Department of Pharmacology

Qualifying Examination (Part I)

STUDENT VERSION

December 15-19, 2008

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.

If you have any questions regarding the examination, please contact Joey Barnett at:

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BEST WISHES FOR YOUR SUCCESSFUL COMPLETION OF THE EXAMINATION!

Psychostimulants (cocaine, amphethamine) stimulate locomotor activity, and the effect is enhanced with repeated drug administration. This enhancement of the behavioral response is known as behavioral sensitization, and it persists months after the drug withdrawal. Most studies of the neurological mechanisms of the behavioral sensitization are focused on the dopaminergic system. However, there is evidence that other neurotransmitter systems are critical for the behavioral sensitization to psychostimulants.

You have obtained the data shown in **Figure 1** on the effect of acute and repeated amphetamine (AMPH) administration on locomotor activity and cortical norepinephrine (NE) release. You have then examined the effect of pretreatment of mice with prazosin (antagonist of α1-adrenoreceptors), SR 46349B (SR; antagonist of 5-HT2A serotonin receptors), or both on behavioral sensitization and cortical NE levels (**Figure 2**). To elucidate the role of the D1 dopamine receptor, you have investigated the effect of its inhibition with SCH23390 (SCH) on cortical NE release (**Figure 3**) and behavior (not shown).

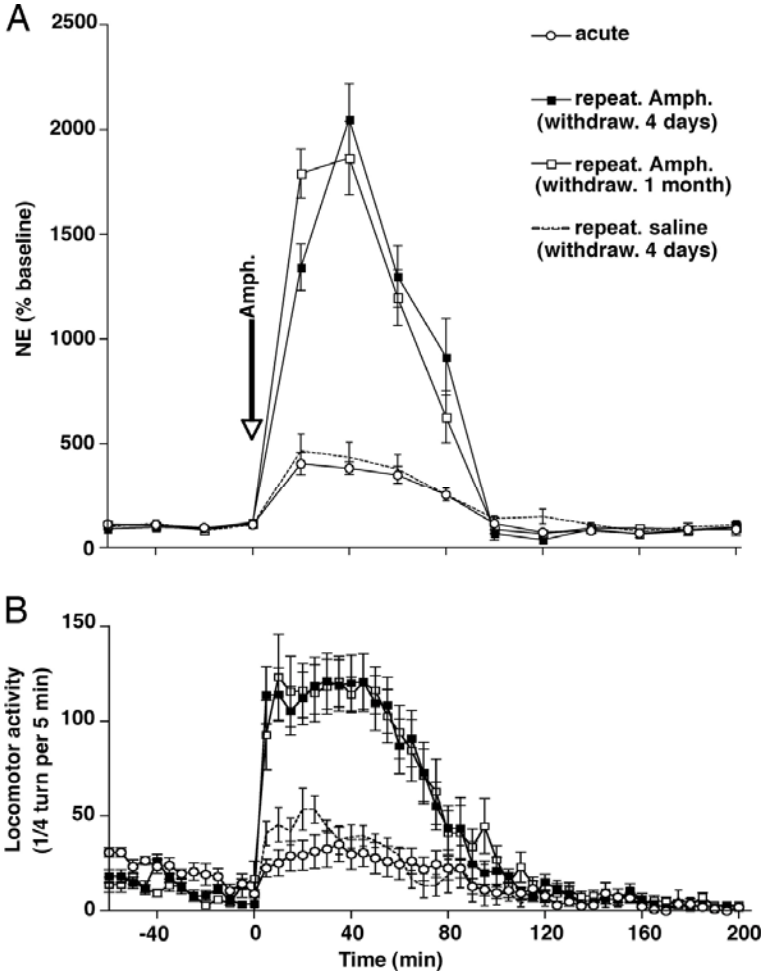


Figure 1. Repeated treatment with amphetamine induces both potentiation of the increase in the extracellular NE levels (**A**) and behavioral sensitization (**B**) in the mouse prefrontal cortex (PFC) that persist for at least 1 month. Cortical NE levels are expressed as percentages of the mean basal level values. The data for acute saline injections, which had no effect on either measure, are not shown for clarity.

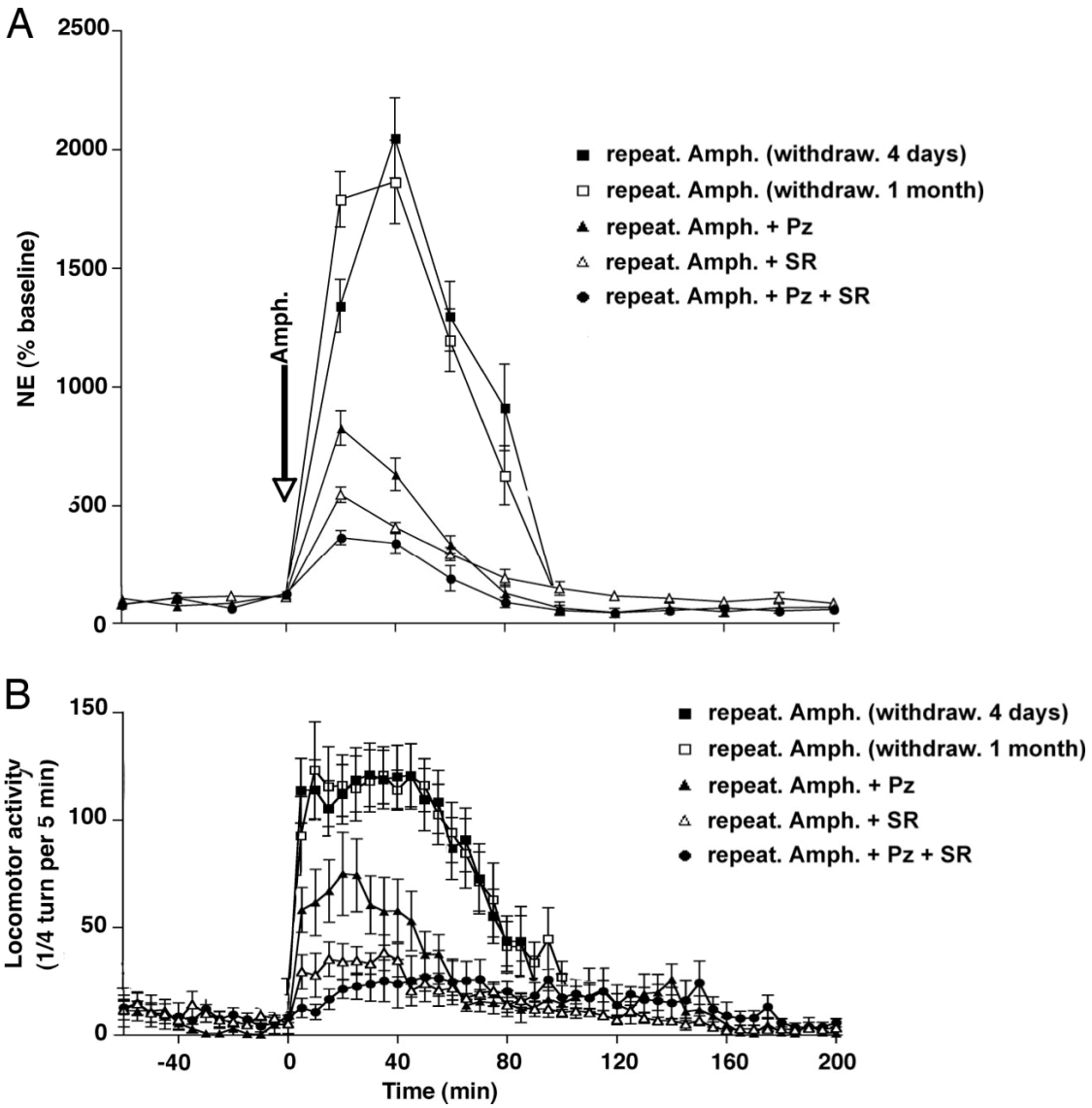


Figure 2. Effects of pretreatment during the sensitization period with prazosin, SR46349B, or a mixture of both on AMPH-induced cortical extracellular NE levels (**A**) and locomotor response (**B**). The animals treated with antagonists were tested 4 days after the last AMPH injection. The effects of acute saline or AMPH injections were not altered by the antagonists and are not shown for clarity.

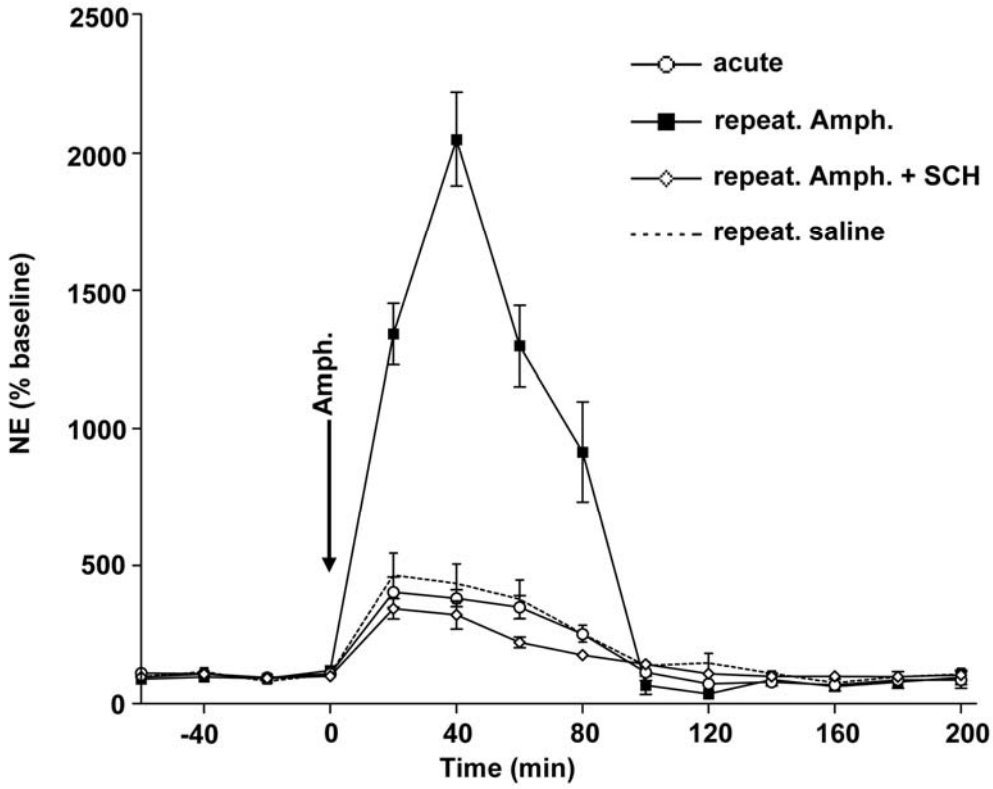


Figure 3. Effects of pretreatment during the sensitization period with D1 receptor antagonist SCH23390 on the AMPH-induced cortical NE release. The data were collected after 4 days withdrawal period. The animals treated with antagonists were tested 4 days after the last AMPH injection. The effects of acute saline injections had no effect and are not shown for clarity. The behavioral sensitization (not shown) was also completely inhibited.

1. **Describe the data. What can you conclude about the relationship between behavioral sensitization to AMPH and cortical NE release? Describe molecular mechanism of AMPH action. What is the common neurochemical effect elicited by most drugs of abuse? What method to measure the neurotransmitter concentration was employed in these experiments? Where in the brain are neurons that supply noradrenergic innervation to the cortex located?**

2. **The activity of all three receptor types (D1, α1, and 5-HT2A) is necessary to produce potentiated NE release and behavioral sensitization. Propose a simple model for the sites of action in the brain of all receptors involved to explain your findings (assume that other parameters not shown in Figures are unchanged). Suggest the site of plastic changes that mediate sensitization of behavioral and molecular effects of AMPH upon repeated administration. Propose experiments to test the model.**

In the following study, some factors affecting the disposition of fexofenadine were examined in wild-type and Pgp knockout mice.

Intravenous infusion. Male wild type and Mdr1a/1b P-gp knockout mice were anesthetized and the tail vein cannulated for the injection of fexofenadine. The bile duct was cannulated for bile collection after ligation of the gall bladder. After an intravenous bolus injection of 1 mg/kg, the mice received a constant infusion of fexofenadine at a rate of 12-14 nmol/min/kg. Bile was collected at 30-min intervals throughout the experiment and blood samples were collected at 75, 105, 135, and 150 min after starting the infusion. The mice were sacrificed after 150 min, and the entire liver and brain were excised immediately, weighed and stored at -80°C until required for assay. Fexofenadine was quantified by LC-MS.

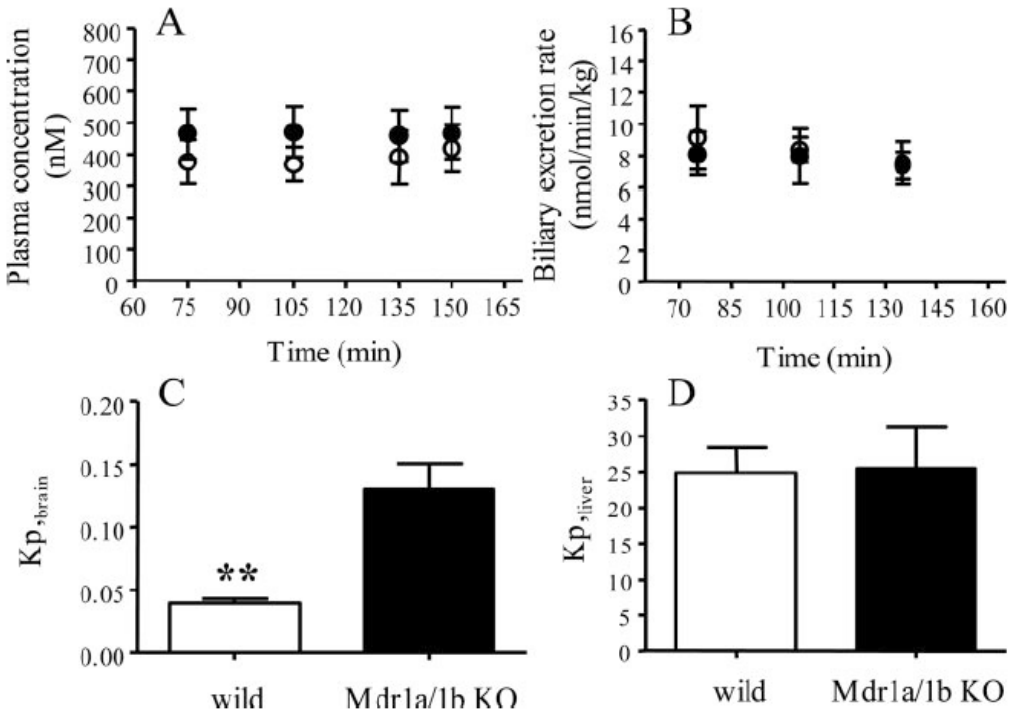


FIG. 1. Concentration ratios of fexofenadine in wild-type (closed circles) and Mdr1a/1b P-gp knockout (open circles) mice during the constant infusion of fexofenadine.
(A) Plasma concentration-time profile
(B) Biliary excretion rate
(C) Brain-to-plasma ratio (*K*p,brain)
(D) Liver-to-plasma ratio (*K*p,liver).
Statistical differences in Mdr1a/1b P-gp knockout mice and wild-type mice were calculated by a two-sided unpaired Student's *t* test with $p<0.05$ as the limit of significance (*, $p< 0.05$; **, $p<0.01$). Each point represents the mean ± S.E. ($n = 3$).

Some pharmacokinetic parameters obtained in the steady state infusion experiment are reported in Table 1. There was no statistical difference between wild-type or knockout mice in steady state plasma concentration, plasma clearance (systemic clearance), or biliary excretion rate, and a slight increase in plasma/bile clearance in the knockout mice. (See Table 1 next page)

TABLE 1

Pharmacokinetic parameters of fexofenadine during constant infusion to wild-type and Mdr1a/1b P-gp knockout mice

Data are expressed as mean ± S.E. (Mdr1a/1b P-gp knockout and wild-type mice, $n = 3$). Statistical differences in Mdr1a/1b P-gp knockout mice and wild-type mice were calculated by a two-sided unpaired Student's t test with $p < 0.05$ as the limit of significance.

	Inss	*Css*	*E*bile	CLplasma	CLbile,plasma
	nmol/min/kg	*nmol/ml*	*nmol/min/kg*	----------*nmol/min/kg*----------	
Mdr1a/1b +/+	14.0	0.46±0.08	7.86±1.45	30.4±2.4	17.1±2.1
Mdr1a/1b -/-	12.0	0.39±0.07	8.28±1.13	30.8±5.4	21.2±2.1*

* $p<0.05$

Inss = infusion rate
*C*ss = steady-state plasma concentration
*E*bile = biliary excretion rate
CLplasma = total plasma clearance (systemic clearance)
CLbile,plasma = biliary clearance

Oral Administration. Both wild-type and Mdr1a/1b P-gp knockout mice received a 10-mg/kg oral dose of fexofenadine. Blood samples (30 μl) were collected from the tail vein at 0.5, 1, 2, 4, 6, and 8 h after oral administration.

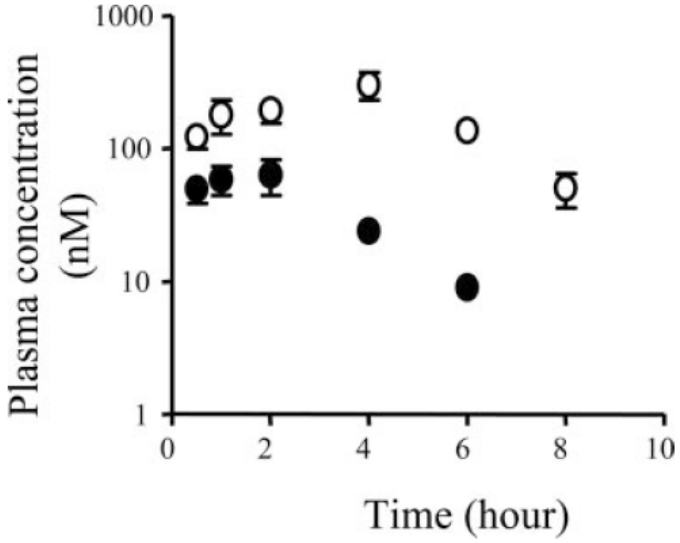


FIG. 2. Plasma concentration-time profiles of fexofenadine after oral administration to wild-type (closed circles) and Mdr1a/1b P-gp knockout mice (open circles) at a dose of 10 mg/kg. Each point represents the mean ± S.E. ($n = 3$).

Questions:

1. **Give an overview of these experiments, explaining the objectives in the protocols, analyzing the data, and drawing the conclusions that can be made at this stage.**

2. **What unanswered questions need to be addressed to complete an understanding of ADME/DMPK of fexofenadine in the mice? Propose additional experiments to examine these issues. Include in your answer a hypothesis to account for biliary excretion and experiments to test it.**

You have recently identified ten unrelated families in which some family members display polydipsia and polyuria. Sequence analyses of genomic DNA from affected individuals revealed missense mutations in the coding region of the gene encoding the arginine vasopressin-neurophysin II (AVP-NPII) precursor as shown in Figure 1. Two families had no mutations within the coding region of the AVP-NPII and further analyses identified no mutations within the entire gene.

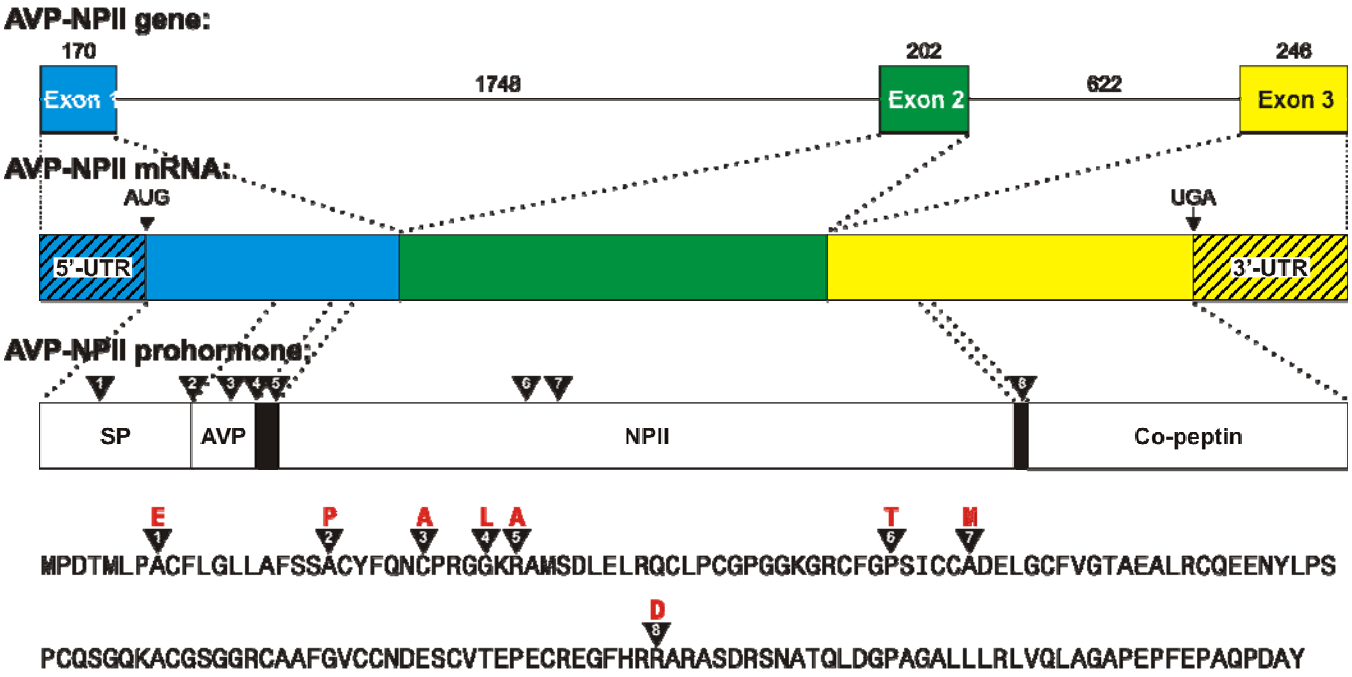


Figure 1. Identification of familial mutations in the human arginine vasopressin-neurophysin II gene. A schematic diagram of the organization of the human AVP-NPII gene is presented indicating the sizes (bp) of introns and exons along with the corresponding structure of the AVP-NPII mRNA and prohormone; UTR, untranslated region; SP, signal peptide, AVP, arginine vasopressin; NPII, neurophysin II. The relative position of identified mutations in the AVP-NPII prohormone for eight affected families is shown; the specific amino acid alterations resulting from these identified missense mutations are indicated in red.

1. **Describe the physiological processes by which mutations in the AVP-NPII gene lead to polydipsia and polyuria.**

2. **Choose three of the eight mutations identified in Figure 1 and describe how a mutation in these regions of the prohormone would affect AVP synthesis and function. Where possible, describe how these specific amino acid changes could result in decreased AVP synthesis and function.**

3. **For the two families in which no mutations were identified in the AVP-NPII gene, propose two additional candidate genes in which mutations could give rise to a similar phenotype. Rather than sequencing these genes directly, propose other experimental approaches that would support the selection of your candidates.**

As part of a high throughput screening strategy a mutagenesis screen was conducted on Jurkat cells, such that only one gene was mutated in a clonal line of cells (designated J-X cells). As part of the screen it was observed that the clinical immunosuppressant FTY720 no longer had pharmacological effects on this particular mutant cell line. It was effective in literally hundreds of other lines where mutations were known to have occurred, but not in the J-X cell line. It was noted that FTY720 is a prodrug and must be taken up and enzymatically modified in cells before it has biological activity.

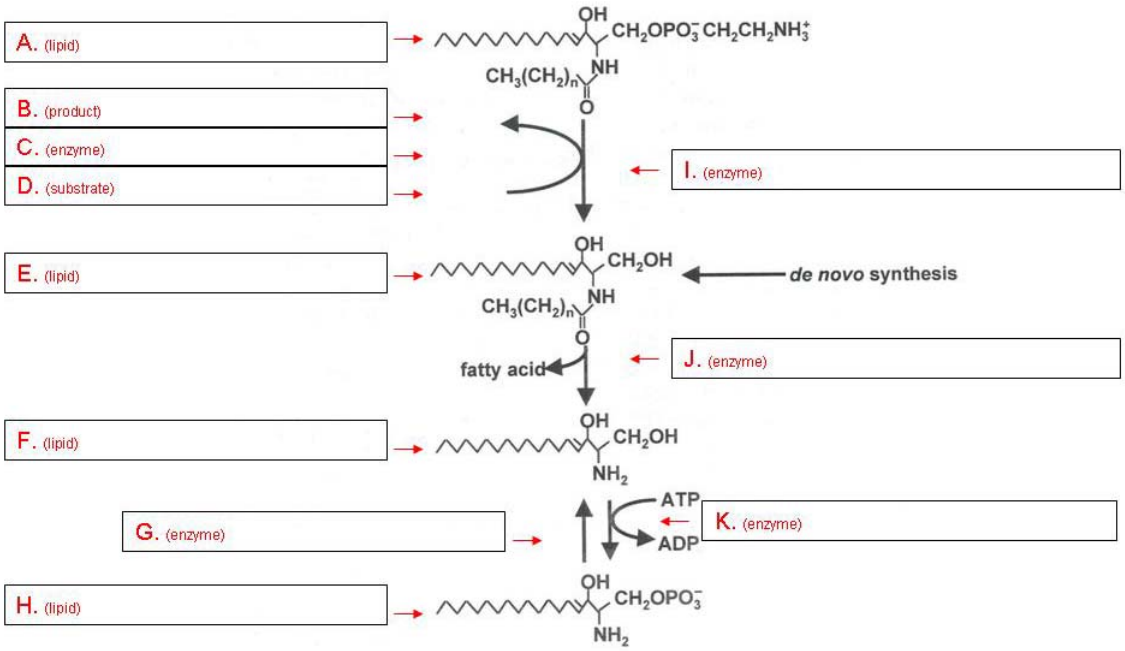
In wild type cells the FTY720 promotes complex biological functions, including stimulation of cell growth, inhibition of apoptosis, and cell motility. In the J-X cell lines these functions have been disregulated by the mutation. You also note that typical cell responses to Tumor Necrosis factor and phorbol esters are disrupted in the J-X cell line.

Taken together there could be more than one explanation and a variety of mutations that could account for these phenotypes, but your incredibly clever graduate student has a hypothesis about the mutated gene target and she wagers with you that she could restore these various functions in the J-X mutant cells by providing an exogenous factor isolated from serum. After a chloroform-methanol:water organic extraction and a subsequent chromatographic separation, she isolates a single peak of biological material present in modest abundance in the serum that when added back to the J-X cells restored all of the previously mentioned receptor mediated and cellular functions. She correctly surmised that this "serum-factor" was a signaling molecule that FTY720 mimics (following its modification inside the cell). Although the addition of this serum factor corrects the short term effects on the mutant cells, it does not alter the longer term effects of the FTY720 (meaning that whatever defect is present is acutely corrected, but the cells still cannot convert the prodrug into an active form).

To confirm her hypothesis she demonstrates that pertussis toxin blocks many of the same responses mediated by this mystery factor in the wild-type cells and also blocks the actions of FTY720 in the wild-type cells. Your student draws the signaling pathway shown below and asks you to fill in the names of the compounds. Based on the information in Figure 1 what is the pathway?

1. **What is the single gene mutated in the J-X cell line?**

2. **Discuss the major components of this lipid metabolic pathway.**



Your research team has identified an apparent orphan G-protein-coupled receptor (G-heart) that is expressed only in the ventricular myocardium. Targeted deletion of the gene reveals apparently normal homozygous null animals. You begin a detailed analysis of these animals including experiments to address any role of this receptor in the response of the myocardium to injury. Experiments were performed with the use of a Langendorff preparation to study the isolated heart. Briefly, mice were anesthetized and hearts were rapidly removed and placed in 4°C Krebs solution. The aorta was cannulated and the heart was perfused (90 mm Hg) with oxygenated (95% O2/5% CO2) Krebs solution (37°C). The hearts beat at a spontaneous rate and myocardial function can be measured. Data were continuously recorded (Fig 1). Examination of several myocardially-derived growth factors revealed alterations in the expression and levels of (Vascular Endothelial-Derived Growth Factor) VEGF (Fig 2). VEGF in this case is made by the myocytes.

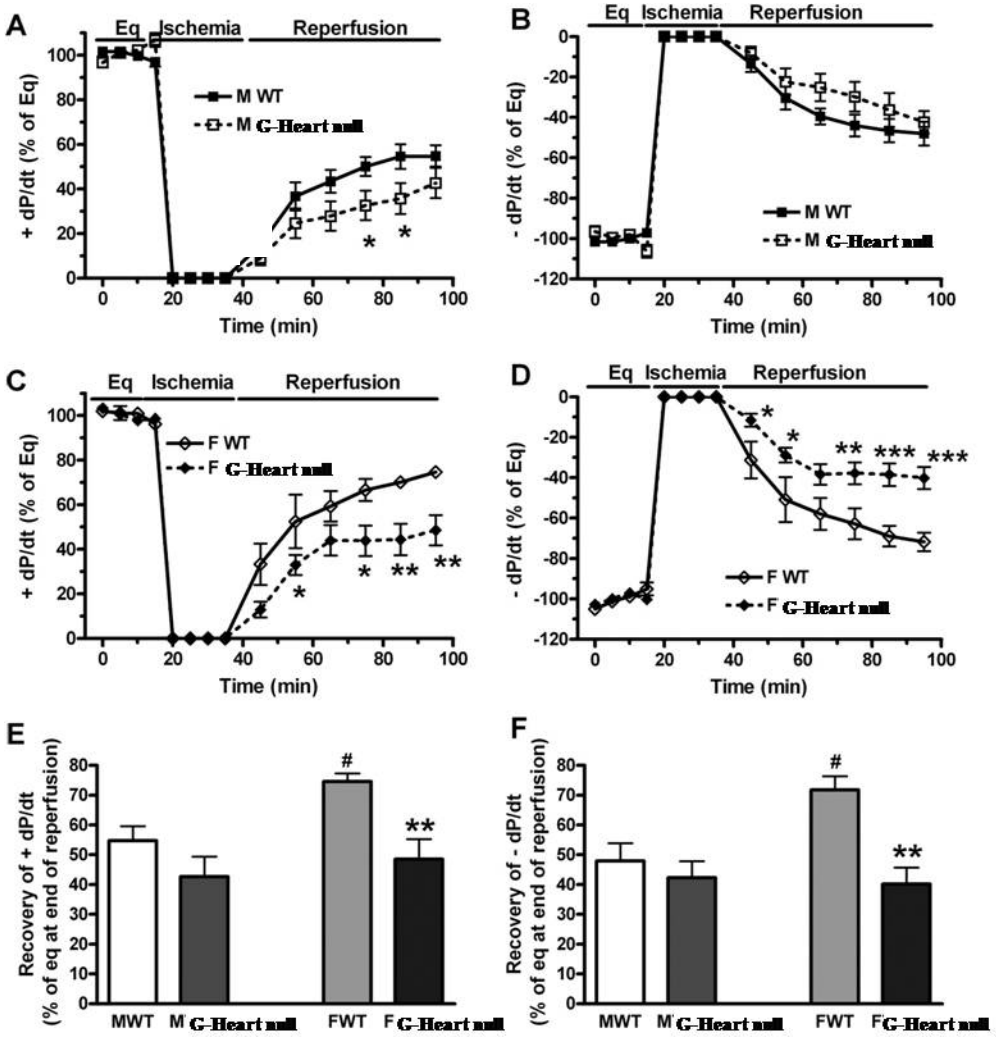


Figure 1. Effect of the loss of G-Heart on myocardial functional recovery after ischemia. Hearts were monitored during a 20 minute equilibrium phase (Eq) prior to 15 minutes of ischemia (no O_2 in the perfusion buffer). During reperfusion O_2 was restored to the buffer. Myocardial function was recorded in male wildtype (WT) (n=6), male G-Heart null (n=5) (A,B) and female WT (n=6), and female G-Heart null (n=5) (C,D) mouse hearts (+dP/dt is positive change in pressure over time which is a measure of contraction. –dP/dt is negative change in pressure over time which is a measure of relaxation). Results are represented as percent of equilibration (Eq). (E,F) +dP/dt,-dP/dt at the end of reperfusion. Results are mean±SEM, **P*<0.05, ***P*<0.01, versus WT.

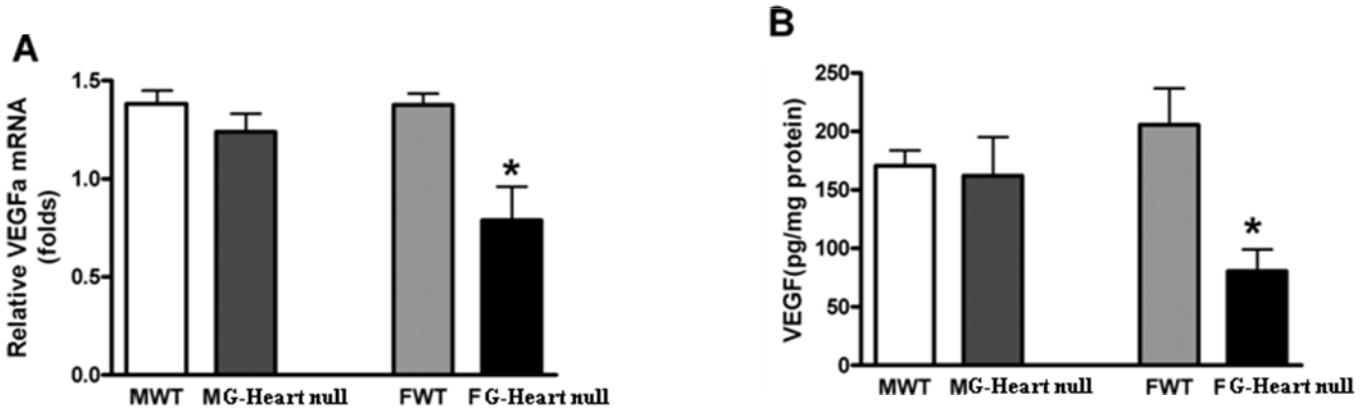


Figure 2. Effects of G-Heart loss on VEGF production after ischemia. Relative quantitation of VEGF mRNA compared with male WT control (real-time reverse transcriptase-polymerase chain reaction) is shown in **A** (WT and G-Heart null hearts). Myocardial VEGF production is analyzed by ELISA in **B** (WT and G-Heart nulls). Results are mean±SEM, n=5 to 6 hearts/group *P<0.05.

1. **Describe these data and your interpretation. Present a hypothesis to explain the role of G-Heart in females. Provide *in vivo* and *in vitro* approaches to test your hypothesis.**

2. **How do you explain the difference noted between males and females? What experiments would you use to test your explanation?**

To find genes controlling blood pressure, a Swedish laboratory performed a phenotype-based screen of mutant mice created using transposon-mediated insertional mutagenesis. The screen identified one strain of animals that when bred to homozygosity (e.g., recessive trait) that exhibited abnormal blood pressure when placed on a low sodium diet (Fig. 1, "Na depletion"). Upon further investigations, the mutant animals (designated as -/-) were found to have other abnormalities (Fig. 1 & 2) including reduced urine Ca^{2+} excretion, increased plasma aldosterone levels, reduced plasma K^+ concentration, and increased urinary K^+ excretion all observed on a low sodium diet. There were no anatomical or histological abnormalities in kidneys of mutant mice and other experiments demonstrated that the animals exhibited intact tubuloglomerular feedback. The investigators postulated that the mutation impairs renal tubular transport.

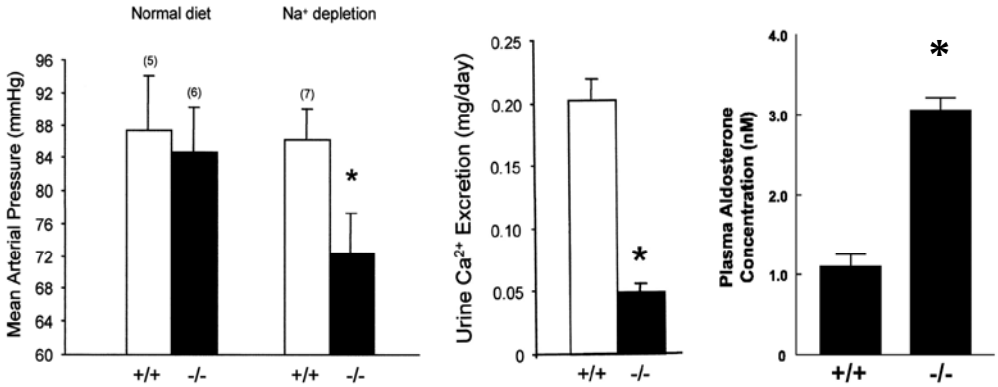


Fig 1 – Comparisons of wildtype (+/+) and mutant (-/-) mice. Statistical significance at the P < 0.01 level is indicated by an asterisk.

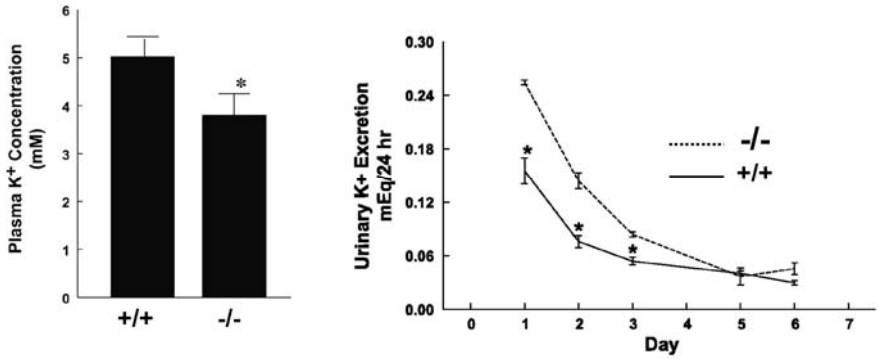


Fig 2 – Comparisons of K^+ balance in wildtype (+/+) and mutant (-/-) mice. Statistical significance at the P < 0.01 level is indicated by an asterisk.

1. **Develop hypotheses to explain the phenotype of these animals.**

2. **Which tubular segment is most likely affected and identify any specific transport activities that you predict to be abnormal. Explain how you would test your ideas.**

3. **Would it be possible to mimic the phenotype of these mutant mice using a pharmacological strategy? Explain.**

The β-adrenoreceptors (βAR) play an important role in the regulation of cardiac function. Two βAR subtypes, β1AR and β2AR are expressed in the heart. You are trying to elucidate relative contribution of these two receptors and the mechanisms translating their activation into changes in contraction rate in primary cultures of cardiomyocytes. You are using wild type (WT) cadiomyocytes, as well as cadiomyocytes from β1AR and β2AR knockout (KO) animals. In all experiments shown below you determine the time course of contraction rate increase over basal level upon stimulation with isoproterenol (Iso). Iso is β-adrenergic agonist that has equal affinity and potency for β1AR and β2AR. You are also using two equally potent inhibitors of cAMP phosphodiesterase (PDE) specific for PDE4 subtype, RS25344 and rolipram (ROL), as well as PKA inhibitor PKI. The labels on the graphs indicate whether WT or knockout myocytes are used. Note that pharmacological inhibition of MAP kinases in these experiments had no effect on contraction rate (data not shown). *In all cases you can assume that there are no secondary effects of knockouts, i.e., all the other proteins are present and expressed at normal levels.* Here is the data you obtained:

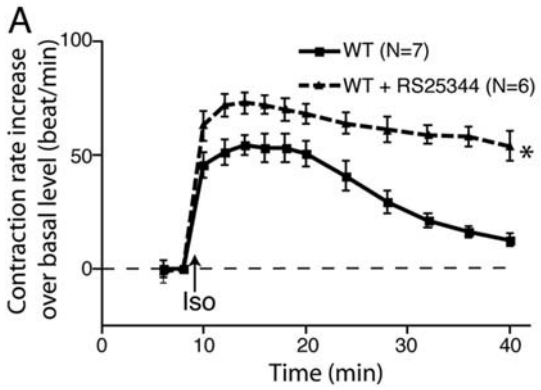


Figure 1. Inhibition of PDE4 affects βAR regulation of the contraction rate in cardiac myocytes. (A) Inhibiting PDE4 with RS25344 enhanced the increase in the contraction rate after stimulation of βAR in WT myocytes. The data represent the mean ± SE of experiments from at least three different myocyte preparations. *, $P < 0.05$; time course significantly different by two-way ANOVA.

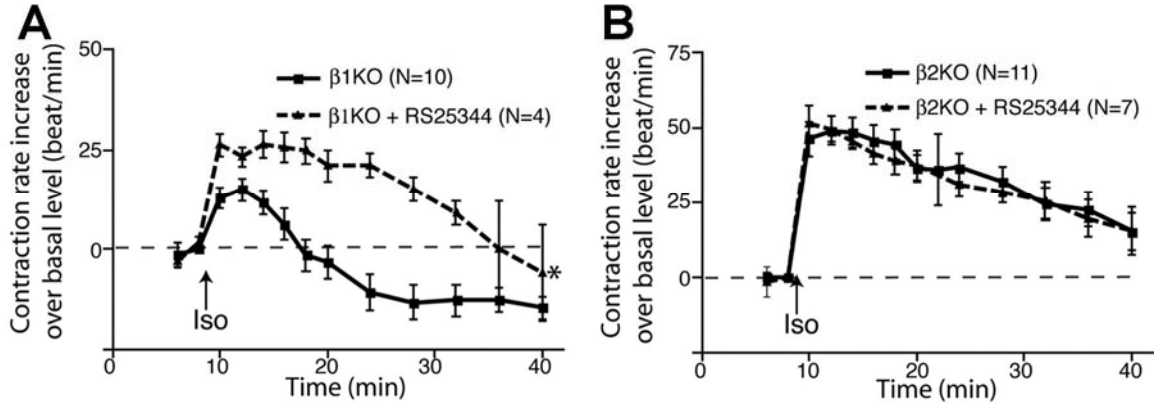
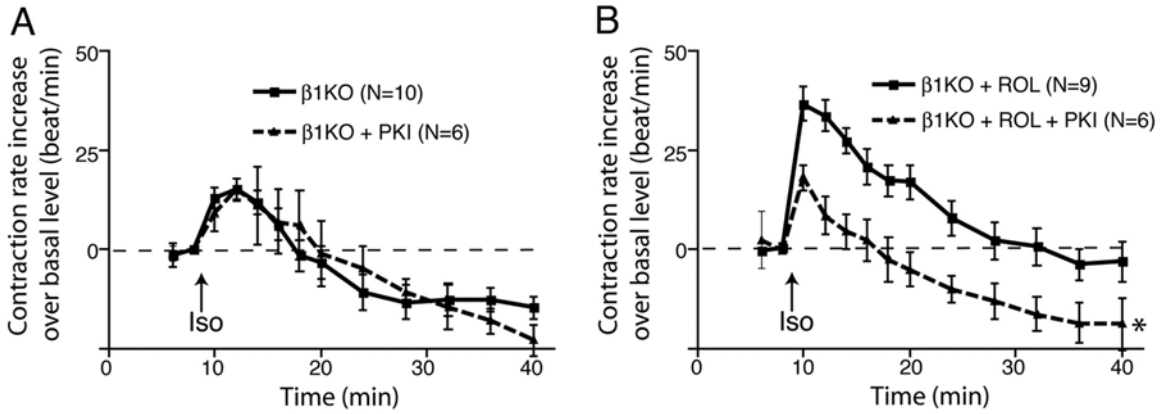


Figure 2. PDE4 is selectively involved in β2AR signaling for contraction rate response in cardiac myocytes. (A) Inhibition of PDE4 activity with RS25344 enhances the contraction rate response to isoproterenol stimulation in β1AR-KO myocytes. (D) Inhibition of PDE4 activity with RS25344 does not alter the contraction rate response to isoproterenol stimulation in β2AR-KO myocytes. The data represent the mean ± SE of experiments from at least three different myocyte preparations. *, $P < 0.05$; time course found to be significantly different by two-way ANOVA.



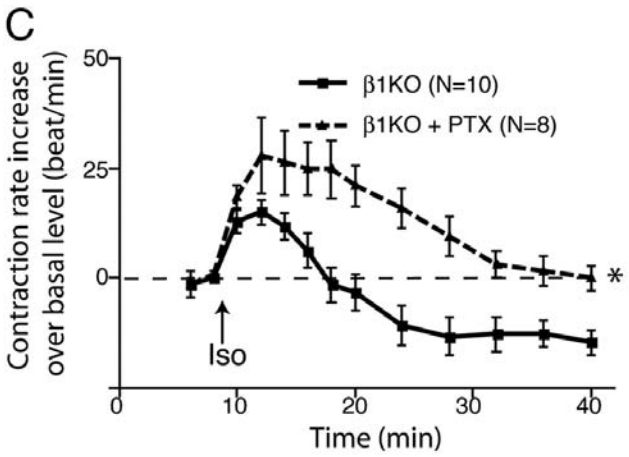


Figure 3. PDE4 inhibition and pertussis toxin evoke an increase in the PKA dependent contraction rate in β1AR KO cardiac myocytes. (A) Effect of PKI on the contraction rate of myocytes from β1AR KO mice. (B) The effect of PKI + PDE4 inhibitor rolipram on the contraction rate of myocytes from β1AR KO mice. (C) Effect of pertussis toxin (PTX) on the contraction rate of myocytes from β1AR KO mice. The data represent the mean ± SE of experiments from at least three different myocyte preparations. *, $P < 0.05$; time course found to be significantly different by two way ANOVA.

Propose the model that explains these findings and propose at least two independent experiments to test your model. Make sure that your model and follow-up experiments answer the following questions:

1. **Based the data, what G protein(s) do β1AR and β2AR couple to and what second messenger(s) are generated upon activation of these receptors. Explain how RS25344 enhances the response. Based on these data, what difference in response would you expect between wild type (WT) and PDE4 knockout cardiomyocytes? What effect of RS25344 on PDE4 knockout myocytes would you expect?**

2. **Explain the difference between the effects of β1AR and β2AR knockout on Iso effect. Which receptor predominantly mediates Iso effect in WT myocytes? What do the effects of RS25344 on knockout myocytes tell you?**

3. **Describe a possible mechanism of PTX effect on signaling via β-adrenergic receptors. Based on the mechanism of the action of PTX and rolipram, do you expect the effects of rolipram and PTX to be additive, non-additive, or to cancel each other out?**