# Supporting Information

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### SI Materials and Methods

**Compounds.** Mouse calcitonin gene-related peptide (CGRP), dynorphin A, and neurotensin were purchased from American Peptide Company.

**Animal Studies.** Insulin-degrading enzyme (IDE) mice (1) were obtained from the mutant mouse regional resource center at University of California Davis. Mice were kept on a 12-h light, 12-h dark schedule and fed ad libitum. For tissue collection, mice were euthanized by $CO_2$ inhalation, their tissue dissected, snap frozen with liquid nitrogen, and stored at −80 °C. All animal care and use procedures were in strict accordance with the standing committee on the use of animals in research and teaching at Harvard University and the National Institutes of Health Guidelines for the Humane Treatment of Laboratory Animals.

**Isolation of Physiological Peptides from Tissue.** Frozen tissues, stored at −80 °C, were boiled in 300 μL boiling water for 10 min to inactivate proteolytic activity. Inactivating proteolytic activity before processing prevents artifacts arising from degradation after tissue homogenization. The aqueous fraction was collected and saved and the tissue was Dounce homogenized in 300 μL 0.25% ice-cold acetic acid in water. The aqueous fraction and the homogenate were combined and centrifuged at 20,000 × *g* for 20 min at 4 °C. The supernatant was then sent through a 10-kDa molecular weight cutoff filter (Microcon; YM-10) and the filtrate containing the peptides was concentrated in a speed vacuum concentrator and then suspended in 0.1% formic acid before analysis by LC–MS/MS.

**Peptide Synthesis and Screening Against the CGRP Receptor.** $CGRP_{1–17}$, $CGRP_{18–37}$, $CGRP_{20–37}$ and heavy-labeled d18-$CGRP_{1–37}$ (deuterated at d8-$Phe_{37}$ and d10-$Leu_{12}$) were synthesized manually using Fmoc chemistry for solid-phase peptide synthesis. Crude peptides were purified by RP-HPLC (Shimadzu) using a C18 column (150 × 20 mm, 10-μm particle size; Higgins Analytical). The HPLC gradient varied depending on the peptide (mobile phase A: 99% $H_2O$, 1% acetonitrile, and 0.1% TFA; mobile phase B: 90% acetonitrile, 10% $H_2O$, and 0.07% TFA). HPLC fractions were analyzed for purity by MALDI-TOF (Waters) using α-cyano-4-hydroxycinnamic acid as the matrix. Pure fractions were combined and lyophilized. Concentrations of the purified peptides were determined by amino acid analysis. The peptides were then submitted to Invitrogen for their SelectScreen profiling service. Using this service, the peptides were tested as agonists against the CGRP receptor (CALCRL:RAMP1) (Table S1).

**Tissue Proteome Fractionation.** Initial fractionation studies determined that CGRP-degrading activity was predominantly in the soluble fraction. One mouse spinal cord was Dounce homogenized in 300 μL PBS on ice. The soluble fraction was obtained by ultracentrifugation at 100,000 × *g* for 45 min at 4 °C. The soluble layer was transferred to a new tube and the pellet was suspended in PBS (membrane fraction). Protein concentration was determined by Bradford assay. The soluble and membrane fractions (1 mg/mL) were separately incubated with CGRP (100 μM final concentration) at 37 °C for 1 h and were quenched with an equal volume of 8 M guanidinium-HCl, followed by heating to 95 °C for 5 min. Degradation of $CGRP_{1–37}$ was analyzed by MALDI-TOF MS and LC–MS/MS using the method outlined in *Materials and Methods*, *MALDI-TOF MS and LC–MS/MS Analysis of in Vitro and in Vivo Peptides*.

The spinal cord proteome was subsequently fractionated by anion exchange chromatography. Two mouse (C57BL/6J) spinal cords were Dounce homogenized in 500 μL 20 mM Tris HCl, pH 8, on ice. The soluble fraction was obtained by ultracentrifugation at 100,000 × *g* for 45 min at 4 °C. The soluble fraction was further fractionated on an ÄKTA FPLC using a HiTrap Capto Q column (GE Healthcare). Buffer A was 20 mM Tris HCl pH 8, and buffer B was 20 mM Tris HCl and 1 M NaCl pH 8. The gradient was 0–50% B over 30 min at a 1 mL/min flow rate. The fractions were concentrated and exchanged into phosphate buffer saline (PBS) using a 10-kDa molecular weight cutoff filter (Microcon YM-10; Millipore) to a final volume of 200 μL. Each fraction was tested for CGRP-degrading activity (i.e., formation of $CGRP_{18–37}$ and $CGRP_{1–17}$). CGRP was added to each fraction to a 100-μM final concentration. The reactions proceeded at 37 °C for 1 h and were quenched with an equal volume of 8 M guanidinium-HCl followed by heating to 95 °C for 5 min. The reactions were analyzed by MALDI and LC–MS using the method outlined in *Materials and Methods, MALDI-TOF MS and LC–MS/MS Analysis of in Vitro and in Vivo Peptides*.

**Recombinant Protein Expression.** Plasmids containing the mouse IDE, thimet oligopeptidase (THOP), and neurolysin (NLN) genes were purchased from Open Biosystems. IDE, THOP, and NLN were each subcloned into the BamHI and EcoRI site of a pTrcHisA vector to create IDE-pTrcHis, THOP-pTrcHis, and NLN-pTrcHis (Invitrogen). The IDE-pTrcHis, NLN-pTrcHis, and THOP-pTrcHis all contained an N-terminal His6 tag fused to the coding sequence. IDE-pTrcHis and NLN-pTrcHis were transformed into Rosetta 2(DE3) pLysS (Stratagene) competent cells. Cells transformed with IDE-pTrcHis or NLN-pTrcHis were grown at 37 °C in Luria broth containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol and expression was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to 1 mM when the $OD_{600}$ of the culture reached 0.4–0.5. Induced cultures were grown overnight or for 6 h at 37 °C for IDE-pTrcHis and NLN-pTrcHis, respectively. BL21-CodonPlus (DE3)-RP (Stratagene) cells transformed with THOP-pTrcHis were grown at 37 °C in Luria broth containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol and expression was induced by the addition of IPTG to 1 mM when the $OD_{600}$ of the culture reached 0.4–0.5. Induced cultures were grown overnight at 25 °C for THOP-pTrcHis.

Recombinant proteins were purified by Ni(II)-affinity chromatography, which permits purification of His6-tagged proteins. Each protein was tested for activity using a known substrate (THOP and NLN cleaved neurotensin, IDE cleaved dynorphin A). Peptides (100 μM) were added to recombinant IDE, NLN, or THOP (0.14 mg/mL, 0.08 mg/mL, 0.05 mg/mL, respectively) in 50 mM Tris HCl pH 7.5 and incubated at 37 °C for 1 h. The reaction was quenched with an equal volume of 8 M guanidinium-HCl followed by heating to 95 °C for 5 min. Reaction products were analyzed by LC–MS and MALDI-MS. Lastly, each recombinant protein was tested for CGRP-cleaving activity. CGRP (100 μM) was added to recombinant IDE, NLN, or THOP (0.05 mg/mL) in 50 mM Tris HCl pH 7.5 and incubated at 37 °C for 1 h. After quenching the reaction with 10% (vol/vol) by volume formic acid, reaction products were analyzed by LC–MS and MALDI-MS.

**Protease Inhibitor Studies.** One mouse spinal cord was Dounce homogenized in 300 μL PBS on ice. The soluble fraction was obtained by ultracentrifugation at 100,000 × *g* for 45 min at 4 °C.

Protein concentration was determined by Bradford assay. Mouse plasma (1 mg/mL) and the soluble fraction of spinal cord lysates (1 mg/mL) were preincubated at 37 °C for 15 min separately with each the following inhibitors: 10 μM E-64, 1 mM iodoacetamide, 10 μM N-ethylmaleimide, 1 mM EDTA, 1 mM *o*-phenanthroline, 10 μM phosphoramidon, 10 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride combined with 1 mM diisopropylfluorophosphate, and vehicle [PBS with 25% (vol/vol) ethanol for 1% final concentration in reaction]. Each reaction also included an aminopeptidase inhibitor mixture with the following inhibitors: 100 μM 2′,3-dinitroflavone-8-acetic acid, 10 μM bestatin, and 10 μM amastatin. After the preincubation with each inhibitor, CGRP was added to 100-μM final concentration. The reactions proceeded at 37 °C for 15 min and were quenched with an equal volume of 8 M guanidinium-HCl. The quenched reactions were analyzed by MALDI-TOF MS and LC–MS/MS for CGRP-degrading activity (i.e., formation of $CGRP_{18–37}$ and $CGRP_{1–17}$). MALDI-TOF MS and LC–MS/MS methods are outlined in *Materials and Methods*, *MALDI-TOF MS and LC–MS/MS Analysis of in Vitro and in Vivo Peptides*.

**Transfection of TT Cells with IDE.** TT cells were maintained in DMEM supplemented with FBS, penicillin, and streptomycin. IDE (from Open Biosystems) was cloned into pcDNA3.1/V5-His-TOPO (Invitrogen) to make IDE-pcDNA3.1, according to the manufacturer's protocol. Transfection of TT cells with either IDE-pcDNA3.1 or pcDNA3.1 (control vector) was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, TT cells were plated at 1.2e7 cells per 10-cm plate the day before transfection. A total of 30 μg of DNA and 60 μL of Lipofectamine 2000 were added to each 10-cm plate during the transfection. Six hours after transfection, the media were changed to phenol red-free media with the same recipe as above. Three days after transfection, the media were collected and the cells were washed with and suspended in PBS. The cells were lysed by sonication using a probe sonicator. Protein concentrations were determined by Bradford assay. Transfection was confirmed by Western blot of the cell lysates. CGRP is secreted by TT cells, so the media were analyzed for CGRP levels. Peptides were extracted from the collected media using the Oasis SepPak 1cc HLB cartridges using methanol to prime the column, water to wash the column, and 1:1 water: acetonitrile to elute the peptides. The eluted fraction was dried in a speed vacuum concentrator and suspended in 0.1% formic acid in water before analysis by LC–MS/MS. LC–MS/MS analysis is outlined in the section using a gradient proceeding from 5 to 50% acetonitrile in water (0.1% formic acid) over 90 min using an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific).

1. Farris W, et al. (2003) Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. *Proc Natl Acad Sci USA* 100:4162–4167.

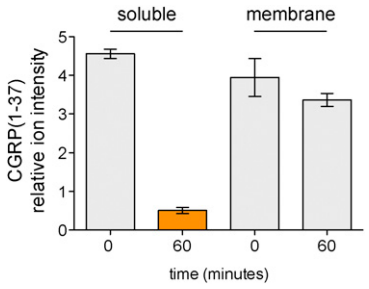



**Fig. S1.** $CGRP_{1–37}$ (100 μM) was incubated with the soluble (1 mg/mL) or membrane (1 mg/mL) fraction of the spinal cord proteome (lysate) for 60 min. The sample was then quenched and analyzed by LC–MS to quantify $CGRP_{1–37}$ levels. A majority of the CGRP-degrading activity was found in the soluble fraction, as evidenced by greatly diminished $CGRP_{1–37}$ levels after 60 min in the soluble fraction, and largely unchanged levels after exposure to the membrane fraction.

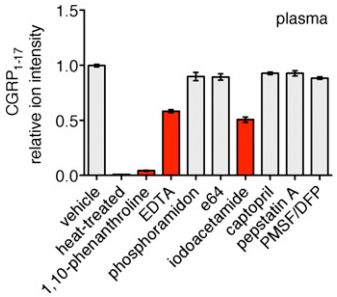



**Fig. S2.** $CGRP_{1–37}$ (100 μM) was added to mouse plasma (1 mg/mL) that had been preincubated with different peptidase inhibitors. After 15 min at 37 °C, the sample was quenched and the levels of the CGRP fragment, $CGRP_{1–17}$, were measured by LC–MS and compared with a vehicle-treated control. Metallopeptidase inhibitors 1,10-phenanthroline and EDTA slowed the production of $CGRP_{1–17}$, as did the cysteine labeling reagent iodoacetamide. Because 1,10-phenanthroline is the most potent inhibitor, the CGRP-degrading enzyme is most likely a metallopeptidase.

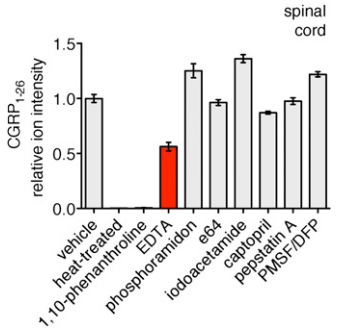



**Fig. S3.** $CGRP_{1–37}$ (100 μM) was added to mouse spinal cord lysate (1 mg/mL, soluble fraction) that had been preincubated with different peptidase inhibitors. After 15 min at 37 °C, the sample was quenched and the levels of $CGRP_{1–26}$ were measured by LC–MS and compared with a vehicle-treated control. Metallopeptidase inhibitors 1,10-phenanthroline and EDTA slowed the production of $CGRP_{1–26}$ the most, indicating that the CGRP-degrading enzyme is likely a metallopeptidase.

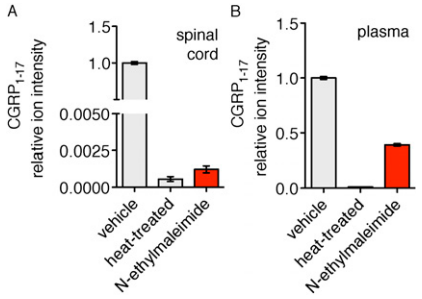



**Fig. S4.** $CGRP_{1–37}$ (100 μM) was incubated with (*A*) spinal cord lysate (1 mg/mL, soluble fraction) or (*B*) plasma (1 mg/mL) for 15 min in the presence or absence of N-ethylmaleimide (NEM), a cysteine-labeling reagent that is also an IDE inhibitor. A heat denatured lysate (heated) served as a control reaction. Samples were quenched and the extent of $CGRP_{1–37}$ degradation was assessed by measuring $CGRP_{1–17}$ levels by LC–MS. NEM effectively blocked $CGRP_{1–17}$ production in (*A*) spinal cord lysate and (*B*) plasma samples, providing additional evidence that IDE is a CGRP-degrading enzyme.

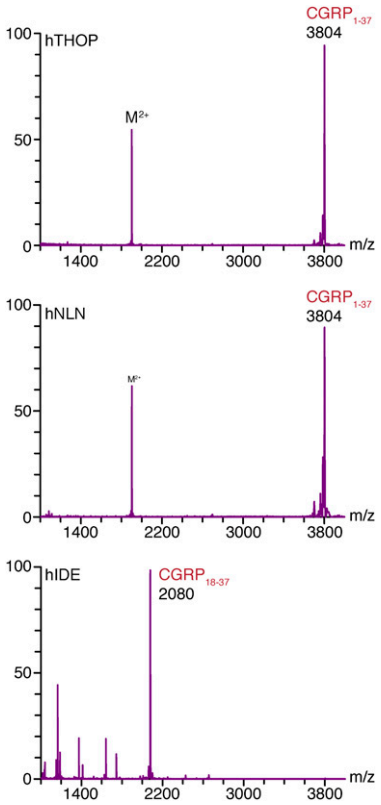
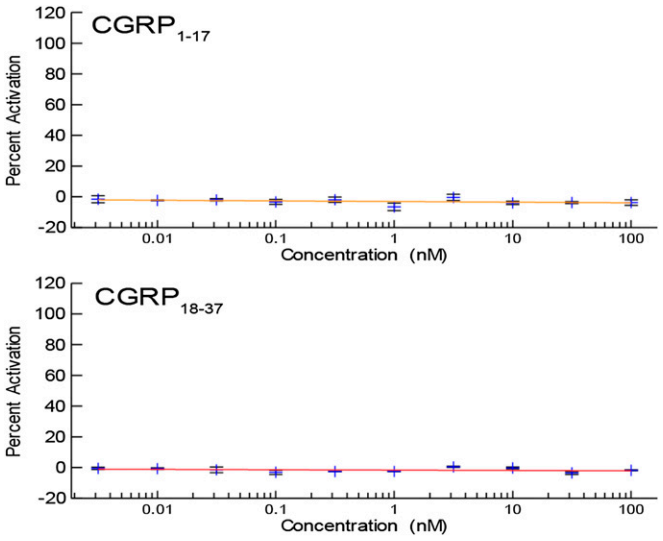



**Fig. S5.** $CGRP_{1–37}$ (100 μM) was incubated with purified human THOP, NLN, or IDE (0.05 mg/mL) at 37 °C for 15 min. The sample was then quenched and analyzed by MALDI-TOF to assess whether $CGRP_{1–37}$ had been cleaved. hTHOP and hNLN showed absolutely no CGRP-degrading activity, whereas $CGRP_{1–37}$ was an excellent substrate for hIDE, consistent with what had been seen with the mouse homologs of these enzymes. Because human IDE is also able to process CGRP, this indicates that the CGRP-degrading activity of IDE is conserved from mouse to human.

**Table S1. Testing the endogenous CGRP fragments $CGRP_{1–17}$ and $CGRP_{18–37}$ against the CGRP receptor (CALCRL:RAMP1) revealed that these peptides are no longer able to activate the receptor**

| Cmpd name | $EC_{50}$, nM | [Cmpd], nM | Control Cmpd name | Control $EC_{50}$, nM |
|---|---|---|---|---|
| CGRP 1–17 | >100 | 100 | CGRP | 0.105 |
| CGRP 1–17 | >100 | 31.6 | CGRP | 0.105 |
| CGRP 1–17 | >100 | 10.0 | CGRP | 0.105 |
| CGRP 1–17 | >100 | 3.16 | CGRP | 0.105 |
| CGRP 1–17 | >100 | 1.00 | CGRP | 0.105 |
| CGRP 1–17 | >100 | 0.316 | CGRP | 0.105 |
| CGRP 1–17 | >100 | 0.100 | CGRP | 0.105 |
| CGRP 1–17 | >100 | 0.0316 | CGRP | 0.105 |
| CGRP 1–17 | >100 | 0.0100 | CGRP | 0.105 |
| CGRP 1–17 | >100 | 0.00316 | CGRP | 0.105 |
| CGRP 18–37 | >100 | 100 | CGRP | 0.105 |
| CGRP 18–37 | >100 | 31.6 | CGRP | 0.105 |
| CGRP 18–37 | >100 | 10.0 | CGRP | 0.105 |
| CGRP 18–37 | >100 | 3.16 | CGRP | 0.105 |
| CGRP 18–37 | >100 | 1.00 | CGRP | 0.105 |
| CGRP 18–37 | >100 | 0.316 | CGRP | 0.105 |
| CGRP 18–37 | >100 | 0.100 | CGRP | 0.105 |
| CGRP 18–37 | >100 | 0.0316 | CGRP | 0.105 |
| CGRP 18–37 | >100 | 0.0100 | CGRP | 0.105 |
| CGRP 18–37 | >100 | 0.00316 | CGRP | 0.105 |




Whereas these fragments had no activity at >100 nM, $CGRP_{1–37}$ activates the receptor at subnanomolar concentrations (0.105 nM), indicating that cleavage of $CGRP_{1–37}$ likely terminates CGRP signaling. Cmpd, compound.

**Table S2. The CGRP-degrading enzymes predicted my MEROPS**

| Peptidase | Enzyme class |
|---|---|
| Ser17-Arg18 cleavage site | |
| Pepsin A | Aspartyl |
| Gastricsin/pepsin C | Aspartyl |
| Memapsin-1/BACE2 | Aspartyl |
| Cathepsin C | Cysteine |
| Calpain-2 | Cysteine |
| **Thimet oligopeptidase** | **Metallo** |
| **MMP19** | **Metallo** |
| Insulin-degrading enzyme | Metallo |
| Nardilysin | Metallo |
| Meprin A | Metallo |
| Elastase-2 | Serine |
| Myeloblastin | Serine |
| Asn26-Phe27 cleavage site | |
| Cathepsin D | Aspartyl |
| Cathepsin E | Aspartyl |
| Neurolysin | Metallo |
| **Thimet oligopeptidase** | **Metallo** |
| MMP1 | Metallo |
| MMP2 | Metallo |
| MMP3 | Metallo |
| MMP7 | Metallo |
| MMP8 | Metallo |
| MMP9 | Metallo |
| MMP13 | Metallo |
| **MMP19** | **Metallo** |
| MMP20 | Metallo |
| Membrane-type matrix metallopeptidase-1 | Metallo |
| ADAMTS1 | Metallo |
| ADAMTS4 | Metallo |
| Cathepsin G | Serine |
| Kallikrein-related peptidase 4 | Serine |

Thimet oligopeptidase and MMP19 (in bold) are the only two enzymes that are predicted to cleave at both CGRP cleavage sites.