Supplemental Data:

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N-arachidonyl glycine (NAGly) does not activate G protein-coupled receptor 18 (GPR18) signaling via canonical pathways

Molecular Pharmacology

Supplementary Figure Legends:

Supplementary Figure 1: GPR18 sequencing results and protein alignment. A) Sequencing results of the mouse GPR18 clone used in this study. Underlined sequence is the open reading frame. Highlighted in yellow is the mismatched nucleotide based on NM_182806.1 (*Mus musculus* GPR18 RefSeq accession number). The mismatch results in a synonymous mutation (CCG → CCA, Proline). B) Partial sequences of tagged GPR18 used in this study, specifically the junction between tagging sequences and GPR18. Underlined sequence is part of the GPR18 sequence. Highlighted in green is the sequence for EGFP and highlighted in orange is the sequence for one HA-tag. Note both sequences are in frame. C) ClustalW protein sequence alignment of GPR18 from mouse (*Mus musculus*), rat (*Rattus norvegicus*) and human (*Homo sapiens*). Highlighted in dark grey are identical sequences and highlighted in light grey are similar sequences. Transmembrane domains (tm1-7) are marked above sequences. Note the greatest divergence occurs in the N-terminus of GPR18.

Supplementary Figure 2: Design strategy for constitutively active mutants of GPR18 and ADRA2A. Mutants were designed based on mutagenesis studies of the α_{1B}-adrenergic receptor, ADRA1B (Kjelsberg et al., 1992; Scheer et al., 1996; Scheer et al., 1997). A) Snake plot diagrams of ADRA1B, ADRA2A and GPR18. Arrows mark residues mutated in GPCRs. B) Alignment of ADRA1B, ADRA2A, and GPR18 protein sequences. The amino acid residues targeted for mutation are bolded in red.

Supplementary Figure 3: Controls for functional coupling of $G\alpha_{15}$ to GPCRs expressed in SCG neurons. For A-F) Left panel: Sample I_{Ca} trace using the double-pulse protocol. Center panel: Time course of I_{Ca} measurements from the double-pulse protocol. ○ represent the prepulse I_{Ca} amplitude, ● represent the postpulse I_{Ca} amplitude. Black dashed line represents NE (10 μM) and grey dashed line represents glutamate (100 μM) application. Right panels: graphs of the magnitude of NE or glutamate responses as measured by the change in FR ($FR_{during} - FR_{before}$) and the response as a % of baseline I_{Ca}. Inhibition of I_{Ca} by mGluR2 coupled to $G\alpha_{15}$ (Figure 8A) was demonstrated with the following controls. A) Suppression of NE-mediated I_{Ca} inhibition by over-expression of $G\alpha_{15}$. This is likely due to the sequestration of all available $G\beta\gamma$. B) Recovery of NE-mediated inhibition of I_{Ca} by expressing $G\beta_1$ and $G\gamma_2$ along with $G\alpha_{15}$. This restores the stoichiometric balance of heterotrimeric G proteins. C) Lack of NE-mediated I_{Ca} inhibition in $G\alpha_{15}\beta_1\gamma_2$-expressing neurons following overnight PTX treatment. This indicates α_2-adrenergic receptors, responsible for mediating NE responses in SCG neurons, remain coupled to $G\alpha_{i/o}$ proteins in the presence of $G\alpha_{15}\beta_1\gamma_2$. D) Functional expression of mGluR2, which couples to $G\alpha_{i/o}$ proteins to produce voltage-dependent inhibition of I_{Ca}. Note the large change in FR and decrease in I_{Ca} following glutamate application. E) Lack of glutamate-mediated I_{Ca} inhibition in mGluR2-expressing neurons following overnight PTX treatment, suggesting mGluR2 coupling exclusively to $G\alpha_{i/o}$ proteins. F) Glutamate-mediated inhibition of I_{Ca} in cells co-expressing mGluR2 and $G\alpha_{15}\beta_1\gamma_2$. There appears to be a voltage-dependent component of I_{Ca} inhibition because of the relief of prepulse I_{Ca} inhibition by the depolarizing conditioning pulse and the change in FR during glutamate application. It is difficult to ascertain what proportion of

mGluR2 signaling is $G\alpha_{15}$-mediated, but there clearly is a $G\alpha_{i/o}$ protein-coupled component. Thus, overnight treatment of cells with PTX is required to observe mGluR2 coupling to $G\alpha_{15}$ because PTX potently uncouples the $G\alpha_{i/o}$ family of proteins from their GPCRs.

A

GPR18 clone:

TCGAGAATTCACCATGGCCACCCTGAGCAATCACAACCAGCTTGATCTTTCTAATGGCTCACACC
CAGAGGAATACAAAATCGCAGCCCTAGTCTTCTACAGCTGCATCTTCCTGATTGGGCTGTTTGTT
AATGTCACTGCGTTGTGGGTTTTCAGCTGTACGACCAAGAAAAGAACCACAGTGACCATCTACAT
GATGAACGTTGCACTACTGGACCTCGTATTTATACTCAGTCTGCCCTTTCGGATGTTTTACTATG
CAAAAGGCGAGTGGCCATTTGGAGAGTACTTCTGCCACATTCTTGGGGCCCTGGTGGTGTTTTAC
CCAAGCCTCGCTCTGTGGCTTCTTGCTTTCATTAGTGCTGACAGATACATGGCCATCGTACAGCC
AAAATATGCCAAGGAGCTGAAGAACACCGGCAAGGCCGTGCTTGCGTGTGGGGGGGTCTGGGTAA
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GCCACCTGCCTGAAGATCTCCGACATCACCCACTTAAAAGCTGTCAACGTGCTCAACTTCACGCG
ACTCATATTTTTCTTCCTGATCCCTTTGTTCATCATGATCGGGTGCTACGTGGTCATCATTCACA
GTCTCCTCCGAGGGCAGACGTCTAAGCTGAAGCCCAAGGTCAAGGAGAAGTCCATACGGATCATC
ATGACCCTCCTGCTGCAGGTGCTCGTCTGCTTCGTGCCCTTCCACATCTGCTTTGCCGTCCTGAT
GCTACAAGGACAGGAGAACAGCTATAGCCCCTGGGGAGCCTTCACCACCTTCCTCATGAACCTCA
GCACCTGTCTCGATGTAGTCCTCTACTACATCGTTTCCAAACAGTTCCAGGCTCGAGTCATCAGC
GTCATGCTGTACCGCAATTACCTTCGCAGTGTTCGCAGAAAAAGTGTCCGATCGGGCAGTTTACG
GTCACTTAGCAACATGAACAGTGAGATGCTTTGAGCGGCCGCTCGAGCAGAC

B

GPR18-EGFP (junction)

- AGT GAG ATG CTT CCG CGG GCC CGG GAT CCA CCG GTC GCC ACC ATG GTG AGC -

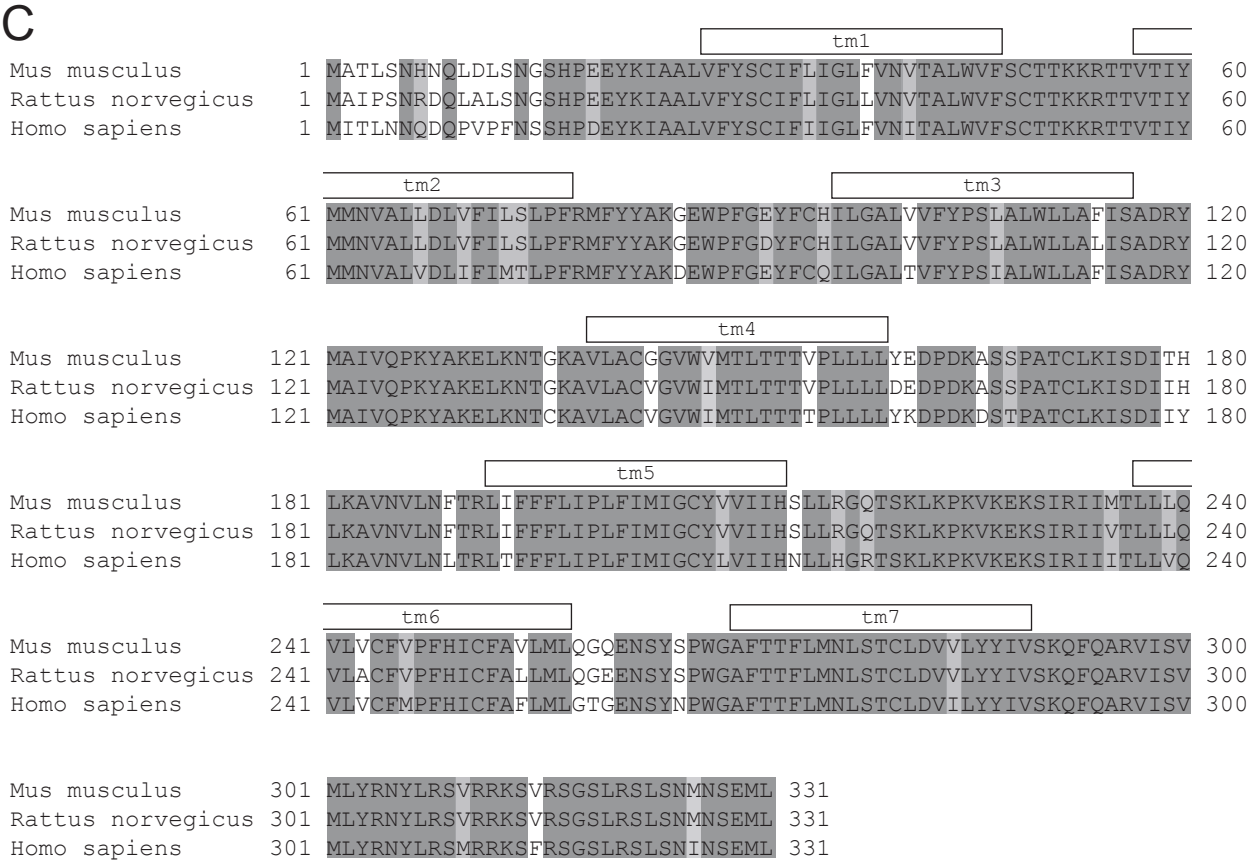
3xHA-GPR18 (junction)

- TAC CCA TAC GAT GTT CCA GAT TAC GCT GAT GCC ACC CTG -

GPR18-3xHA (junction)

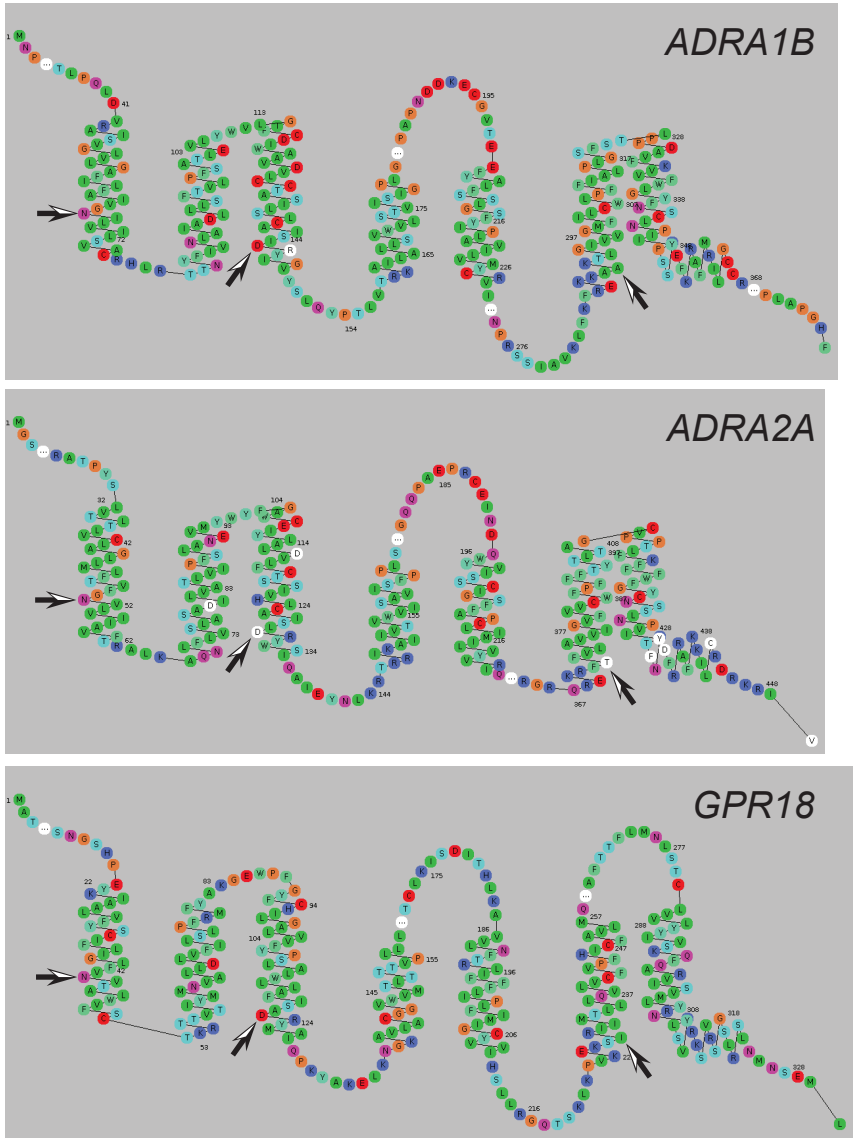
- AGT GAG ATG CTT GAT ATC TAC CCA TAC GAT GTT CCA GAT TAC GCT -

C



Supplemental Figure 1

A. Snake plot diagrams



B. Sequence alignment

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ADRA1B    MNPDLDTGHNTSAPAHWGELKDDNFTGPNQTSSNSTLPQLDVTRAISVGL
ADRA2A    -----------MGSLQPEAGNASWNGTEAPGGGARATPYSLQVTLTLVC
GPR18     ------------------MATLSNHNQLDLSNGSHPEEYKIAALVFYSC

ADRA1B    VLGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIADLLLSFTVLP
ADRA2A    LAGLLMLFTVFGNVLVIIAVFTSRALKAPQNLFLVSLASADILVATLVIP
GPR18     IF----LIGLFVNVTALWVFSCTTKKRTTVTIYMMNVALLDLVFILS-LP

ADRA1B    FSATLEVLGYWVLGRIFCDIWAAVDVLCCTASILSLCAISIDRYIGVRYS
ADRA2A    FSLANEVMGYWYFGKAWCEIYLALDVLFCTSSIVHLCAISLDRYWSITQA
GPR18     FRMFYYAKGEWPFGEYFCHILGALVVFYPSLALWLLAFISADRYMAIVQP

ADRA1B    LQYPTLVTRRKAILALLSVWVLSTVISIGPLLGWKEPAPNDDKE-----C
ADRA2A    IEYNLNRTPRRIKAIIVTVWVISAVISFPPLISIEKKAGGGGQQPAEPRC
GPR18     KYAKELKNTGKAVLACGGVWVMTLTTTVPLLLLYEDPDKASSPATCLKIS

ADRA1B    GVTEEPFYALF---SSLGSFYIPLAVILVMYCRVYIVAKRTTKNLEAGVM
ADRA2A    EINDQKWYVIS---SCIGSFFAPCLIMILVYVRIYQIAKRRTR-------
GPR18     DITHLKAVNVLNFTRLIFFFLIPLFIMIGCYVVIIHSLLR----------

ADRA1B    KEMSNSKELTLRIHSKNFHEDTLSSTKAKGHNPRSSIAVKLFKFSREKKA
ADRA2A    ----------------------------VPPSRRGPDAAAALPGGAERRPN
GPR18     ------------------------------------GQTSKLKPKVKEKS

ADRA1B    AKTLGIVVGMFILCWLPFFIALPLGSLFSTLKPPDAVFKVVFWLGYFNSC
ADRA2A    GLGPERGVGRVGAEAEPLPVQLNGAPGEPAPAGPRDADGLDLEESSSSEH
GPR18     IRIIMTLLLQVLVCFVPFHICFAVLMLQGQENSYSPWGAFTTFLMNLSTC

ADRA1B    LNPIIYPCSSKEFKRAFMRILGCQCRGGRRRRRRRRLGGCAYTYRPWTRG
ADRA2A    AERPPGPRRSERGPRAKSKARASQVKPGDSLPRRG-------------PG
GPR18     LDVVLYYIVSKQFQARVISVMLYRN-------------------------

ADRA1B    GSLERSQSRKDSLDDSGSCMSGSQRTLPSASPSPGYLGRGTQPPVELCAF
ADRA2A    APGPGAPATGAGEERGGVAKASRWRGRQNREKRFTFVLAVVIGVFVVCWF
GPR18     ---------YLRSVRRKSVRSGSLRSLSNMNSEML---------------


ADRA1B    PEWKPGALLSLPEPPGRRGRLDSGPLFTFKLLGDPESPGTEGDTSNGGCD
ADRA2A    PFFFTYTLTAVG---CSVPPTLFKFFFWFGYCNSSLNPVIYTIFNHDFRR
GPR18     --------------------------------------------------

ADRA1B    TTTDLANGQPGFKSNMPLAPGHF
ADRA2A    AFKKILCRGDRKRIV--------
GPR18     ----------------------
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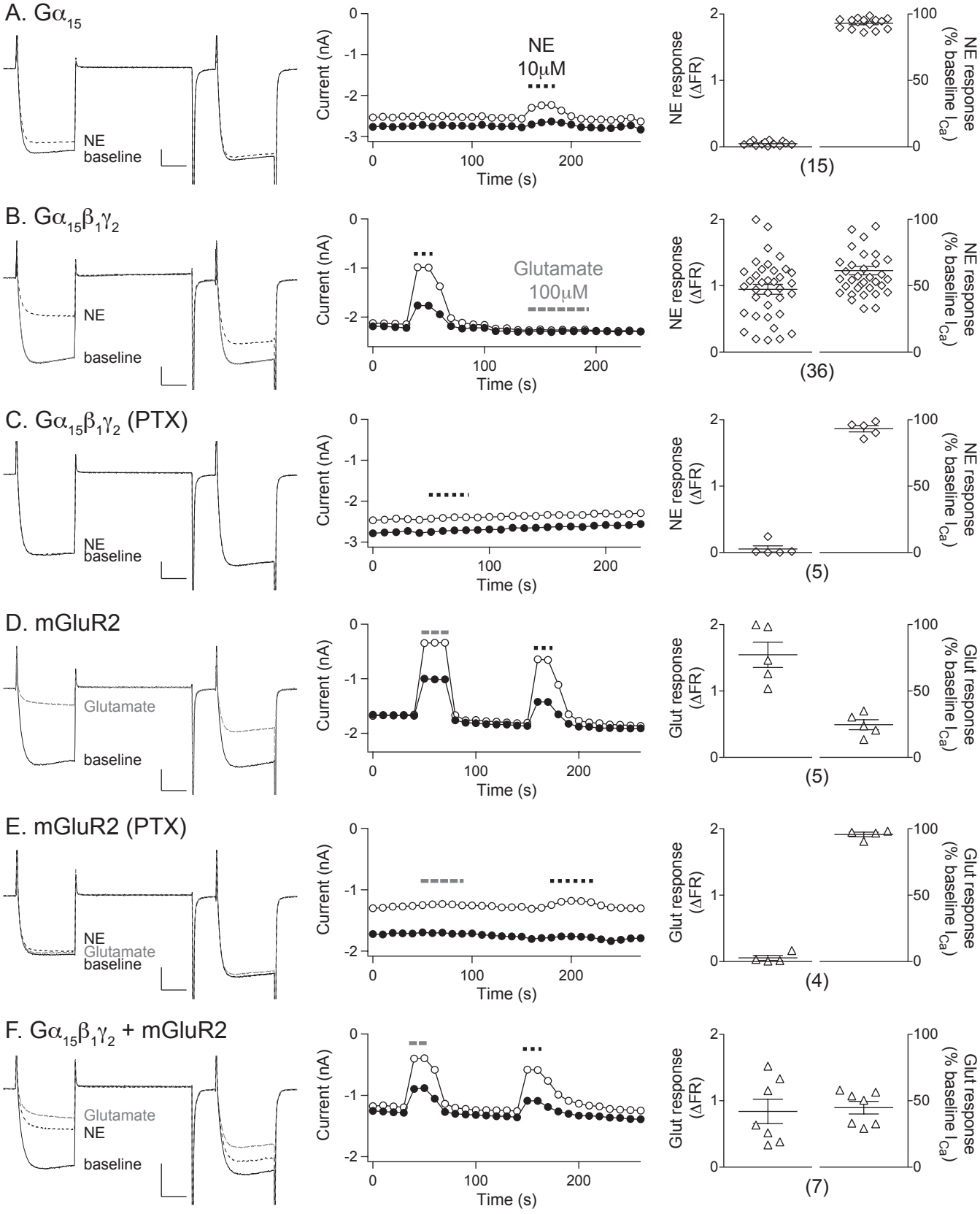
Supplemental Figure 2



Supplemental Figure 3