Supporting information: Pharmacological characterisation of mouse calcitonin and calcitonin receptor-like receptors reveals differences compared to human receptors

Michael L. Garelja^{1,2}, Rebekah L Bower², Margaret A. Brimble^{2,3,4}, Shanan Chand², Paul W.R. Harris^{2,3,4}, Muhammad Aqfan Jamaluddin², Jakeb Petersen², Andrew Siow^{2,4}, Christopher S. Walker^{2,3}, Debbie L. Hay^{*1,2,3}.

- 1. Department of Pharmacology and Toxicology, University of Otago, Dunedin, 9016, New Zealand
- 2. School of Biological Sciences, University of Auckland, Auckland, 1010, New Zealand
- Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland, 1010, New Zealand
- 4. School of Chemical Sciences, University of Auckland, Auckland, 1010, New Zealand

Figure count: 29 (22 Figures, 7 Schemes)

Contents

Supplemental biology, Pages 2 – 8 (Supplementary Figures 1-8)

Supplemental chemistry, Pages 9-31 (Supplementary Figures 9-22, Schemes 1-7)

References, Page 32

Supplemental Biology



Figure S 1. Heat-maps showing the relative potency of different peptides at human and rat CLR-based receptors. Panel **A** shows results from cells transfected with human CLR and human RAMPs, while panel **B** shows results from cells transfected with rat CLR and rat RAMPs. Panel **C** shows results from this publication. Results presented as fold difference relative to a reference ligand. A positive number indicates that the peptide is more potent than the reference ligand (species matched α CGRP at CLR:RAMP1, and species matched AM at CLR:RAMP2 and CLR:RAMP3), while a negative number indicates the peptide has a lower potency than reference ligand. White boxes displaying a cross indicate that the ligand/receptor combination has not been tested, while a grey box indicates that no curve could be fit to this ligand:receptor combination. Human and rat results are fold-difference between the mean pEC₅₀ values derived from multiple previously published studies using transfected cells, values were recently compiled in (Hay et al., 2018) and (Garelja et al., 2020).



Figure S 2 Heat-maps showing the potencies of peptides at CTR-based receptors, relative to the speciesmatched Amy (comparisons within receptors). Panel **A** shows results from cells transfected with human receptor components, while panel **B** shows the results from cells transfected with rat receptor components. Panel **C** shows results from this publication. Results presented as fold-difference between the named peptide and the species matched Amy. A positive number indicates that the peptide is more potent than Amy, while a negative number indicates the peptide has a lower potency than Amy. White boxes displaying a cross indicate that the ligand/receptor combination has not been tested. Human and rat results are fold-difference between the mean pEC₅₀ values derived from multiple previously published studies using transfected cells, values were recently compiled in (Hay et al., 2018).



Figure S 3. Heat-maps showing the relative potency of peptides at CTR:RAMP complexes compared to CTR alone. Panel **A** shows results from cells transfected with human CTR and human RAMPs, while panel **B** shows results from cells transfected with rat CTR and rat RAMPs. Panel **C** shows results from this publication. Results presented as fold change relative to the potency at CTR alone. A positive number indicates an increase in peptide potency when cells were transfected with a RAMP in combination with CTR compared to cells transfected with CTR alone. White boxes displaying a cross indicate that the ligand/receptor combination has not been tested. Human and rat results are fold-difference between the mean pEC₅₀ values derived from multiple previously published studies using transfected cells, values were recently compiled in (Hay et al., 2018).

| a) h-CTR m-CTR r-CTR | y v v m R F T F T S R C L A L F L L L N H P T P I L P A F S N Q T Y P T I E P K P M R F T F T S R C L A L F L L L N H P T P I L P A F S N Q T Y P T I E P K P M T P R R S R V K R R N L R K P K M R F L L V N R F T L L L L L V S P T P V L Q A P T N L T D S G L D Q E P M R F L L L N R F T L L L L L L V S P T P V L Q A P T N L T D S G L D Q E P |
|---|--|
| h-CTR m-CTR r-CTR | FLYV ^v GRKKMMDAQ ⁷ KCYDRMQQL ^p AYQGEGPYC ^N RTWDGWLCW ¹⁰ DTPAGYLSY ¹⁰ FLYLVGRKKLLDAQYKCYDRIHQLPSYEGEGL <u>YCNRTWDGWMCWDDTPAG</u> ATAYQ FLYLVGRKKLLDAQYKCYDRIIQQLPPYEGEGPYCNRTWDGWMCWDDTPAGATAYQ |
| h-CTR m-CTR r-CTR | F C P D Y F P D F D P S E K V T K Y C D E K G V W F K H P E N N R T W S N Y T M C N A F T P E K L K N A Y V L H C P D Y F P D F D T A E K V S K Y C D E N G E W F R H P D S N R T W S N Y T L C N A F T S E K L Q N A Y V L H C P D Y F P D F D P T E K V S K Y C D E N G E W F R H P D S N R T W S N Y T L C N A F T P D K L H N A Y V L H C P D Y F P D F D P T E K V S K Y C D E N G E W F R H P D S N R T W S N Y T L C N A F T P D K L H N A Y V L H C P D Y F P D F D P T E K V S K Y C D E N G E W F R H P D S N R T W S N Y T L C N A F T P D K L H N A Y V L H C P D Y F P D F D P T E K V S K Y C D E N G E W F R H P D S N R T W S N Y T L C N A F T P D K L H N A Y V L H C P D Y F P D F D P T E K V S K Y C D E N G E W F R H P D S N R T W S N Y T L C N A F T P D K L H N A Y V L |
| h-CTR m-CTR r-CTR | YYLAİVĞHSLSIFTLVİSLĞLFVFFRSLĞCQRVTLHKNMFLTYLNSMIIIHLV YYLALVĞHSLSIAALVASML FWIFKNLSCQRVTLHKHMFLTYLNSIIIIHLV YYLALVĞHSLAALIASMĞLFFFKNLSCQRVTLHKNMFLTYLNSIIIIHLV 240 - 240 |
| h-CTR m-CTR r-CTR | E V V P N G E L V R R D P V S C K I L H F F H Q Y M M A C N Y F W M L C E G I Y L H T L I V V A V F T E K Q R E V V P N G D L V R R D P I S C K V L H F L H Q Y M M S C N Y F W M L C E G I Y L H T L I V M A V F T D E Q R E V V P N G D L V R R D P I S C K I L H F F H Q Y M M A C N Y F W M L C E G I Y L H T L I V M A V F T E D Q R |
| h-CTR m-CTR r-CTR | L R W Y Y L L G W G F P L V P T T I H A I T R A V Y F N D N C W L S V E T H L L Y I I H G P V M A A L V V N F L R W Y Y L L G W G F P I V P T I I H A I T R A L Y Y N D N C W L S A E T H L L Y I I H G P V M V A L V V N F L R W Y Y L L G W G F P I V P T I I H A I T R A V Y Y N D N C W L S T E T H L L Y I I H G P V M A A L V V N F |
| h-CTR m-CTR r-CTR | F F L L N I V R V L V T K M R E T H E Å E S H M Y L K A V K A T M I L V P L L G I Q F V V F P W R P S N K M L F F L L N I V R V L V T K M R Q T H E A E S Y M Y L K A V K A T M V L V P L L G I Q F V V F P W R P S N K V L F F L L N I V R V L V T K M R Q T H E A E A Y M Y L K A V K A T M V L V P L L G I Q F V V F P W R P S N K V L |
| h-CTR m-CTR r-CTR | G K L Y D Y V M H S L I H F O G F F V A T L Y C F C N N E V Q T T V K R Q W A Q F K L Q W N Q R W G R R P G K L Y D Y L M H S L I H F O G F F V A T L Y C F C N H E V Q V T L K R Q W T Q F K L Q W S Q R W G R R R R P G K L Y D Y L M H S L I H F O G F F V A T L Y C F C N H E V Q V T L K R Q W A Q F K L Q W S N R W G R R R R P |
| h-CTR m-CTR r-CTR | SNR SARAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA |
| h-CTR | |
| m-CTR r-CTR | |
| m-CTR r-CTR b) h-CLR m-CLR r-CLR | A A MEKKCTLNELVLLPFFMILVTAELEESPEDSLQLGVTRNKIMTAQYECYQKIMQ MDKKHILCELVLLPLNMALISAESEEGVNQT-DLGVTRNKIMTAQYECYQKIMQ MMDKKCTLCELFLLLLNMALIAAESEEGANQT-DLGVTRNKIMTAQYECYQKIMQ |
| m-CTR r-CTR b) h-CLR m-CLR r-CLR h-CLR m-CLR r-CLR | MEKKCTINÉ VILPFFMIL V TAELEESPËDSUQLGVTRNKIMTAQYECYQKIMQ MDKKHILCELVLLPFFMIL V TAELEESPËDSUQLGVTRNKIMTAQYECYQKIMQ MDKKHILCELVLLPLNMALISAESEEGVNQT-DLGVTRNKIMTAQYECYQKIMQ MDKKCTICELFLLLLNMALIAAESEEGANQT-DLGVTRNKIMTAQYECYQKIMQ DPIQQAEGVYCNRTWDGWLCWNDVAAGTESMQLCPDYFQDFDPSEKVTKICDQDG DPIQQAEGLYCNRTWDGWLCWNDVAAGTESMQYCPDYFQDFDPSEKVTKICDQDG DPIQQEGLYCNRTWDGWLCWNDVAAGTESMQYCPDYFQDFDPSEKVTKICDQDG |
| m-CTR r-CTR b) h-CLR m-CLR r-CLR h-CLR r-CLR h-CLR m-CLR r-CLR | A MEKKCTINELVLLPFFMILVTAELEESPEDSUQLGVTRNKIMTAOYECYOKIMO MDKKHILCELVLLPFFMILVTAELEESPEDSUQLGVTRNKIMTAOYECYOKIMO MDKKCTLCELVLLPLNMALISAESEEGVNQT-DLGVTRNKIMTAQYECYQKIMO MMDKKCTLCELFLLLLNMALIAAESEEGANQT-DLGVTRNKIMTAQYECYQKIMO DPIQQAEGVYCNRTWDGWLCWNDVAAGTESMQLCPDYFQDFDPSEKVTKICDQDG DPIQQAEGLYCNRTWDGWLCWNDVAAGTESMQYCPDYFQDFDPSEKVTKICDQDG DPIQQAEGLYCNRTWDGWLCWNDVAAGTESMQYCPDYFQDFDPSEKVTKICDQDG DPIQQAEGLYCNRTWDGWLCWNDVAAGTESMQYCPDYFQDFDPSEKVTKICDQDG NWFRHPASNRTWTNYTQCNYNTHEKVKTALNLFYLTIGHGLSASLLSLIFF NWFRHPDSNRTWTNYTLCNNSTHEKVKTALNLFYLTIGHGLSASL |
| m-CTR r-CTR b) h-CLR m-CLR r-CLR h-CLR m-CLR r-CLR h-CLR r-CLR h-CLR r-CLR h-CLR r-CLR | A MEKKCTLNELVLPFFMILLYTAELEESPEDSOUGVTRNKIMTAOYECYOK MO MDKKHLICELVLPLPLNMALISAESEGVNQT-DLGVTRNKIMTAOYECYOK MO MDKKCTLCELFLLLNMALIAAESEGANQT-DLGVTRNKIMTAOYECYOK MO MDKKCTLCELFLLLNMALIAAESEGANQT-DLGVTRNKIMTAOYECYOK MO 100 0 P 1 QQAEGVYCNRTWDGWLCWNDVAAGTESMQLCPDYFQDFDPSEKVTKICDODG 100 0 P 1 QQAEGLYCNRTWDGWLCWNDVAAGTESMQYCPDYFQDFDPSEKVTKICDODG 100 100 100 100 100 100 100 10 |
| m-CTR r-CTR b) h-CLR m-CLR r-CLR h-CLR r-CLR h-CLR r-CLR h-CLR r-CLR h-CLR r-CLR h-CLR r-CLR h-CLR r-CLR h-CLR r-CLR | A MEKKCTINELVILPEFMILLYTAELEESPEDSUQUGVTRNKIMTAOYECYOK MO MDKKKTICEEVILPLNMAALISAESEEGVNQT-DLGVTRNKIMTAOYECYOK MO MDKKKICTICEEVILPLNMAALISAESEEGVNQT-DLGVTRNKIMTAOYECYOK MO MDKKKICTICEEVILPLNMAALISAESEEGANQT-DLGVTRNKIMTAOYECYOK MO MDKKKICTICEEVILPLNNMAALISAESEEGANQT-DLGVTRNKIMTAOYECYOK VICIO DPIOQAEGUYCNRTWDGWLCWNDVAAGTESMOYCPDYFODFDPSEKVTKICOODG DPIOQAEGUYCNRTWDGWLCWNDVAAGTESMOYCPDYFODFDPSEKVTKICOODG DPIOQAEGUYCNRTWDGWLCWNDVAAGTESMOYCPDYFODFDPSEKVTKICOODG NWFRHPASNRTWTNYTLCNNSTHEKVKTALNIEYLTIGHGLSIASLIISLI HWFRHPDSNRTWTNYTLCNNSTHEKVKTALNIEYLTIGHGLSIASLIISLI VFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPVSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTAVANNOALVATNPUSCKVSOFTH YFKSLSCORITLHKNIEFSFYCNSIVTIHLTA |
| m-CTR r-CTR b) h-CLR m-CLR r-CLR h-CLR r-CLR r-CLR h-CLR r-CLR | MEKKCTINN 10 10 10 10 10 10 10 10 10 10 |
| m-CTR r-CTR b) h-CLR r-CLR | A |
| m-CTR r-CTR b) h-CLR r-CLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR h-CCLR r-CLR | A |

Figure S 4. Amino acid sequence alignment of human, mouse, and rat (a) $CTR_{(a)}$ and (b) CLR. Sequence alignment created using Geneious Prime. Dark blue background with white text indicates an exact match, medium blue background indicates greater than 80% similarity at a given position, and light blue background indicates a similarity between 60% and 80% at a given position.

| a) | 1 10 | 20 | 30 | 40 50 | |
|-------------------------------|--|--|--|---|------------------|
| h-RAMP1 m-RAMP1 r-RAMP1 | MARALCRLPRRGLWL MAPGLRGLPRCGLWL MALGLRGLPRRGLWL | LLAHHLFMVT LLAHHLFMVT LVHHLFMVT | A C Q E À N Y G A L A C R D P D Y G T L A C R D P D Y G T L | LRELCLTQFQVDMEÅVGE IQELCLSRFKENMETIGK IQELCLSRFKEDMETIGK | |
| h-RAMP1 m-RAMP1 r-RAMP1 | L W C D W G R T R S Y R E L L W C D W G K T Q S Y G E L L W C D W G K T G S Y G E L 110 | A D C T W H M A E K T Y C T K H V A H T T H C T K L V A N K | L G C F W P N A E V I G C F W P N P E V I G C F W P N P E V | DIR F F L A V H G R Ý F R S C P I S DIR F F I A V H H R Y F S K C P I S DIK F F I A V H H R Y F S K C P V S 148 | G G G G |
| h-RAMP1 m-RAMP1 r-RAMP1 | R Á V R D P P G S I L Ý P F I R A L R D P P N S I L C P F I R A L R D P P N S I L C P F I | V V P I T V Ť L L V A L <u>P I T V T L L</u> M V L P I T V T L L M | T A L V V W Q S K R T A L V V W R S K R T A L V V W R S K R | T E G I V T E G I V T E G I V | |
| b) | | 22 | | 10 | |
| h-RAMP2 m-RAMP2 r-RAMP2 | MASLRVERA – GGPRL MAPLRVERAPGGSRL MAPLRVERAPGGSQL | PRTRVGRPAA GVTRAQRPTA AVTSAQRPAA | | L L L L G A V L N P H E AL L L L L G A V S A S P E S L N Q S L L L L G A V S T S P E S L | |
| h-RAMP2 m-RAMP2 r-RAMP2 | A Q P L P T T G T P G P E S Q N Q S H P T E D S L V N Q S H P T E D S L L | ŠEGGTVKNYE SK - GKMEDYE SK - GKMEDYE 130 | TAVQFCWNHY THVLPCWYEY TNVLPCWYYY | C D Q M D P I E K D W A M S K S C M D S V - K D W T L S K T S M D S V - K D W T L S K T S M D S V - K D W T L S 160 140 <td< th=""><th>R R R</th></td<> | R R R |
| h-RAMP2 m-RAMP2 r-RAMP2 | P Ý S T L R D C L E H F A E L H Y S D L Q N C L E Y N A D K Y Y S N L R Y C L E Y E A D K | FDLGFPNPLA FGLGFPNPLA FGLGFPNPLA | | H F A N C S Ĺ V Q P T F S D P P É D H F A N C S L V Q P T F S D P P E D H F A N C S L V Q P T F S D P P E D | |
| h-RAMP2 m-RAMP2 r-RAMP2 | L L A M I I A P I C L I P F L L L A M I I A P I C L I P F L L L A M I I A P I C L I P F L | T L V V W R S K D V T L V V W R S K D V T L V V W R S K D | S E A Q A S D A Q A G D A Q A | | |
| c) | 1 10 | 20 | 30 | 40 50 | |
| h-RAMP3 m-RAMP3 r-RAMP3 | METGALRRPQLLPLL MKTPA - QRLHLLPLL MATPA - QRLHLLPLL | L L C G G C P R A L L C G E C A Q V L L C G E C A Q V | G G C N È T G M L E F C G C N E T G M L E F C G C N E T G M L E F | R L P L Č G K A F A D M M G Ř V D V R L P R C G K A F A D M M Q K V A V R L P R C G K A F A D M M Q K V A V R L P R C G K A F A E M M Q K V D V | W W W |
| h-RAMP3 m-RAMP3 r-RAMP3 | KWCNLSEFIVYYESF KWCNLSEFIVYYESF KWCNLSEFIVYYESF | T N C T E M E A N V T N C T E M E T N I T N C T E M E T N I T N C T E M E T N I | W G C Y W P N P L A M G C Y W P N P L A V G C Y W P N P L A | Q G F I T G I H R Q F F S N C T V D S F I T G I H R Q F F S N C T V D S F I T G I H R Q F F S N C T V D | R R R |
| h-RAMP3 m-RAMP3 r-RAMP3 | V H L E D P P D E V L I P L I T H W E D P P D E V L I P L I T H W E D P P D E V L I P L I | V I P V V L T V A M A V P V V L T V A M A V P V L L T V A M | A G L V V W R S K R A G L V V W R S K H A G L V V W R S K H A G L V V W R S K R | | |

Figure S 5. Amino acid sequence alignment of human, mouse, and rat (a) RAMP1, (b) RAMP2, and (c) RAMP3. Sequence alignment created using Geneious Prime using the uniprot accepted sequences. Dark blue background with white text indicates an exact match, medium blue background indicates greater than 80% similarity at a given position, and light blue background indicates a similarity between 60% and 80% at a given position. Note that the m-RAMP2 used in this study encodes a threonine in position ten.

| | 1 | 10 | 20 | 30 | 40 | 50 | 60 | | 70 | 80 | 90 |
|--------------|---------------------------|--------------------------|------------------------------------|-----------------|---|-----------|-------------------|---------------------|-----------|-----------|---|
| XP 021069211 | MAPLRVER | APGGSRLG\ | /TR <mark>A</mark> QRPA/ | ALCLPPLLM | <u>ALLLLLLLAGA</u> | VSTSPESLN | QSFPENLN | SSPESLN | QSHPTEDSI | VSKGKMEDY | ETNVLPCWYYY |
| XP_021033447 | MAPLRVER | S <mark>P</mark> GGSRLG\ | /TRTQRPA/ | ALCLPP | LLLLLLLGA | VSTSPESLN | R <mark>SL</mark> | PESLN | QSHPTEDSL | VSKGKMEDY | ET <mark>H</mark> VLPCWY <mark>E</mark> Y |
| AAH69992 | MAPLRVER | ATGGSRLG\ | TR <mark>A</mark> QR P | ALCLPPL- | LLLLLLLGA | VSASPESLN | Q <mark>SL</mark> | PESQN | QSHPTEDSL | VSKGKMEDY | ET <mark>H</mark> VLPCWY <mark>E</mark> Y |
| AF146523 1 | MAPLRVER | APGGSRLG\ | /TR <mark>A</mark> QR P T / | ALCLPPL | LLLLLLLGA | VSASPESLN | Q <mark>SL</mark> | PESQN | QSHPTEDSL | VSKGKMEDY | ETHVLPCWYEY |
| AF209906_1 | MAPLRVER | AP <mark>GGSRLG\</mark> | /TR <mark>A</mark> QR P T / | ALCLPPL- | LLLLLLLGA | VSASPESLN | Q <mark>SL</mark> | <mark>PESQ</mark> N | QSHPTEDSL | VSKGKMEDY | ET <mark>H</mark> VLPCWY <mark>E</mark> Y |
| CAB59512 | MAPLRVER | AP <mark>GGSRLG\</mark> | /TR <mark>A</mark> QR P T / | ALCLPPL- | LLLLLLLGA | VSASPESLN | Q <mark>SL</mark> | PESQN | QSHPTEDSL | VSKGKMEDY | ET <mark>H</mark> VLPCWY <mark>E</mark> Y |
| EDL03901 | MAPLRVER | APGGSRLG\ | /TR <mark>A</mark> QR P T / | ALCLPPL- | LLLLLLLGA | VSASPESLN | Q <mark>SL</mark> | <mark>PESQ</mark> N | QSHPTEDSL | VSKGKMEDY | ET H VLPCWY <mark>E</mark> Y |
| NP 062317 | MAPLRVER | AP <mark>GGSRLG\</mark> | /TR <mark>A</mark> QRP T / | ALCLPPL- | LLLLLLLGA | VSASPESLN | Q <mark>SL</mark> | PESQN | QSHPTEDSL | VSKGKMEDY | ET <mark>H</mark> VLPCWY <mark>E</mark> Y |
| - | 100 | 110 | 120 | 1 | 30 14 | 0 1 | 50 | 160 | 170 | 180 | 190 |
| XP 021069211 | KSGMDSVK | | RYYSNLO | | K F <mark>G</mark> LGF PNP L A | ESIILEAHT | I HFANCSL | /QPTFSDP | PEDVLLAM | IAPICLIPF | LVTLVWRSKD |
| XP_021033447 | K S <mark>S</mark> MDSVKI | DWCNWTL I S | RYYSDLQ | CLEYKAD | K F E L G F P N P V A | ESIILEAH | I HF ANCSL | /QPTFSDF | PEDVLLAM | IAPICLIPF | LVTLVWRSKD |
| AAH69992 | KSCMDSVK | DWCNWTL I S | RHYSDLQ | NCLEYNAD | K F <mark>G</mark> LGF PNP <mark>L</mark> A | ENTILEAHL | I HF ANCSL | /QPTFSDP | PEDVLLAM | IAPICLIPF | LVTLVVWRSKD |
| AF146523 1 | K SCMDSVKI | DWCNWTL I S | SRHYSDLQ | NCL E YNAD | K F <mark>G</mark> LGF PNP <mark>L</mark> A | ENTILEAHL | I HF ANCSL | /QPTFSDP | PEDVLLAM | IAPICLIPF | LVTLVVWRSKD |
| AF209906_1 | KSCMDSVK | DWCNWTL I S | SRHYSDLQ | ICLEYNAD | K F <mark>G</mark> LGF PNP <mark>L</mark> A | ENILEAHL | IHFANCSL\ | /QPTFSDP | PEDVLLAM | IAPICLIPF | LVTLVVWRSKD |
| CAB59512 | K SCMDSVKI | DWCNWTL I S | SRHYSDLQ | NCL EYNAD | K F <mark>G</mark> LGF PNP <mark>L</mark> A | ENILEAHL | I HF ANCSL | /QPTFSDP | PEDVLLAM | IAPICLIPF | LVTLVVWRSKD |
| EDL03901 | KSCMDSVK | DWCNWTL I S | SRHYSDLQ | NCLEYNAD! | K F <mark>G</mark> LGF PNP <mark>L</mark> A | ENILEAHL | I HF ANCSL | /QPTFSDP | PEDVLLAM | IAPICLIPF | LVTLVVWRSKD |
| NP 062317 | KSCMDSVK | DWCNWTLIS | RHYSDLQ | NCLEYNAD! | K F <mark>G</mark> LGF PNP <mark>L</mark> A | ENILLEAHL | I HF ANCSL | /QPTFSDF | PEDVLLAM | IAPICLIPF | LVTLVVWRSKD |
| - | 199 | | | | | | | | | | |
| XP 021069211 | SDAQA | | | | | | | | | | |
| XP_021033447 | SDAQA | | | | | | | | | | |
| | SDAQA | | | | | | | | | | |

Figure S 6. Amino acid sequence of m-RAMP2 from different studies. NCBI protein accession numbers are listed on the left. Sequences alignment performed in Geneious Prime. Dark blue background with white text indicates an exact match, medium blue background indicates greater than 80% similarity, and light blue indicates a similarity between 60% and 80%. The sequence used in this study was AAH69992.



Figure S 7 cAMP production in Cos7 cells transfected with m-CLR:pcDNA and stimulated with m/r- α CGRP or m-AM. Results are the mean \pm s.e.m. of 3 independent experiments performed in duplicate. The black dotted line indicates 0, while the colour coded lines indicate the mean of the unstimulated point for each peptide.



Figure S 8. Distribution of pEC₅₀ values from agonist concentration-response experiments in Cos7 cells transfected with m-CTR:pcDNA (**A**), m-CTR:m-RAMP1 (**B**), m-CTR:m-RAMP2 (**D**), m-CTR:m-RAMP3 (**F**), m-CLR:m-RAMP1 (**C**), m-CLR:m-RAMP2 (**E**), or m-CLR:m-RAMP3 (**G**). NC indicates no curve could be fit to the data. Each individual point represents an independent experiment. Bars show the mean \pm s.e.m. of experiments. Significance determined using one-way ANOVA with Tukey's post-hoc test comparing peptides within a receptor. For presentation, only differences to a reference agonist are shown in this figure (m-CT at m-CTR:pcDNA, m/r-Amy for m-CTR in complex with m-RAMP1, 2, or 3, m/r- α CGRP at m-CLR:m-RAMP1, and m-AM at m-CLR:m-RAMP2 or 3). See also **Table 1**.

General Procedure

All reagents were purchased as reagent grade and used without further purification. N,N-Diisopropylethylamine *N*,*N*'-diisopropylcarbodiimide (DIPEA). piperidine, (DIC). 1,2-ethanedithiol (EDT), triisopropylsilane (TIPS) and 4-methylmorpholine (NMM) were purchased from Sigma-Aldrich (St. Louis, Missouri). O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium Fmoc-Arg(Pbf)-OH hexafluorophosphate (HATU). Fmoc-Ala-OH. (Pbf 2.2.4.6.7-= pentamethyIdlhydrobenzofuran-5-sulfonyl), Fmoc-Asn(Trt)-OH (Trt = triphenylmethane), Fmoc-Asp(*t*Bu)-OH (*t*Bu = *tert*-butyl), Fmoc-Cys(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-lle-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH (Boc = tertbutoxycarbonyl), Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH and 4-[(2,4-Dimethoxyphenyl)(Fmocamino)methyl]phenoxyacetic acid (Rink amide linker) were purchased from CS Bio (Shanghai, China). 6-Chloro-1-hydroxybenzotriazole (6-Cl-HOBt), Fmoc-Ser(tBu)-Ser(Psi(Me,Me)pro)-OH, Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH, Fmoc-Leu-Thr(Psi(Me,Me)pro)-OH, Fmoc-Asp-Ser(Psi(Me,Me)pro)-OH, Fmoc-Gly-Thr(Psi(Me,Me)pro)-OH were purchased from Apptec (Louisville, Kentucky). Aminomethyl polystyrene resin was purchased from Rapp Polymere (Tübingen, Germany). Guanidine hydrochloride (Gu·HCl) was purchased from AK Scientific (Union City, California). Yields refer to chromatographically homogeneous materials. Microwave reactions were carried out on a Biotage® Initiator+ AlstraTM (Uppsala, Sweden) automated peptide synthesizer. Semi-preparative/analytical RP-HPLC was performed on a Thermo Scientific (Waltham, MA) Dionex Ultimate 3000 HPLC equipped with a four channel UV detector at 210, 225, 254 and 280 nm using either an analytical column (Waters (Milford, MA) XTerra[®] MS C18, (5 μ m; 4.6 × 150 mm) at a flow rate of 1 mL min⁻¹, or a Phenomenex[®] semi-preparative column (Torrance, CA), Gemini C18, (5 µm; 10 × 250 mm) at a flow rate of 4 mL min⁻ ¹. A suitably adjusted gradient of 5% B to 95% B was used, where solvent A was 0.1% TFA in H₂O and B was 0.1% TFA in acetonitrile. LC-MS spectra were acquired using Agilent Technologies (Santa Clara, CA) 1260 Infinity LC equipped with an Agilent Technologies 6120 Quadrupole mass spectrometer. An analytical column (Agilent C3, 3.5 μ m; 3.0 \times 150 mm) was used at a flow rate of 0.3 mL min⁻¹ using a linear gradient of 5% B to 95% B over 30 min, where solvent A was 0.1% formic acid in H₂O and B was 0.1% formic acid in acetonitrile.

General Methods

Method 1: General procedure for attachment of Fmoc Rink amide to the resin:

To aminomethyl polystyrene resin (80 mg, 0.1 mmol, loading: 1.26 mmol/g) pre-swollen in CH₂Cl₂ (5 mL, 20 min), was added 4-[(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid (Rink amide linker) (220.2 mg, 4 equiv., 0.4 mmol) and 6-Cl-HOBt (70.0 mg, 3.5 equiv., 0.35 mmol) dissolved in DMF (1.5 mL) followed by addition of DIC (62 μ L, 4 equiv., 0.4 mmol). The reaction mixture was gently agitated at room temperature for 24 h. The resin was filtered and washed with DMF (3 × 3 mL) after which a negative ninhydrin test confirmed successful coupling.

Method 2: General procedure for removal of N^{α} -Fmoc-protecting group:

Peptidyl resin was treated with a solution of 20 vol % piperidine in DMF (v/v, 4 mL) and the mixture was agitated on the Biotage[®] Initiator Alstra for 2 × 5 min at room temperature. The resin was filtered and washed with DMF (3 × 3 mL).

Method 3: General coupling procedure for Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH Fmoc-Gly-OH, Fmoc-lle-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Trr(*t*Bu)-OH, Fmoc-Val-OH:

Couplings were performed using the Biotage[®] Initiator Alstra with the appropriate Fmoc-protected amino acid (0.2 M, DMF, 5 equiv.), HATU (0.5 M, DMF, 4.75 equiv.) and NMM in DMF (2 M, 8equiv.) using a single coupling cycle at 75 °C, 110 W for 5 min. The resin was filtered and washed with DMF (3×3 mL).

Method 4: General coupling procedure for Fmoc-Arg(Pbf)-OH:

Double coupling cycles of Fmoc-Arg(Pbf)-OH were carried out with the Biotage[®] Initiator Alstra using Fmoc-Arg(Pbf)-OH (0.2 M, DMF, 5 equiv.), HATU (0.5 M, DMF, 4.75 equiv.) and NMM in DMF (2 M, 8 equiv.) with first coupling at room temperature for 25 min, followed by a second coupling cycle at 72 °C, 110 W for 5 min. The resin was filtered and washed with DMF (3×3 mL).

Method 5: General coupling procedure for Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH:

Double coupling cycles of Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH were carried out with the Biotage[®] Initiator Alstra using Fmoc protected amino acid (0.2 M, DMF, 5 equiv.), HATU (0.5 M, DMF, 4.75 equiv.) and NMM in DMF (2 M, 8 equiv.) with first coupling at room temperature for 15 min, followed by 43 °C, 110 W for 10 min. The resin was filtered and washed with DMF (3×3 mL).

Method 6: Coupling of Fmoc-dipeptide(Psi(Me,Me)pro)-OH:

Deprotected resin was treated with the appropriate Fmoc-dipeptide(Psi(Me,Me)pro)-OH (5 equiv., 0.5 mmol) and HATU (171.1 mg, 4.5 equiv., 0.45 mmol) in 1.5 mL DMF followed by addition of NMM (88 μ L, 8 equiv., 0.8 mmol) and agitated for 30 min, before being filtered, washed with DMF (3 × 3 mL) and the procedure repeated again with fresh reagents. The resin was filtered and washed with DMF (3 × 3 mL).

Method 7: General procedure for the capping of free amino groups:

Fmoc-protected peptidyl resin was treated with 5 M Ac₂O in DMF (0.47 mL, 2.5 equiv.) and NMM in DMF (2 M, 8 equiv.) using the Biotage[®] Initiator Alstra at room temperature for 10 min. The resin was filtered and washed with DMF (3×3 mL).

Method 8: General procedure for TFA-mediated resin cleavage and global deprotection:

Peptidyl resin was treated with a mixture of TFA/H₂O/TIPS/EDT (94:2.5:2.5:1, v/v/v/v, 10 mL) for 120 min. The filtrate was partially concentrated under a gentle stream of N₂, then cold diethyl ether was then added to form a precipitate. The mixture was centrifuged, and the solution was carefully decanted off and discarded, before dissolving the solid pellet in H₂O:acetonitrile containing 0.1% TFA (1:1, v/v, 25 mL) and lyophilised.

Method 9: General procedure for oxidation between cysteines at position 2 and 7:

To purified peptide was added a pre-made solution of (6 M Gu·HCl/ 0.2 M Na₂HPO₄/ 0.1 mM EDTA/ 8 mM cysteine/ 1 mM cysteine) in H₂O adjusted to pH 8.2- 8.3 using 5 M HCl and 10 M NaOH to make a peptide concentration of 1 mM. Once the peptide had been added and dissolved the solution was subsequently diluted with H₂O to a final peptide concentration 0.25 mM and the resulting solution was sonicated for 1 min then agitated for 3 h at room temperature. The crude reaction mixture was quenched with 5 M HCl (40 μ L) and the crude solution of peptide was immediately purified batchwise by semi-preparative RP-HPLC.

Synthesis of m-βCGRP using Fmoc-SPPS.

Scheme 1. Synthesis of m- β CGRP.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin 1 using Method 1 followed by Fmocremoval using Method 2. Direct attachment of Fmoc-Phe-OH at position 37 to resin bound Rink amide 2 was achieved using Method 3. Method 2 was used for all subsequent N^{α} -Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ¹⁸Arg indicated in Scheme 1 using Method 3. All Fmoc-Arg(Pbf)-OH residues are coupled using Method 4. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using Method 5. Capping of free amino groups was conducted throughout the synthesis using Method 7. Coupling of Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH dipeptide at position 18 was achieved using Method 6. Linear synthesis of the peptide was continued up to Ser at position 1 using appropriate Methods (2-5, 7). Peptide 3 was liberated from resin using Method 8 affording 4. Crude 4 was purified batchwise by semipreparative RP-HPLC on a Phenomenex[®] Gemini C18, $(10 \times 250 \text{ mm}, 5 \mu \text{m})$ using a linear gradient of 5% to 95% over 90 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford 4 as a white amorphous solid (90 mg, 24% yield based on 0.1 mmol scale). Oxidation of 4 (40 mg, 1.0×10^{-5} mol) was achieved using Method 9. The crude 5 reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex[®] Gemini C18 column (10×250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford the compound 5 (m-\beta CGRP) as a white amorphous solid (11 mg, 28% yield based on 1.0×10^{-5} mol), $t_{\rm R} = 34.4$ min, >98% purity by HPLC (Figure S 9, Figure S10).



Figure S 9. Analytical RP-HPLC chromatogram of purified peptide **5**, $t_R = 34.4$ min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra[®] MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H₂O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).



Figure S 10. LC-MS profile of purified peptide **5**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS. $(m/z \text{ [M+3H]}^{3+} \text{ calcd: } 1294.1; \text{ found: } 1293.7; \text{ [M+4H]}^{4+} \text{ calcd: } 970.8; \text{ found: } 970.7; \text{ [M+5H]}^{5+} \text{ calcd: } 776.8; \text{ found: } 776.8. \text{ Mass deconvolution calculated at } 3878.63 \text{ Da with standard deviation of } 0.47; \text{ theoretical mass calculated at } 3879.34 \text{ Da.}$

Synthesis of AC187 using Fmoc-SPPS.

Scheme 2. Synthesis of AC187.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmocremoval using **Method 2**. Direct attachment of Fmoc-Tyr(*t*Bu)-OH at position 25 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α} -Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ¹Val indicated in **Scheme S2** using appropriate **Methods** (**2-5**, **7**). All Fmoc-Arg(Pbf)-OH residues are coupled using **Method 4**. All Fmoc-His(Trt)-OH residues are coupled using **Method 5**. Capping of free amino groups was conducted throughout the synthesis using **Method 7**. Peptide **6** was liberated from resin using **Method 8** which was subsequently purified batchwise by semi-preparative RP-HPLC on a Phenomenex[®] Gemini C18, ($10 \times 250 \text{ mm}$, $5 \mu \text{m}$) using a linear gradient of 5% to 95% over 95 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m/z* were combined and lyophilised to afford the compound **7** (AC187) as a white amorphous solid (84 mg, 29% yield based on 0.1 mmol scale), *t*_R = 32.3 min, >99% purity by HPLC; (**Figure S 11, Figure S 12**).



Figure S 11. Analytical RP-HPLC chromatogram of purified peptide **7**, $t_R = 32.3$ min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra[®] MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H₂O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).



Figure S 12. LC-MS profile of purified peptide **7**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS (m/z [M+2H]²⁺ calcd: 1446.13; found: 1445.8; [M+3H]³⁺ calcd: 964.4; found: 964.2; [M+4H]⁴⁺ calcd: 723.6; found: 723.4. Mass deconvolution calculated at 2889.60 Da with standard deviation of 0.00; theoretical mass calculated at 2890.26 Da.

Synthesis of m-AM using Fmoc-SPPS.

Scheme 3 Synthesis of m-AM.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin 1 using Method 1 followed by Fmocremoval using **Method 2**. Direct attachment of Fmoc-Tyr(tBu)-OH at position 50 to resin bound Rink amide 2 was achieved using Method 3. Method 2 was used for all subsequent N^{α} -Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ³³Asp indicated in Scheme S3 using Method 3. All Fmoc-Arg(Pbf)-OH residues are coupled using Method 4. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using Method 5. Capping of free amino groups was conducted throughout the synthesis using Method 7. Coupling of Fmoc-Leu-Thr(Psi(Me,Me)pro)-OH dipeptide at position 33 was achieved using Method 6. Linear synthesis of the peptide was continued up to Cys at position 19 using appropriate Methods (2-5, 7). Coupling of Fmoc-Gly-Thr(Psi(Me,Me)pro)-OH dipeptide at position 19 was achieved using Method 6. Linear synthesis of the peptide was continued up to Tyr at position 1 using appropriate Methods (2-5, 7). Peptide 8 was liberated from resin using Method 8 affording 9. Crude 9 was purified batchwise by semi-preparative RP-HPLC on a Phenomenex[®] Gemini C18, (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 90 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford 9 as a white amorphous solid (60 mg, 10% yield based on 0.1 mmol scale). Oxidation of 9 (40 mg, 6.9×10^{-6} mol) was achieved using Method 9. The crude 10 reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex[®] Gemini C18 column (10×250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford the compound 10 (m-AM) as a white amorphous solid (8 mg, 20% yield based on 6.9 × 10⁻⁶ mol), $t_{\rm R}$ = 30.4 min, >97% purity by HPLC; (Figure S 13, Figure S 14)



Figure S 13. Analytical RP-HPLC chromatogram of purified peptide **10**, $t_R = 30.4$ min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra[®] MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H₂O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).



Figure S 14. ESI-MS profile of purified peptide **10**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS (m/z [M+3H]³⁺ calcd: 1909.1; found: 1908.8; [M+4H]⁴⁺ calcd: 1432.1; found: 1431.7; [M+5H]⁵⁺ calcd: 1145.8; found: 1145.7; [M+6H]⁶⁺ calcd: 955.0; found: 954.9; [M+7H]⁷⁺ calcd: 818.7; found: 818.8. Mass deconvolution calculated at 5723.54 Da with standard deviation of 0.65; theoretical mass calculated at 5724.44 Da.

Synthesis of m-CT using Fmoc-SPPS.

Scheme 4. Synthesis of m-CT.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin 1 using Method 1 followed by Fmocremoval using Method 2. Direct attachment of Fmoc-Pro-OH at position 32 to resin bound Rink amide 2 was achieved using Method 3. Method 2 was used for all subsequent N^{α} -Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ¹Cys indicated in Scheme S4 using Method 3. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using **Method 5**. Capping of free amino groups was conducted throughout the synthesis using Method 7. Peptide 11 was liberated from resin using Method 8 affording 12. Crude 12 was purified batchwise by semi-preparative RP-HPLC on a Phenomenex[®] Gemini C18, $(10 \times 250 \text{ mm}, 10 \times 250 \text{ mm})$ 5 µm) using a linear gradient of 5% to 95% over 90 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford 12 as a white amorphous solid (47 mg, 13%) yield based on 0.1 mmol scale). Oxidation 12 (47 mg, 1.3×10^{-5} mol) was achieved using Method 9. The crude 13 reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex[®] Gemini C18 column (10×250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford the compound 13 (m-CT) as a white amorphous solid (14.7) mg, 31% yield based on 1.3×10^{-5} mol), $t_{\rm R} = 32.2$ min, >99% purity by HPLC (Figure S 15, Figure S 16).



Figure S 15. Analytical RP-HPLC chromatogram of purified peptide **13**, $t_R = 32.2$ min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra[®] MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H₂O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).



Figure S 16. LC-MS profile of purified peptide **13**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS (m/z [M+2H]²⁺ calcd: 1736.9; found: 1736.6; [M+3H]³⁺ calcd: 1158.3; found: 1158.0; [M+4H]⁴⁺ calcd: 868.9; found: 868.8. Mass deconvolution calculated at 3471.13 Da with standard deviation of 0.12; theoretical mass calculated at 3471.93 Da.

Synthesis of m-AM247 mouse using Fmoc-SPPS.

Scheme 5 Synthesis of m-AM2₄₇.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin 1 using Method 1 followed by Fmocremoval using Method 2. Direct attachment of Fmoc-Tyr(tBu)-OH at position 47 to resin bound Rink amide 2 was achieved using Method 3. Method 2 was used for all subsequent N^{α} -Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ³⁷Ala indicated in Scheme S5 using Method 3. All Fmoc-Arg(Pbf)-OH residues are coupled using Method 4. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using Method 5. Capping of free amino groups was conducted throughout the synthesis using Method 7. Coupling of Fmoc-Asp-Ser(Psi(Me,Me)pro)-OH dipeptide at position 37 was achieved using Method 6. Linear synthesis of the peptide was continued up to His at position 22 using appropriate Methods (2-5, 7). Coupling of Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH dipeptide at position 22 was achieved using Method 6. Linear synthesis of the peptide was continued up to Pro at position 1 using appropriate Methods (2-5, 7). Peptide 14 was liberated from resin using Method 8. Crude 15 was purified batchwise by semipreparative RP-HPLC on a Phenomenex[®] Gemini C18, $(10 \times 250 \text{ mm}, 5 \mu\text{m})$ using a linear gradient of 5% to 95% over 90 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford 15 as a white amorphous solid (50 mg, 10% yield based on 0.1 mmol scale). Oxidation of 15 (50 mg, 9.6×10^{-6} mol) was achieved using Method 9. The crude 16 reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex[®] Gemini C18 column (10×250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford the compound 16 (m-AM2₄₇) as a white amorphous solid (15 mg, 30% yield based on 9.6 × 10⁻⁶ mol), $t_{\rm R}$ = 32.6 min, >98% purity by HPLC (Figure S 17, Figure S 18).



Figure S 17. Analytical RP-HPLC chromatogram of purified peptide **16**, $t_{\rm R} = 32.6$ min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra[®] MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H₂O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).



Figure S 18. LC-MS profile of purified peptide **16**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS (m/z [M+4H]⁴⁺ calcd: 1301.1; found: 1301.1; [M+5H]⁵⁺ calcd: 1041.1; found: 1041.0; [M+6H]⁶⁺ calcd: 867.7; found: 867.8; [M+7H]⁷⁺ calcd: 743.9; found: 743.9; [M+8H]⁸⁺ calcd: 651.1; found: 651.0 [M+9H]⁹⁺ calcd: 578.8; found: 578.8. Mass deconvolution calculated at 5200.28 Da with standard deviation of 0.30; theoretical mass calculated at 5200.45 Da.

Synthesis of m-AM240 mouse using Fmoc-SPPS.

Scheme 6. Synthesis of m-AM2₄₀.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin 1 using Method 1 followed by Fmocremoval using Method 2. Direct attachment of Fmoc-Tyr(tBu)-OH at position 47 to resin bound Rink amide 2 was achieved using Method 3. Method 2 was used for all subsequent N^{α} -Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ³⁷Ala indicated in Scheme S6 using Method 3. All Fmoc-Arg(Pbf)-OH residues are coupled using Method 4. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using Method 5. Capping of free amino groups was conducted throughout the synthesis using Method 7. Coupling of Fmoc-Asp-Ser(Psi(Me,Me)pro)-OH dipeptide at position 37 was achieved using Method 6. Linear synthesis of the peptide was continued up to His at position 22 using appropriate Methods (2-5, 7). Coupling of Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH dipeptide at position 22 was achieved using Method 6. Linear synthesis of the peptide was continued up to Val at position 8 using appropriate Methods (2-5, 7). Peptide 17 was liberated from resin using Method 8. Crude 18 was purified batchwise by semipreparative RP-HPLC on a Phenomenex[®] Gemini C18, $(10 \times 250 \text{ mm}, 5 \mu\text{m})$ using a linear gradient of 5% to 95% over 90 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford 18 as a white amorphous solid (75 mg, 17% yield based on 0.1 mmol scale). Oxidation of 18 (75 mg, 1.7×10^{-5} mol) was achieved using Method 9. The crude 19 reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex[®] Gemini C18 column (10×250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford the compound 19 (m-AM 2_{40}) as a white amorphous solid (30 mg, 40% yield based on 1.7×10^{-5} mol), $t_{\rm R} = 32.3$ min, >98% purity by HPLC (Figure S 19, Figure S 20).



Figure S 19. Analytical RP-HPLC chromatogram of purified peptide **19**, $t_{\rm R} = 32.3$ min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra[®] MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H₂O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).



Figure S 20. LC-MS profile of purified peptide **19**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS (m/z [M+3H]³⁺ calcd: 1462.6; found: 1462.1; [M+4H]⁴⁺ calcd: 1097.2; found: 1097.1; [M+5H]⁵⁺ calcd: 877.9; found: 877.8; [M+6H]⁶⁺ calcd: 731.8; found: 731.8; [M+7H]⁷⁺ calcd: 627.4; found: 627.3. Mass deconvolution calculated at 4384.12 Da with standard deviation of 0.55; theoretical mass calculated at 4384.98 Da.

Synthesis of m/r-aCGRP using Fmoc-SPPS.

Scheme 7 Synthesis of m/r-aCGRP



Fmoc-Rink amide was attached to aminomethyl polystyrene resin 1 using Method 1 followed by Fmocremoval using Method 2. Direct attachment of Fmoc-Phe-OH at position 37 to resin bound Rink amide 2 was achieved using Method 3. Method 2 was used for all subsequent N^{α} -Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ¹⁸Arg indicated in Scheme 7 using Method 3. All Fmoc-Arg(Pbf)-OH residues are coupled using Method 4. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using Method 5. Capping of free amino groups was conducted throughout the synthesis using Method 7. Coupling of Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH dipeptide at position 18 was achieved using Method 6. Linear synthesis of the peptide was continued up to Ser at position 1 using appropriate Methods (2-5, 7). Peptide 20 was liberated from resin using Method 8 affording 21. Crude 21 was purified batchwise by semipreparative RP-HPLC on a Phenomenex[®] Gemini C18, $(10 \times 250 \text{ mm}, 5 \mu\text{m})$ using a linear gradient of 5% to 95% over 90 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford 21 as a white amorphous solid (74 mg, 19% yield based on 0.1 mmol scale). Oxidation of 21 (20 mg, 5.2×10^{-6} mol) was achieved using Method 9. The crude 22 reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex[®] Gemini C18 column (10×250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (ca. 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct m/z were combined and lyophilised to afford the compound 22 (m/r-aCGRP) as a white amorphous solid (3 mg, 15% yield based on 5.2×10^{-6} mol), $t_{\rm R} = 31.1$ min, >99% purity by HPLC (Figure S 21, Figure S 22).



Figure S 21. Analytical RP-HPLC chromatogram of purified peptide **22**, $t_R = 31.1$ min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra[®] MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H₂O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).



Figure S 22. LC-MS profile of purified peptide **22**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS. $(m/z \ [M+3H]^{3+} \text{ calcd}: 1269.7; \text{ found}: 1269.5; \ [M+4H]^{4+} \text{ calcd}: 952.6; \text{ found}: 952.5; \ [M+5H]^{5+} \text{ calcd}: 762.2; \text{ found}: 762.1. Mass deconvolution calculated at 3805.67 Da with standard deviation of 0.29; theoretical mass calculated at 3806.30 Da.$

References

- Garelja, M. L., Au, M., Brimble, M. A., Gingell, J. J., Hendrikse, E. R., Lovell, A., Prodan, N., Sexton, P. M., Siow, A., Walker, C. S., Watkins, H. A., Williams, G. M., Wootten, D., Yang, S. H., Harris, P. W. R., & Hay, D. L. (2020, Apr 10). Molecular Mechanisms of Class B GPCR Activation: Insights from Adrenomedullin Receptors. ACS Pharmacol Transl Sci, 3(2), 246-262. https://doi.org/10.1021/acsptsci.9b00083
- Hay, D. L., Garelja, M. L., Poyner, D. R., & Walker, C. S. (2018, Jan). Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. Br J Pharmacol, 175(1), 3-17. https://doi.org/10.1111/bph.14075