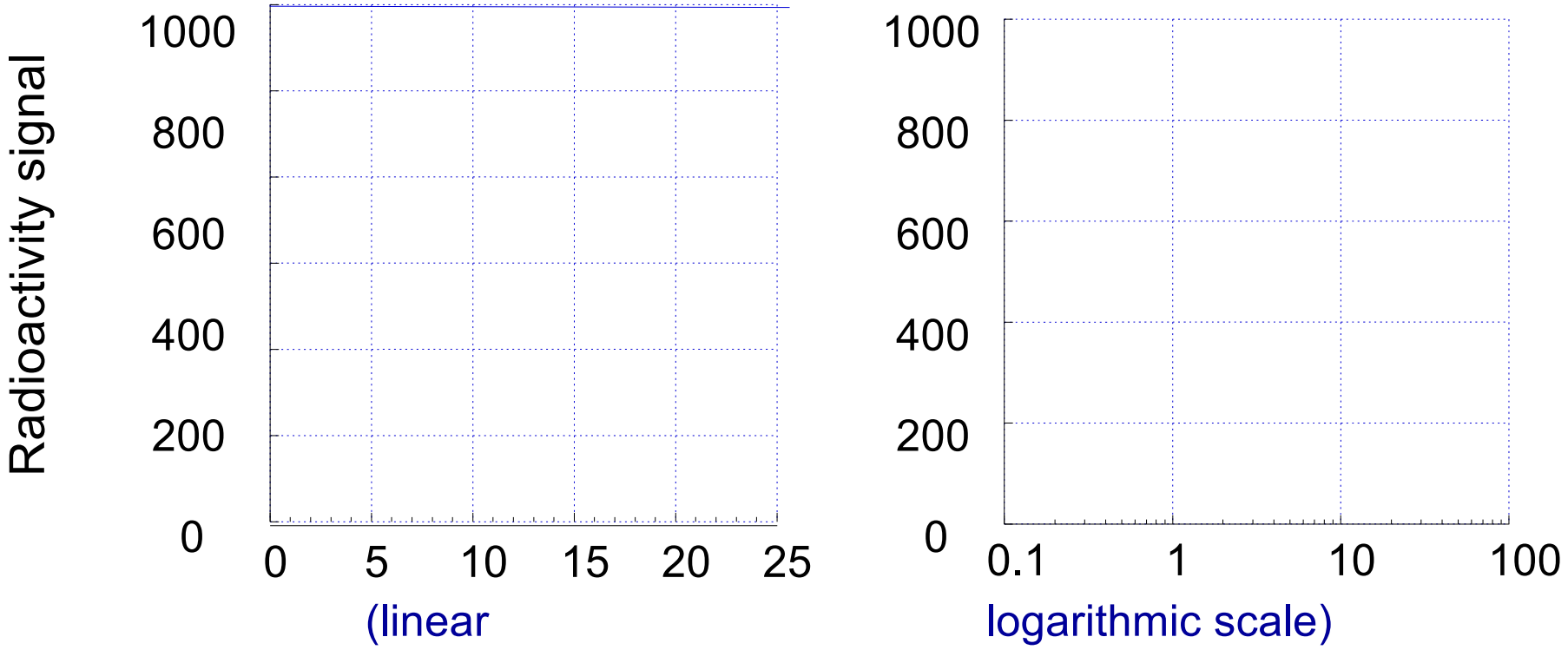
Analysis of the binding measurement

Radioligand: [3H]Ro-15-1788, ligand of GABAA receptors, binding to brain tissue

concentration radioligand (nM)	0.1	0.3	1	2	4	6	8	12	16	20
radioactivity bound (e.g. in cpm)	36.2	104	277	426	573	677	739	824	897	964



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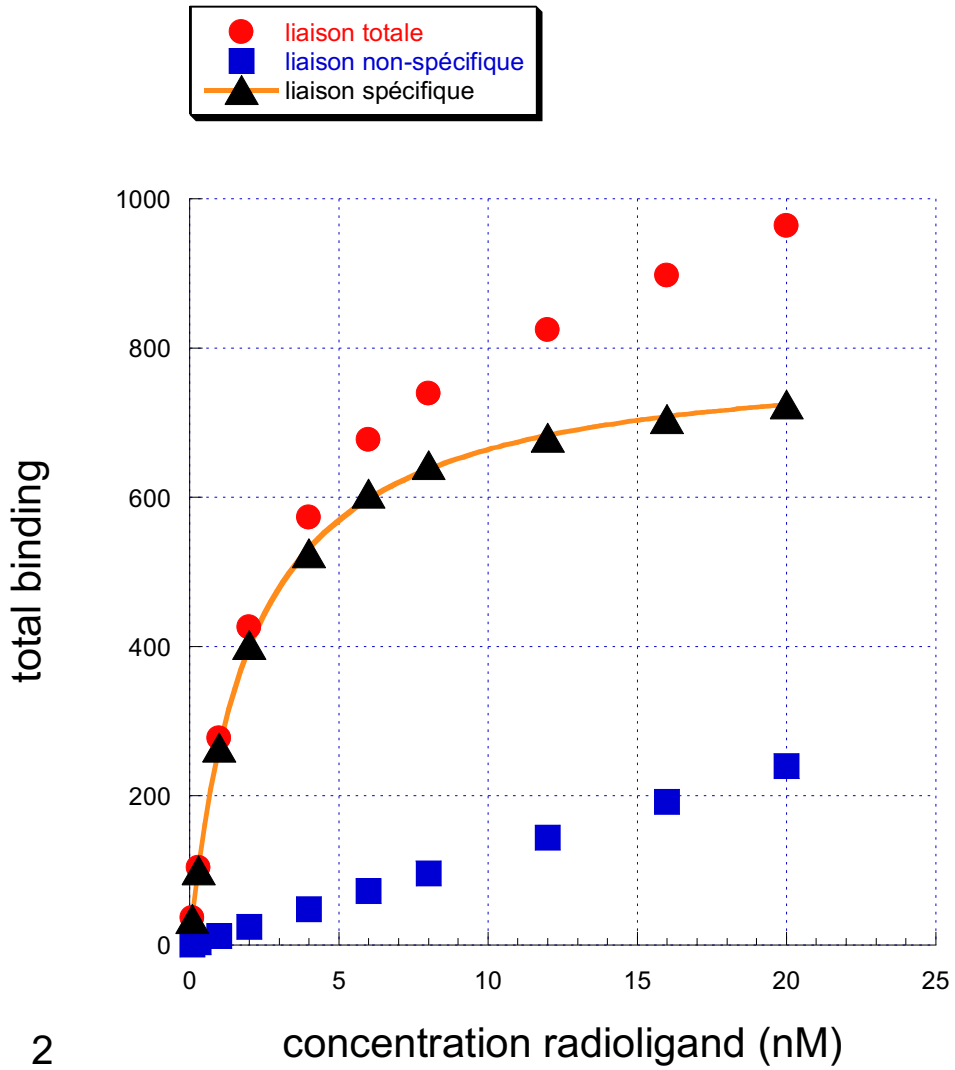
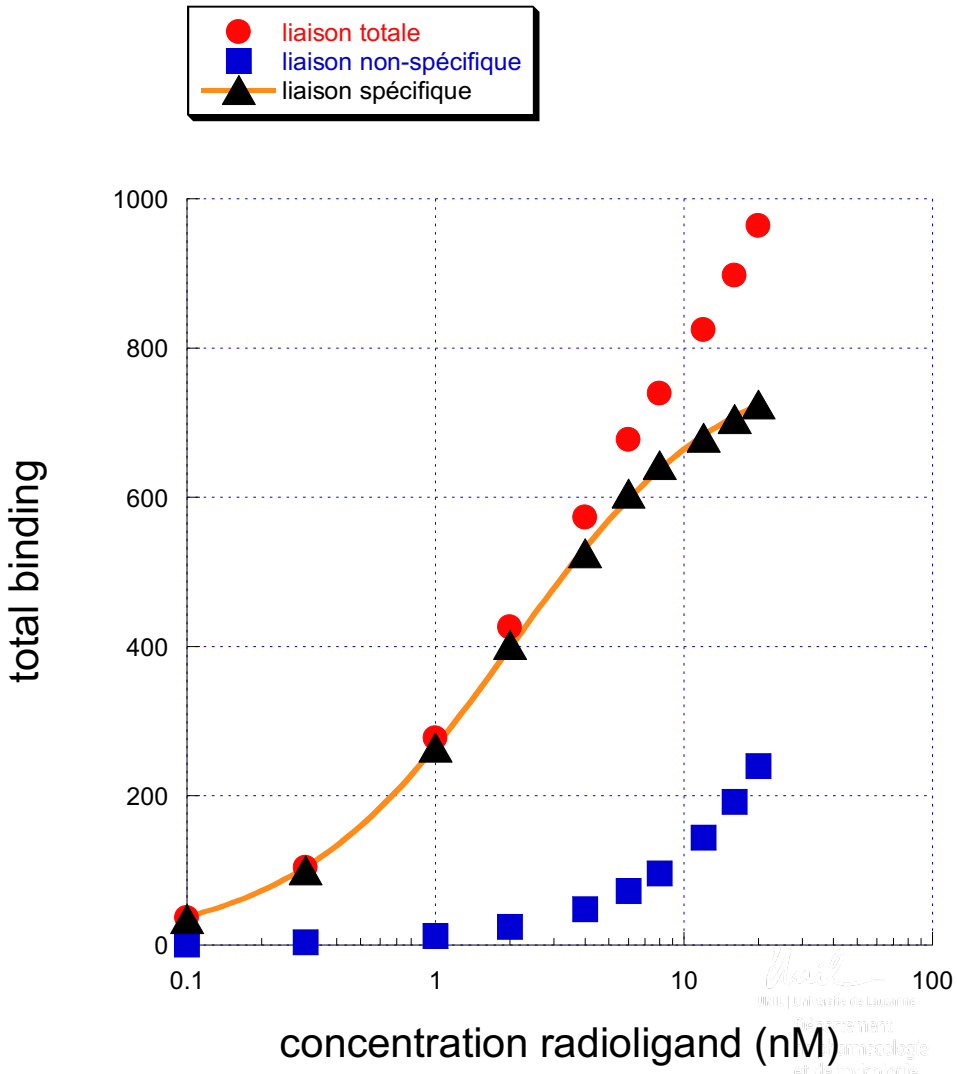
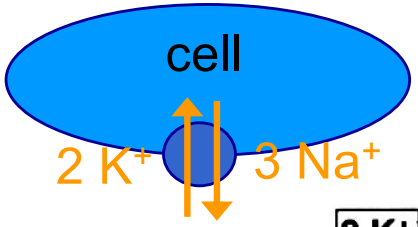
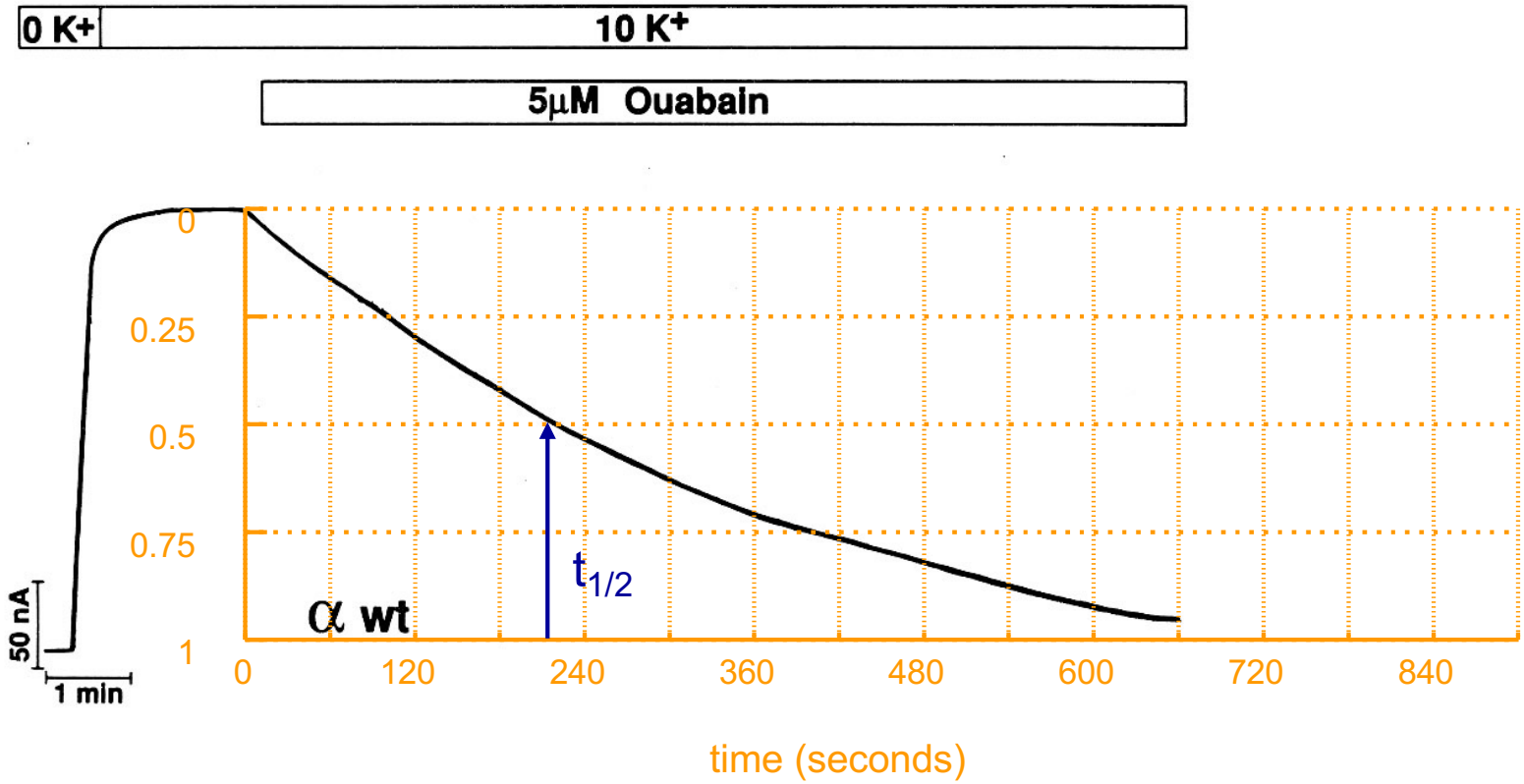
radioactivity bound (p.e. en cpm	36.2	104	277	426	573	677	739	824	897	964
non-specific binding	1.2	4	12	24	48	72	96	144	192	240
specific binding	**35**	**100**	**265**	**402**	**525**	**605**	**643**	**680**	**705**	**724**





Examples of the kinetics of an effect: measurement of k_{on} by the appearance of an effect



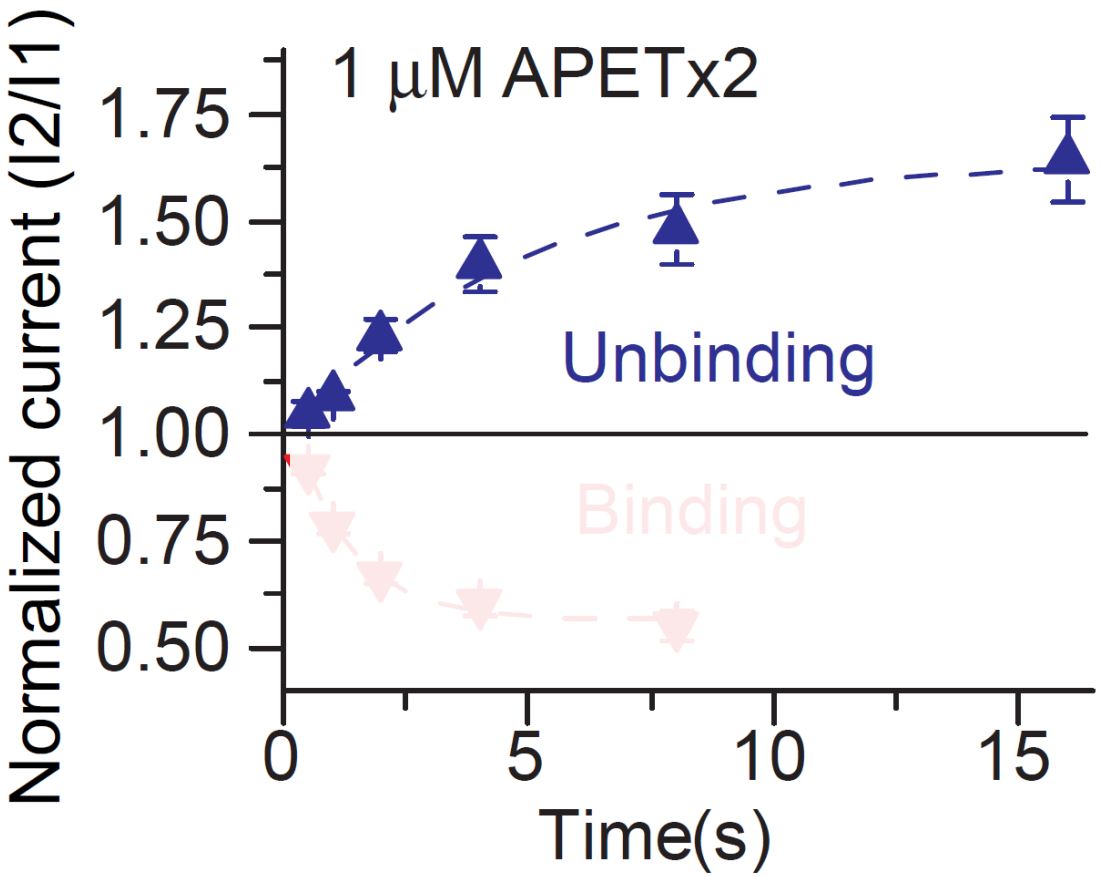


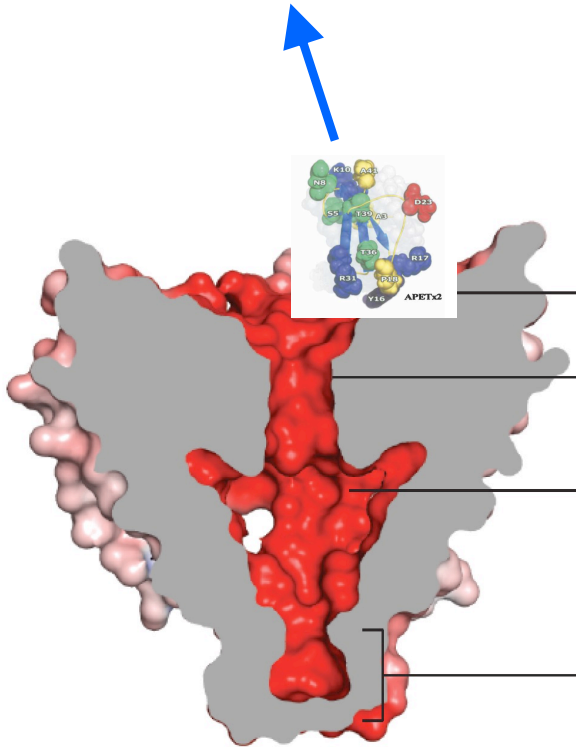
Appearance of the inhibitory effect of 5 µM ouabain on the current generated by the Na,K-ATPase : determine the k_{on} *(Assumption: current inhibition occurs when ouabain binds; and $k_{off} << k_{on} * x_A$).*

Solution: t1/2 = 210 s (from graph); the $k_{observed} = \ln(2)/t_{1/2} = 0.0033\ s^{-1}$; Since $k_{observed} = kon * x_A$, and x_A=5 µM ($5*10^{-6}$M), kon =660.14 $s^{-1}\ M^{-1}$

Off rate k_{off}

APETx2, a 40 aa toxin, and Nav1.8



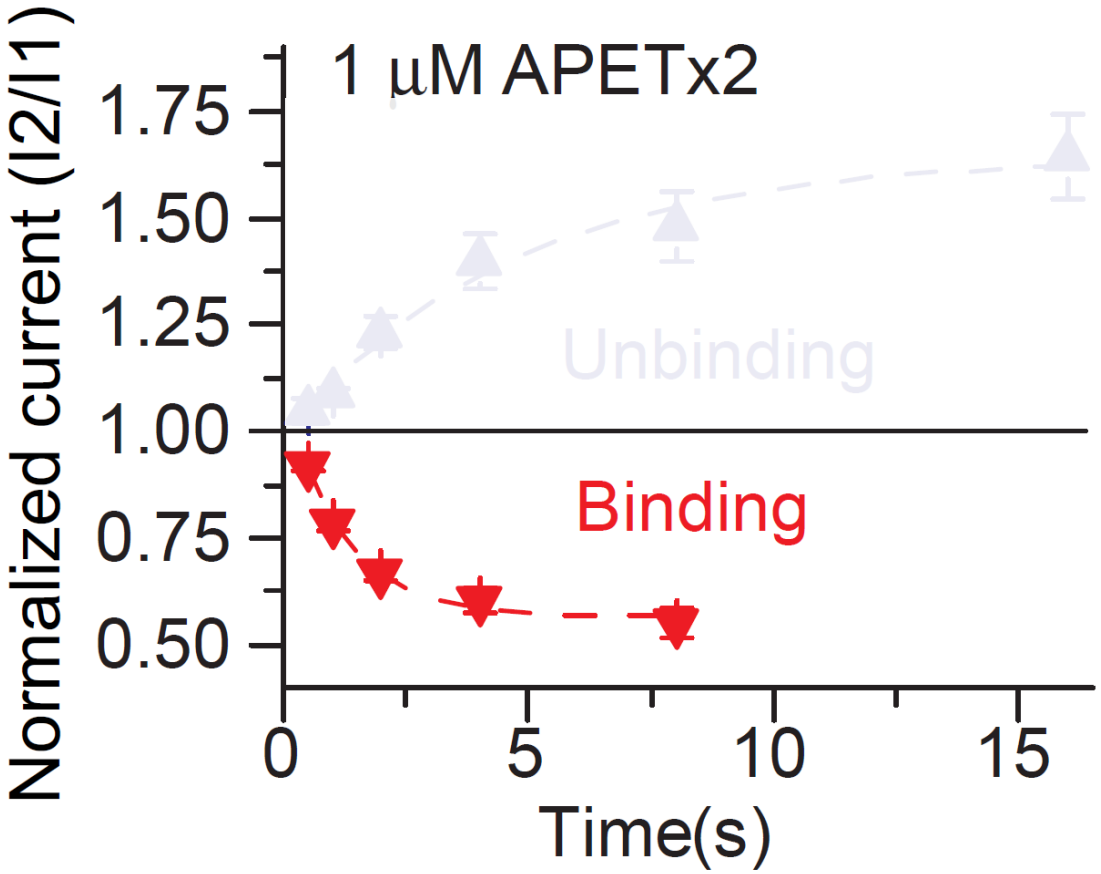


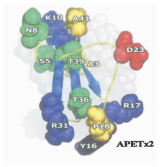
t1/2 = 2.5 s

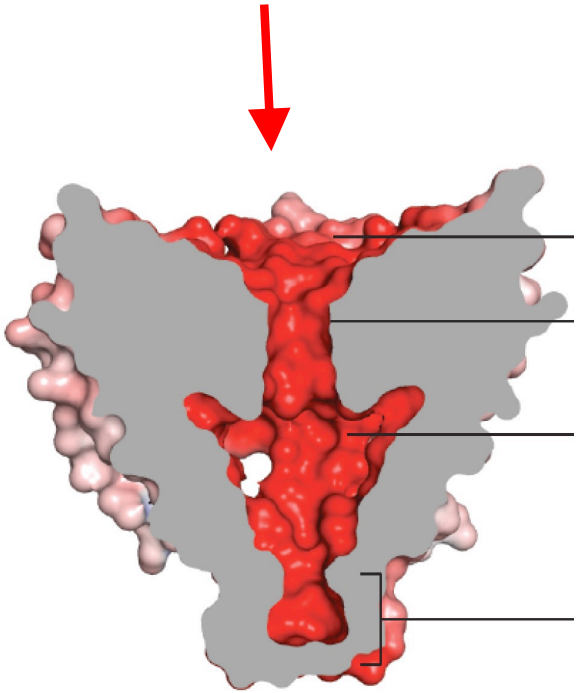
k_{off} (dissociation rate constant) = $\ln(2)/ t_{1/2}$ =0.28 s^{-1}

On rate k_{on}

APETx2, a 40 aa toxin, to Nav1.8







k_{obs} (at 1 µM) = 0.67 s^{-1}

$k_{obs} = x_A * k_{on} + k_{off}$ (where x_A = concentration)

$k_{on} = (k_{obs}-k_{off})/x_A$ = 400000 s^{-1} M^{-1}

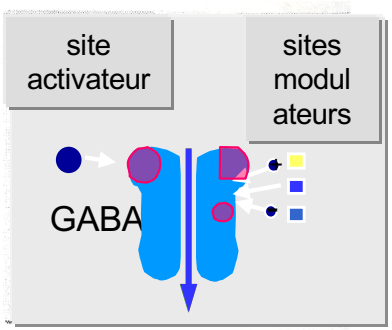
Exercise Glutamate

In synapses of the CNS, high concentrations of the neurotransmitter glutamate are reached in the synaptic cleft after presynaptic stimulation. Glutamate acts on post-synaptic receptors, as e.g. NMDA receptors. Glutamate is rapidly cleared from the synaptic cleft, and it is estimated that glutamate stays less than 1 ms at these high concentrations in the synaptic cleft. The removal of glutamate from the synaptic cleft is faster than its unbinding from the postsynaptic receptors, therefore the unbinding (koff) of glutamate determines the current decrease. On high affinity NMDA receptors glutamate has a Kd of 1 μM, and its k_{on} is $2 \cdot 10^8$ M^{-1} s^{-1}.

→ Calculate the time course ($t_{1/2}$) of glutamate unbinding from the NMDA receptor.

Solution: Kd=koff/kon; from this, koff= 200 s^{-1}. Since t1/2=ln(2)/k, t1/2 of the unbinding is 3.5 ms

Exercice agonistes/antagonistes récepteur GABA



Expérience

interprétation

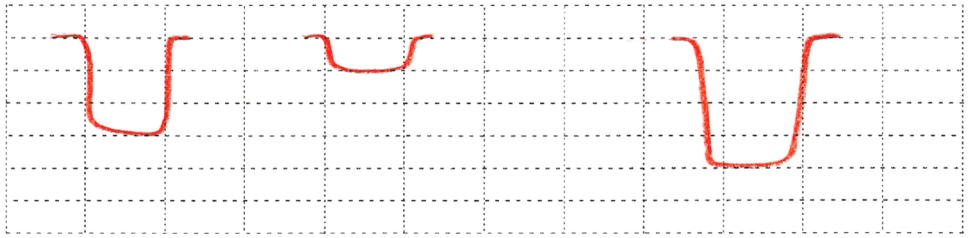
(agoniste, etc; site activateur/modulateur)

GABA Substance A GABA + substance A

exemple

A is an agonist at the modulatory site

GABA Substance B GABA + substance B

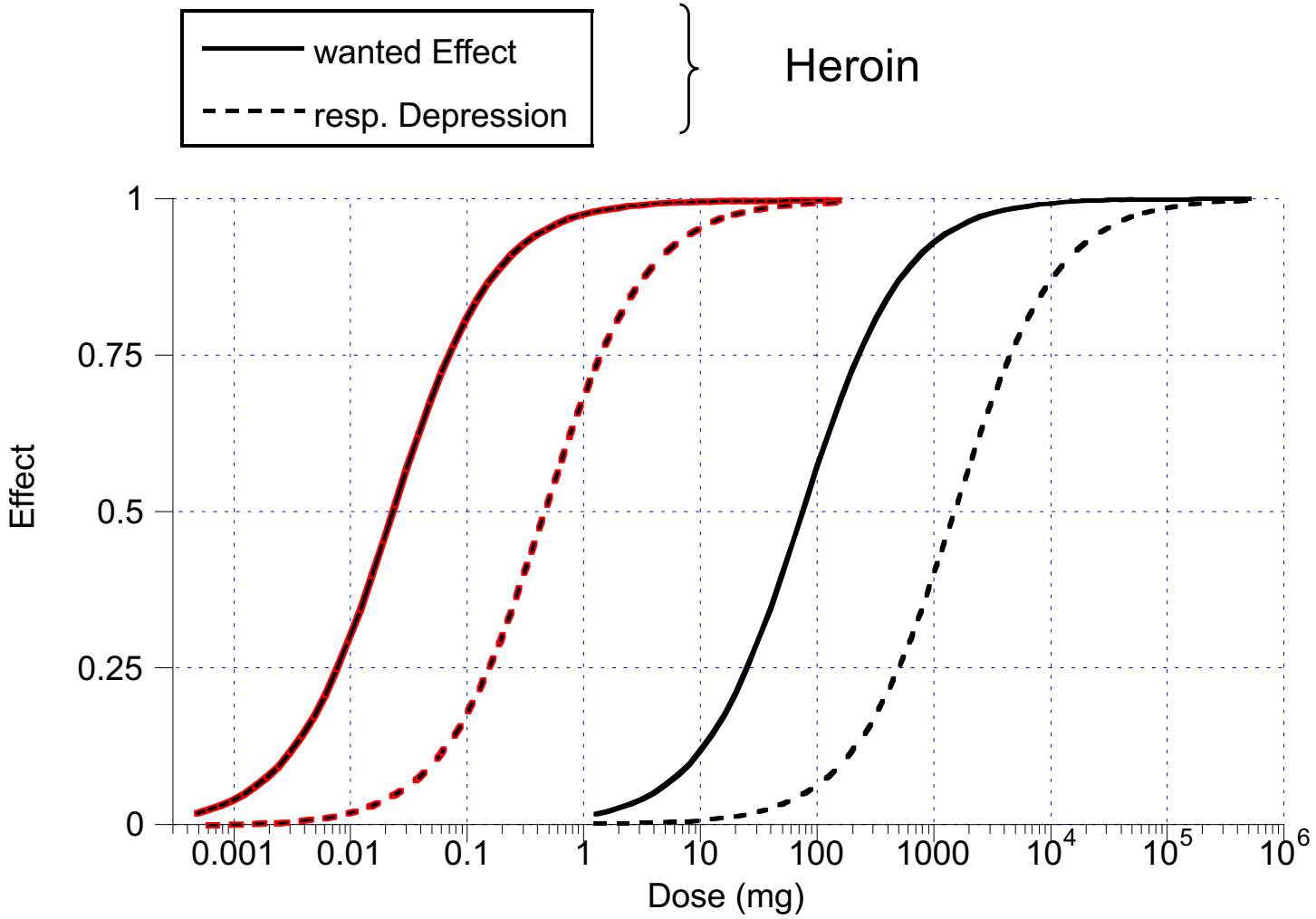


B is an agonist (possibly a partial agonist) at the activator site. We can not exclude from the experiment that it does not also act as agonist at the modulatory site

GABA Substance C GABA + substance C GABA + Diazepam GABA + Diazepam + substance C

C is an antagonist at the modulatory site

Exercise Heroin and 3-methyl fentanyl

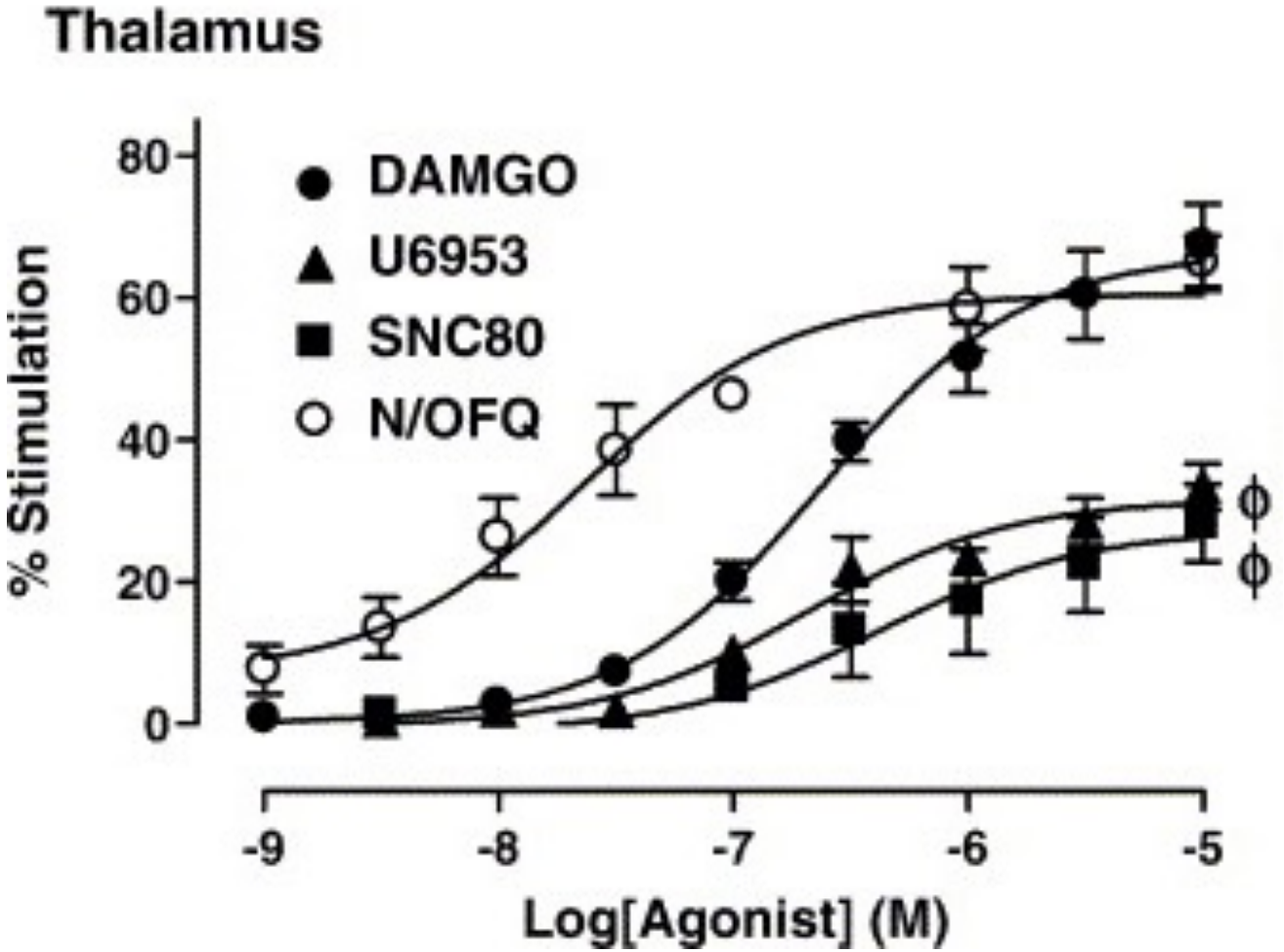


Heroin is usually measured in doses of 25-mg bags. Unsuspecting users of China White, who might have achieved a desired “high” with three bags of heroin, died after injecting one bag of 3-methyl fentanyl. Assuming that 3-methyl fentanyl has a 3000x higher potency than heroin, for the wanted and unwanted effects,

- draw the two corresponding curves for 3-methyl fentanyl
- explain why the drug users died

Solution: see red curves. They are both shifted by 3000-fold to lower doses. The drug users died because at the dose taken (25 mg), they had maximal unwanted effects.

Compare the efficiency and the potency of these agonists

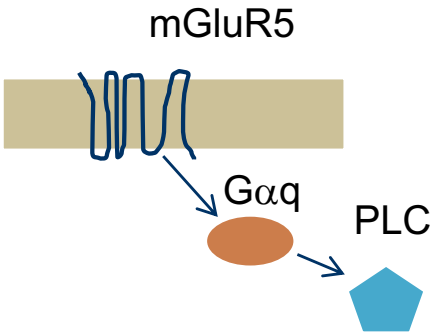


	EC 50	Max stimulation (%)
DAMGO		
U6953		
SNC80		
N/OFQ		

N/OFQ has the highest potency, DAMGO has the highest efficacy

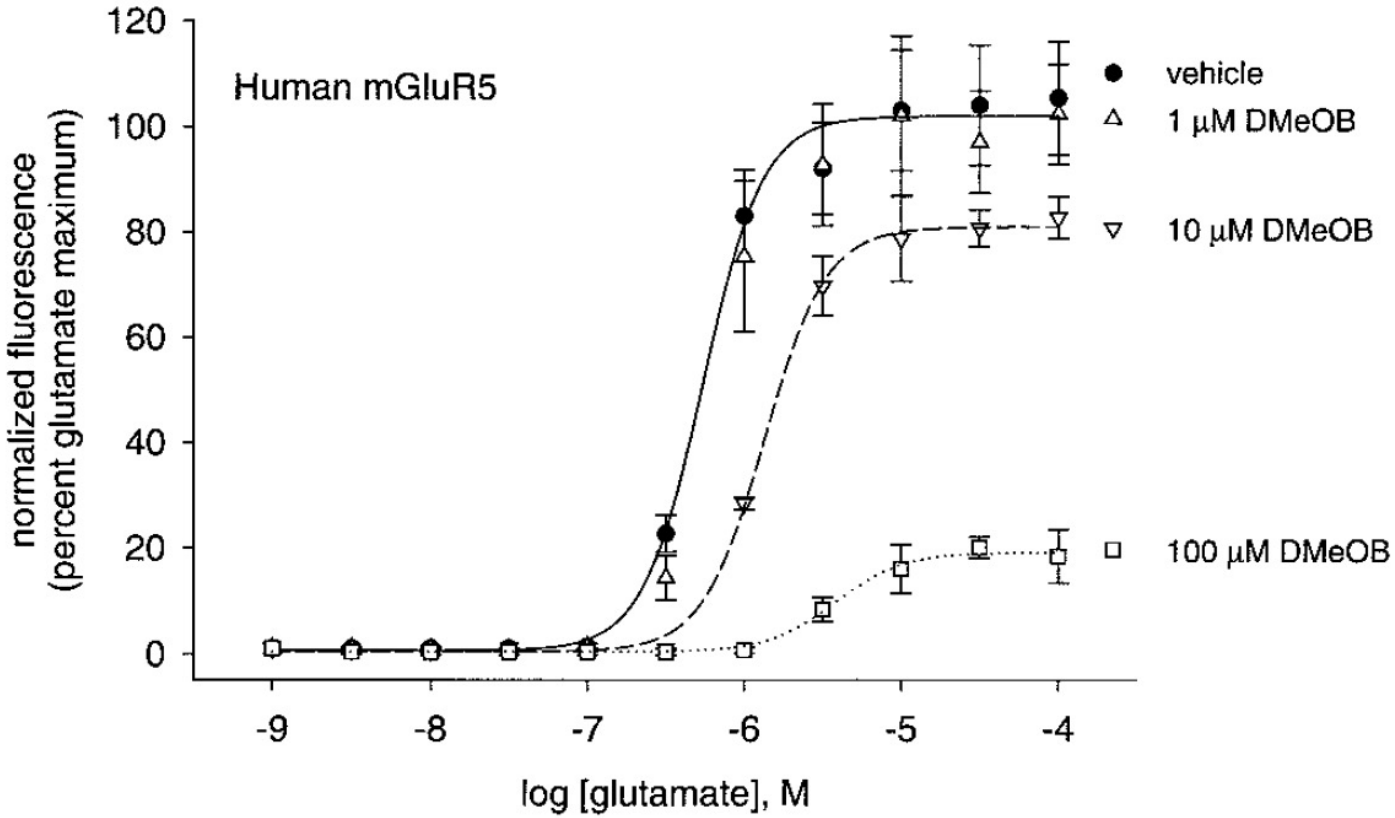
Fig. 1. Stimulation of [^{35}S]GTPγS binding by selective μ (DAMGO), δ (SNC80), κ (U69593) or ORL$_1$ (N/OFQ) agonists in frontal cortical membrane homogenates or thalamic membrane homogenates of the dog. Concentration–effect curves were generated using 15–20 μg membrane protein and of 0.1 nM [^{35}S]GTPγS as described in Experimental procedures. Data are expressed as mean values ± SEM from experiments performed in tissues from two female dogs and one male dog repeated twice in duplicate. *P < 0.001 versus U69595, P < 0.01 versus N/OFQ, P < 0.05 versus SNC80; ϕP < 0.001 versus DAMGO and N/OFQ.
(Brain Res. 1073-1074, 290)

Example: antagonism of mGluR5 receptor



→ Calcium release from intracellular stores

→ *measured by fluorescence*



Question: what type of antagonist is DMeOB? Answer: a non-competitive antagonist

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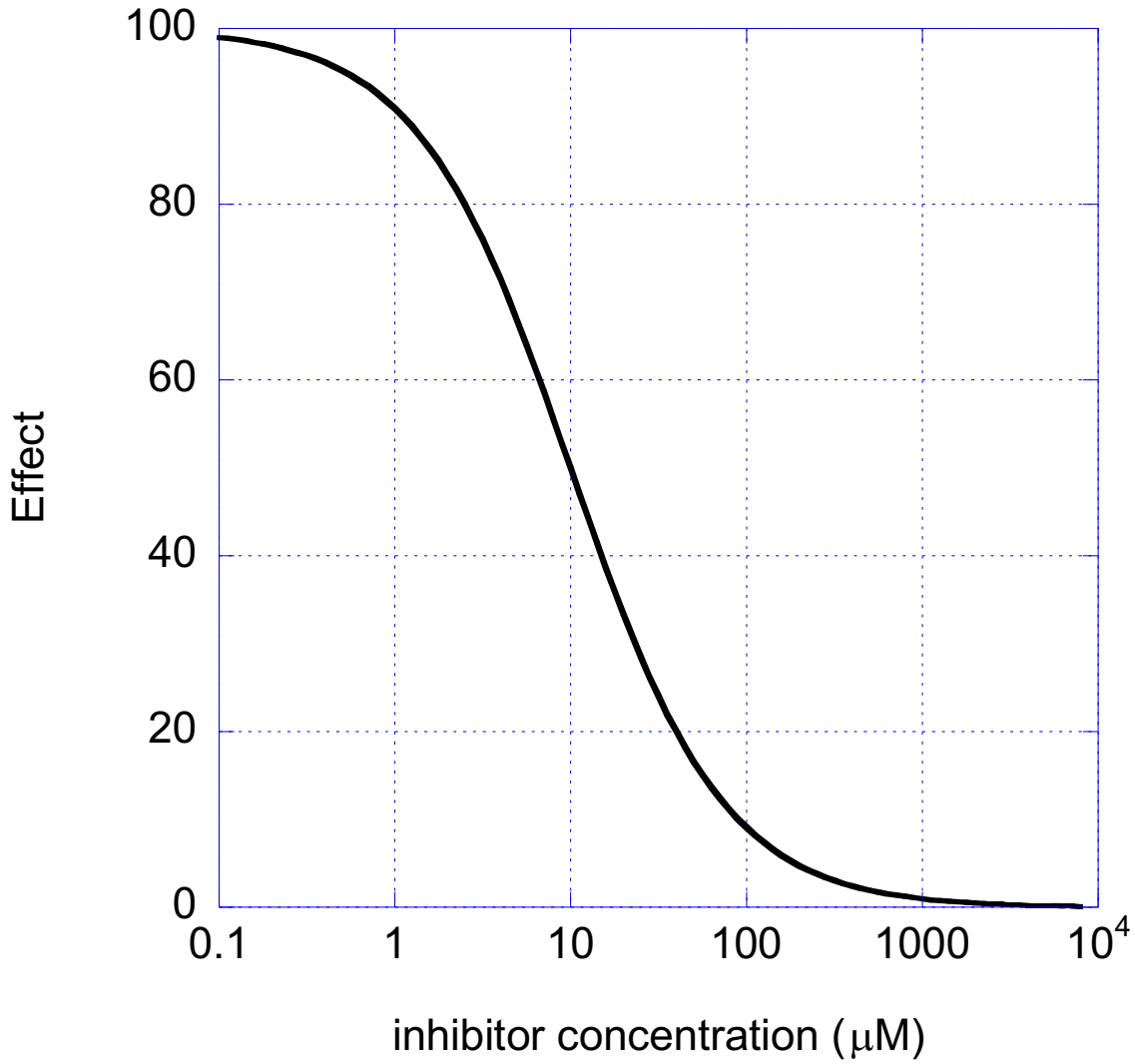


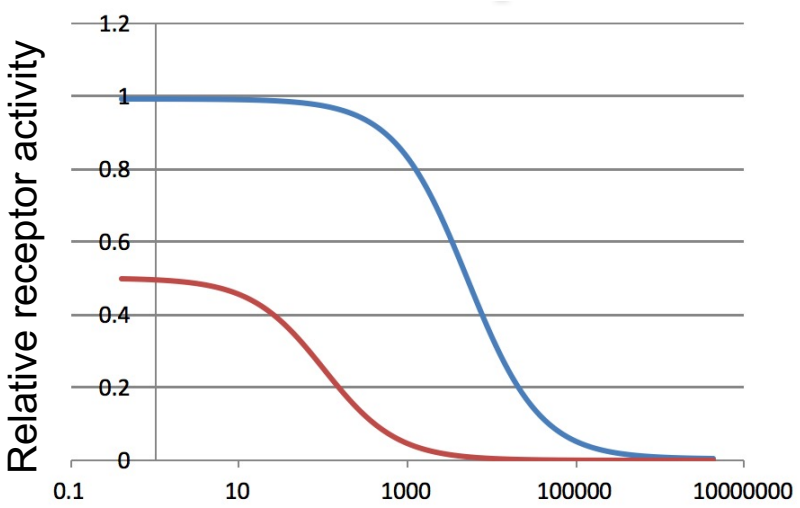
The inhibition curve is measured with increasing concentrations of antagonist, and at one given concentration of agonist (always the same for this curve)
Imagine now that you would carry out an inhibition curve in the same system (receptor, agonist, antagonist), but using a lower agonist concentration, which, in the absence of any antagonist (thus on the left end of the graph), would induce 50% of the effect.

- Draw this curve, for the case of a competitive, and for a non-competitive antagonist
- Will the IC50 be the same as in the existing curve in the graph (obtained at the higher agonist concentration)?

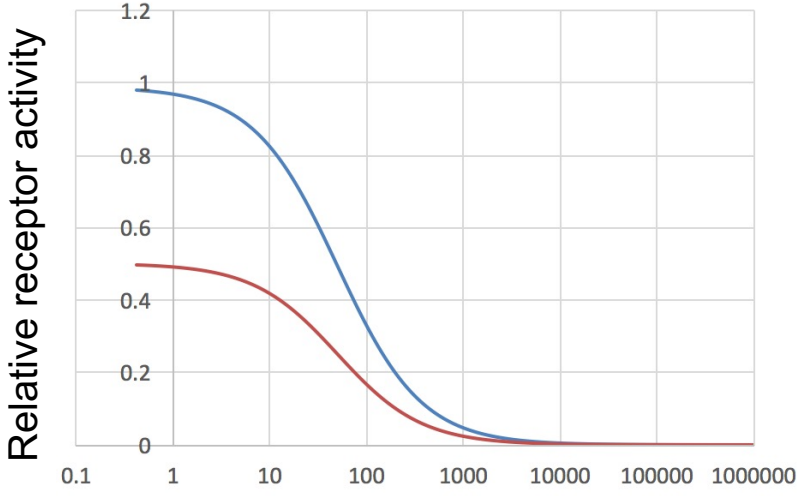
Inhibition curves at two agonist concentrations

For the same pair of agonist and antagonist, an inhibition curve (response to a series of different antagonist concentrations) is measured at two different agonist concentrations. It the antagonist is competitive, the observed IC50 will be different at the two conditions. If the antagonist is non-competitive, the observed IC50 will not be influenced by the agonist concentration.

Competitive Antagonist



Non-competitive Antagonist



At the lower agonist concentration, the IC50 of inhibition by a competitive antagonist is higher than it is at the higher agonist concentration.

(blue curve = higher, red curve = lower agonist concentration)

1. At μ-opioid receptors, Morphine has a Kd of 2.5 nM (K_A), while the antagonist Naloxone has a Kd of 4.4 nM (K_B). Calculate the occupancy of the receptor by Morphine (or 3-methyl-fentanyl)

- in the presence of 10 nM Morphine (or 3-methyl-fentanyl) (x_A) (without antagonist), and
- in the presence of 10 nM Morphine (or 3-methyl-fentanyl) (x_A) and 50 nM Naloxone (x_B)

(*assuming a K_A for China White of 0.4 pM = 0.0004 nM*)

$$pA = \frac{x_A/K_A}{x_A/K_A + x_B/K_B + 1} \quad (17)$$

Exercise: binding antagonism				
Morphine or ChinaWhite displacement by the antagonist Naloxon				
what we know:			unit	
Morphine	Kd	2.5	nM	
Naloxon	Kd	4.4	nM	
ChinaW	Kd	0.0004	nM	
		Agonist		
calculated:		conc		pA
Morphine		10	nM	0.8
Morphine 10 nM, with Naloxone 50 nM		10	nM	0.244444444
ChinaW		10	nM	0.999958335
ChinaW 10 nM, pA with Naloxone 50 nM		10	nM	0.999485114

Additional exercises

1. At μ-opioid receptors, Morphine has a Kd of 2.5 nM, while the antagonist Naloxone has a Kd of 4.4 nM. Calculate the occupancy of the receptor by Morphine

- in the presence of 10 nM Morphine (without antagonist), and
- in the presence of 10 nM Morphine and 50 nM Naloxone

Solution: previous slide

2. Omeprazol is an inhibitor of the gastric H,K-ATPase that binds to its receptor by forming an –S-S- bridge between a cystein residue of the receptor and an –SH group of the ligand. Its K_{on} for binding to the H,K-ATPase is 2000 $s^{-1}.M^{-1}$.
After how much time will an inhibition of 90% be obtained at an omeprazol concentration of 100 nM?

3. Using an electrophysiology approach you measure ionic currents induced by GABA in cells expressing $GABA_A$ receptors. The currents that you measure during application of the following concentrations of GABA are:

GABA Concentration	0.2 μM	1 μM	2μM	6 μM	12 μM	40 μM	100 μM
current (pA)	440	1750	2800	4700	5650	6500	6800

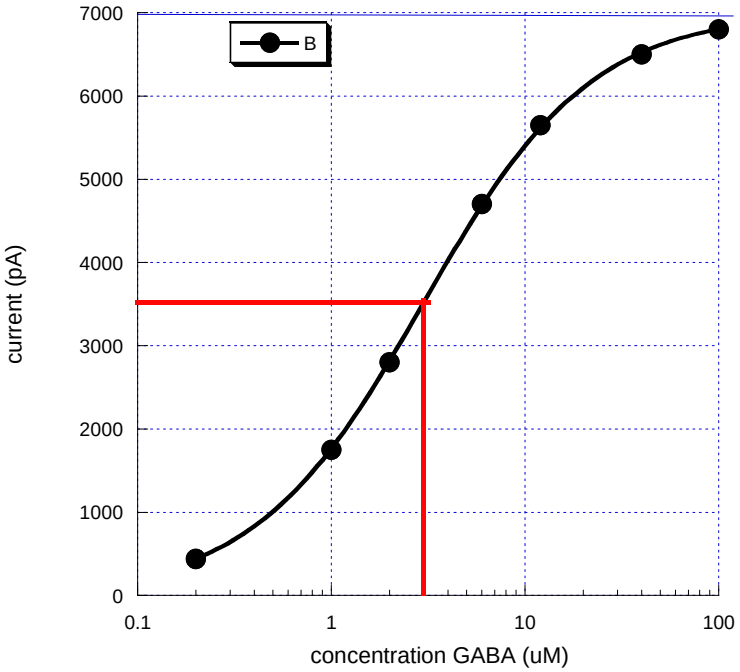
Draw a concentration-effect curve and determine the EC50 and the maximal effect.

4. Using the agonist A at a concentration of 10 μM you obtain an effect that equals 75% of the maximal effect. Which concentration of the agonist A do you need to apply to obtain the same activation level (75% of the maximal effect) in the presence of 500 nM of a competitive inhibitor whose Ki is 100 nM?

Strategies/Answers for exercises Pharmacodynamics by Stephan Kellenberger

Question Nr.	Situation at equilibrium?	Strategy/Answer
1	yes	For the situation without the antagonist, the occupancy is calculated according to the binding equation of the class, as pA=1/(1+(Kd/xA))= 1/(1+(2.5/10)=0.8. For the situation with the Antagonist present, the equation for competitive antagonism has to be used, pA= (xA/KA)/[(xA/KA)+(xB/KB)+1], with KA being the dissociation constant of the agonist and xA its concentration, and KB being the dissociation constant of the antagonist and xB its concentration. This will result in an occupancy of morphine (pA) in the presence of 50 nM Naloxone of 0.24.
2	No	you need to use the exponential equation that describes the association of the drug to its receptor. This equation is: $pA = 1-e^{-kt}$. pA is the occupancy, that you want to reach (90% --> pA=0.9), k can be calculated from the concentration of the drug and the Kon ($k= 2000s^{-1}M^{-1} \times 10^{-7}$ M = $2x\ 10^{-4}$ s^{-1}. t, is the time to reach an inhibition of 90%, thus what you want to know. You have to solve the equation for t. This will yield $t = -(\ln(1-pA))/k = -\ln(0.1)/2.10^{-4}s^{-1}$ = 11513s = 3.2 h.
3	Yes	Draw a concentration-response curve as discussed in the class. Determine the maximal response (maximal current = ~7000pA, blue line in Fig.) and determine the EC50 (red line in figure), which is 3 μM.
4	Yes	<u>Strategy 1</u> 1. From the situation in the absence of the antagonist you have all the information to determine the K_A, the dissociation constant of the agonist, by using equation 8. 2. You use the equation of competitive antagonism to solve this problem. You have all the information, you just have to solve the equation for xA. Kd is 3.3 μM, xA1 (=concentration of agonist for a pA of 0.75 in absence of antagonist) is 10 μM, and xA2 is 60 μM. <u>Strategy 2 (more direct)</u> xA1 is the concentration of agonist for a pA of 0.75 in absence of antagonist, xA2 is the concentration of agonist for a pA of 0.75 in presence of antagonist. Equation 8, with xA1, equals equation of p.70, with xA2. This is solved for xA2/xA1, as shown below. --> see more detailed calculation below

Exercise 3: Figure



Ex. 4

x_A = concentration of agonist A

$x_{A1} = 10\,\mu M$

x_{A2} = conc. of Agonist A, necessary to obtain a pA of 0.75 in the presence of the antagonist

x_B = concentration of competitive antagonist

K_B = Dissociation constant of the competitive antagonist

K_A = Dissociation constant of the Agonist

$$pA = 0.75 = \frac{1}{1 + K_A/x_{A1}} = \frac{x_{A2}/K_A}{x_{A2}/K_A + x_B/K_B + 1}$$

$\frac{1}{1 + K_A/x_{A1}}$: Agonist alone

$\frac{x_{A2}/K_A}{x_{A2}/K_A + x_B/K_B + 1}$: presence of agonist and antagonist

$$x_{A2}/K_A + (x_B/K_B) + 1 = (x_{A2}/K_A)(1 + K_A/x_{A1}) \quad \text{re-shuffle}$$

$$= \frac{x_{A2}}{K_A} + \frac{x_{A2} \cdot K_A}{K_A \cdot x_{A1}} \quad \Big| - \frac{x_{A2}}{K_A}$$

$$(x_B/K_B) + 1 = \frac{x_{A2}}{x_{A1}}$$

$$\frac{500nM}{100nM} + 1 =$$

$$6 =$$

From $x_{A1} = 10\,\mu M$ it follows that x_{A2} is $60\,\mu M$