Department of Pharmacology

Qualifying Examination (Part I)

July 19-22, 2011

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Tuesday, July 19	Exam – 9:00 – 11:00
	Exam – 11:00 – 1:00
	Exam – 2:00 – 4:00
	Exam – 4:00 – 6:00
Wednesday, July 20	Exam – 11:00 – 1:00
	Exam – 2:00 – 4:00
	Exam – 4:00 – 6:00
Thursday, July 21	Exam – 9:00 – 11:00
	Exam – 11:00 – 1:00
	Exam – 2:00 – 4:00
Friday, July 22	Exam – 9:00 – 11:00
	Exam – 11:00 – 1:00
	Exam – 2:00 – 4:00

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.

Qualifying Exam – July 2011
Question 1

Cocaine is an abused psychostimulant that interacts with monoamine (dopamine, norepinephrine, serotonin) transporters blocking the reuptake of these neurotransmitters. Neuroadaptations in the nucleus accumbens (NAC), a central component of the mesolimbic dopamine (DA) system, has been implicated in the development of cocaine-induced psychomotor sensitization and relapse to cocaine seeking. Administration of mGluR agonists induces long-term depression (LTD), i.e. persistent reduction in the evoked synaptic currents, in NAC neurons.

Experiments were conducted to investigate the effect of withdrawal from chronic cocaine on mGluR-dependent LTD. Some of the results are presented in Figures 1 and 2 below. LTD recording were conducted in brain slices prepared from animals chronically treated with saline, cocaine, or cocaine in combination with other drugs as indicated in the legends to Figures.

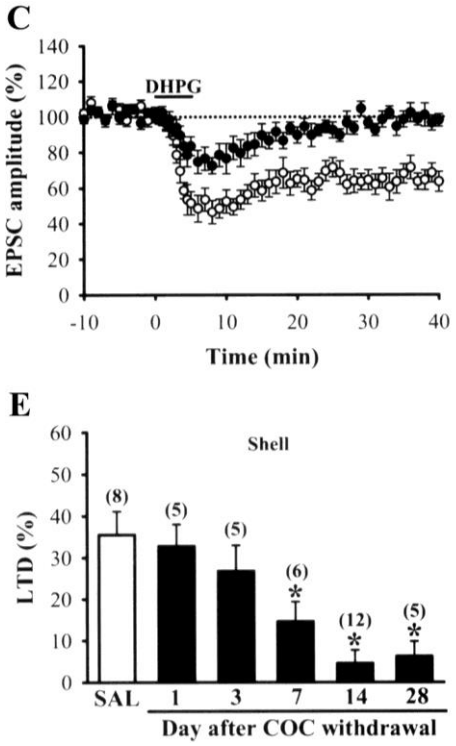

Figure 1. Effect of cocaine withdrawal on the induction of LTD by a non-selective group I mGluR agonist DHPG in NAC. Example (**C**) LTD in NAC and summary (**E**) of normalized EPSC (excitatory postsynaptic current) amplitude before and after brief application of DHPG (100 µm) for 5 min in saline (SAL)- or cocaine (COC)-treated mice on withdrawal day 14. The magnitude of LTD was measured 30 min after washout of DHPG. Horizontal bar denotes the period of delivery of DHPG. Dashed line indicates baseline level. *$p < 0.05$ compared with SAL control, unpaired Student's t test.

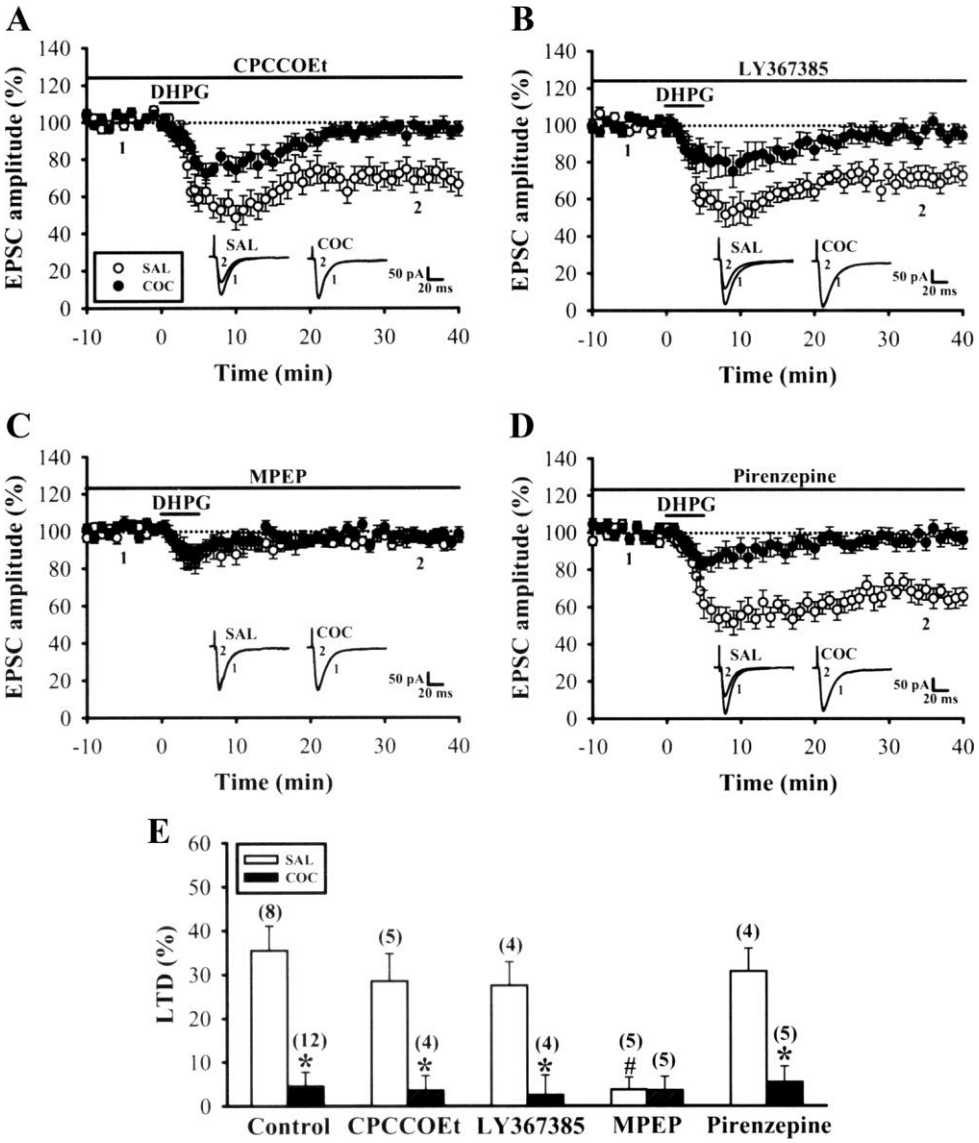

Figure 2. mGluR-mediated the LTD in NAC. Graphs show normalized EPSC amplitude before and after brief application of DHPG (100 µm) for 5 min in the NAC. Drugs were applied 10 min prior the application of DHPG. Summary of experiments showing the effects of mGluR1 antagonists, CPCCOEt (**A**) or LY367385 (**B**), on DHPG-LTD induction in the NAC in slices from saline (SAL)- or cocaine (COC)-treated mice examined on withdrawal day 14. Summary of experiments showing the effect of mGluR5 antagonist MPEP on DHPG-LTD induction in the NAC in slices from SAL- or COC-treated mice (**C**). Summary of experiments showing the effect of M1 muscarinic acetylcholine antagonist pirenzepine on DHPG-LTD induction (**D**). Summary of experiments showing the effects of different mGluR antagonists on DHPG-LTD induction for data in A–D (**E**). #$p < 0.05$ compared with SAL-control; *$p < 0.05$ compared with SAL-drug alone, unpaired Student's t test.

Qualifying Exam – July 2011
Question 1

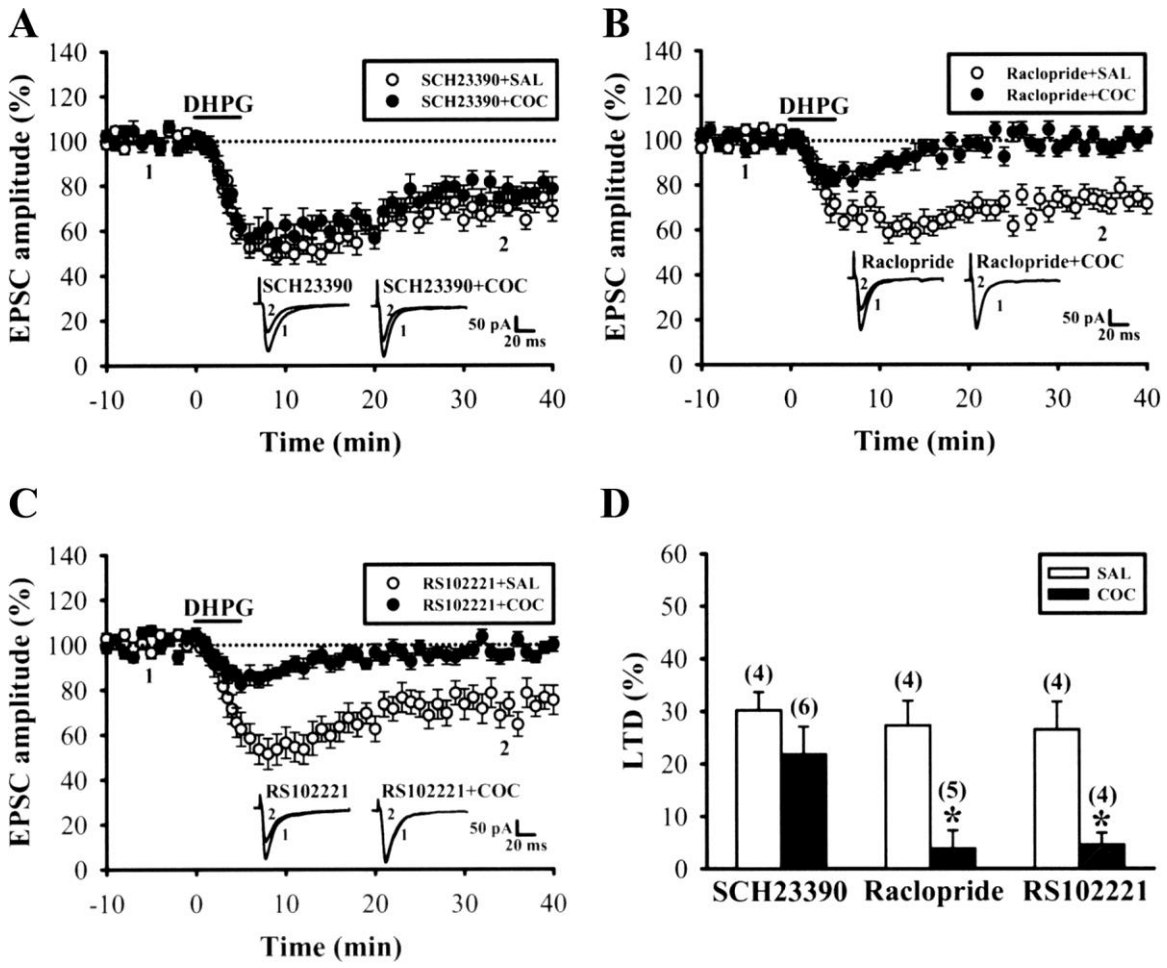

Figure 3. The role of dopamine receptors in LTD in NAC. Summary of experiments showing the induction of DHPG-LTD in the NAC in slices from mice receiving D1 dopamine receptor antagonist SCH23390 15 min before saline (SAL) or cocaine (COC) injections for 5 d and then examined on withdrawal day 14 (**A**). Summary of experiments showing the induction of DHPG-LTD in the NAC in slices from mice receiving D2 receptor antagonist raclopride before SAL or COC injections (**B**). Summary of experiments showing the induction of DHPG-LTD in the NAC in slices from mice receiving a specific 5-HT2C receptor antagonist RS102221 before SAL or COC injections (**C**). Summary of experiments showing the effects of different antagonist pretreatment on the development of cocaine-induced impairment of DHPG-LTD for data in A–C (**D**). *$p < 0.05$ compared with SAL-drug alone, unpaired Student's t test.

QUESTIONS:

1. Describe the study as you see it from the figures presented. Describe the main objective of the study and the steps. Provide the rationale for each step and for the use of the specific drugs.

2. Based on the data presented, do you think the objective has been achieved? If not, propose additional experiments to obtain necessary information and propose a model to account for all findings.

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Question 2

An exploratory compound, VUXY (MW = 300 g/mol) displayed an *in vitro* IC_{50} of 200 nM in antagonizing a receptor expressed in a rodent cell line. VUXY was subsequently evaluated *in vivo* (single oral dose of 10 mg/kg) and found to be ineffective in a rodent pharmacological model that is sensitive to antagonism of the receptor. It is well established that efficacy associated with this particular rat pharmacological model correlates with a Cmax obtained that is equal to, or exceeds the *in vitro* IC_{50}. An *in vitro* DMPK appraisal indicated that VUXY was highly protein bound in rat plasma producing a fraction unbound (F_u) of 0.03 and was metabolized in rat liver microsomes with a predicted hepatic clearance (CL_{HEP}) of 35 mL/min/kg (Figure 1). The metabolism of VUXY in rat microsomes is NADPH-dependent. Displayed in Table 1 are the resulting pharmacokinetic parameters of VUXY calculated from data obtained following an IV and PO dose of the test article in rats.

Figure 1. Metabolism of VUXY in rat liver microsomes employing substrate depletion approach.

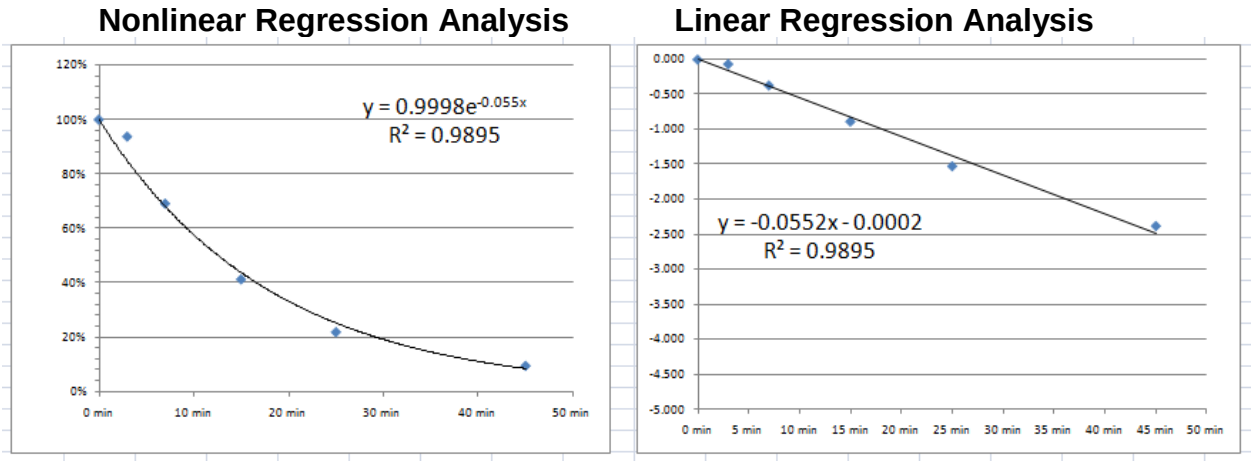


Table 1. VUXY PK in Sprague-Dawley Rats

Data compiled from a **1 mg/kg IV** and **10 mg/kg PO** study	**PK parameters**
Cl (mL/min/kg)	34
Vss (L/kg)	5.6
$T_{½}$ (min)	114
AUC (I.V., 1 mg/kg) (ng.hr/mL)	485
Cmax, systemic (ng/mL)	1410
Cmax, portal vein (ng/mL)	2310
Tmax (hr)	1.5
AUC (PO, 10 mg/kg) (ng.hr/mL)	2625

QUESTIONS:

1. Interpret the PK data from Figure 2 & Table 1, indicating how the Clearance and Volume of distribution values were obtained. Estimate the bioavailability of VUXY in rats. Please include in your discussion a definition of the statement that "half-life is a dependent PK parameter". Discuss the basic design of an *in vivo* PK experiment, quantitative analysis of compound levels in blood, plasma or serum and the basics of PK analysis.

2. Davies and Morris (*Pharmaceutical Research* **1993**, *10(7)*, 1093-5) report the blood flow to the liver (Q_H) of male rats is 55 mL/min/kg. Using pertinent data from the *in vitro* DMPK assessment, calculate the predicted hepatic extraction ratio (ER) of VUXY in rat? Is the relationship between $Cmax_{systemic}$ and $Cmax_{portal\ vein}$ consistent with your calculated ER for VUXY? Please explain.

3. Using the information available to you, how do you account for the lack of efficacy in the rodent model described?

4. Based on the *in vitro* pharmacology facts associated with VUXYand the corresponding *in vitro* and *in vivo* DMPK properties of the compound, propose an experiment that would enable the investigator to test the efficacy of VUXY in the rat pharmacological model.

Qualifying Exam – July 2011
Question 3

You have a new job at a pharmaceutical company. They ask you do design an oral contraceptive pill for women.

QUESTIONS:

1. Other than the ovary, what tissues or organs contribute to regulation of the female reproductive cycle?

2. Starting at the hypothalamus, describe hormones made by this axis that control the reproductive cycle –use a drawing to help

3. What is the difference between the first half of the menstrual cycle and the second half? How do hormone changes result in ovulation of a single oocyte?

4. With this hypothalamic-pituitary-ovarian axis in mind, what hormones would you include in an oral contraceptive pill, and how would you expect this to prevent ovulation?

Qualifying Exam – July 2011
Question 4

c-KIT is a member of platelet derived growth factor receptor (PDGFR) family (Figure 1), and is activated by stem cell factor (SCF) that regulates hematopoiesis. Mutations in c-KIT have been found in human mastocytosis, which is characterized by increased mast cells. In adult sporadic mastocytosis, three types of mutations, D816V, D816Y, and D816F were detected in c-kit. Mutations such as E839K also were found in patients with anemia with reduced red blood cells. To understand how these mutations lead to mastocytosis and anemia, each mutation was introduced into full-length c-kit cDNA, and mutant cDNA was transfected into COS cells for functional characterization. Results are shown in Figures 2 and 3.

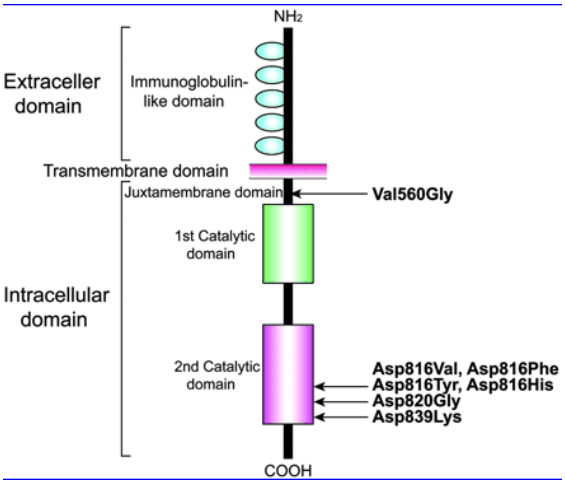


Figure 1. *A schematic diagram depicting structure of c-kit and mutations found in patients with mastocytosis and anemia.*

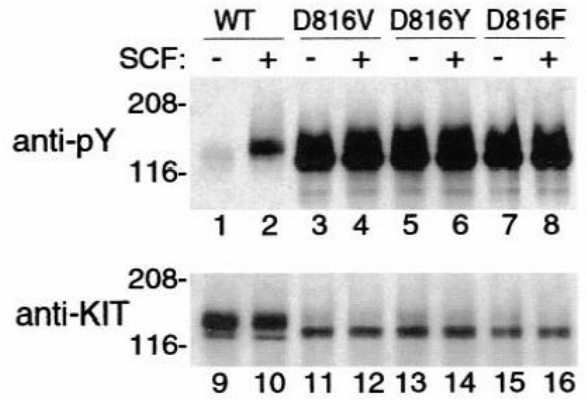


Figure 2. *Wild-type and mutant c-KIT phosphorylation in COS cells.* Shown is the Western blotting (top, anti-phosphotyrosine, bottom, anti-KIT antibodies) of immunoprecipitates using anti-KIT antibodies.

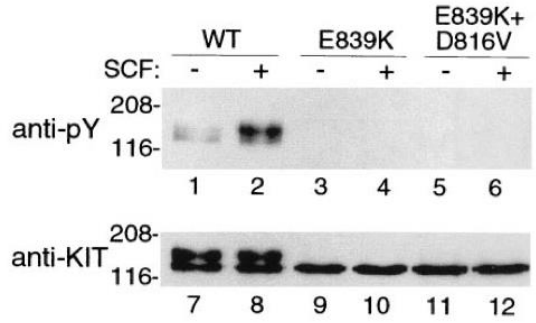


Figure 3. *Phosphorylation of c-KIT (E839K) and c-KIT (E839K, D816V) in COS cells.*

QUESTIONS:

1. Explain the experimental details and results as shown in Figures 2 and 3. Speculate how each mutation (D816V, D816Y, D816F, and E839K) affects the normal activity of c-KIT.

2. Based on your understanding regarding how c-KIT is regulated, design additional experiments to support your hypothesis in relation to how mutant c-KIT functions to cause mastocytosis and anemia.

3. What are known downstream targets following c-KIT activation? Which pathway is most likely to cause the observed mastocytosis phenotype? Design at least two independent experiments to support your hypothesis.

Qualifying Exam – July 2011
Question 5

The genes encoding the β1-adrenergic receptor (β1AR) and Gαs have been targeted in the mouse. Both heterozygous null mice are viable and have no significant difference in cardiac contractility as measured by echocardiography. Wild type and heterozygous null cardiac myocytes were analyzed below.

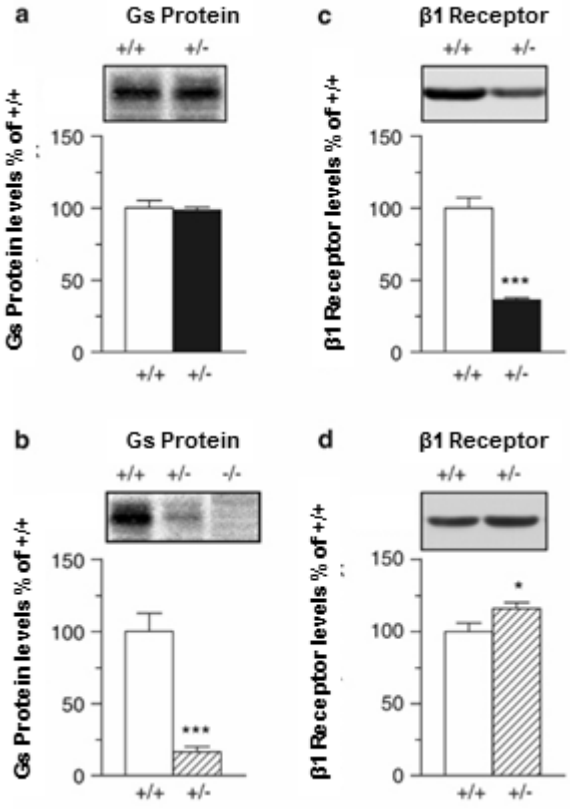
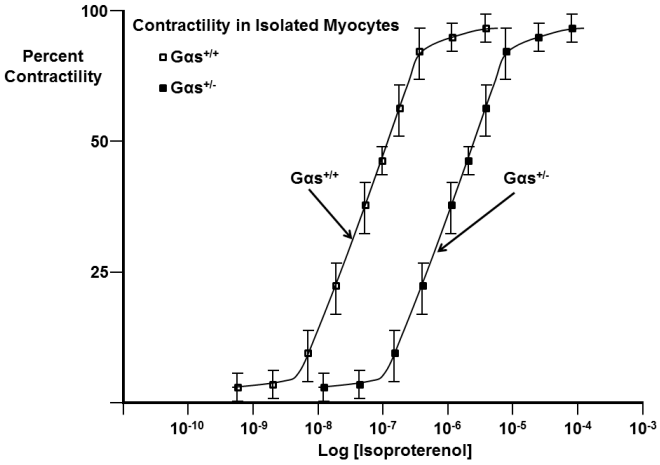
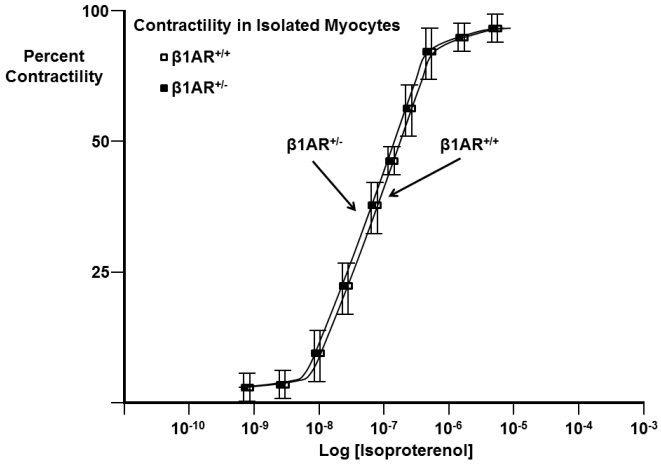


Figure 1. β1AR, Gαs levels of *Gαs+/-* and *β1AR+/-* mice. (a–d) β1AR mice (a, c) and Gαs mice (b, d) protein levels were measured by immunoblotting in heart muscle extracts of *Gα*s +/- (*n*=5, b, d) and *β1AR*+/- (*n*=6, a, c) mice and expressed as percentages of the levels found in the myocytes of their wild-type littermates (*n*=5–6). Student's *t*-test: * $p<0.05$, *** $p<0.001$.

Figure 2, below. Contractility in isolated myocytes from wildtype and *β1AR+/-* or wildtype and *Gαs+/-* mice. Left panel – Myocytes isolated from 3 month old littermates. N=5 for each genotype. Right panel – Myocytes isolated from 3 month old littermates. N=5 for each genotype.





QUESTIONS:

1. Diagram the pathway whereby the β1-adrenergic receptor regulates cardiac contractility.

2. How do you explain the above data? Present hypotheses for data in Figure 2, left and right panels, with experiments to test them.

3. How do you explain the lack of altered contractility in intact heterozygous null animals?

Qualifying Exam – July 2011
Question 6

Several factors mediate the regulation of glomerular filtration rate (GFR) such as angiotensin II and tubuloglomerular feedback (TGF). Recently, investigators at the Panamanian Institute for Glomerular Science (PIGS) have uncovered a novel mechanism of GFR regulation involving feedback from the connecting tubule (CNT) – a short segment of the distal nephron locating between the end of the distal convoluted tubule and the cortical collecting duct. Changes in tubular fluid flow rate or solute delivery to the CNT evoke changes in GFR analogous to TGF, but in the opposite direction. Specifically, a high rate of fluid delivery to the CNT evokes vasodilation of the afferent arteriole and a rise in GFR (*see* **Figure 1**). This CNT-glomerular feedback (CTGF) requires a functional amiloride-sensitive sodium channel (ENaC) in the CNT. The investigators have now tested the hypothesis that CTGF can modulate the magnitude of TGF by performing the experiments illustrated in **Figure 2**. Specifically, stop-flow pressure (surrogate for GFR) was measured in superficial rat cortical nephrons while changes in the rate of perfusion of the adjacent thick ascending limb were made. Benzamil, a more selective and more potent derivative of amiloride was used in one set of experiments.

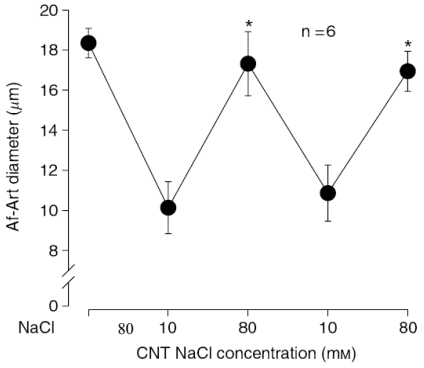


Fig 1 – Effect of perfusing CNT with low or high NaCl concentration.
*, $p < 0.05$, high vs low NaCl.

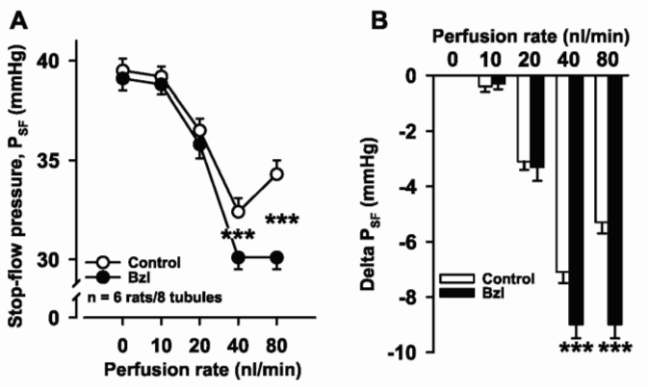


Fig 2 – TGF measurements in rat cortical nephrons. (A) Stop-flow pressure (P_{SF}) in response to changes in thick ascending limb perfusion rate in the presence or absence of benzamil (Bzl). (B) Change (delta) in P_{SF} calculated for each perfusion rate relative to 0 nl/min. ***, $p<0.001$

QUESTIONS:

1. Propose at least one hypothesis or model to explain the behavior of P_{SF} under the control condition, especially at the highest perfusion rates. Explain the effect of benzamil with your model.

2. Predict how the behavior of P_{SF} under control conditions might change if the experimental animals were pretreated for 1 week with a supra-physiological dose of aldosterone.

3. Predict how the behavior of P_{SF} under control conditions might change if the experimental animals were pretreated with a selective adenosine 1A receptor antagonist.

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Question 7

β1- and β2-adrenergic receptors (βARs) are highly homologous and both activate Gs, but they play distinct roles in cardiac physiology and pathology. Myocyte contraction, for instance, is stimulated by β1AR but not β2AR signaling, and chronic stimulation of the two receptors has opposing effects on myocyte survival. Differences in the assembly of macromolecular signaling complexes could explain the distinct biological effects elicited via activation of these receptors. Although prior studies have demonstrated that agonist occupancy of the β2AR initiates recruitment of a preformed complex consisting of β-arrestin and a cAMP-specific phosphodiesterase (PDE4D5), no data are available on the potential interaction of PDEs with the β1AR. To address this question, a group of investigators performed the experiments described below.

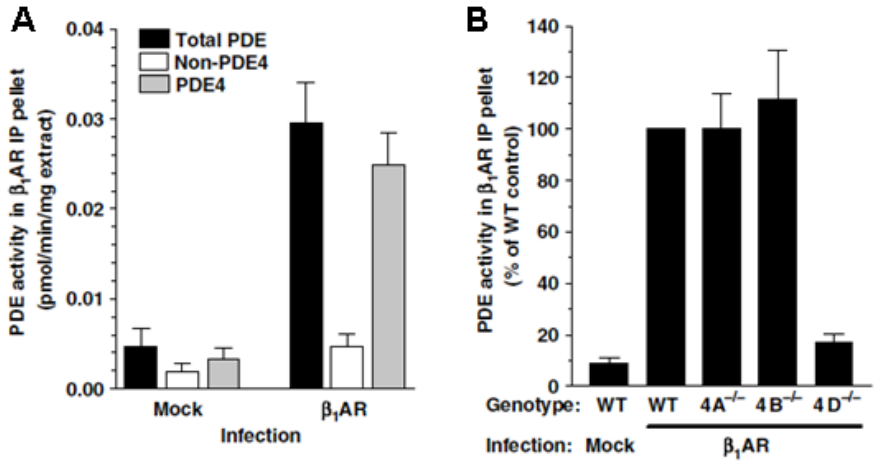


Figure 1. *A*) Mouse neonatal cardiomyocytes were infected with 'control' adenovirus (Mock) or an adenovirus encoding Flag-tagged β1AR. Flag immunoprecipitations were performed from the cell extracts and assayed for PDE activity. Non-PDE4 activity represents the activity measured in the presence of a PDE4-specific inhibitor. *B*) Flag immune complexes from wild type (WT) and PDE4A-, PDE4B-, and PDE4D-deficient mouse neonatal cardiomyocytes infected with 'control' adenovirus (Mock) or Flag-β1AR adenovirus were assayed for PDE activity.

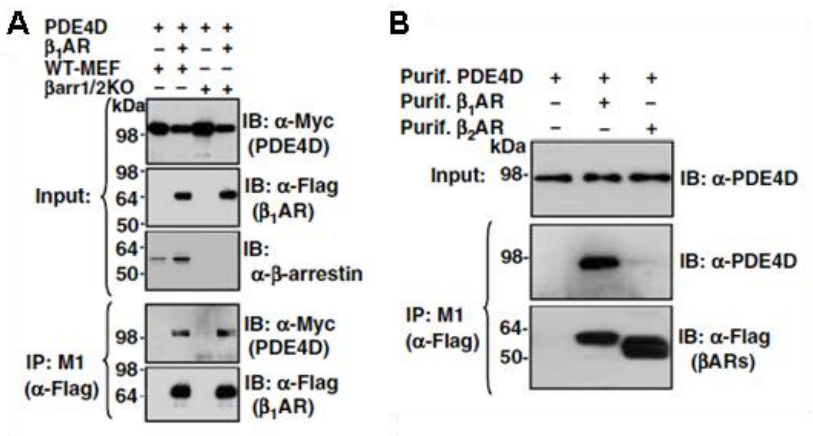


Figure 2. *A*) Mouse embryonic fibroblasts derived from mice deficient in β-arrestin 1 and 2 (βarr1/2KO) or from wild type controls (WT-MEF) were infected with Myc-PDE4D8 adenovirus together with (+) or without (-) Flag-β1AR adenovirus. The cell lysates (Input) and Flag immune complexes (IP:M1) were subjected to SDS-PAGE and immunoblotted (IB) with the indicated antibodies. *B*) PDE4D8, Flag-β1AR, and Flag-β2AR were purified after expression in baculovirus. Purified PDE4D8 was incubated in the absence (-) or presence (+) of the indicated purified βAR, and the receptor was immunoprecipitated using anti-Flag resin and subjected to immunoblot (IB) analysis.

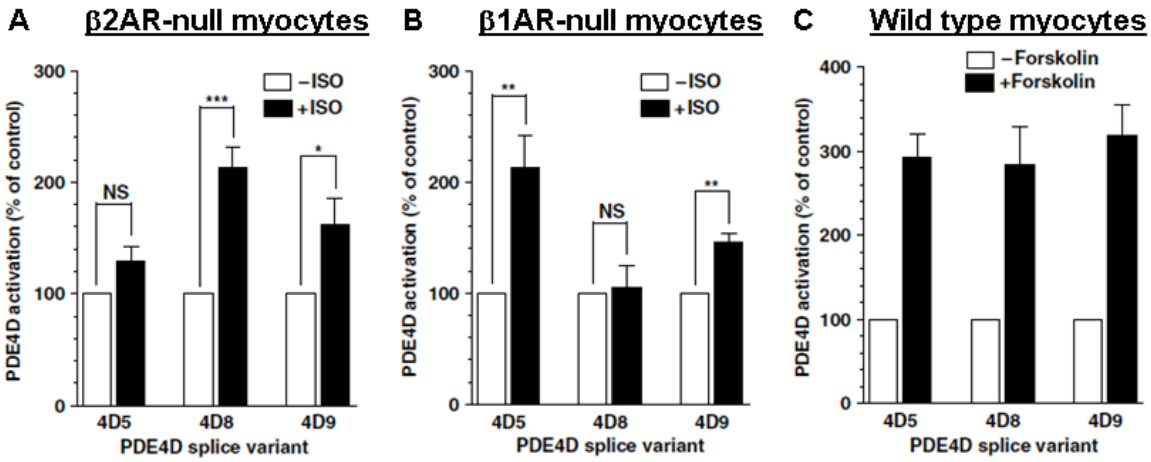


Figure 3. Neonatal cardiac myocytes derived from β2AR-null mice (*A*) and β1AR-null mice (*B*) were treated for 3 min with (+) or without (-) 10 μM isoproterenol (ISO). At the end of the incubation, PDE4D5, PDE4D8, and PDE4D9 were immunopurified from the cell lysates with isoform-specific antibodies and assayed for PDE activity. *C*) The indicated PDE4D splice variant was immunopurified from extracts of wild type neonatal cardiac myocytes treated with (+) or without (-) forskolin and subsequently assayed for PDE activity. Data shown are expressed as the means +/- s.e.m. of at least three experiments. NS (P>0.05); * (P<0.05); ** (P<0.005); *** (P<0.0005).

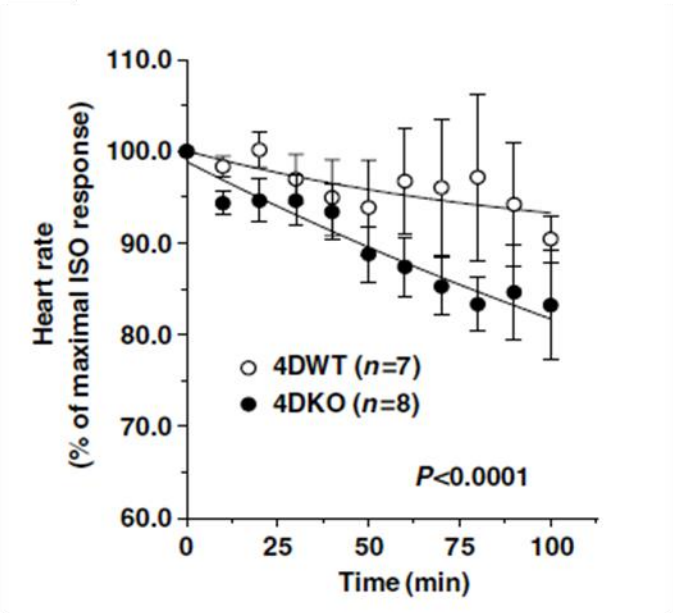
QUESTIONS:

1. What do the data in Figs. 1 and 2 tell you about the interaction of PDE4Ds with the β1AR?

2. How would you interpret the data shown in Fig. 3?

To assess the role of PDE4D in β1AR function in a more physiological context, the investigators monitored changes in the heart rate of mice following β-adrenergic stimulation, as it is known that *in vivo* contraction rate is primarily controlled by β1AR. Wild type mice (4DWT) and PDE4D-null mice (4DKO), matched by age, sex, and genetic background, were sedated using isoflurane. While their heart rate was continuously measured, the mice were injected with a submaximal dose of isoproterenol (ISO). *Wild type mice and PDE4D-null mice showed no significant differences in either basal heart rate (4DWT = 410+/-52 and 4DKO = 400+/-45 beats/min) or maximal heart rate after the ISO injection (4DWT = 540+/-25 and 4DKO = 550+/-20 beats/min).* However, when the investigators monitored the decline in heart rates of 4DWT and 4DKO mice as a function of time after ISO injection, they observed the following results.

Qualifying Exam – July 2011
Question 7



3. What conclusions can be drawn from the above data about the role of PDE4D in the control of heart rate?

4. Based on the data shown in Figs. 1-4, propose a reasonable hypothesis that might explain the molecular events underlying PDE4D regulation of β1AR signaling/function. Design an experiment that would allow you to further test your hypothesis.