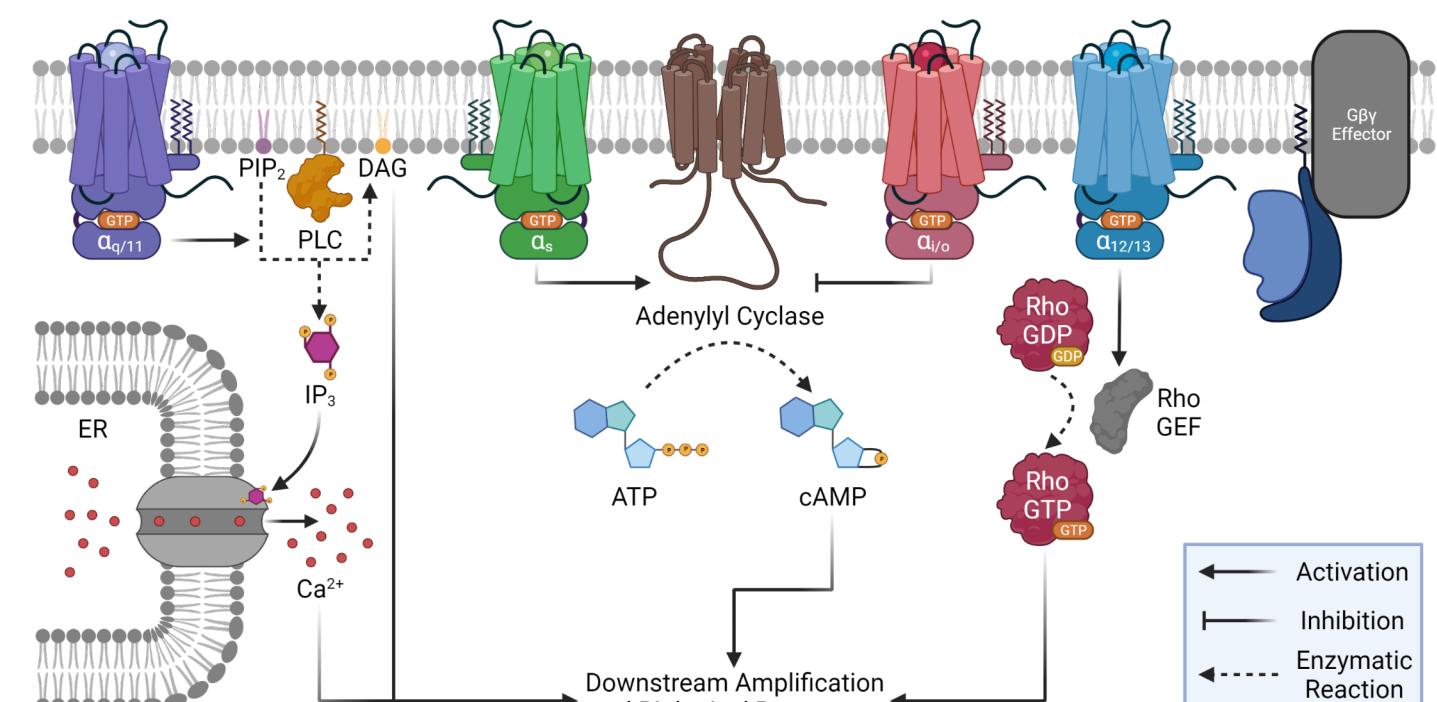


Understanding the Mechanisms of GPCR-G Protein Coupling

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INTRODUCTION

The **neurotensin receptor 1 (NTSR1)** is a G-protein coupled receptor (GPCR) which couples promiscuously to all four G protein family subtypes (G_q, G_{i/o}, G_s, and G_{12/13}). Three prototypical homologues - Human NTSR1, Rat NTSR1, and enNTSR1 - makes it a versatile tool in determining G protein coupling mechanisms.



Although structural information delineates some molecular mechanisms underlying GPCR-G protein promiscuity, the kinetic and temporal contexts are largely unexploited.

However, a G protein biosensor tool to interrogate GPCR-G protein coupling from a wide-view cell-based assay down to a fine-grain cell-free assay is lacking.

Through the project, we aimed to characterise our G protein bioluminescence resonance energy transfer (BRET) biosensors and begin assay development of a cell-free GPCR-G protein assay.

METHODS

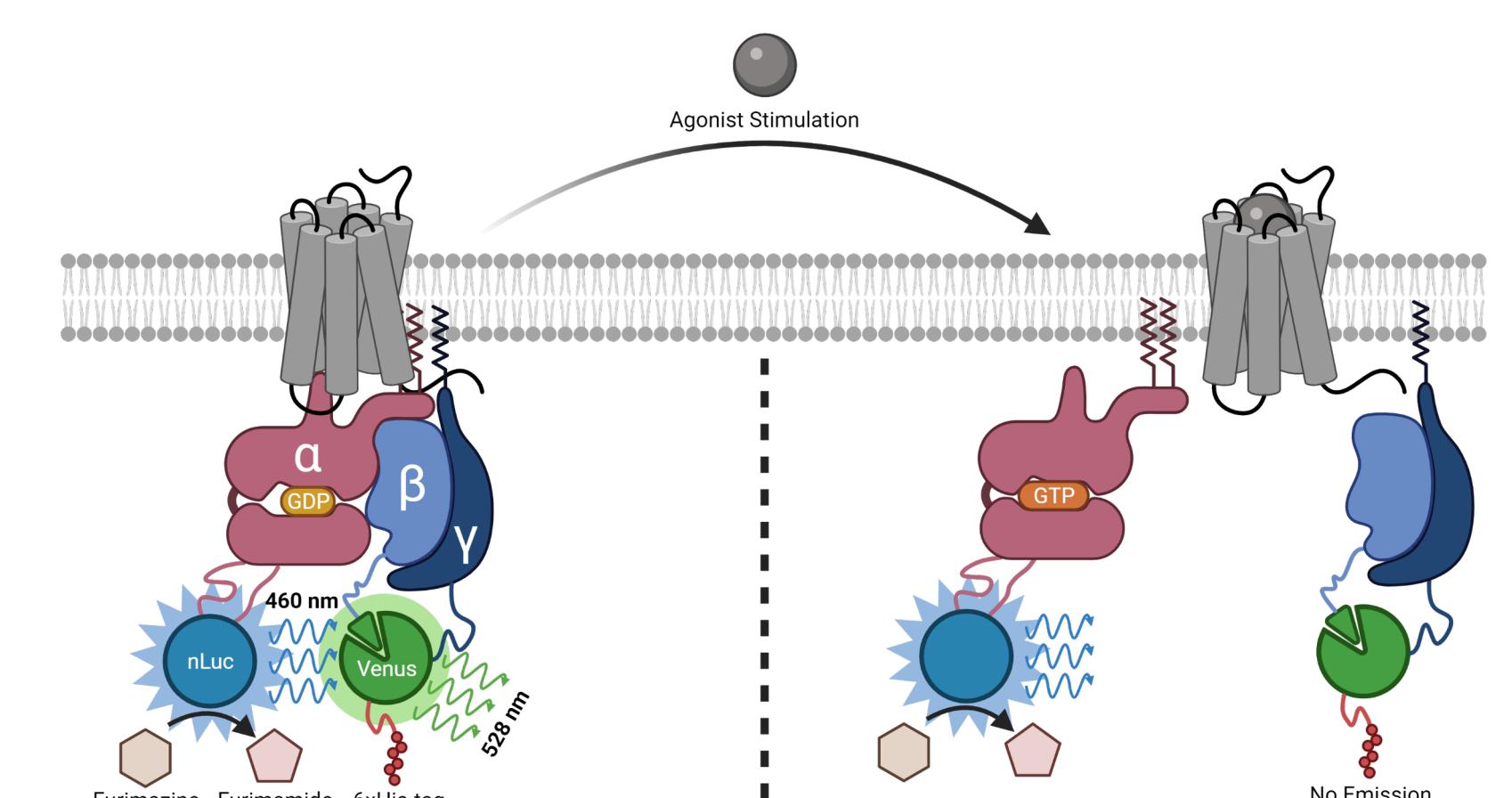
G Protein BRET Biosensors Constructs

Novel G Protein tricistronic BRET constructs were generated as G_{αi3} and G_{αq} variants. Both contained a nanoluciferase donor fused to the α subunit, a split-Venus acceptor fused to the β₁-γ₂ subunits, and 6xHis-tag attached to the split-Venus (γ₂ subunit).



Live-Cell G Protein BRET Assay

HEK293-F cells were maintained in DMEM and plated in 6-well plates at a density of 500,000 cells per well. Cells were transfected 24 hours later with G protein biosensor (250 ng) and receptor (750 ng). 24 hours post-transfection, cells were replated in 96-well plates at a density of 40,000 cells per well in phenol red free DMEM. Measurements were conducted in a PHERAstar® FSX microplate reader. Plates were incubated with Nano-Glo® Luciferase Assay Substrate for several minutes prior to ligand addition. Donor (Nanoluciferase) and acceptor (Venus) emission were measured simultaneously using a LUM 535 450 optics module. BRET ratios were calculated as acceptor/donor emission.



Membrane Preparation BRET Assay

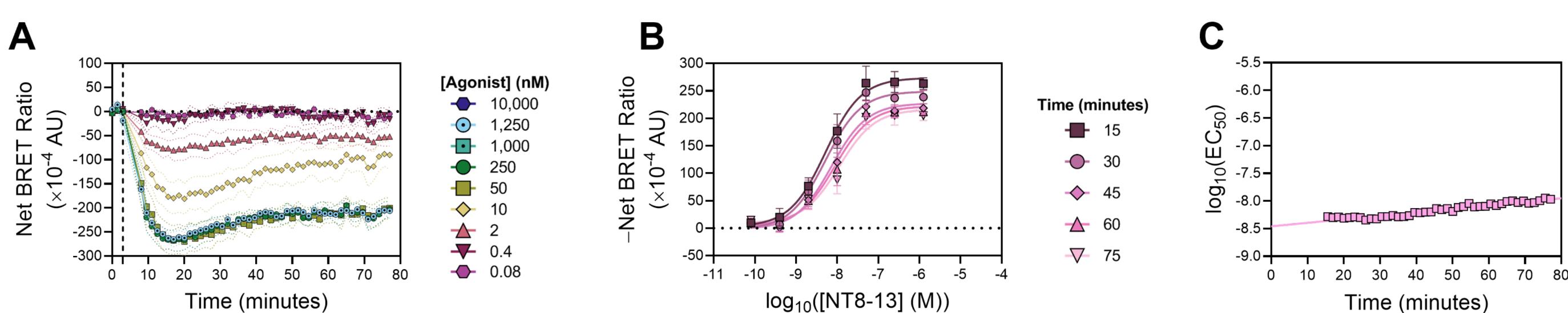
HEK293-F cells were transfected with receptor:biosensor DNA proportional to the cell count as previously mentioned. Cells were harvested 72 hours post-transfection and homogenised in hypotonic lysis buffer supplemented with GDP, MgCl₂, and EDTA. Membrane pellets were resuspended in buffer lacking the latter three components. Assays were conducted in 96-well OptiPlates™. 10 µg membrane preparations were supplemented with 1.25 µM GDP, 10 mM MgCl₂, 1 mM DTT, and Nano-Glo® Luciferase Assay Substrate. Assays were conducted as previously mentioned (Live-Cell G Protein BRET Assay).

G Protein BRET Biosensor Production and Purification

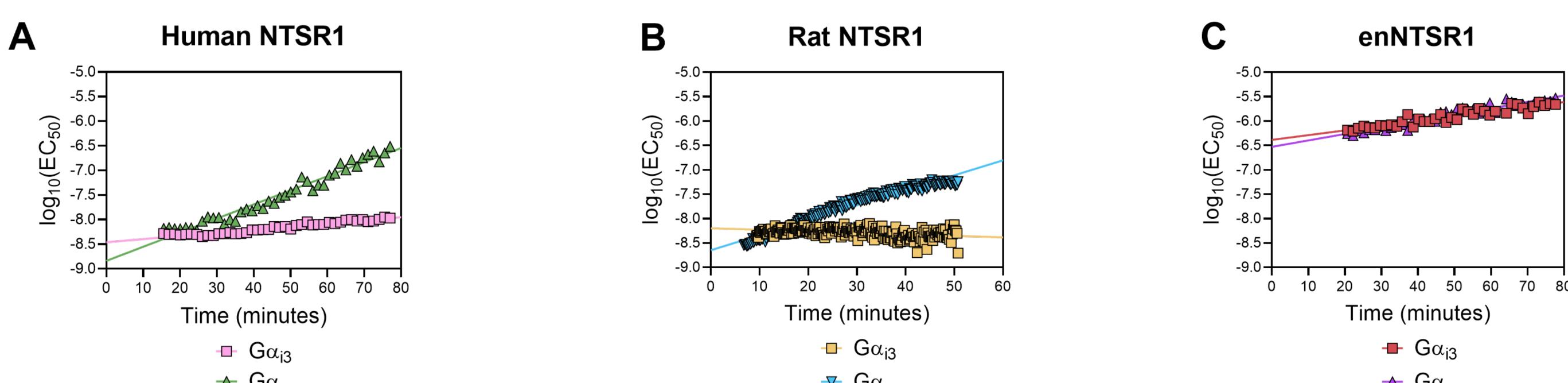
HEK293-T cells were utilised to produce lentiviral particles packaged with G_{αi3} biosensor RNA. HEK293-T cells were transfected with biosensor DNA, pMDL, pRSV-Rev, and pCMV-VSV-G plasmids. Viral media was collected 48 hours post-transfection and purified via ultracentrifugation (final volume 40 µL PBS). HEK293-F cells were transduced with 1 µL lentivirus per 10⁶ cells. Cells were resuspended in fresh PRF-DMEM and allowed to grow for 72 hours. Cells were lysed in hypotonic lysis buffer supplemented with MgCl₂, GDP, β-mercaptoethanol, and protease inhibitors. Proteins were solubilised in HEPES buffer supplemented with DDM and sodium cholate. Solubilised G protein was then extracted utilising TALON® metal affinity resin and eluted using imidazole.

RESULTS

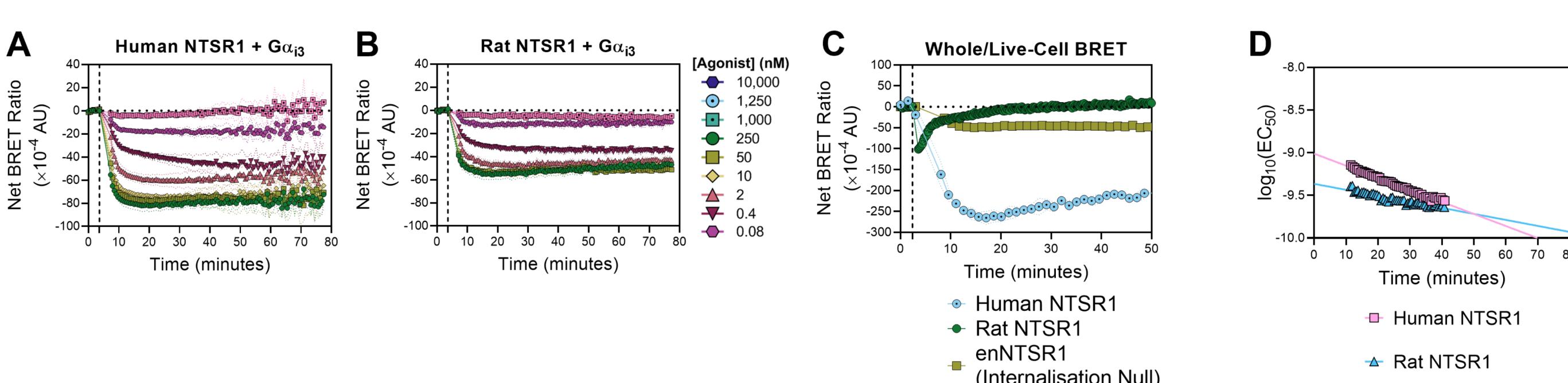
The novel tricistronic G protein BRET biosensors are functional with human NTSR1 when assayed with neurotensin 8-13 (NT8-13).



Human NTSR1, rat NTSR1, and enNTSR1 couple to their primary and secondary G protein partners with subtle kinetic differences.



The pseudo-reductionist format membrane preparation G protein BRET-based assay highlights removal of internal components, such as β-arrestin-mediated internalisation, thereby revealing facets of GPCR-G protein coupling not clear in live-cell BRET assays.



Our G_{αi3} BRET-based biosensor is functional when purified, paving the way for a reductionist format GPCR-G protein BRET assay.

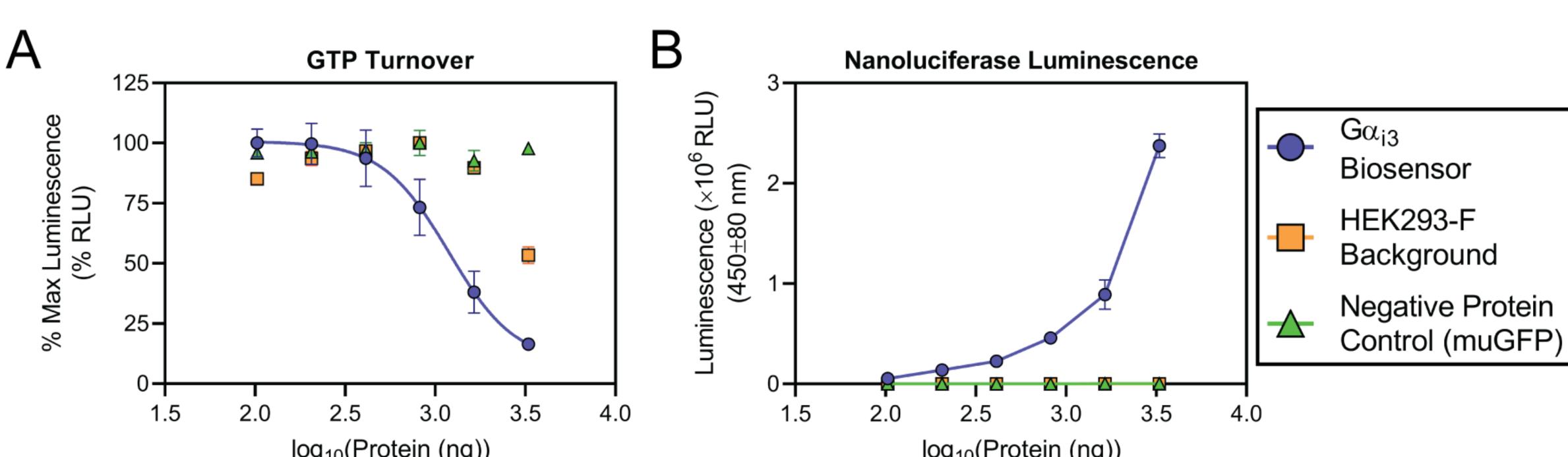


Figure 4. Determining the functionality of our novel G_{αi3} BRET-based biosensor. (A) Functionality of G protein subunits was determined via a GTP turnover assay using Promega's GTPase™-Glo Assay Kit. (B) BRET fluorophore fusion protein functionality was determined using Nano-Glo® Luciferase.

CONCLUSION

Through the project, we have laid the foundations for developing a reductionist format GPCR-G protein resonance energy transfer assay for proximal measurements of coupling. Additionally, each layer of the assay system reveals vastly different information on GPCR-G protein temporal kinetics. In particular, the three NTSR1 are homologous, however, show differences in temporal association with their primary and secondary G protein coupling partners (G_q and G_{i3}, respectively). Moving forward, a reductionist format assay would require a high-expressing receptor (e.g. enNTSR1) for assay optimisation.

