Inhibition of the angiotensin-converting enzyme N-terminal catalytic domain prevents endogenous opioid degradation in brain tissue

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Abstract

Angiotensin-converting enzyme (ACE) regulates blood pressure by cleaving angiotensin peptides in the periphery, and can also regulate endogenous opioid signaling by degrading enkephalin peptides in the brain. ACE has two catalytic domains, located in the N-terminal or C-terminal region of the protein, but little is known about the roles that these two catalytic domains play in regulating endogenous opioid degradation in brain tissue. Using acute brain slice preparations from mice of both sexes, we developed methods to study degradation of Met-enkephalin-Arg-Phe (MERF) by ACE, and investigated the role of each ACE catalytic domain in MERF degradation. Using mutant mouse lines with functional inactivation of either the N-terminal domain or the C-terminal domain, we incubated acute brain slices with exogenous MERF at a saturating concentration. The degradation MERF to produce Met-enkephalin was only reduced by N-terminal domain inactivation. Additionally, application of a selective N-terminal domain inhibitor (RXP 407) reduced degradation of both exogenously applied and endogenously released MERF, without affecting degradation of endogenously released Met-enkephalin or Leu-enkephalin. Taken together, our results suggest that the ACE N-terminal domain is the primary site of MERF degradation in brain tissue, and that N-terminal domain inhibition is sufficient to reduce degradation of this specific endogenous opioid peptide. Our results have exciting implications for the development of novel pharmacotherapies that target the endogenous opioid system to treat psychiatric and neurological disorders.

Introduction

Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase, cleaving two amino acids from the C-terminus of its peptide substrates (1). It is primarily known for its role in blood pressure regulation via the cleavage of angiotensin I to produce angiotensin II. We have recently shown that ACE has another role in the brain (2): it modulates synaptic transmission and plasticity via the cleavage and degradation of a specific enkephalin peptide, Met-enkephalin-Arg-Phe (MERF). Among the enkephalins, MERF has a uniquely high binding affinity for the mu-opioid receptor (3), where it exhibits G protein signaling bias (4). MERF is also degraded by ACE with high efficiency (2, 5). ACE inhibition can thus prevent MERF degradation and selectively engage mu opioid receptor signaling in brain regions like the nucleus accumbens and dorsal striatum, which have conjointly high expression of ACE, MERF, and the mu opioid receptor. The ability of ACE inhibition to modulate striatal function opens a promising therapeutic avenue for substance use disorders (2), mood disorders (6, 7), and other psychiatric and neurological conditions involving striatal dysfunction, thus calling for the development of central ACE inhibition as a novel therapeutic strategy. However, the mechanism by which ACE cleaves MERF in the brain is not completely understood. ACE has two catalytic domains, the N-terminal domain and the C-terminal domain, each with a distinct profile of substrate specificity (8). Previous studies have used biochemical methods and recombinant ACE to study the roles of the two catalytic domains in enkephalin degradation *in vitro* (5), but are limited in the fact that they do not replicate the unique chemical milieu that regulates ACE function in the brain, such as the glycosylation state of ACE (9) and the concentration of ions like zinc (10). These variables affect the function of ACE, making them an important consideration for analysis of its catalytic efficiency. In this study, we investigated the roles of the two catalytic domains of ACE in MERF cleavage in acute brain slices harvested from mice, thereby capturing the native environment of brain ACE and enkephalins.

Results

To study how ACE degrades MERF in the brain, we developed an assay that quantifies enkephalin concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in mouse brain slices (Fig. 1A). In this assay, acute coronal brain slices containing the mouse striatum are incubated in artificial cerebrospinal fluid (aCSF) containing a saturating concentration of exogenous MERF (10-50 nM), which is cleaved and degraded by ACE to produce Met-enkephalin. Since spontaneous release of Met-enkephalin is negligible under baseline conditions in the absence of stimulation (2, 11), these assay conditions enable us to use Met-enkephalin production to measure degradation of exogenous MERF by ACE. We used this assay to separately interrogate the role of each catalytic domain of ACE in MERF degradation, by using mutant mouse lines that carry mutations in the active site of either the N-terminal catalytic domain (12) (NKO) or the C-terminal catalytic domain (13) (CKO; Fig. 1B). Crucially, the mutations that inactivate each catalytic domain are amino acid substitutions that do not change the expression level of ACE and preserve the function of the intact domain (12, 13), allowing us to independently study each domain without affecting the rest of the protein.

Functional inactivation of the N-terminal domain resulted in a 36.95% reduction in Met-enkephalin production (Fig. 1C), indicating a reduction in MERF degradation. In contrast, functional inactivation of the C-terminal domain did not alter MERF degradation, as there was no difference in Met-enkephalin production between CKO mice and their wild-type littermates (Fig. 1D). These results prompted us to further investigate the role of the N-

terminal domain in MERF degradation, by asking whether we can achieve the same effect using acute pharmacological inhibition rather than genetic manipulation. We incubated brain slices from wild-type mice with RXP 407 (0.1-10 μM), a small molecule ACE inhibitor with a high selectivity for the N-terminal domain (14), and measured Met-enkephalin production from MERF degradation (Fig. 1E). RXP 407 reduced Met-enkephalin production in a dose-dependent manner, with a maximum effect of 54.63% reduction from baseline (Fig. 1F), recapitulating our findings with NKO mice. Taken together, these results point to an important role of the N-terminal domain of ACE in MERF degradation.

To build on our analysis of exogenous MERF degradation by ACE, we next used an assay that more closely resembles physiological conditions, measuring endogenous MERF that is produced and released by striatal neurons. To this end, we stimulated the release of endogenous peptide using a high concentration (50 mM) of potassium chloride in aCSF (Fig. 2A), as previously described (2, 15). To test the hypothesis that N-terminal domain inhibition is sufficient to reduce MERF degradation in the brain, we stimulated brain slices from wild-type mice in aCSF containing different concentrations of RXP 407 (Fig. 2B). We observed a dose-dependent increase in MERF concentration (Fig. 2C), but no change in the concentration of endogenous Met-enkephalin (Fig. 2D) or Leu-enkephalin (Fig. 2E), suggesting that N-terminal domain inhibition is sufficient to selectively block MERF degradation in the brain. To confirm that RXP 407 was specifically acting via inhibition of the ACE N-terminal catalytic domain, we repeated the same experiment in NKO mice. In this case, we hypothesized that inhibition of the N-terminal domain with RXP 407 would have no further effect when the N-terminal domain was genetically inactivated (Fig. 2F). As expected, RXP 407 had no effect in NKO mice at the same concentrations that reduced MERF degradation in wild-type mice (Fig. 2G), ruling out non-specific effects of RXP 407 on other potential targets. These results further support the conclusion that inhibition of the N-terminal catalytic domain of ACE is sufficient to reduce MERF degradation.

Discussion

Our findings point to the untapped therapeutic potential of inhibiting the ACE N-terminal catalytic domain, as a means to prevent MERF degradation and engage endogenous opioid signaling in the brain. Critically, we previously showed that central ACE inhibition does not have obvious rewarding effects, and in fact attenuates the rewarding properties of exogenous opioids like fentanyl (2). The engagement of endogenous opioid signaling without corresponding risk of misuse or abuse is a holy grail for opioid-based pharmacotherapy, with tremendous potential for the treatment of diverse neuropsychiatric conditions. Our current work will thus guide the development of more specific ACE inhibitors that target the primary site of MERF degradation in the brain, resulting in better efficacy and fewer side effects. By analogy to reuptake mechanisms for other neurotransmitters, domain-selective ACE inhibitors may enhance signaling by specific neuropeptides in a manner that parallels selective reuptake inhibitors, which have substantial therapeutic value.

Materials and Methods

Experiments with C57Bl/6J mice, NKO mice (12), and CKO mice (13) were approved by the University of Minnesota Institutional Animal Care and Use Committee, and conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Preparation of acute brain slices and liquid chromatography-tandem mass spectrometry were conducted as previously described (2), with details regarding data collection and analysis provided as Supplemental Information. All data are included in the article, with graphs displaying mean ± SEM as well as individual data points from female and male mice shown as open and closed circles, respectively.

Acknowledgments

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References

1. K. E. Bernstein *et al.*, A modern understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme. *Pharmacol Rev* **65**, 1-46 (2013).

2. B. H. Trieu *et al.*, Angiotensin-converting enzyme gates brain circuit-specific plasticity via an endogenous opioid. *Science* **375**, 1177-1182 (2022).

3. A. Mansour, M. T. Hoversten, L. P. Taylor, S. J. Watson, H. Akil, The Cloned Mu-Receptors, Delta-Receptors and Kappa-Receptors and Their Endogenous Ligands - Evidence for 2 Opioid Peptide Recognition Cores. *Brain Res* **700**, 89-98 (1995).

4. I. Gomes *et al.*, Biased signaling by endogenous opioid peptides. *Proc Natl Acad Sci U S A* **117**, 11820-11828 (2020).

5. P. A. Deddish, H. L. Jackman, R. A. Skidgel, E. G. Erdos, Differences in the hydrolysis of enkephalin congeners by the two domains of angiotensin converting enzyme. *Biochem Pharmacol* **53**, 1459-1463 (1997).

6. G. S. Zubenko, R. A. Nixon, Mood-elevating effect of captopril in depressed patients. *Am J Psychiatry* **141**, 110-111 (1984).

7. W. J. Giardina, D. M. Ebert, Positive effects of captopril in the behavioral despair swim test. *Biol Psychiatry* **25**, 697-702 (1989).

8. K. E. Bernstein *et al.*, Different in vivo functions of the two catalytic domains of angiotensin-converting enzyme (ACE). *Curr Opin Pharmacol* **11**, 105-111 (2011).

158 9. S. M. Strittmatter, E. A. Thiele, M. S. Kapiloff, S. H. Snyder, A rat brain isozyme of
159 angiotensin-converting enzyme. Unique specificity for amidated peptide substrates.
160 *J Biol Chem* **260**, 9825-9832 (1985).

161 10. P. Bunning, J. F. Riordan, The functional role of zinc in angiotensin converting
162 enzyme: implications for the enzyme mechanism. *J Inorg Biochem* **24**, 183-198
163 (1985).

164 11. B. K. Atwood, D. A. Kupferschmidt, D. M. Lovinger, Opioids induce dissociable
165 forms of long-term depression of excitatory inputs to the dorsal striatum. *Nat*
166 *Neurosci* **17**, 540-548 (2014).

167 12. S. Fuchs *et al.*, Role of the N-terminal catalytic domain of angiotensin-converting
168 enzyme investigated by targeted inactivation in mice. *J Biol Chem* **279**, 15946-
169 15953 (2004).

170 13. S. Fuchs *et al.*, Angiotensin-converting enzyme C-terminal catalytic domain is the
171 main site of angiotensin I cleavage in vivo. *Hypertension* **51**, 267-274 (2008).

172 14. V. Dive *et al.*, RXP 407, a phosphinic peptide, is a potent inhibitor of angiotensin I
173 converting enzyme able to differentiate between its two active sites. *Proc Natl Acad*
174 *Sci U S A* **96**, 4330-4335 (1999).

175 15. R. Al-Hasani *et al.*, In vivo detection of optically-evoked opioid peptide release. *Elife*
176 **7** (2018).

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178 **Figures and Figure Legends**

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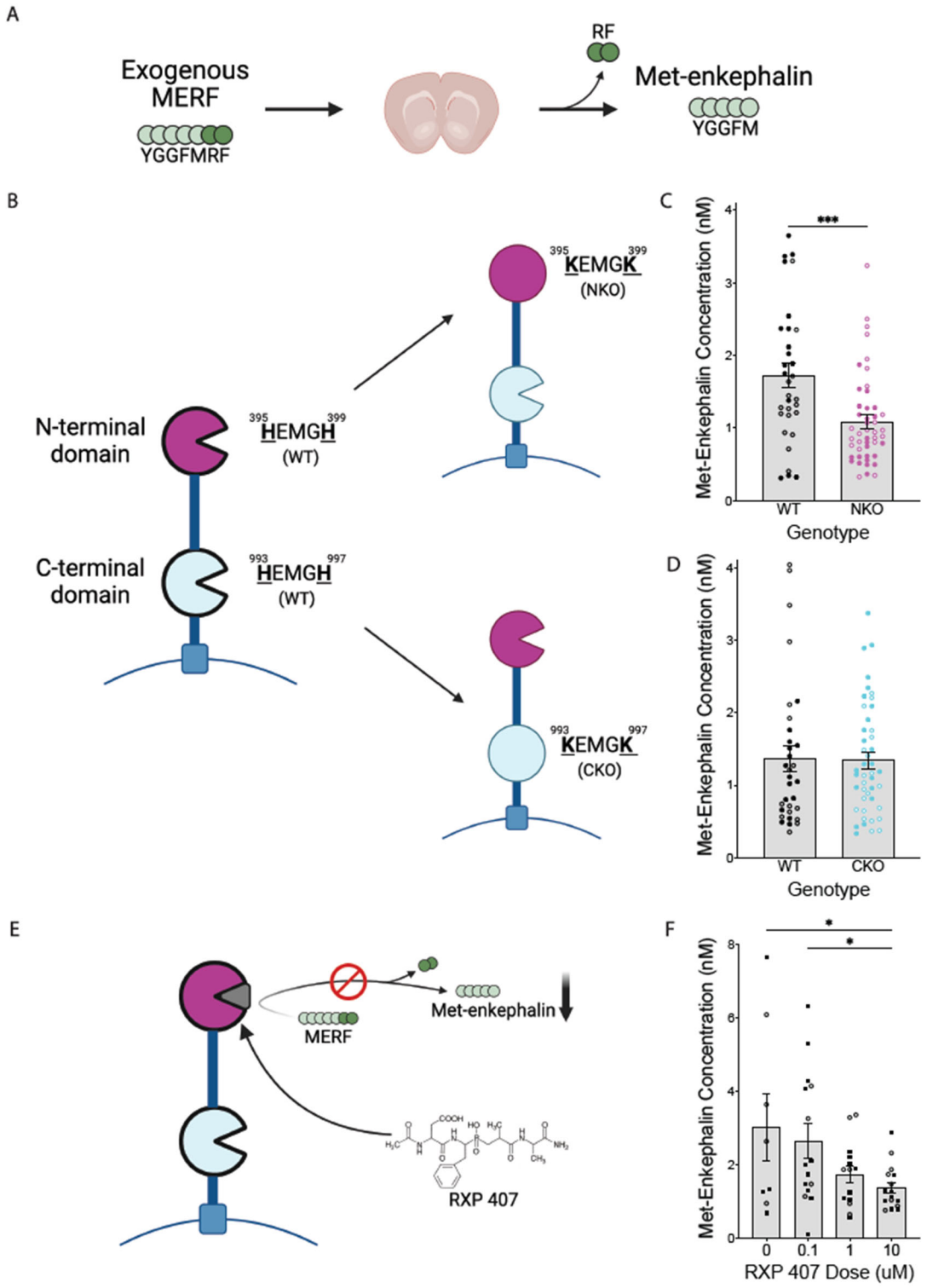

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182 **Figure 1. Production of Met-enkephalin via degradation of exogenous Met-**
183 **enkephalin-Arg-Phe (MERF) by the N-terminal catalytic domain of angiotensin-**
184 **converting enzyme (ACE).** (A) Experimental design: exogenous MERF was applied to

coronal brain slices containing the striatum, and cleaved by ACE to produce Met-enkephalin. (B) Schematic showing genetic manipulation of each individual catalytic domain of ACE: histidine was substituted with lysine at residues 395 and 399 for the N-terminal domain in the “NKO” mouse line, and at residues 993 and 997 for the C-terminal domain in the “CKO” mouse line. (C) Met-enkephalin production via MERF degradation by brain slices from NKO (n=47) and wild-type (WT) littermates (n=32). ANOVA main effect of Genotype: $F_{1,75} = 14.22$, $p = 0.0003$. (D) Met-enkephalin production via MERF degradation by brain slices from CKO (n=45) and WT littermates (n=32). ANOVA main effect of genotype; $F_{1,73} < 1$. (E) Schematic of RXP 407 selectively inhibiting the N-terminal domain of ACE, preventing Met-enkephalin production via MERF degradation. (F) Effects of RXP 407 on Met-enkephalin production via MERF degradation by brain slices from WT mice (n=8=16 per dose). ANOVA main effect of Dose: $F_{3,45} = 3.25$; $p=0.031$; $^*p<0.05$, Fisher’s LSD post-hoc test.

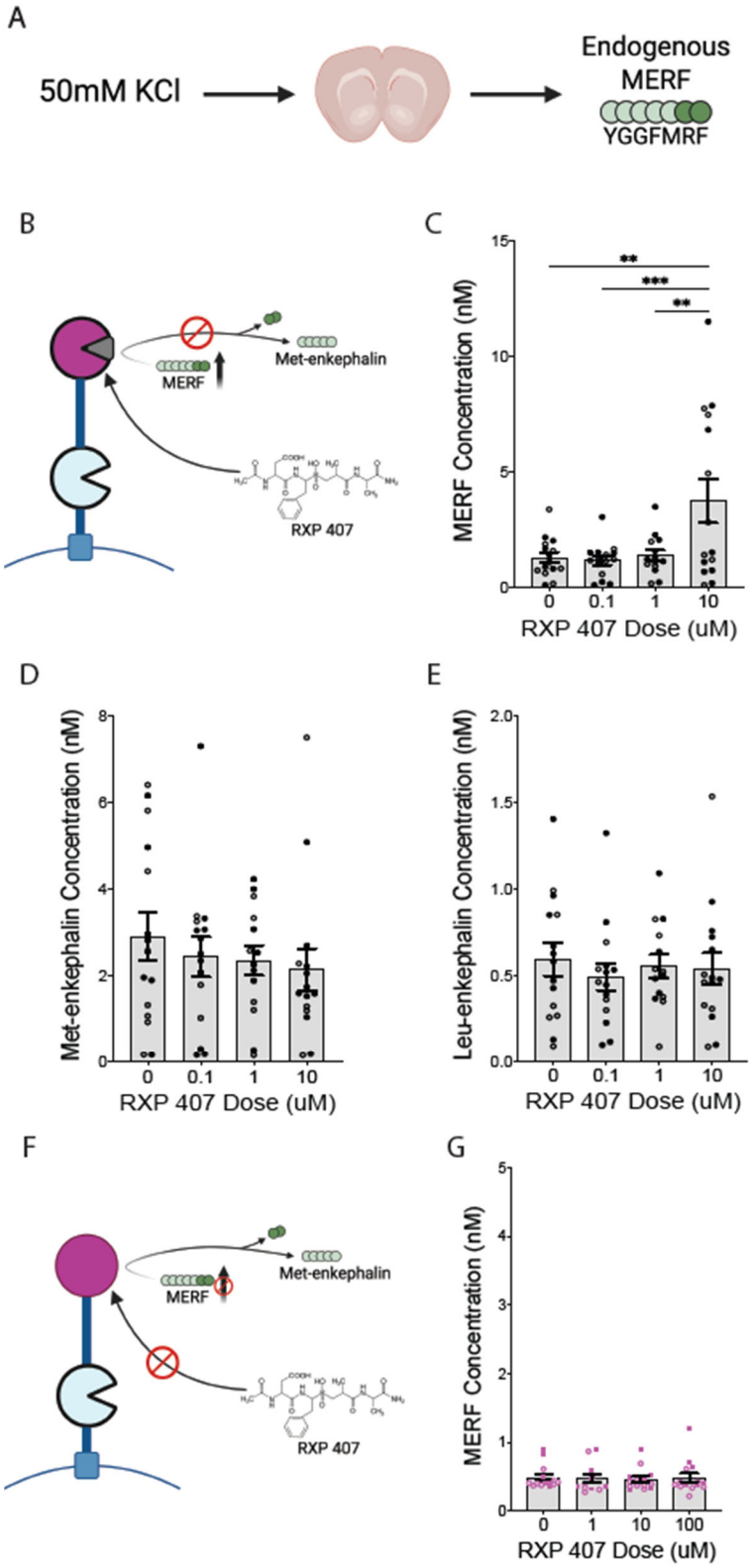

Figure 2. Degradation of endogenous Met-enkephalin-Arg-Phe (MERF), but not Met- or Leu-enkephalin, by the N-terminal catalytic domain of angiotensin-converting enzyme (ACE). (A) Experimental design: endogenous MERF was released by stimulating

203 coronal brain slices with a high concentration of KCl. (B) Schematic of RXP 407 selectively
204 inhibiting the N-terminal domain of ACE, preventing degradation of endogenous MERF.
205 (C-E) Effects of RXP 407 on extracellular concentration of MERF (C; ANOVA main effect
206 of Dose; $F_{3,51}$=5.38; p=0.003), Met-enkephalin (D; ANOVA main effect of Dose: $F_{3,51}$<1),
207 and Leu-enkephalin (E; ANOVA main effect of Dose: $F_{3,51}$<1) in brain slices from wild-type
208 mice (n=15-16 per dose). (F) Schematic showing RXP 407 has no effect on the ACE N-
209 terminal domain in NKO mice. (G) Effects of RXP 407 on extracellular MERF
210 concentration in brain slices (n=15-16 per dose) from NKO mice (ANOVA main effect of
211 Dose; $F_{3,46}$<1). *p<0.05, **p<0.005, ***p<0.0005, Fisher's LSD post-hoc test.