

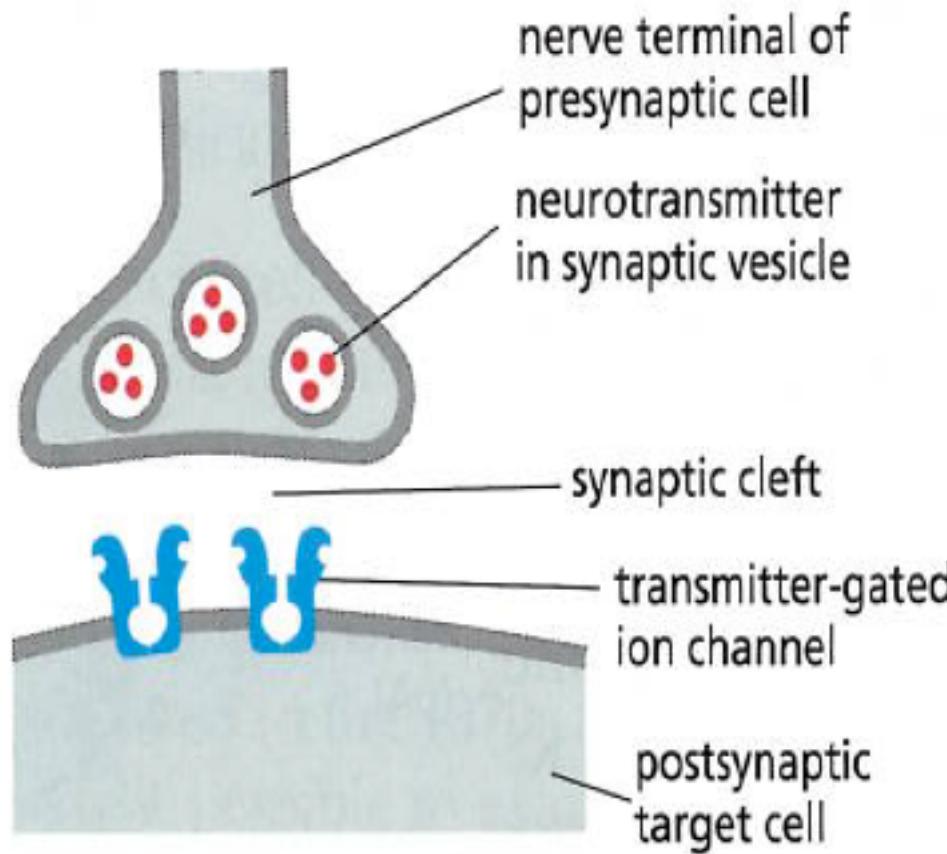
# **Scaffold Protein “Gephyrine”**

**Literature Seminar  
M1 Miura Kensuke**

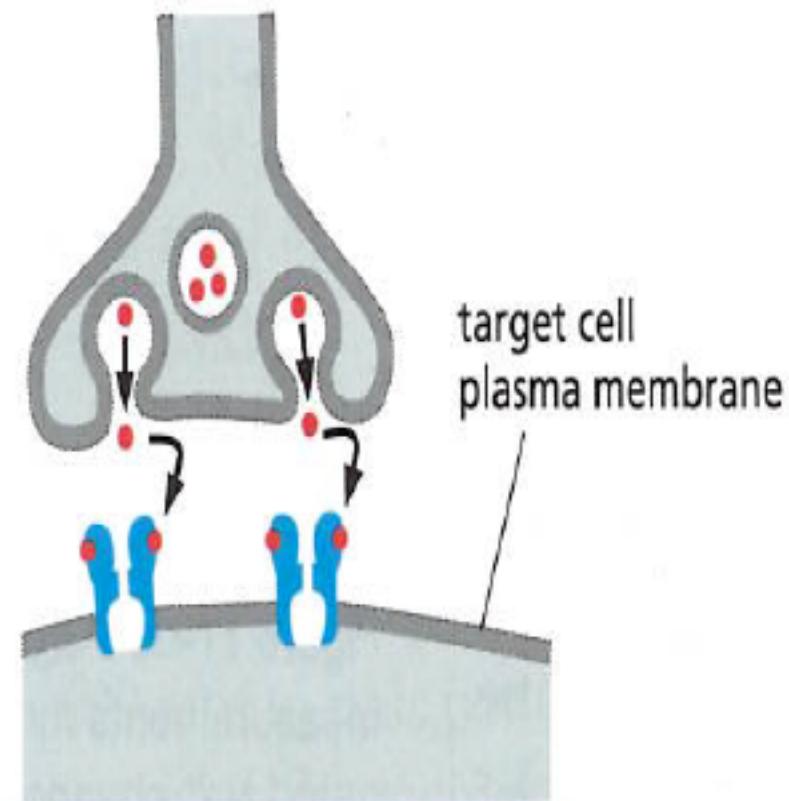
# **Outline**

- **Introduction of Neurotransmission**
- **Gephyrine**
- **Main topic:**  
“Gephyrine-binding peptides visualize postsynaptic sites and modulate neurotransmission”  
(Maric, M. C.; Hausrat, J. T.; Neubert, F; Dalby, O. N; Doose, S; Sauer, M; Kneussel, M; Stromgaard, K. *Nat. ChemBio.* 2016, 13, 153-160.)

# Neurotransmission



RESTING CHEMICAL SYNAPSE



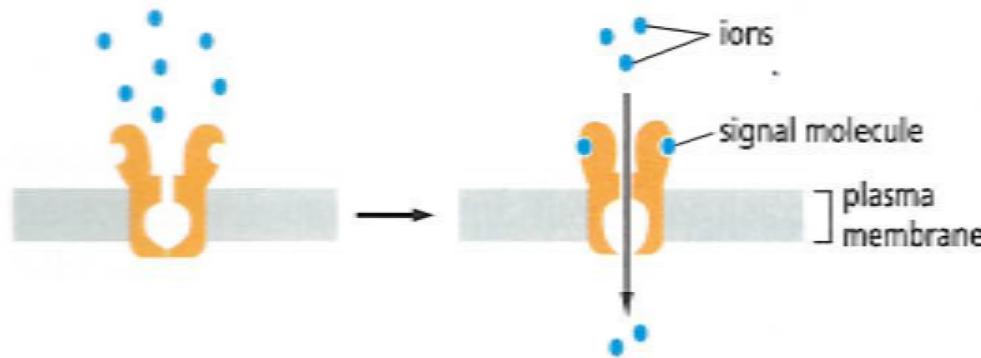
ACTIVE CHEMICAL SYNAPSE

- Neurotransmitter is a type of chemical messenger which transmits signals
- Neurotransmission is divided to 4 classes

# Fast-Slow Neurotransmission

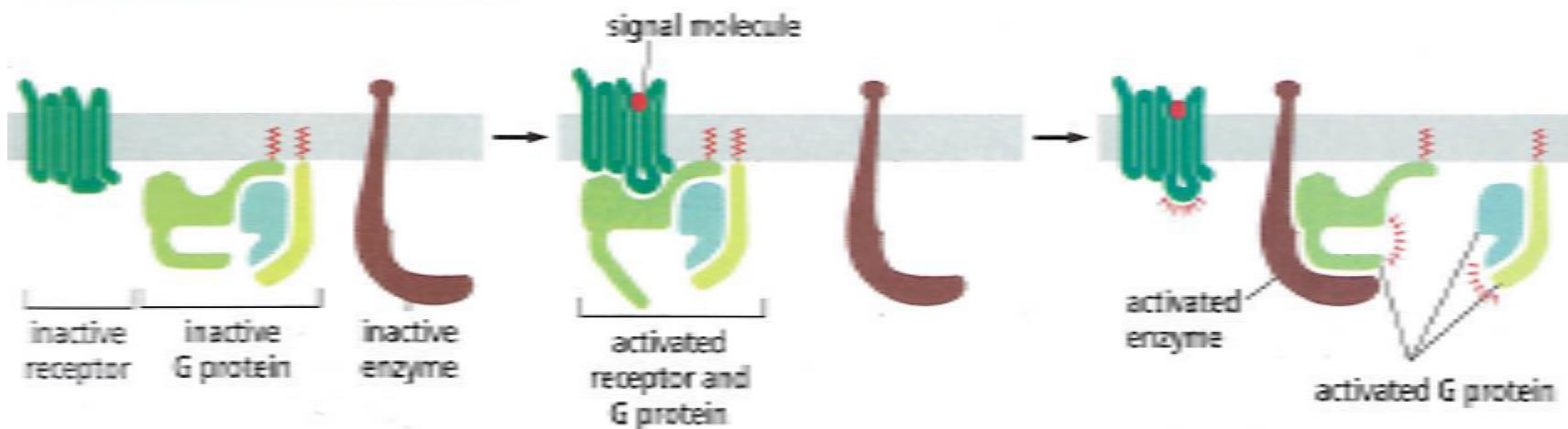
- Fast neurotransmission;  $\sim$  several tens of millisecond

(A) ION-CHANNEL-COUPLED RECEPTORS



- Slow neurotransmission; several hundreds of millisecond  $\sim$

(B) G-PROTEIN-COUPLED RECEPTORS



# **Excitatory-Inhibitory Synapse**

## **■ Excitatory Synapse**

- $\text{Na}^+$ ,  $\text{Ca}^{2+}$  ion channel
- Depolarization
- Excitatory postsynaptic potential; EPSP

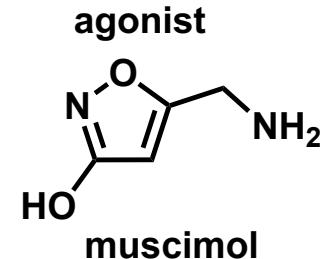
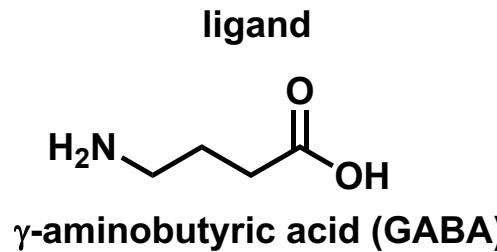
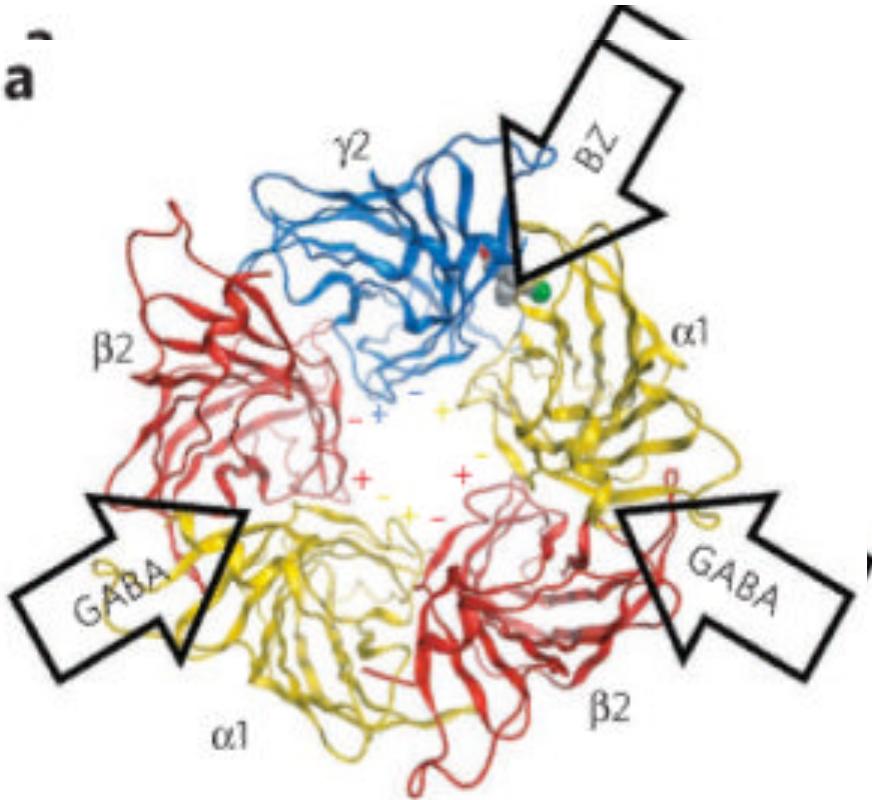
## **■ Inhibitory Synapse**

- $\text{Cl}^-$  ion channel
- hyperpolarization
- Inhibitory postsynaptic potential; IPSP

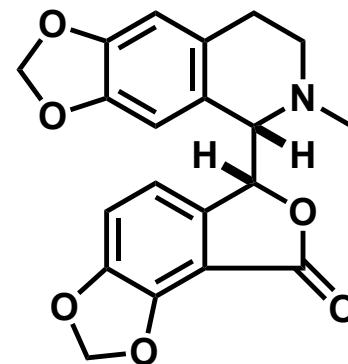
1. Excitatory-fast synaptic transmission: nicotinic acetylcholine receptor; nAChR
2. Excitatory-slow synaptic transmission: muscarinic acetylcholine receptor; mAChR
3. Inhibitory-fast synaptic transmission: **GABA<sub>A</sub> receptor, glycine receptor**
4. Inhibitory-slow synaptic transmission: GABA<sub>B</sub> receptor

# GABA<sub>A</sub> Receptor

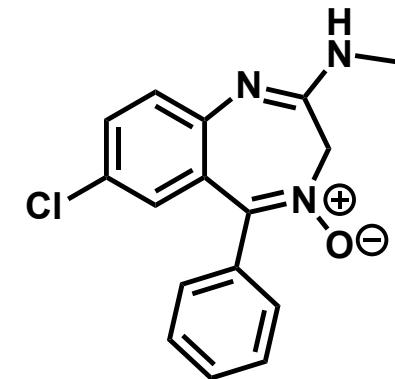
a



antagonist



benzodiazepine

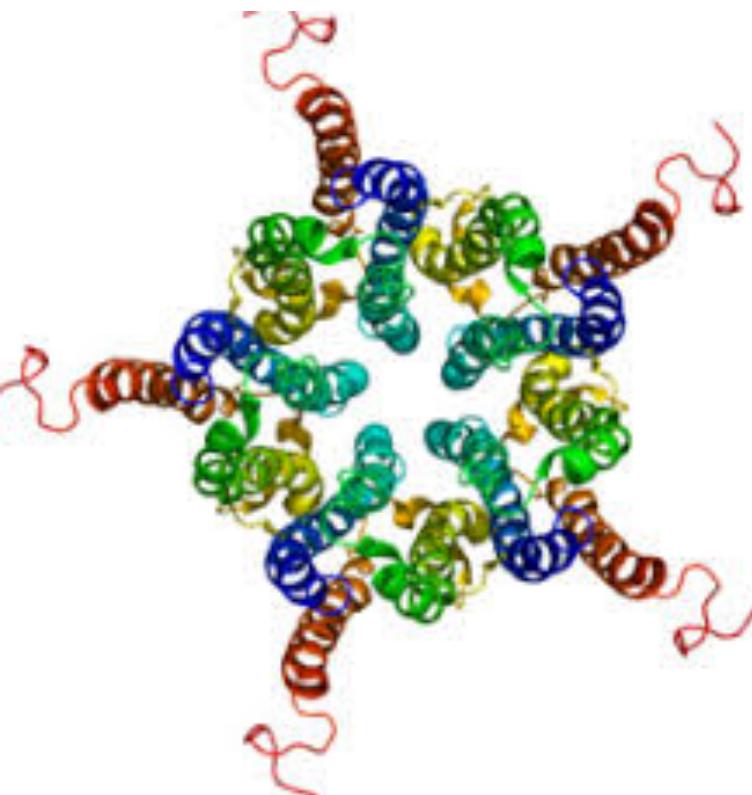


- α subunits × 2, β subunits × 2, γ subunits × 1

- Related disease
  - epilepsy

1) Johnston, G. A. R. *Pharmacology and Therapeutics*. 1996, 69, 173–198.

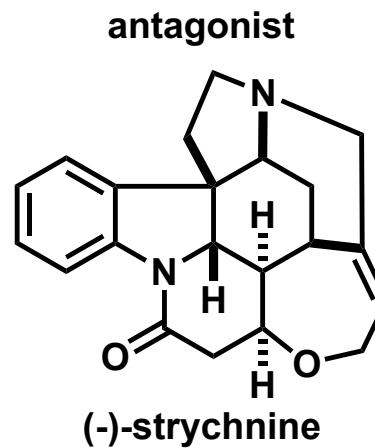
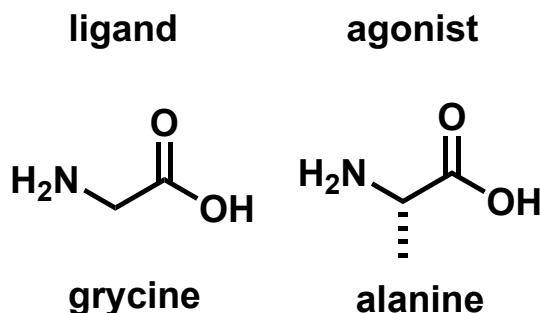
# Glycine Receptor



- $\alpha$  subunits  $\times$  5 (non-synaptic)
- $\alpha$  subunits  $\times$  2,  $\beta$  subunits  $\times$  3 (synaptic)
- $\alpha$  subfamily ( $\alpha 1 \sim \alpha 4$ )

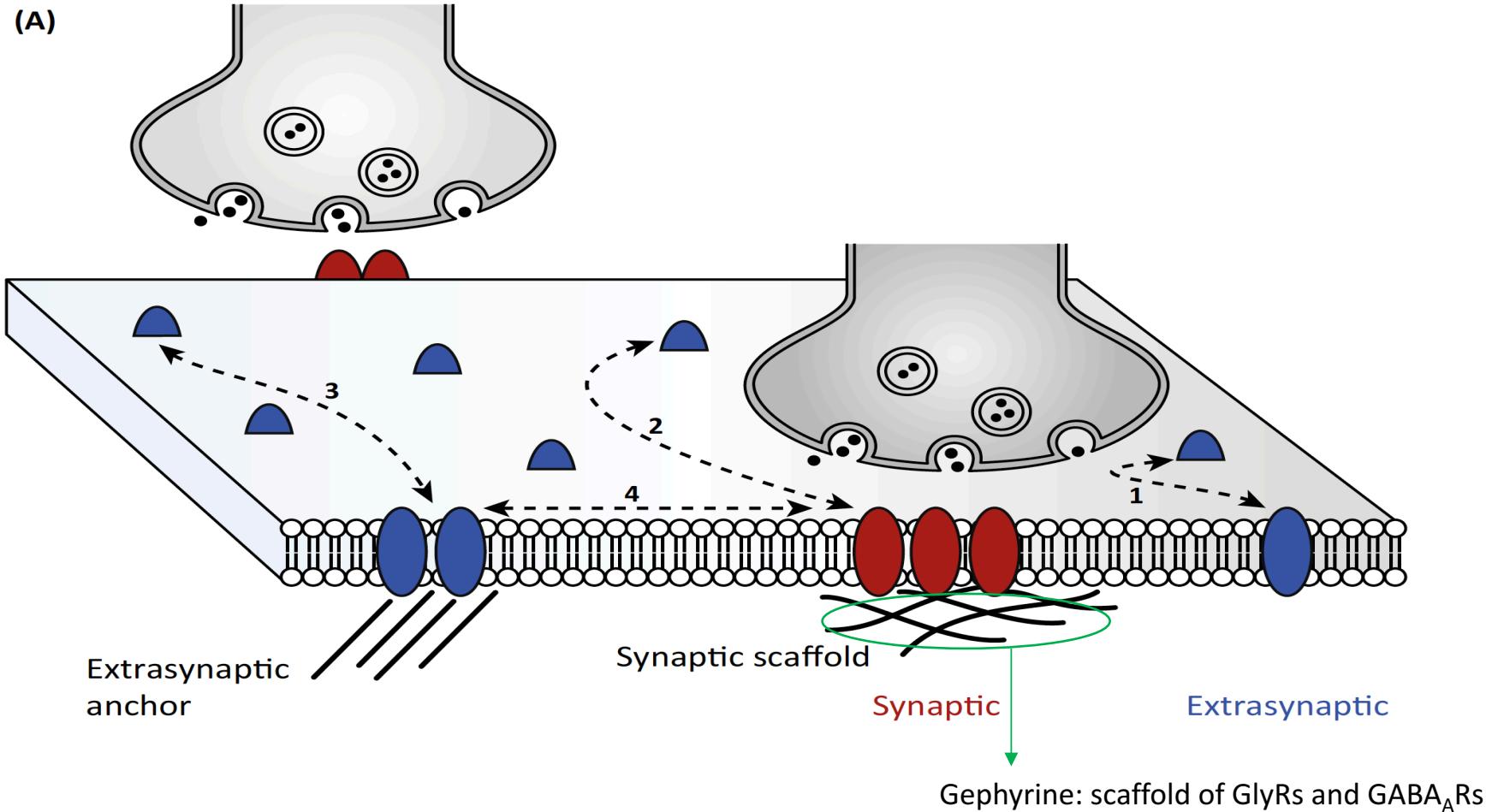
## ■ Related disease

- myoclonus
- hyperekplexia



1) Mullhardt, C.; Fischer, M.; Gass, P.; Chazottes, S. D.; Guenet, L. S.; Kuhse, J.; Betz, H.; Becker, M. C. *Neuron*. 1994, 13, 1003

# Clustering of Receptors

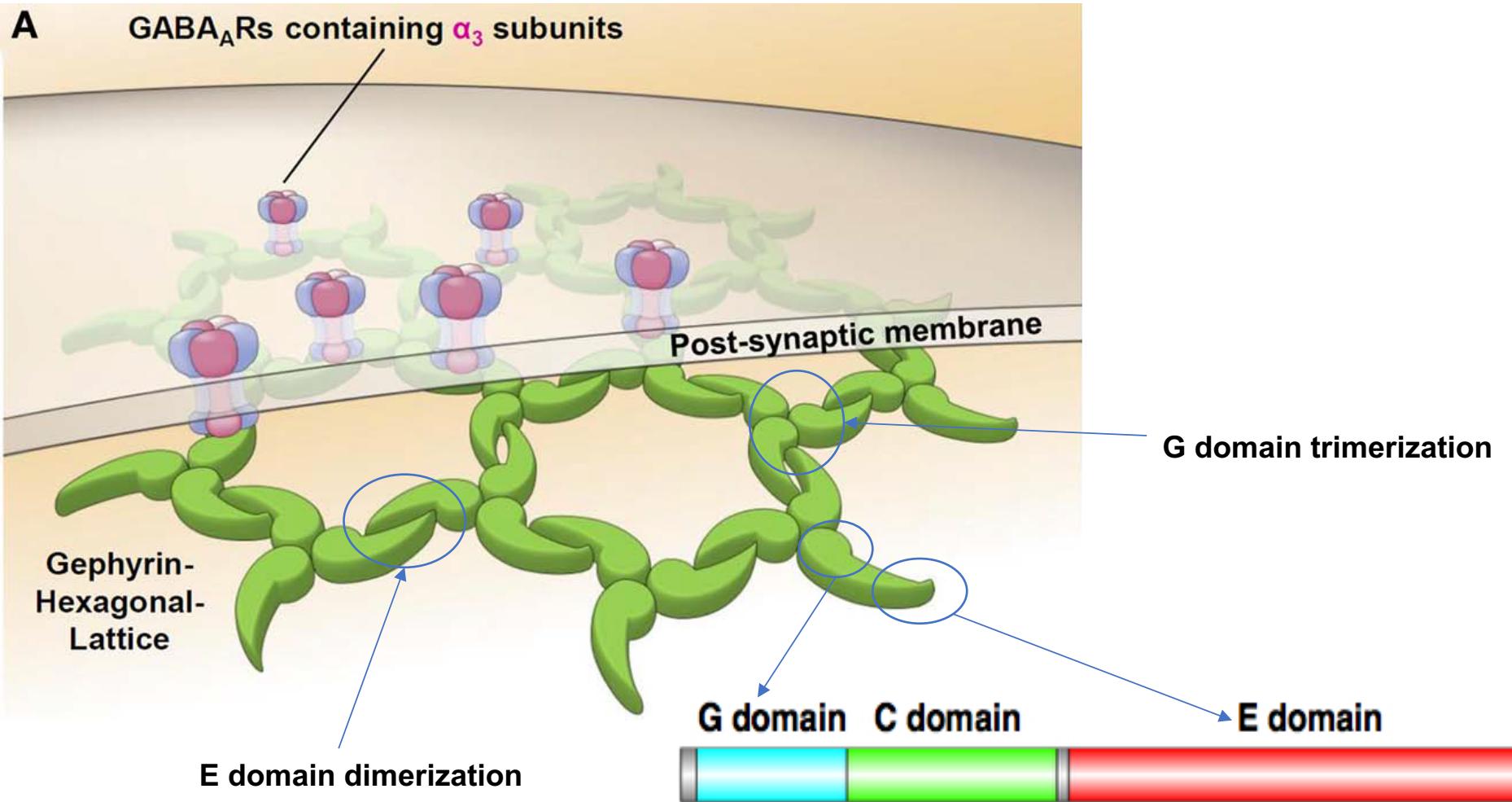


- Diffusion of mobile extrasynaptic receptor
- Clustering on synaptic scaffold

# **Outline**

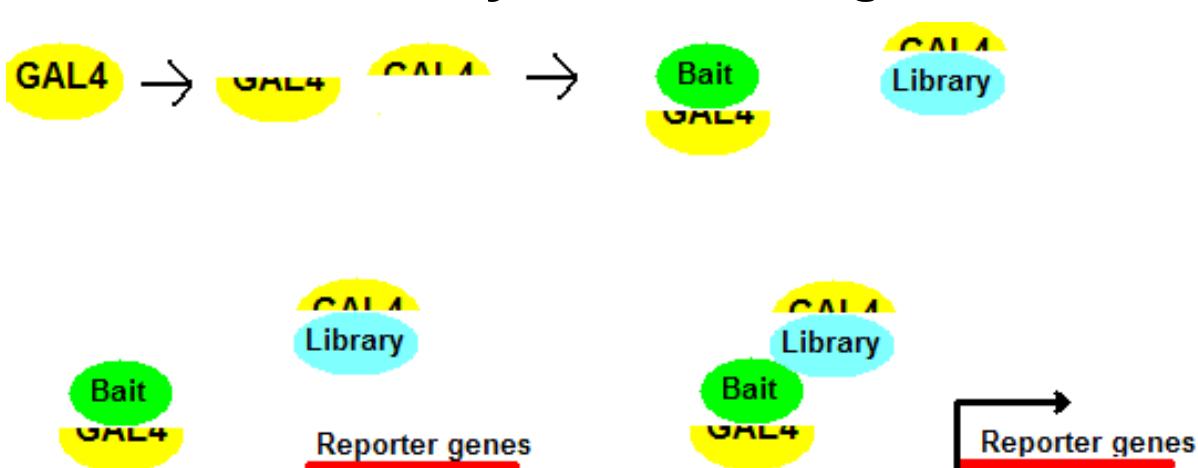
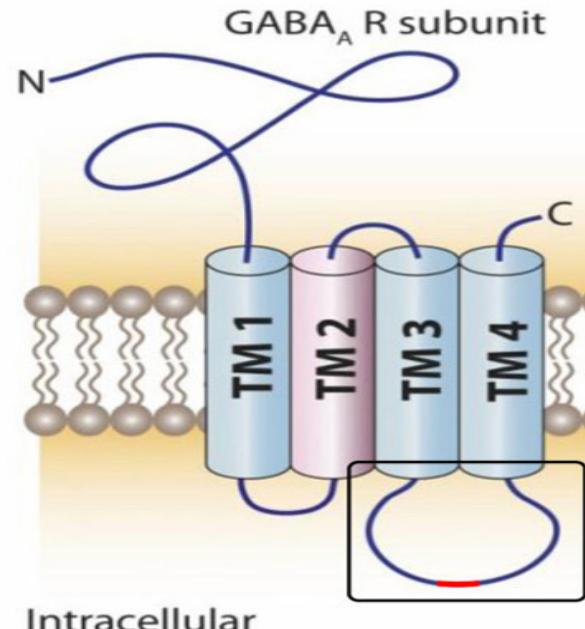
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# Gephyrine

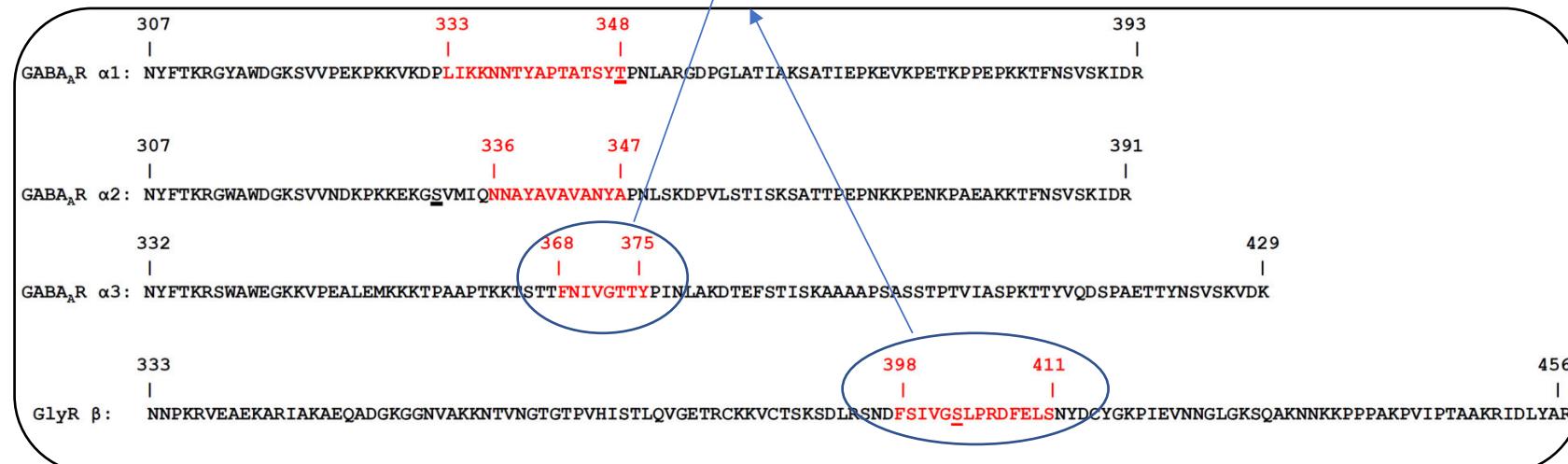


# Binding Motif of GlyRs and GABA<sub>A</sub>Rs

## Two-hybrid screening

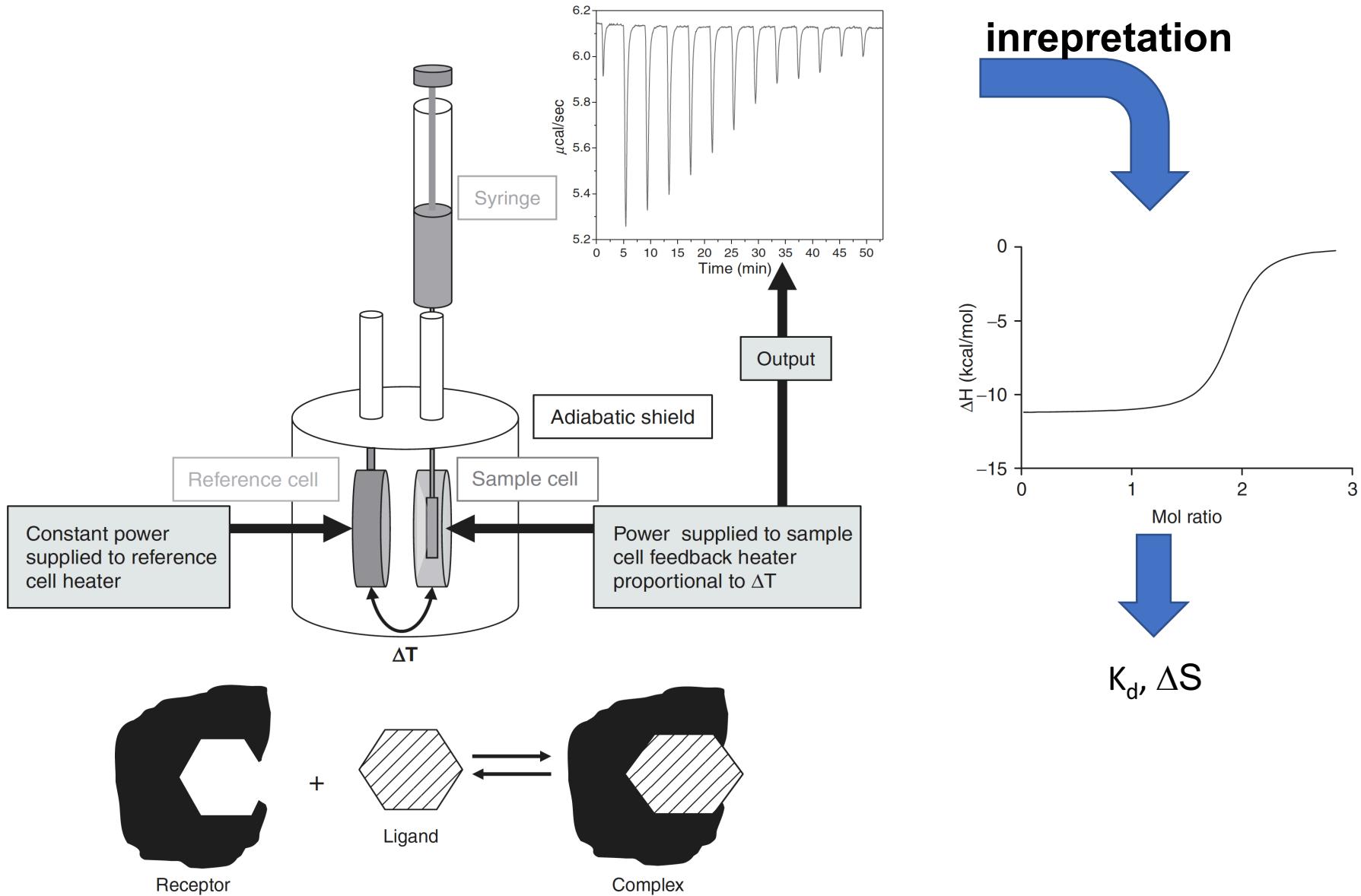


GABA<sub>A</sub>Rs α3: 368 **FNIVGTTYPIN**<sup>378</sup>  
Glyβ: 398 **FSIVGSLPRDF**<sup>408</sup>

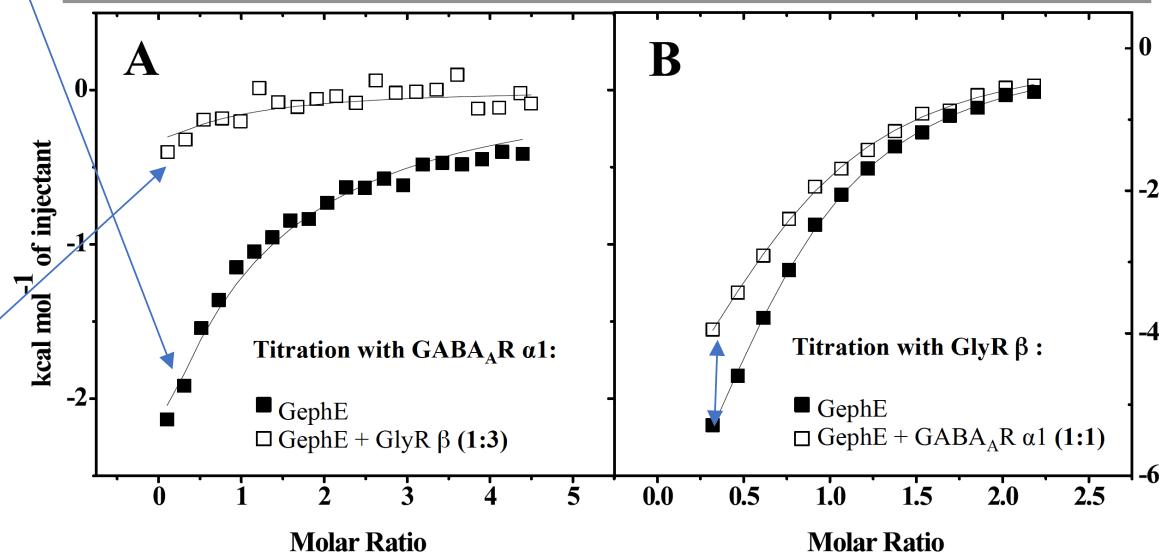
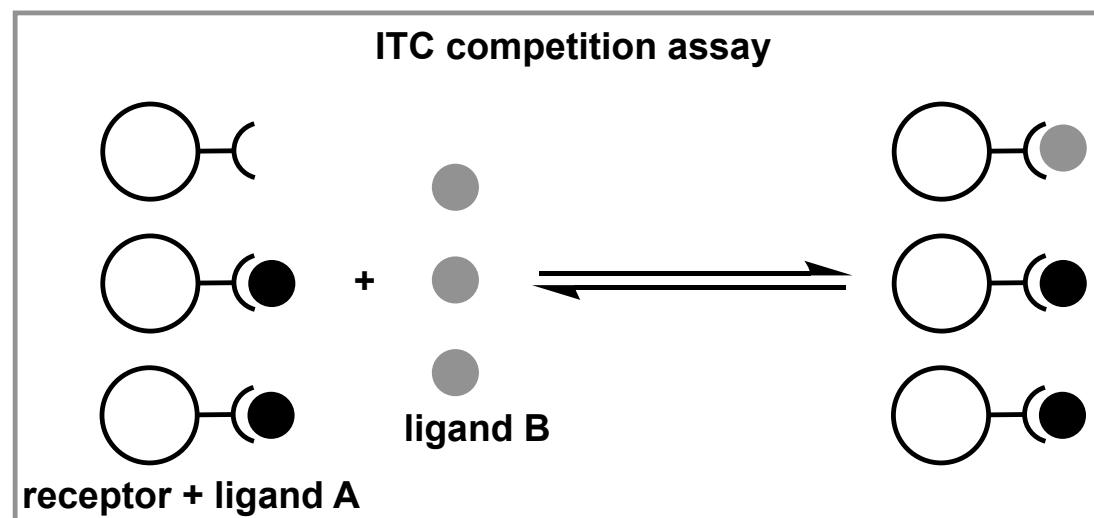
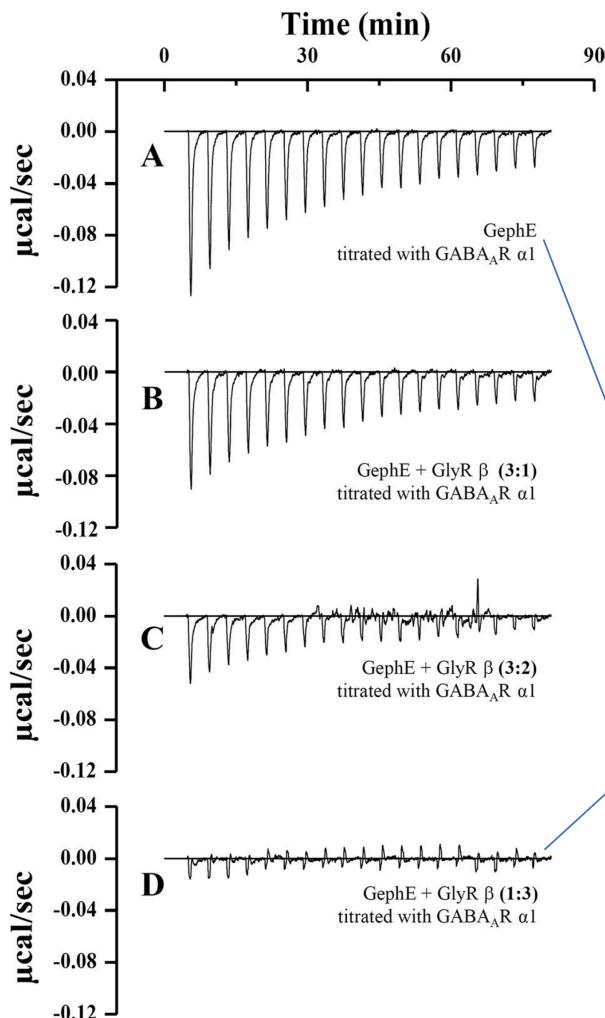


- 1) Verena, T.; Hans, M. M.; Jayanta, M.; Hermann, S.; Werner, S.; Stephan, J. M.; *Front. Cell. Neurosci.* 2012, 6, 23.

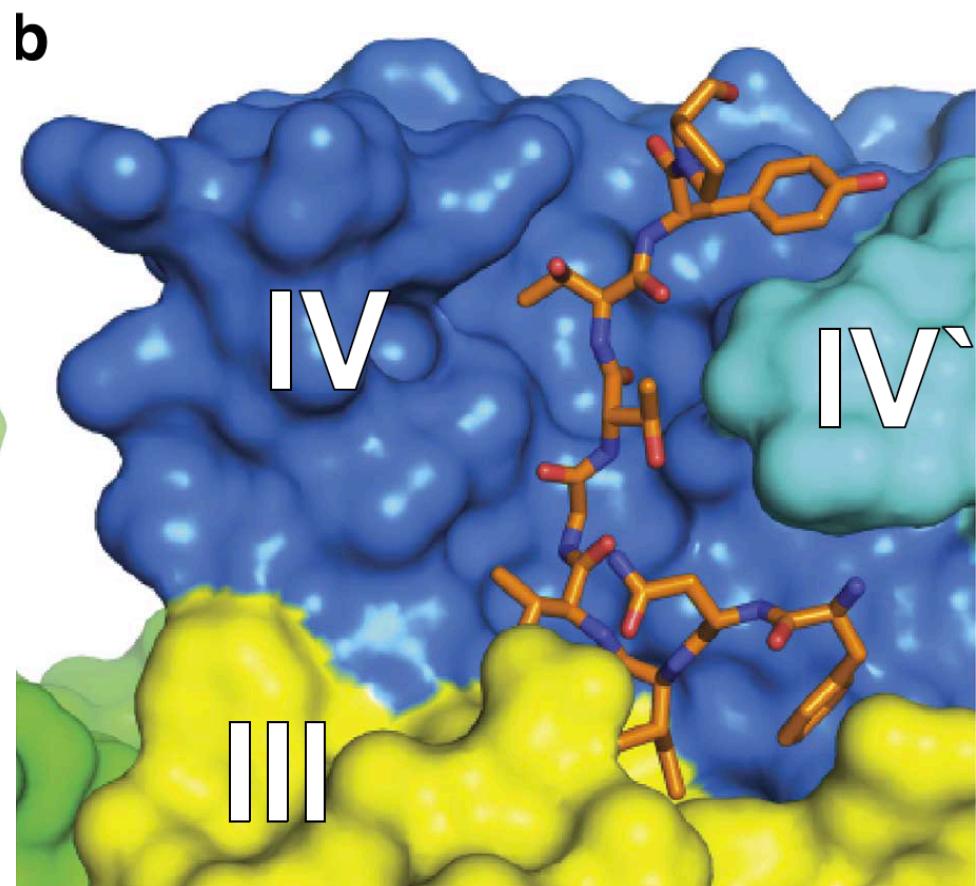
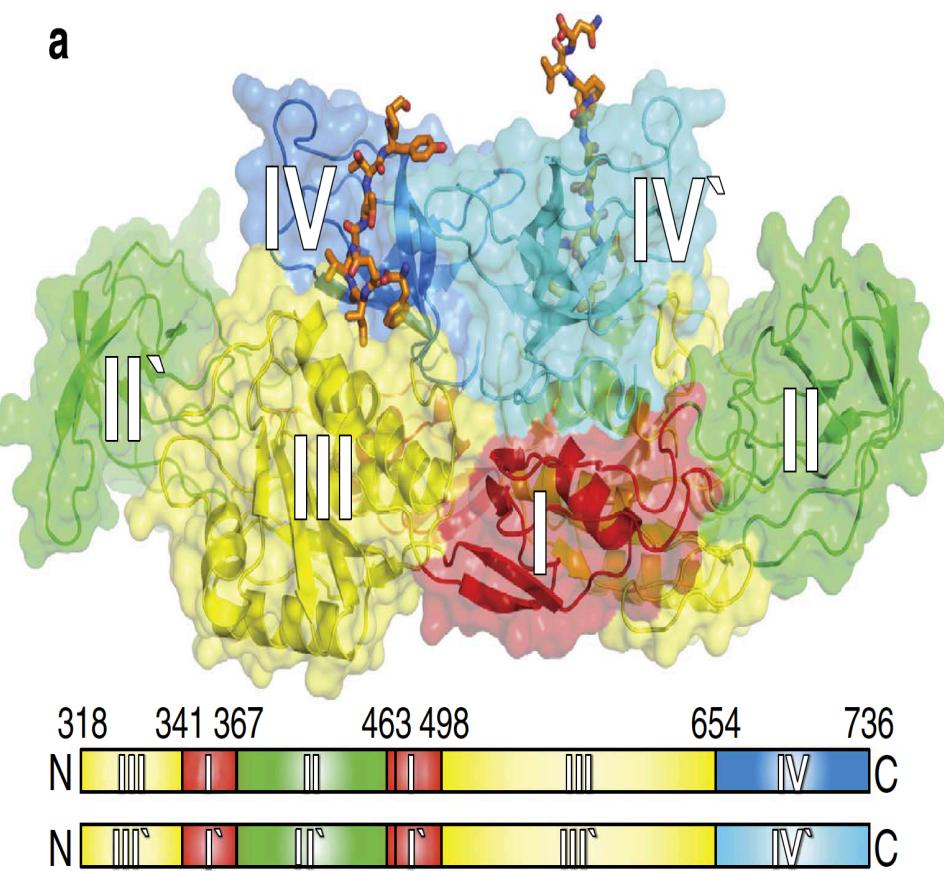
# Isothermal Titration Calorimetry (ITC)



# Common Binding Site of GephE



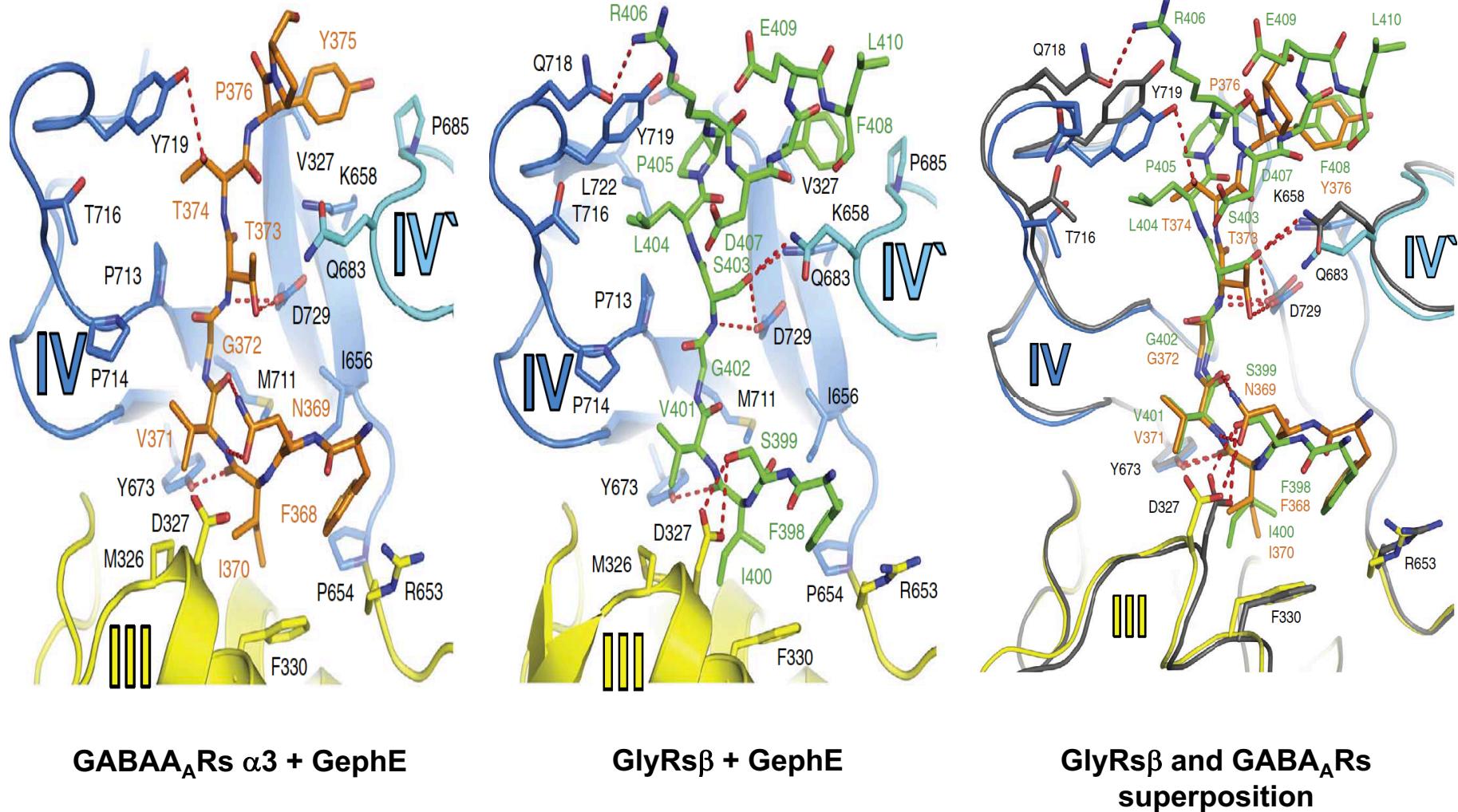
# GephE in Complex with GABA<sub>A</sub>Rs



**Subdomain III, IV, IV' are involved in the interaction with GlyRs and GABA<sub>A</sub>Rs**

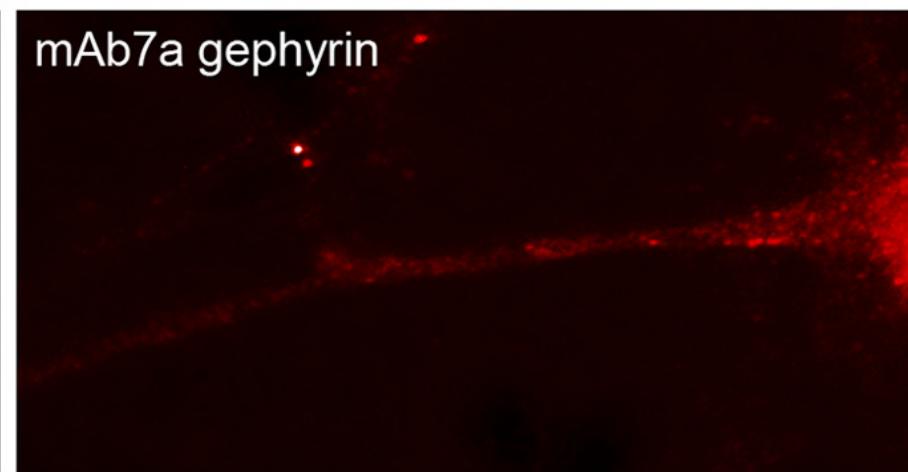
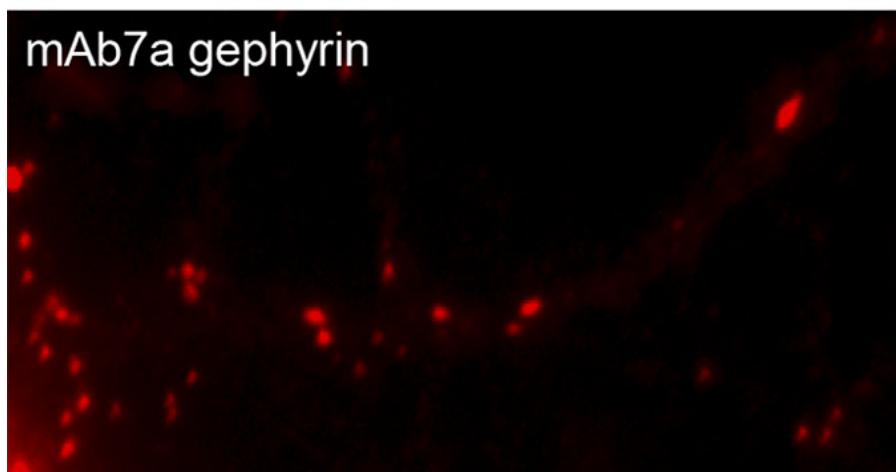
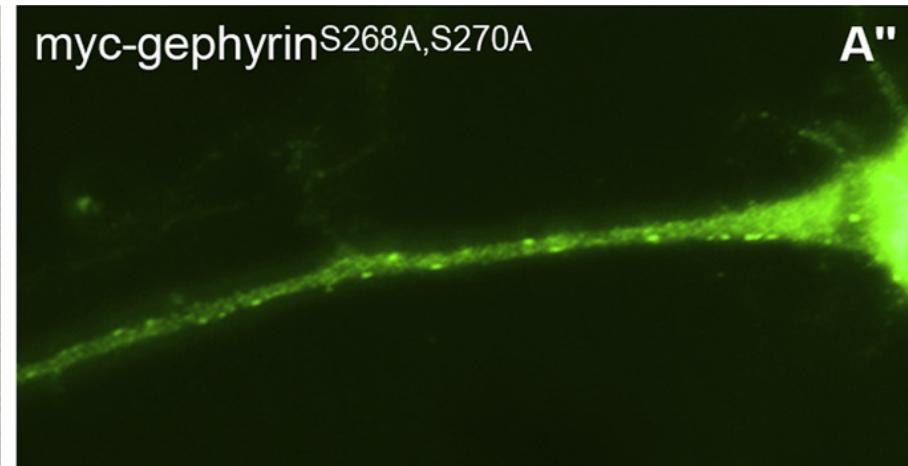
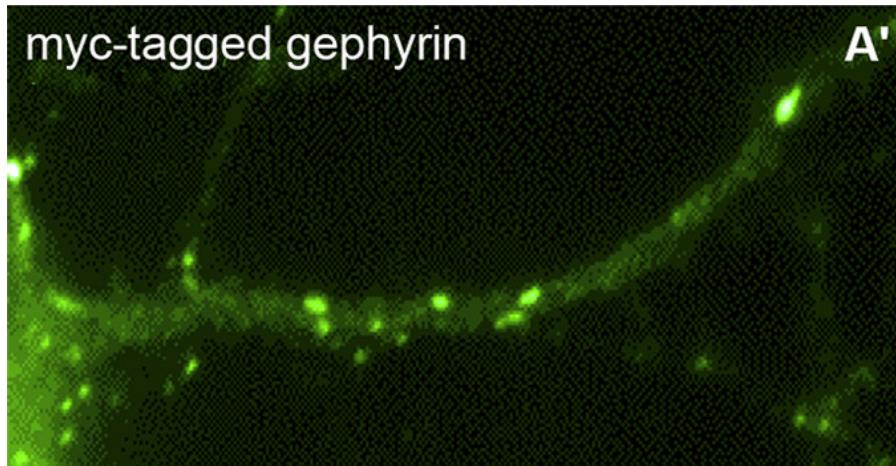
1) Hans, M. M.; Vikram, B. K.; Torben, J. H.; Matthias, K.; Veren, T.; Kristian, S.; Hermann, S. *Nat. Comm.* 2014, 28, 1356-1365.

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1) Hans, M. M.; Vikram, B. K.; Torben, J. H.; Matthias, K.; Veren, T.; Kristian, S.; Hermann, S. *Nat. Comm.* **2014**, 28, 1356-1365.

# Visualizing Gephyrine with Monoclonal Antibody(1)



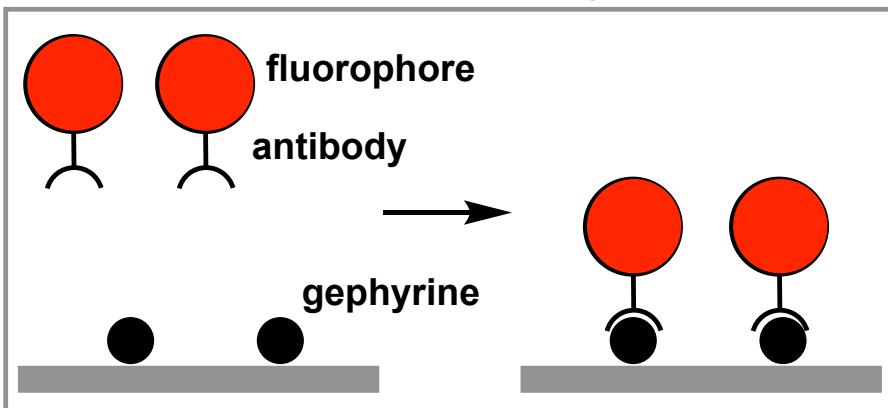
**Signal of mAb7a decreased when S268 and S270 of gephyrine were mutated**

1) Jochen, K.; Heba, K.; Andrea, S.; Susanne, M.; Ralph, N.; Joachim, K. *J. Biol. Chem.* **2012**, 287, 9389-9393.

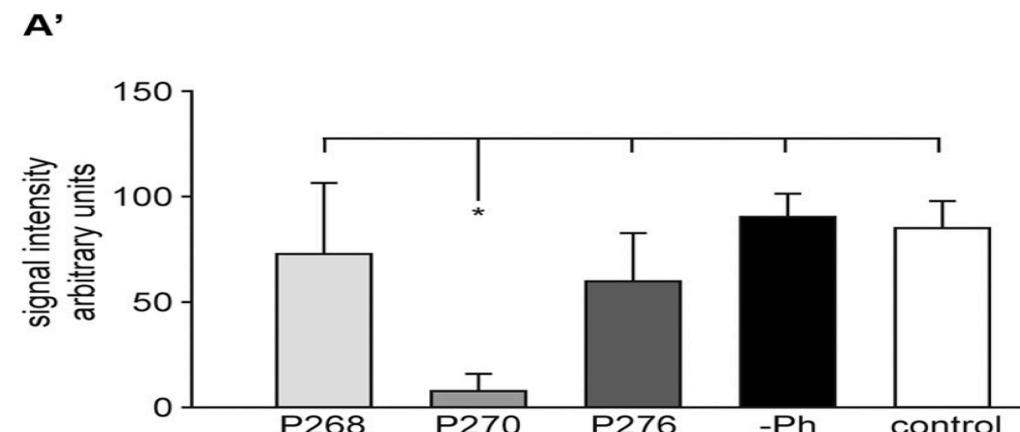
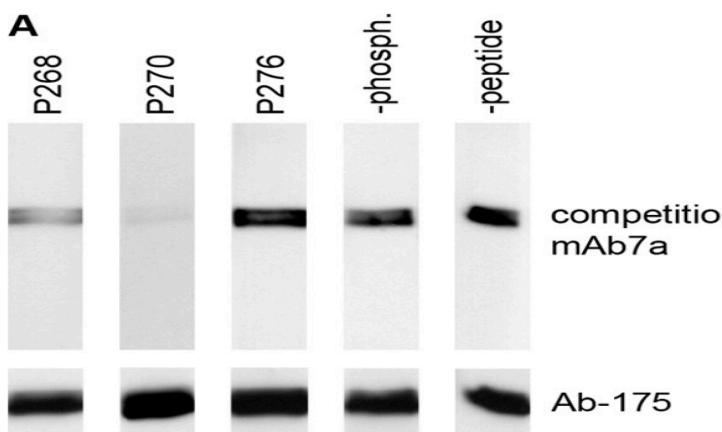
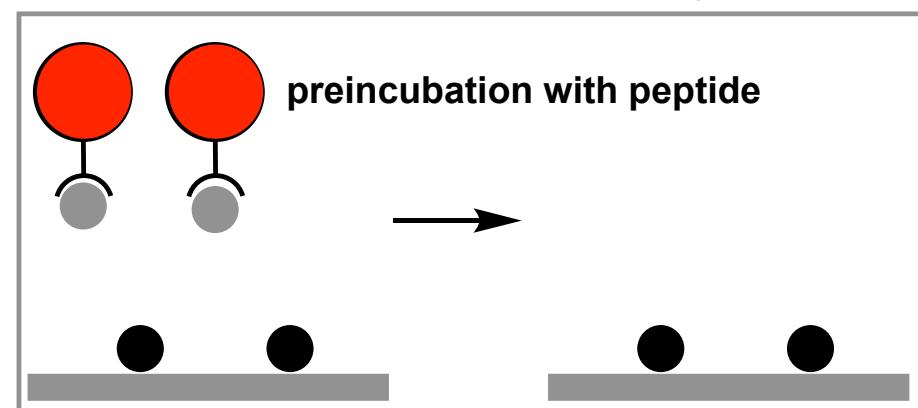
# Visualizing Gephyrine with Monoclonal Antibody (2)

## ■ Pepide competition assay

Immunostaining



Peptide competition assay



- Gephyrine detected with mAb7a is phospholylated at S270
- mAb7a requires phosphorylation at S270 of gephyrine

# **Difficulties in Visualizing Gephyrine**

## **■ Limitation of immunocytochemistry**

- **Membrane permeability** is a major obstacle
  - Staining of cytoplasmic proteins requires fixed cells
  - Experiment that uses living cell is precluded
- These limitations are overcomed with the cloning of the gene encoding GFP

## **■ Limitation of GFP**

- Introduced GFP-fusion proteins may fail to localize properly, due to saturation of targetting machinery
- Overexpression of proteins can have dramatic morphological and functinal effect on cells

1) Garret, G. G.; Jason, A. J.; Rudy, J. M.; Hyung, B. K.; Anders, C. O.; Terry, T. T.; Emily, R. L.; Graham, C. R.; Aaron, W. M.; Bernardo, L. S.; Richard, W. R.; Don, B. A. *Neuron*. 2013, 78, 971-985.

# **Difficulties in Visualizing Gephyrine**

## **■ Circumvention of limitation of GFP**

- Tagged protein is introduced into a knockout background**
- GFP is introduced into the locus of the endogenous gene**

## **■ Remained problems of using GFP**

- Two or more proteins in the same cell cannot be labelled**
- It is difficult to confine the expression of the tagged proteins to a genetically defined subset of cells**
- They do not allow any analysis of either posttranslational modifications or specific protein conformations**

1) Garret, G. G.; Jason, A. J.; Rudy, J. M.; Hyung, B. K.; Anders, C. O.; Terry, T. T.; Emily, R. L.; Graham, C. R.; Aaron, W. M.; Bernardo, L. S.; Richard, W. R.; Don, B. A. *Neuron*. 2013, 78, 971-985.

# **Short Summary**

## **■ Gephyrine**

- **Scaffold protein of GlyRs and GABA<sub>A</sub>Rs**
- **Dimerization of E-domain, trimerization of G-domain**
- **Common binding site for GlyRs and GABA<sub>A</sub>Rs subunit**
- **New therapeutic target in modulation of fast synaptic inhibition**

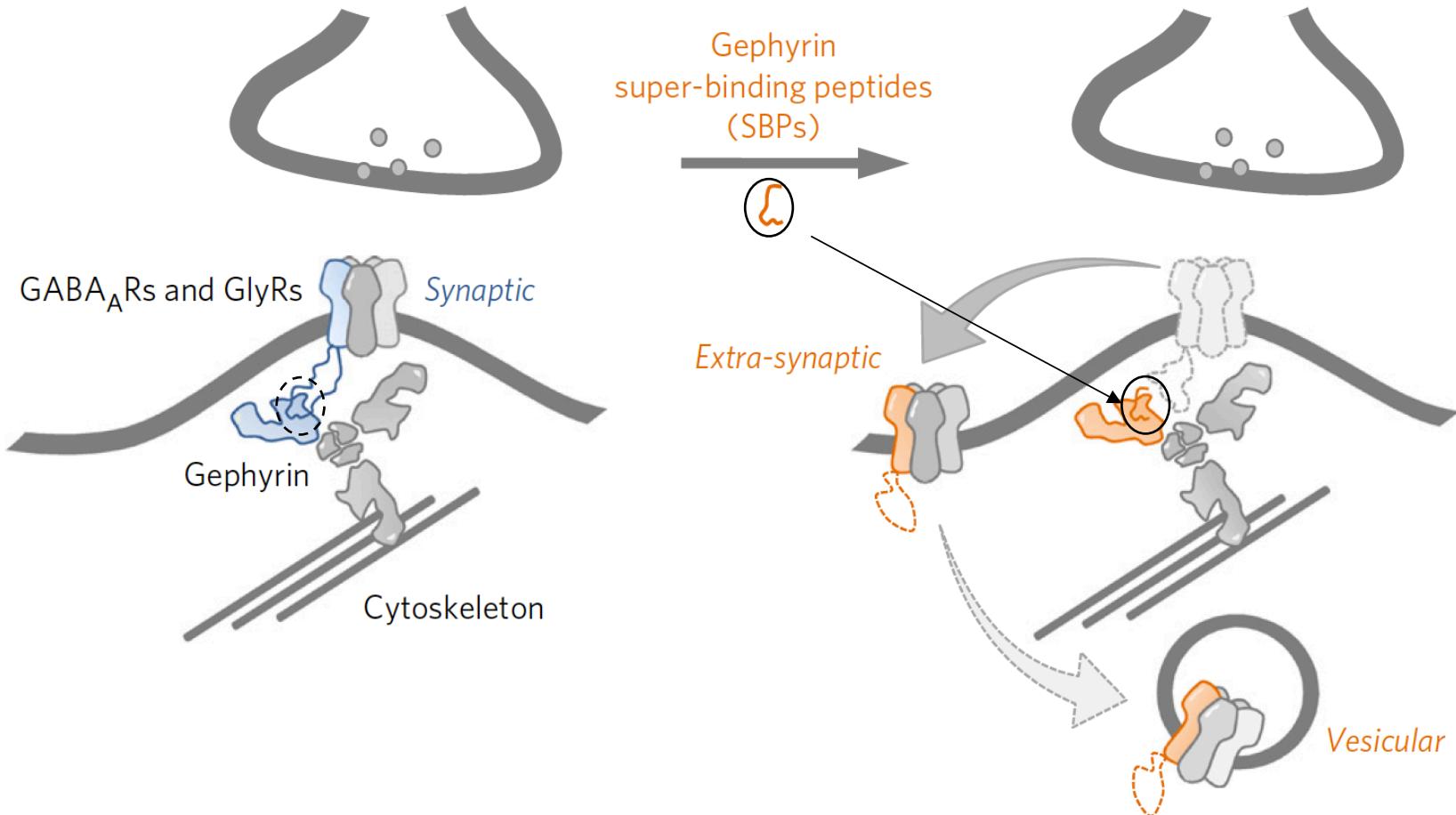
## **■ Visualization**

- **Difficulties in visualization with antibody**
- **Necessity of selective, high-affinity markers for visualization of gephyrine**

# **Outline**

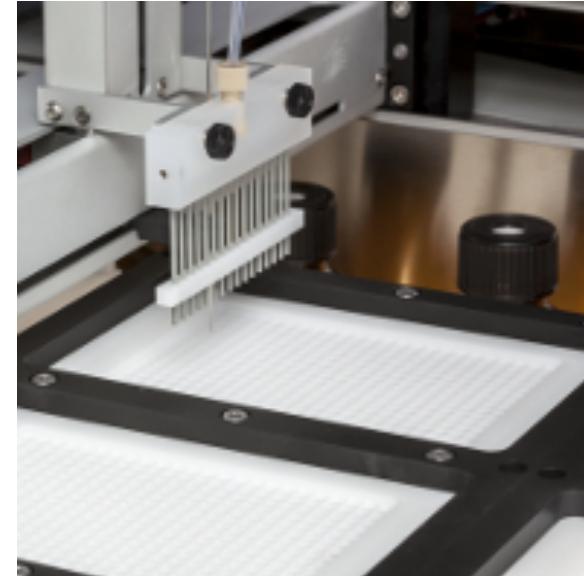
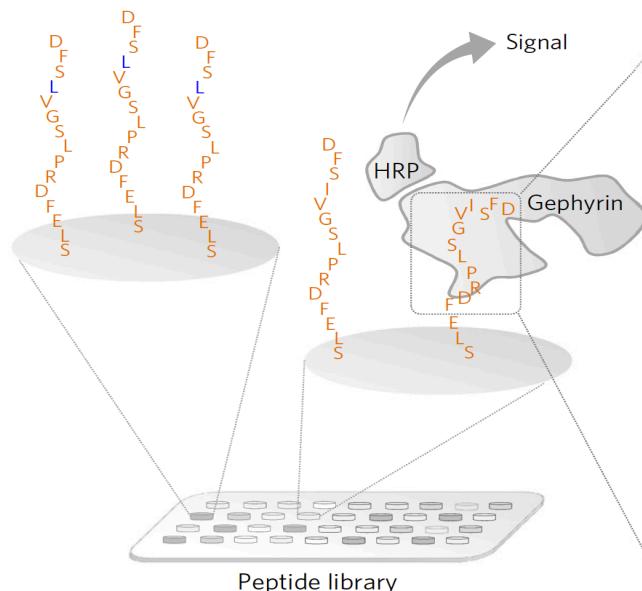
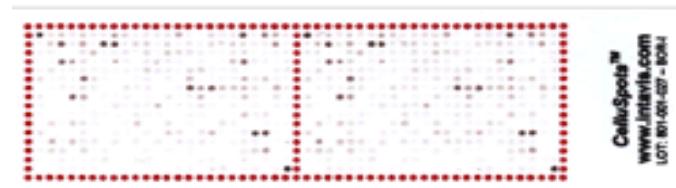
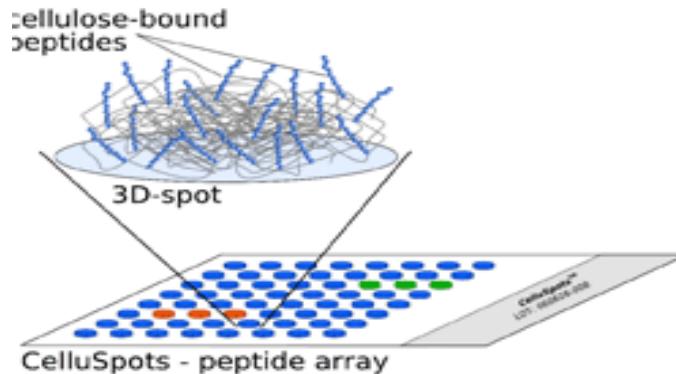
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# Author's Working Hypothesis



- SPBs occupy the binding site of GABA<sub>A</sub>Rs and GlyRs
- SPBs modulate clustering of GABA<sub>A</sub>Rs and GlyRs

# Screening SBPs by $\mu$ SPOT (1)



CelluSpots

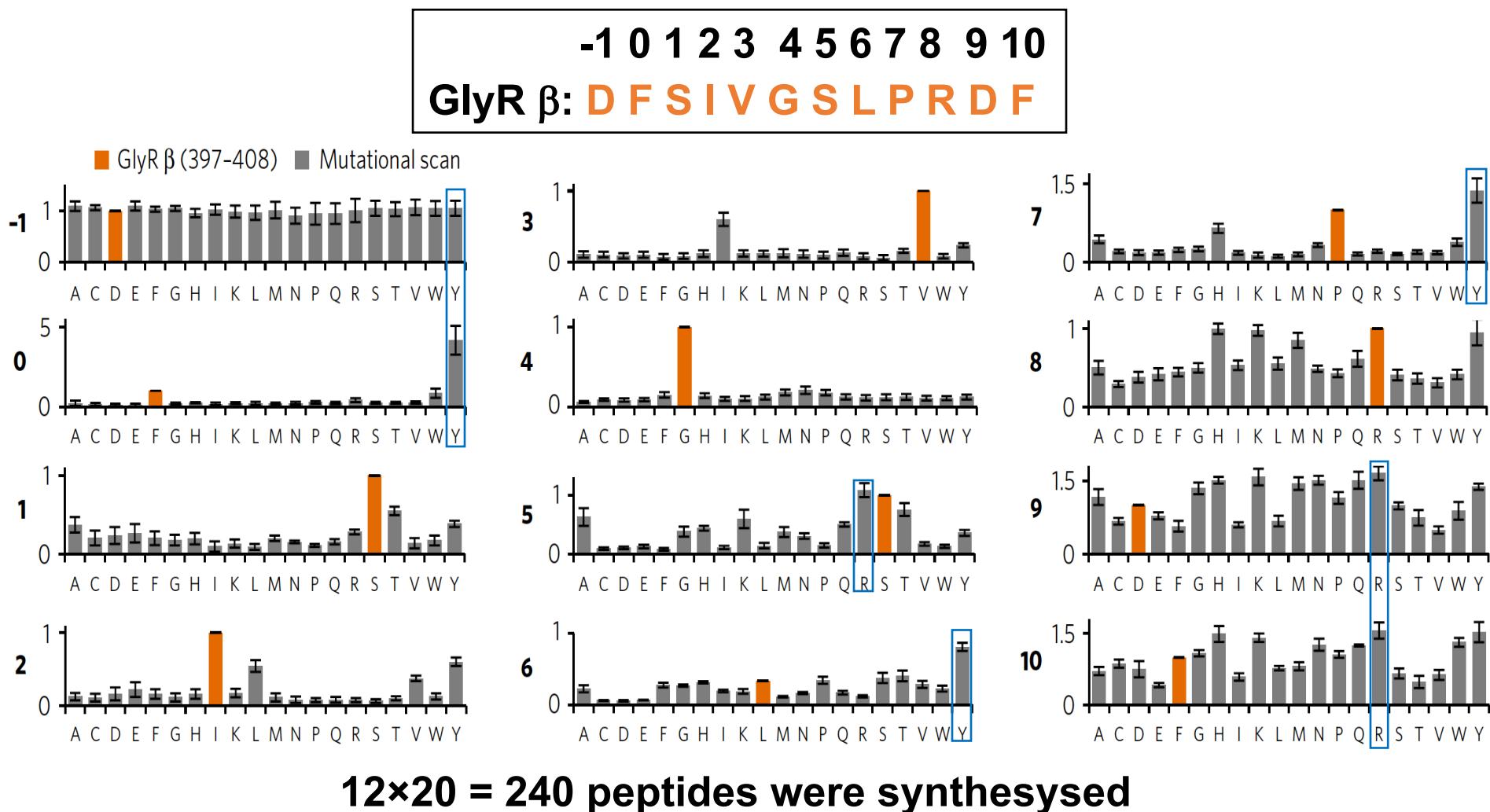
## ■ Similarity to SPPS on beads

One cycle of amino acid addition

1. Spotting of activated Fmoc-amino acid derivative
2. Wash
3. Fmoc-cleavage

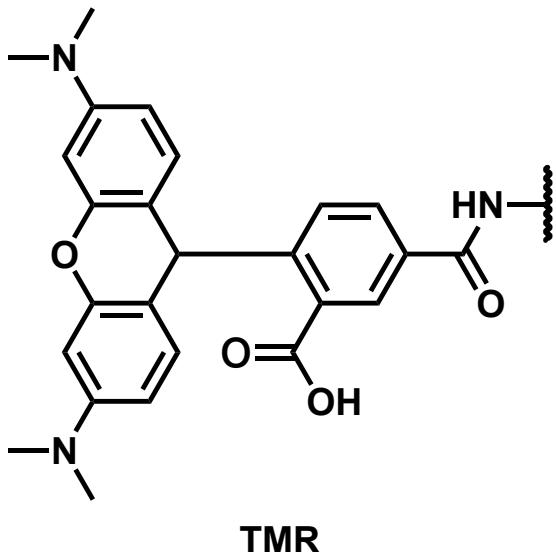
HRP: Horseradish Peroxidase

# Screening SBPs by $\mu$ SPOT (2)



**12×20 = 240 peptides were synthesised**

# Developing SBPs by ITC (1)



**Arg<sub>8</sub>: cell penetrating moiety**

Cpd.	AA Sequence	K <sub>D</sub> [μM]
	-1 0 1 2 3 4 5 6 7	
<b>2a</b> 8	F S I V G S L P R D F E	8.3±0.1
<b>2b</b> 8	F S I V G S L Y R D F E	4.7±0.8
<b>2c</b> 8	F S I V G S L P R R R R	3.0±0.2
<b>2d</b> 8	F S I V G S Y Y R R R R	n.s.
<b>2e</b> 8	D F S I V G S L P R D F E	74±11
<b>2f</b> 8	Y Y S I V G R Y Y R R R R	1.9±0.2
<b>2g</b> 9	F S I V G R Y P R R R R	0.14±0.04
<b>2h</b> 9	F S I V R G Y P R R R R	> 500
<b>2i</b>	F S I V G R Y P R R R R R R R R R R	0.5±0.4
<b>TMR-2i</b>	<b>TMR-F S I V G R Y P R R R R R R R R</b>	0.3±0.1
<b>2j</b>	F S I V R G Y P R R R R R R R R R R	> 500
<b>3a</b>	F S I V G R Y P R R R C*	0.18x10 <sup>-3</sup> ±0.1x10 <sup>-3</sup>
<b>3b</b>	F S I V G S L P R D F E C*	25x10 <sup>-3</sup> ±3x10 <sup>-3</sup>
<b>3c</b>	F N I V G T T Y P I N L	183±38

**2a: GlyR-derived fragment**

**3c: GABA<sub>A</sub>R-derived fragment**

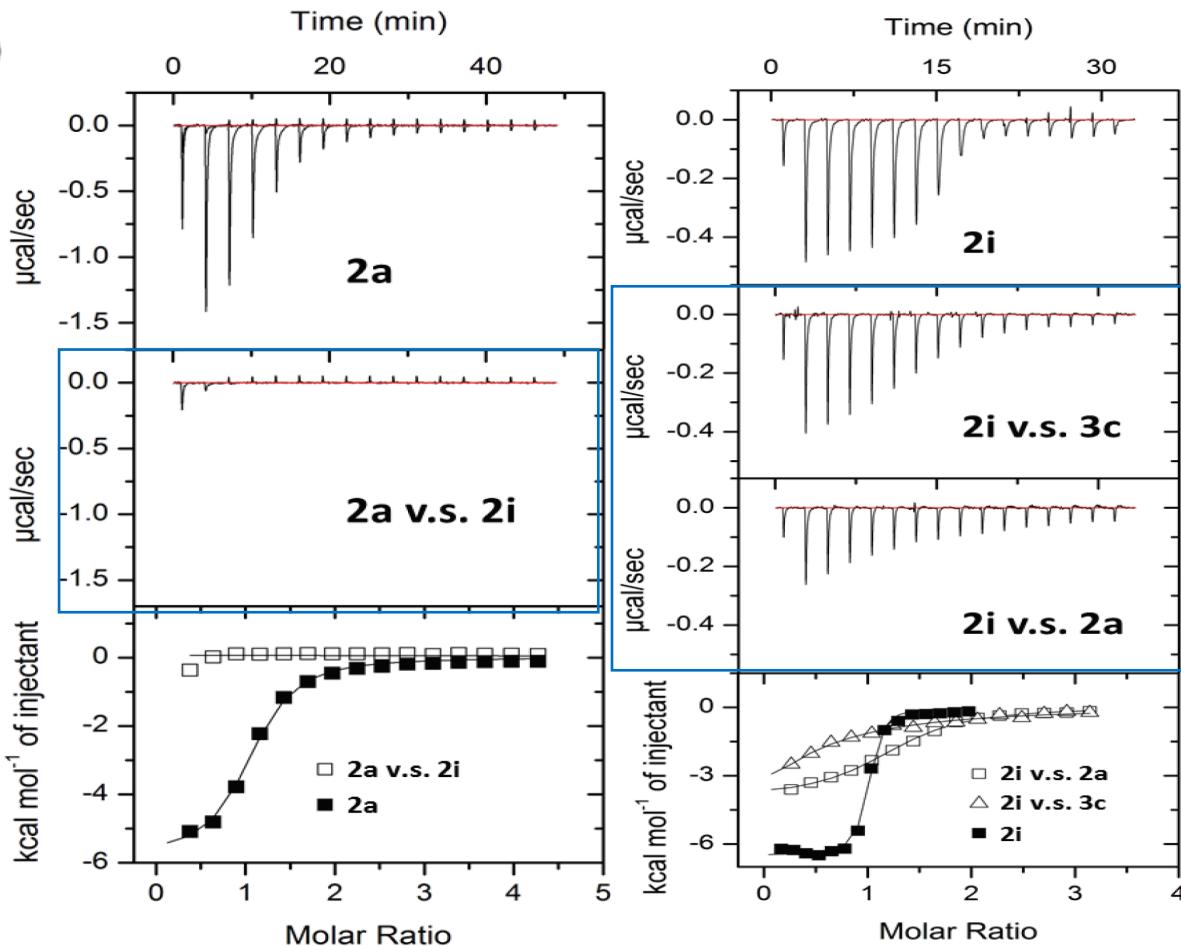
**2f: optimized peptide by μSPOT**

**2g: optimized peptide by ITC**

**TMR-2i: optimized peptide with a fluorophore and cell-penetrating moiety**

# Developing SBPs by ITC (2)

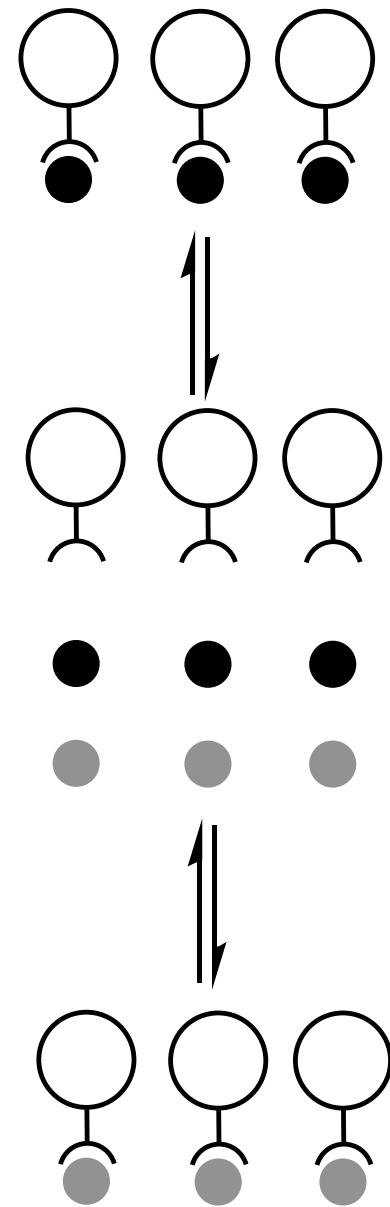
## ITC competition assay



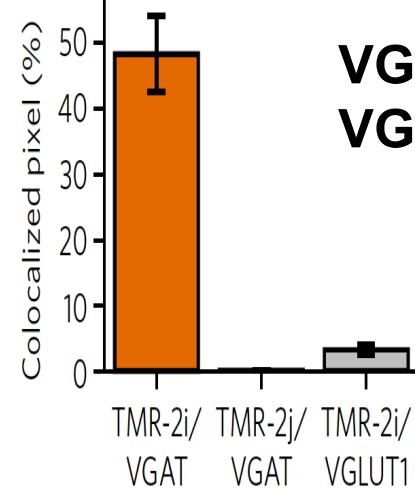
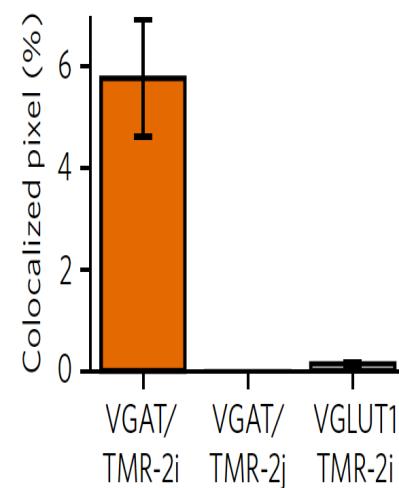
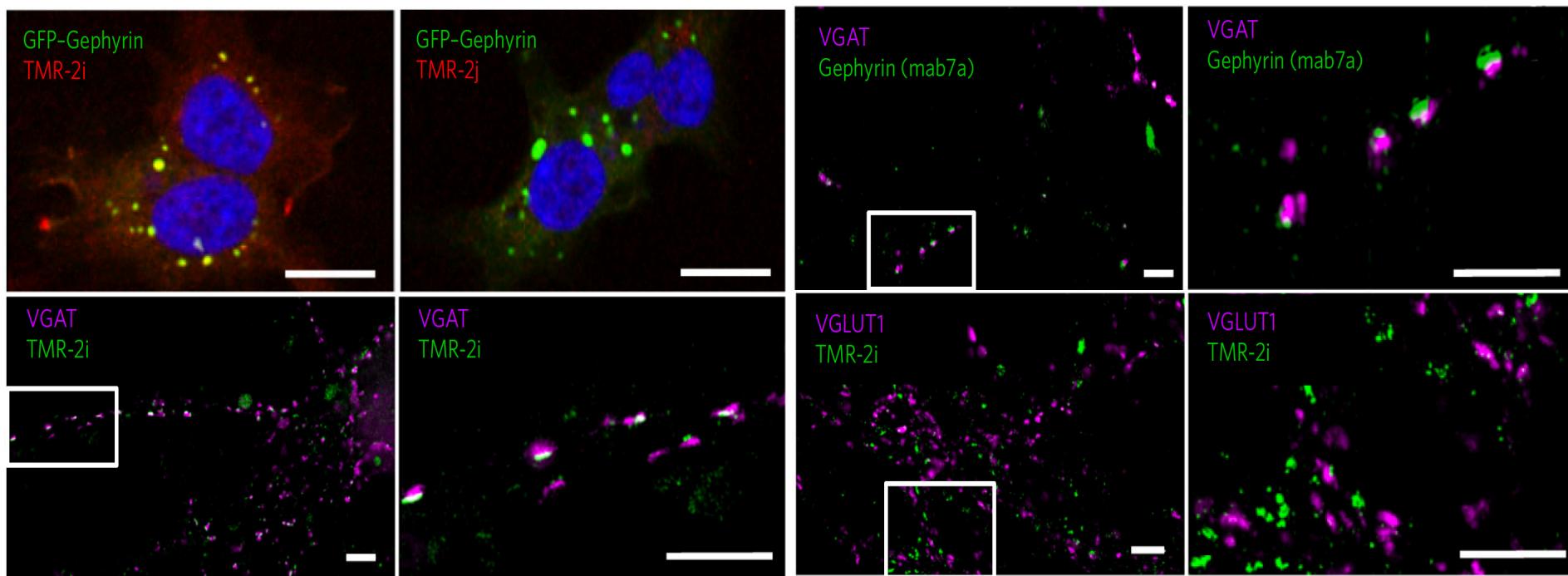
**2a:** GlyR-derived fragment

**3c:** GABA<sub>A</sub>R-derived fragment

**2i:** optimized peptide by  $\mu\text{SPOT}$



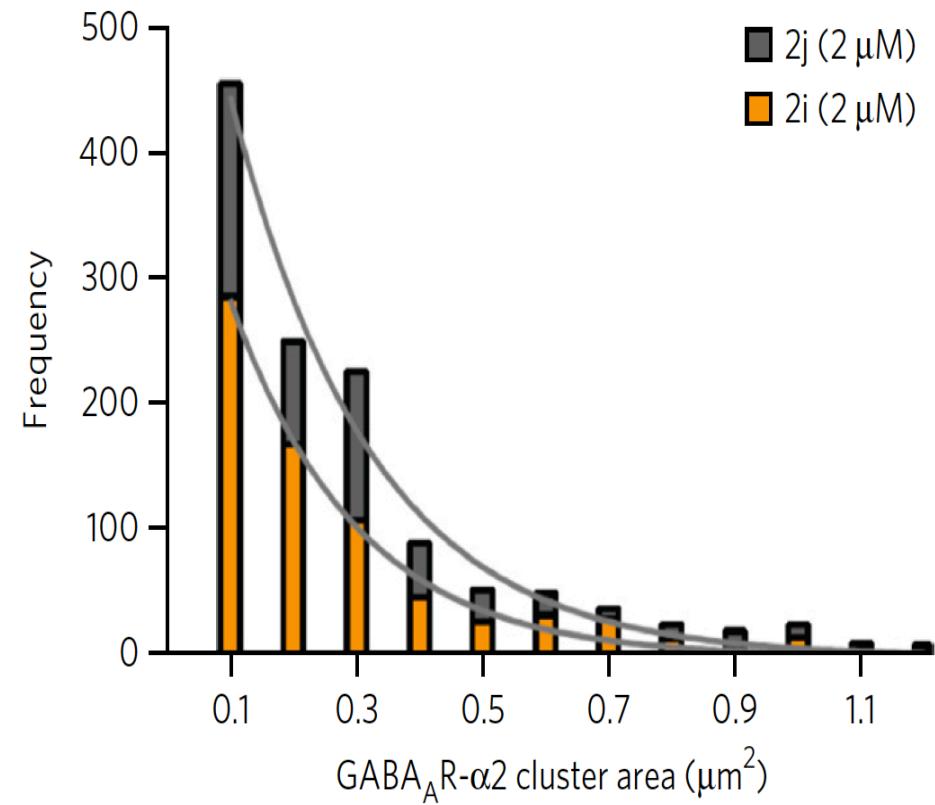
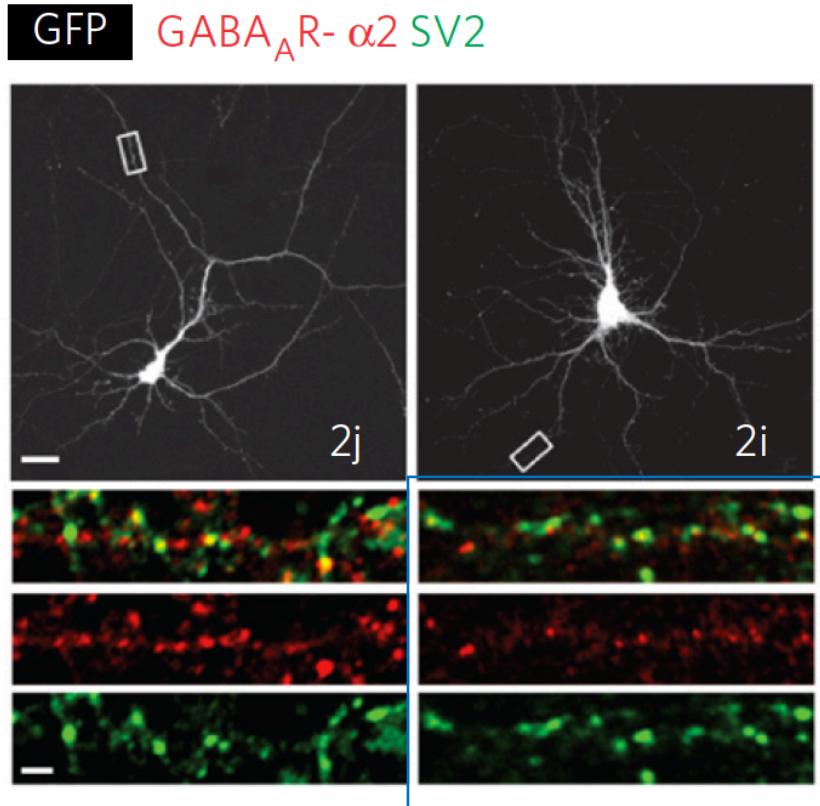
# Visualization of Gephyrine by SBPs



**VGAT: Vesicular GABA Transporter**  
**VGLUT1: Vesicular Glutamate Transporter 1**

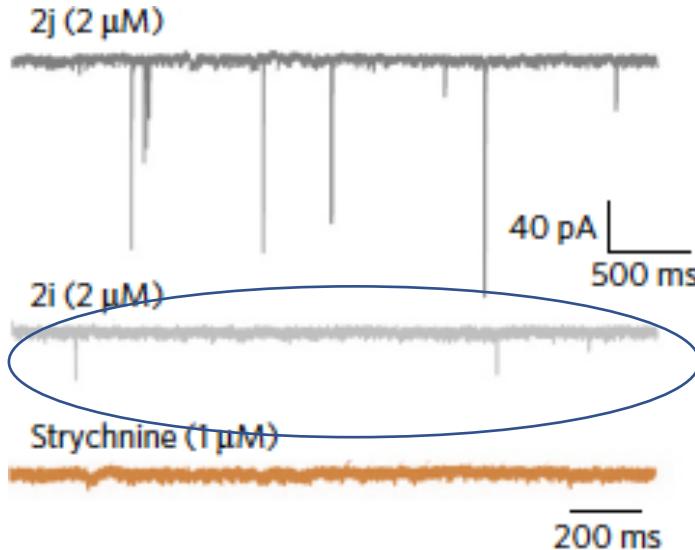
**TMR-2i labels GABAergic synapses selectively**

# Modulation of fast synaptic inhibition by SPBs (1)

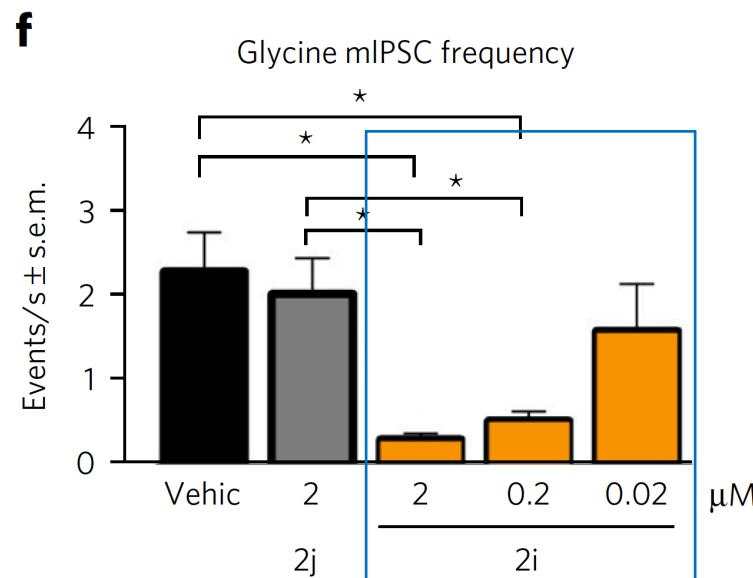
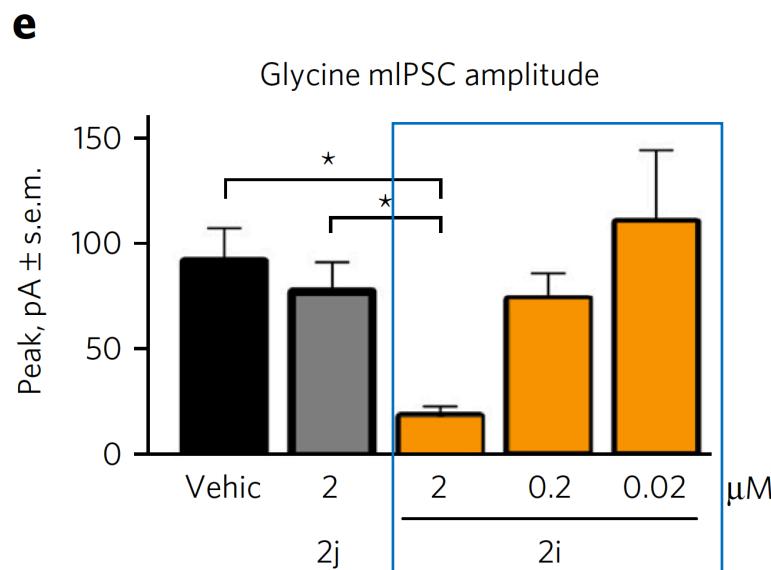


2i inhibited clustering of  $\text{GABA}_A\text{Rs}$

# Modulation of fast synaptic inhibition by SPBs (2)



- Inhibition of GlyRs-mediated inhibitory postsynaptic currents (mIPSCs)
- Dose-dependent effect of 2i on the amplitude and frequency of mIPSCs



# Summary

## ■ Summary of main paper

- SBPs was identified by  $\mu$ SPOT and ITC
- SBPs could be a novel tool for modulation of inhibitory-fast postsynaptic transmission and labeling of gephyrine

