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Pharmacological characterization of a novel non-AT1, non-AT2 angiotensin binding site identified as neurolysin

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Abstract

The discovery of a novel non-AT_1, non-AT_2 binding site for angiotensins in the rodent brain and testis that is unmasked by the organomercurial compound para-chloromercuribenzoic acid (PCMB) has catalyzed efforts to purify and characterize this protein. We recently reported that this protein is neurolysin and now report upon the specificity of this binding site for various neuropeptides. Competition binding assays in rat brain and testis used ^{125}I-Sar^1, Ile^8 angiotensin II (Ang II) as the radioligand in the presence of saturating concentrations of AT_1 and AT_2 receptor antagonists and 100 μM parachloromercuribenzoate. Primary screening of 36 peptides and other compounds at 10 μM concentration revealed seven peptides that inhibited specific binding > 50%: ghrelin, Tyr^1 S36057 (a melanin-concentrating hormone receptor ligand), orphanin FQ and its congeners (Tyr^1 and Tyr^{14}), Dynorphin A (1–8), and Ang (1–9). The selective neurolysin inhibitor Proline-Isoleucine dipeptide was inactive at 1 mM. These results suggest that the ability of PCMB to unmask high affinity binding of Ang II to neurolysin is a pharmacological effect and that neurolysin may significantly affect the activity of the reninangiotensin system.

Keywords

Neurolysin; Angiotensin II; Renin-Angiotensin System; p-chloromercuribenzoic acid (PCMB); neuropeptides; ^{125}I-Sarcosine^1, isoleucine^8 angiotensin II; Radioligand binding; Brain; Testis

Introduction

Research studies have long determined that angiotensin II (Ang II) plays a significant role in the development of cardiovascular disease and hypertension. According to the Centers for Disease Control and Prevention, the leading cause of death in the U.S. in 2010 was heart disease (595,444 deaths) and the fourth leading cause was cerebrovascular disease (129,180 deaths) (http://www.cdc.gov/nchs/data/nvsr/nvsr60/nvsr60_04.pdf, Accessed October 4, 2012). Ang II is a major effector octapeptide, critical for the maintenance of blood pressure

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Conflict of Interest

Neither Dr. Robert C. Speth, Kira L. Santos, nor Jamala D. Swindle have any conflicts of interests or competing interests with respect to the publication of our manuscript: “Pharmacological characterization of a novel non-AT1, non-AT2 angiotensin binding site identified as neurolysin” which we have submitted to Endocrine for consideration for publication.

and plasma Na^+ concentration. The primary role of the renin-angiotensin system (RAS) in the body is to maintain blood volume and electrolyte balance. Dysregulation of the RAS causes several pathophysiological states, of which hypertension is most prominent [1].

Dysregulation can result from altered redox state in the bloodstream which can increase the conversion of angiotensinogen to Ang I [2]. Overexpression of renin, as well as prorenin, also increases Ang I formation. In the classic RAS, angiotensin-converting enzyme (ACE) then converts Ang I into Ang II, the major effector peptide of the RAS. Its well-known roles include stimulation and release of aldosterone, leading to retention of sodium and an increase in blood pressure by vascular smooth muscle contraction and increased sympathetic outflow. Additionally, excessive demand on the heart leads to hypertrophy while direct effects of Ang II on the heart cause fibrosis which compromises cardiac output. Stress on vascular cell walls combined with proinflammatory effects of Ang II lead to an increased deposition of plaque on blood vessel walls leading to atherosclerosis [3]. Ang II exerts these effects primarily on the angiotensin II type I (AT_1) subtype receptor. Ang II is further converted to the heptapeptide Ang III by glutamyl aminopeptidase A. Ang III, which also acts upon the AT_1 subtype, is then converted to the hexapeptide Ang IV by the cleavage of the Arg at its N-terminal by membrane alanyl aminopeptidase N [4]. Ang IV binds to a pleiotropic protein known as the AT_4 receptor, insulin-regulated aminopeptidase, vasopressinase and oxytocinase (E.C. 3.4.11.3) [5].

A novel angiotensin binding protein, distinct from the known receptors for angiotensin peptides was discovered in the rat brain [6,7]. It is membrane-bound, unmasked in the presence of parachloromercuribenzoic acid (PCMB), not N-glycosylated nor does it behave similarly to most G protein coupled receptors, including the AT_1 receptor, and has an isoelectric point of ~7 [8]. In addition, it has been localized in mouse testis and other structures [9]. These studies led to the identification of this binding site as the metallopeptidase neurolysin (E.C. 3.4.24.16) [10]. Of note, a soluble angiotensin binding protein isolated from porcine liver [11] was later shown to be neurolysin [12], which has 90% homology to rat neurolysin and 91% homology to mouse neurolysin. While the latter study [10] of the angiotensin binding protein was in progress, the present study examined the ability of this protein to interact with other brain peptides, analogs of Ang II and a putative dipeptide inhibitor of neurolysin.

Materials and Methods

Tissue Preparation

Male Sprague-Dawley rats were sacrificed with fluorothane inhalation and brains and testes were harvested, frozen at −20° C and subsequently stored at −80° C. The frozen rat brains and testes were weighed and homogenized for 5–10 seconds in 25 mL of ice-cold, 20 mM $NaPO_4$, pH 7.4 (hypotonic buffer) by a mechanical homogenizer. Homogenates were centrifuged at 40–48,000× g for 20 minutes and the supernatants were decanted. The membrane pellets were resuspended by homogenization for 5–10 seconds in the assay buffer AM5 (150 mM NaCl, 5mM EDTA, 0.1 mM bacitracin, 50 mM $NaPO_4$). The homogenates were recentrifuged as before. The final pellet was resuspended in AM5 to give a tissue concentration of 50 mg/mL (initial wet weight). AT_1 and AT_2 blockers, PD 123319 and Losartan (each having a final concentration of 10 μM), and PCMB (final concentration of 100 μM) were added to the tissue homogenate approximately 10 minutes before incubation. Tissue was kept on ice prior to incubation at room temperature. Animal procedures were approved by the IACUCs of Nova Southeastern University and the University of Florida.

Competition Binding Assay Procedure

Competition binding assays were carried out in two steps. For the primary screenings 50 μL membrane preparations were incubated with 0.25 nM ^{125}I-SI Ang II and either 10 μM of the test peptide, 3 μM Ang II (all final concentrations) or without any competing ligand "total binding". Specific binding was determined as "total binding" minus the amount of binding in the presence of 3 μM Ang II "non-specific binding". All peptides were run in triplicate or more for each assay, and each peptide was assayed in 2 or more separate assays. Peptides were obtained from Phoenix Pharmaceuticals, Bachem, American Peptides, Peninsula, Sigma-Aldrich, and other sources. Ang I Pro^{11}, D-Ala^{12} was synthesized by Dr. Gerhard Munskie at Washington State University, HPP SDKP was provided by Dr. Nour-Eddine Rhaleb at Henry Ford Hospital, Detroit MI. In addition, the carboxypeptidase inhibitor DL-mercaptomethyl-3-guanidinoethylthiopropanoic acid (Plummer's inhibitor) was screened. The tissue homogenate was incubated for one hour at room temperature. Studies carried out in primary cortical neurons obtained from mouse brain indicate that ^{125}I-SI Ang II is stable for incubation periods up to 180 min, with > 90% of equilibrium binding occurring by 60 minutes [13]. Membrane bound ^{125}I-SI Ang II was collected on glass fiber filters prewetted with 1mg/mL bovine serum albumin using a cell harvester (Model M24R, Brandel, Gaithersburg, MD). Incubation tubes and filters were rinsed three times with 50 mM $NaKPO_4$, pH 7.4 to minimize non-specific binding to the filters. Bound radioligand maintained on the filter disks was assayed with a COBRA II gamma counter. For three of the peptides that exhibited >50% inhibition of specific ^{125}I-SI Ang II binding at 10 μM, as well as proline-isoleucine dipeptide (Pro-Ile), reported to be specific for neurolysin [14], secondary screenings were performed at five different concentrations ranging from 0.1 μM up to 1 mM. Log IC_{50} was determined by nonlinear regression curve fitting of % inhibition of specific binding data to a one-site competition model algorithm constrained for 100 % specific binding (Prism,Graphpad Software, San Diego, CA). Graphical representation of the data also used Prism software. Values presented are averages ± SEM. Statistical comparisons of Log IC_{50} values between brain and testis were based on 95% confidence intervals.

Results

Primary screening of thirty-six peptides, endocrine peptides, angiotensin-related peptides, the putative substrates for the AT_4 receptor (vasopressin and oxytocin), the putative neurolysin specific inhibitor, Pro-Ile, and Plummer's inhibitor, revealed seven peptides with IC_{50} values < 10 μM for both brain and testis. Three of the peptides were orphanin FQ, Y^1 orphanin FQ, and Y^{14} orphanin FQ. Of the three orphanin FQ peptides, only native orphanin FQ was selected for a representative secondary screen. Secondary screenings were also performed on ghrelin, S36057, and Pro-Ile (Figure 1B, C, and D). Primary amino acid sequences for these neuropeptides and angiotensin peptides were aligned for comparison (Table 2). In addition, IC_{50} values for Ang II and SI Ang II were determined for comparison. Only Ang II and SI Ang II showed IC_{50} values less than 1 μM (Figure 1A). Percent specific binding of ^{125}I-SI Ang II in the presence of each of these peptides was determined at concentrations, ranging from 0.1 μM to 1mM. The IC_{50} values for the peptides were similar in brain and testis (Table 1) with overlapping 95% confidence intervals for Log IC_{50} values.

Dynorphin A (1–8) exhibited 69±9% and 82±3% inhibition of ^{125}I-SI Ang II binding in brain and testis, respectively. Assuming a one-site competition model the extrapolated IC_{50} values are 4.5 and 2.2 μM for brain and testis, respectively. Ang (1–9) exhibited 59±3 and 75±2% inhibition of ^{125}I-SI Ang II binding in brain and testis, respectively. Assuming a one-site competition model the extrapolated IC_{50} values are 7.0 and 3.3 μM for brain and testis, respectively.

The 3–7 fragment of Ang II showed less than 10% inhibition of ^{125}I-SI Ang II binding in brain and testis at 10 μM. The selective neurolysin inhibitor Pro-Ile also failed to inhibit ^{125}I-SI Ang II binding in brain and testis at concentrations up to 1 mM. This value is well above the reported K_I value (90 μM [14]) of Pro-Ile for neurolysin.

Discussion

The main purpose of this study was to further characterize the pharmacological specificity of the novel non-AT_1, non-AT_2 binding site in the rodent brain and testis [6,9] which has been subsequently identified as neurolysin [10]. Competitive binding assays were performed using thirty-six different peptides and a specific peptidase inhibitor to determine whether this binding site is specific for angiotensins or if it has broader ligand/substrate specificity. Primary screening of the thirty-six peptides showed only S36057, orphanin FQ and its analogs, ghrelin, dynorphin A (1–8), and angiotensin (1–9) to inhibit greater than 50% at 10μM in both brain and testis. Secondary screenings performed on three of these peptides indicated IC_{50} values between 1 and 10 μM, except for orphanin FQ in the brain which was slightly greater than 10 μM.

All tested peptides (Table 1) are grouped according to physiological relevance. Orphanin FQ, also known as nociceptin is a heptadecapeptide which has a primary structure reminiscent of that of opioid peptides[15] and has structural homology with the dynorphin family [16].Orphanin FQ is an endogenous ligand that also inhibits adenylyl cyclase activity. The name nociceptin was derived from its ability to induce enhanced reactivity to noxious thermal stimuli. Studies on rats have shown that orphanin FQ decreases systemic arterial pressure in a dose–related manner [17]. Tyr^1 orphanin FQ, an analog of nociceptin, was also found to behave similarly in terms of vasodepressor activity and potency [16].

Ghrelin is a 28 amino acid peptide that acts as an endogenous orexigenic hormone. This peptide is widely distributed in various tissues, such as the intestines, hypothalamus, and pancreas, along with its mRNA. Studies have also shown ghrelin to have multiple biological functions, including vasodilation and energy homeostasis [18]. S36057 has been classified as a potent ligand for the melanin-concentrating hormone receptor [19]. Melanin-concentrating hormone also has orexigenic actions, as well as reproductive and stress-related signaling activity [20].

The amino acid sequences of orphanin FQ, ghrelin and S36057 do not reveal a recognition motif identical to known neurolysin cleavage sites [21–23], however, these peptides like most neurolysin substrates are primarily basic, and have aromatic amino acids and/or prolines. Given the extensive diversity of amino acids that can mimic the P_3, P_2, P_1, P_1`, $P_2$` and P_3` sites of the substrates of neurolysin, these peptides might easily be previously unidentified substrates of neurolysin.

Dynorphin A (1–8) whose first 5 amino acids contain the structure of leucine-enkephalin, is reported to be a substrate of neurolysin [23–26]. Dynorphin A (1–8) is reported to be cleaved at the 5–6 bond by neurolysin to form the endogenous opioid peptide leucine-enkephalin[23,25,26]. As such, only 5 amino acids must be accommodated in the putative substrate binding channel (as described from its crystal structure [22]) distal to the active site.

Angiotensin (1–9) is a likely substrate of neurolysin in view of the high affinity binding of Ang I and Ang II to this protein [6]. Angiotensin (1–9) is formed by the action of angiotensinconverting enzyme 2 (ACE2) albeit at a relatively slow rate compared to the formation of Ang (1–7) from Ang II [27,28]. It is not certain whether neurolysin forms Ang (1–7) or Ang (1–4) and Ang (5–9) from Ang (1–9). If the cleavage site is at the 4–5 bond,

then only 4 amino acids must be accommodated in the putative substrate binding channel [22] distal to the active site. In contrast, the 3–7 fragment of Ang II showed negligible inhibition of ^{125}I-SI Ang II binding in brain and testis, consistent with a previous observation in mouse brain [7]. This suggests that the arginine^{2} at the P_3 position relative to neurolysin is of critical importance for Ang II binding. It is noteworthy that the acidic carboxy terminal of PCMB has the potential to form an ionic bond with a basic amino acid, such as arginine. This potential for ionic bond formation may be compromised by the aspartic acid^{1} of Ang II based upon the higher binding affinities of Ang III and Sar^{1}, Ile^{8}Ang II for this protein relative to Ang II [6,7]. Additionally, the Ang (3–7) and (1–9) data suggests that having a phenylalanine or isoleucine as the carboxy terminal amino acid at the $P`_4$ position is also critical to high affinity binding of Ang II to neurolysin.

In contrast, several neuropeptides reported to be substrates for neurolysin; urocortin, neuromedin B [29], and somatostatin [23,30] inhibited ^{125}I-SI Ang II binding less than 25% at a concentration of 10 μM. Of note, there is one report claiming that human neurolysin does not metabolize somatostatin [31]. Orexin B, which is also reported to be a substrate of neurolysin [29] displayed a slightly greater inhibition of ^{125}I-SI Ang II binding, 31 and 41% in brain and testis, respectively.

The putative cleavage sites for urocortin, neuromedin B, somatostatin and orexin B by neurolysin are 13, 9, 6 or 10, and 6 amino acids from the amino terminal of the parent peptide, respectively [23,29]. This portion of these peptides may not be able to fit into the bottom of the ligand binding channel [22] when PCMB is bound to a cysteine sulfhydryl distal to the active site of neurolysin as proposed (Santos et al., submitted for publication).

Pro-Ile has been characterized as a specific albeit low affinity inhibitor of neurolysin. Its K_I is reported to be 90 μM and has been used at concentrations up to 5 mM to selectively inhibit neurolysin activity relative to other endopeptidases [14]. Surprisingly, Pro-Ile did not inhibit ^{125}I-SI Ang II binding at concentrations up to 1mM. This observation is reminiscent of previous studies of this non-AT_1, non-AT_2 binding protein in mouse brain in which it was shown that another neurolysin inhibitor, JA-2, also did not compete for ^{125}I-SI Ang II binding [7].

Previous studies of the affinity of this PCMB unmasked Ang II binding site for substrates of neurolysin also failed to reveal a specificity characteristic of neurolysin [6]. This suggests that the alteration of neurolysin by PCMB such as to unmask its ability to bind Ang II with high affinity is a pharmacological effect to enhance its affinity for Ang II, while diminishing its affinity for other substrates and Pro-Ile.

Originally described as an inhibitor of angiotensinase C (now known as prolylcarboxypeptidase) [32], PCMB has also been reported to inhibit the catalytic activity of neurolysin [33]. Neurolysin has been shown to metabolize Ang II as well as Ang I [22,23,25,34,35]. The products of Ang II metabolism by neurolysin are the inactive, (1–4) and (5–8) fragments of Ang II [22,23]. The product of Ang I metabolism by neurolysin is Ang (1–7) [25,36]. By degrading Ang II as well as forming Ang (1–7), neurolysin can antagonize the well-known pathophysiological effects of Ang II mediated by the AT_1 receptor [37]. However, there are two reports [26,36] that Ang II is not a substrate of neurolysin despite having K_I values of 4.9 and 2.4 μM for rat and human neurolysin. This suggests that Ang II could be an endogenous inhibitor of neurolysin. Thus, neurolysin may play an important role in the regulation of the RAS. Conversely, neurolysin may be regulated by the RAS, thereby affecting the functionality of other peptidergic systems in the brain and other tissues. Both mechanisms warrant further study of the interactions of the renin-angiotensin system with neurolysin.

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Reference List

1. Tamura K, Umemura S, Fukamizu A, Ishii M, Murakami K. Recent advances in the study of renin and angiotensinogen genes: from molecules to the whole body. Hypertens. Res. 1995; 18:7–18. [PubMed: 7584914]
2. Zhou A, Carrell RW, Murphy MP, Wei Z, Yan Y, Stanley PL, Stein PE, Pipkin FB, Read RJ. A redox switch in angiotensinogen modulates angiotensin release. Nature. 2010; 468:108–111. [PubMed: 20927107]
3. Schmieder RE, Hilgers KF, Schlaich MP, Schmidt BM. Renin-angiotensin system and cardiovascular risk. Lancet. 2007; 369:1208–1219. [PubMed: 17416265]
4. Wright JW, Harding JW. Brain renin-angiotensin--a new look at an old system. Prog. Neurobiol. 2011; 95:49–67. [PubMed: 21777652]
5. Albiston AL, McDowall SG, Matsacos D, Sim P, Clune E, Mustafa T, Lee J, Mendelsohn FA, Simpson RJ, Connolly LM, Chai SY. Evidence that the angiotensin IV (AT4) receptor is the enzyme insulin regulated aminopeptidase . J. Biol. Chem. 2001; 276:48623–48626. [PubMed: 11707427]
6. Karamyan VT, Speth RC. Identification of a novel non-AT1, non-AT2 angiotensin binding site in the rat brain. Brain Res. 2007; 1143:83–91. [PubMed: 17306233]
7. Karamyan VT, Gembardt F, Rabey FM, Walther T, Speth RC. Characterization of the brain-specific non-AT(1), non-AT(2) angiotensin binding site in the mouse. Eur. J Pharmacol. 2008; 590:87–92. [PubMed: 18571643]
8. Karamyan VT, Arsenault J, Escher E, Speth RC. Preliminary biochemical characterization of the novel, non-AT1, non-AT2 angiotensin binding site from the rat brain. Endocrine. 2010; 37:442–448. [PubMed: 20960166]
9. Rabey FM, Karamyan VT, Speth RC. Distribution of a novel binding site for angiotensins II and III in mouse tissues. Regul. Pept. 2010; 162:5–11. [PubMed: 20171994]
10. Wangler NJ, Santos KL, Schadock I, Hagen FK, Escher E, Bader M, Speth RC, Karamyan VT. Identification of membrane-bound variant of metalloendopeptidase neurolysin (EC 3.4.24.16) as the non-AT1, non-AT2 angiotensin binding site. J. Biol. Chem. 2012; 287:1014–1022.
11. Sugiura N, Hagiwara H, Hirose S. Molecular cloning of porcine soluble angiotensinbinding protein. J. Biol. Chem. 1992; 267:18067–18072. [PubMed: 1517239]
12. Kato A, Sugiura N, Saruta Y, Hosoiri T, Yasue H, Hirose S. Targeting of endopeptidase 24.16 to different subcellular compartments by alternative promoter usage. J. Biol. Chem. 1997; 272:15313–15322. [PubMed: 9182559]
13. Rashid M, Arumugam TV, Karamyan VT. Association of the novel non-AT1, non- AT2 angiotensin binding site with neuronal cell death. J. Pharmacol. Exp. Ther. 2010; 335:754–761. [PubMed: 20861168]
14. Dauch P, Vincent JP, Checler F. Specific inhibition of endopeptidase 24.16 by dipeptides. Eur. J. Biochem. 1991; 202:269–276. [PubMed: 1761032]
15. Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ Jr. Civelli O. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Sci. 1995; 270:792–794.
16. Champion HC, Kadowitz PJ. [Tyr1]-nociceptin, a novel nociceptin analog, decreases systemic arterial pressure by a naloxone-insensitive mechanism in the rat. Biochem. Biophys. Res. Commun. 1997; 234:309–312. [PubMed: 9177265]
17. Champion HC, Kadowitz PJ. Nociceptin, an endogenous ligand for the ORL1 receptor, has novel hypotensive activity in the rat. Life Sci. 1997; 60:L-5.

18. Sato T, Nakamura Y, Shiimura Y, Ohgusu H, Kangawa K, Kojima M. Structure, regulation and function of ghrelin. J. Biochem. 2012; 151:119–128. [PubMed: 22041973]
19. Audinot V, Lahaye C, Suply T, Beauverger P, Rodriguez M, Galizzi JP, Fauchere JL, Boutin JA. [125I]-S36057: a new and highly potent radioligand for the melanin-concentrating hormone receptor. Br. J. Pharmacol. 2001; 133:371–378. [PubMed: 11375253]
20. Griffond B, Baker BI. Cell and molecular cell biology of melanin-concentrating hormone . Int. Rev. Cytol. 2002; 213:233–277. [PubMed: 11837894]
21. Shrimpton CN, Smith AI, Lew RA. Soluble metalloendopeptidases and neuroendocrine signaling . Endocr. Rev. 2002; 23:647–664. [PubMed: 12372844]
22. Brown CK, Madauss K, Lian W, Beck MR, Tolbert WD, Rodgers DW. Structure of neurolysin reveals a deep channel that limits substrate access. Proc. Natl. Acad. Sci. U. S. 2001; A 98:3127–3132.
23. Dahms P, Mentlein R. Purification of the main somatostatin-degrading proteases from rat and pig brains, their action on other neuropeptides, and their identification as endopeptidases 24.15 and 24.16. Eur. J. Biochem. 1992; 208:145–154. [PubMed: 1355047]
24. Barelli H, Vincent JP, Checler F. Peripheral inactivation of neurotensin. Isolation and characterization of a metallopeptidase from rat ileum. Eur. J. Biochem. 1988; 175:481–489. [PubMed: 3409880]
25. Rioli V, Kato A, Portaro FC, Cury GK, te KK, Vincent B, Checler F, Camargo AC, Glucksman MJ, Roberts JL, Hirose S, Ferro ES. Neuropeptide specificity and inhibition of recombinant isoforms of the endopeptidase 3.4.24.16 family: comparison with the related recombinant endopeptidase 3.4.24.15. Biochem. Biophys. Res. Commun. 1998; 250:5–11. [PubMed: 9735321]
26. Barelli H, Vincent JP, Checler F. Rat kidney endopeptidase 24.16. Purification, physico-chemical characteristics and differential specificity towards opiates, tachykinins and neurotensin-related peptides. Eur. J. Biochem. 1993; 211:79–90. [PubMed: 8425555]
27. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9. Circ. Res. 2000; 87:E1–E9. [PubMed: 10969042]
28. Rice GI, Thomas DA, Grant PJ, Turner AJ, Hooper NM. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. Biochem. J. 2004; 383:45–51. [PubMed: 15283675]
29. Kadonosono T, Kato M, Ueda M. Substrate specificity of rat brain neurolysin disclosed by molecular display system and putative substrates in rat tissues. Appl. Microbiol. Biotechnol. 2007; 75:1353–1360. [PubMed: 17401561]
30. Mentlein R, Dahms P. Endopeptidases 24.16 and 24.15 are responsible for the degradation of somatostatin, neurotensin, and other neuropeptides by cultivated rat cortical astrocytes. J. Neurochem. 1994; 62:27–36. [PubMed: 7903352]
31. Krause DR, Piva TJ, Brown SB, Ellem KAO. Characterization and localization of mitochondrial oligopeptidase (MOP) (EC 3.4.24.16) activity in the human cervical adenocarcinoma cell line HeLa. J. Cell Biochem. 1997; 66:297–308. [PubMed: 9257187]
32. Tan F, Morris PW, Skidgel RA, Erdos EG. Sequencing and cloning of human prolylcarboxypeptidase (angiotensinase C). Similarity to both serine carboxypeptidase and prolylendopeptidase families. J. Biol. Chem. 1993; 268:16631–16638. [PubMed: 8344943]
33. Serizawa A, Dando PM, Barrett AJ. Characterization of a mitochondrial metallopeptidase reveals neurolysin as a homologue of thimet oligopeptidase. J. Biol. Chem. 1995; 270:2092–2098. [PubMed: 7836437]
34. Barelli H, Girard F, St PS, Kitabgi P, Vincent JP, Checler F. Further characterization of a neurotensin-degrading neutral metalloendopeptidase from rat brain. Neurochem. Int. 1988; 12:351–359. [PubMed: 20501238]
35. Kato A, Sugiura N, Hagiwara H, Hirose S. Cloning, amino acid sequence and tissue distribution of porcine thimet oligopeptidase. A comparison with soluble angiotensinbinding protein. Eur. J. Biochem. 1994; 221:159–165. [PubMed: 8168506]

36. Vincent B, Vincent JP, Checler F. Purification and characterization of human endopeptidase 3.4.24.16. Comparison with the porcine counterpart indicates a unique cleavage site on neurotensin. Brain Res. 1996; 709:51–58. [PubMed: 8869556]

37. Stegbauer J, Coffman TM. New insights into angiotensin receptor actions: from blood pressure to aging. Curr. Opin. Nephrol. Hypertens. 2011; 20:84–88. [PubMed: 21076298]

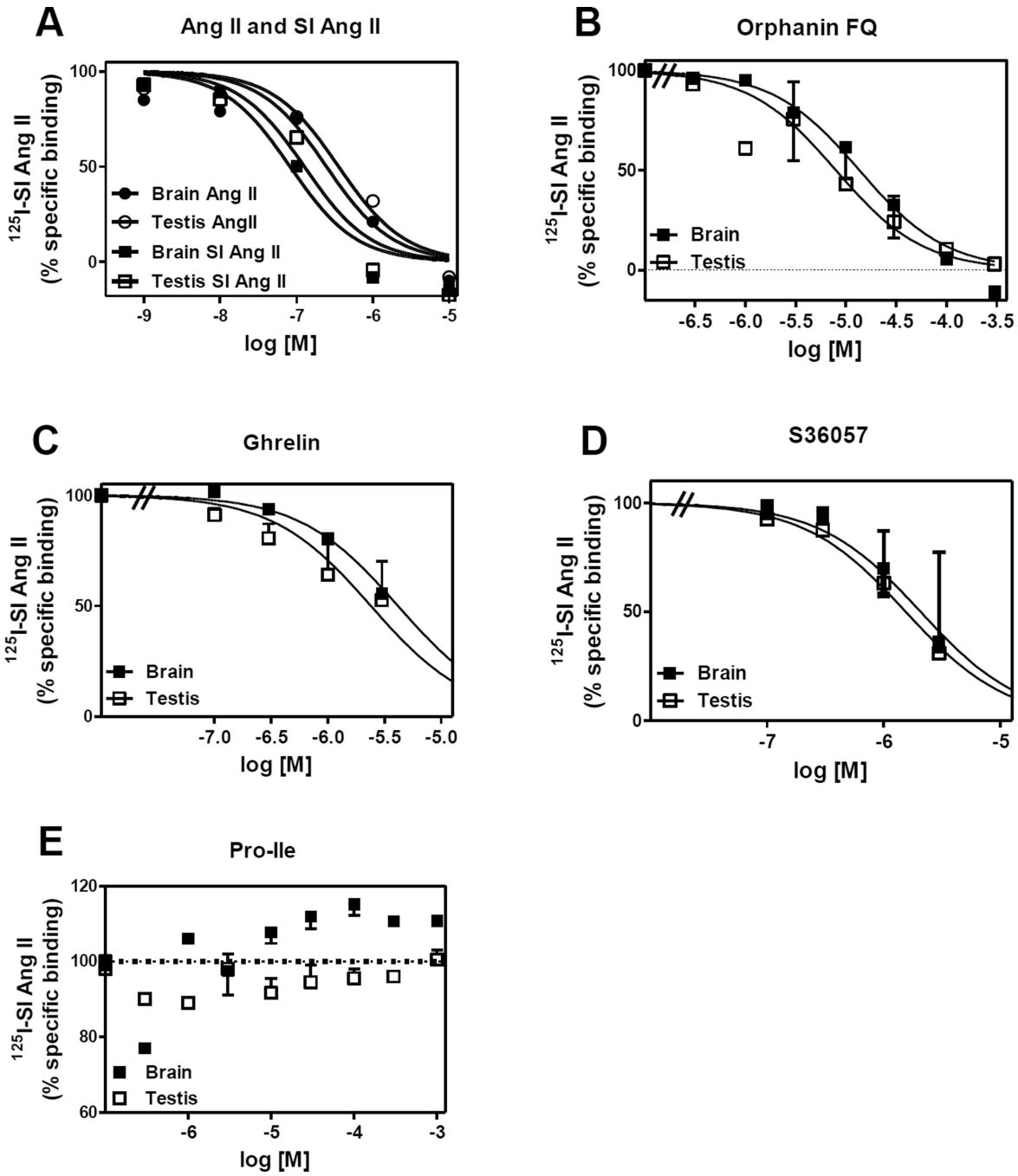

Figure 1. Competition for ^{125}I-SI Ang II binding to novel non-AT$_1$, non-AT$_2$binding site

Panel A: IC_{50} values for Ang II were 246 nM in brain and 356 nM in testis. The IC_{50} values for SI Ang II were 82.2 nM in brain and 126 nM in testis. The respective Log IC_{50} values for Ang II in brain and testis were −6.61 ± 0.21 and −6.46 ± 0.17. The respective Log IC_{50} values for SI Ang II in brain and testis were −7.08 ± 0.18 and −6.90 ± 0.22. Panel B: IC_{50} values for orphanin FQ were 13.6 μM in brain and 7.95μM in testis. The respective Log IC_{50} values in brain and testis were −4.87 ± 0.14 and −5.10 ± 0.135. Panel C: IC_{50} values for ghrelin were 4.15 μM in brain and 2.39 μM in testis. The respective Log IC_{50} values in brain and testis were −5.38 ± 0.07 and −5.62 ± 0.13. Panel D: IC_{50} values for Tyr^1 S36057 were 2.07 μM in brain and 1.54 μM in testis. The respective Log IC_{50} values in brain and testis

are −5.68 ± 0.11 and −5.81 ± 0.23. Panel E: Competition curves for Pro-Ile showed no inhibition of ^{125}I-SI Ang II binding at concentrations up to 1 mM. Standard error bars for testis are shown in an upward direction and for brain they are shown in a downward direction. Solid symbols represent competition for brain binding, and open symbols represent competition for testis binding.

Table 1

Summary of tested neuropeptides and respective IC_{50} determinations

IC_{50} Determinations for Thirty-six peptides and related compounds		
	Brain (μM)	**Testis (μM)**
Neuropeptides and Analogs *		
Oxytocin	>10	>10
Vasopressin	>10	>10
Orphanin FQ	13.6	7.95
Tyr^{14} Orphanin FQ	**<10**	**<10**
Tyr^{1}Orphanin FQ	**<10**	**<10**
Neuropeptide Y (NPY)	>10	>10
Calcitonin Gene-Related Protein (CGRP)	>10	>10
Galanin-like Peptide (GALP)	>10	>10
Met enkephalin	>10	>10
[d-Pen^{2},d-Pen^{5}] Enkephalin (DPDPE) **	>10	>10
Dynorphin A (1–8)	**<10**	**<10**
Pancreatic Polypeptide (PPP)	>10	>10
Somatostatin	>10	>10
Melanin-related		
Y^{1}S36057	2.07	1.54
MCH (D Phe^{13}tyr)	>10	>10
Endocrine-related		
Ghrelin	4.15	2.39
alpha-Melanocyte Stimulating Hormone (α-MSH)	>10	>10
Cholecystokinin-8 (CCK-8)	>10	>10
Thyroid Releasing Hormone (TRH)	>10	>10
HPP-HOE 140	>10	>10
D-Ala^{6} des Gly^{10} LHRH	>10	>10
YFMRF amide	>10	>10
FMRF amide	>10	>10
ArgPheethylamide	>10	>10
ArgPhe	>10	>10
Tyr^{0} C-type natriuretic peptide (Y^{0} CNP-22)	>10	>10
Angiotensins		
Angiotensin 1 Pro^{11}, D-Ala^{12}	>10	>10
Angiotensin 3–7	>10	>10
Angiotensin 1–9	<10	<10
HPP SDKP	>10	>10
Neurolysin substrates		

IC_{50} Determinations for Thirty-six peptides and related compounds		
	Brain (μM)	**Testis (μM)**
Orexin B	>10	>10
Urocortin	>10	>10
Neuromedin B	>10	>10
Others		
Plummer's inhibitor	>10	>10
Pro-Ile	>10	>10
Tyrosine	>10	>10

Peptides in bold font exhibited > 50% inhibition at 10 μM but were not evaluated in a secondary screen.

*Functions of many of these peptides can include pituitary and peripheral signaling.

**Oxidized form

Table 2

Primary Amino Acid Sequences of Selected Peptides

Amino Acid Order	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Tyr[1] S36057	Tyr	*ADO	Arg	Cys	Met	Leu	Gly	Arg	Val	Phe	Arg	Pro	Cys	Trp			
Orphanin FQ	Phe	Gly	Gly	Phe	Thr	Gly	Ala	Arg	Lys	Ser	Ala	Arg	Lys	Leu	Ala	Asn	Gln
Tyr[1] Orphanin FQ	Tyr	Gly	Gly	Phe	Thr	Gly	Ala	Arg	Lys	Ser	Ala	Arg	Lys	Leu	Ala	Asn	Gln
Tyr[14] Orphanin FQ	Phe	Gly	Gly	Phe	Thr	Gly	Ala	Arg	Lys	Ser	Ala	Arg	Lys	Tyr	Ala	Asn	Gln
Dynorphin A (1–8)	Tyr	Gly	Gly	Phe	Leu	Arg	Arg	Ile									
Ang I	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu							
Ang (1–9)	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His								
Ang II	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe									
Sar[1], Ile[8] Ang II	Sar	Arg	Val	Tyr	Ile	His	Pro	Ile									
Pro-Ile							Pro	Ile									
Ghrelin	Gly	Ser	Ser (octanoyl)	Phe	Leu	Ser	Pro	Glu	His	Gln	Lys	Ala	Gln	Gln	Arg	Lys	Glu
Amino Acid Order	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**						
Ghrelin continued	Ser	Lys	Lys	Pro	Pro	Ala	Lys	Leu	Gln	Pro	Arg						

*ADO is 8-amino-3, 6-dioxy octanoyl