Department of Pharmacology

Qualifying Examination (Part I)

July 15-19, 2013

ALL EXAMS TAKE PLACE IN THE BASS CONFERENCE ROOM, 436 RRB

Date	Time
Tuesday, July 16th	10:00 am – 12:00 pm – Exam #1
	1:00 pm – 3:00 pm – Exam #2
	3:00 pm – 5:00 pm – Exam #3
Wednesday, July 17th	10:00 am – 12:00 pm – Exam #4
	1:00 pm – 3:00 pm – Exam #5
	3:00 pm – 5:00 pm – Exam #6
Thursday, July 18th	1:00 pm – 3:00 pm – Exam #7
	3:00 pm – 5:00 pm – Exam #8
Friday, July 19th	10:00 am – 12:00 pm – Exam #9
	1:00 pm – 3:00 pm – Exam #10
	4:00 pm ***(Results given to students – Pharm South Conf. Rm.)***

Please remember that this is a closed-book examination. You must be prepared to answer 4 of the 7 questions. Although not necessary, you may prepare written answers, overhead figures, or any type of materials that you think might be useful in the presentation of your answers. You may bring such preparation materials with you to the examination. The oral examination itself will not extend beyond two hours.

Prefrontal acetylcholine (ACh) release increases with attentional effort. The loss of prefrontal ACh afferents substantially lowers cue detection in attention tasks. The cortico-thalamic neurons of layer VI are very sensitive to nicotinic stimulation and are thought to control attentional performance. Layer VI neurons express the relatively rare $\alpha5$ nicotinic accessory subunit, in addition to the $\alpha4$ and $\beta2$ subunits that form the high-affinity nicotinic receptors. In layer VI neurons, the $\alpha5$ subunit is incorporated into the $\alpha4\beta2$ subtype of nicotinic receptors, greatly enhancing their conductance and currents.

The study examined the role $\alpha5$ and $\beta2$ subunits of the nicotinic receptor in cholinergic excitation of layer VI neurons and in attentional performance.

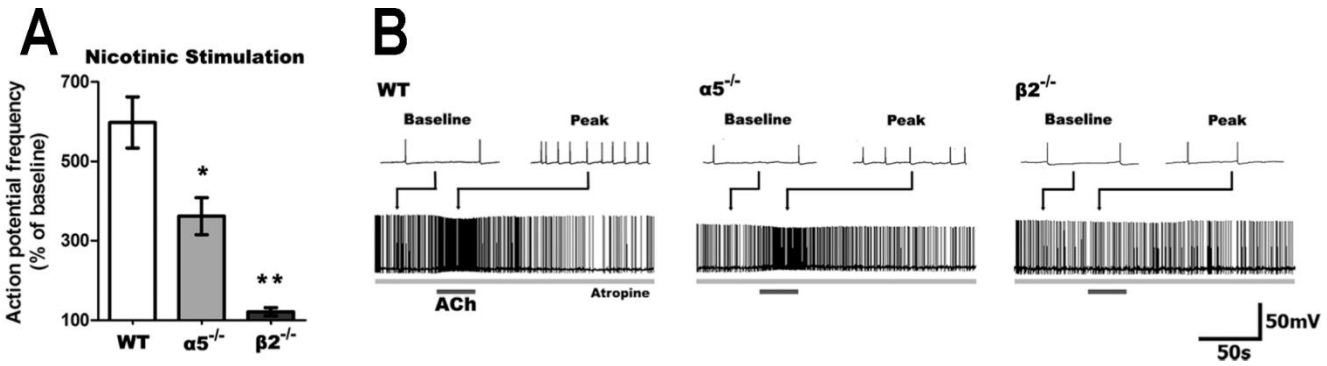


Figure 1. Nicotinic excitability of layer VI neurons in $\alpha5$-/- and $\beta2$-/- mice. (**A**) The effect of stimulation of only nicotinic receptors following blockade of muscarinic receptors by atropine (200nM) on firing frequency of layer VI neurons in different genotypes (*, p< 0.05 ; **, p<0.0001). (**B**) Sample traces show nicotinic responses in neurons across all genotypes.

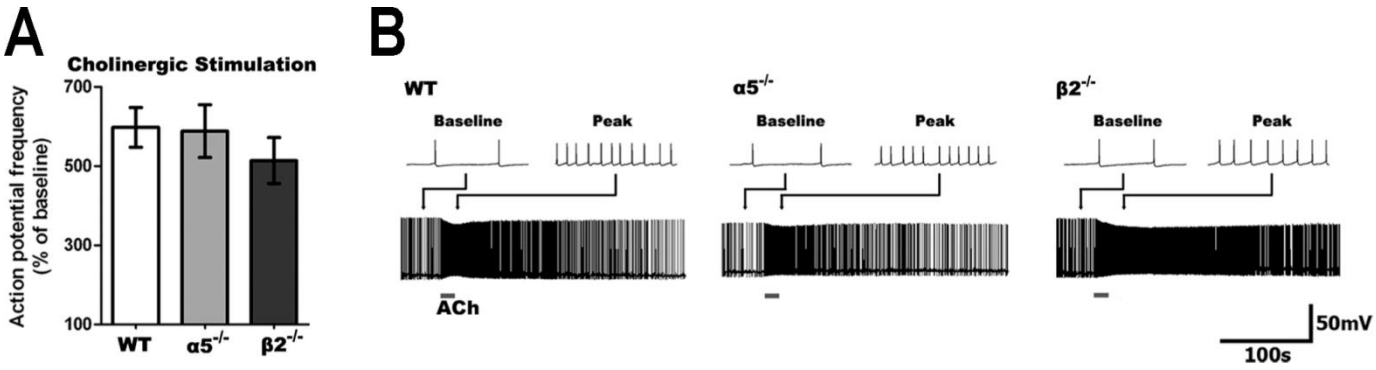


Figure 2. Cholinergic (nicotinic and muscarinic receptor) excitability in different genotypes. (**A**) The effect of cholinergic stimulation on layer IV neurons spike frequency in different genotypes (**B**) Sample traces showing cholinergic responses in neurons of all genotypes.

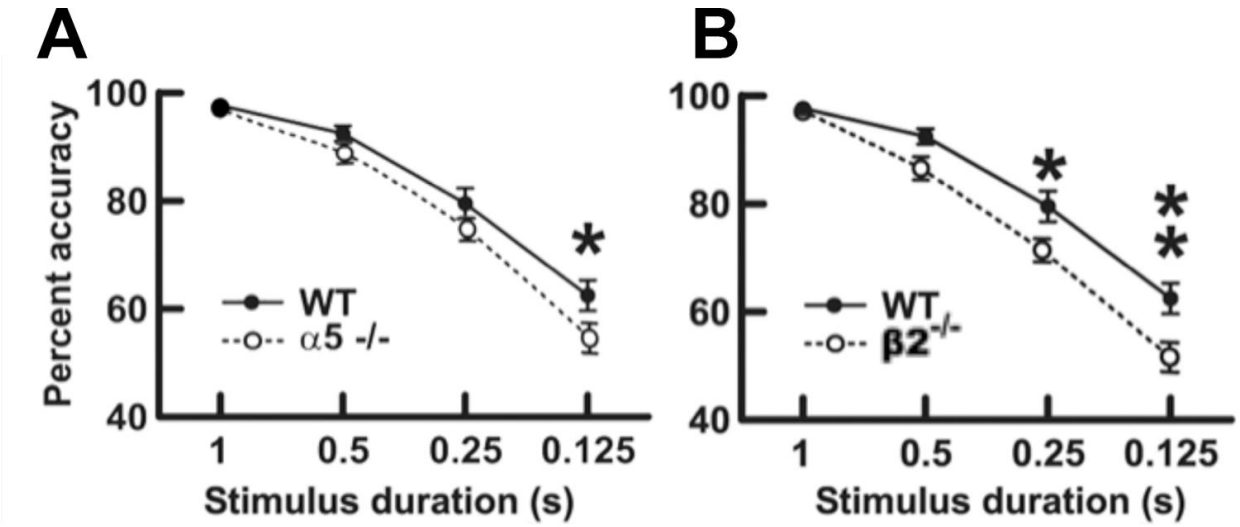


Figure 3. Attentional performance in mice lacking nicotinic receptor α5 or β2 subunit. WT mice and mice in which the α5 or β2 subunits had been genetically deleted (α5-/- or β2-/-) were trained on the five-choice serial reaction time test for sustained visual attention (**A**). Percent accuracy of WT and α5-/- mice depending on the difficulty of the task (lower stimulus duration makes the task more challenging), *p<0.05; (**B**) Percent accuracy of WT and β2-/- mice depending on the difficulty of the task, ** - p<0.01, * - p<0.05.

1. **Describe the salient features of the responses to nicotinic (Fig. 1) and cholinergic (Fig. 2) stimulation of layer VI neurons in mice lacking α5 or β2 subunits of the nicotinic receptor as compared to WT mice.**

2. **Propose a mechanism that explains the response of layer VI neurons in α5-/- and β2-/- mice to cholinergic stimulation.**

3. **How does the lack of α5 and β2 subunits affect attentional performance? Propose a mechanistic model explaining the behavioral and electrophysiological data. Propose experiments to test the model.**

Compound 1 is a potent ALK kinase inhibitor in vitro with potential anti-cancer activity. It was selected as a candidate to advance into efficacy studies in mice.

However, Compound 1 underwent enzymatic hydrolysis in mouse plasma or liver microsomes to an inactive primary amine product (Figure 1). Several lines of evidence indicated that metabolism/elimination of Compound 1 in murine tissues was saturable.

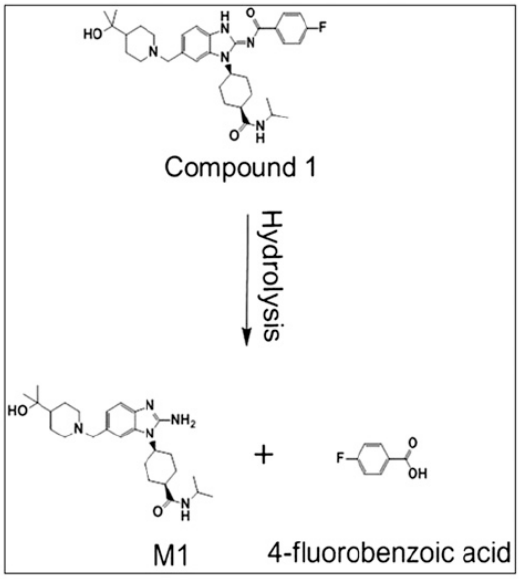


Figure 1: Structure of Compound 1 and its hydrolysis products

It was found that this hydrolysis is mouse-specific (Table 1).

Oral dose escalation studies in mice indicated that higher doses achieved sufficient exposures above in vitro IC50 for inhibition of ALK kinase (Figure 2, Table 2).

Analogs were synthesized with bulky groups designed to block enzymatic hydrolysis (Compounds 2, 3, and 4). These were compared with Compound 1 in vivo in mice (Figure 3).

TABLE 1
Intrinsic liver microsomal clearance (CL_{int}, μl/min.mg) of Compound 1 in liver microsomes

Concentration	Mouse +NADPH	Rat +NADPH	Dog +NADPH	Human +NADPH	Mouse −NADPH
0.1 μM	891	14	51	93	805
1 μM	110	13	33	67	50

CL_{int} in rat, dog, and human −NADPH was <5 μl/min·mg.

Fig. 2. Plasma concentrations of compound 1 after its oral administration to mice.

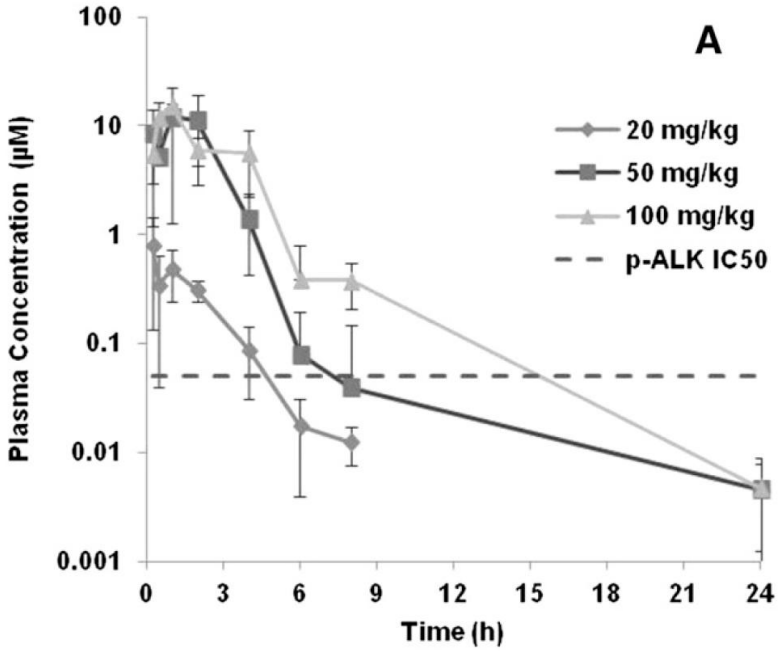


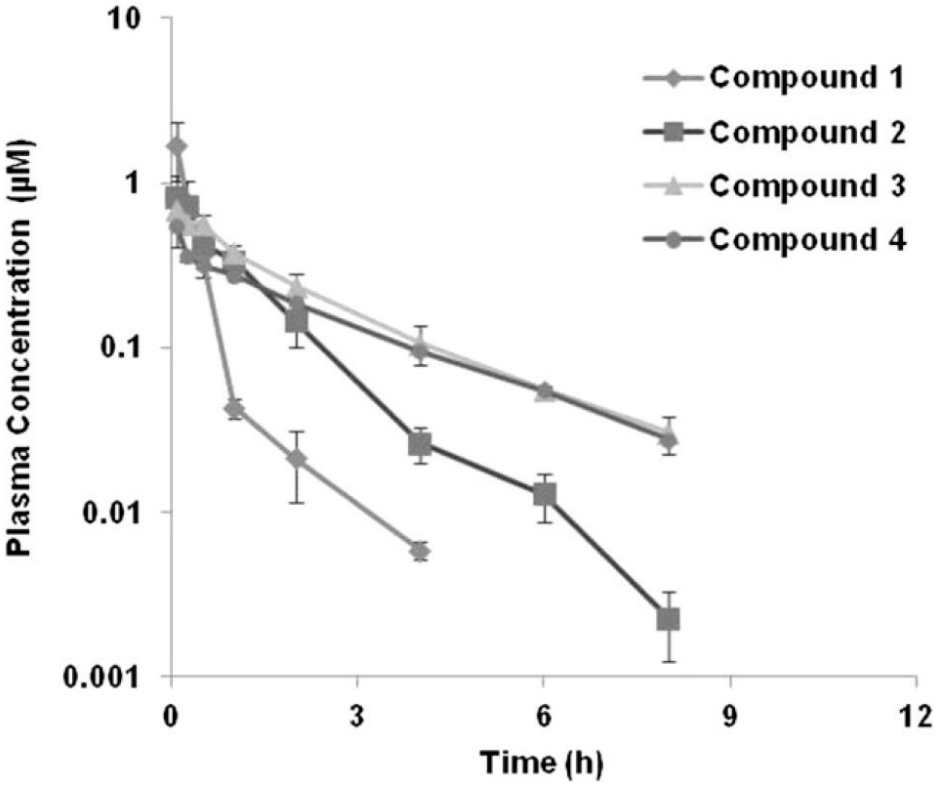
TABLE 2
Mean noncompartmental pharmacokinetics parameters for compound 1 after its oral administration to mice

A total of 24 animals (3 per time point) were used to generate the data for each dose group.

	Dose	C_{max}	T_{max}	$t_{1/2}$	$AUC_{0\text{-}inf}$	CL/F	F^a
	mg/kg	*μM*	*h*	*h*	*μM·h*	*l/h·kg*	*%*
Compound 1	20	0.782	0.25	1.23	1.31	26.2	21.1
	50	10.7	1.0	2.30	23.7	3.65	151
	100	15.2	1.0	2.17	37.4	4.63	120

Tmax is defined as the time at which the maximum concentration of drug in plasma (Cmax) is observed.
F is bioavailability. (F^a is bioavailability relative to 2 mg/kg i.v. dose).

Fig. 3. Plasma concentrations of ALK inhibitor analogs after 2 mg/kg i.v. administration to mice.



1. **A. Interpret the data in Table 1.**

   B. Interpret the data in Figure 2 and Table 2.

2. **Briefly evaluate the DMPK characteristics of Compounds 2, 3 and 4 compared to Compound 1.**

3. **Present your prediction of the appearance of the in vitro enzyme kinetics for metabolism of Compound 1 in mouse liver microsomes in the presence and absence of NADPH (Michaelis-Menten, initial rate versus substrate concentration). On the same graph, overlay your predicted result for the metabolism of Compound 4 under the same conditions.**

A young couple is seen in a fertility clinic for infertility. Examination of the man reveals gynecomastia (enlarged breast tissue), and he is found to have a very low sperm count. He has a brother with a similar condition.

Based on your knowledge of gonadal endocrinology, you hypothesize that the patient has a genetic defect in either A) testosterone production or B) androgen sensitivity.

1. **For each of these possible explanations, indicate the likely result in the measured level of the hormone listed (high, low, high in one case and low in the other case, or no change):**

	Testosterone	LH	FSH
A			
B			

2. **Choose either condition A or B and indicate the potential physiological and molecular mechanisms of the defect (proven or theoretical) and how you would test for them.**

3. **List 3 additional clinical manifestations (exam findings or symptoms) that might be seen with (A) and (B).**

Prostaglandin E_2 (PGE_2) has been implicated in the growth of colonic polyps and cancers. In gastrointestinal mucosa, activation of the EGFR switches on mitogenic signaling. Moreover, EGFR is up-regulated in colon cancer. To investigate how PGE_2 transactivates EGFR, several experiments were carried out in human colon cancer cells (Caco-2) and results are shown in Figures 1 and 2.

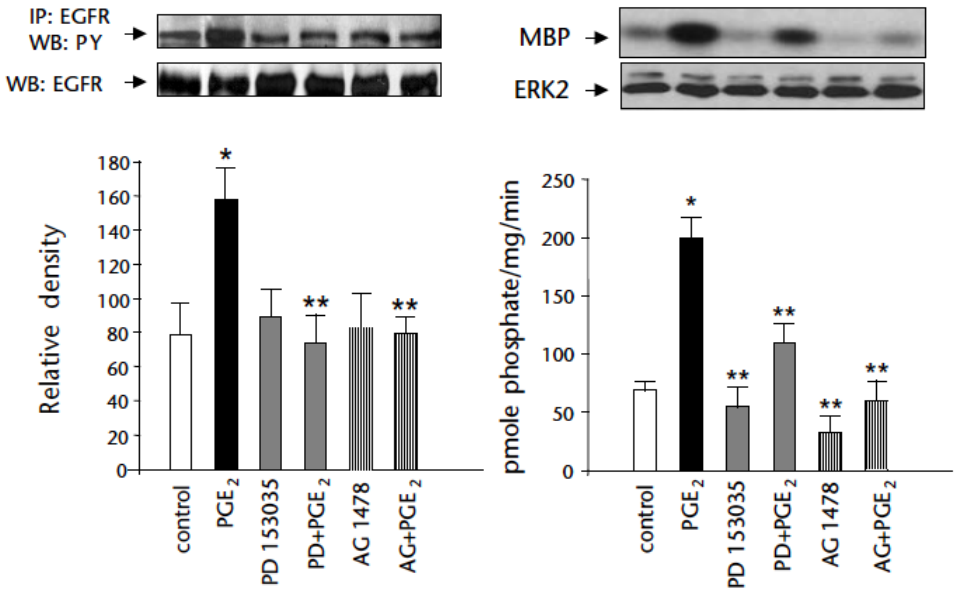


Figure 1: Effects of PGE_2 on the activation of EGFR (left). Cells were treated with PGE_2 or/and EGFR inhibitors as indicated below. Total EGFR or phosphorylated EGFR is determined by Western blotting (top). The ratios between phosphorylated EGFR and EGFR under different experimental conditions were quantified and compared (bottom). Both PD153035 and AG1478 are EGFR inhibitors. PGE_2 triggers ERK2/MAPK signaling (right). The ERK2 activity was analyzed by in vitro kinase assay (top) following immunoprecipitation from cell lysates using MBP (myosin basic protein) as a substrate in the presence of ^{32}P-ATP. Phosphorylation of MBP was compared (bottom). *, $p < 0.05$ versus control; **, $p < 0.001$, versus PGE_2.

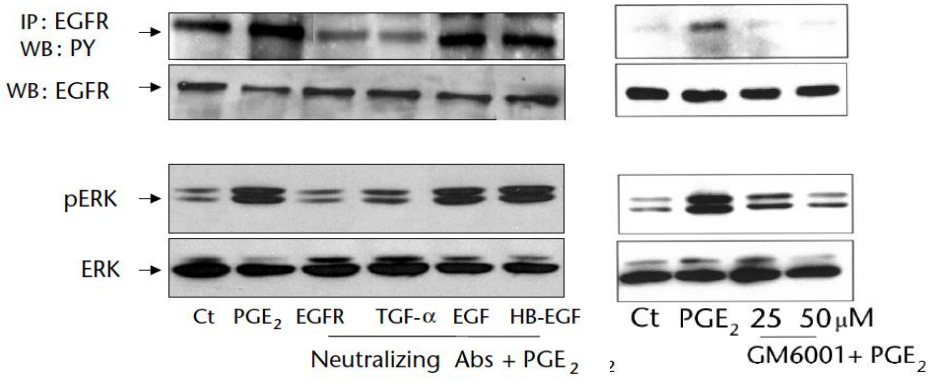


Figure 2: The effect of PGE_2 to transactivate EGFR in the presence of neutralizing antibodies against EGFR, TGF-α, EGF and HB-EGF) (left) and metalloprotease inhibitor, GM6001 (right). Ct, control

1. **Explain the experiments and results in Figures 1 and 2.**

2. **Based on these results, propose a hypothesis that links PGE_2 to the activation of EGFR and cell proliferation (use a diagram).**

3. **There are four EGF receptors, EGFR1-4. Provide two independent strategies/experiments to support that both EGFR1 and EGFR4 are critical for the PGE_2–mediated proliferation.**

Epacs (exchange proteins directly activated by cAMP) are guanine-nucleotide-exchange factors for the Ras-like small GTPases Rap1 and Rap2. Epacs are response elements for the second messenger cAMP acting in parallel to protein kinase A (PKA) via the small-G protein, Rap, and phospholipase-Cε. Cardiac Epac activation with the cAMP analog 8-CPT increases the frequency of brief, spontaneous openings of RyR2 sarcoplasmic reticulum Ca release channels (**Ca sparks**). Epacs are upregulated in heart failure and may contribute to the pathogenesis of cardiac hypertrophy, heart fibrosis and arrhythmia. However, the function of Epacs in heart are not completely certain because studies using the Epac activator 8-CPT are confounded by off-target effects of this compound. The following experiments were performed in isolated ventricular cardiomyocytes to elucidate the importance of two Epac isoforms in heart for regulating RyR2 Ca release channels using Ca sparks as the main readout.

Abbreviations and reagents:
CaSpF = Ca spark frequency, a measure of RyR2 Ca release channel activity
ISO = Isoproterenol
8-CPT = cAMP analog that activates both Epac 1 and Epac 2
H89 = PKA inhibitor
KN93 = Calmodulin kinase II (CaMKII) inhibitor
WT = Wild-type mice
KO = Knock-out mice
Epac DKO = Double knock out mice lacking both Epac isoforms
S2814A = RyR2 mutant mice lacking the CaMKII phosphorylation site on RyR2
S2814D = RyR2 mutant mice with phosphomimetic residue at the RyR2 CaMKII phosphorylation site
CaMKII KO = Mice lacking the CaMKIIδ isoform

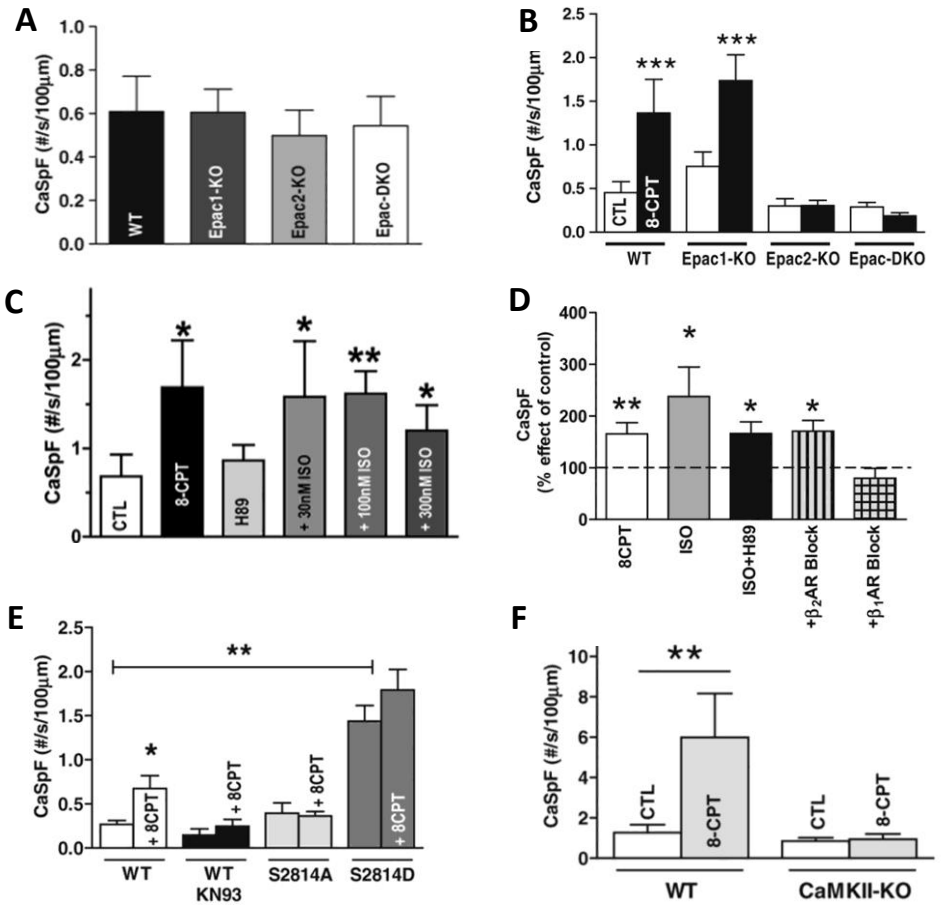


Figure legend: Mean Ca spark frequency (CaSpF) as number of sparks per 100 µm/s measured using confocal microscopy in isolated ventricular cardiomyocytes.

A. Mean CaSpF in baseline conditions. WT (n=25), Epac1-KO (n=14), Epac2-KO (n=39), and Epac-DKO (n=34).

B. Mean CaSpF in presence or absence of 8-CPT in WT (n=7), Epac1-KO in presence or absence of 8-CPT (n=13), Epac2-KO in presence or absence of 8-CPT (n=21), and Epac-DKO in presence or absence of 8-CPT (n=20).

C. Mean CaSpF in wild-type myocytes before and after 8-CPT, and after physiological activation of Epac (ISO + 2 µmol/L H89). Control (CTL; n=8), 8-CPT (n=8), H89 alone (n=8), 30 nmol/L ISO + H89 (n=4), 100 nmol/L ISO + H89 (n=6), and 300 nmol/L ISO + H89 (n=6).

D. Mean CaSpF in wild-type myocytes under 8-CPT (white, n=7), 100 nmol/L isoproterenol (ISO; n=5), 100 nmol/L ISO + 2 mmol/L H89 (n=7), ISO + H89 + 300 nmol/L CGP-20712A (β2-AR block; n=8), and ISO + H89 + 50 nmol/L ICI-118.551 (β1-AR block; n=7). Data are percent relative to control.

E. Mean CaSpF from wild-type (WT; n=16), S2814D (n=9), and S2814A mice (n=10) before and after 8-CPT in the absence or presence of KN93 (n=9) for WT.

F. Mean CaSpF from WT (n=10) and CaMKIIδ-knockout (KO; n=10) before and after 8-CPT

1. **Explain how Epacs regulate the RyR2 channel under baseline conditions and during beta-adrenergic stimulation.**

2. **Explain the signaling mechanisms upstream and downstream of Epacs involved with RYR2 regulation.**

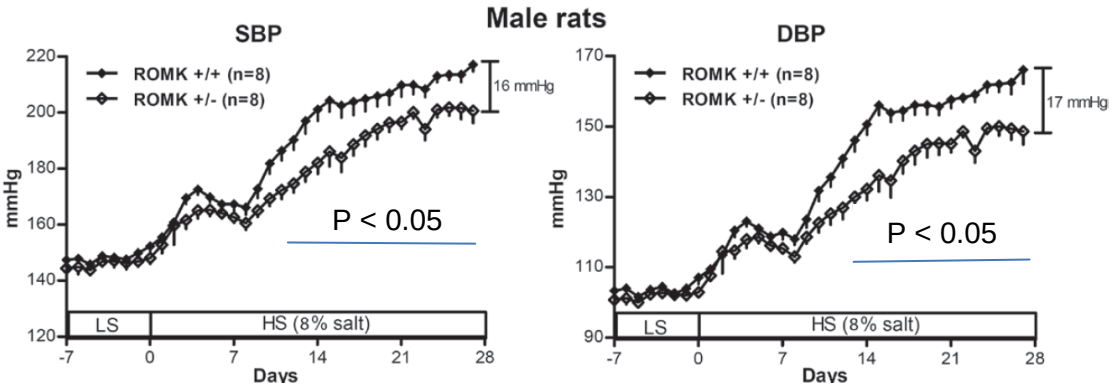
3. **What are potential pathological consequences of Epac activation and the resultant increase in Ca spark frequency? Design experiments that will test your hypotheses in vivo.**

Human hypertension is a genetically complex trait. Recent evidence indicates that genetic variants in a renal potassium channel (ROMK) may protect against hypertension. Using the Dahl salt-sensitive hypertension rat model, investigators at a Swiss pharmaceutical company introduced a loss-of-function variant into the rat ROMK gene and then assessed effects on blood pressure, blood electrolytes and other traits. Table 1 illustrates some of their data obtained from male rats. Blood pressure was assessed first on a low salt diet (LS), then on an 8% NaCl diet (high salt, HS). Figure 1 illustrates their observations in male rats.

Table 1

Parameter	Wild-type rats	ROMK +/- rats	ROMK -/- rats
Body weight, g	295 ± 4	266 ± 14	143 ± 2*
Blood potassium	4.8 ± 0.1	4.7 ± 0.2	6.4 ± 0.4*
Hematocrit (%)	45 ± 3	46 ± 4	52 ± 3*
Blood urea nitrogen	8.7 ± 1.1	10.1 ± 0.7	49.8 ± 2*

Figure 1 – Systolic (SBP) and diastolic (DBP) blood pressure in wild-type (ROMK +/+) and heterozygous knock out (ROMK +/-) Dahl rats on low (LS) or high (HS) salt diets. The horizontal line indicates the range of days when there were significant differences in blood pressures between groups.



1. **Propose mechanisms to explain the differences in baseline parameters (Table 1) between the various rat genotypes. Explain your hypotheses based on physiological principles.**

2. **Propose a mechanism to explain the difference responses to high salt diet between ROMK +/+ and ROMK +/- Dahl rats (Figure 1). Explain your hypothesis based on physiological principles.**

3. **Predict the effect of major classes of diuretic agents on ROMK +/+ Dahl rats and explain similarities and differences with the ROMK +/- Dahl rats.**

Investigators are studying signaling by dopamine D2 and D3 receptors, focusing on the inhibition of intracellular cAMP and the activation of ERK1/2 (as judged by its phosphorylation). They manipulate cell signaling by knockdown of individual components with siRNA, cell treatment with pertussis toxin, and the expression of WT and mutant G protein α-subunits, as well as Gβγ-scavenger βARK-CT (the C-terminus of GRK2 that has Gβγ-binding element). The results of these experiments are shown in Figs. 1 and 2.

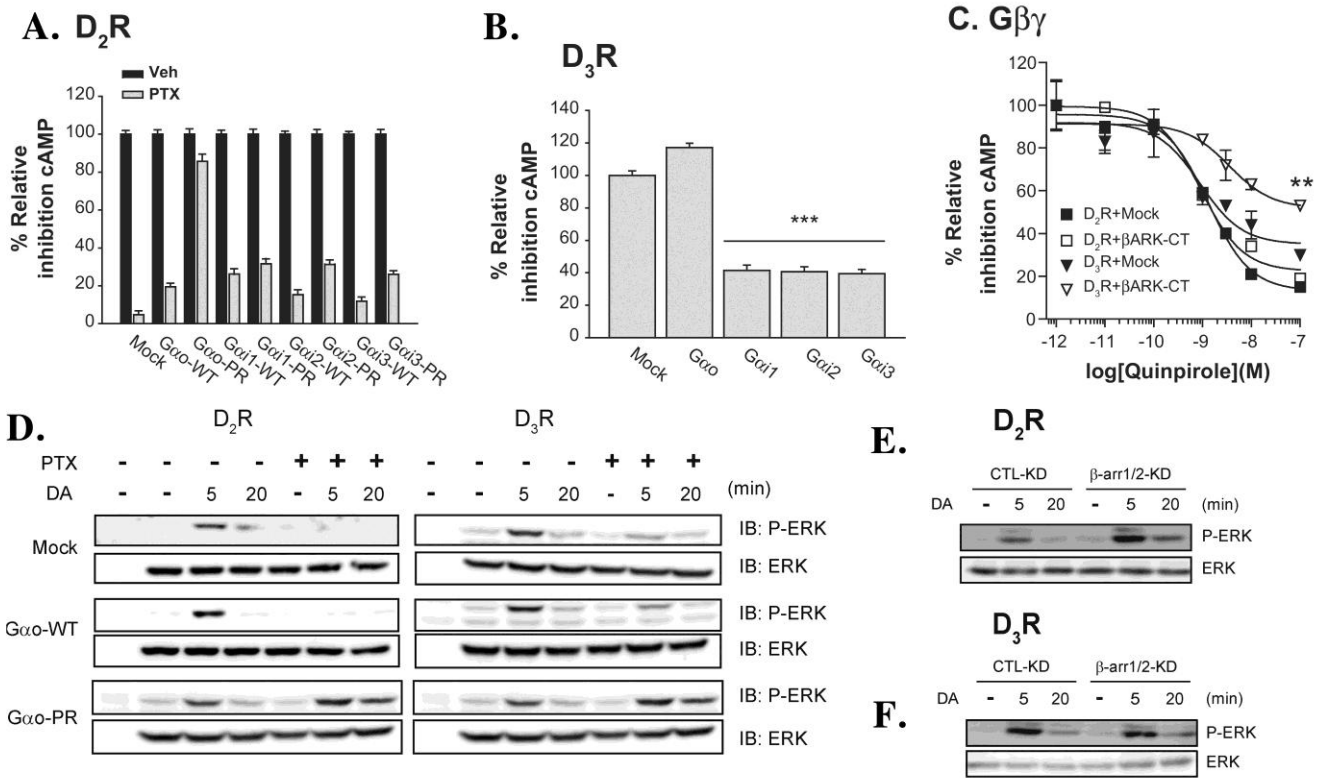


Fig. 1. The role of different signaling pathways in cAMP and p-ERK1/2 responses to D2 and D3 dopamine receptor stimulation in HEK293 cells. A. The effect of pertussis toxin (PTX) treatment and over-expression of wild type (WT) and pertussis toxin-resistant (PR) versions of indicated G protein a-subunits on inhibition of cAMP response via D2 receptors (Mock – control cDNA). **B.** The effect of siRNA knockdown of indicated G protein α-subunits on inhibition of cAMP response via D3 receptors (Mock – control siRNA). **C.** The effect of βARK-CT over-expression on D2- or D3-induced inhibition of forskolin-stimulated cAMP production (Mock – control cDNA). **D.** The effect of pertussis toxin on ERK1/2 activation via D2 and D3 receptors (DA – dopamine; time of treatment is shown in min; PTX – pertussis toxin; P-ERK – active phosphorylated ERK1/2, ERK, total ERK1/2; Gαo-WT, over-expression of WT Gαo; Gao-PR, over-expression of pertussis toxin-resistant Gαo). **E, F.** The effect of simultaneous knockdown of β-arrestin1/2 on ERK1/2 phosphorylation by the activation of D2 (**E**) or D3 (**F**) receptors by dopamine (DA). Where indicated, statistical analysis was performed by ANOVA with correction for multiple comparisons. **, $p<0.01$; ***, $p<0.001$.

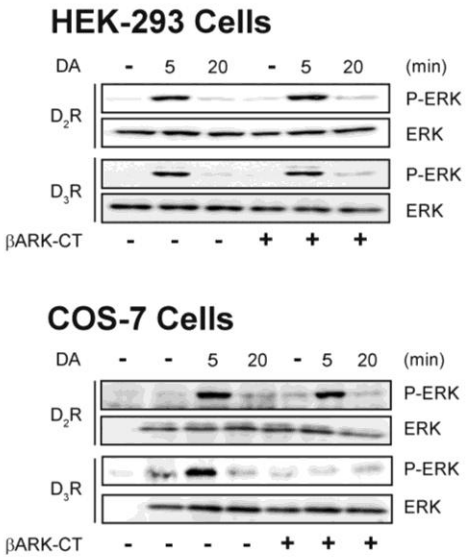


Fig. 2. Signaling differences between HEK293 and COS-7 cells. The effect of over-expression of βARK-CT on D2- or D3-induced ERK1/2 phosphorylation in HEK-293 (upper panel) or COS-7 (lower panel) cells.

Questions (in each case, indicate the evidence supporting your answer in the data given):

1. **What G proteins do D2 and D3 receptors couple to?**
2. **What subunit(s) of these G proteins mediate cAMP inhibition by D2 and D3 receptors?**
3. **Describe two main signaling pathways between GPCR activation and adenylyl cyclase inhibition.**
4. **Via which pathway(s) do D2 and D3 receptors promote ERK1/2 activation in HEK-293 and COS-7 cells, and which signaling proteins mediate these effects?**
5. **What possible pathway of ERK1/2 activation is tested in Fig.2? What conclusions can you draw based on the data?**
6. **What pathways of ERK1/2 activation by GPCRs do you know that were <u>not</u> tested here? Were there good reasons not to test those pathways? Propose experiments testing the involvement of at least two additional pathways between D2 or D3 receptors and ERK1/2 activation that should have been tested.**