3D-specific chicken antibodies targeting GPCR extracellular loops

Simon Gaudreau[1], Marie-Edith Nepveu-Traversy[1,2], Sandra Morissette[3], Pierre-Luc Boudreault[2], Hassan Trabloussi[2], Paul Held[4], Peter Banks[4], Eric Marsault[2], Philippe Sarret[2], Fernand-Pierre Gendron[2], Billy Breton[3] & Mathieu Arcand[1]

[1] Immune Biosolutions, Sherbrooke, Québec, Canada. [2] FMSS Université de Sherbrooke, Sherbrooke, Québec, Canada. [3] Domain Therapeutics NA, Montréal, Québec Canada, [4] BioTek Instruments, Winooski, Vermont, USA.

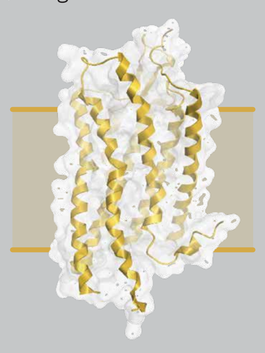
ABSTRACT

GPCR recognition by antibodies remains a challenge mainly because of their unique anchored membrane structure containing loop steric constraints and their difficult expression and stabilization for immunization. To overcome these bottlenecks, we developed a novel approach using our Nebula antibody platform. As proof-of-concept, novel antibodies targeting the neurotensin receptor 1 (NTS1r). This G-protein-coupled receptor (GPCR) is expressed by several cell types within the gastrointestinal tract and the nervous system, and is involved in many complex biological processes such as pain perception, gastrointestinal motility, cancer and obesity. The Nebula antibody platform overcomes the fundamental limitations in generating anti-GPCR antibodies. First, the immunization of chicken with Spatial Peptides that mimic the 3D conformational structures of the external loops of GPCRs, overcomes issues of GPCRs expression, purification and stabilization for host immunization. Secondly, at the core of the platform, the immune system of chickens provides an untapped antibody repertoire that circumvents homology issues of conserved mammalian proteins. This repertoire is fully exploited by the use of phage display technologies that allow a high-throughput selection and validation of the best binders. Finally, these chicken antibodies in their recombinant format can be humanized using well established human antibody frameworks for a potential therapeutic use. Using the Nebula platform, we have produced several specific antibodies recognizing NTS1r in its native form. These antibodies were characterized by various in vitro and ex vivo methodologies. Moreover, we were able to demonstrate in functional assays that some antibodies generated can modulate NTS1r signaling. These functional antibodies will be tested for several indications in pertinent animal models. These results show that the strategy developed with the Nebula antibody platform can generate antibodies leads with great therapeutic potential. Immune Biosolutions is currently seeking partners to explore novel targets and expand its tridimensional GPCRs-specific antibody portfolio.

INTRODUCTION

Limitations in antibody targeting of GPCRs

Although G Protein-coupled receptors (GPCRs) remain intensely studied targets, their detection and modulation by antibodies has remained a constant challenge. This is due to receptor properties including membrane proximity and spanning domains, as well as topological and steric constraints, along with limitations in the traditional ways with which antibodies are designed. Polyclonal antibodies are typically raised in rabbits against a recombinant protein. However, generation of soluble full-length recombinant GPCRs has also proved challenging. On the other hand, monoclonal antibodies could be used but they are typically raised against a short linear peptide, which differs from the native GPCR conformation. To overcome these classic hurdles, antibodies were raised in chickens against three dimensionally-constrained antigenic peptides. They are here characterized for detection and functional properties.



MATERIAL & METHODS

Antibody generation & purification Hens were immunized with the corresponding peptides and eggs were harvested for 28 days. Total immunoglubulin was extracted form egg yolk and concentrated in phosphate-buffered saline (PBS) prior to loading on a column containing an agarose bead-conjugated immunogenic peptide. After high salt washing, the immunoaffinty-purified antibodies were eluted in 0.1M Glycine pH 2.4 and submitted to buffer exchage in PBS. Elution was monitored by following A280.

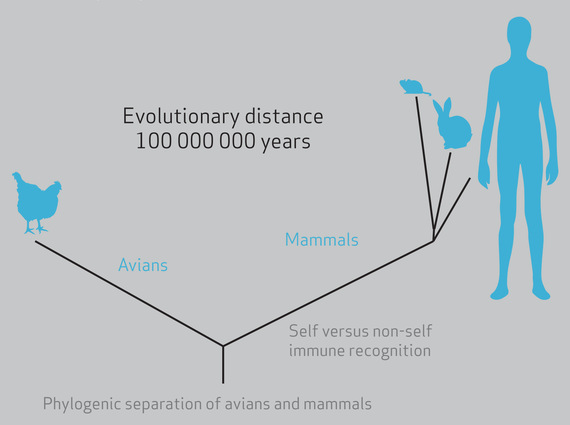
Immunofluorescence microscopy Two days post-transfection, HEK293 cells were seeded on glass coverslips. The following day, cells were washed and fixed in 3.7% paraformaldehyde, blocked with 10% normal goat serum and incubated with Atto 488-conjugated antibody. Anti-HA primary and secondary antibodies were diluted 1:200. Samples were washed before mounting in a solution containing DAPI. Imaging was performed with Cytation™ reader (BioTek Instruments), with parameters set to identical values.

Immunohistochemistry Paraformaldehyde-fixed wild-type Brown-Norway rat brain slices were blocked with NGS blocking buffer (G Bioscience). After PBS washing, the samples were incubated with 1:100-diluted primary antibody. After a second series of washes, horseradish peroxydase-conjugated Alpaca anti chicken IgY secondary antibody was incubated. Samples were additionally washed prior to revelation for optical microscopy.

Receptor signaling assays BRET2 proximity assays using BioSens-All™ technology were performed by Domain Therapeutics NA. In short, HEK293 cells were PEI-transfected with DNA encoding Renilla luciferase- (RLuc) or GFP-tagged probes. 48 h later, compounds were incubated with the cells at the indicated concentrations for 30 min prior to addition of coelantrazine. The bioluminescence resonance energy transfer between the probes in proximity (< 10 nm) was measured using the BRET2 filter module on the Synergy™ NEO (BioTek Instruments) in a ratiometric manner.

PRINCIPLE

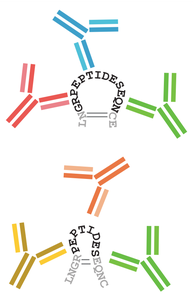
Comibining avian antibody diversity
& 3D-constrained epitopes



IMMUNOGENIC PEPTIDES & ANTIBODIES

Y00128

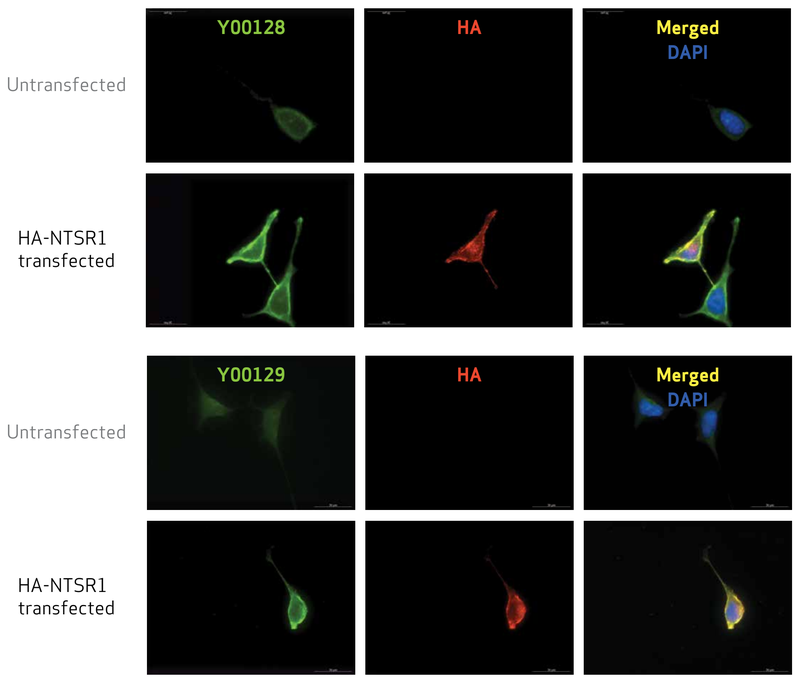
Linear
Polyclonal antibody raised against linear peptide encompassing the sequence of the extracellular loop.



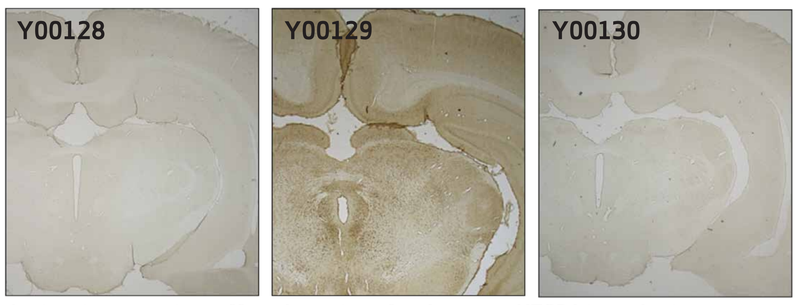
Y00129
Y00130
Y00238

Spatial
Polyclonal antibody raised against the three dimensionally-constrained peptide mimicking the GPCR's extracellular loop. Different constraints are imposed so the antibodies recognize different target conformations.

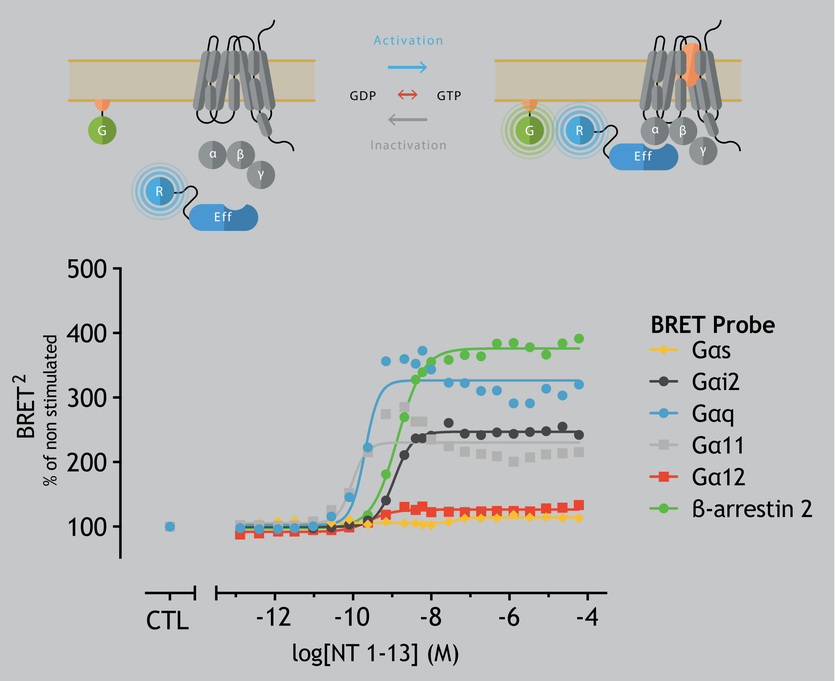
RESULTS



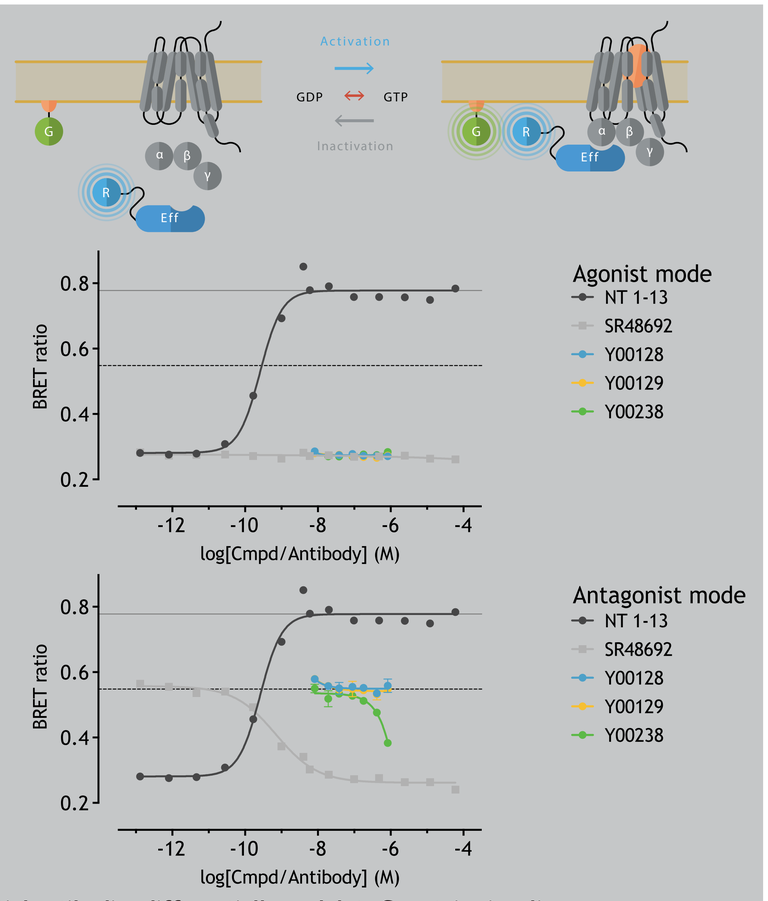
Antigenic constraints affect antibody selectivity
HeLa cells were transfected with HA-tagged NTSR1 and fixed. Double immunofluorescence staining was performed using the IgY Spatial antibodies (green) and an anti-HA (red) with Atto 488 and Atto 543-conjugated secondary antibodies, respectively. The Spatial Y00129 displays high selectivity, whereas the linear Y00128 is more promiscuous.



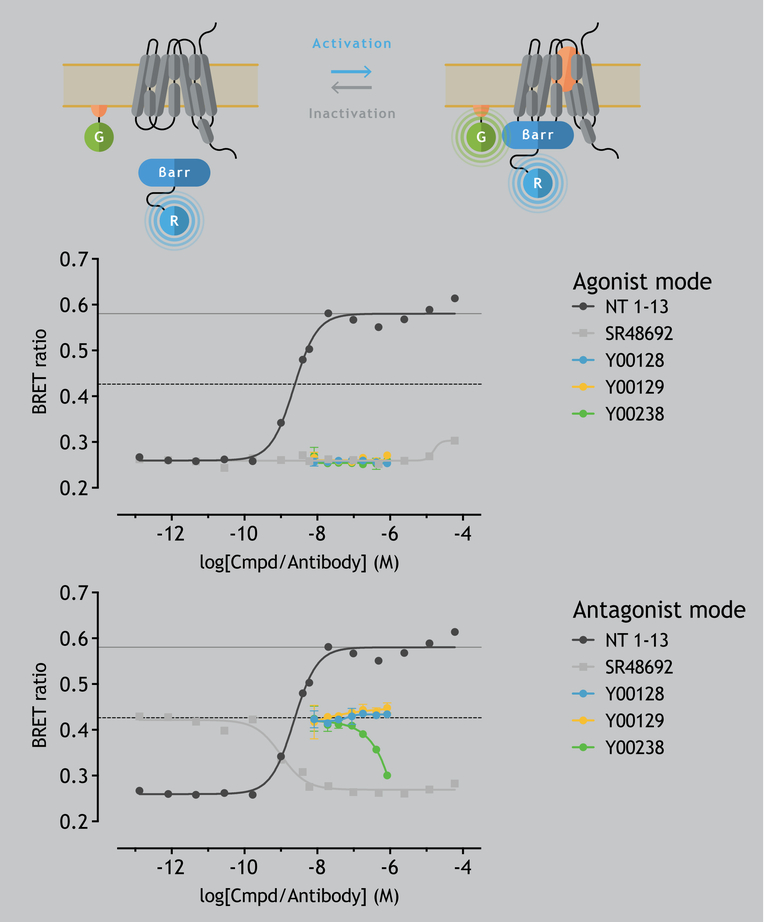
Antigenic constraints affect detection of endogenous NTSR1 in rat brain
Wild type rat brain slices were fixed with paraformaldehyde and immuno-histochemistry was performed using anti-NTSR1 antibodies. HRP-conjugated alpaca anti-chicken was used as secondary antibody. The Spatial antibodyY00129 can stain native NTSR1 in contrast with linear (Y00128) and Spatial (Y00130) counterparts.



NTSR1 engages multiple effector pathways
BRET2 assay principles with probe for G protein activation (top). Gα with βγ subunits are recruited by the receptor. The membrane proximity of the tagged Gα–binding effector (Eff) allows for the resonance energy to transfer from the RLuc-catalyzed coelantrazine to the membrane-anchored GFP which then becomes fluorescent. Receptor engagement data (bottom) confirms that NTSR1 activates the Gq, Gi ,G11 and arrestin pathways, but not Gs and G12 upon nerotensin (NT 1-13) stimulation . NTSR1 also engages GoB, G13, G14, G15, Gz and β-arrestin 1 (data not shown). A different set of probes was used for each assay.



Spatial antibodies differentially modulate G protein signaling
BRET2 assay with Gq engagement probe (top) was performed by titrating the antibodies in absence (agonist mode; middle) or presence (antagonist mode; bottom) of half maximal effective concentration (dashed line) of neurotensin. Orthosteric antagonist SR48692 serves as negative control. Contrarily to Y00128 and Y00129, Y00238 decreases neurotensin signaling to Gq but also Gi, GoB, Gz and G13 (data not shown).



Spatial antibody Y00238 inhibits neurotensin signaling
BRET2 assay with β-arrestin 2 recruitment probe (top) was performed by titrating the antibodies in absence (agonist mode; middle) or presence (antagonist mode; bottom) of half maximal effective concentration (dashed line) of neurotensin.

SUMMARY

3D-specific antibodies were raised through the Nebula platform by immunizing chickens with peptides encompassing the primary sequence of an extracellular loop of NTSR1. The peptides were either linear or spatially-constrained to mimic different conformations of the receptor. The different constraints in their antigens confer the antibodies with different selectivities, as exemplified by the detection of ectopic (immunofluorescence) and endogenous (immunohistochemistry) NTSR1.

Upon stimulation with neurotensin, NTSR1 selectively activates multiple pathways including Gq and β–arrestin. In absence of ligand, the antibodies tested do not engage receptor effectors. However, Y00238 blocks ligand-induced NTSR1 signaling, whereas Y00128 and Y00129 do not.

The results show that subtle changes in the 3D constraints of the antigens can modulate antibody selectivity. More so, these data open a potential for therapeutic antibodies with GPCR-modulating activity.

PERSPECTIVES

The confromational antibodies raised through the Nebula platform display distinct target recognition and modulation properties. Mechanistic studies will determine whether Y00238 is an orthosteric or allosteric inhibitor. Spatial peptides are beign used to genrate recombinant antibodies with high potential to serve as therapeutics in obesity and Gi tract diseases. Immune Biosolutions is actively seeking partners for R&D collaborations and licensing opportunities.

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