Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: Predominant role of angiotensin III in the control of vasopressin release

(hypothalamus/mercapto-inhibitors/mouse/renin-angiotensin system/zinc metalloproteases)

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ABSTRACT Angiotensin (Ang) II and Ang III are two peptide effectors of the brain renin-angiotensin system that participate in the control of blood pressure and increase water consumption and vasopressin release. In an attempt to delineate the respective roles of these peptides in the regulation of vasopressin secretion, their metabolic pathways and their effects on vasopressin release were identified in vivo. For this purpose, we used recently developed selective inhibitors of aminopeptidase A (APA) and aminopeptidase N (APN), two enzymes that are believed to be responsible for the N-terminal cleavage of Ang II and Ang III, respectively. Mice received [3H]Ang II intracerebroventricularly (i.c.v.) in the presence or absence of the APA inhibitor, EC33 (3-amino-4-thiobutyl sulfonate) or the APN inhibitor, EC27 (2-amino-pentan-1,5-dithiol). [3H]Ang II and [3H]Ang III levels were evaluated from hypothalamus homogenates by HPLC. EC33 increased the half-life of [3H]Ang II 2.6-fold and completely blocked the formation of [3H]Ang III, whereas EC27 increased the half-life of [3H]Ang III 2.3-fold. In addition, the effects of EC33 and EC27 on Ang-induced vasopressin release were studied in mice. Ang II was injected i.c.v. in the presence or absence of EC33, and plasma vasopressin levels were estimated by RIA. While vasopressin levels were increased 2-fold by Ang II (5 ng), EC33 inhibited Ang II-induced vasopressin release in a dose-dependent manner. In contrast, EC27 injected alone increased in a dose-dependent manner vasopressin levels. The EC27-induced vasopressin release was completely blocked by the coadministration of the Ang receptor antagonist (Sar2-Ala6) Ang II. These results demonstrate for the first time that (i) APA and APN are involved in vivo in the metabolism of brain Ang II and Ang III, respectively, and that (ii) the action of Ang II on vasopressin release depends upon the prior conversion of Ang II to Ang III. This shows that Ang III behaves as one of the main effector peptides of the brain renin-angiotensin system in the control of vasopressin release.

Several lines of evidence support that a renin-angiotensin system (RAS) is present in the central nervous system and controls cardiovascular functions and body fluid homeostasis. All components of this system, including precursors and enzymes required for the formation and degradation of angiotensins (Angs), as well as Ang receptors, have been identified within the brain. However, their physiological functions are far from being completely understood. Two of the main bioactive peptides of the RAS are believed to be Ang(1–8) (Ang II) and its direct metabolite, Ang(2–8) (Ang III), both exhibiting the same affinity for type 1 (AT1) and type 2 (AT2) Ang II receptors (for reviews, see refs. 1 and 2).

If injected into cerebral ventricles, Ang II and Ang III cause pressor and dipogeneric effects, and salt appetite and also stimulate pituitary hormone release (3). However, other Ang fragments such as Ang(1–7) and Ang(3–8) could be effectors of the brain RAS (4, 5). Ang immunoreactive nerve cells in the subfornical organ send efferent fibers, directly or via the median preoptic nucleus, to synapse in the paraventricular nucleus and the supraoptic nucleus (6, 7). Subfornical organ stimulation may result in the release of peptides from angiotensinergic nerve terminals in these two nuclei, suggesting a role of Angs as neurotransmitter (8, 9). One consequence of the injection of Angs in the paraventricular and the supraoptic nucleus is the stimulation of magnocellular neurons projecting to the posterior pituitary, thus releasing vasopressin in the peripheral circulation (10–13). The respective roles of Ang II and Ang III in the control of these biological responses have not been clarified. Several studies suggest that Ang III could be the true effector of this system and that Ang II must be converted to Ang III to exert its biological effects (14–17). However, no definitive proof of this hypothesis has been provided.

Among the enzymes able to hydrolyze Ang II and Ang III, two aminopeptidases, aminopeptidase A (APA, EC 3.4.11.7) and aminopeptidase N (APN, EC 3.4.11.2), could be good candidates. Both are two zinc metalloproteases that belong to thermolysin-like enzyme group with significant identity between their amino-acid sequences (18, 19). In vitro, APA hydrolyzes the N-terminal asparagine of Ang II to generate Ang III (20–22), whereas APN hydrolyzes the N-terminal arginine of Ang III to generate Ang IV (20, 23). Infused intracerebroventricularly (i.c.v.), APA and APN produced changes in blood pressure, suggesting their involvement in the metabolism of cerebral Angs (24). In an attempt to define whether APA and APN are able to hydrolyze in vivo Angs in the brain, we have recently developed highly selective aminopeptidase inhibitors: the compound EC33(18U.S.C. §1734) specifically inhibits APA while the compound EC27 [(S)-2-amino-pentan-1,5-dithiol] specifically inhibits APN (25, 26) (Fig. 1). Using these new tools, we examined (i) the physiological involvement of APA and APN in the metabolism of Ang II and Ang III in the mouse brain and (ii) the respective roles of Ang II and Ang III in the central regulation of vasopressin release.

Abbreviations: Ang, angiotensin; AT1 and AT2, angiotensin receptor type 1 and type 2, respectively; APA and APN, aminopeptidase A and N, respectively; i.c.v., intracerebroventricularly; RAS, renin-angiotensin system.

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Angiotensin II
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

EC33

Angiotensin III
Arg-Val-Tyr-Ile-His-Pro-Phe

EC27

Angiotensin IV
Val-Tyr-Ile-His-Pro-Phe

EC33, APA inhibitor; EC27, APN inhibitor.

MATERIALS AND METHODS

Drugs. EC33 ([R,S]-3-amino-4-thiol-butyl sulfonate) and EC27 ([S]-2-amino-pentan-1,5-dithiol) were synthesized by the laboratory of B.P.R. as described (25, 26). [3H]Ang II ([19oxyl-
3,5-3H]Ang II, 60 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham. (Sar1-Ala8)-Ang II, human Ang II, and human Ang III were purchased from Sigma.

Animals. Male Swiss mice (Iffa Credo) weighing 18-20 g were kept under artificial light (12 h light/12 h dark cycle) with food and water ad libitum. Experiments were performed between 9 and 11 a.m. Drugs were administered in the lateral ventricle by i.c.v. route in a volume of 5 µl per mouse according to the method of Haley and McCormick (27).

In Vivo Metabolism Studies. Intracerebroventricular injections. For each experiment, 6-8 mice were used for each condition. A mixture of 2.106 cpmp [3H]Ang II and 30 µg unlabeled Ang II was administered under a volume of 5 µl, either alone or with EC33 or EC27 (30 µg). Drugs were dissolved in isotonic saline solution adjusted to pH 7.4 with 0.01 M NaOH. At different times after injection, mice were decapitated, their brains were immediately removed, and the hypothalamus was dissected out and homogenized by sonication (Ultra-sons, Annemasse, France) in 10 vol of ice-cold 0.1 M HCl. The homogenate was centrifuged 12,000 × g × 20 min at 4°C and the supernatant was collected and kept at −80°C until analysis.

Separation of Ang II and Ang III by cation-exchange chromatography. To determine the amount of [3H]Ang II and [3H]Ang III present in the hypothalamus, we used the procedure of Ledwith et al. (28) with slight modifications. Briefly, SP-Sepharose Fast Flow (1 ml bed volume, Pharmacia) was packed in 3-ml syringe columns and equilibrated at room temperature in 15 ml of equilibration buffer (10 mM sodium acetate/50 mM NaCl, pH 5.0). After thawing, hypothalamic extracts containing about 30,000 cpmp were diluted in 2 ml of equilibration buffer, loaded onto columns, and washed with 1 ml of equilibration buffer. A first fraction (F1) containing [3H]Ang II and other [3H]Ang metabolites was eluted with 7 ml of 10 mM sodium acetate/80 mM NaCl/5% acetonitrile, pH 5. A second fraction (F2) containing [3H]Ang III alone was eluted with 3 ml of 3 M HCl. F1 and F2 fractions were then analyzed by HPLC for their contents of [3H]Ang II and [3H]Ang III, respectively.

C18 Sep-Pak purification. For HPLC analysis, F1 and F2 were initially concentrated by extraction on C18 Sep-Pak Cartridges (Waters). Trifluoroacetic acid (1% final in water) was added to F1 that was loaded onto the Sep-Pak that was first equili-

brated with 1% trifluoroacetic acid in water. Radioactivity was eluted with 1.5 ml 100% acetonitrile. Under this condition, the recovery for Ang II was 75%. F2 was loaded onto the Sep-Pak first equilibrated with 2 ml 100% methanol followed by 5 ml H2O. The radioactivity was eluted with 1.5 ml 100% methanol. Recovery of Ang III was then 92%. After Sep-Pak elution, F1 and F2 were lyophilized, dissolved in 0.2 ml of 0.01 M acetic acid with 20 ng Ang II and Ang III as internal standards, and analyzed by HPLC.

HPLC analysis. HPLC was carried out using an Hypersil ODS-3 µm reverse phase column (Shandon, Pittsburgh) heated at 45°C followed by isocratic elution at a flow rate of 0.6 ml/min. The mobile phase consisted of 86 mM H3PO4 adjusted to pH 3 with triethylamine, plus 17.5% acetonitrile. The HPLC system had pumps, an autosampler, and a UV detector from Waters. After sample injection (0.15 ml), fractions (0.2 ml) were collected for 12 min using a Gilson 202 fraction collector, and their radioactivity was estimated with a beta spectrometer. Standards [Ang II, Ang III, Ang II-(4-8), and Ang II-(3-8); 50 ng in 0.01 M acetic acid] were routinely run after every sixth sample. Under these conditions, standard retention times were 7 min for Ang II-(4-8), 7.8 min for Ang III, 9.5 min for Ang II, and 10.7 min for Ang II-(3-8).

Effect of i.c.v. Injection of Ang II or Ang III on Plasma Vasopressin Levels. Intracerebroventricular injections. Mice (n = 5 to 10 animals per group) received a single dose of 5 µl of saline or the following drugs: Ang II or Ang III (0.5–5 ng), EC33 (3–30 µg), or EC27 (3–100 µg). Mice were decapitated 1 min after the injection, and trunk blood (0.5–1 ml) was collected in chilled tubes containing 50 µl of 0.3 M EDTA. Samples were stored on ice for a maximum of 15 min before centrifugation (1600 × g for 15 min) at 4°C. Plasma (0.2 ml) was collected and transferred to polypropylene tubes containing 50 µl of 3 M HCl and kept at −80°C until vasopressin RIA.

Vasopressin RIA. Vasopressin concentrations were estimated from 0.2 ml of plasma using a specific RIA kit (Peninsula Laboratories) using (3-[125I]iodotyrosyl-2)vasopressin-[Arg8], 2000 Ci/mmol) as a tracer and a polyclonal antiserum specific for vasopressin-[Arg8] with no cross reactivity with oxytocin. Vasopressin was extracted from the plasma with C18 Sep-Pak cartridges and measured in duplicate according to the protocol of the kit. The limit of detection of the vasopressin was 0.2 pg per tube.

Data and Statistical Analysis. For in vivo metabolism experiments, [3H]Ang II and [3H]Ang III levels were expressed as the percentage of total radioactivity present in the hypothalamus. Estimation of the kinetic parameters was performed by fitting the data to a nonlinear regression model (least-squares criterion, Gauss Newton algorithm) using a commercially available software (29). Half-lives of disappearance of labeled Angs were calculated from the elimination rate constants (k1) according to the following equation: ln(1/k1 2/k1. Plasma vasopressin concentrations were estimated in pg/ml plasma and results were expressed as the percentage of the control values. Results were expressed in terms of mean ± SEM. Statistical comparisons were performed using a one-way ANOVA followed by Fisher’s test.

RESULTS

Separation of Ang II and III by Cation-Exchange Chromatography and HPLC Analysis. Initial experiments were designed to determine elution conditions on SP-Sepharose that allowed the separation of [3H]Ang III from [3H]Ang II-like peptides. For this purpose, mice were injected with [3H]Ang II; 2 min later, the mice were killed and their hypothalamic extracts were prepared. The tissue extract containing [3H]Ang II and its metabolites was loaded onto the SP-Sepharose column in the presence of a mixture of synthetic standard peptides [Ang II, Ang III, Ang III-(3-8), and Ang II-(4-8)].
Two fractions, F1 and F2, were eluted, F1 with 80 mM NaCl and F2 with 3 M HCl. HPLC analysis of F1 showed that [3H]Ang II coeluted with Ang II-(4–8) and Ang II-(3–8) standards (Fig. 24), and also that [3H]Ang II coeluted with other unidentified radioactive Ang fragments (Fig. 2B). In contrast, the Ang III standard and [3H]Ang III were clearly eluted in the F2 fraction (Fig. 2 C and D). Therefore, in further experiments, [3H]Ang II levels were routinely estimated directly by measuring the radioactivity in the F2 fraction, whereas [3H]Ang II levels were estimated in the F1 fraction after HPLC analysis.

In Vivo Study of Ang II and Ang III Metabolism in the Mouse Hypothalamus. Injection of the inhibitors EC33 or EC27 did not modify the total amount of radioactivity found in the hypothalamus. The time course of [3H]Ang II levels in the hypothalamus after i.c.v. injection of [3H]Ang II, in the absence (control values), or presence of EC33 (the APA inhibitor) is shown in the Fig. 3. In control mice, [3H]Ang II levels decreased rapidly after 1.5 min and were undetectable after 7 min. In the presence of EC33, [3H]Ang II levels were significantly higher between 0.5 min to 7 min, reaching about 10-fold the control levels. The semilogarithmic representation of the rate of disappearance of [3H]Ang II levels, in the presence of EC33 versus the control is shown in Fig. 3 Insert. The best fit of both kinetics corresponded to a first-order elimination model ($r = 0.994$ and $0.998$ for the control and EC33, respectively). In animals treated with EC33, the [3H]Ang II half-life ($5.38 \pm 0.21$ min) was significantly increased 2.61-fold when compared with the control value ($2.06 \pm 0.23$ min, $P < 0.01$). The time course of [3H]Ang III levels in the hypothalamus after i.c.v. injection of [3H]Ang II, in the absence (control values) or presence of EC33 or EC27 (the APN inhibitor) is shown in Fig. 4. In control mice, [3H]Ang III levels could be detected in the hypothalamus from 0.5 min. The formation of [3H]Ang III was maximal at 1 min and then levels decreased progressively until 10 min. In contrast, with EC33, the formation of [3H]Ang III was immediately blocked and very low levels were measured over the time period of the experiment. Conversely, the presence of EC27 significantly increased [3H]Ang III levels, when compared with control values, the effect was maximal ($+150\%$) between 7–10 min. The semilogarithmic representation of the rate of disappearance of [3H]Ang III in the presence of EC27 versus the control is shown in the Fig. 4 Insert. Both kinetics corresponded to a first-order elimination model ($r = 0.974$ and 0.989 for the control and EC27, respectively). The weak amounts of [3H]Ang III found in the presence of EC33 did not allow the determination of its half-life. In animals treated with EC27, the [3H]Ang III half-life ($7.40 \pm 0.63$ min) was significantly increased 2.26-fold when compared with control value ($3.28 \pm 0.56$ min, $P < 0.01$). After 7 min, when [3H]Ang II was injected with EC27 (30 \mu g) alone, hypothalamic [3H]Ang III levels decreased rapidly after 1.5 min and were undetectable after 7 min. In the presence of EC33, [3H]Ang II levels were significantly higher between 0.5 min to 7 min, reaching about 10-fold the control levels. The semilogarithmic representation of the rate of disappearance of [3H]Ang II levels, in the presence of EC33 versus the control is shown in Fig. 3 Insert. The best fit of both kinetics corresponded to a first-order elimination model ($r = 0.994$ and $0.998$ for the control and EC33, respectively).
whereas by purification of [3H]Ang II (2 × 10^6 cpm) with 30 μg of unlabeled Ang II were injected i.c.v. in the absence (control) or in presence of 30 μg EC33 or 30 μg EC27. Animals were killed at various times after injection, and [3H]Ang III levels were measured in hypothalamic extracts after purification by cation-exchange chromatography. [3H]Ang III levels are expressed as the percentage of total radioactivity found in the hypothalamus. Mean ± SEM of 6–8 determinations for each condition. *P < 0.01 versus control. (Inset) Semilogarithmic representation of [3H]Ang II levels between 1–10 min. Elimination rate constants (k−1) were found 0.211 ± 0.036 min−1 and 0.094 ± 0.008 min−1 for the control (C) and EC27, respectively.

levels increased 2.53 ± 0.16-fold as compared with the control, whereas when EC33 (30 μg) and EC27 (30 μg) were coadministered, [3H]Ang III levels were not significantly increased (1.20 ± 0.07-fold, data not shown). This indicated that the presence of EC33 could block the EC27-induced [3H]Ang III accumulation in the hypothalamus.

Effect of Intracerebroventricular Injections of Ang II and Ang III on Plasma Vasopressin Levels. First, to test the responsiveness of the mouse vasopressinergic system to Angs, mice were i.c.v. injected with either Ang II or Ang III, and plasma vasopressin levels were evaluated 1 min after injection. After physiological saline administration, basal vasopressin levels were routinely found around 20–40 pg/ml plasma. Ang II at the dose of 5 ng (Fig. 5) as well as 5 ng Ang III (Fig. 6) significantly increased plasma vasopressin levels by 94% and 127%, respectively, both effects being dose-dependent with maximal responses found around 5–10 ng for Ang II and Ang III (data not shown). Injection of EC33 (3–30 μg) significantly inhibited in a dose-dependent manner the Ang II-induced vasopressin release, whereas EC33 administered alone did not change the basal vasopressin levels (Fig. 5). Injection of a low dose of Ang III (0.5 ng) did not modify vasopressin release. By contrast, Ang III (0.5 ng) coinjected with EC27 (3–30 μg) elicited a dose-dependent increase in plasma vasopressin release (Fig. 6). This increase induced by coadministration of Ang III (0.5 ng) with EC27 (30 μg) was significantly higher than that obtained with a maximal dose of Ang III (5 ng) or of EC27 (30 μg) injected alone (Fig. 6). Injection of EC27 (3–100 μg) alone elicited a dose-dependent increase in plasma vasopressin levels (Fig. 7). The EC27-induced vasopressin release was completely abolished by the coadministration of the Ang receptor antagonist (Sar^4-Ala^8)-Ang II or the APA inhibitor EC33 (Fig. 7). To ensure that the vasopressin release induced by EC27 was not due to a systemic effect, the APN inhibitor (30 μg) or saline were administered by intravenous route. In this condition, EC27 did not modify plasma vasopressin levels, compared with control values (data not shown).

**DISCUSSION**

The present study provides the first demonstration that (i) aminopeptidases A and N are involved in vivo in the metabolism of Ang II and Ang III, respectively, and (ii) Ang III is the main effector peptide of the RAS, as compared with Ang II, in regulating vasopressin secretion.

Harding and colleagues (30, 31) were the first to investigate the metabolism of Ang II and Ang III in the brain. They shown that i.c.v. injection of aminopeptidase inhibitors such as amastatin or bestatin lengthened the half-lives of Ang II and Ang III and that iontophoretically applied bestatin on paraventricular neurons potentiated the stimulatory effect of Ang II and Ang III, whereas amastatin diminished Ang II-dependent activity (32, 33). However, these compounds are nonselective aminopeptidase inhibitors. Amastatin that was initially described as a specific APA inhibitor, is in fact 40-fold more potent on APN (34, 35) and bestatin exhibits a broad specificity on various aminopeptidases (35, 36). Therefore, the development of specific and potent APA and APN inhibitors was crucial for studying the in vivo metabolism of Angs in the brain. Based on the structure of the glutamate thiol described by Wilk and Thurston (37) as a potent but nonselective inhibitor of APA and APN, and on the similarities in the active sites of thermolysin-like enzymes (38), we designed the compound EC33 where the carbonylate of the side chain of the glutamate thiol was replaced by a sulfonate, and the compound EC27 that derived from the homogluathiol where the side chain carbonylate group was changed to a thiol. In vitro studies performed with purified APA and APN revealed the inhibitory potency of EC33 was nearly 100-fold better for APA (K_i = 0.29 μM) than for APN, while the inhibitory potency of
EC27 was 100-fold better for APN (K_i = 0.032 μM) than for APA (26).

We show that the APA inhibitor EC33 largely reduced the metabolism of [3H]Ang II and abolished the formation of [3H]Ang III in the hypothalamus. In contrast, application of the APN inhibitor EC27 induced an accumulation of [3H]Ang III and lengthened its half-life. Finally, the coadministration of both APA and APN inhibitors reduced the EC27-induced [3H]Ang III accumulation. Thus, the decrease in [3H]Ang III levels induced by the APA inhibitor did not result from an enhancement of [3H]Ang III metabolism by APN, but was really due to a blockade of the conversion of [3H]Ang II in [3H]Ang III. These results demonstrate that APA and APN could metabolize exogenous Ang II and Ang III in the hypothalamus. Both enzymes are present in mouse and rat brain tissues, especially in cerebral microvessels of circumventricular organs (20, 21, 39, 40) and in several hypothalamic nuclei, including the subfornical organ, the supraoptic and the paraventricular nuclei (S.Z. and C.L.-C., unpublished work). In these nuclei, the presence of AT1 receptors and Ang nerve terminals have been visualized (7, 41). Consequently, these data strongly suggest that, like Ang converting enzyme, APA and APN are important components of the brain RAS.

To determine the respective biological roles of Ang II and Ang III, we explored the physiological importance of the metabolism of endogenous Angs in vivo, by studying the effect of APA and APN inhibitors on Ang-induced vasopressin release. Previously, in the rat, central application of low doses of Ang II, Ang III, or 24–48 h water deprivation produced a rapid and robust increase in plasma vasopressin levels (42, 43). In the mouse, we observed that i.c.v. Ang II or Ang III injections also induced a rapid increase in plasma vasopressin levels, as well as 24 h water deprivation (S.Z. and C.L.-C., unpublished observations). We showed that coadministration of Ang II with increasing doses of the APA inhibitor (EC33) elicited a dose-dependent inhibition of Ang II-induced vasopressin release. This suggested that conversion of Ang II to Ang III or another Ang III metabolite was required to stimulate vasopressin secretion. To ensure that Ang III was really an endogenous brain RAS effector on the control of vasopressin release, we conducted a set of experiments. First, when Ang III at a low dose (ineffective by itself) was coadministered with increasing doses of the APN inhibitor (EC27), a progressive enhancement of plasma vasopressin levels was observed. This effect was higher than that produced by Ang III alone at a maximal dose, suggesting that EC27 inhibits degradation of both exogenous and endogenous Ang III. Second, this is corroborated by the fact that EC27 administered alone enhanced plasma vasopressin levels in a dose-dependent manner, indicating that EC27 inhibits endogenous Ang III degradation by APN. Third, the fact that EC33 can block the EC27-induced vasopressin release confirmed that APA is responsible for the transformation of endogenous Ang II into Ang III. At last, the abolition of the EC27-induced vasopressin release by the Ang receptor antagonist (Sar¹–Ala⁸)-Ang II demonstrates the specificity of action of APN on Ang III metabolism: by blocking the action of APN, EC27 induces an increase in endogenous Ang III levels, resulting in vasopressin release through interaction with Ang receptors. Together, these results clearly demonstrated that Ang III was one of the main effector peptides of the RAS controlling vasopressin release.

Although until now no specific APA or APN inhibitors were available, the concept that Ang II was not the only centrally active form of Angs emerged progressively from several studies. Initially, Ang receptors were shown to have a higher affinity for Ang III than Ang II in brain membranes (44). Subsequently, it was observed that Ang III was nearly as equipotent as Ang II in inducing dipsogenic and pressor responses (15, 45, 46) and that Ang III was more potent than its parent compound when applied iontophoretically in paraventricular neurons to increase the firing rate (16). Between
the two aminopeptidases resistant Ang analogs, [d-Asp1]Ang II and [d-Asp1]Ang III, the latter was the more potent pressor agent after i.c.v. infusion (17). Subsequently, bestatin was shown to potentiate the dipsogenic and pressor responses of centrally administered Ang III (33, 47), and also to be dipso-
genic by itself (48). Finally, Harding et al. (8) demonstrated in a push-pull cannula study that 93% of the Ang release in the paraventricular nucleus, after stimulation by veratridine or water deprivation, was comprised of Ang III. Our observations are in complete agreement with these results and strongly suggest that Ang III could act as a regulatory peptide in the brain.

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