Supplemental Data

Membrane tethered bursicon constructs as heterodimeric modulators of the D*rosophila* GPCR rickets

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Molecular Pharmacology

Figure Legends

Supplemental Figure 1. Nucleotide sequence encoding the TNFα type II transmembrane tethered ligand backbone.

All bursicon MTL constructs were generated using the same transmembrane domain and linker sequence. All backbone sequences included a TNFα transmembrane domain (blue shading), a repetitive glycine-asparagine linker (red shading) and a myc epitope tag (green shading). The expression plasmid pcDNA1.1 was used as the vector. Bursicon subunits were cloned in frame into the construct using BamHI and EcoRI restrictions sites (gray shading).

Supplemental Figure 2. Tethered bursicon subunits are expressed independent of complementary subunit

A) Quantification of cell surface expression of tBur-α in the presence or absence of tBur-β. Forty eight hours after transfection ELISA was performed using an antibody directed against an HA epitope. B) Quantification of cell surface expression of tBur-β in the

presence or absence of tBur-α. Forty eight hours after transfection ELISA was performed using an antibody directed against a c-myc epitope. Data represent the mean ± SEM from 3 independent experiments, each performed in triplicate. Abbreviations: tBur= bursicon MTL subunit cDNA

Supplemental Figure 3. Active conditioned media cannot be made from expression of tethered bursicon constructs.

Activity of media isolated from cells expressing indicated bursicon MTLs. Method: Conditioned media was made from cells transfected in 6-well dishes with 75ng/well of each indicated tethered or soluble bursicon cDNA construct. Twenty four hours after transfection, the dishes were aspirated and 1.2mL of serum free DME was added. Media was then conditioned for 48 hours and prepared for signaling assays as described in Materials and Methods. The conditioned media was transferred to cells transfected with cDNAs encoding rk, a 6X-CRE-luc reporter gene, and a β-galactosidase control gene. After 4 hours, luciferase activity was quantified. The activity values were normalized relative to maximal stimulation of rk with bursicon conditioned media made by co-expression of soluble α and β subunits. Data represent the mean ± SEM from 3 independent experiments, each performed in triplicate. Abbreviations. sBur = soluble Bursicon subunit cDNA, tBur= bursicon MTL subunit cDNA, CM=conditioned media.

Supplemental Figure 4. dLGR1and dLGR3 are not activated by soluble or tethered bursicon.

A) dLGR1 is not activated by bursicon conditioned media B) Bursicon MTLs do not activate dLGR1. C) dLGR3 is not activated by bursicon conditioned media. D) Bursicon MTLs do not activate dLGR3. Method: HEK293 cells were transiently co-transfected with cDNAs encoding dLGR1 or dLGR3, a 6X-CRE-Luc reporter gene, and a β-galactosidase control gene. For assessment of tethered construct activity, cDNAs encoding bursicon MTL constructs were also co-transfected as indicated. The x-axes denote the amount transfected for each cDNA subunit (B, D). The cells were transfected for twenty hours, followed by stimulation with bursicon conditioned media for 4 hours if no MTLs were co-expressed (A, C). Luciferase activity was then determined and corrected for variability using the β-galactosidase control gene. Data represent the mean ± SEM from 3 independent experiments, each performed in triplicate. Abbreviations. tBur= bursicon MTL subunit cDNA.

Supplemental Figure 5. Agonist of tBur-α-β is maintained despite serial C-terminal deletions .

Assessment of tBur-α-β C-terminal deletion agonist activity. HEK293 cells were transiently co-transfected with cDNAs encoding: rk, a 6X-CRE-Luc reporter gene, the indicated bursicon MTL, and a β-galactosidase control gene. Twenty four hours after transfection, luciferase activity was quantified and normalized relative to maximal stimulation of rk by full length tBur-α-β. Data represent the mean ± SEM from 3

independent experiments, each performed in triplicate. Abbreviations: tBur= bursicon MTL subunit cDNA.

Supplemental Figure 6. Soluble Chimeric Bursicon conditioned media activates rk. Concentration dependent activation of rk with bursicon and soluble chimeric bursicon conditioned media. A series of ten fold dilutions of conditioned media (1=undiluted conditioned media) was added to cells 20 hours after transfection; the duration of ligand stimulation was 4 hours. Data represent the mean ± SEM from 3 independent experiments, each performed in triplicate. Abbreviations: sBurβ-α= genetically fused α and β bursicon subunit cDNA

Supplemental Figure 7. Soluble Chimeric Bursicon deletion mutants are not potent rk antagonists.

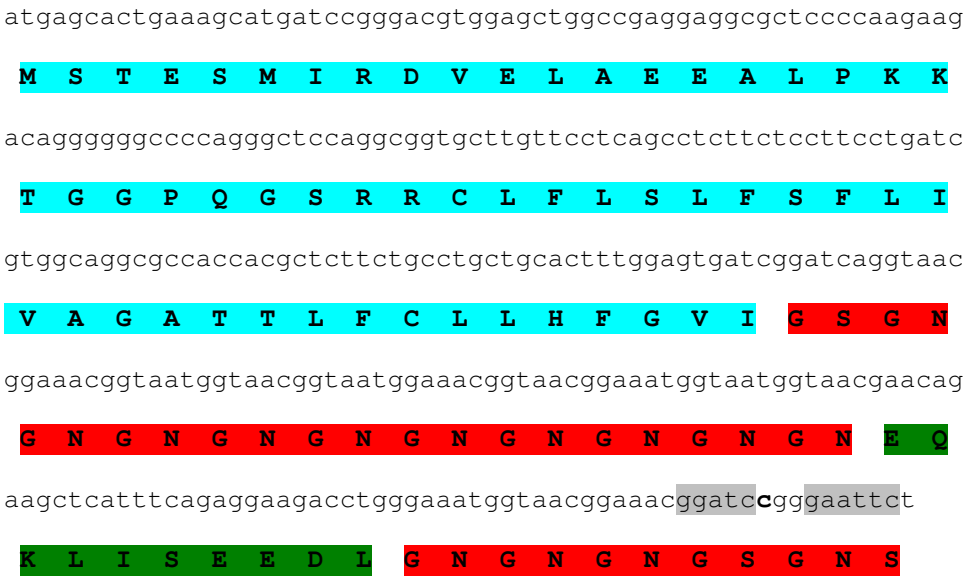
Co-expression of rk and indicated soluble chimeric bursicon C-terminal deletion constructs do not block

receptor activation by soluble bursicon. HEK293 cells were transiently transfected with 2ng of the indicated deletion construct, rk, a 6X-CRE-Luc reporter gene, and a β-galactosidase control gene. Twenty hours following transfection bursicon conditioned media was added at ten-fold serial dilutions (1=undiluted conditioned media). Following a four-hour incubation with bursicon conditioned media, luciferase activity was quantified and normalized relative to maximal stimulation of rk by bursicon conditioned media in the absence of a potential inhibitor. Data represent the mean ± SEM from 3

independent experiments, each performed in triplicate. Abbreviations: sBurβ-α= genetically fused α and β bursicon subunit cDNA

Supplemental Figures

Type II Membrane Tethered Ligand backbone sequence



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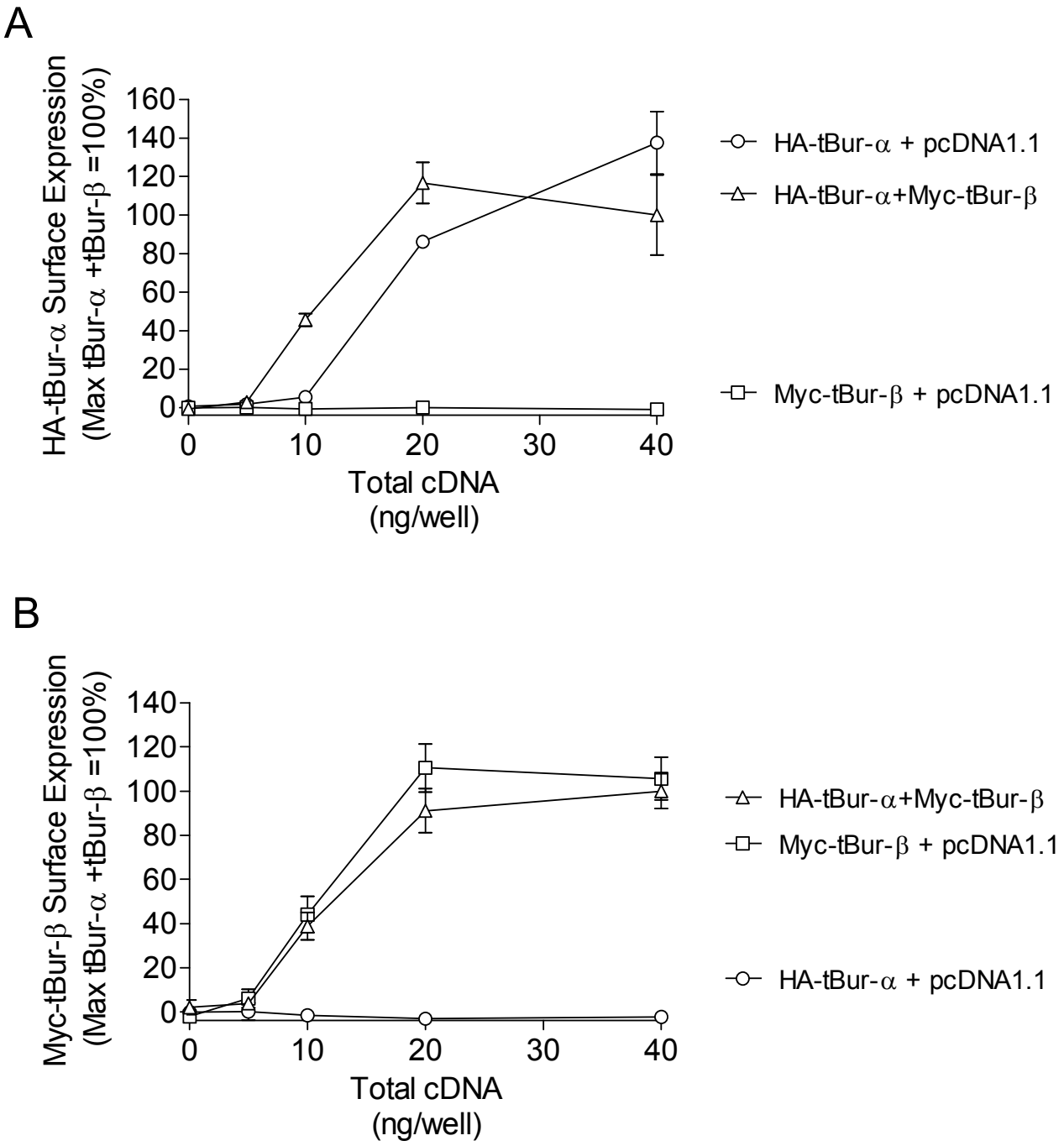

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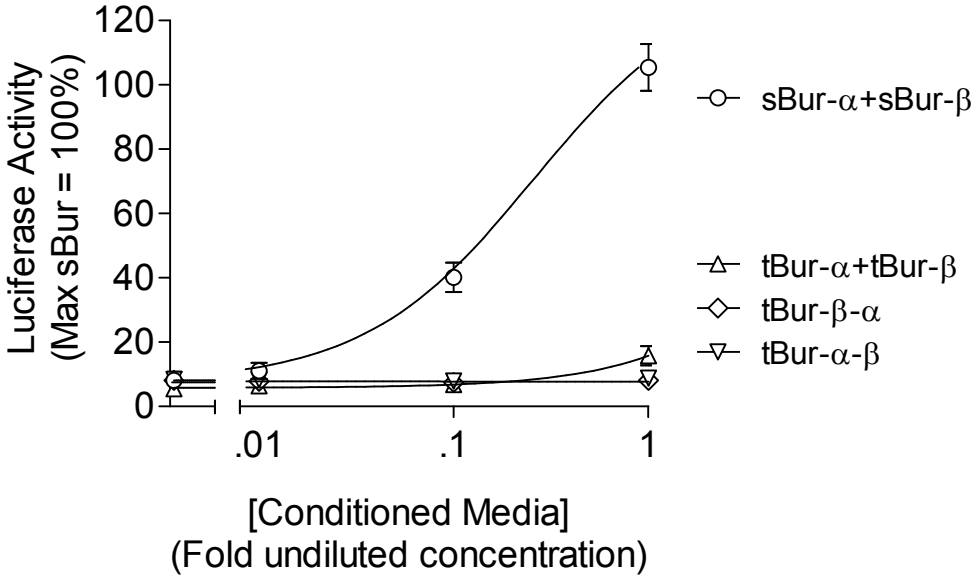

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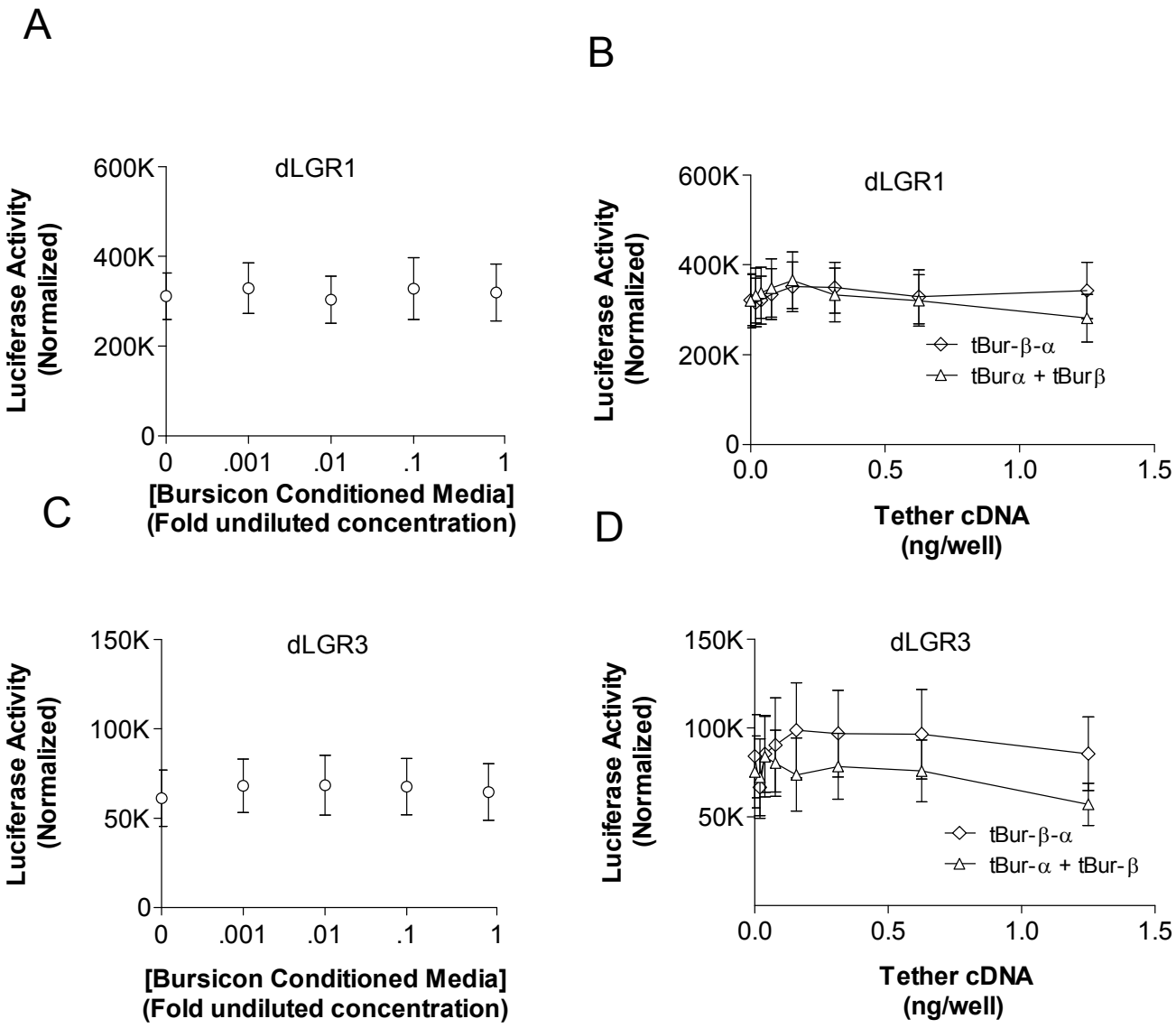

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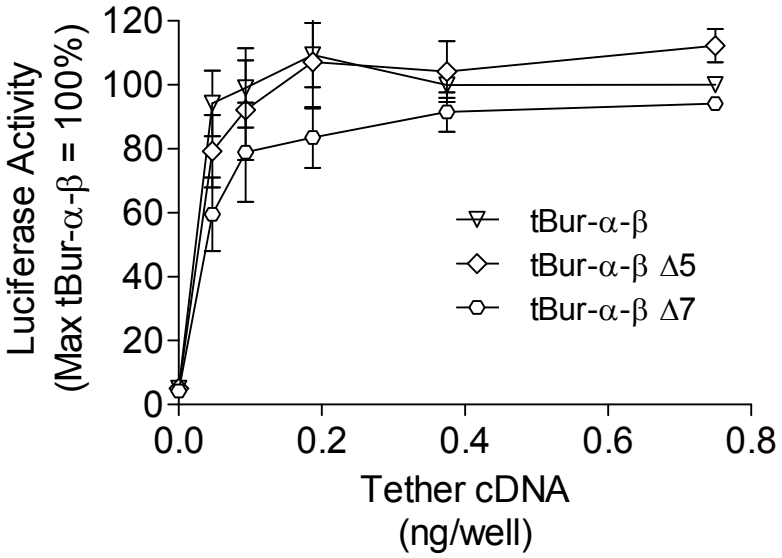

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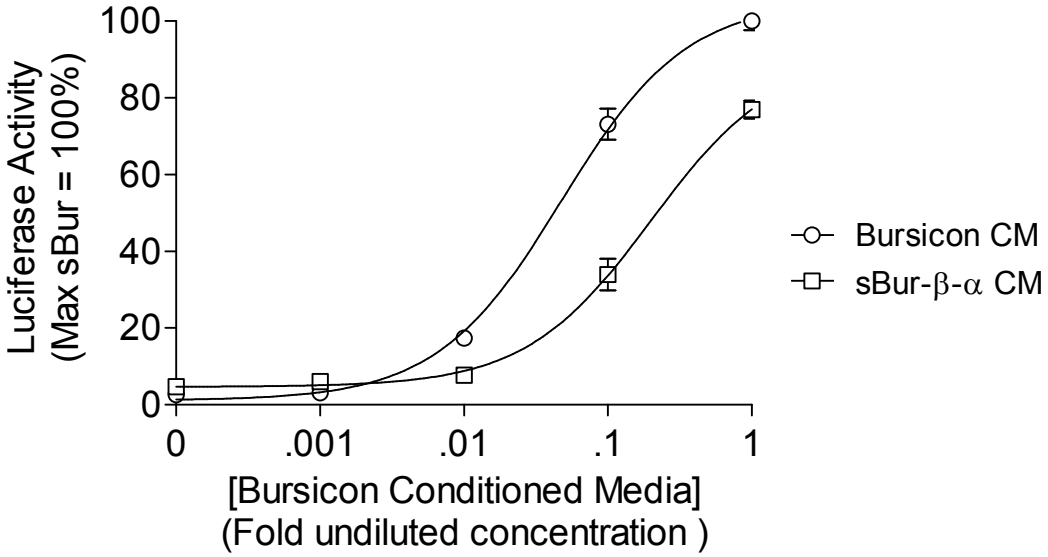
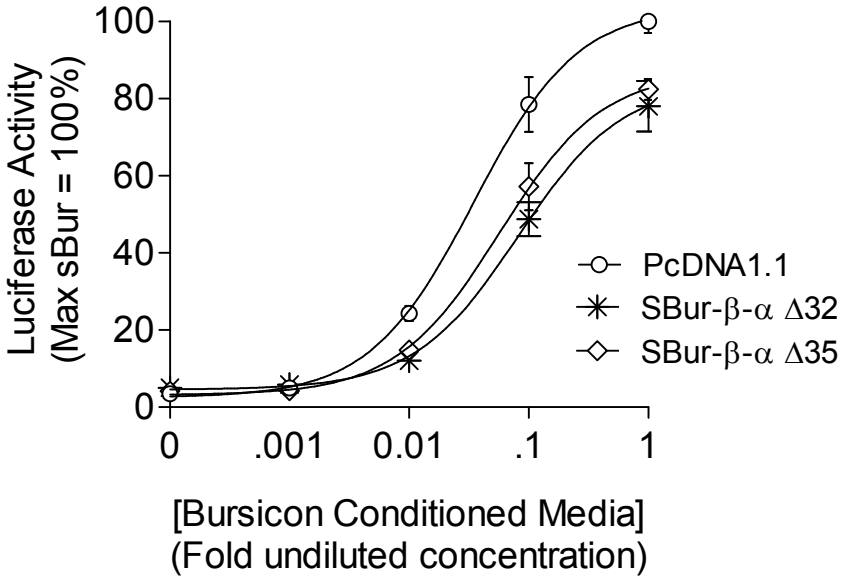


Supplemental Figure 6.



Supplemental Figure 7.