SDS-polyacrylamide gel cut in ~0.3-cm slices.

and comparison of total and nonspecific samples to avoid tracking of nonspecifically photoradiolabeled protein(s).

To obtain sufficient material for purification and identification of the binding protein, multiple preparative SDS-PAGE separations were performed using photoradiolabeled mouse forebrain membranes. Gel slices from the ~75-kDa region were pooled together from all preparative runs, and proteins were extracted, concentrated, and separated by two-dimensional gel electrophoresis (Fig. 2*a*). The photoradiolabeled protein was reproducibly detected by autoradiography after two-dimensional gel electrophoresis (Fig. 2*b*). To further purify the photoradiolabeled protein, the concentrate of proteins from the 75-kDa regions was subjected to preparative isoelectric focusing on one-dimensional pH 3–10 gradient strips. Next, slices of strips containing the radioactive signal (pH ~5.5–7.0) were collected and combined. Proteins were extracted and concentrated, followed by two-dimensional gel electrophoresis of the sample (Fig. 2*c*). Upon reproducible detection of the photoradiolabeled protein following the purification steps (Fig. 2*d*), the region of the two-dimensional gel containing the radioactive signal was cut out and used for identification of proteins by LC-MS/MS analysis.

Five independent LC-MS/MS analyses were performed using the two-dimensional gel excisions, where 32–54 proteins were identified in each (supplemental Table 1). Because all two-dimensional gel excision samples contained radioactive signal (*i.e.* photoradiolabeled non-AT₁, non-AT₂ binding site), we expected to see the protein of interest detected in all LC-MS/MS analyses. For this reason, only the proteins repeatedly identified in all five LC-MS/MS runs were shortlisted for further analysis (Table 1). These proteins included neurolysin (EC 3.4.24.16, accession number gi|28077051), collapsin response mediator protein 2 (accession number gi|40254595), thimet oligopeptidase (EC 3.4.24.15, accession number gi|239916005), α-adducin (accession number gi|6851286), collapsin response mediator protein 1 (accession number gi|74186635), G-protein signaling modulator 1 (accession number gi|32189378), long-chain fatty acid-CoA ligase (accession number gi|16716465), ezrin (accession number gi|74179741), and serine protease 1 (accession number gi|16716569). Of these nine proteins, serine protease 1 had an estimated molecular mass of 26.8 kDa and was considered unlikely to be the angiotensin-binding protein and consequently excluded

from further analysis. The remaining eight protein candidates were ranked using their Mascot “Score” and “Peptide query” (or spectral counts) values. These data indicated consistently higher amounts of neurolysin, thimet oligopeptidase, collapsin response mediator protein 2, and α-adducin in the analyzed samples. Therefore, these proteins were prioritized for investigation to test the candidacy of the non-AT₁, non-AT₂ binding site (supplemental Fig. 1).

The results of immunoprecipitation studies using specific antibodies against the top four candidates (neurolysin, thimet oligopeptidase, collapsin response mediator protein 2, and α-adducin) in photoradiolabeled mouse forebrain membranes are summarized in Fig. 3. “Total” samples of immunoprecipitates with anti-neurolysin antibody contained a substantial amount of radioactive signal (Fig. 3*a*), indicating that the non-AT₁, non-AT₂ angiotensin binding site is probably neurolysin. Total samples of anti-thimet oligopeptidase immunoprecipitates contained a marginal level of radioactivity, which could be due to cross-reactivity of the anti-thimet oligopeptidase antibody with neurolysin or a small degree of radiophotolabeling of thimet oligopeptidase. Total samples of collapsin response mediator protein 2 and α-adducin showed negligible amounts of radioactive signal. To ensure that the proteins of interest were indeed immunoprecipitated from photoradiolabeled membrane samples, their presence in the immunoprecipitates was documented by Western blotting (Fig. 3, *b–e*).

Stable cell lines overexpressing mouse neurolysin or thimet oligopeptidase in HEK293 cells were established to further test the identity of the angiotensin-binding protein. Overexpression of both proteins in HEK293 cells was confirmed by Western blotting (Fig. 4, *a* and *b*). Radioligand binding assays in membrane preparations of these cells using ¹²⁵I-SI-Ang II showed dramatically increased density of the novel angiotensin binding sites only in HEK293 cells overexpressing neurolysin ($B_{\max} = 533 \pm 69$ fmol/mg protein) compared with non-transfected, empty vector-transfected, and thimet oligopeptidase-overexpressing HEK293 cell membranes (108 ± 25.2 , 94.6 ± 20.1 , and 90.4 ± 7.9 fmol/mg protein respectively; $n = 3$) (Fig. 4, *c* and *d*). Notably, affinity of the radioligand for the novel binding site did not differ significantly among the four groups ($K_d = 3.35 \pm 0.41$, 2.11 ± 0.57 , 1.75 ± 0.42 , and 2.53 ± 0.58 nM,

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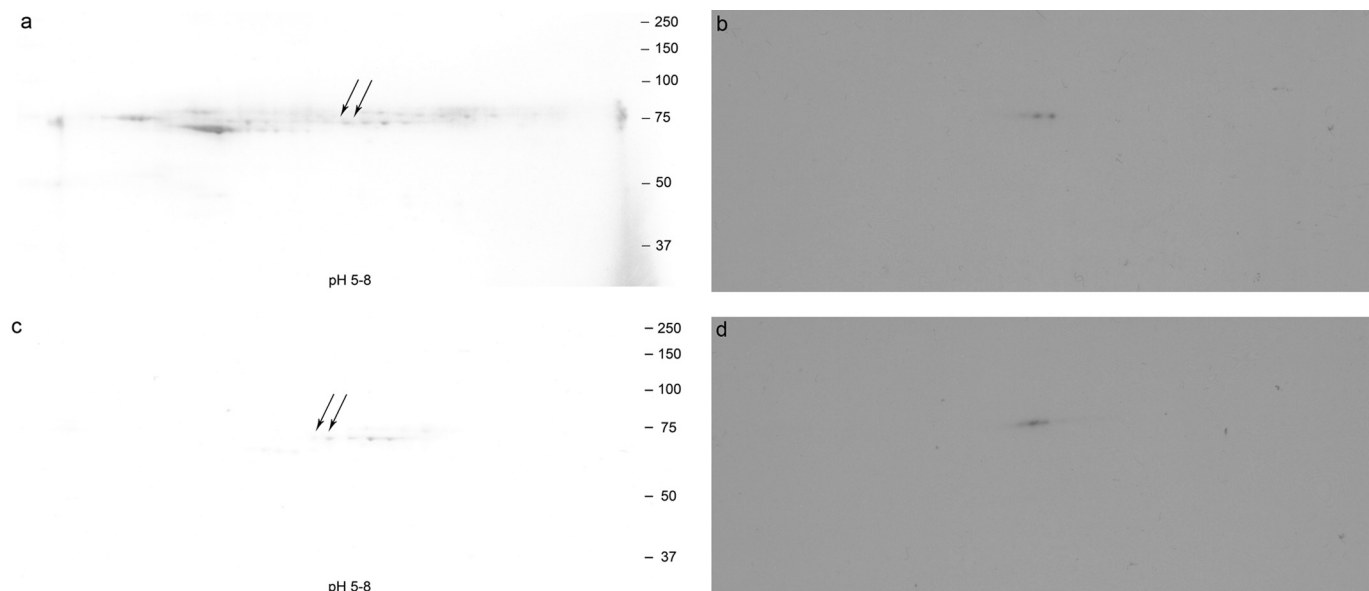


FIGURE 2. Two-dimensional gel electrophoretic analyses of partially purified, photoradiolabeled non-AT₁, non-AT₂ angiotensin binding site from P10 mouse forebrain membranes. Shown are the first (a) and second (c) purification steps. Their corresponding autoradiograms are shown in b and d, respectively. Arrows indicate the location of radioactive signal in Coomassie Blue-stained gels (note that because of low amount of protein, no distinct Coomassie Blue staining was observed in the location of the radioactive signal).

TABLE 1

Mass spectrometry identification of proteins repeatedly identified in all two-dimensional gel samples

Protein ^a	Accession No.	Sample 1		Rank ^b (emPAI) ^c				
		Score	Peptide queries ^d	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Neurolysin	gi 28077051	1310	73	1 (3.36)	3 (3.36)	10 (0.8)	2 (3.0)	2 (3.4)
CRMP2	gi 40254595	1307	55	2 (2.56)	2 (4.33)	6 (1.8)	1 (8.5)	1 (12)
Thimet OP	gi 239916005	874	50	3 (1.88)	9 (0.44)	1 (2.8)	6 (0.9)	11 (0.4)
α -Adducin	gi 6851286	726	36	4 (0.88)	4 (1.05)	4 (0.7)	3 (1.0)	3 (0.9)
CRMP1	gi 74186635	517	21	5 (0.85)	1 (2.33)	8 (0.8)	5 (0.8)	6 (1.5)
Gpsm1	gi 32189378	453	27	8 (1.39)	10 (0.4)	3 (2.4)	9 (0.8)	13 (0.7)
LCFA-CoA-L	gi 16716465	439	16	9 (0.71)	18 (0.1)	2 (1.8)	32 (0.05)	42 (0.09)
Ezrin	gi 74179741	415	33	10 (1.0)	5 (2.14)	5 (1.0)	35 (0.1)	25 (0.4)
Serine protease 1	gi 16716569	94	6	26 (0.3)	24 (0.3)	23 (0.3)	8 (0.14)	12 (0.2)

^a Proteins were as follows: neurolysin (EC 3.4.24.16); collapsin response mediator protein 2 (dihydropyrimidinase-related protein 2) (CRMP2); thimet oligopeptidase (EC 3.4.24.15) (Thimet OP); collapsin response mediator protein 1 (CRMP1); G-protein signaling modulator 1 (Gpsm1); long-chain fatty acid-CoA ligase (LCFA-CoA-L); ezrin, and serine protease 1.

^b Rank number reflects top Mascot score position in a sample.

^c emPAI value reflects protein abundance, using spectral counts and normalizing by protein size and expected peptide coverage.

^d Also known as spectral counts.

respectively). Photoradiolabeling experiments carried out in membrane preparations of the same cells also indicated substantially higher photoradiolabeling of the binding site in cell membranes overexpressing neurolysin compared with thimet oligopeptidase or control groups (Fig. 4, e and f).

Forebrain membranes from neurolysin knock-out (KO) mice were compared with wild-type (WT) mice to determine if the non-AT₁, non-AT₂ binding site was neurolysin. Absence of neurolysin in the neurolysin KO mouse forebrains was documented by Western blotting (Fig. 5a). Saturable binding of ¹²⁵I-SI-Ang II to the non-AT₁, non-AT₂ angiotensin binding site in neurolysin KO forebrain membranes was not statistically significantly different from zero based on 95% confidence interval values (Fig. 5b). In contrast, there was a substantial amount of high affinity, saturable binding of ¹²⁵I-SI-Ang II ($K_d = 1.13 \pm 0.21$ nM, $B_{max} = 54.4 \pm 12.7$ fmol/mg protein, $n = 3$) in wild-type forebrain membranes (Fig. 5c). Photoradiolabeling experiments in the same membrane preparations confirmed the presence of the expected radioactive signal from WT control

samples and the absence of radioactive signal in neurolysin KO forebrain membranes (Fig. 5, d and e). *In vitro* receptor autoradiography studies in coronal sections of neurolysin KO and WT mouse forebrains also verified these observations, indicating high intensity binding of ¹²⁵I-SI-Ang II in WT and a paucity of binding in neurolysin KO brains (supplemental Fig. 2).

The presence of classic AT₁ and AT₂ receptors in neurolysin KO and WT animal brains was tested by photoradiolabeling and radioligand binding experiments. Ang II and/or ZD7155 (AT₁ receptor antagonist) and PD123319 (AT₂ receptor antagonist) blocked photoradiolabeling of a diffuse band of protein at ~55–60 kDa by ¹²⁵I-SBpa-Ang II (supplemental Fig. 3). The presence of AT₁ and AT₂ receptors in neurolysin KO and WT mouse brains was clearly distinguished by radioligand binding experiments using ¹²⁵I-SI-Ang II (supplemental Fig. 4).

DISCUSSION

The recently discovered non-AT₁, non-AT₂ angiotensin binding site in rodent and human brain membranes has dis-

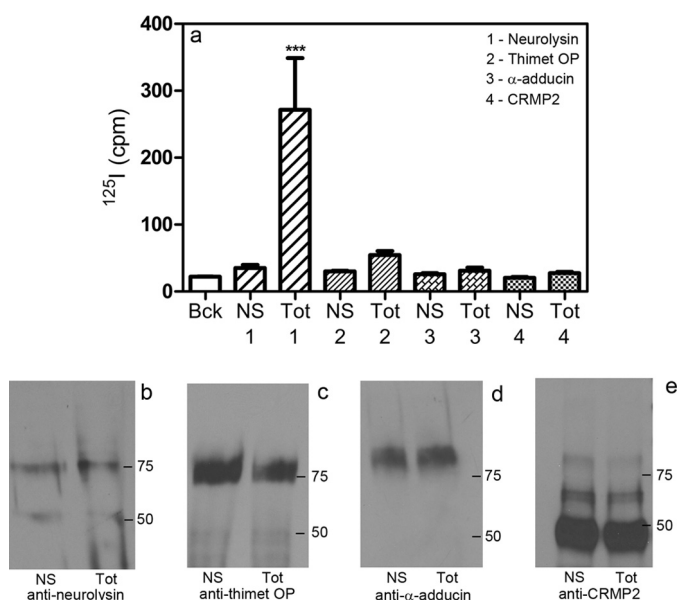


FIGURE 3. Immunoprecipitation of neurolysin (EC 3.4.24.16), thimet oligopeptidase (thimet OP; EC 3.4.24.15), α -adducin, and collapsin response mediator protein 2 (CRMP2) from photoradiolabeled P10 mouse forebrain membrane preparations. Immunoprecipitation of each protein was carried out from parallel nonspecific (NS) and total (Tot) photoradiolabeled groups as detailed in the legend to Fig. 1 and under "Experimental Procedures." *a*, equal amounts of NS and total sample of each protein immunoprecipitate were used to count the radioactive signal. *Bck*, background radioactivity; ***, $p < 0.001$ versus all other groups; $n = 3-4$. *b-e*, representative Western blot confirmation of successful immunoprecipitation of each candidate protein from NS and total samples.

tinctly different biochemical properties and tissue distribution from classic angiotensin receptors and key angiotensin-processing enzymes (15–19). Many features of this protein (high affinity and pharmacological specificity for angiotensins; conservation in brains of numerous species, including humans; localization on the plasma membrane, where it can interact with extracellular ligands; and association of expression of this protein with neuronal cell death and neurogenic hypertension) strongly favor its functional significance (16, 20). However, the unknown identity of the novel angiotensin binding site has limited our understanding of the (patho)physiological function of this hypothesized new member of the RAS.

The photoaffinity probe ¹²⁵I-SBpa-Ang II specifically and covalently photoradiolabeled the non-AT₁, non-AT₂ angiotensin binding site in mouse forebrain membranes (Fig. 1). The radioactive signal was tracked and purified through electrophoretic procedures based on molecular size and isoelectric point of the binding protein (Fig. 2). Two-dimensional gel electrophoresis and LC-MS/MS analysis of the radioactive protein spot revealed eight candidates (Table 1 and supplemental Table 1), of which the four most abundant (neurolysin (EC 3.4.24.16), thimet oligopeptidase (EC 3.4.24.15), collapsin response mediator protein 2, and α -adducin) were immunoprecipitated from photoradiolabeled mouse forebrain membranes. Immunoprecipitates of neurolysin contained substantial radioactive signal, in contrast to negligible radioactivity in the other three protein immunoprecipitates (Fig. 3).

Radioligand binding and photoradiolabeling experiments independently showed "gain of phenotype" (*i.e.* significantly increased density of the angiotensin binding site) in mem-

branes of HEK293 cells overexpressing neurolysin but not thimet oligopeptidase or control groups (Fig. 4).

Radioligand binding, photoradiolabeling, and *in vitro* receptor autoradiography experiments performed in neurolysin KO and WT mouse forebrains revealed a "loss of phenotype" (*i.e.* loss of high affinity specific binding to the angiotensin binding site) in neurolysin KO animals (Fig. 5 and supplemental Fig. 2), further establishing membrane-bound neurolysin as the non-AT₁, non-AT₂ angiotensin binding site. Although the existence of another angiotensin binding protein cannot be excluded, our data indicate that any other binding site has much lower affinity for angiotensins and is expressed in substantially lower levels than neurolysin, precluding its quantification by the methods used in our study.

Separate photoradiolabeling (in the absence of PCMB) and radioligand binding assays indicated that AT₁ and AT₂ receptors were still present in the neurolysin knock-out mouse brains, endorsing earlier observations distinguishing this novel binding site from classic Ang II receptors (16).

Additional evidence supporting neurolysin as the angiotensin binding site is the lack of glycosylation of neurolysin (30, 31) and the non-AT₁, non-AT₂ angiotensin binding site (25), similar pI values for both proteins (31), and its presence in membrane preparations of primary neurons but not astroglial cells (20, 32).

A minor photoradiolabeling of a ~50-kDa protein was observed in addition to the major ~75-kDa protein (Figs. 1*b*, 4*d*, and 5*d*). For a number of reasons, it is likely that the photoradiolabeled protein at ~50 kDa is a degradation product or a shorter form of neurolysin. First, similar to the ~75-kDa protein, the radioactive signal of the ~50-kDa photoradiolabeled protein was substantially increased in membrane preparations of HEK cells overexpressing neurolysin compared with all other groups (Fig. 4*d*). Second, the radioactive signal of the ~50-kDa photoradiolabeled protein, like the ~75-kDa protein, was negligible in membrane preparations of neurolysin KO mouse forebrains (Fig. 5*d*). Third, at least four sequences for mouse neurolysin are published to date (NCBI protein database) with 752-, 704-, 683-, and 533-amino acid-long sequences and 85.3-, 80.4-, 77.9-, and 60.7-kDa predicted masses, indicating that different isoforms of this protein may exist.

Neurolysin (EC 3.4.24.16) is a zinc metalloendopeptidase belonging to the M3 family that is ubiquitously distributed in central nervous system and peripheral tissues of mammals (33). It was first detected and purified from rat brain synaptic membranes (34) and is also known as liver-soluble angiotensin-binding protein, mitochondrial oligopeptidase, microsomal endopeptidase, oligopeptidase M, and thimet oligopeptidase II (35). Neurolysin exists primarily as a cytosolic protein; however, depending on cell type, it can also be secreted, bound to the plasma membrane, or targeted to mitochondria (30–32, 36). Neurolysin has >60% amino acid sequence identity with thimet oligopeptidase (EC 3.4.24.15) (33). These oligopeptidases share several substrates, including neurotensin, bradykinin, angiotensins, opioids, substance P, somatostatin, and hemoglobin-derived peptides, and hydrolyze many of them at the same peptide bond, at comparable rates (33, 37–40). Additionally, accumulating evidence indicates the role of these peptidases in the intracellular metabolism of peptides (41, 42). Functional studies revealed the role of both peptidases in neurotensin-

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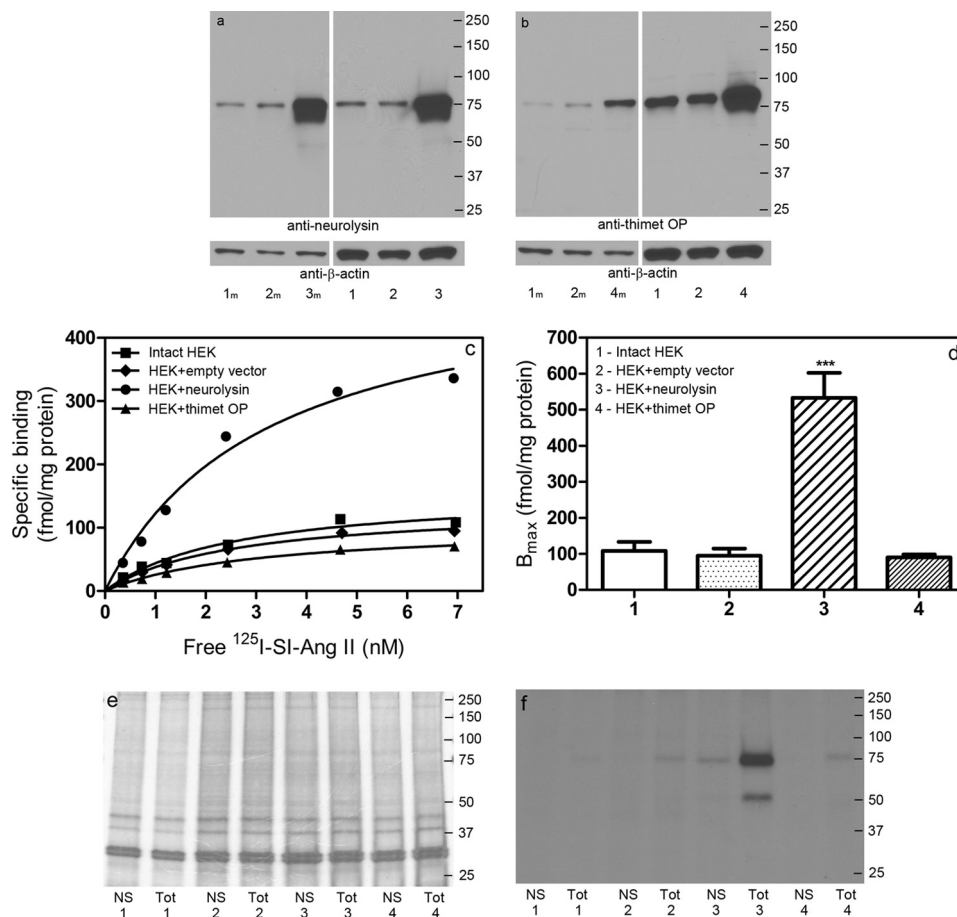


FIGURE 4. Summary of experiments conducted in HEK293 cells. Demonstration of overexpression of neurolysin (EC 3.4.24.16) (a) and thimet oligopeptidase (thimet OP; EC 3.4.24.15) (b) in HEK cell membranes (m) and whole cells by Western blotting (1, intact HEK; 2, HEK + empty vector; 3, HEK + neurolysin; 4, HEK + thimet oligopeptidase). c, representative saturation binding isotherms of specific (10 μ M Ang II displaceable) 125 I-SI-Ang II binding to the non-AT₁, non-AT₂ angiotensin binding site in HEK293 cell membranes. Cell membranes were incubated in the presence of 20 μ M PD123319, 2 μ M ZD7155, and 150 μ M PCMB for 1 h at 24 $^{\circ}$ C with or without 10 μ M Ang II for estimation of the nonspecific binding. In non-transfected HEK cells (Intact HEK), B_{\max} = 158 \pm 19.6 fmol/mg protein, and K_d = 2.61 \pm 0.75 nM; in HEK cells transfected with empty vector (HEK + empty vector), B_{\max} = 135 \pm 7.3 fmol/mg protein, and K_d = 2.58 \pm 0.32 nM; in HEK cells overexpressing neurolysin (HEK + neurolysin), B_{\max} = 514 \pm 56 fmol/mg protein, and K_d = 3.2 \pm 0.76 nM; in HEK cells overexpressing thimet oligopeptidase (HEK + thimet OP), B_{\max} = 104 \pm 6.0 fmol/mg protein, and K_d = 3.03 \pm 0.39 nM. d, comparison of B_{\max} values in the same experimental groups from three independent radioligand binding experiments (***, p < 0.001 versus groups 1, 2 and 4). Error bars, S.E. e, representative SDS-PAGE analysis of HEK293 cell membranes after photoradiolabeling of the non-AT₁, non-AT₂ angiotensin binding site with 125 I-SBpa-Ang II in parallel nonspecific (NS) and total (Tot) groups (Coomassie Blue-stained gel). f, autoradiogram corresponding to c (1, intact HEK; 2, HEK cells transfected with empty vector; 3, HEK cells overexpressing neurolysin; 4, HEK cells overexpressing thimet oligopeptidase). Only group 3 total showed elevated amounts of the photoradiolabeled protein.

related nociception (43, 44), bradykinin-mediated regulation of blood pressure and microvascular permeability (45, 46), and antigen presentation through major histocompatibility complex class I (MHC-I) (47–49). Cell type-dependent membrane association of neurolysin, constituting \sim 10% of total activity of this peptidase, was documented in several studies (31, 32, 34, 50–53). However, membrane-anchoring mechanism(s) and how the membrane-bound, mitochondrial, and cytoplasmic variants differ are not fully understood.

Some similarities of the non-AT₁, non-AT₂ angiotensin binding site to the liver-soluble angiotensin-binding protein (*i.e.* cytoplasmic neurolysin) (54–56) were noted in our first publication documenting the existence of the novel angiotensin binding site in rat brain membranes (15). However, we could not conclude whether the angiotensin binding site was a membrane-bound variant of neurolysin or thimet oligopeptidase or a different protein because of a number of differences between our observations and observations of the soluble angiotensin-binding protein (pharmacological specificity, insensitivity

toward selective neurolysin/thimet oligopeptidase inhibitor JA-2, and time sensitivity toward sulfhydryl reagents) and conflicting reports on the identity of the soluble angiotensin-binding protein as neurolysin or thimet oligopeptidase (57) as well as tissue distribution (31, 58, 59) and cellular localization (32, 52, 60–62) of these two proteins (15).

In this study, biochemical, molecular-genetic, and receptor pharmacological approaches identify the non-AT₁, non-AT₂ angiotensin binding site as a membrane-bound variant of metalloendopeptidase neurolysin (EC 3.4.24.16). A key question regarding the identity of the novel angiotensin binding site is thus resolved. As such, neurolysin may be a key enzyme responsible for sequestration or metabolic processing of angiotensins at certain (patho)physiological conditions, perhaps even more important than other peptidases. This notion is supported by recent observations indicating significantly decreased density of this angiotensin binding site in brain cardiovascular centers of spontaneously hypertensive rats (21). Thus, neurolysin may serve to limit the

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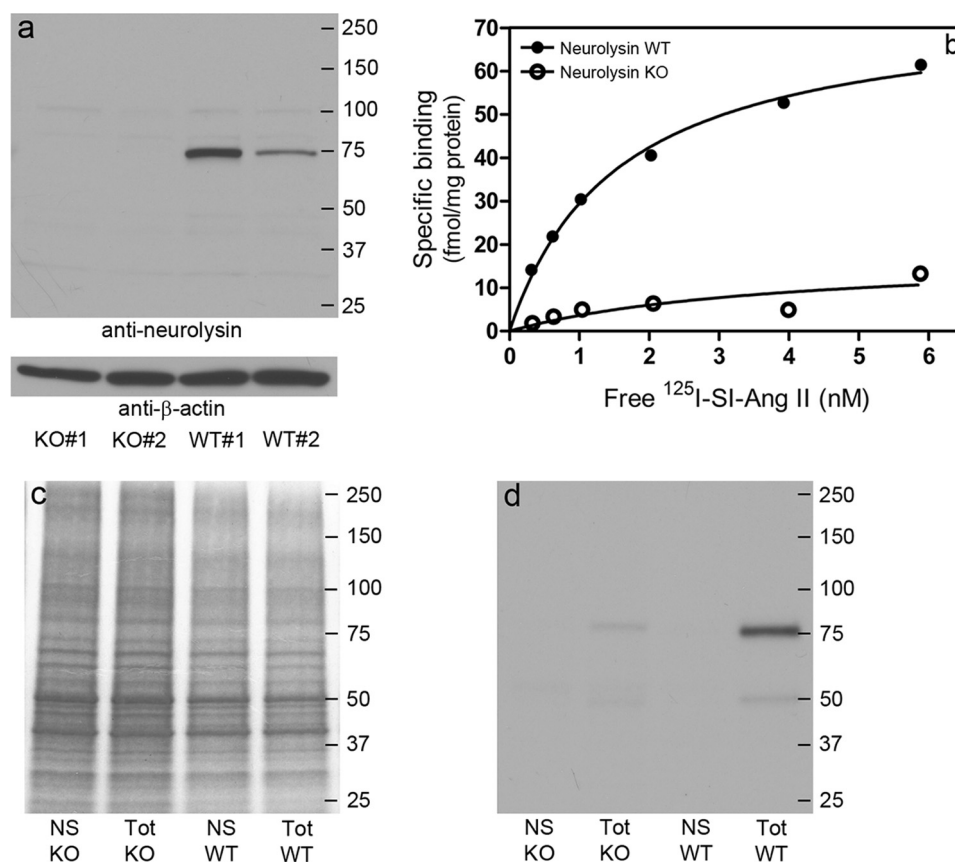


FIGURE 5. Summary of experiments conducted in neurolysin (EC 3.4.24.16) knock-out and wild-type mice. *a*, representative Western blot of neurolysin in knock-out (KO#1 and KO#2) and wild-type (WT#1 and WT#2) mouse forebrain membranes. *b*, representative saturation binding isotherms of specific (10 μ M Ang II displaceable) ¹²⁵I-SI-Ang II binding to the non-AT₁, non-AT₂ angiotensin binding site in neurolysin KO and WT mouse forebrain membranes (1-h incubation at 24 °C in the presence of 20 μ M PD123319, 2 μ M ZD7155, and 150 μ M PCMB with or without 10 μ M Ang II for estimation of the nonspecific binding). In neurolysin WT, $B_{max} = 75.5 \pm 3.2$ fmol/mg protein, and $K_d = 1.56 \pm 0.17$ nM; in neurolysin KO, B_{max} and K_d values were not significantly different from 0: $B_{max} = 17.7 \pm 11.1$ fmol/mg protein, 95% confidence interval = -13.2–48.6; $K_d = 3.87 \pm 4.7$ nM, 95% confidence interval = -9.14–16.9. *c*, representative SDS-PAGE analysis of neurolysin KO and WT forebrain membranes after photoradiolabeling of the non-AT₁, non-AT₂ angiotensin binding site with ¹²⁵I-SBpa-Ang II in parallel nonspecific (NS) and total (Tot) groups (Coomassie Blue-stained gel). *d*, autoradiogram corresponding to *c*.

access of Ang II to AT₁ receptors, and its reduction might contribute to the well documented overactivation of brain RAS in spontaneously hypertensive rats. Future studies focusing on metabolic processing of angiotensins by neurolysin will provide insights about the role of this peptidase related to the function of the renin-angiotensin system.

It is noteworthy that the non-AT₁, non-AT₂ binding site was found in high densities not only in brain areas known to contain AT₁ and AT₂ receptors (*e.g.* hypothalamic subnuclei, thalamic nuclei, circumventricular organs, nucleus of solitary tract, ventrolateral medulla, and amygdala) but also in regions with low/negligible density of classic angiotensin receptors (*e.g.* cerebral cortex, choroid plexus, hippocampus, nucleus accumbens, substantia nigra, and substantia innominata) (18). This correlates well with Ang II immunoreactivity in the brain (63), indicating possible AT₁ and AT₂ receptor-independent and non-cardiovascular functions of RAS (*e.g.* neurogenesis, plasticity and memory, and cognition). The density of the binding site is substantially higher than AT₁ and AT₂ receptors (*e.g.* in paraventricular nucleus of hypothalamus, which is rich in AT₁ receptors, density of the binding site is ~15 times higher compared with the AT₁ receptor) (18). As mentioned above, neurolysin processes other neuropeptides; hence, it is possible that some

functions of angiotensins involve inhibition of the ability of neurolysin to process those neuropeptides. Such a phenomenon is documented for angiotensin IV and its receptor, insulin-regulated aminopeptidase, which metabolizes several bioactive peptides, including vasopressin, CCK-8, oxytocin, and somatostatin (64). Another possibility could be activation of intracellular signaling pathways by membrane-bound neurolysin in response to angiotensins similar to several other peptidases (65).

Two important questions remaining unanswered that warrant detailed investigations include the following. What molecular mechanism(s) unmask the high affinity angiotensin binding characteristics of neurolysin in (patho)physiological conditions? What is the functional significance of this interaction?

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