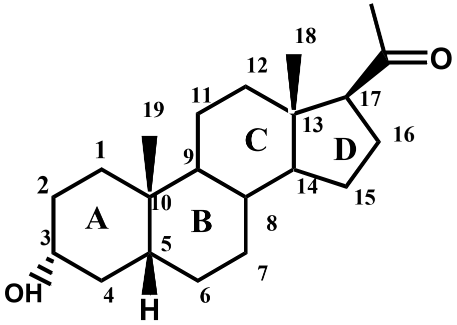
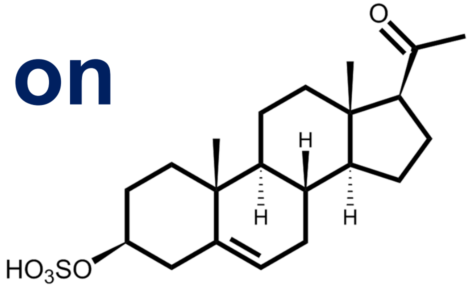


Neurosteroid Binding Sites and Actions on $GABA_A$ Receptors



International Society of Anesthetic Pharmacology
New Orleans, Louisiana
October 21, 2022

Alex S. Evers, M.D.

Henry E. Mallinckrodt Professor of Anesthesiology
Professor of Developmental Biology (Pharmacology) and Internal Medicine
Washington University in St. Louis

Disclosures: I receive research and educational funding from the:
National Institutes of Health:(R01GM108799, P50MH122379, T32GM108539)
The Taylor Family Foundation for Innovative Psychiatry

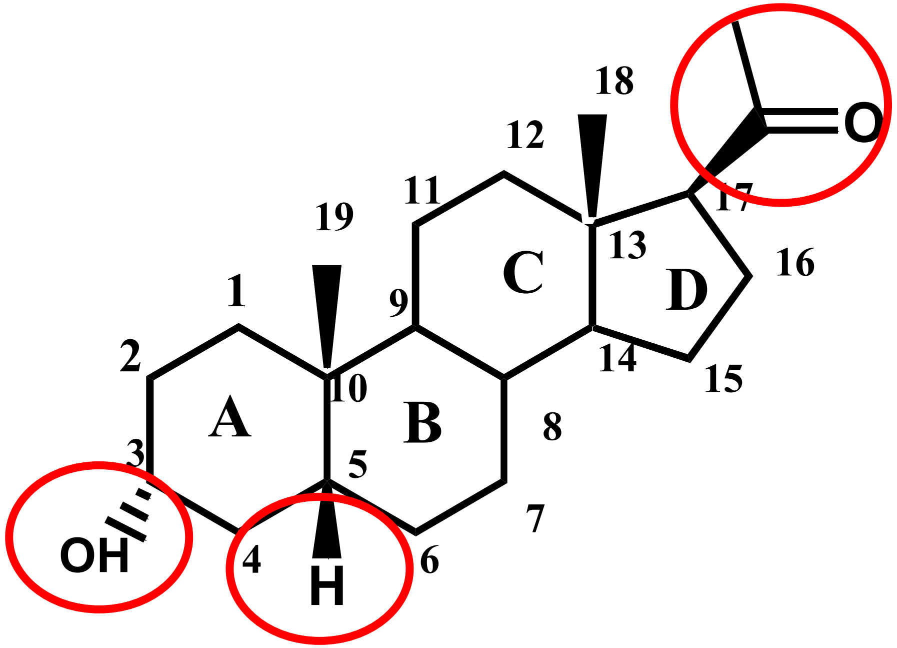
What are neurosteroids?

Neurosteroids are steroids synthesized within the brain that modulate neuronal excitability by rapid non-genomic actions

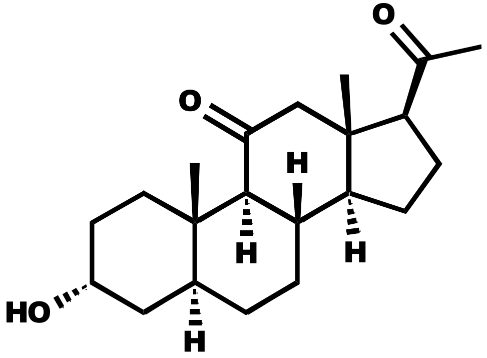
Endogenous steroids that modulate CNS behavior

- *Mood*, neuroprotection, excitability

Therapeutically used as *i.v.* *anesthetics*, anti-depressants and anti-epileptics


18
12
11
19
13
17
16
C
D
1
9
2
10
A
B
14
15
8
3
5
OH
4
H
6
7
O

Clinically used steroid anesthetics



Alphaxolone

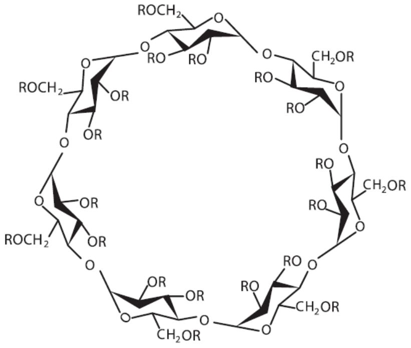
1971-1985

- **Althesin**- Alphaxolone/Alphadolone in Cremophor EL
- The most widely used i.v. anesthetic in Europe/Canada
- Minimal adverse effects on CV or respiratory
- Histamine reactions/anaphylaxis due to Cremophor
- Withdrawn from Canada and Europe in early 1980’s

Therapeutic indices

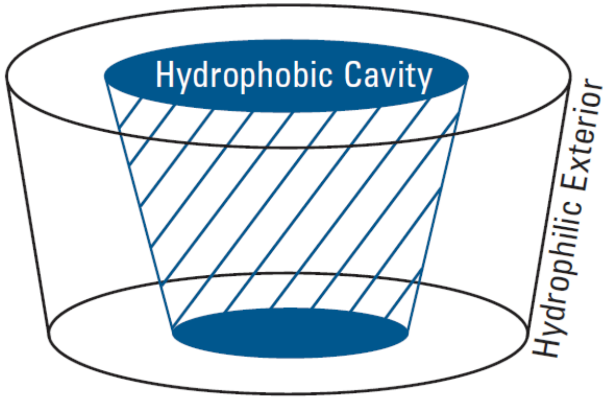
	Mice	Rats
Hydroxydione	17.3	-----
Althesin	30.6	28.7
Pregnanolone	-----	40.0
Thiopental	6.9	4.6
Propofol	-----	5.1
Midazolam	-----	6.8

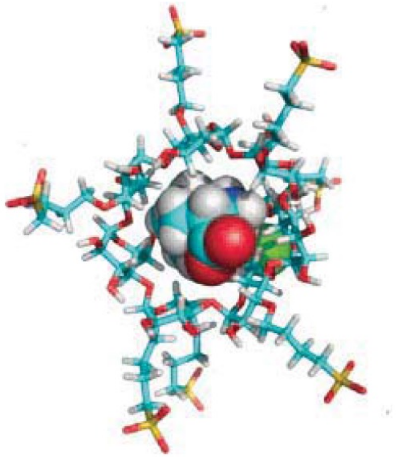
Alphaxalone Reformulated: A Water-Soluble Intravenous Anesthetic Preparation in Sulfobutyl-Ether-β-Cyclodextrin



$R=(H)_{21-n}$ or $(CH_2CH_2CH_2CH_2SO_2ONa)_n$
where n=6.2 to 6.9

7-sulfobutyl-ether-β-cyclodextrin





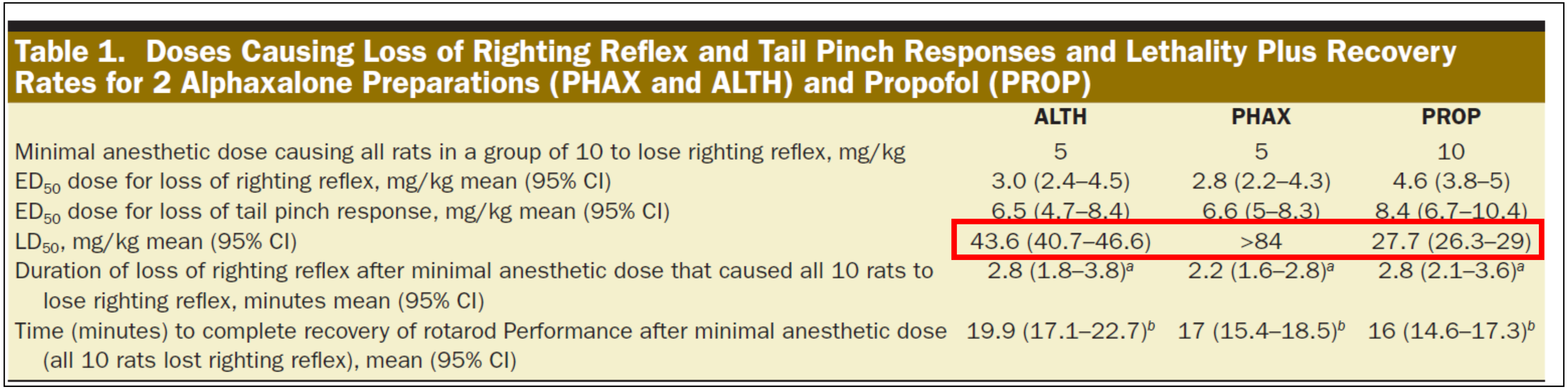


Table 1. Doses Causing Loss of Righting Reflex and Tail Pinch Responses and Lethality Plus Recovery Rates for 2 Alphaxalone Preparations (PHAX and ALTH) and Propofol (PROP)

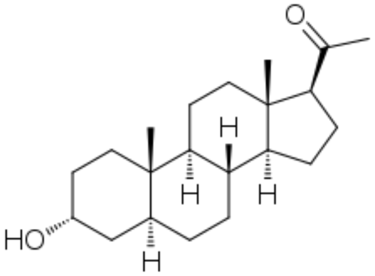
	ALTH	PHAX	PROP
Minimal anesthetic dose causing all rats in a group of 10 to lose righting reflex, mg/kg	5	5	10
ED_{50} dose for loss of righting reflex, mg/kg mean (95% CI)	3.0 (2.4–4.5)	2.8 (2.2–4.3)	4.6 (3.8–5)
ED_{50} dose for loss of tail pinch response, mg/kg mean (95% CI)	6.5 (4.7–8.4)	6.6 (5–8.3)	8.4 (6.7–10.4)
LD_{50}, mg/kg mean (95% CI)	43.6 (40.7–46.6)	>84	27.7 (26.3–29)
Duration of loss of righting reflex after minimal anesthetic dose that caused all 10 rats to lose righting reflex, minutes mean (95% CI)	2.8 (1.8–3.8)[a]	2.2 (1.6–2.8)[a]	2.8 (2.1–3.6)[a]
Time (minutes) to complete recovery of rotarod Performance after minimal anesthetic dose (all 10 rats lost righting reflex), mean (95% CI)	19.9 (17.1–22.7)[b]	17 (15.4–18.5)[b]	16 (14.6–17.3)[b]

Anesth Analg. 2015 120(5):1025-31.

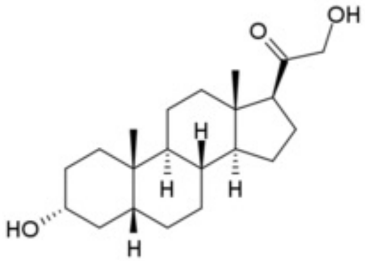
Neurosteroid (NS) actions on GABA_AR

- ✓ Neurosteroids are endogenous modulators of neuronal excitability and nervous system development
- ✓ GABA_ARs are the primary molecular targets of neurosteroid action
- ✓ **Neurosteroid enhancement of GABA_A currents is the primary mechanism of anesthetic action**

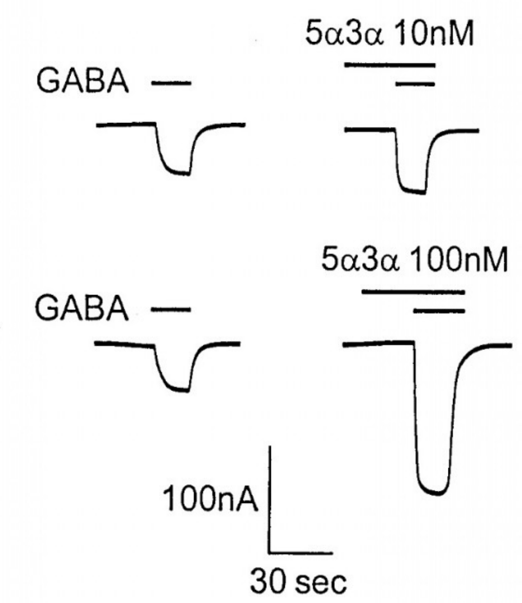
- ✓ **Positive allosteric modulators (PAMs)**:
  Allopregnanolone
  Tetrahydrodeoxycorticosterone (THDOC)



Allopregnanolone



THDOC



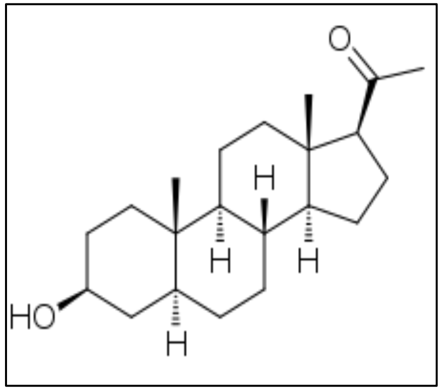
Neil Harrison, Mike Simmonds, Jeff Barker

Neurosteroid (NS) actions on GABA$_A$R

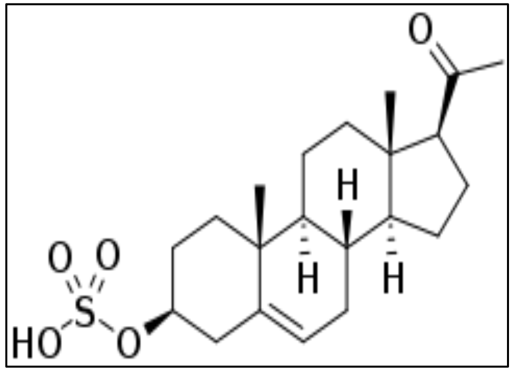
- ✓ Neurosteroids are endogenous modulators of neuronal excitability and nervous system development
- ✓ GABA$_A$Rs are the primary molecular targets of neurosteroid action
- ✓ **Neurosteroid enhancement of GABAA currents is the primary mechanism of anesthetic action**

✓ Negative allosteric modulators (NAMs):

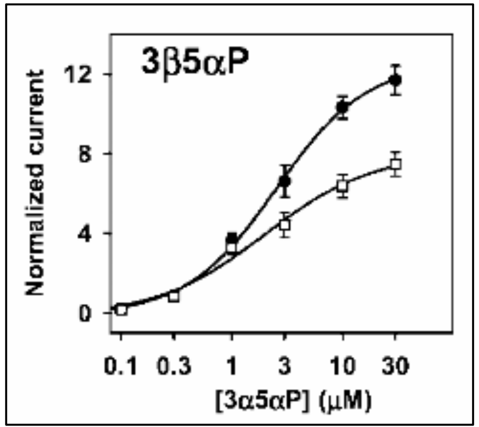
Pregnenolone sulfate (PS)
Epi-Allopregnanolone



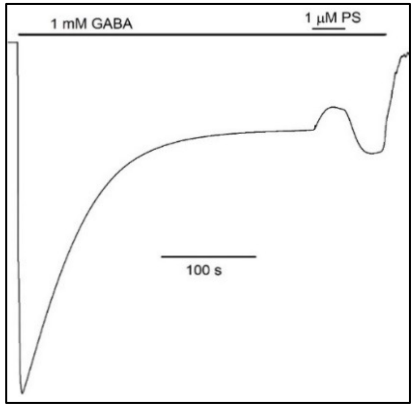
Epi-Allopregnanolone



Pregnenolone sulfate

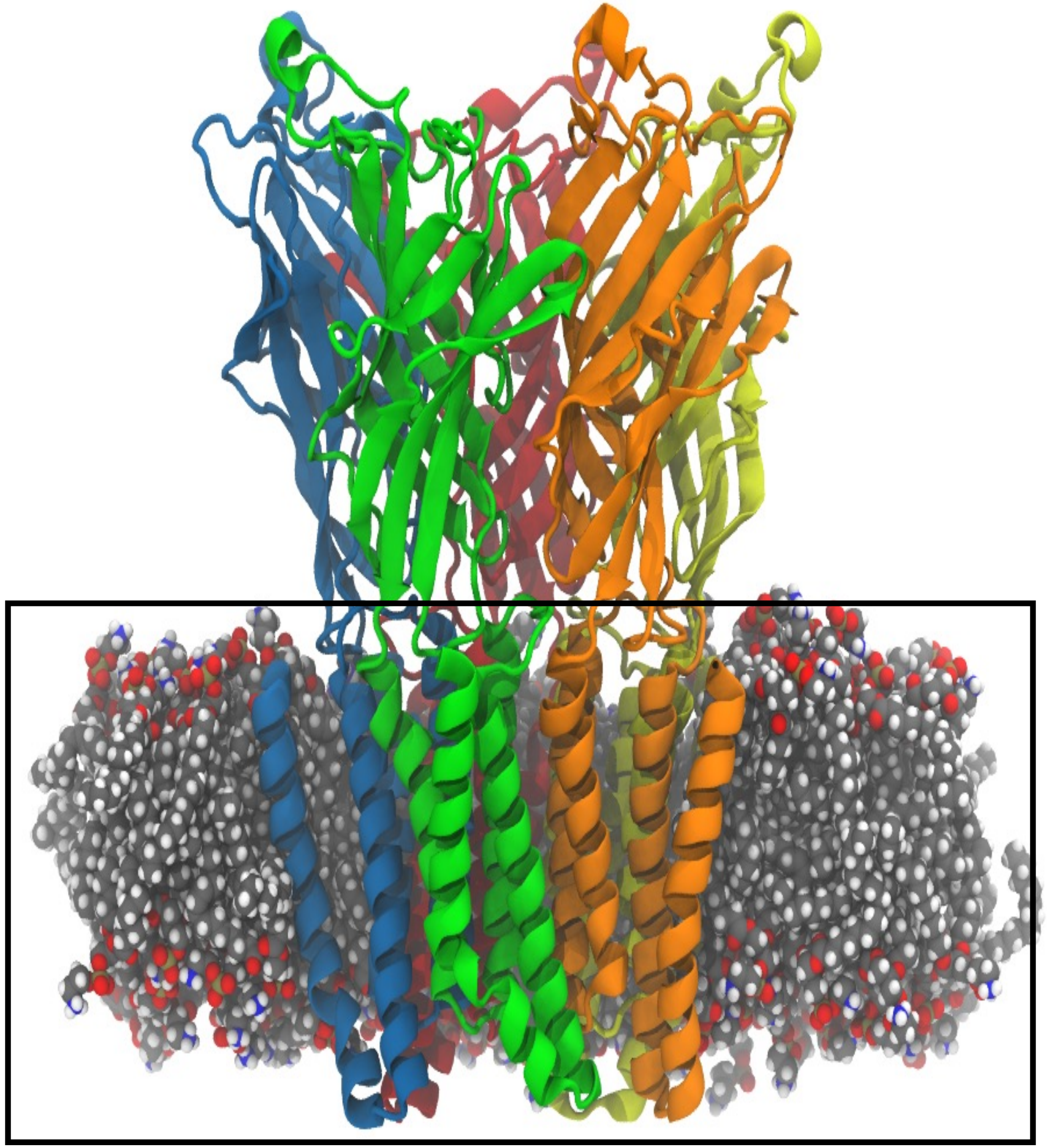


Wang M et. al. (2002) J Neurosci.

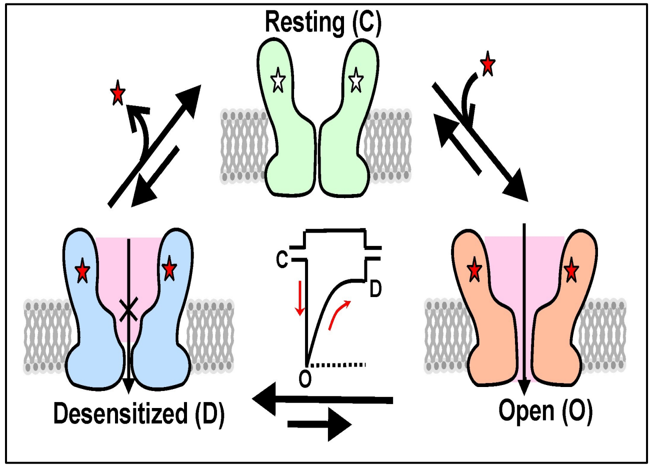


Akk, G

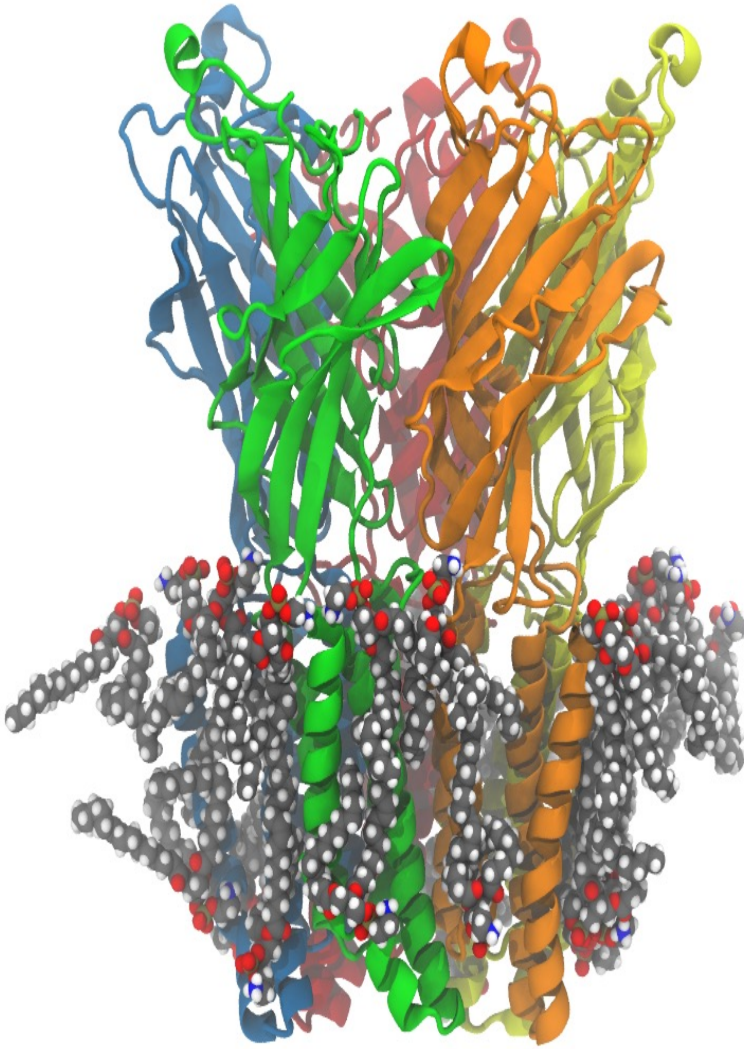
Pentameric Ligand-gated ion Channels: Targets of Anesthetic Action

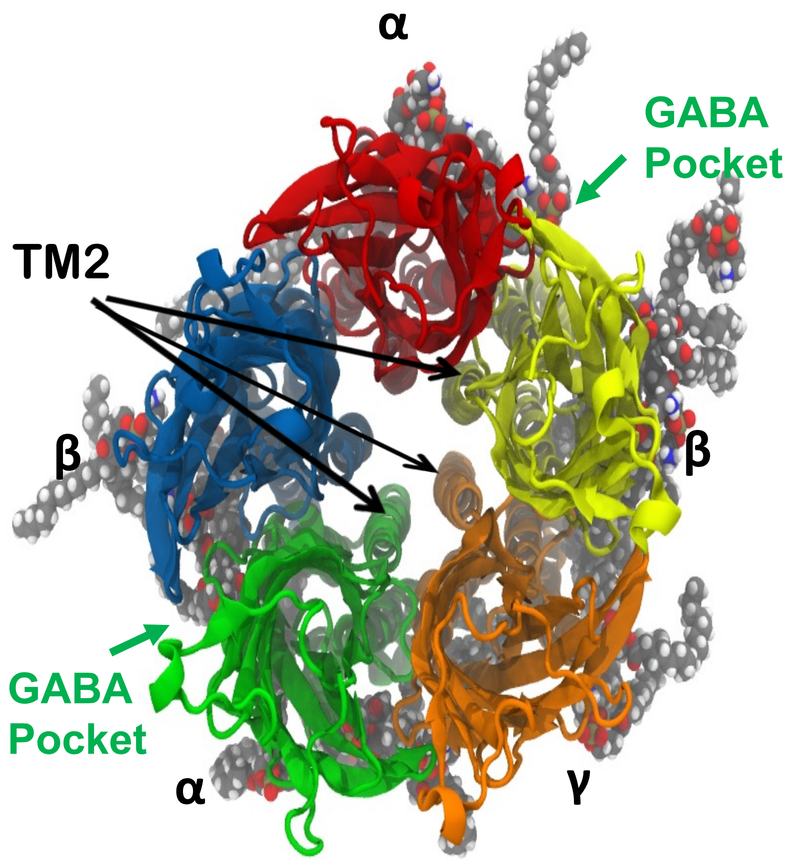


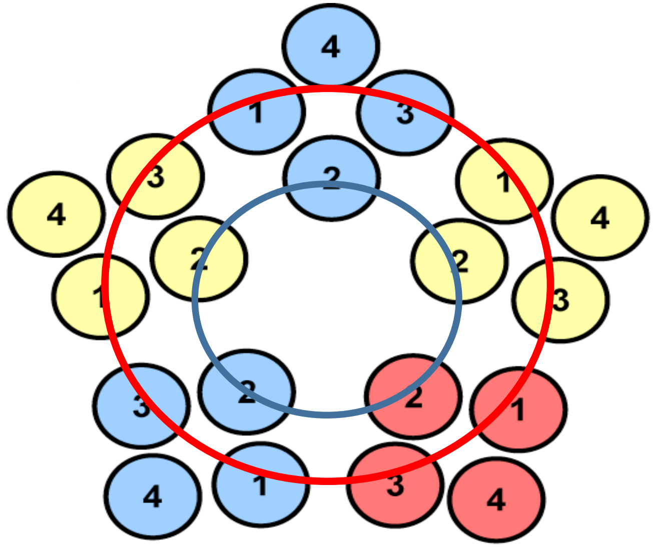
GABA
Glycine
Acetylcholine
Serotonin


Resting (C)
C
D
O
Desensitized (D)
Open (O)

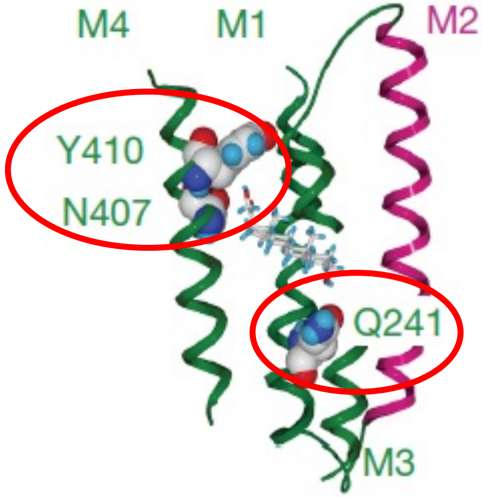
Anionic Phospholipids
Cholesterol
Polyunsaturated Fatty Acids
Neurosteroids



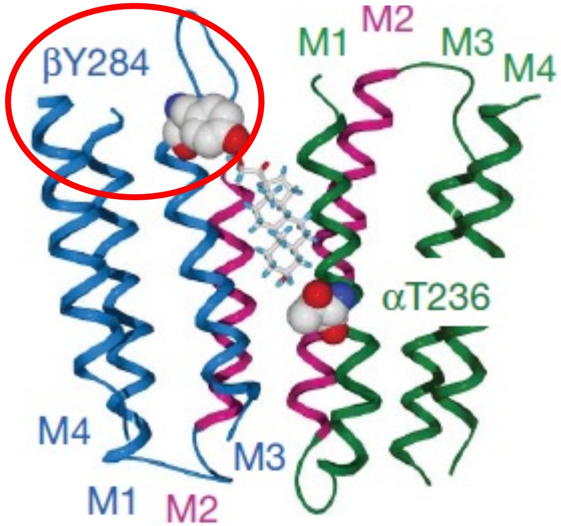

α
GABA
Pocket
TM2
β
β
GABA
Pocket
α
γ


4
1
3
3
2
1
4
2
2
4
1
3
3
2
2
1
4
1
3
4

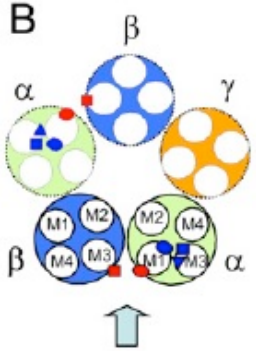
Sites identified by mutagenesis

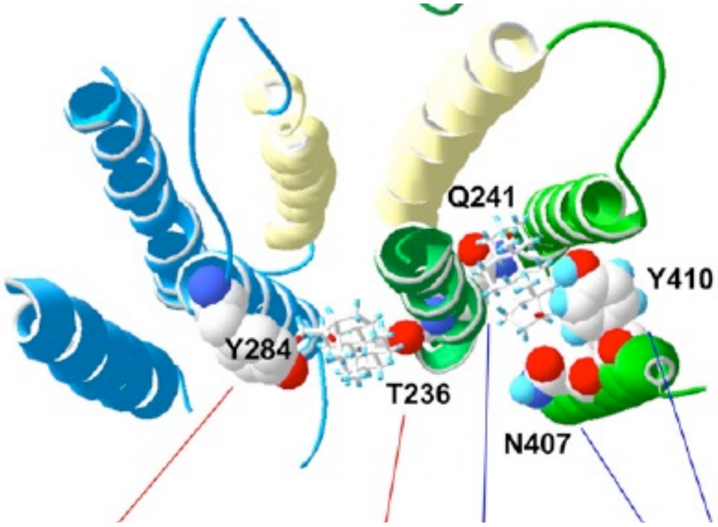

M4
M1
M2
Y410
N407
Q241
M3

Potentiation


βY284
M1
M2
M3
M4
αT236
M4
M3
M1
M2

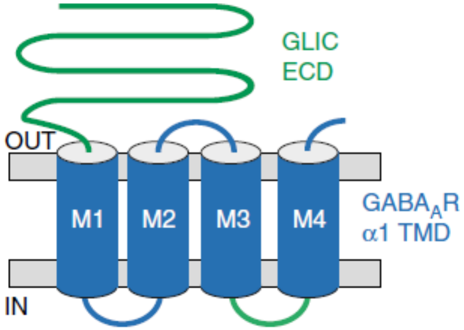
Direct activation

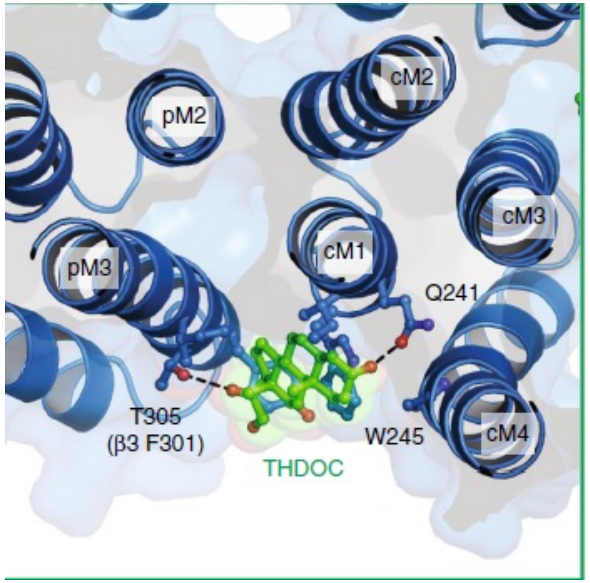

B
β
α
γ
M1
M2
M4
M3
M2
M4
M1
M3
β
α


Q241
Y410
Y284
T236
N407

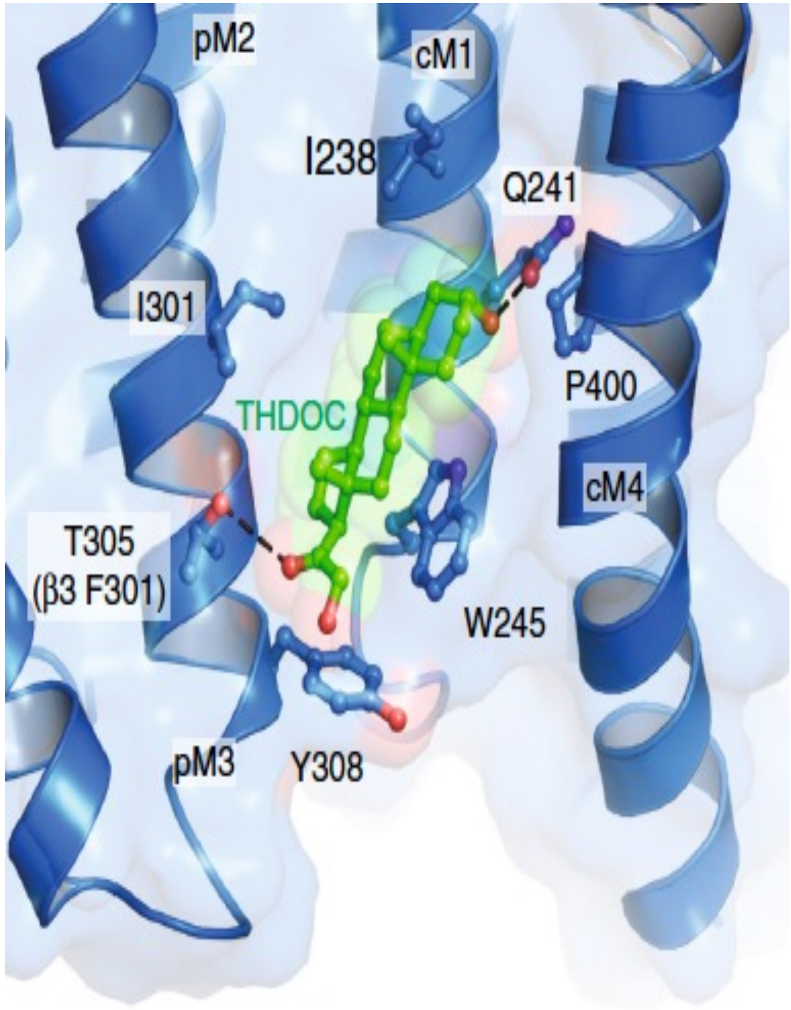
Hosie AM et. al, Nature, 2006

Neurosteroid binding to GLIC-GABA$_A$ chimera


GLIC
ECD
OUT
M1
M2
M3
M4
GABA$_A$R
α1 TMD
IN


cM2
pM2
cM3
pM3
cM1
Q241
T305
(β3 F301)
W245
cM4
THDOC

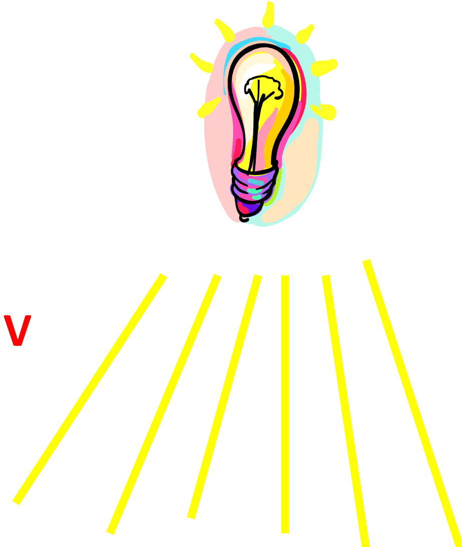
GLIC-a1GABA$_A$R

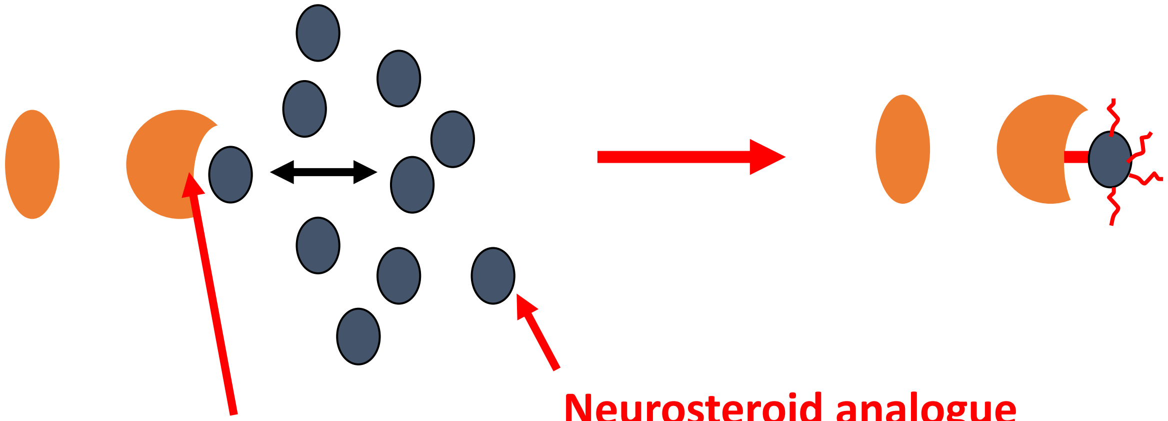

pM2
cM1
I238
Q241
I301
P400
THDOC
cM4
T305
(β3 F301)
W245
pM3
Y308

Laverty D (2017) Nat Struct Mol Biol 11:977

Photoaffinity labeling to identify neurosteroid binding sites

Doug Covey, PhD

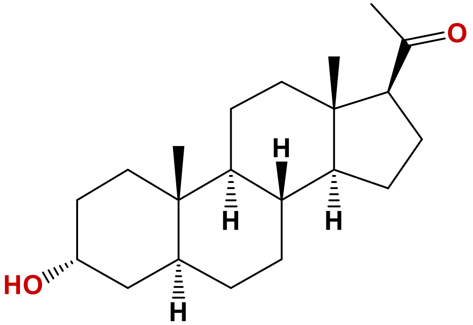




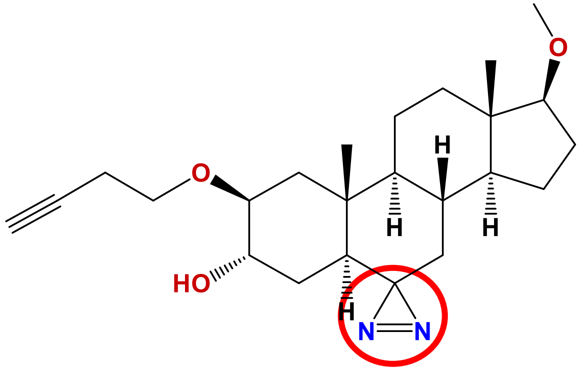
UV

Binding Site

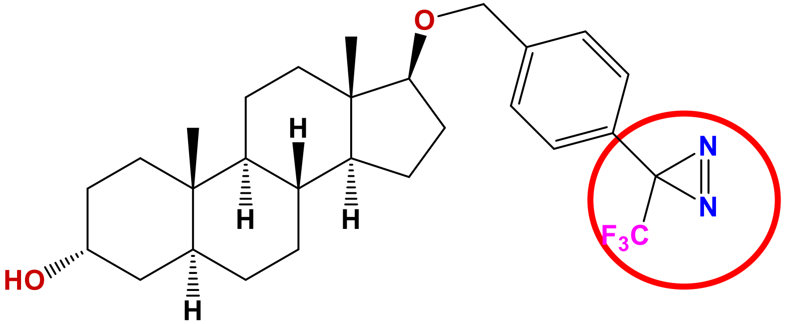
Neurosteroid analogue photolabeling reagent



Allopregnanolone

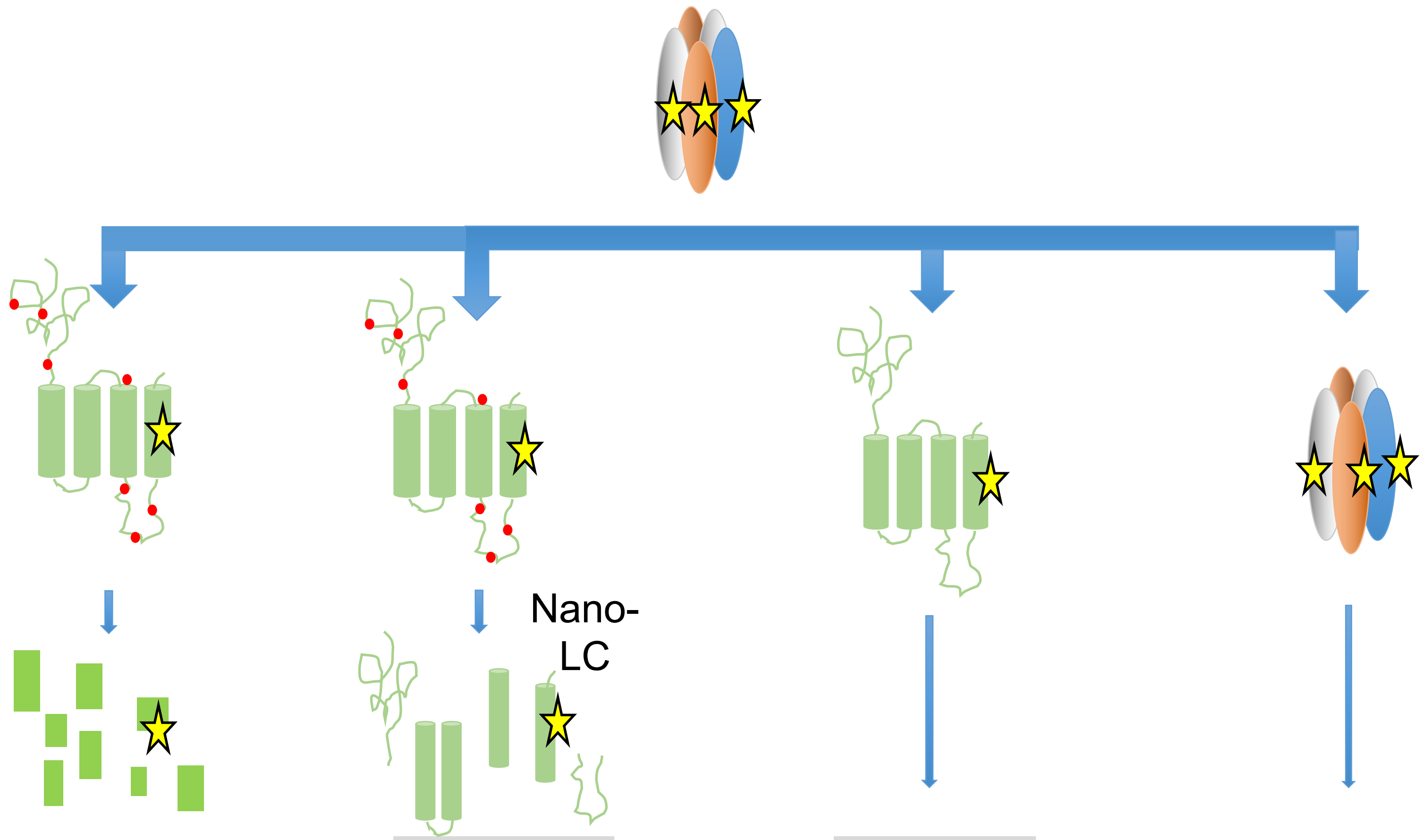


KK123



KK200

Mass spectrometric analysis of pLGICs


Nano-LC

Bottom-up

- Sites

Middle-down

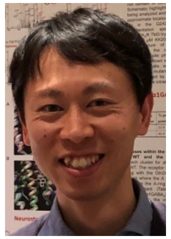
- Sites
- Efficiency

Top-down

- Stoichiometry
- Efficiency

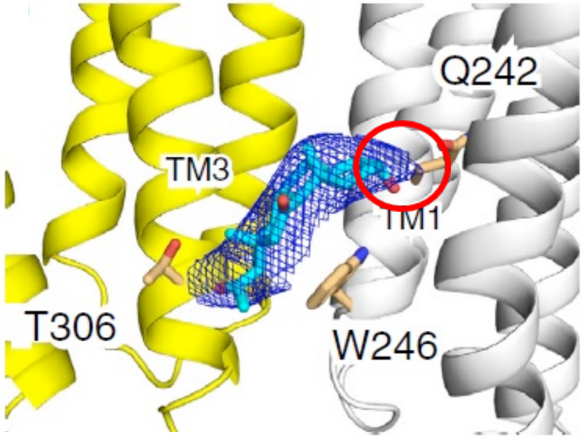
Native

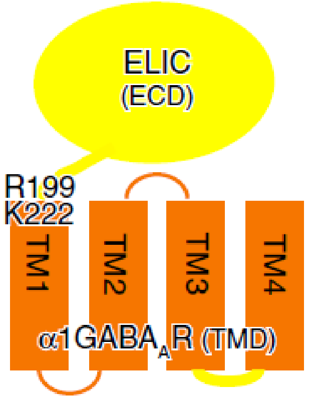
- Stoichiometry
- Cooperativity

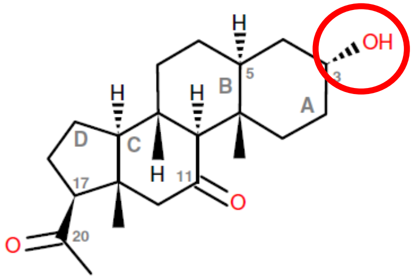


Yusuke Sugasawa, MD PhD

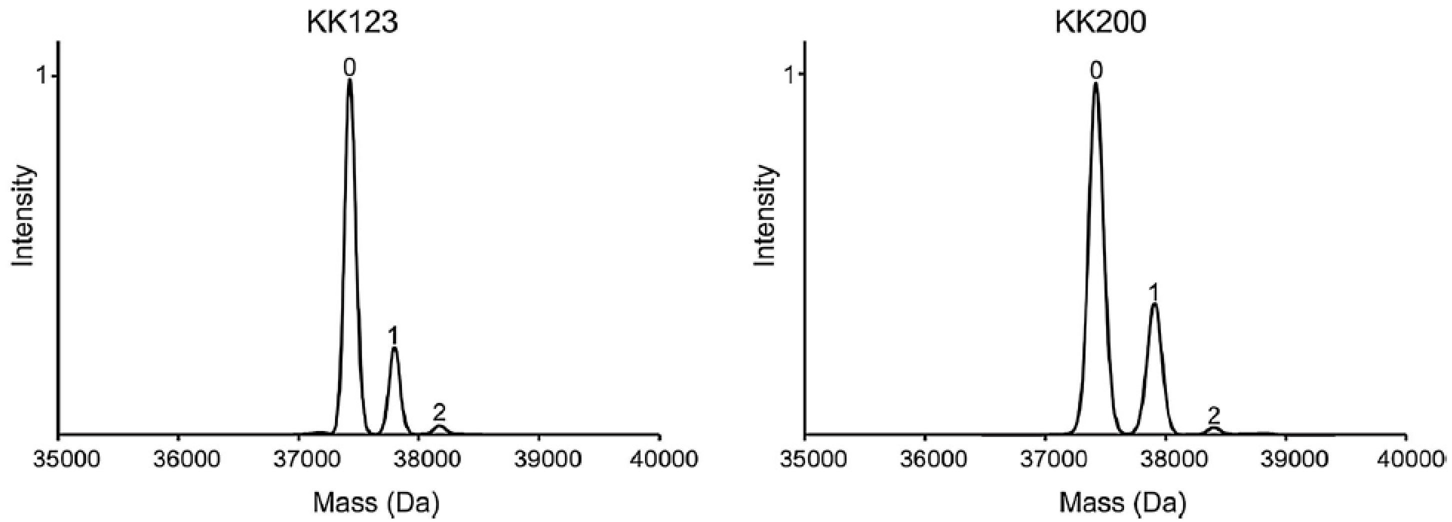
Neurosteroid binding to ELIC-α1 Chimera


Q242
TM3
TM1
T306
W246

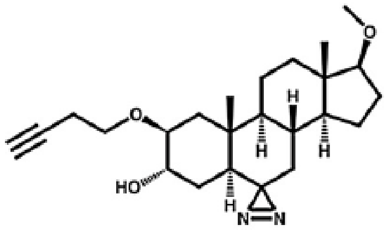

ELIC
(ECD)
R199
K222
TM1
TM2
TM3
TM4
α1GABA$_A$R (TMD)


H
OH
H
H
B
5
3
A
D
C
H
11
17
O
O
20

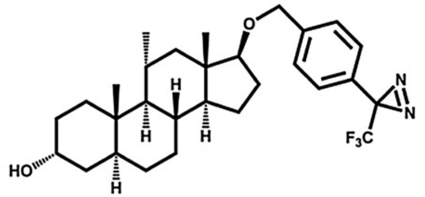
Alphaxolone



KK123


O
O
H
HO
H
H
H
H
N=N

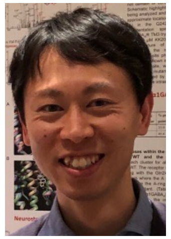
KK200


O
H
H
H
HO
H
N
N
F_3C

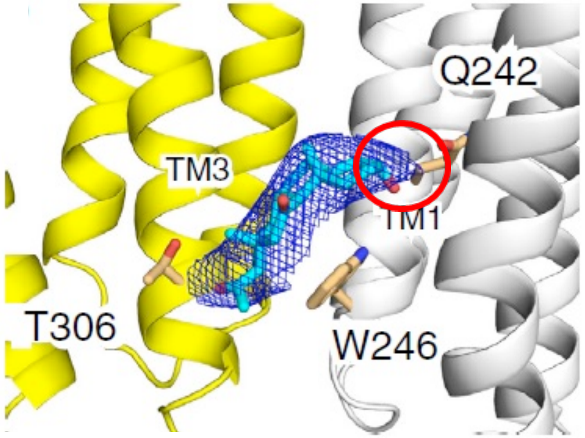
Chen Q (2018) Nat Commun.

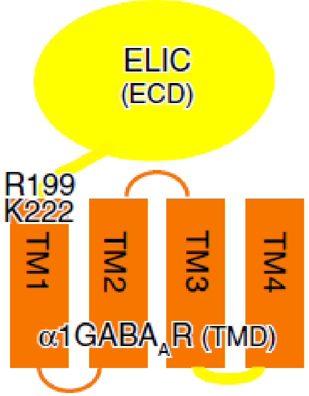
Sugasawa Y (2019) J Steroid Biochem Mol Biol

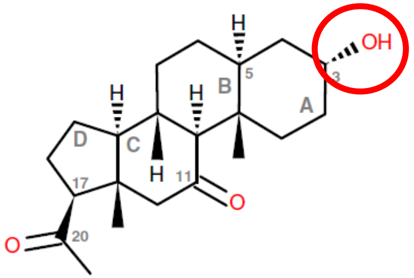
Two neurosteroid binding sites per α1 Subunit



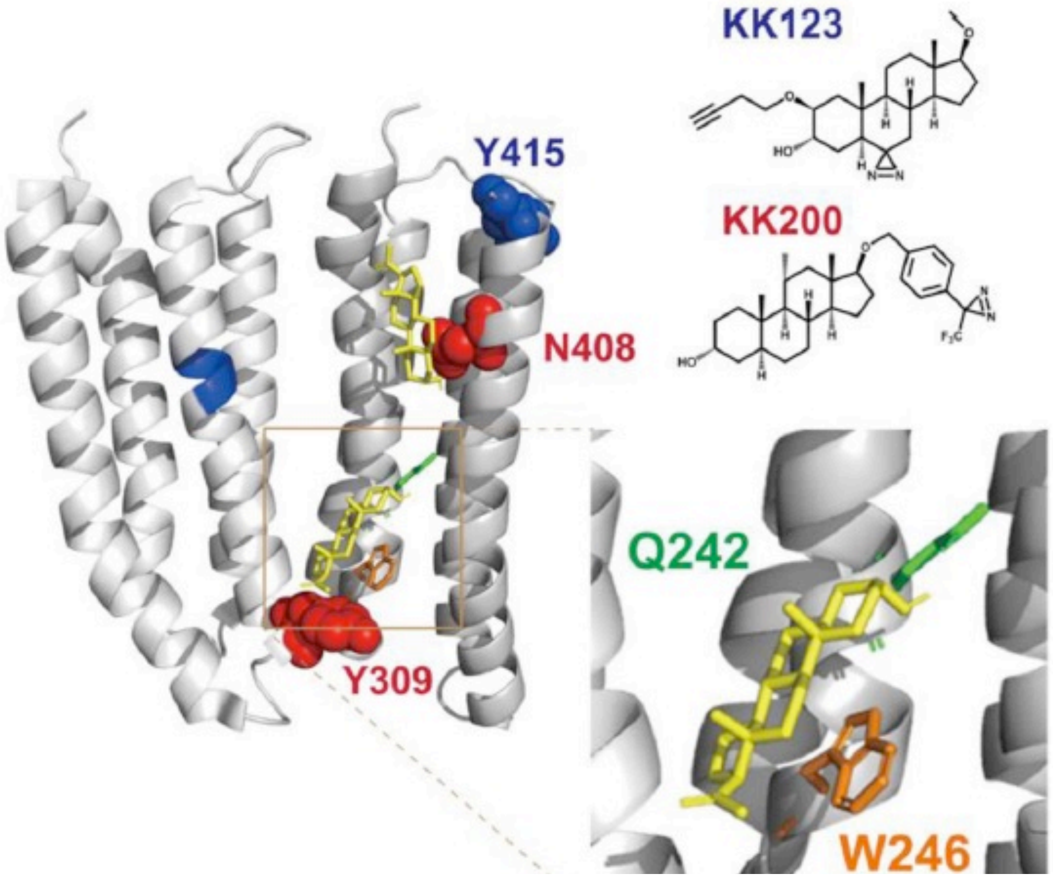
Yusuke Sugasawa,
MD PhD


Q242
TM3
TM1
T306
W246


ELIC
(ECD)
R199
K222
TM1
TM2
TM3
TM4
α1GABA$_A$R (TMD)


H
OH
H
H
B
5
3
A
D
C
H
11
17
O
O
20

Alphaxolone

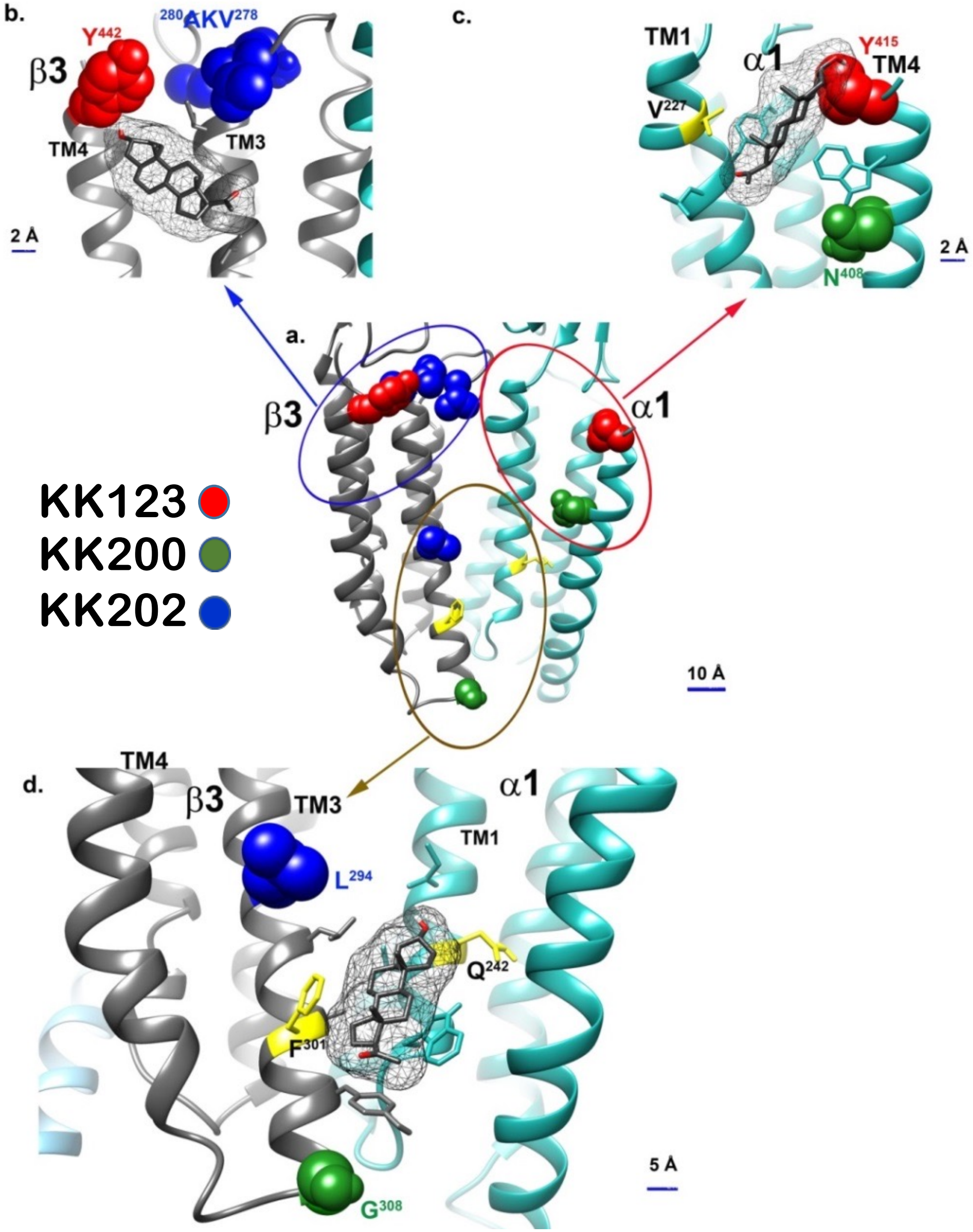

KK123
Y415
KK200
N408
Y309
Q242
W246

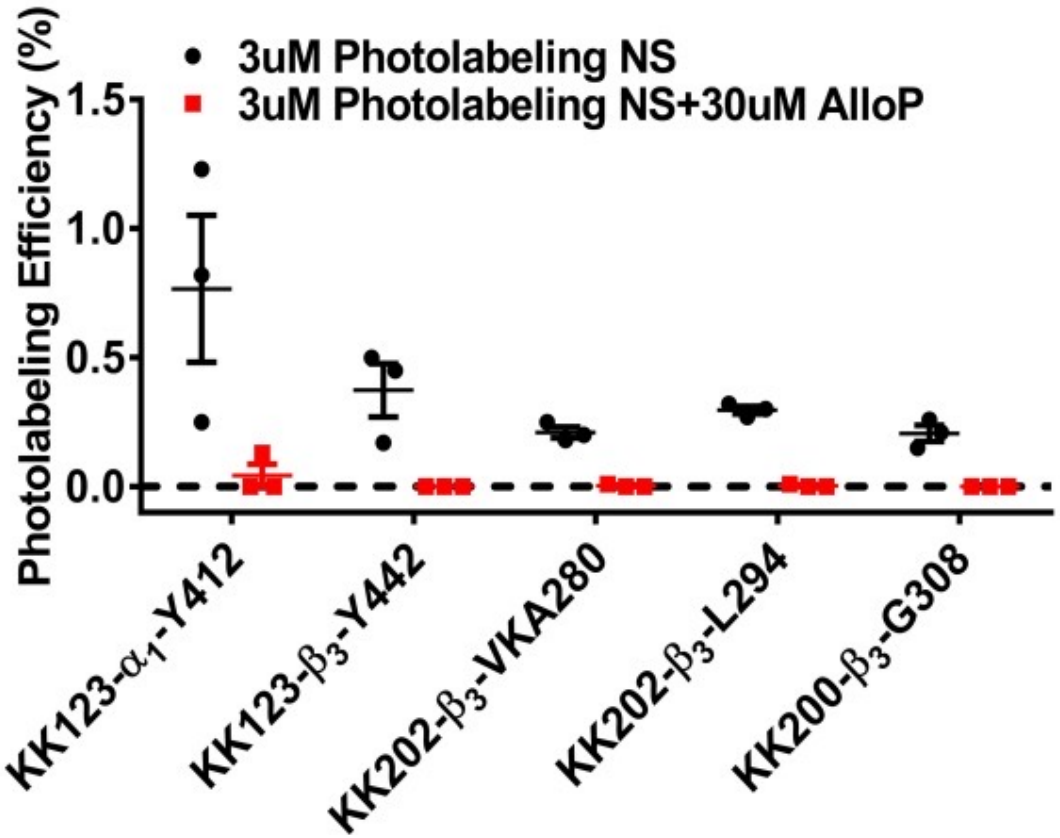
Chen Q (2018) Nat Commun.

Sugasawa Y (2019) J Steroid Biochem Mol Biol

Specific NS binding sites

ZiWei Chen, MD PhD

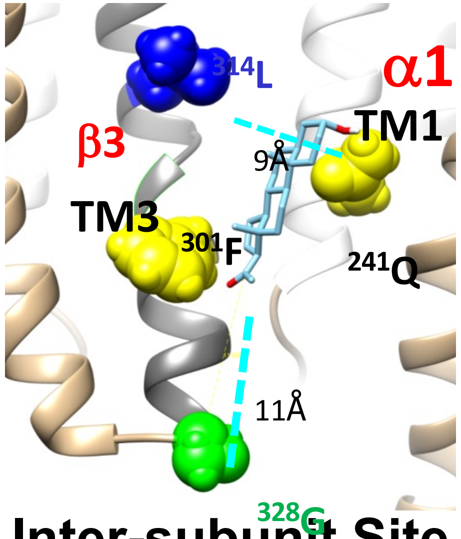

b.
Y442
280AKV278
β3
TM4
TM3
2 Å
c.
TM1
α1
Y415
TM4
V227
N408
2 Å
a.
β3
α1
KK123 ●
KK200 ●
KK202 ●
10 Å
d.
TM4
β3
TM3
α1
TM1
L294
Q242
F301
G308
5 Å



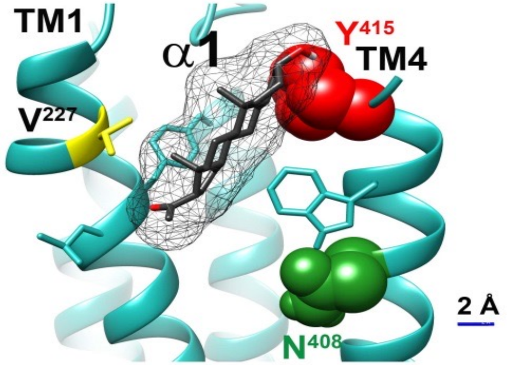
Chen ZW et al, PLoS Biol 2019

Both inter- and α_1 intra-subunit sites contribute to neurosteroid potentiation of $GABA_A$ receptors

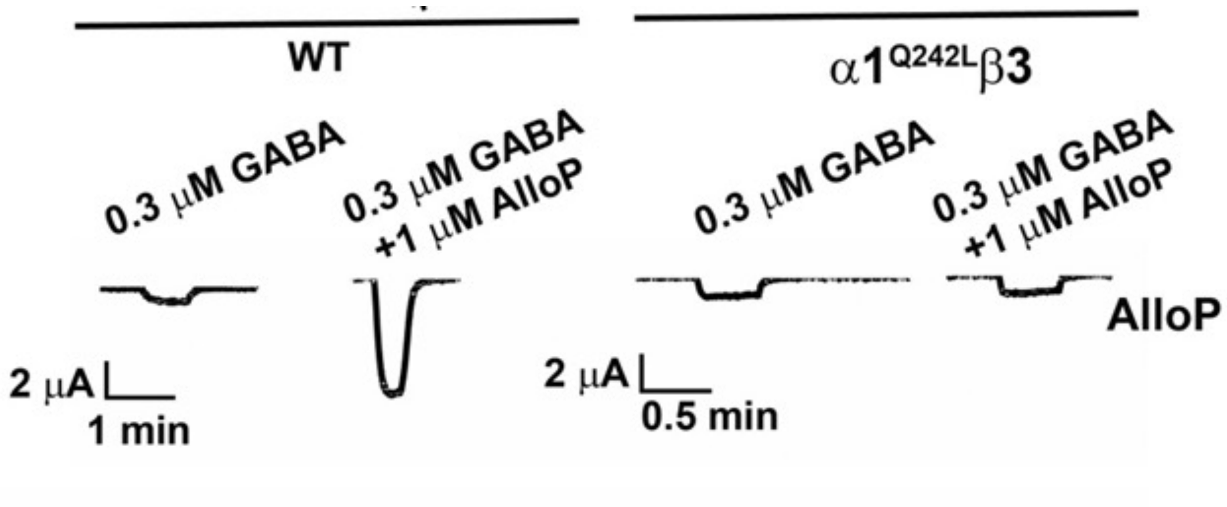
ZiWei Chen, MD PhD

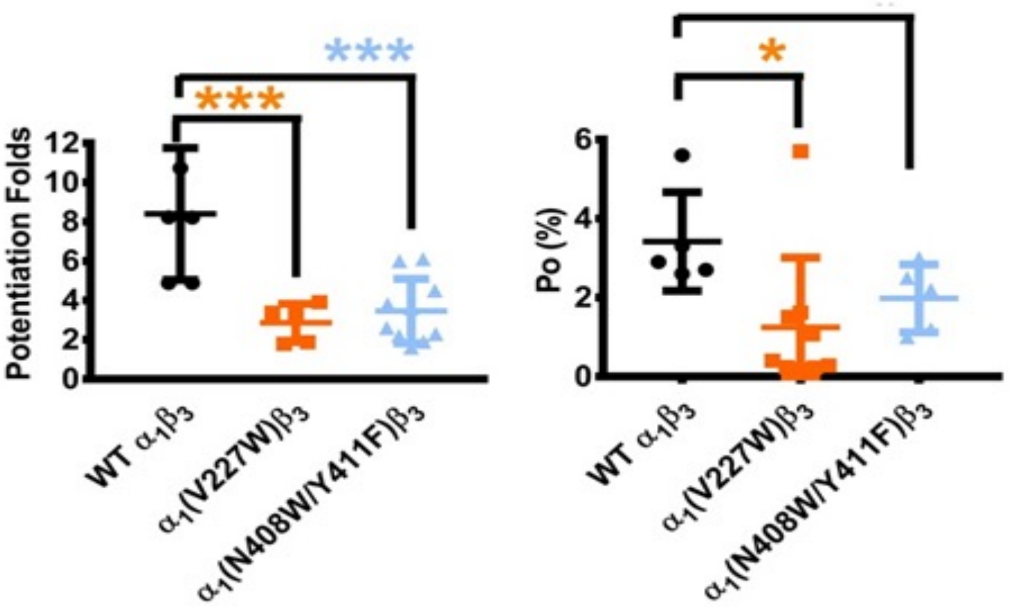

314L
α1
TM1
β3
9Å
TM3
301F
241Q
11Å
328G

Inter-subunit Site


TM1
α1
Y415
TM4
V227
2 Å
N408

α1-Intra-subunit Site



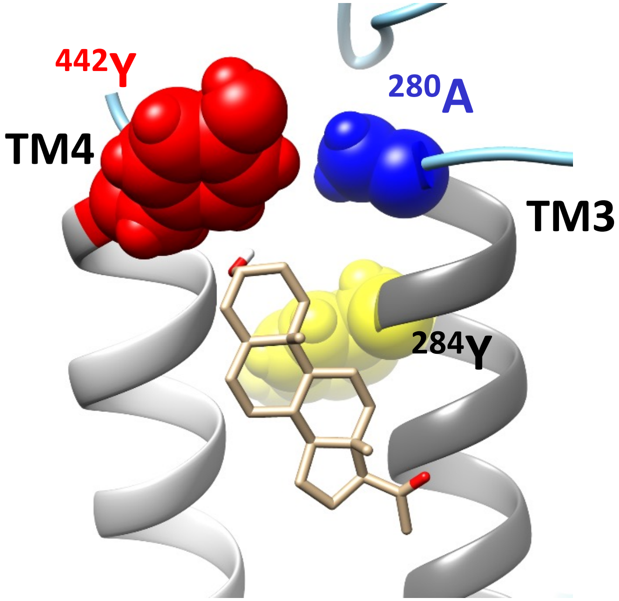


Chen ZW et al, PLoS Biol 2019

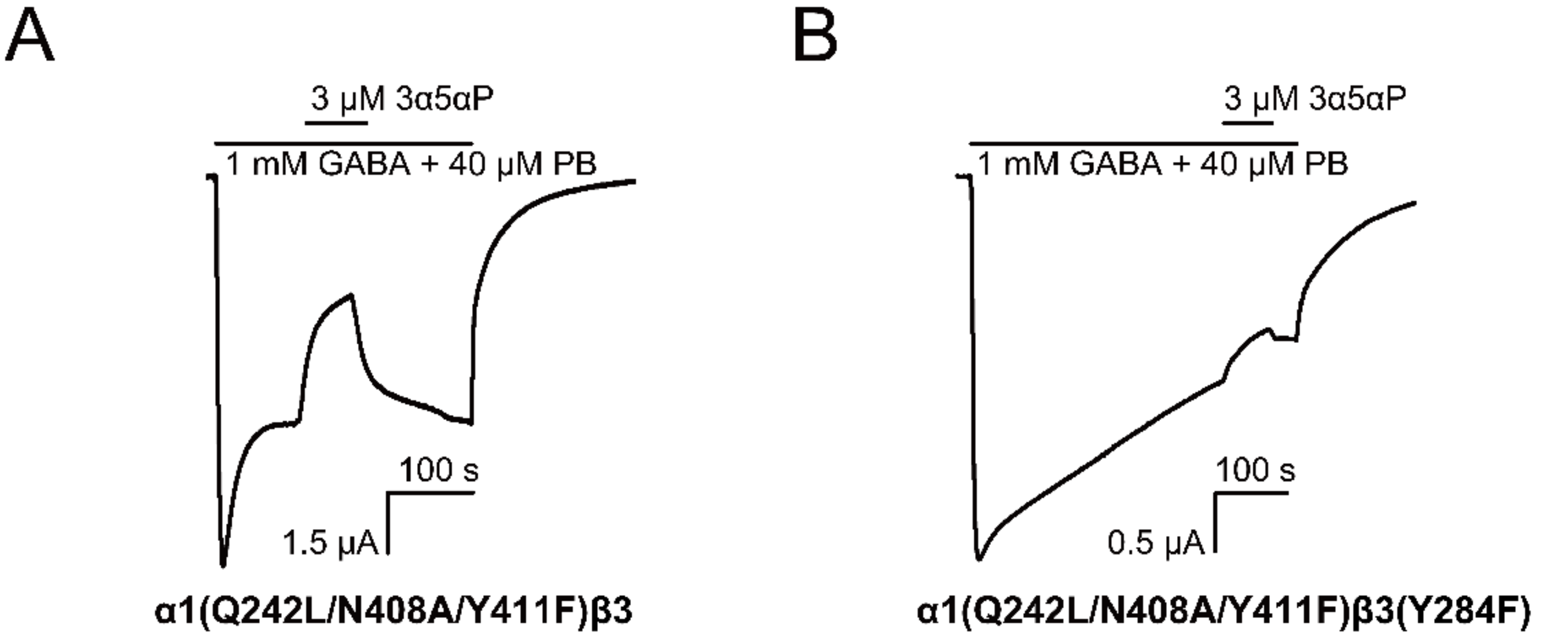
Gustav Akk, PhD

Allopregnanolone binding at β_3 intrasubunit site inhibits GABA$_A$-R

β_3 intrasubunit site



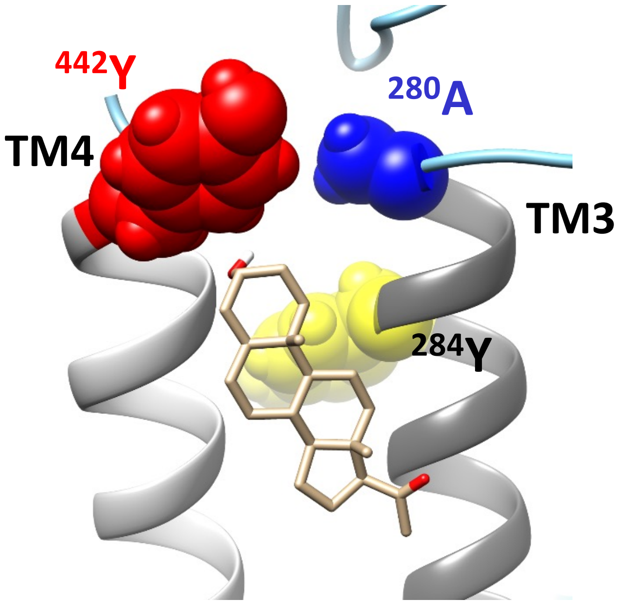
442Y
280A
TM4
TM3
284Y



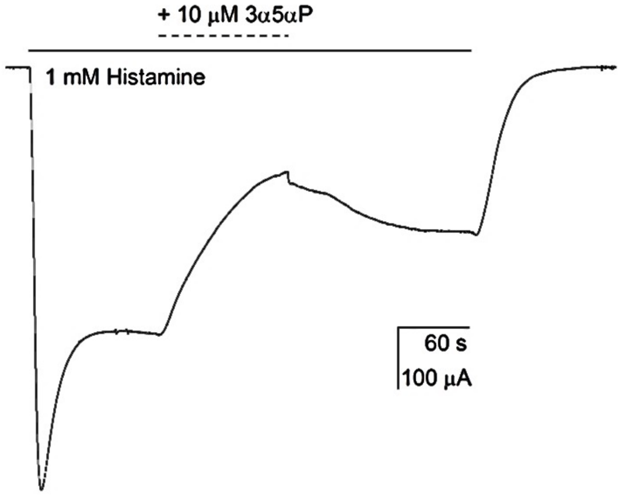
Sugasawa (2020) eLife e55331. doi: 10.7554/eLife.55331

Allopregnanolone binding at β_3 intrasubunit site inhibits GABA$_A$-R: β_3-homomeric receptors

β_3 intrasubunit site

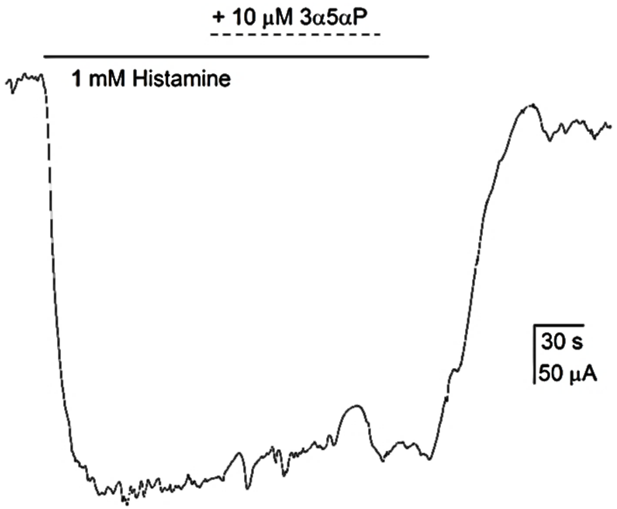


Allopregnanolone



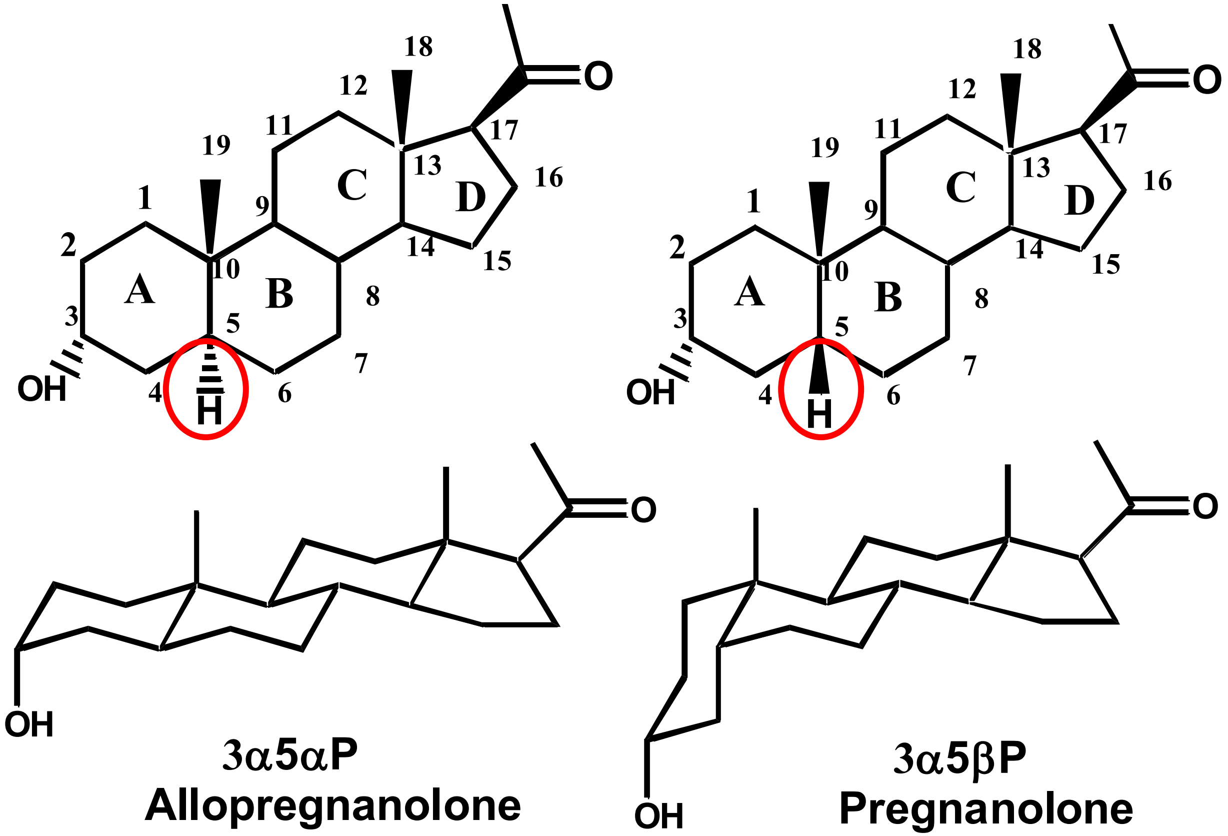
WT β_3 receptor

Allopregnanolone



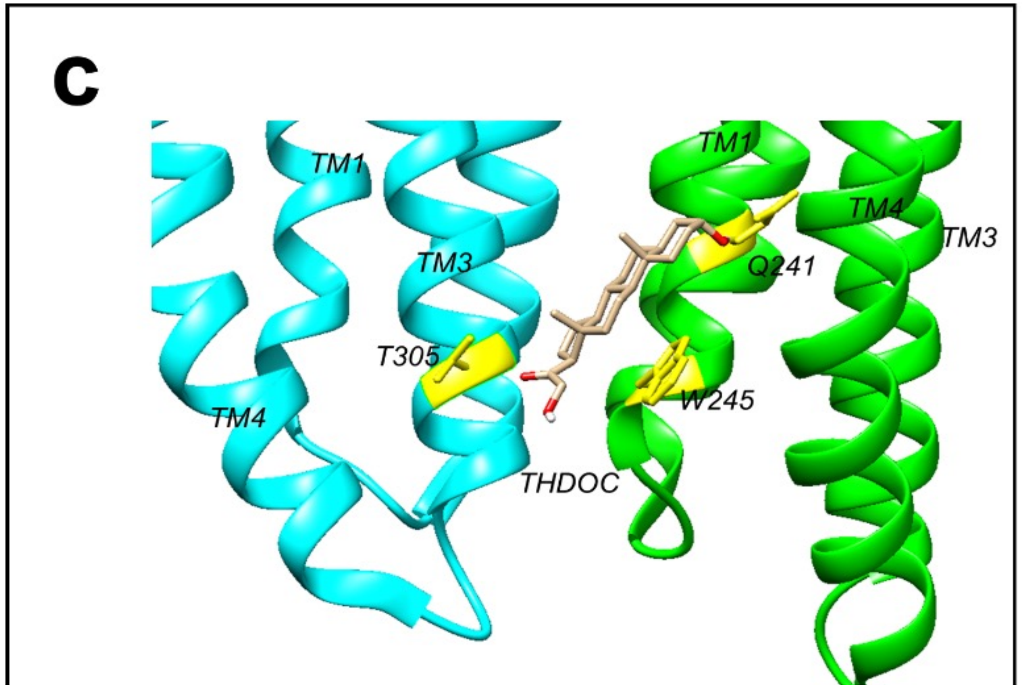
B_3 Y284F receptor

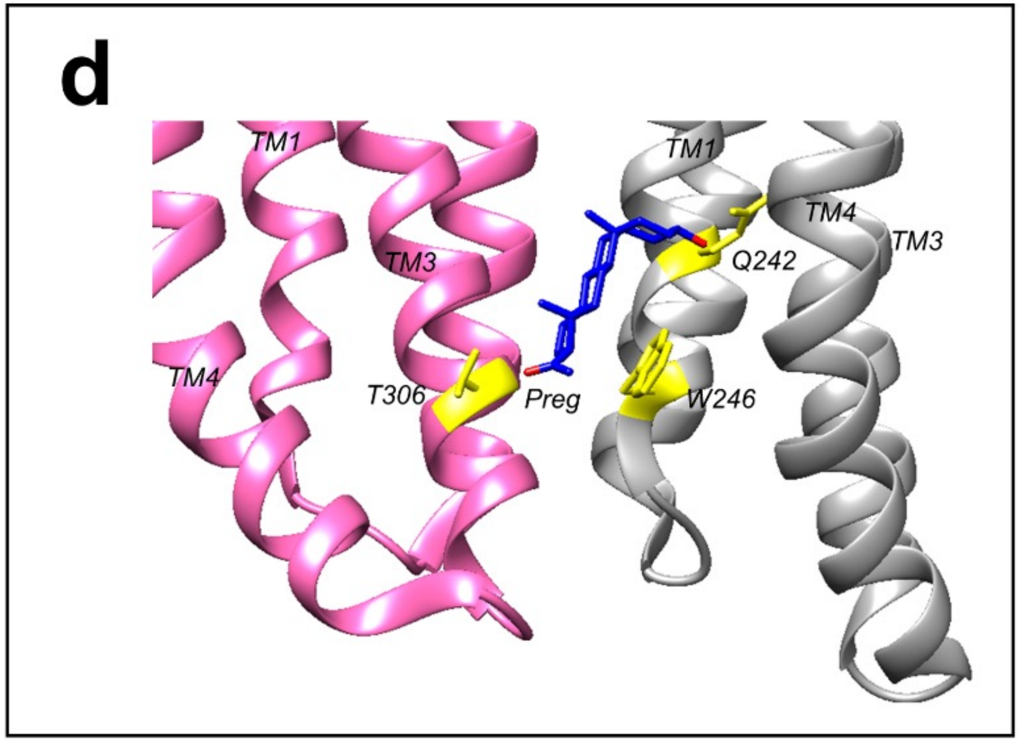
Unpublished


A
B
C
D
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
OH
H
O

3α5αP
Allopregnanolone
DHP

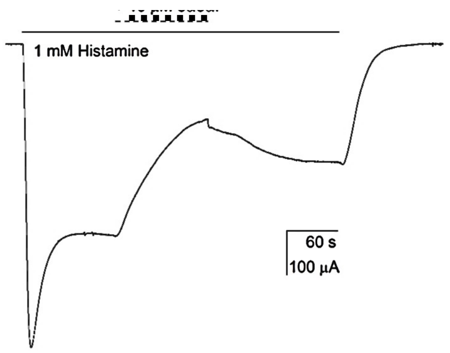
3α5βP
Pregnanolone
5β-DHP


c
TM1
TM1
TM4
TM3
TM3
Q241
T305
W245
TM4
THDOC


d
TM1
TM1
TM4
TM3
TM3
Q242
TM4
T306
Preg
W246

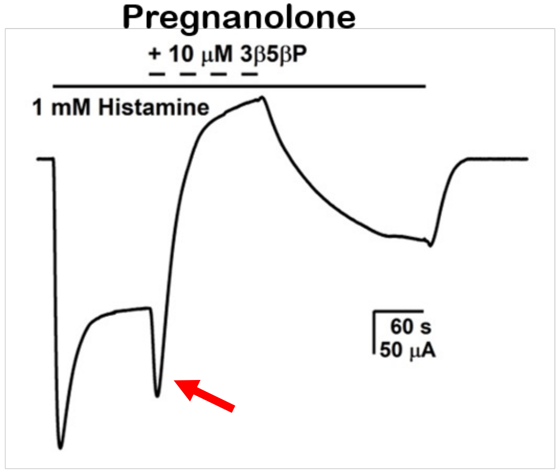
Pregnanolone binds to β_3-β_3 intersubunit site

Gustav Akk, PhD



Allopregnanolone

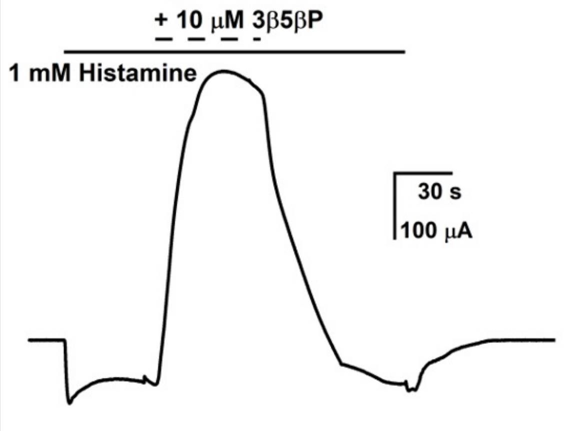
+ 10 μM 3α5αP



Pregnanolone

+ 10 μM 3β5βP

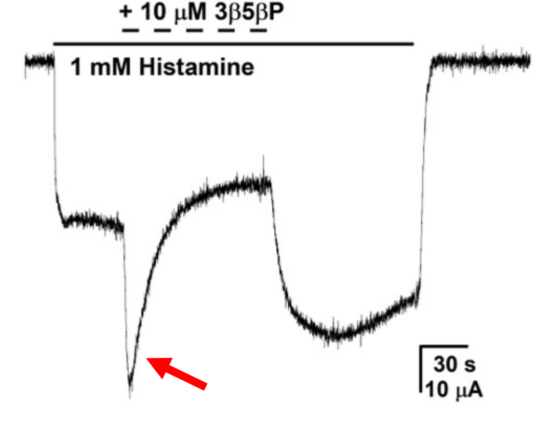
WT β_3 receptor



Pregnanolone

+ 10 μM 3β5βP

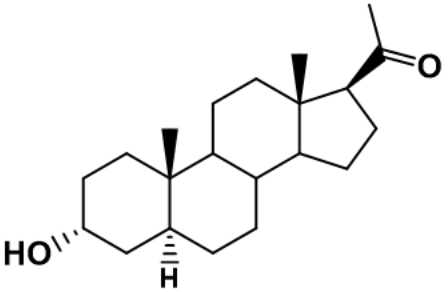
β_3 F301S receptor



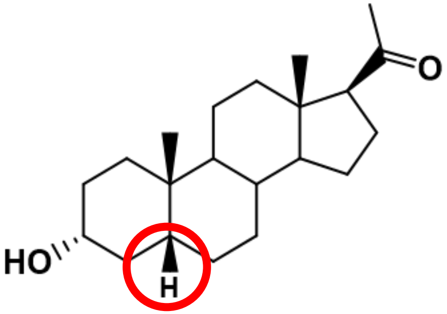
Pregnanolone

+ 10 μM 3β5βP

β_3 Y284F receptor



Allopregnanolone

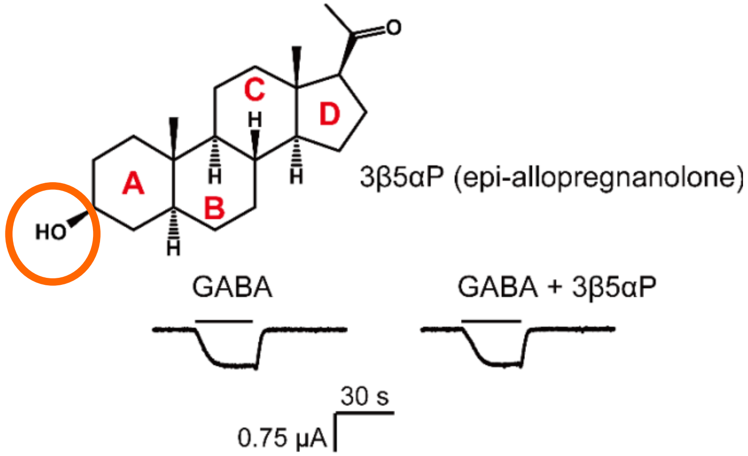


Pregnanolone

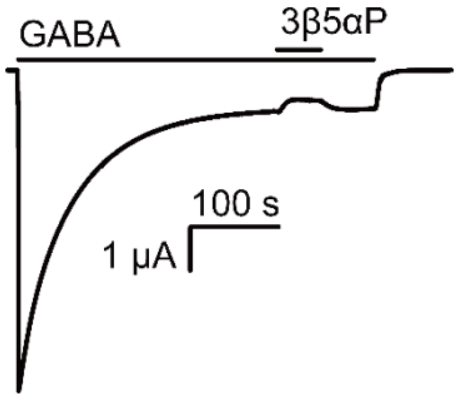
Unpublished

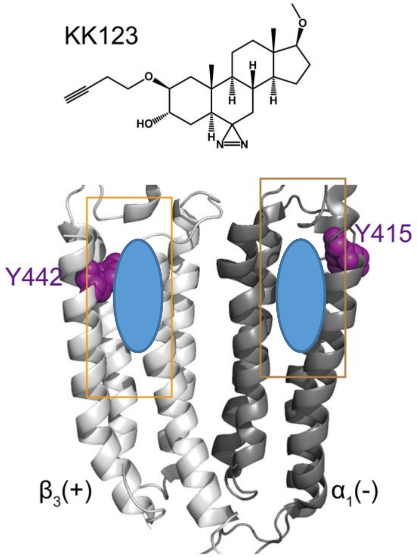
Where does epi-allopregnanolone (3β) bind?

Yusuke Sugasawa, MD PhD

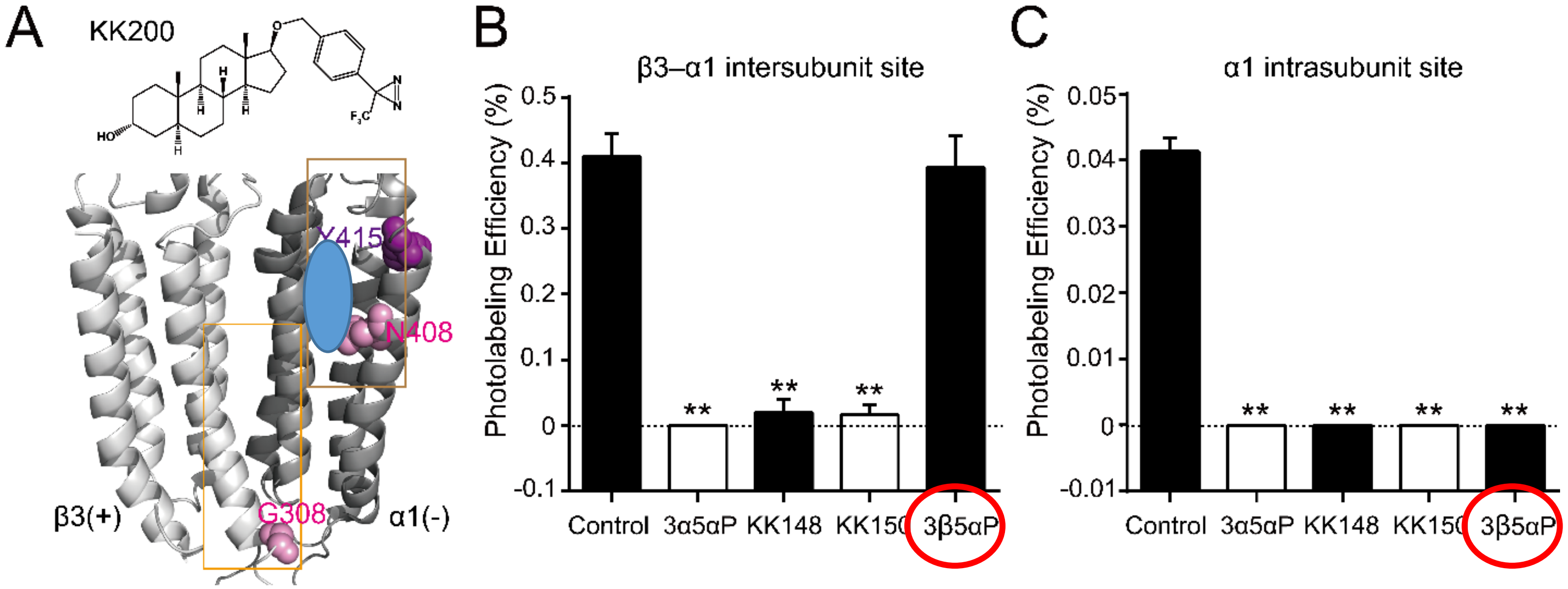


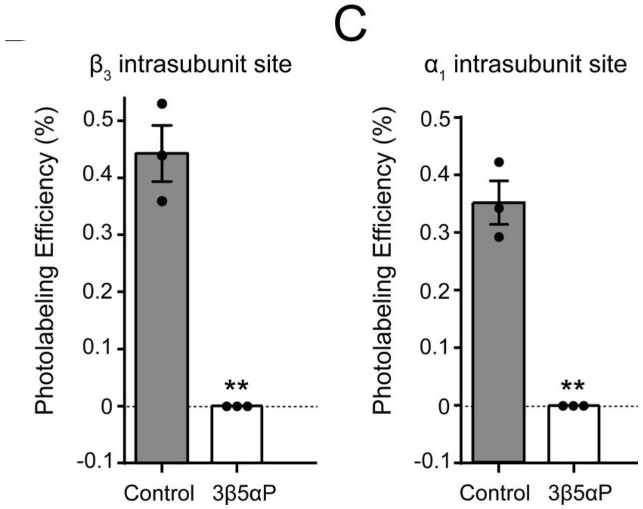
HO
A
B
C
D
H
3β5αP (epi-allopregnanolone)
GABA
GABA + 3β5αP
30 s
0.75 μA





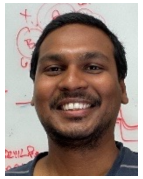
KK123
Y442
Y415
β3(+)
α1(-)



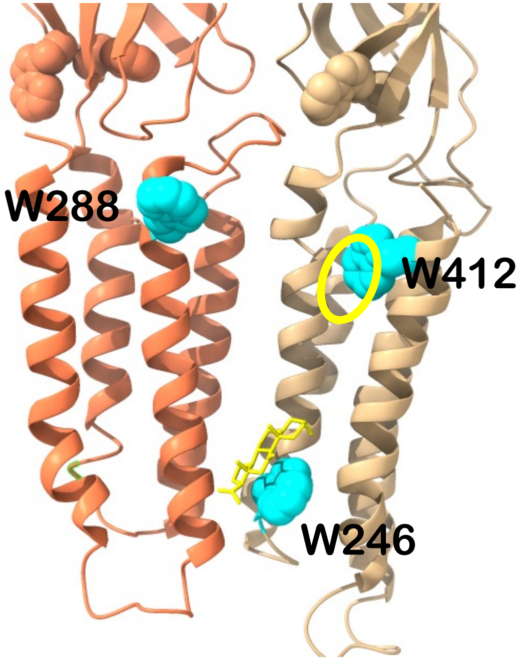


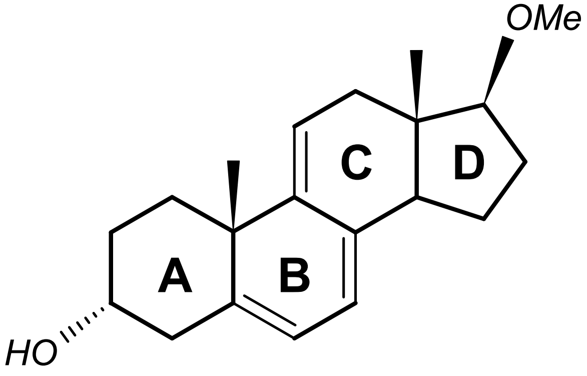
Sugasawa (2020) eLife e55331. doi: 10.7554/eLife.55331

Neurosteroid binding affinity: A FRET- based binding assay

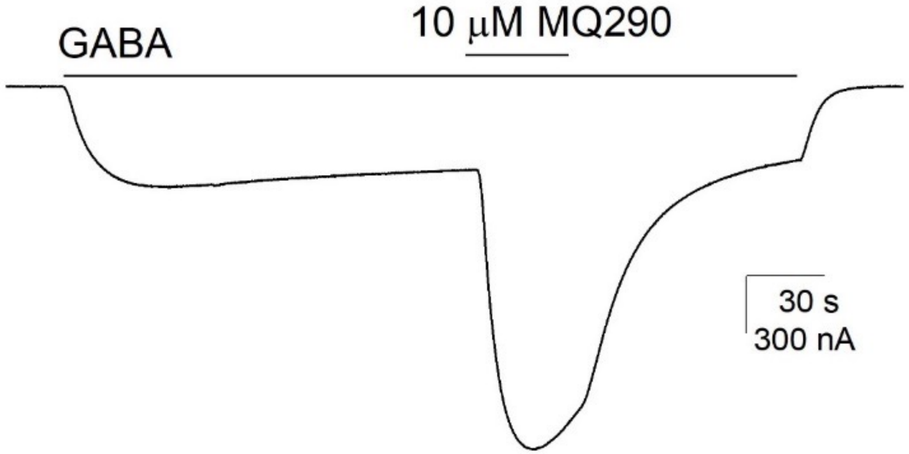


Hiroki Tateiwa MD, PhD
Murthy Chintala, PhD

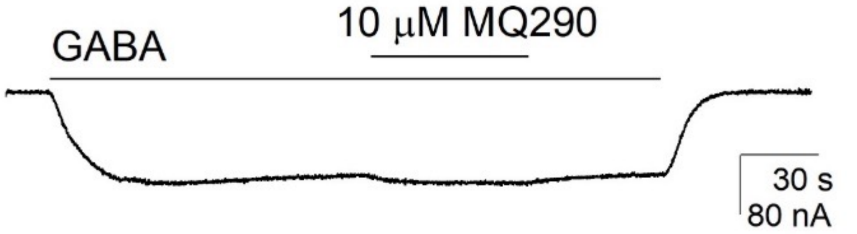

W288
W412
W246



MQ290



Wild-type
$\alpha_1\beta_3$

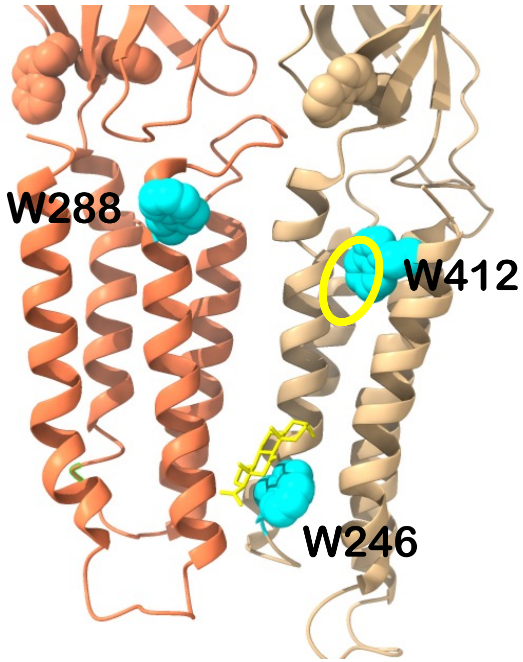


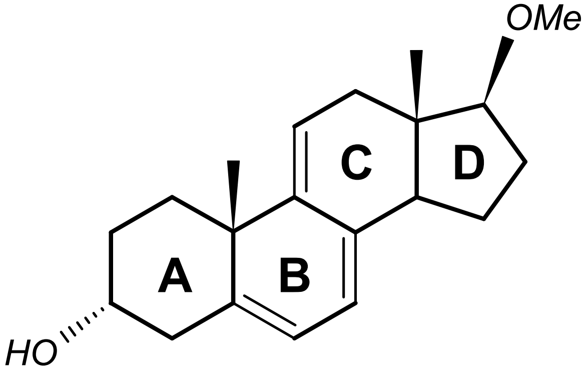
$\alpha_1(Q241L)\beta_3$

Unpublished

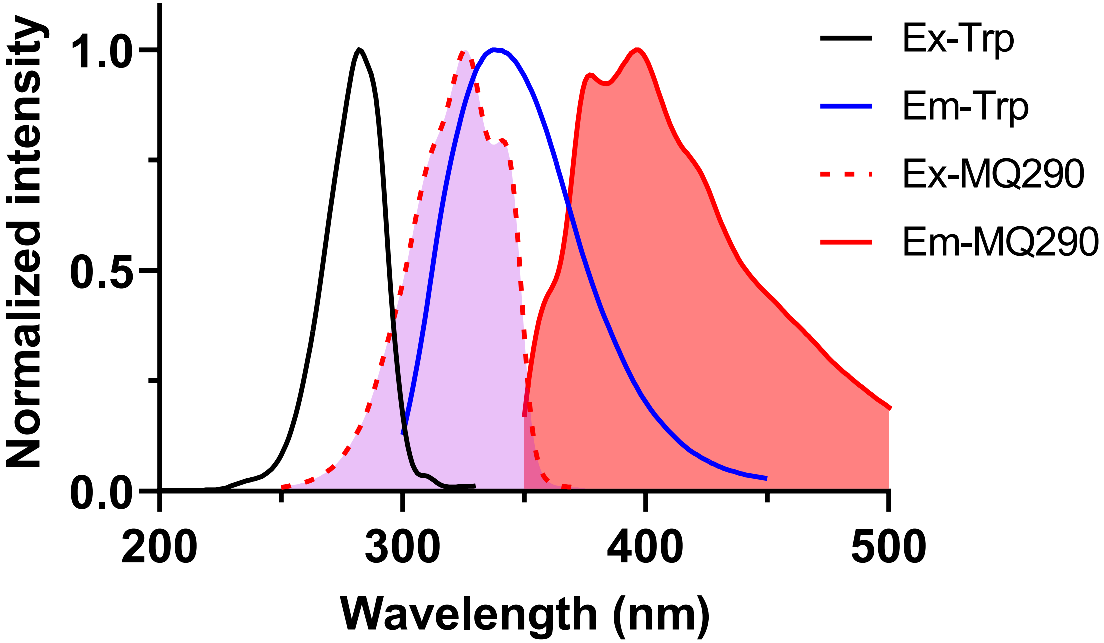
Neurosteroid Binding Affinity: A FRET- based binding assay

Hiroki Tateiwa MD, PhD
Murthy Chintala, PhD


W288
W412
W246

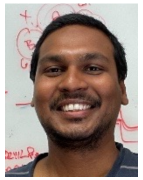


MQ290

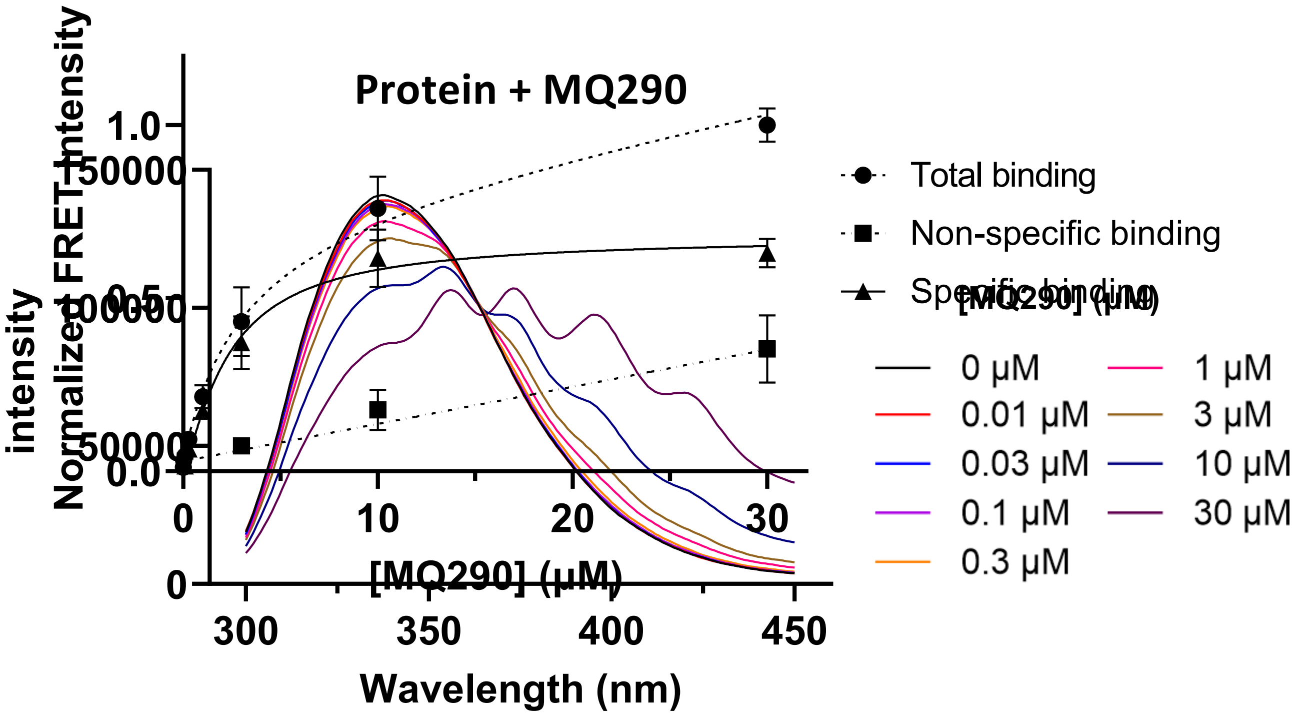


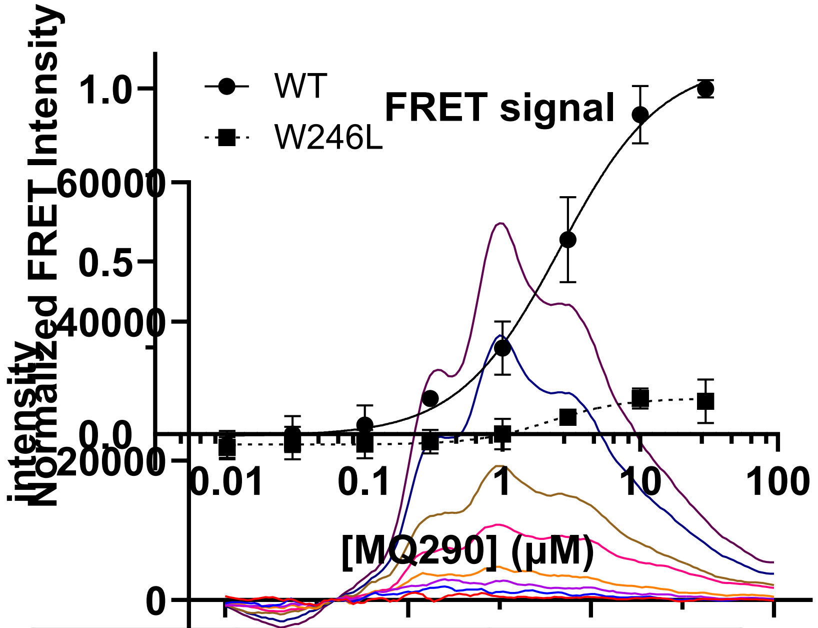
Unpublished

MQ290 FRET- based binding assay (ELIC-α1)



Hiroki Tateiwa MD, PhD
Murthy Chintala, PhD



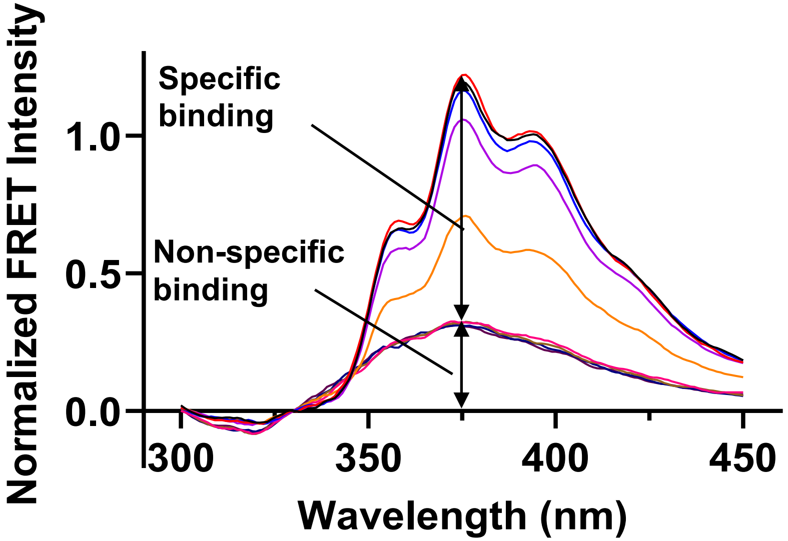


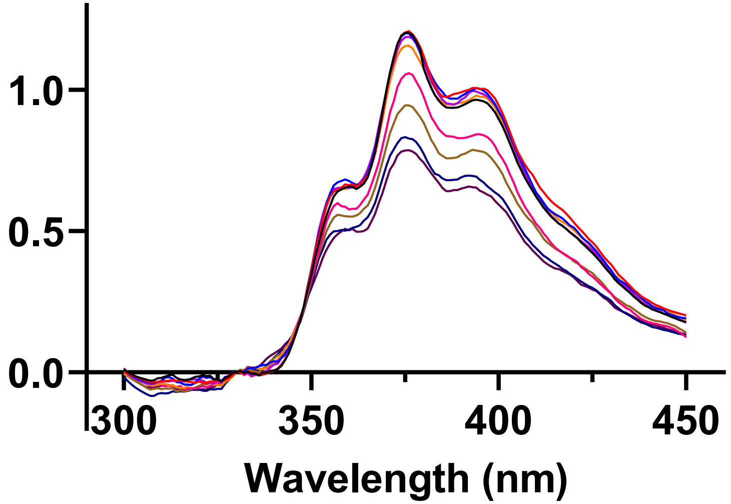
	K_d (μM ±SEM)	B_{max} (±SEM)
WT	2.61 ± 0.41	1.08 ± 0.06
W246L	2.10 ± 0.76	0.11 ±0.10

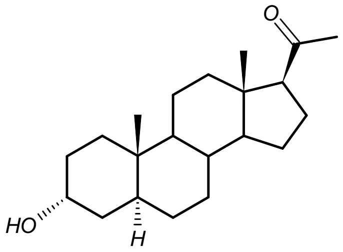
Unpublished

Inhibition of MQ290 FRET

Hiroki Tateiwa MD, PhD
Murthy Chintala, PhD



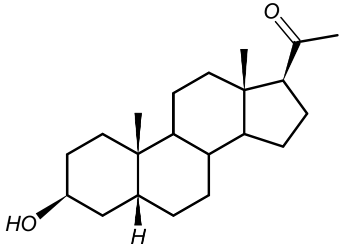




Allopregnanolone

[AlloP] (μM)

- 0 μM
- 0.01 μM
- 0.03 μM
- 0.1 μM
- 0.3 μM
- 1 μM
- 3 μM
- 10 μM
- 30 μM



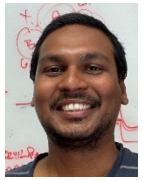
Epi-Pregnanolone

[Epi-AlloP] (μM)

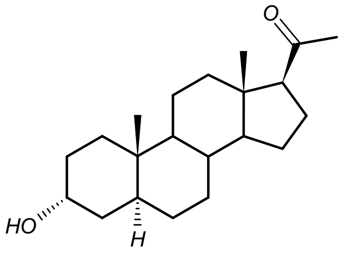
- 0 μM
- 0.01 μM
- 0.03 μM
- 0.1 μM
- 0.3 μM
- 1 μM
- 3 μM
- 10 μM
- 30 μM

Unpublished

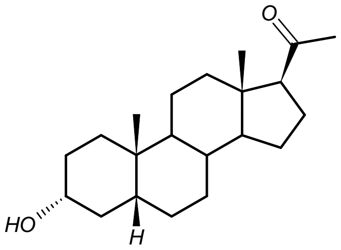
PAM-neurosteroid inhibition of MQ290 FRET



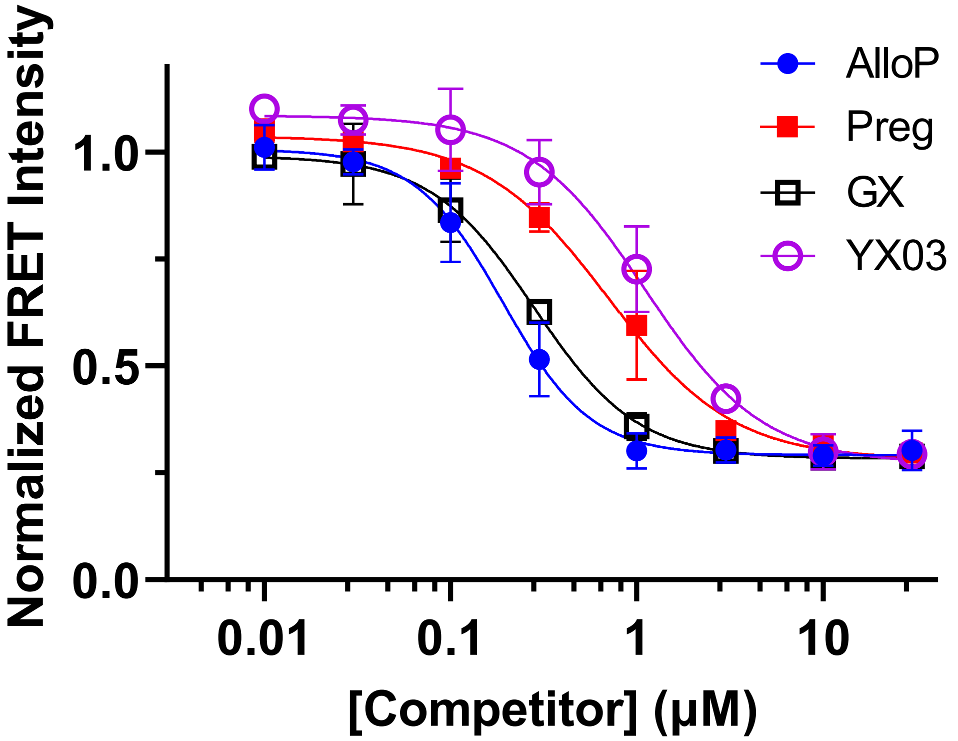
Hiroki Tateiwa MD, PhD
Murthy Chintala, PhD

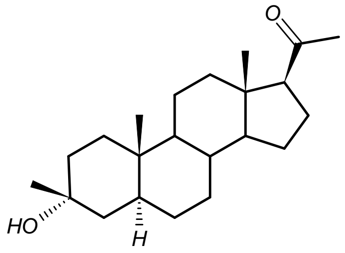


Allopregnanolone

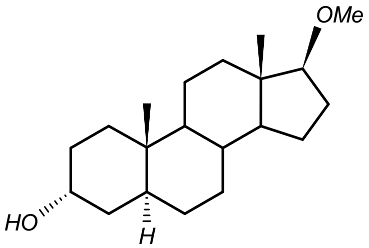


Pregnanolone





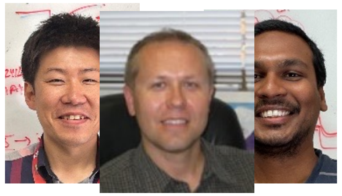
Ganaxolone



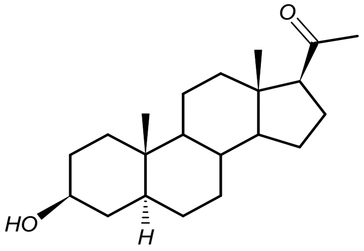
YX03

Unpublished

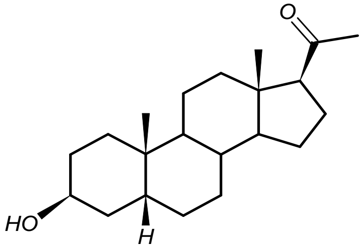
NAM-neurosteroid potentiation of GABA$_A$R ($\alpha_1\beta_3$)



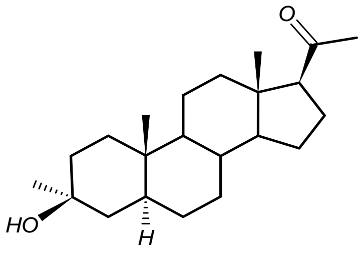
Hiroki Tateiwa, MD, PhD
Gustav Akk, PhD
Murthy Chintala, PhD



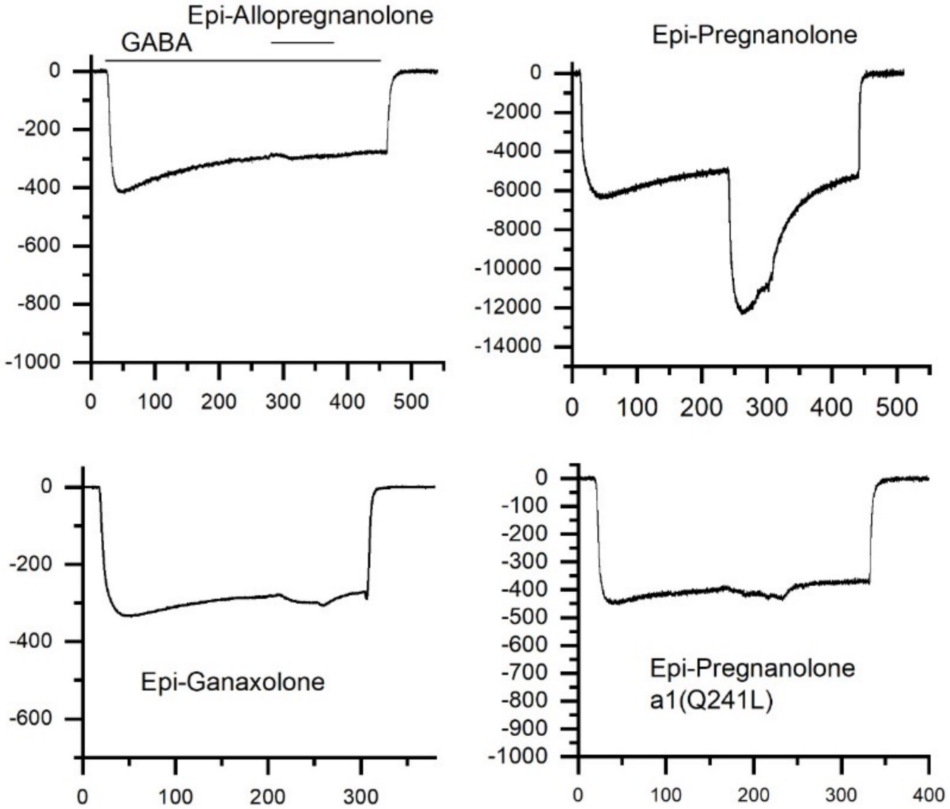
Epi-Allopregnanolone

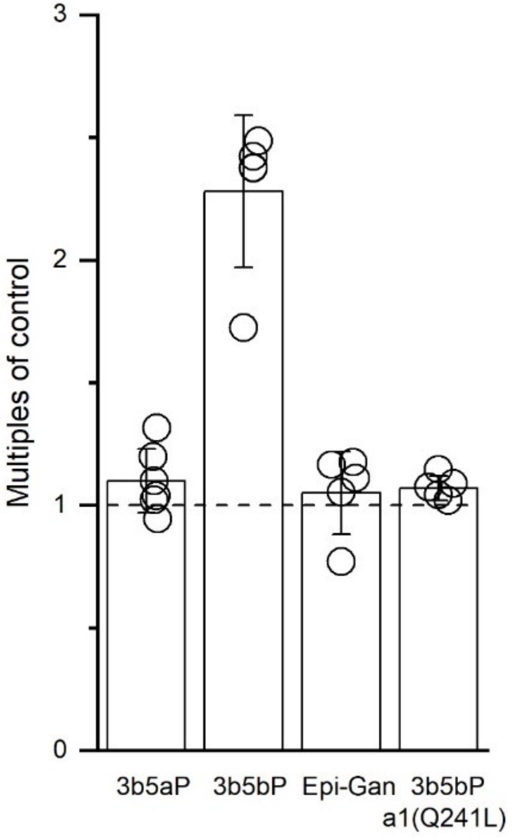


Epi-Pregnanolone



Epi-Ganaxolone





Unpublished

	IC$_{50}$ (μμM, ± SEM)	**Hill Slope** (± SEM)	**K$_i$** (μM, ± SEM)
3α-NS: Intersubunit Site			
AlloP	0.19 ± 0.02	-1.84 ± 0.28	**0.08 ± 0.01**
Preg	0.70 ± 0.09	-1.30 ± 0.20	**0.31 ± 0.04**
GX	0.28 ± 0.03	-1.57 ± 0.23	0.12 ± 0.01
YX03	1.11 ± 0.17	-1.38 ± 0.28	**0.48 ± 0.08**
3β-NS: Intrasubunit Site			
Epi-AlloP	0.69 ± 0.04	-0.82 ± 0.32	**0.50 ± 0.03**
Epi-Preg	1.81 ± 0.94	-0.56 ± 0.16	**1.31 ± 0.68**
Epi-GX	1.35 ± 0.38	-1.22 ± 0.40	0.98 ± 0.28
YX04	2.62 ± 3.00	-0.63 ± 0.38	**1.89 ± 2.17**

Hiroki Tateiwa MD, PhD
Murthy Chintala, PhD

Summary

- Neurosteroids bind to multiple sites on the $GABA_A$ receptor
- Different neurosteroids bind to overlapping subsets of these sites
- Different neurosteroids can be PAMs or NAMs at a given site
- There may be additional neurosteroid binding sites in $GABA_A$ receptors with different subunit composition and cyclic order

Conclusions

- The matrix of multiple endogenous neurosteroids bathing GABA_A receptors and the array of subunit compositions expressed with cellular and anatomic specificity indicates a complex endogenous pharmacology in which neurosteroids fine tune neuronal excitability and regulate behavior (mood)
- The structural complexity of neurosteroids and (and their binding sites) presents an **opportunity to develop neurosteroid analogues that are** site-specific agonists and antagonists for fine-tuning sedation, anesthesia and mood

Acknowledgements

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Gustav Akk Lab

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David Reichert

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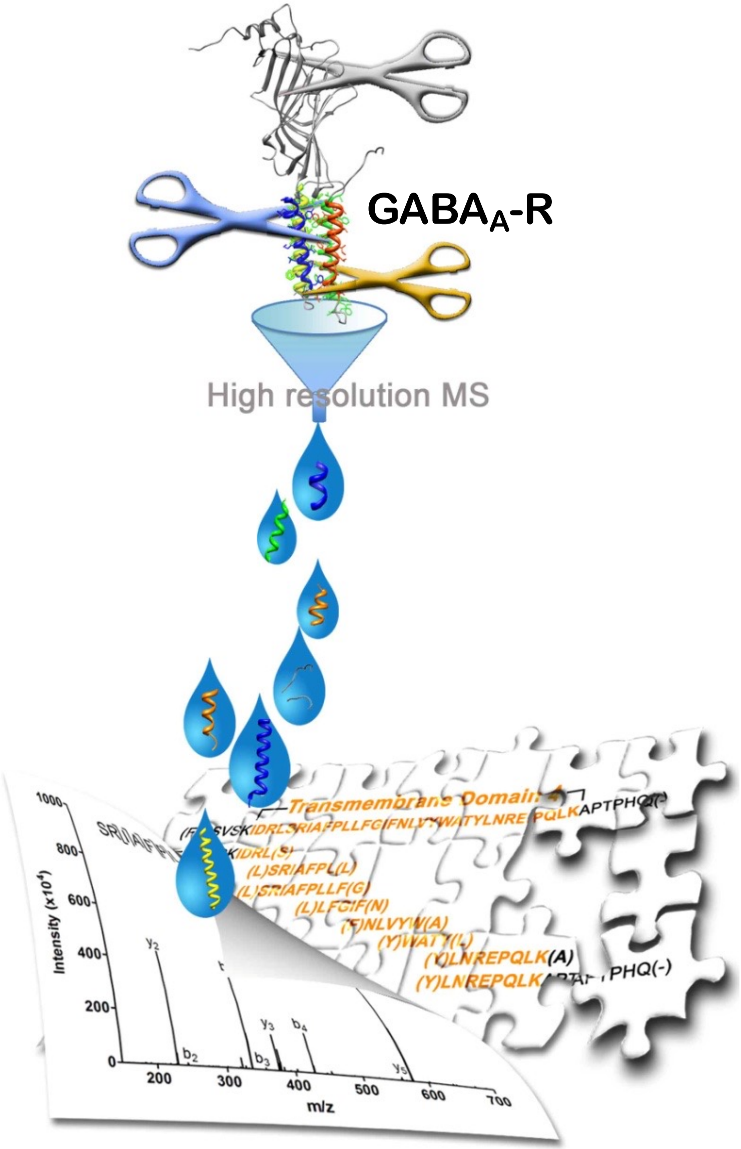
GABA$_A$-R
High resolution MS
Transmembrane Domain
Intensity (x10^4)
1000
800
600
400
200
0
200
300
400
500
600
700
m/z
y_2
b_2
b_3
y_3
b_4
y_5
(Y)LNREPQLK(A)
(Y)LNREPQLK
PHQ(-)