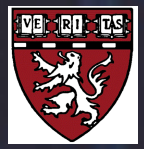
Case Study: HTS of **GPR-54** with IP-One Assay

Marcie Glicksman, PhD
Senior Director of Leads Discovery
Laboratory for Drug Discovery in Neurodegeneration
Brigham and Women's Hospital & Harvard Medical School

Assays and Cellular Targets
October 31, 2006

Harvard Center for Neurodegeneration and Repair



Outline

- What is LDDN?
- GPR54 Background
- Why IP-One
- HTS protocol
- Summary

Laboratory for Drug Discovery in Neurodegeneration

Mission of the LDDN is to ...

Create a new model for drug discovery that integrates the best of industry and academics.

Discover chemical agents that can be used as lead structures in the development of drugs to treat neurodegenerative diseases.

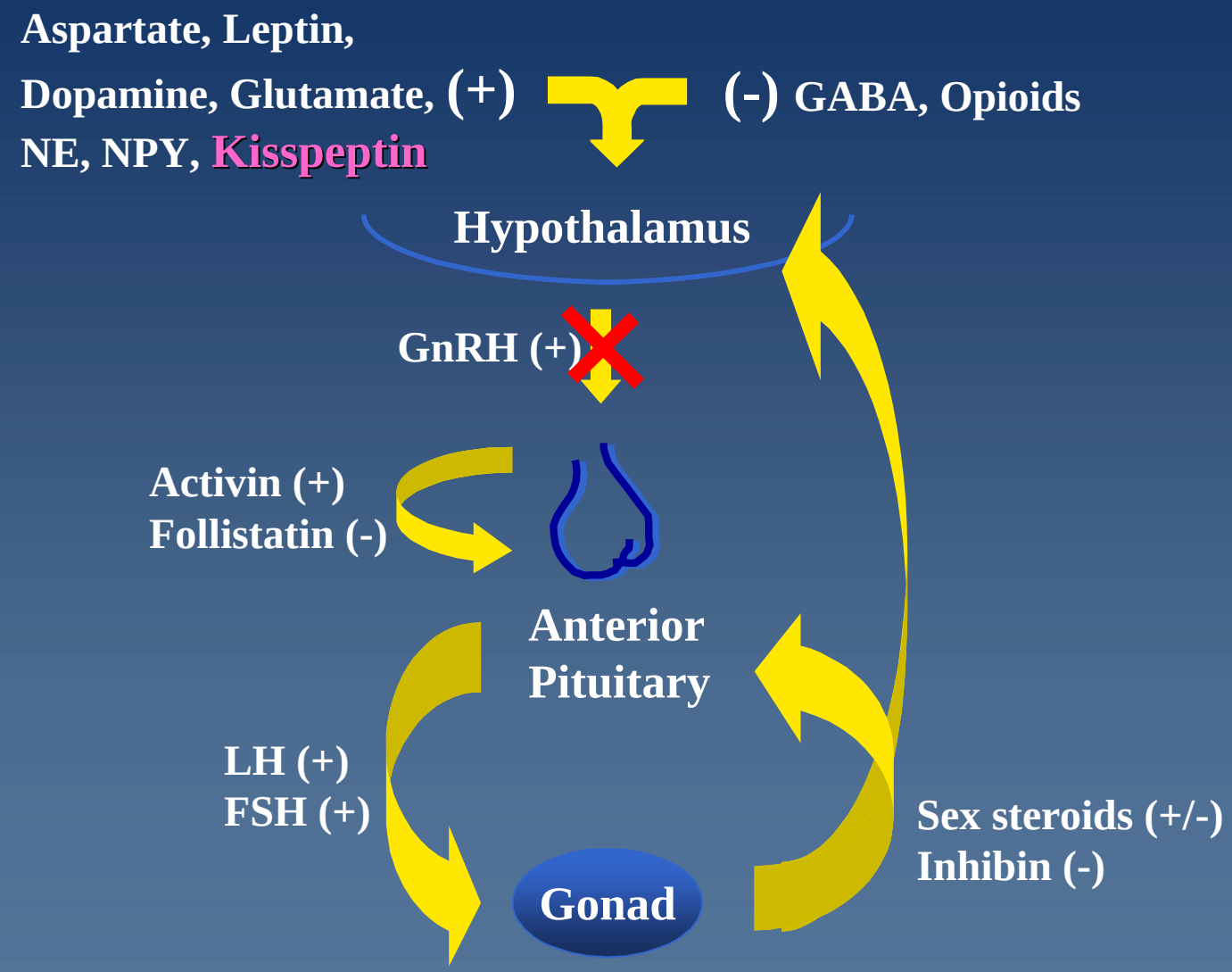
Laboratory for Drug Discovery in Neurodegeneration

Features of the LDDN model …

- Hypothesis-driven, screening-based approach.
- Managed by industry-seasoned professionals.
- Programs based on tight academic collaborations.
- Resourced for success – Commercialization of disease modifying therapeutics.
- Cover a broad range of targets in neurodegeneration.
- Forward-expansion – New collaborators and new disease areas.

GPR54: Case Study for LDDN

The Hypothalamic-Pituitary-Gonadal Axis



AIM

To identify GPR54 agonists, antagonists, and enhancers for therapeutic and research uses.

Agonists:

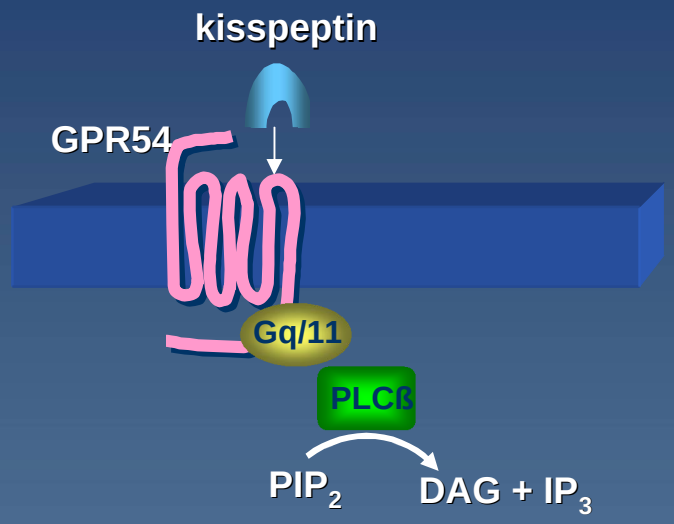
delayed puberty, infertility

Antagonists:

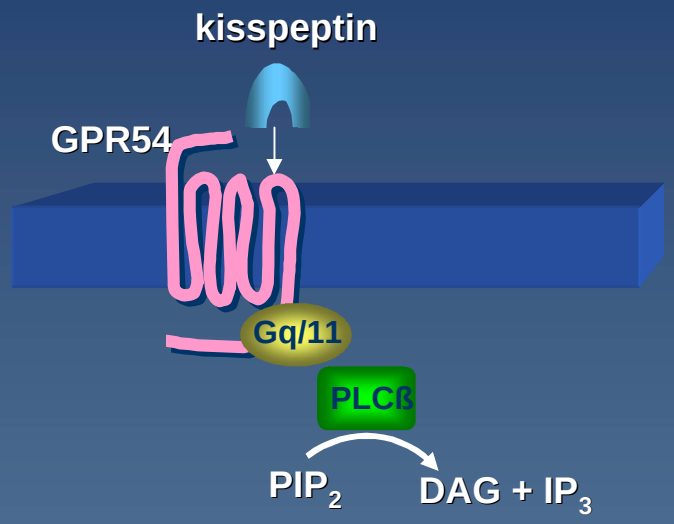
hormone-dependent cancers, precocious puberty

GPR54, A G Protein-Coupled Receptor

- GPR54 is expressed primarily in brain, pituitary, and placenta.

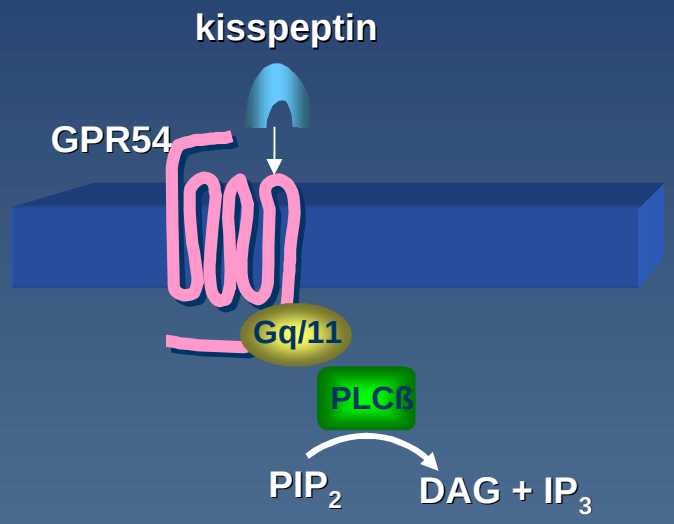


GPR54, A G Protein-Coupled Receptor



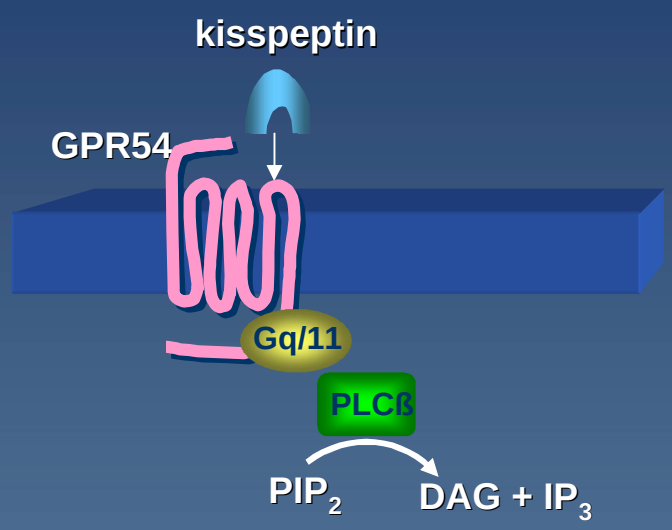
- GPR54 is expressed primarily in brain, pituitary, and placenta.
- Natural ligand of GPR54 = kisspeptin, encoded by gene KiSS-1

GPR54, A G Protein-Coupled Receptor



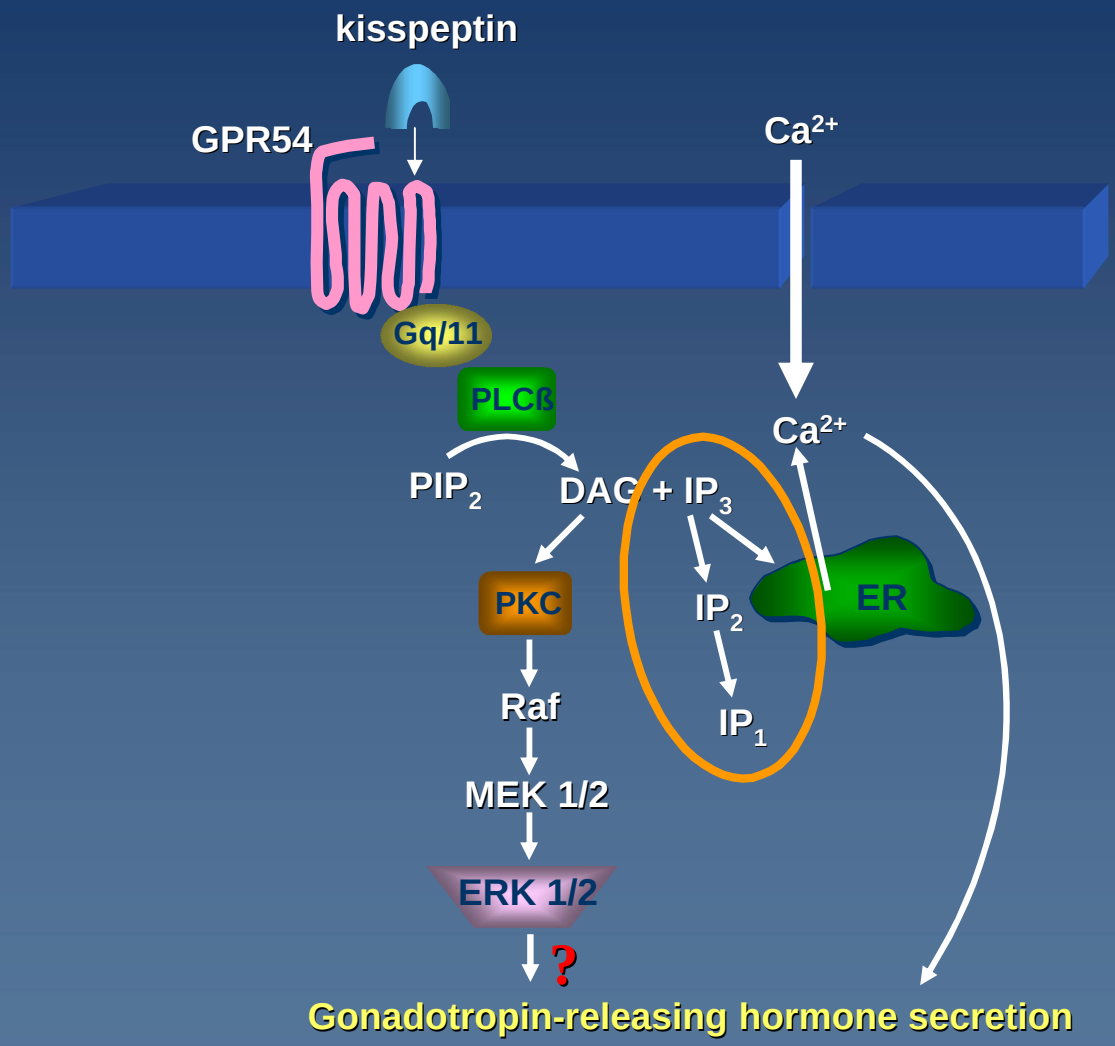
- GPR54 is expressed primarily in brain, pituitary, and placenta.
- Natural ligand of GPR54 = kisspeptin, encoded by gene KiSS-1
- KiSS-1 is expressed in hypothalamus and placenta.

GPR54, A G Protein-Coupled Receptor



- GPR54 is expressed primarily in brain, pituitary, and placenta.
- Natural ligand of GPR54 = kisspeptin, encoded by gene KiSS-1
- KiSS-1 is expressed in hypothalamus and placenta.
- Kisspeptin-54 was named metastin for its ability to inhibit tumor metastasis.
- GPR54 knockouts do not undergo puberty and antibodies will block LH surge

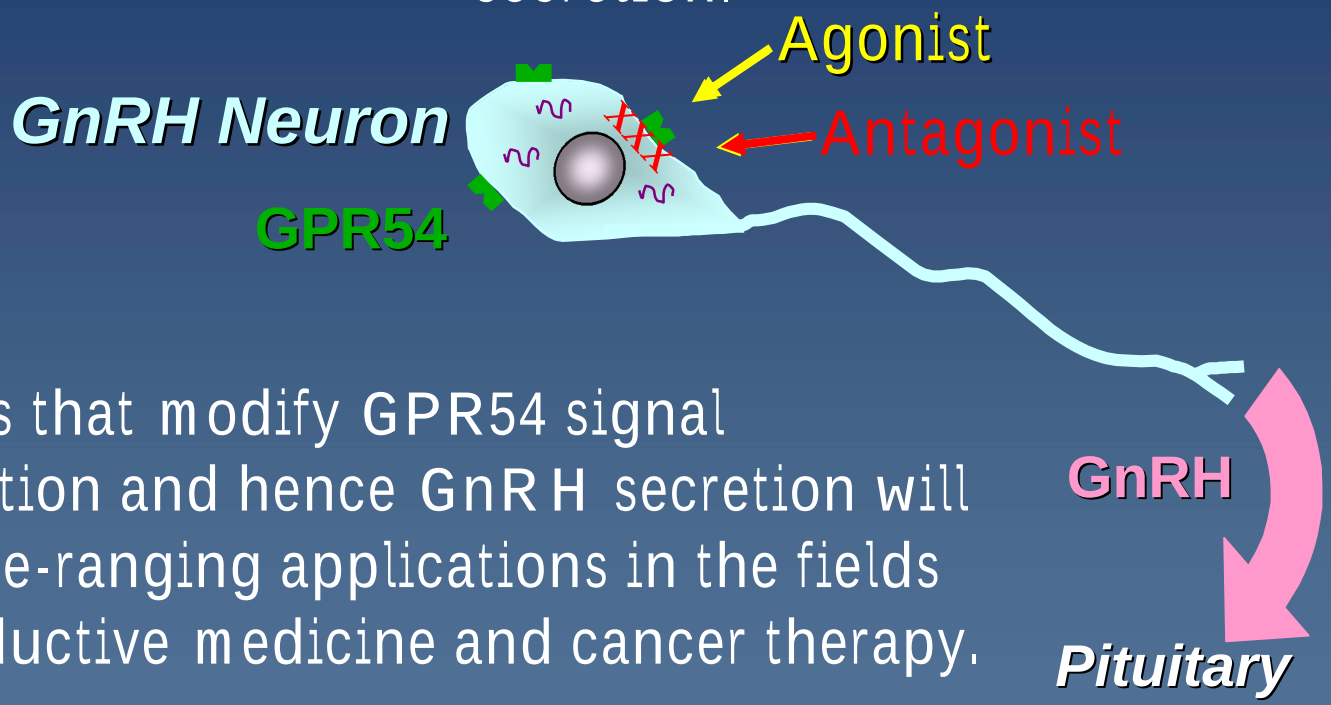
GPR54-Coupled Signal Transduction Pathways



Laboratory for Drug Discovery in Neurodegeneration

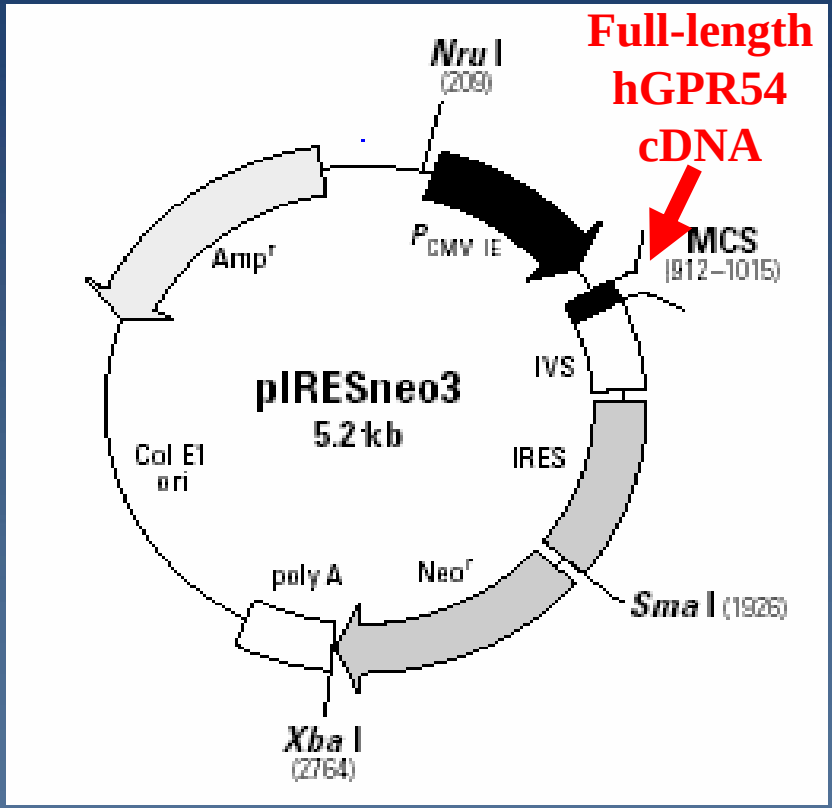
HYPOTHESIS

Agonists and enhancers of GPR54 will promote GnRH secretion, while GPR54 antagonists will suppress GnRH secretion.



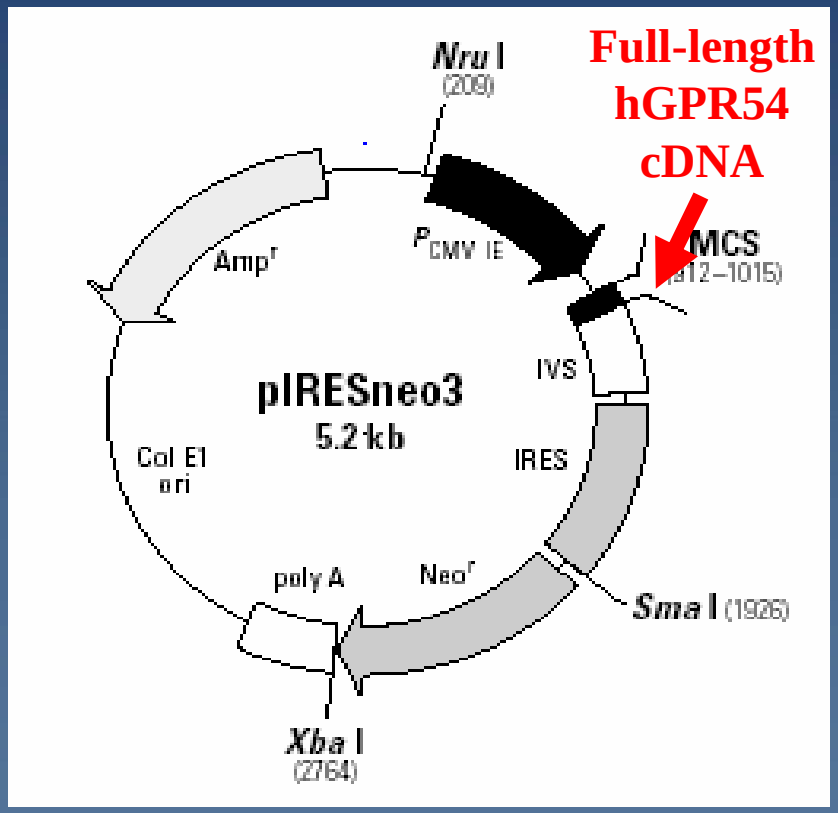
Reagents that modify GPR54 signal transduction and hence GnRH secretion will have wide-ranging applications in the fields of reproductive medicine and cancer therapy.

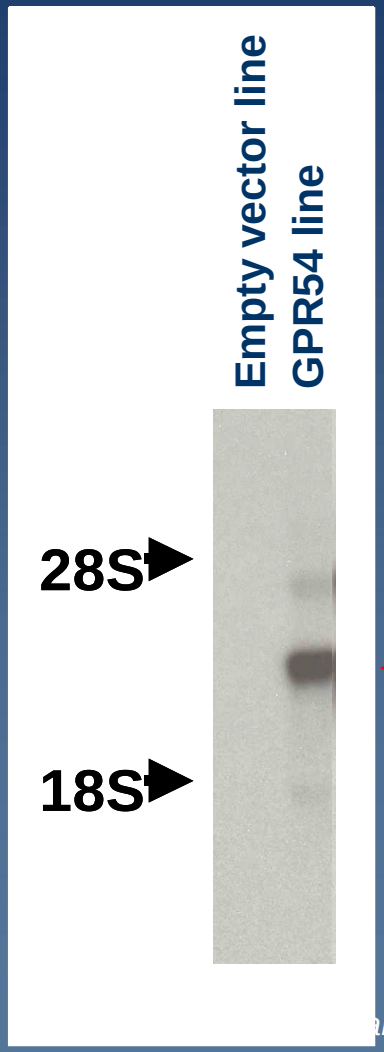
Generation of Stably Transfected GPR54 Cell Lines



- Chinese hamster ovary (CHO) cell line
  - Used in other published GPR54 stable cell lines
  - Growth pattern simplifies clone selection
- Construct backbone – pIRESneo3 (Clontech)
  - Bicistronic vector – gene of interest and selection marker are under control of same promoter, favoring selection of clones with higher expression of transgene

Generation of Stably Transfected GPR54 Cell Lines

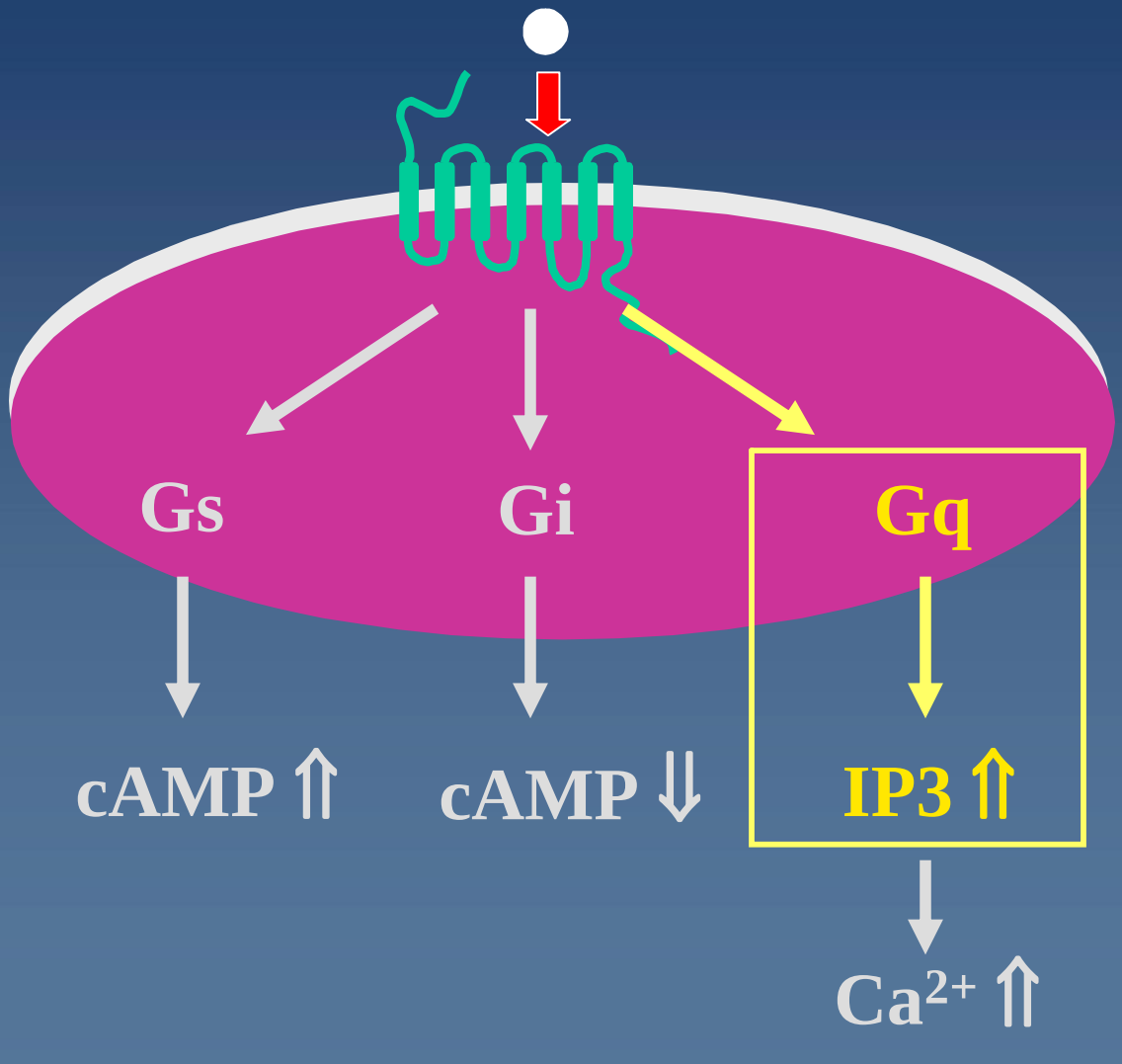




HCNR

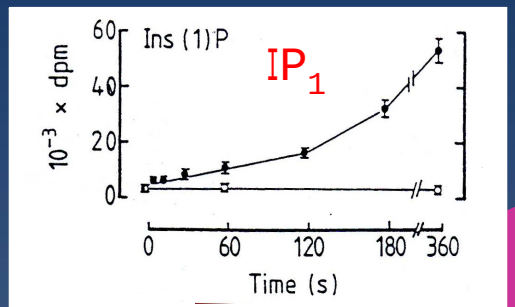
aboratory for Drug Discovery in Neurodegeneration

GPR54 is a Gq coupled GPCR

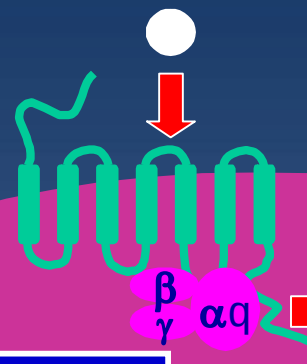


Laboratory for Drug Discovery in Neurodegeneration

Gq coupled GPCR signalling pathway



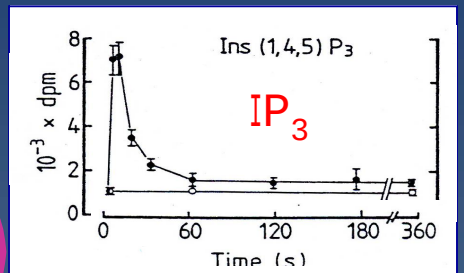
In presence of LiCl, IP1 accumulation upon GPCR activation



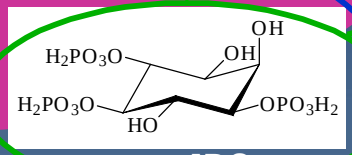
PLC

PIP2

Radioactive detection

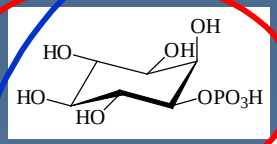


IP3 lifetime is very short

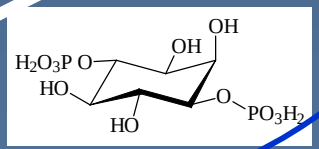


IP3

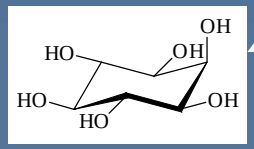
Inositol cascade



IP1



IP2



Myo-inositol

Inhibition by LiCl

E.R.

Ca^{2+}

Fluorescent sensing

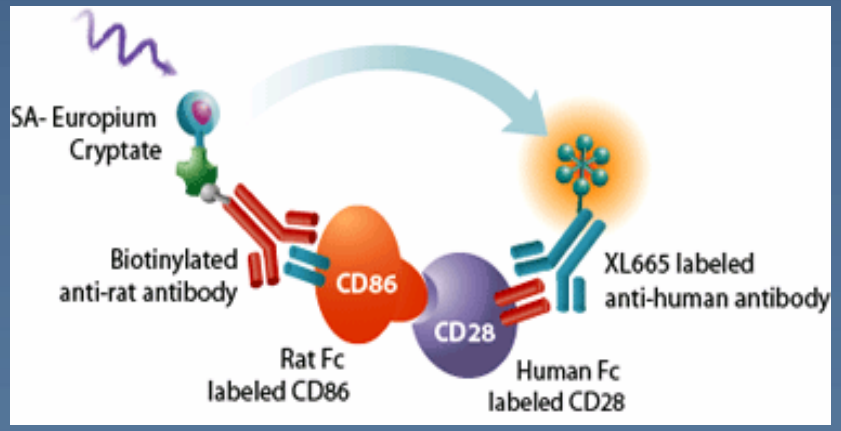
Laboratory for Drug Discovery in Neurodegeneration

Why Choose IP-One for HTS

- **Did not have access to a FLIPR for measuring Ca^{++} flux**
- **Miniaturization to 384-well format to reduce reagent consumption**
- **Minimal number of steps to facilitate automation and maximize speed and efficiency**
- **Non-radioactive detection for throughput, safety, and waste disposal considerations**
- **Must have satisfactory sensitivity, accuracy, and reproducibility (Z factor)**

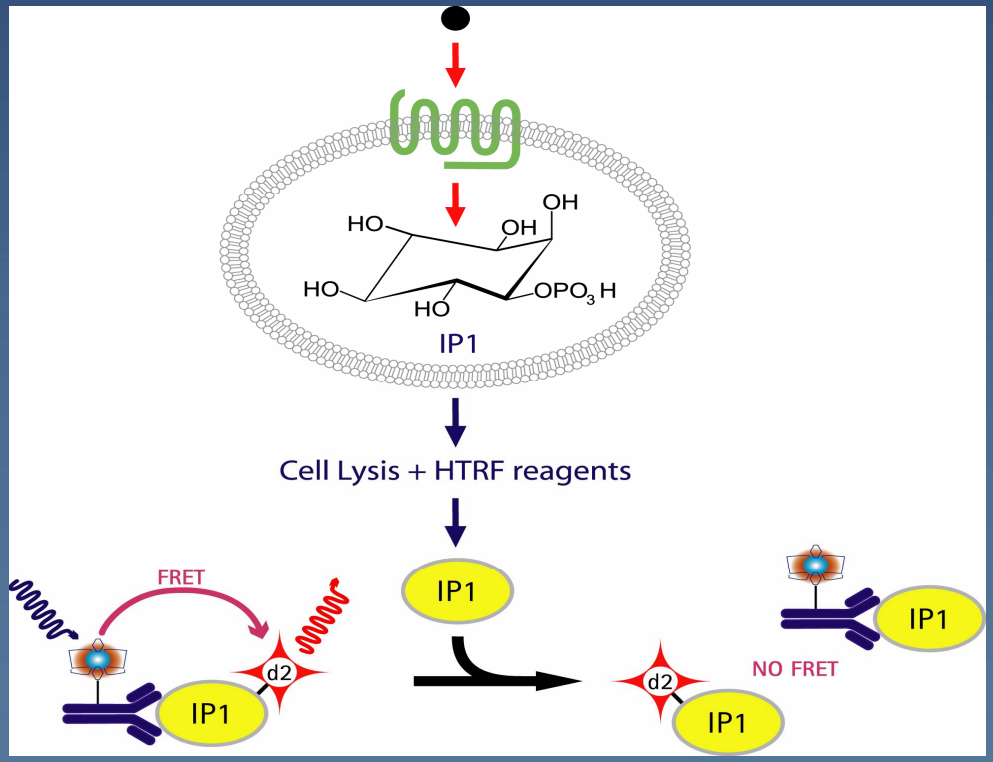
Homogeneous Time Resolved Fluorescence

- HTRF® is a technology based on TR-FRET, a combination of FRET chemistry and the use of fluorophores with long emission half-lives.
- HTRF uses lanthanide with an extremely long half-life (Europium), conjugation of Eu3+ to cryptate, an entity which confers increased assay stability
- Use of a ratiometric measurement that allows correction for quenching and sample interferences.
  - Simplified assay miniaturization
  - Tolerant of additives e.g. DMSO & EDTA
  - Cell-based functional assay



The IP-One assay

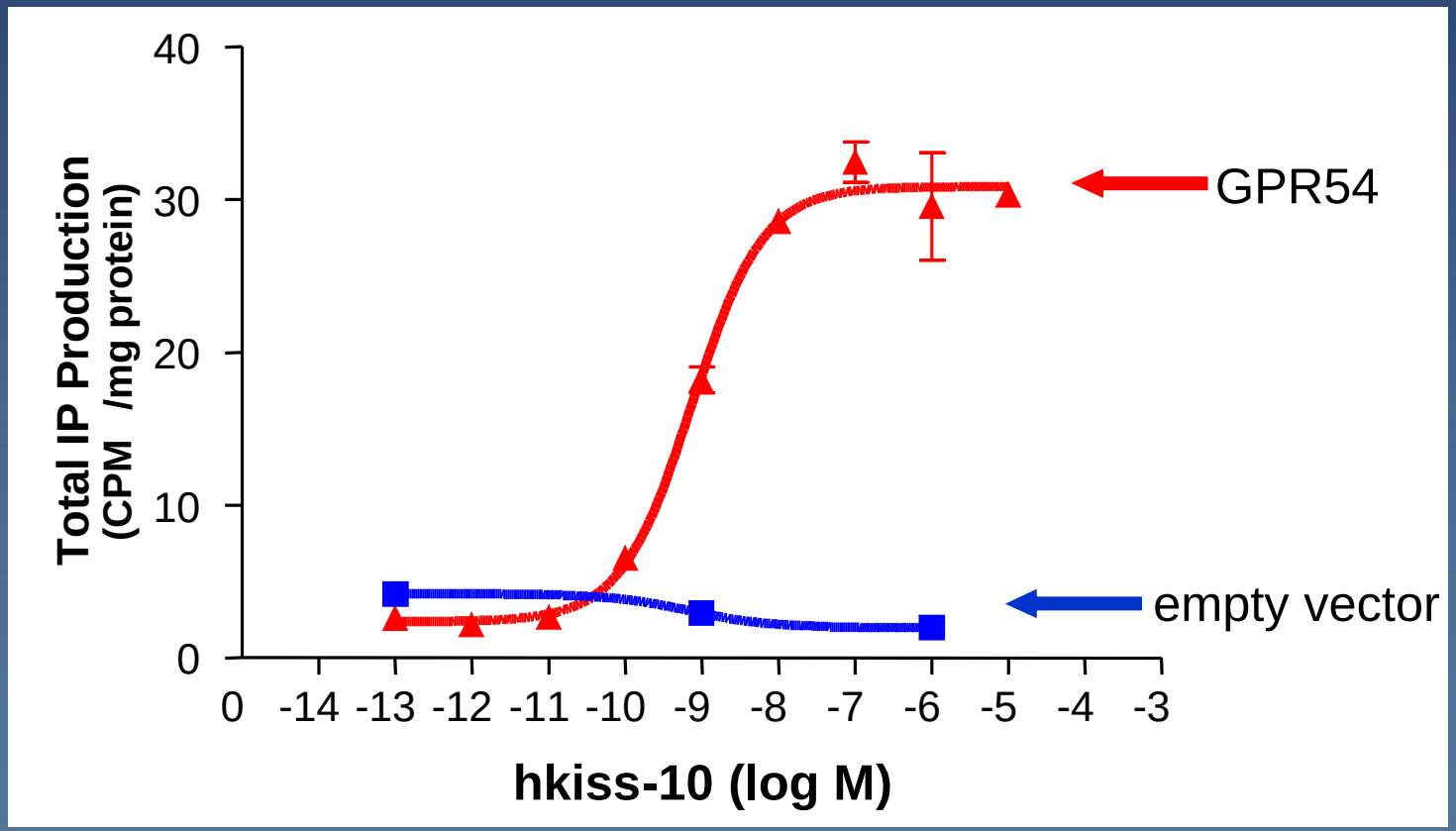
- **Competitive immunoassay with cryptate-labeled anti-IP1 monoclonal antibody and d2-labeled IP1.**
- **LiCl is used causing the accumulation of IP1 upon receptor activation.**
- **The assay can be run in a single microplate and requires only a single 1 hour incubation following cell stimulation.**
- **No cross-reactivity with 50µM of (phospho) inositides phosphates**



Laboratory for Drug Discovery in Neurodegeneration

Kisspeptin Stimulation

- **Kisspeptin Stimulates IP Accumulation In a Dose-Dependent Manner in GPR54 Stably Transfected Cells**



IP-One assay miniaturization

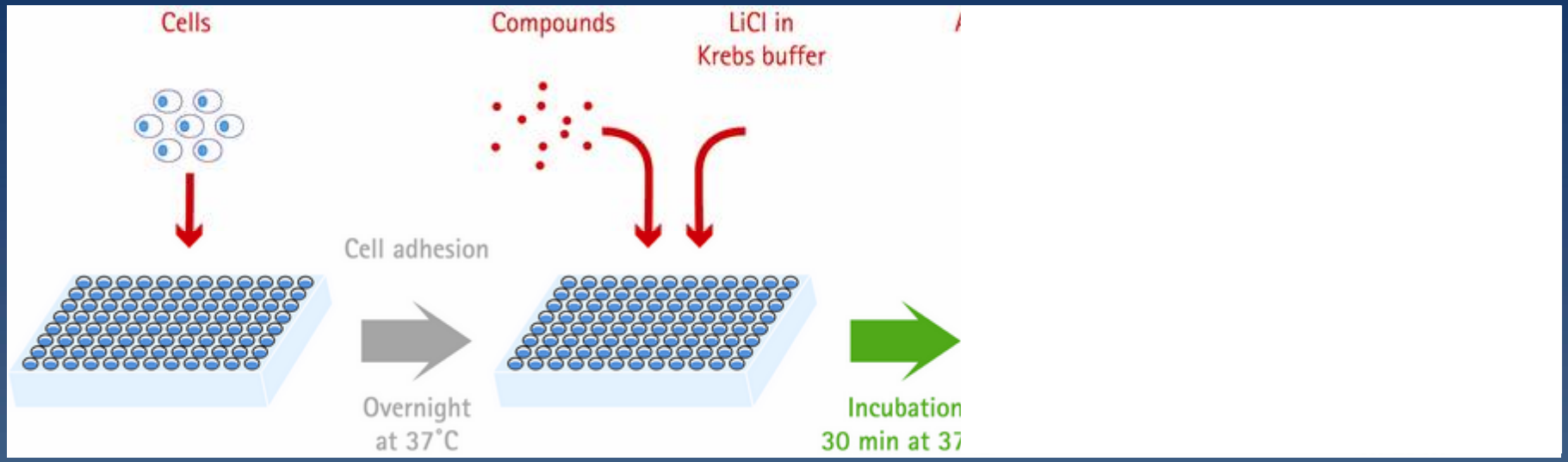
Plate type	96	96 1/2	384	384 sv
Cell number	80,000	40,000	15,000 to 30,000	8,000
Total volume (µl)	100	50	20 to 40	10

IP-One assay protocol

30,000 cells/40ul/well in
white Nunc 384-plates

Seal with aeroSeals

IP-One assay protocol



30,000 cells/40ul/well in white Nunc 384-plates

Seal with aeroSeals

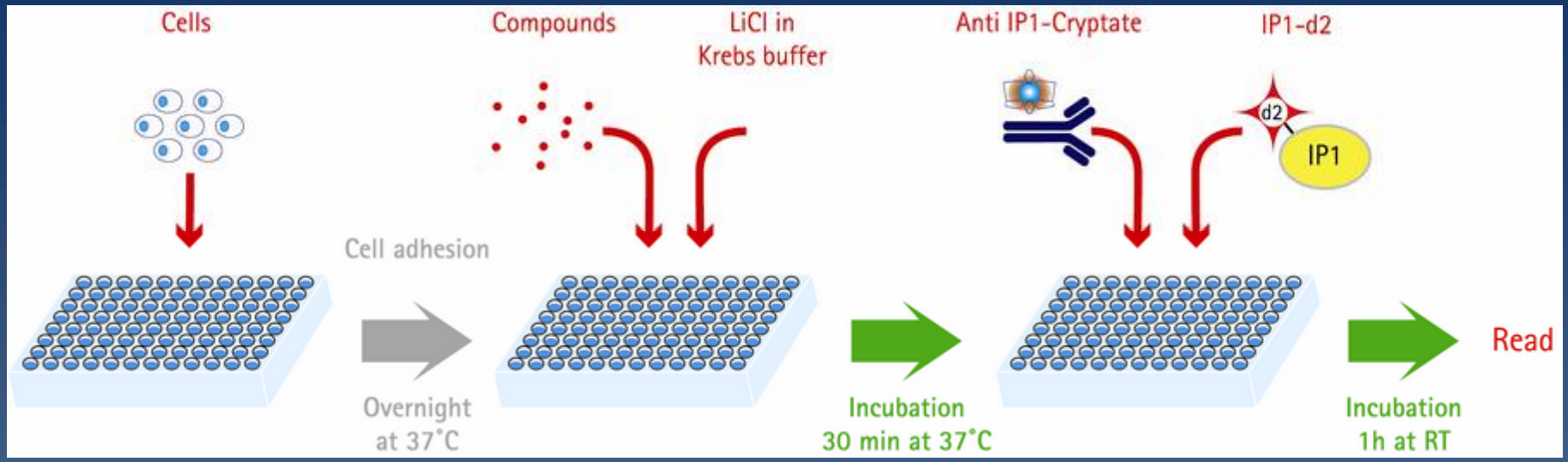
Remove media

Add compound together with stimulation buffer

(add Kisspeptin for antagonist screen)

Seal with aeroSeals

IP-One assay protocol



30,000 cells/40ul/well in white Nunc 384-plates

Seal with aeroSeals

Remove media

Add compound together with stimulation buffer

(add Kisspeptin for antagonist screen)

Seal with aeroSeals

Add IP-One kit D2

Add IP-One Cryptate reagent

Seal with aluminum seals

1 h-24 h

Laboratory for Drug Discovery in Neurodegeneration

Robotics Used for the Screen

- Beckman FX core system
- BMG PHERAstar or PerkinElmer Envision
- High energy xenon flashlamp excitation
- Simultaneous dual wavelength measurement for HTRF
- Integrated into robotic system

Laboratory for Drug Discovery in Neurodegeneration

Compound Library (110,000 compounds)

➢ **Computational filters applied to select compounds with an increased probability of oral bio-availability and blood brain barrier penetration**

3000 Peakdale
7000 Bionet
5000 CEREP
16,000 Maybridge
50,000 ChemDiv
6000 Enamine
6000 IF Lab

- All small molecules adhere to Lipinski's rules.
- Low proportion of toxicophores
- Low proportion of unwanted functionalities.
- Maximization of molecular diversity.

1670 FDA-approved drugs from Prestwick
480 purified natural products
4,100 N-Ac-tetrapeptide amides
1570 compounds from academic organic chemists
800 proprietary compounds synthesized by LDDN

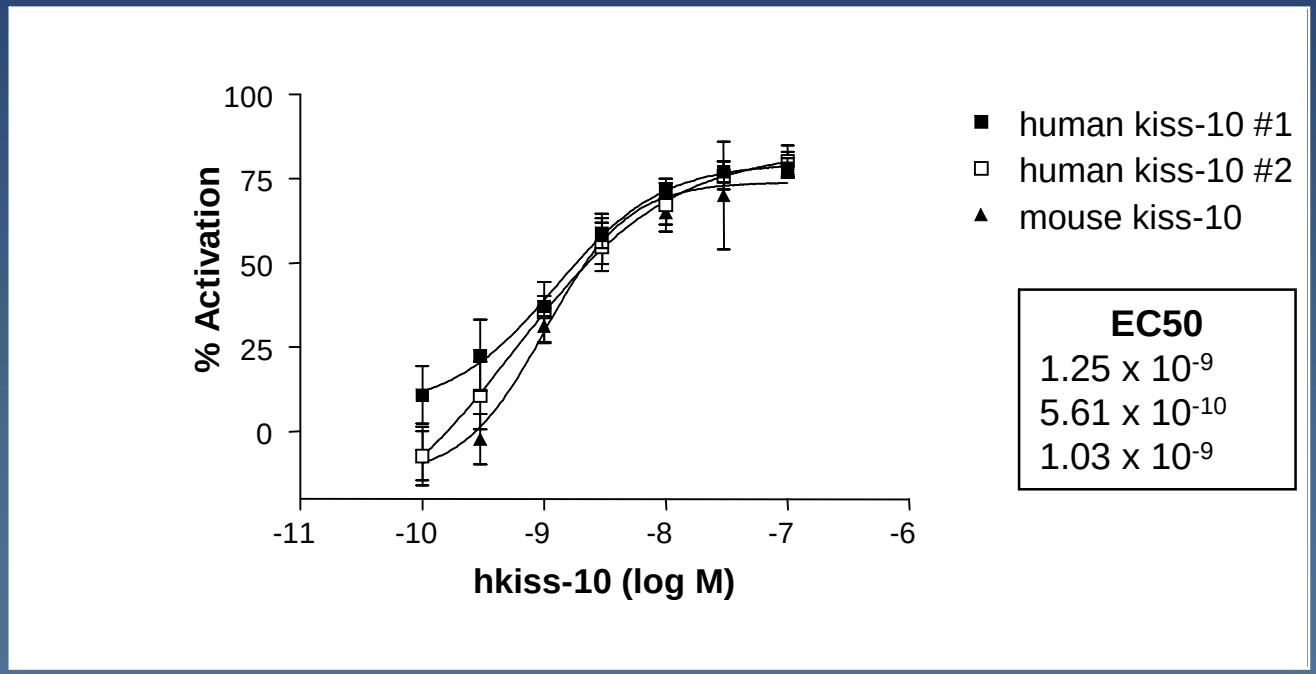
➢ ***Plus* . . . Program to collect additional compounds from academia.**
➢ **Additional compounds being purchased.**

H C
N R

Laboratory for Drug Discovery in Neurodegeneration

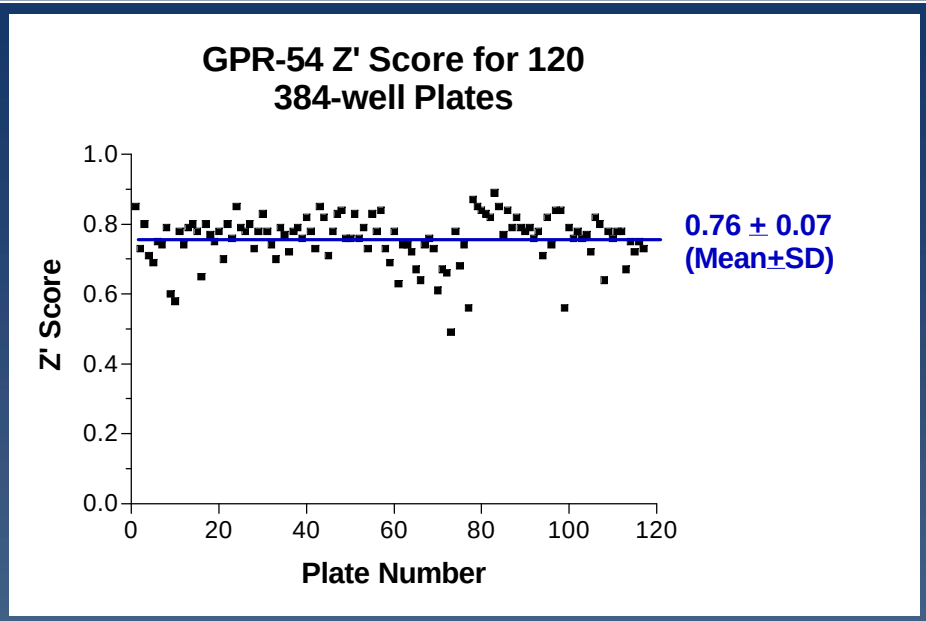
Control for Automation

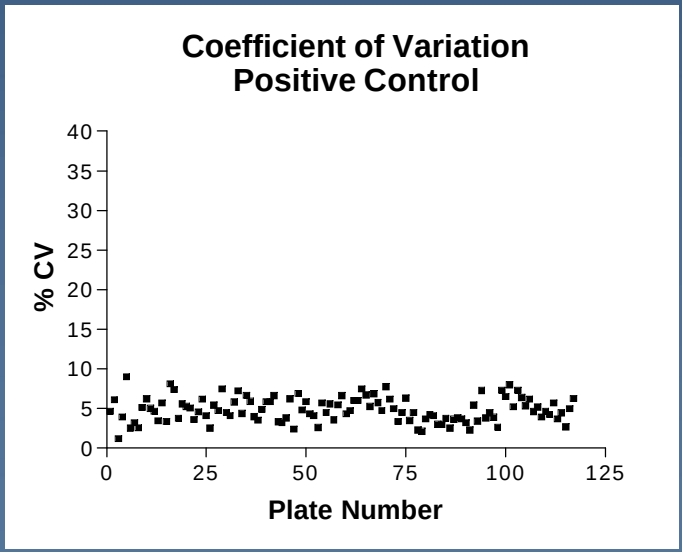
- % Activation with Kisspeptin Dose-Response



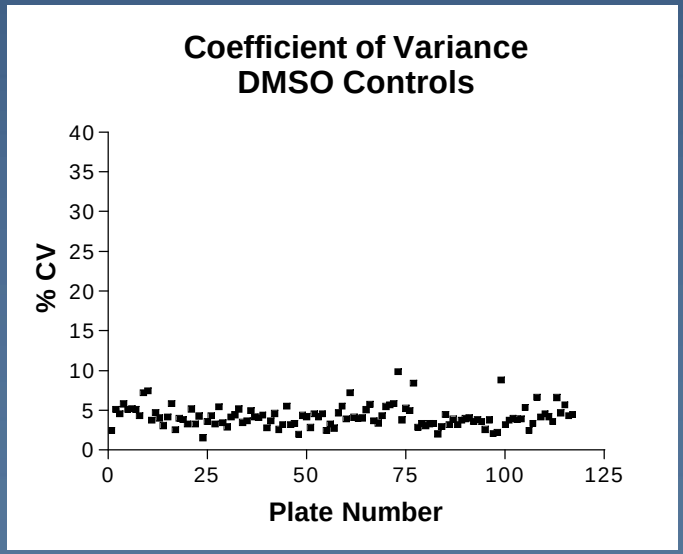
Screening Statistics

Z’ = 0.7
S:B = 4-5





Maximal kisspeptin



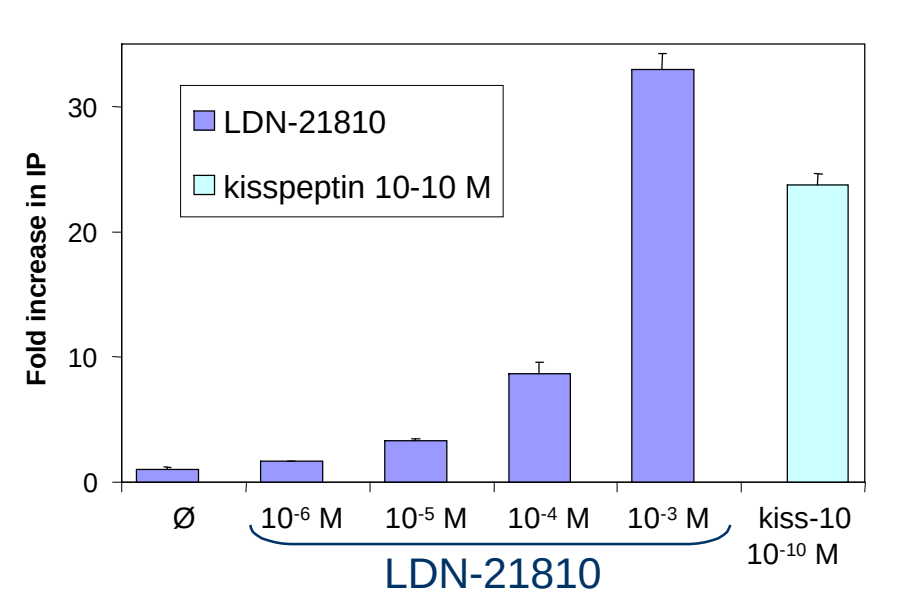
DMSO

Laboratory for Drug Discovery in Neurodegeneration

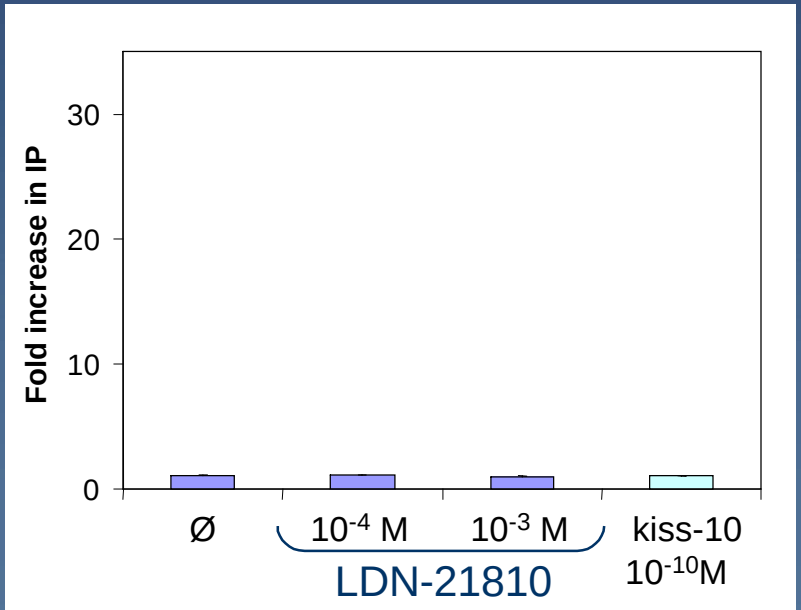
LDN-21810 In Follow-up Assays

- **LDN-21810 Stimulates IP Production in a GPR54-Specific and Dose-Dependent Manner**

GPR54 Line



Empty Vector Line



Laboratory for Drug Discovery in Neurodegeneration

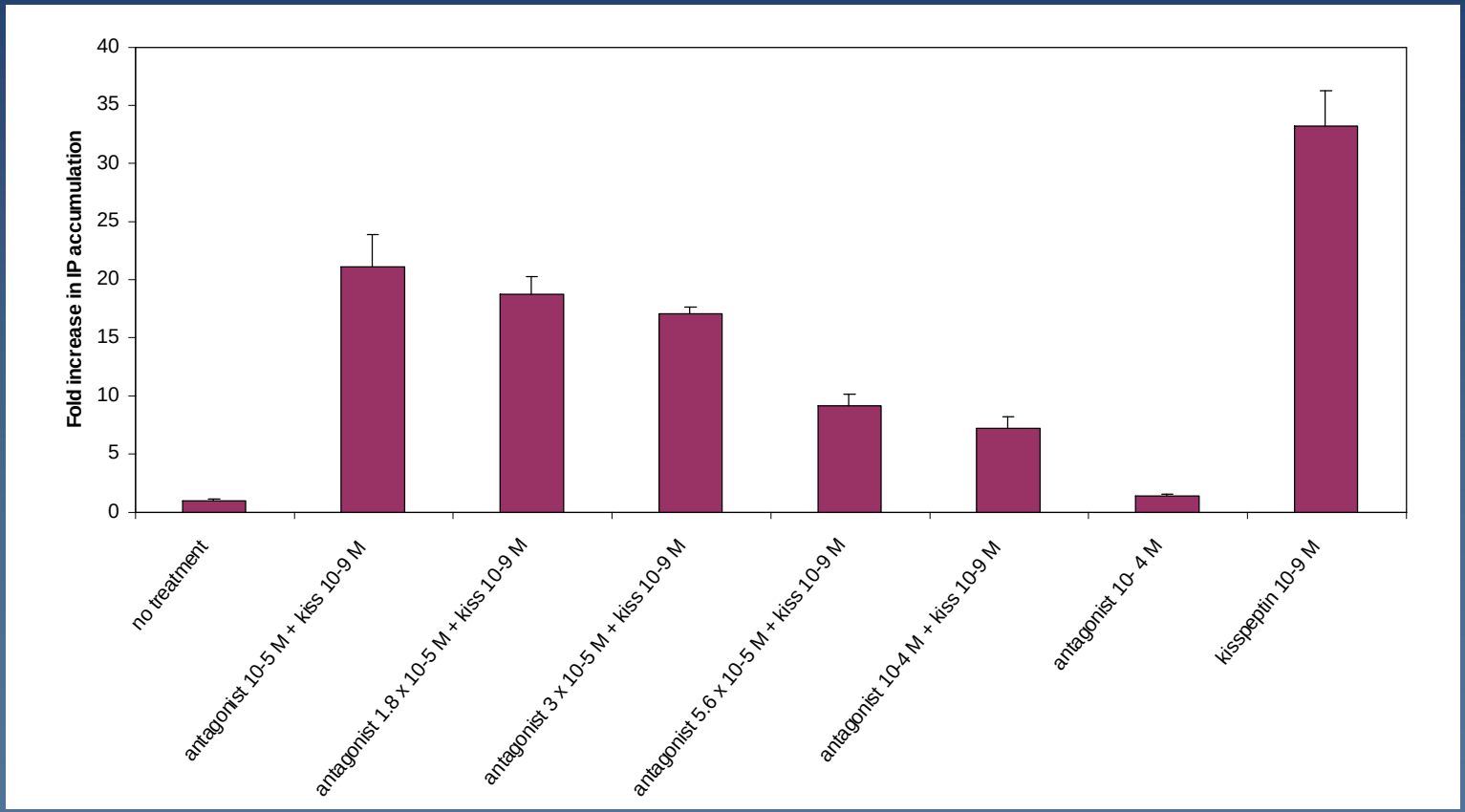
Agonist Screening Results

- The LDDN small molecule library of 110,000 compounds has been screened for GPR54 agonists.
- Currently characterizing one of the hits
  - LDN-21810.
- Unfortunately, HTS did not identify many hits. This is not surprising with peptide-ligand receptors.

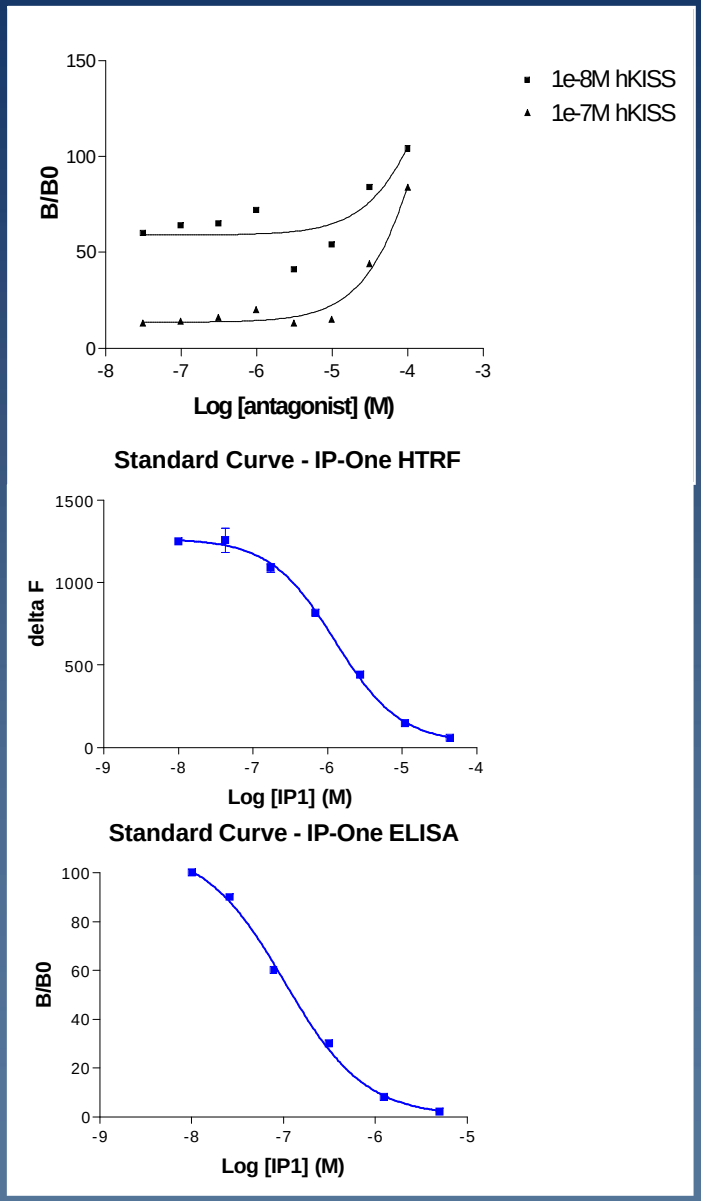
Strategy for Antagonist Screening

- The PLC inhibitor U-73122 used as a positive control.
- Negative controls: kisspeptin without U-73122 and no D_2 wells
- Currently undergoing optimization

Radioactive IP assay with kisspeptin and U-73122 (antagonist)



IP-One ELISA assay of GPR54 with kisspeptin



- ELISA is more sensitive then HTRF
- Data very similar
- Allows labs without fancy readers the ability to use IP-One

Laboratory for Drug Discovery in Neurodegeneration

SUMMARY

- A GPR54 stably transfected cell line was generated for screening and was found to respond appropriately in functional assays.
  - IP accumulation
  - ERK phosphorylation

SUMMARY

- A GPR54 stably transfected cell line was generated for screening and was found to respond appropriately in functional assays.
  - IP accumulation
  - ERK phosphorylation
- An assay based on measurement of IP1 accumulation was optimized for high-throughput screening.

SUMMARY

- A GPR54 stably transfected cell line was generated for screening and was found to respond appropriately in functional assays.
  - IP accumulation
  - ERK phosphorylation
- An assay based on measurement of IP1 accumulation was optimized for high-throughput screening.
- We completed an initial screen of an entire small molecule library for GPR54 agonists.

SUMMARY

- A GPR54 stably transfected cell line was generated for screening and was found to respond appropriately in functional assays.
  - IP accumulation
  - ERK phosphorylation
- An assay based on measurement of IP1 accumulation was optimized for high-throughput screening.
- We completed an initial screen of an entire small molecule library for GPR54 agonists.
  - Positive hits from ligand screening were selected and verified using the screening assay (IP-One) and secondary functional assays.
  - The compound verified on secondary assays is now being tested in an animal model.

SUMMARY

- A GPR54 stably transfected cell line was generated for screening and was found to respond appropriately in functional assays.
  - IP accumulation
  - ERK phosphorylation
- An assay based on measurement of IP1 accumulation was optimized for high-throughput screening.
- We completed an initial screen of an entire small molecule library for GPR54 agonists.
  - Positive hits from ligand screening were selected and verified using the screening assay (IP-One) and secondary functional assays.
  - The compound verified on secondary assays will then be tested in an animal model.
- Repeat screening of the library for GPR54 antagonists and enhancers is underway.

Laboratory for Drug Discovery in Neurodegeneration

Laboratory for Drug Discovery in Neurodegeneration

Ross Stein, PhD, *Director* LDDN
Marcie Glicksman, *Sr.Director* Leads Discovery
Greg Cuny, *Sr.Director* Chemistry

- Jake Ni
- April Case
- Mickey Huang

In collaboration with:

Brigham and Women's Hospital

- Wendy Kuohung
- Ursula Kaiser
- Deepa Mukhtyar

Massachusetts General Hospital

- William Crowley, Jr.
- Stephanie Seminara
- Samuel Posner

Harvard Center for Neurodegeneration and Repair