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# High Receptor Reserve and GPCR Cross Talk in a mGluR2 FLIPR Assay

The Lilly logo is written in a red, cursive script font.

Answers That Matter.

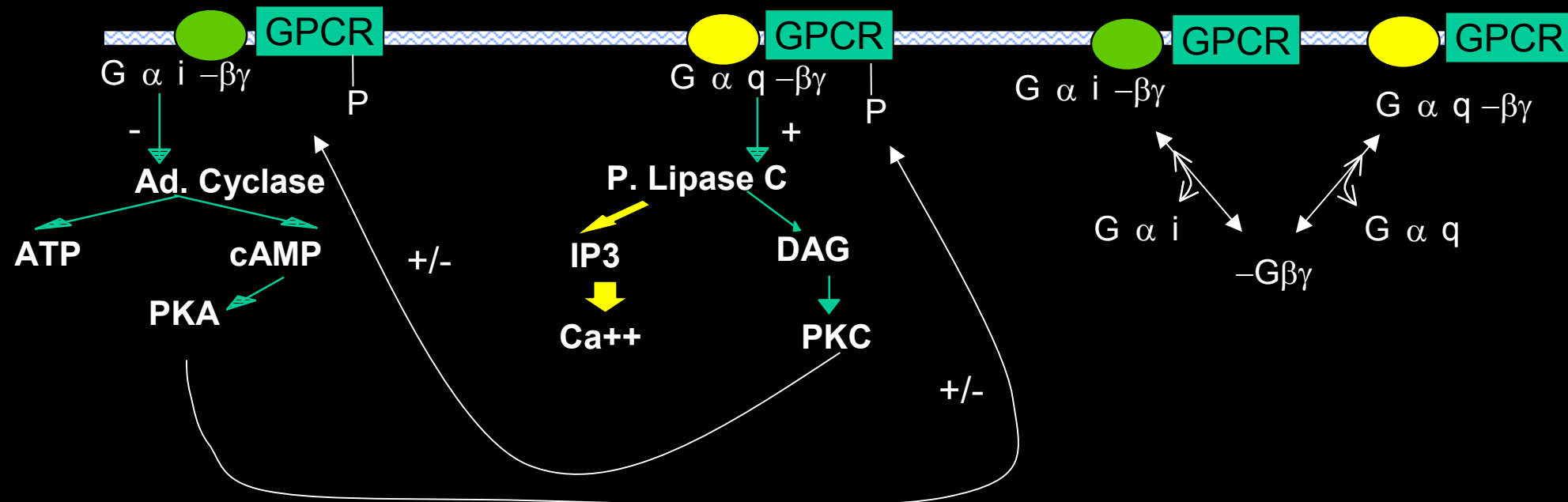
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# Screening GPCR's with Functional Assays

- Receptor Cross Talk

- Expression and/or activation of one receptor changing the coupling characteristics of another.

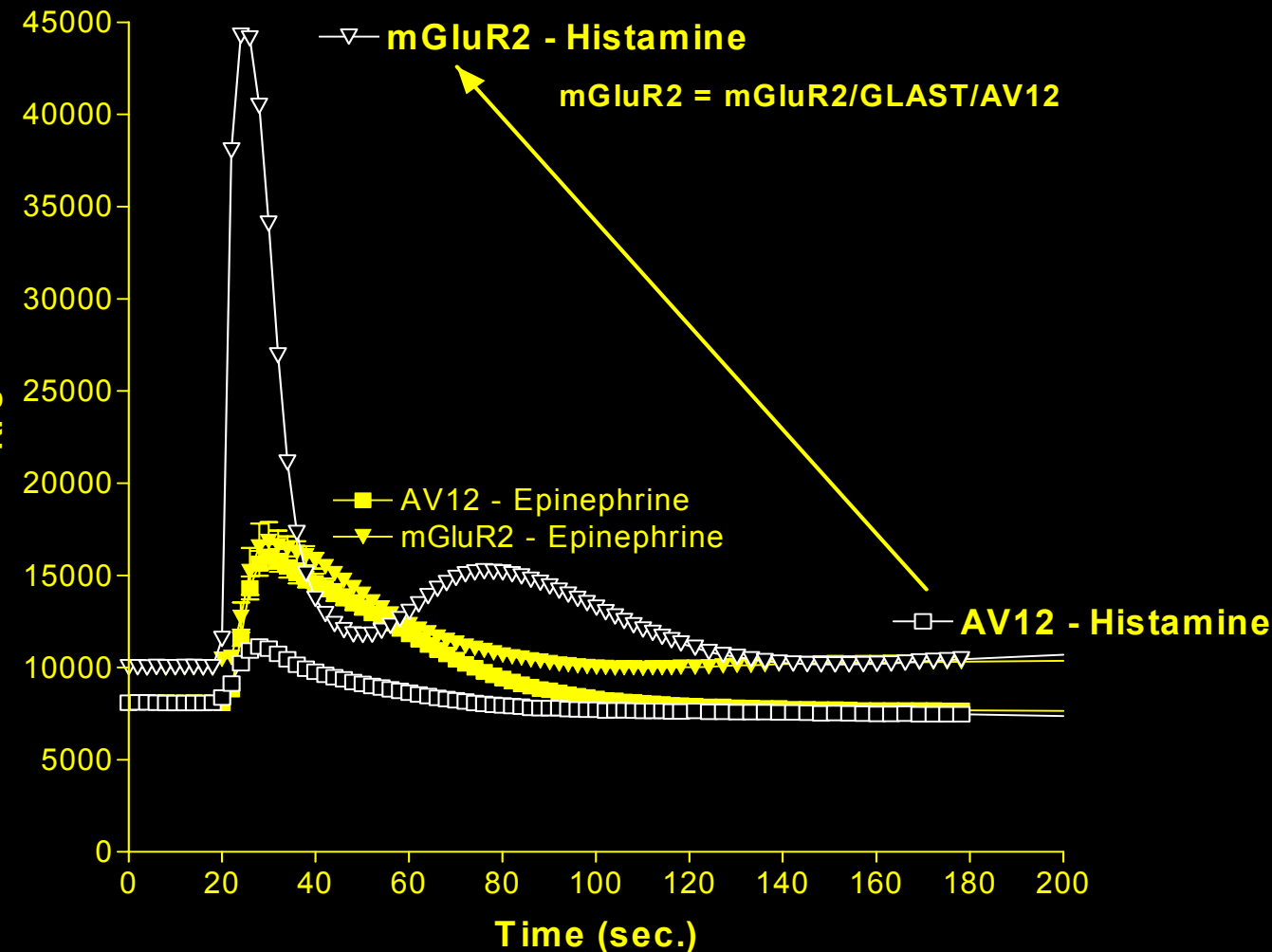
- Induces changes in Phosphorylation states of the GPCR or G-proteins.
- Competition for limited  $\alpha$ ,  $\beta$  and/or  $\gamma$  G-protein subunits.



# Expression of the G $\alpha$ i-coupled mGlu receptors greatly amplifies a histamine-response.

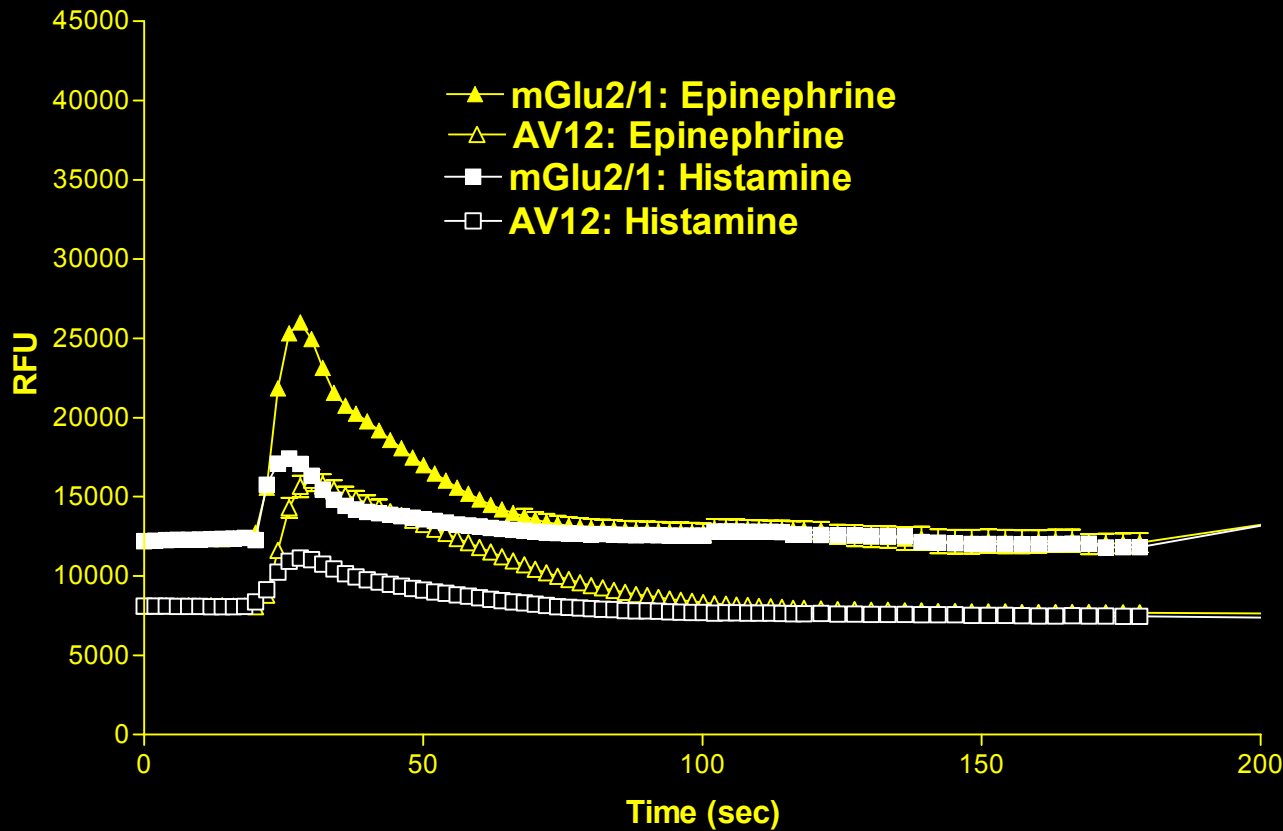
Shown to occur in cell lines expressing the G $\alpha$ i-coupled mGlu receptors:  
i.e.  
mGlu2, mGlu3  
mGlu4, mGlu6, mGlu7, &  
mGlu8

Pharmacology of the histamine response, and mRNA expression levels indicates this is an H1-mediated Gq-coupled response, and that the same levels of mRNA for H1 are found in all AV-12 lines.



# Expression of the G $\alpha$ q-coupled mGlu receptors does not increase the histamine-response.

mGlu2/1: epi & hist. signal vs. AV-12 line

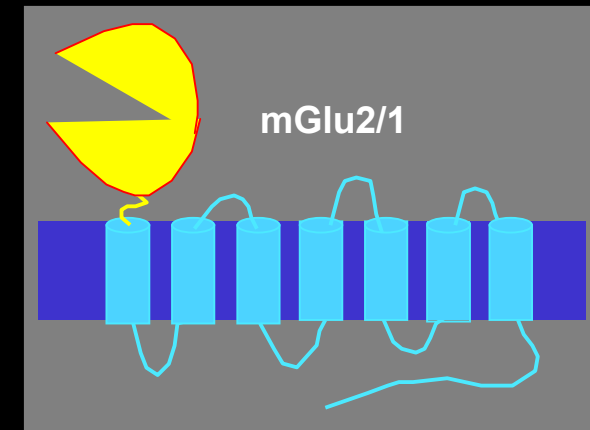


No amplification of histamine response in:

mGlu1 or mGlu5

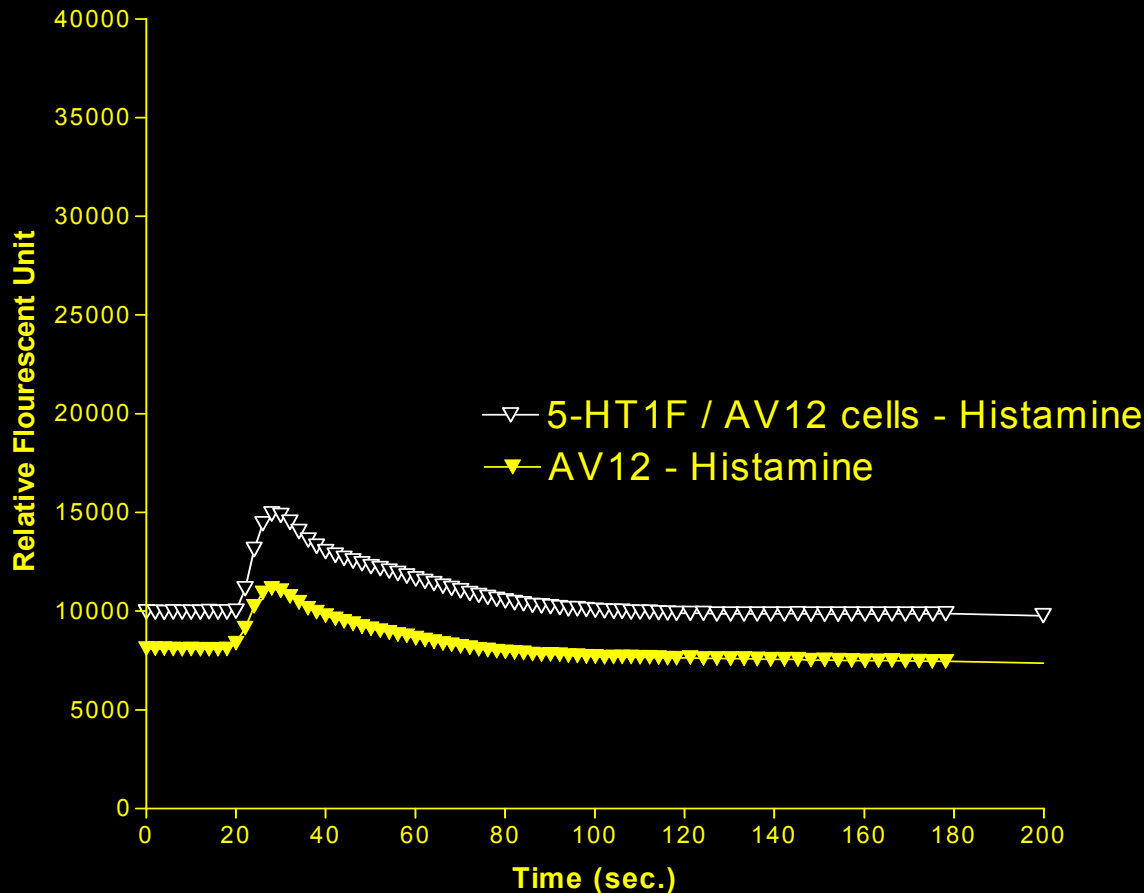
or

chimeric mGlu receptors that have the coupling domain of mGlu1



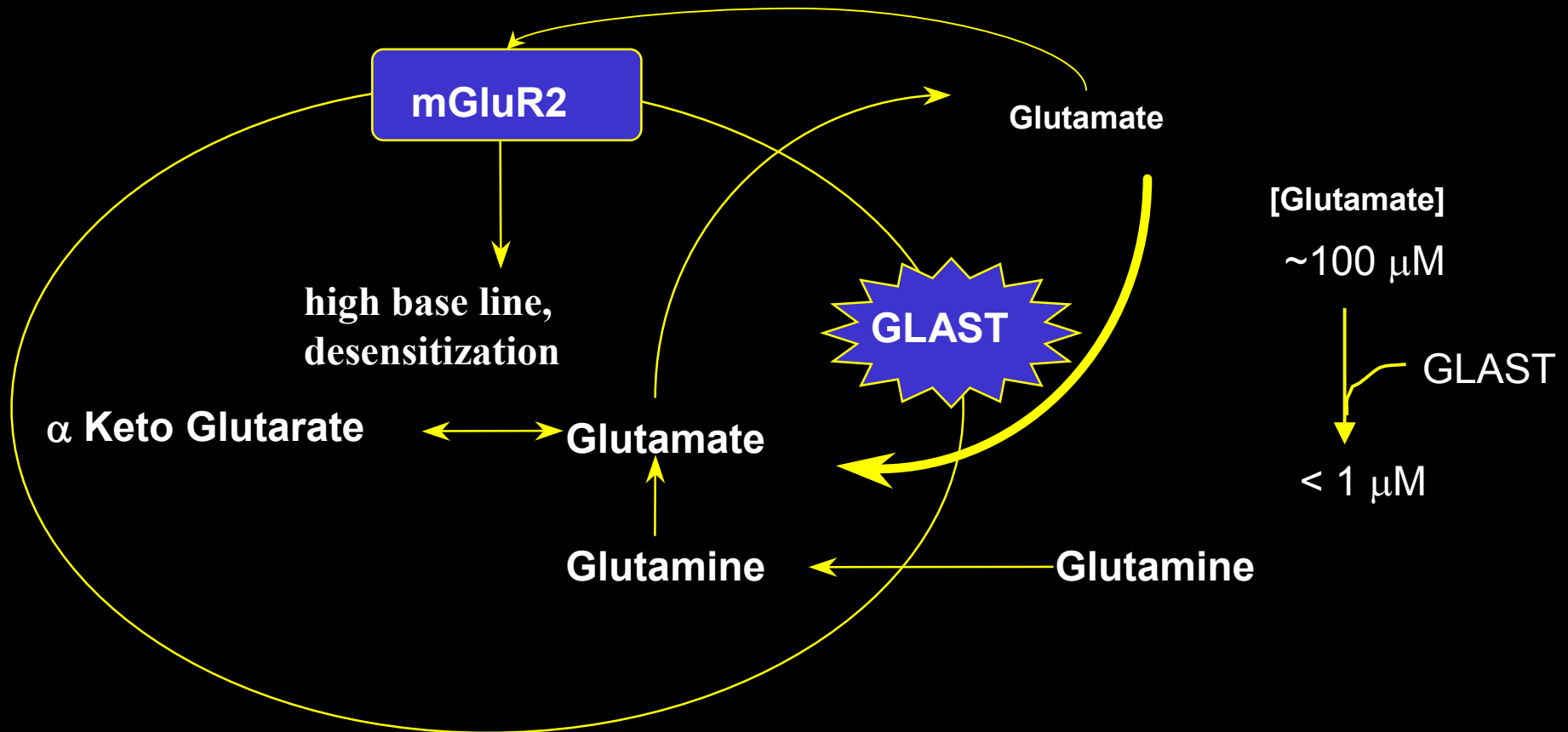
# Induction of histamine response not seen with all $G\alpha_i$ -coupled GPCR's

Effect of  $G\alpha_i$ -coupled 5-HT receptor on Histamine



In high expressing serotonin 5-HT1F AV-12 cells, a receptor known to couple well to  $G\alpha_i$  pathway, there is no apparent induction of the H1-mediated histamine signal.

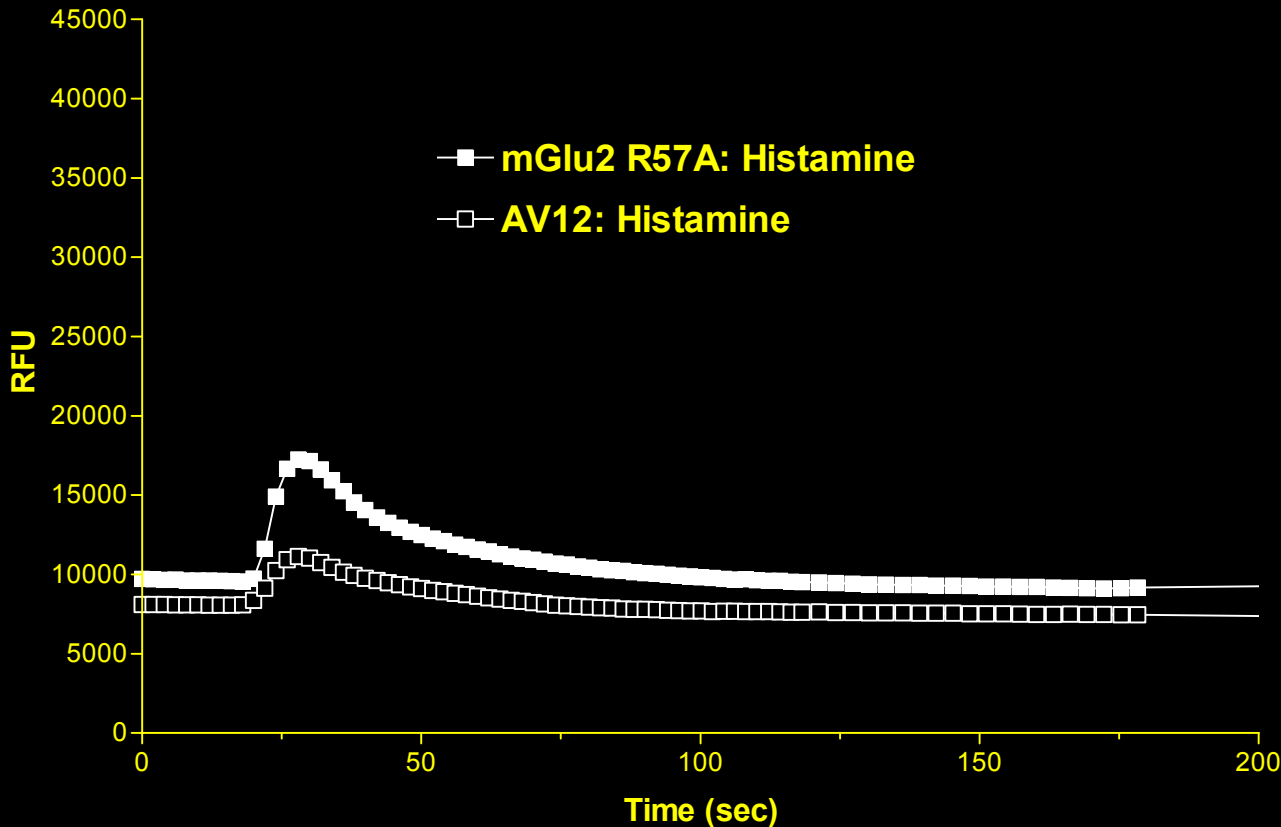
# AV12's synthesis and release glutamate



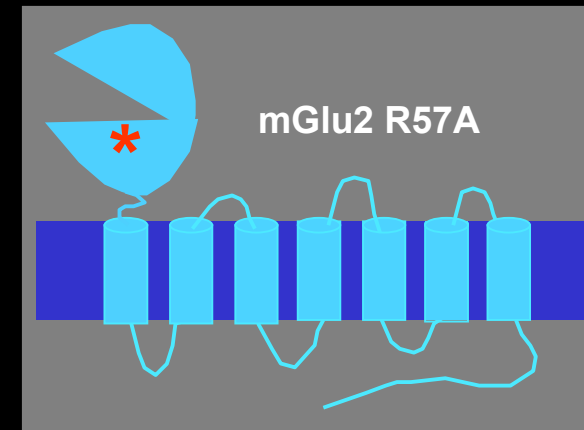
**Glutamate Transporter decreases extracellular glutamate levels**

# A single point mutation in the glutamate binding pocket reverses the increased histamine response

mGlu2 R57A: Histamine Response



No amplification of histamine response when a single amino acid is changed in glutamate binding pocket... Thus decreasing glutamate affinity by at least 100 x

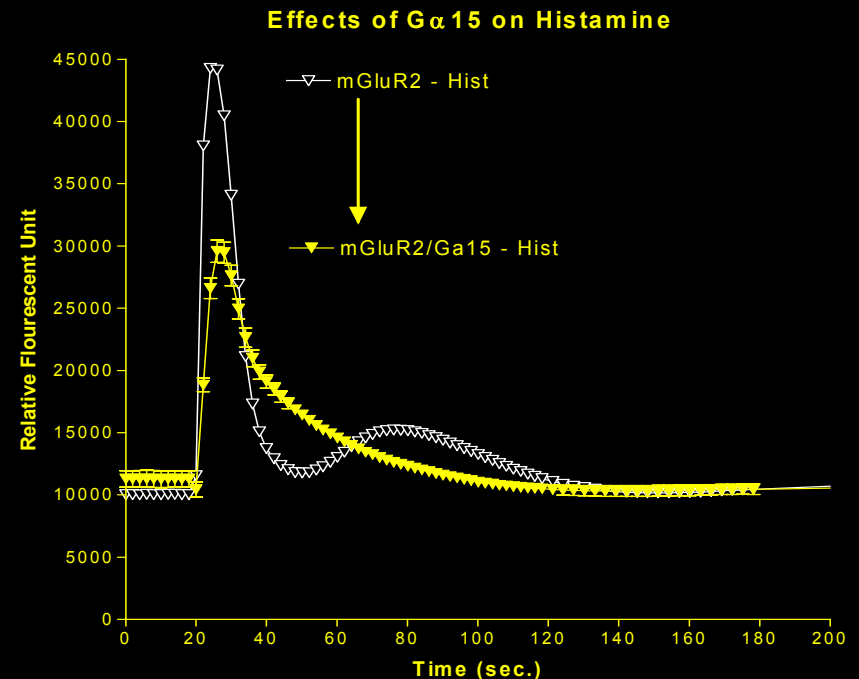


# Histamine Induction is not PKA, or PKC mediated but is attenuated by $G\alpha 15$ expression.

No Effect of pretreatment with:

- Pertussis Toxin (inhibits  $G\alpha i$ )
- Bisindoylmaleimide (Protein Kinase C inhibitor)
- Phorbol 12-myristate 13-acetate (Protein Kinase C activator)

Histamine signal is diminished by ~ 40% with Expression of the promiscuous  $G\alpha$  protein,  $G\alpha 15$ , which allows  $G\alpha i$ -linked GPCR's to activate the Phospholipase C pathway.

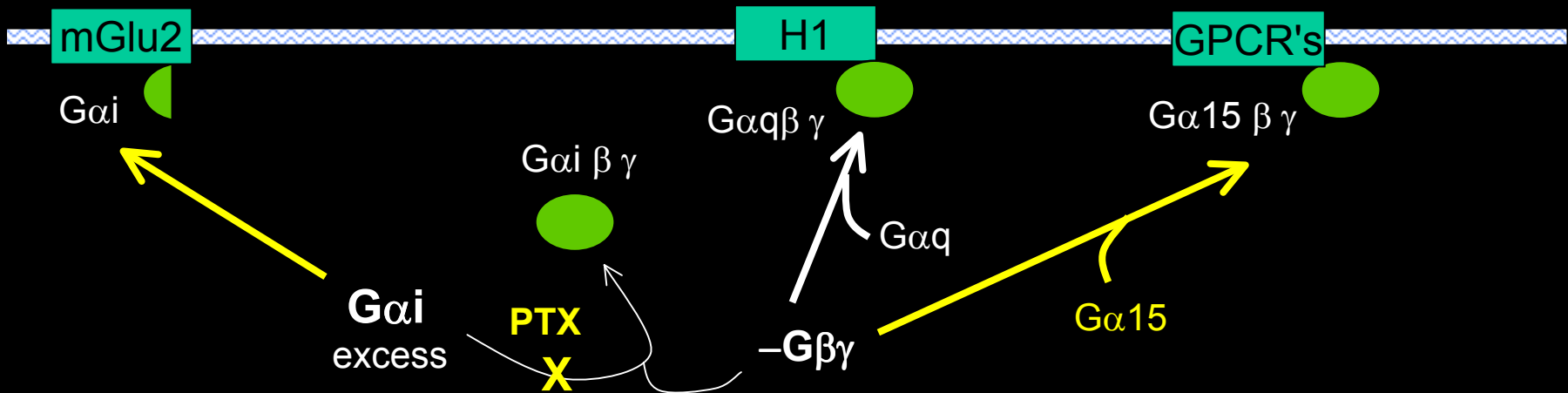


# Summary...so far

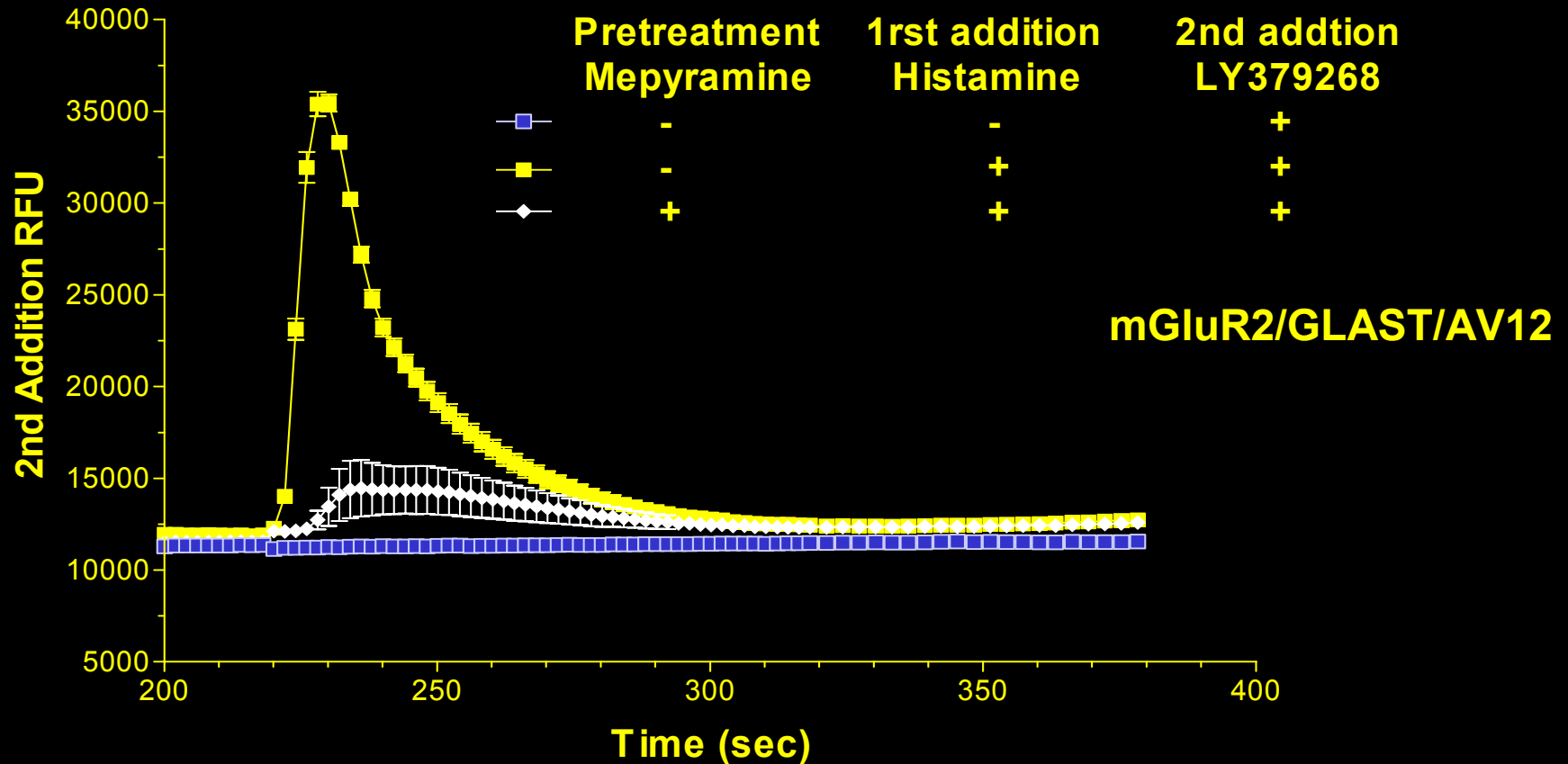
- There is an induction in the signal generated from H1 stimulation.
  - Dependent upon expression of  $G\alpha_i$ -coupled mGlu receptors.
    - Not  $G\alpha_q$ -coupled mGlu receptors.
    - Not other  $G\alpha_i$ -coupled GPCR's.
  - Does not appear to involve protein phosphorylation states.
    - Not protein kinase C dependent.
    - Not cAMP-dependent protein kinase dependent.
  - Glutamate affinity/activation dependent
    - Prevented by mutations in Glutamate binding (caveat are receptor expression levels the same in the two lines?)
  - Not altered by Pertussis toxin pretreatment.

# Competition for $\beta\gamma$ -G-proteins?

- Possible Explanation for induction of histamine response?
  - The  $G\alpha_q$  required for histamine response competes with  $G\alpha_i$  for required  $G\beta\gamma$  subunits.
    - Free  $G\beta\gamma$  protein can directly activate Phospholipase C.
    - Overexpression of mGluR depletes  $G\alpha_i$ . Dependent upon expression levels and receptors affinity for free  $G\alpha$  and glutamate activity?
    - Expression of a competing  $G\alpha_{15}$  diminishes the  $G\beta\gamma$  needed for histamine response.



# Histamine pretreatment 'unmasks' mGlu receptor agonist-mediated increase in $Ca^{2+}$ .

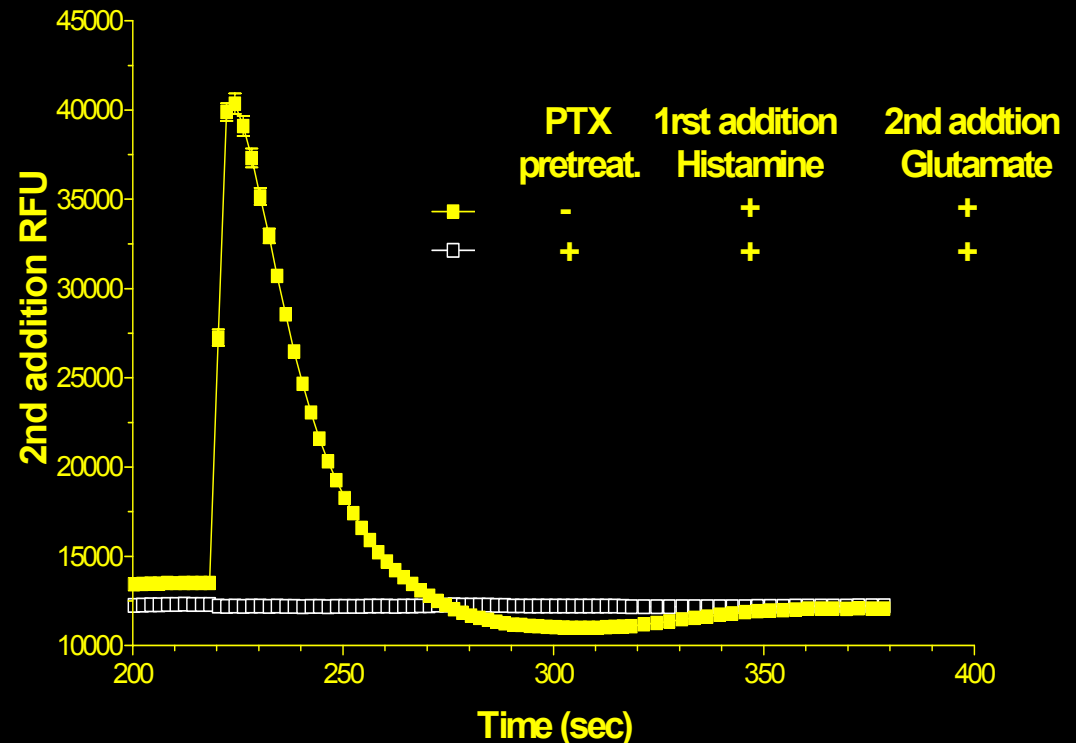


# Glutamate response is dependent upon $G\alpha_i$ activation but not PKC or PKA activation.

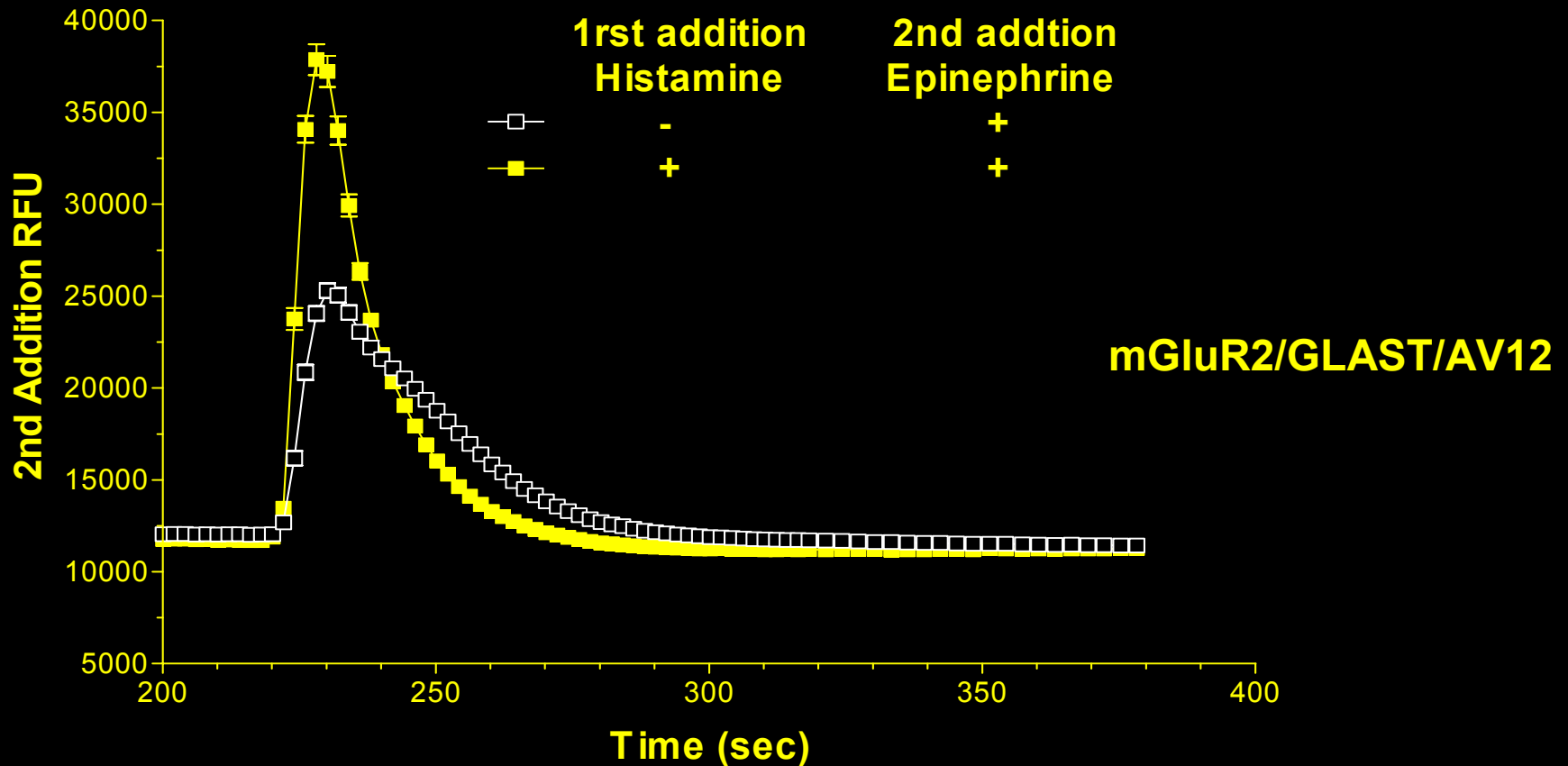
No Effect of pretreatment with:

- Bisindoylmaleimide (Protein Kinase C inhibitor)
- Phorbol 12-myristate 13-acetate (protein kinase C activator)

Pertussis Toxin 16 hr pretreatment completely eliminates the Glutamate response (without effecting the histamine response)

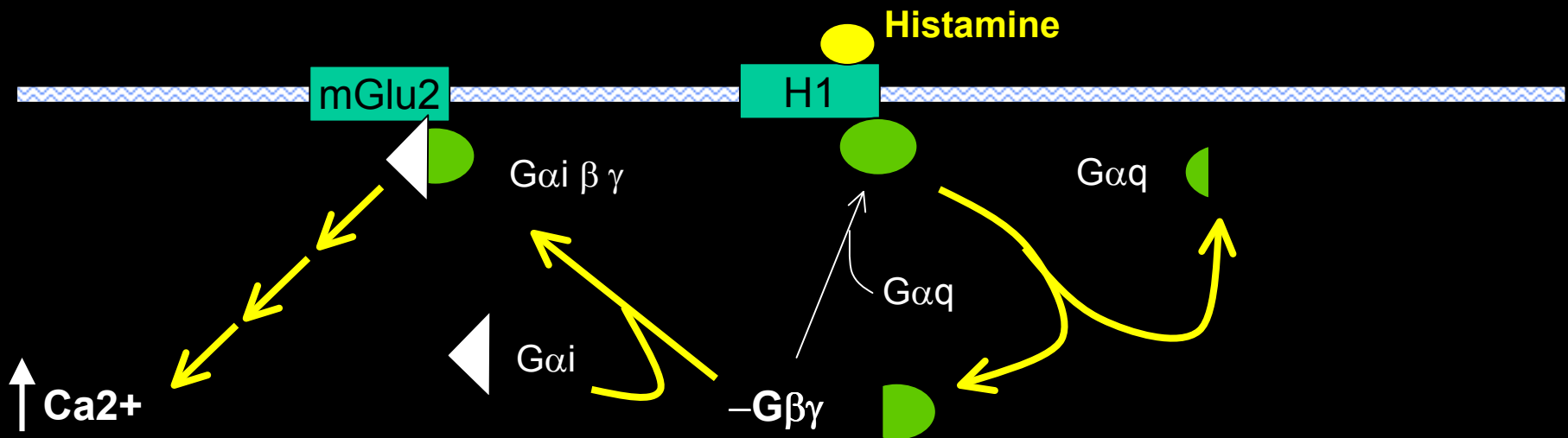


# Histamine can also increase epinephrine response.

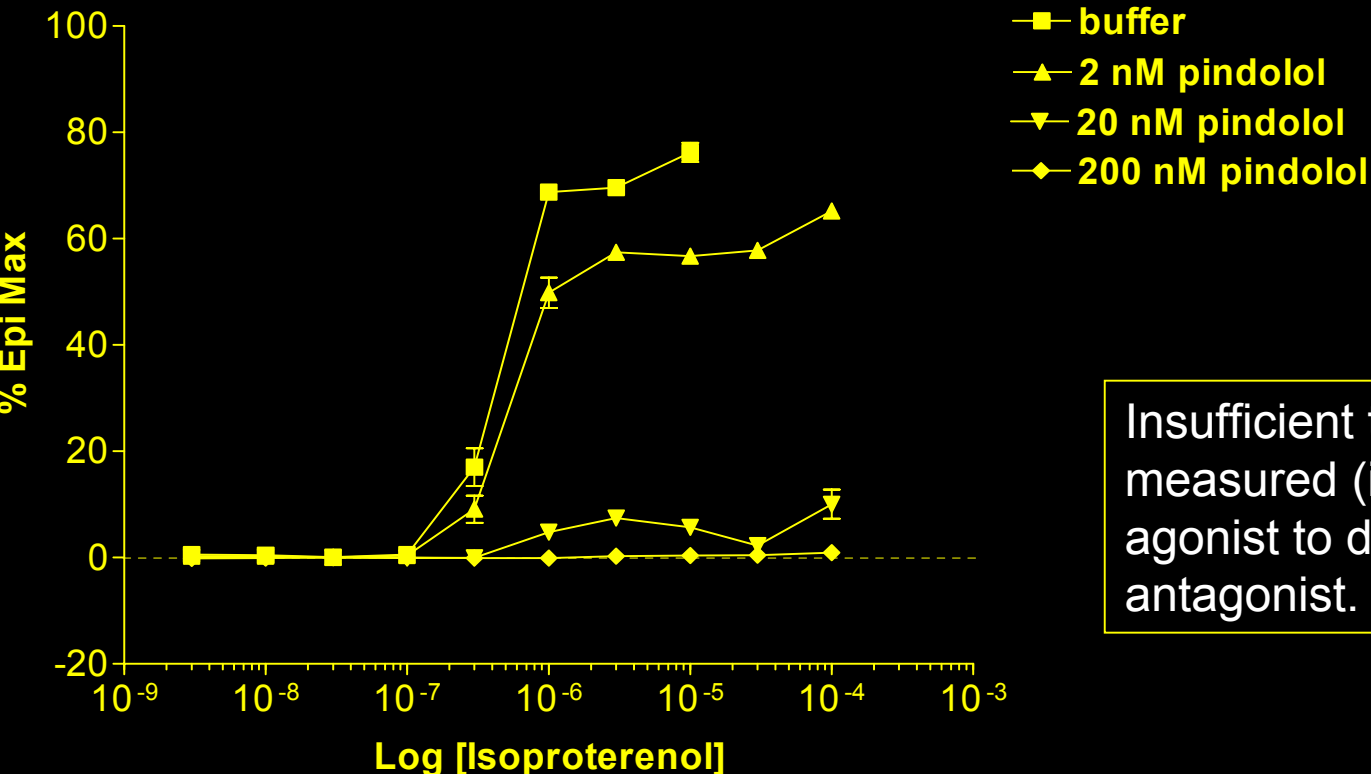


# Competition for $\beta\gamma$ -G-proteins again?

- Why activation by histamine changes the coupling of mGlu2
  - Histamine receptor must be activated, desensitized and free appropriate  $G\beta\gamma$  subunits so that a  $G\alpha_i$  can complex, and be available to mediate a glutamate-dependent  $Ca^{2+}$  response.
    - Limiting factor in the pathway is  $\beta\gamma$  G-proteins. Receptor and  $G\alpha_i$  are in excess.
    - The appropriate  $\beta\gamma$  subunit has a stronger interaction with  $G\alpha_q$  than  $G\alpha_i$  but can still be activated by  $G\alpha_i$



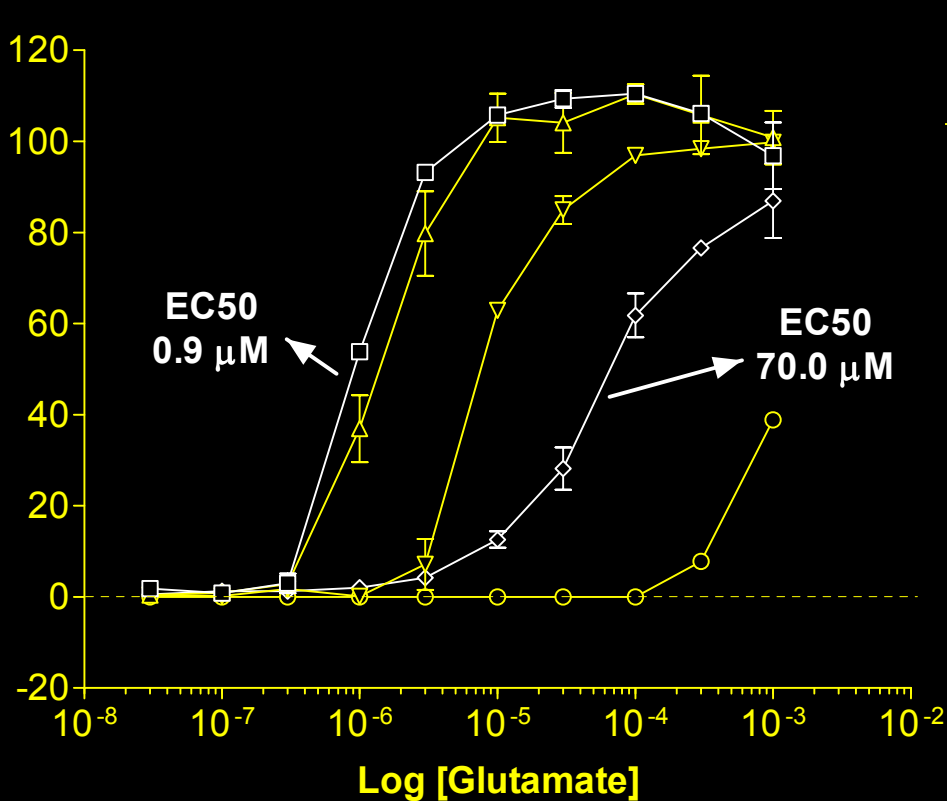
# Competitive Antagonists act as if they are non-competitive in FLIPR system.



Insufficient time prior to response measured (i.e. Intracellular Ca<sup>2+</sup>) for agonist to displace even a competitive antagonist.

# Apparent potency of antagonists can vary greatly with the assay system utilized.

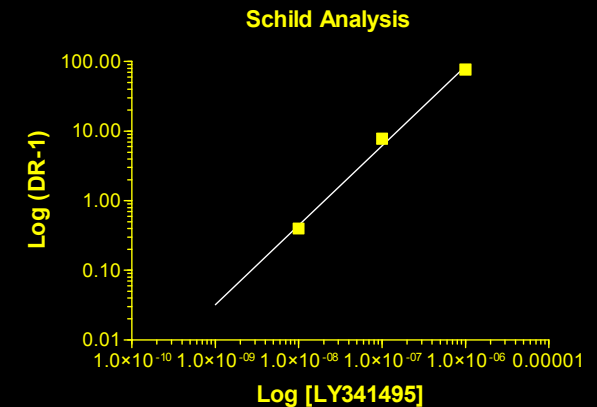
Shift in Glutamate Dose/Response in  
mGluR2/GLAST/G $\alpha$ 15/AV12 line



LY341495

Binding Kd = 1.67 nM

FLIPR IC50 = 21.0 - 160.0 nM



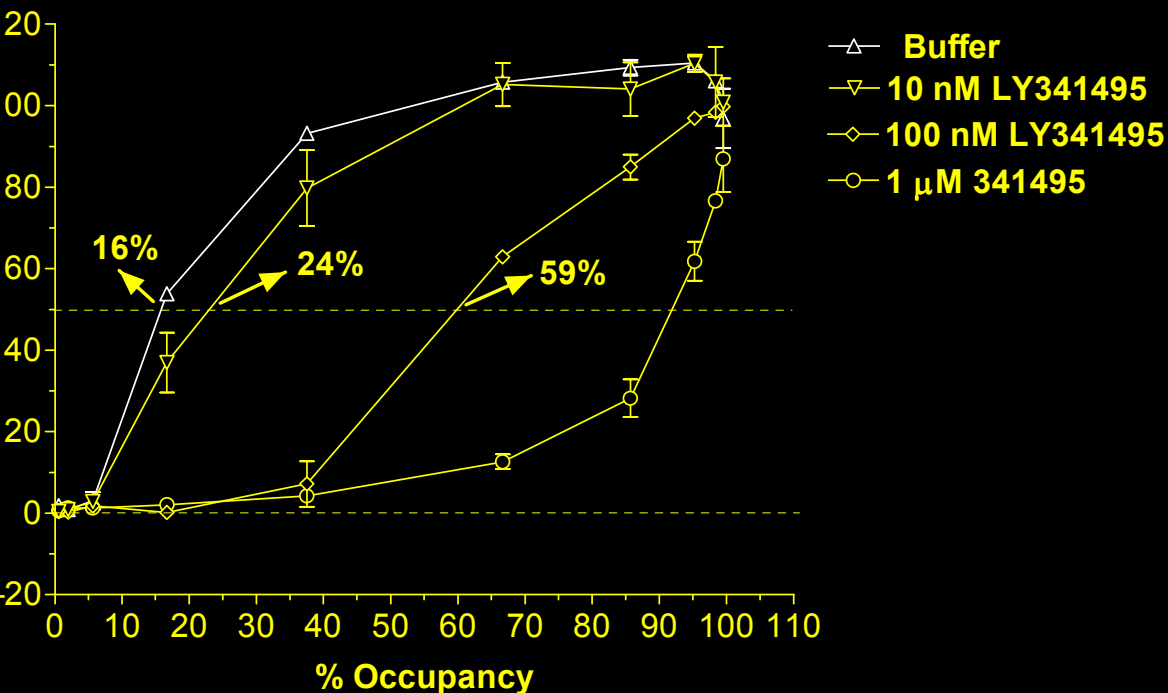
Schild plot analysis K<sub>b</sub> = 20 nM

# What Receptor Reserve can do to agonist and antagonist functional assay dose-response curves.

## 'Receptor Reserve'

is defined here as only a fraction of the available receptors needing to be activated in order to achieve a maximal response.

% Occupancy Required for Effect



Under equilibrium conditions...

- Partial agonists can be fully efficacious
- Agonist dose-response curves will be shifted to the left (more potent).
- Alkylating agents or irreversible inhibitors will shift agonist curves to the right until the receptor reserve is eliminated and then decrease the maximal effect of the agonists.

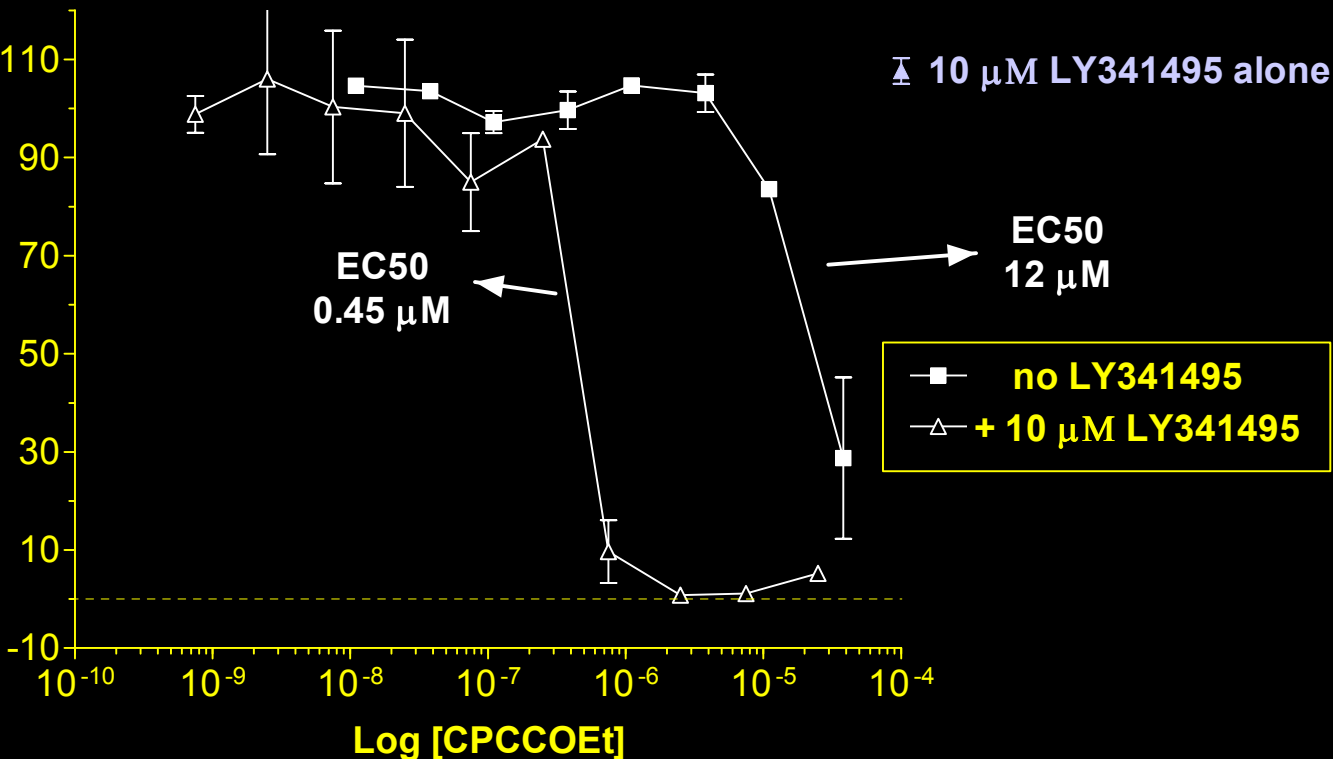
# Antagonists are at equilibrium but agonists are not in a rapid measurement system such as the FLIPR.

- Antagonists - If there is a sufficient pretreatment time...
  - Can calculate % occupancy based on  $K_d$  from receptor binding experiments.
    - i.e. **10 to 100 nM** LY341495 will occupy **86 to 98%** of the mGlu2 receptors.
- Agonists
  - Will only have to activate the fraction of receptors required for a given effect.
    - i.e. only **15%** of the receptors are occupied by glutamate to give a **50%** response.
- All antagonists (whether competitive or noncompetitive) will act as if they are irreversible.
  - Antagonist IC<sub>50</sub> values will correspond to the dose of the antagonists that binds all of the receptor reserve (e.g. 60% of total receptor) plus half of the remaining receptor required to give a maximal response (e.g. 1/2 of 40%, for a total of 80% occupancy).
    - i.e. if 86 - 98% of the receptors are in reserve, a predicted IC<sub>50</sub> value for LY341495 would be **19 to 165 nM**: experimental finding... 21 - 160 nM IC<sub>50</sub>.

# Eliminating receptor reserve

Shift in non-competitive antagonist curve when pretreated with a competitive antagonist.

mGlu1 / GLAST / AV-12 line



- All Antagonists behave as alkylating agents.

- Pretreat with a 'competitive antagonist' at a threshold dose (i.e. a dose that inhibits < 10% of the response)

- e.g. 10  $\mu$ M LY341495

- Then a run dose-response of a non-competitive antagonist and the IC50 value will correspond to the  $K_i$  (50% occupancy).

- e.g. CPCCOEt

# Summary

- GPCR Cross talk:
  - Complex relationship between receptors expressed, constitutive activity, and ability to interact and/or stimulate multiple  $G\alpha$  proteins can complicate interpretation of results.
    - Lack of agonist activity in the host cell line alone does not confirm that the target of the compound is the protein of interest.
      - Expression alone of the new GPCR (such as an orphan) might dramatically change the coupling capabilities of other unknown receptors in the host cell.
    - Stimulation of one GPCR in the cell system can alter the coupling of a subsequent activation of a separate receptor subtype.
      - Through either alterations in receptor/G-protein phosphorylation states and/or by changing the 'pool' of  $\alpha$ ,  $\beta$ , and/or  $\gamma$  G-proteins that are available to couple.
- Antagonists and the FLIPR:
  - Nature of system is such that most antagonists will behave as if irreversible.
    - High 'receptor reserve' cell lines will limit the potency of antagonists.
    - Non-conventional methods to eliminate receptor reserve can be employed.