Department of Pharmacology
Qualifying Examination (Part I)
December 12, 2002

***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 material 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 936-1722 (w) or 385-4396 (h)

GOOD LUCK!

QUESTION 1:

Previous work has demonstrated that various isoforms of nitric oxide synthase (NOS) are expressed in thick ascending limb (TAL) cells including the endothelial (eNOS), and inducible (iNOS) forms of the enzyme. By contrast, only neuronal NOS (nNOS) is expressed in macula densa cells. A recent report from investigators in Michigan demonstrated interesting effects of an nNOS specific inhibitor (7-nitroindazole or 7-NI) on afferent arteriole diameter in isolated, perfused rabbit glomeruli with adherent tubular segments (consisting of TAL, macula densa and early distal convoluted tubule). Video microscopy was used to monitor the diameter of the afferent arteriole during different maneuvers such as tubular perfusion with either high or low NaCl containing tubular fluid. There is no leakage of tubular fluid in this preparation. The following data were collected during **retrograde** perfusion of the macula densa (ie. perfused from distal convoluted tubule).

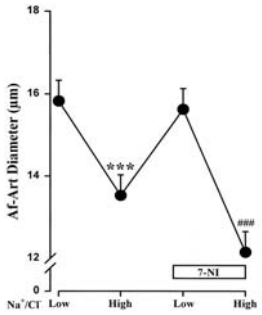


Fig. 1 - Afferent arteriole (Af-Art) diameter was measured during **retrograde** perfusion of the macula densa with fluid containing either **Low** or **High** NaCl concentration with or without the specific nNOS inhibitor 7-NI.

Part A

A. Ignoring the effects of 7-NI, describe the basic phenomenon observed in this experiment, and explain the responsible mechanism(s).

B. How would you describe the effect of NO in this phenomenon?

The investigators next examined the effects of the non-specific NOS inhibitor (L-NAME) on afferent arteriole diameter in the presence of 7-NI. In the experiment depicted in Fig. 2A, L-NAME was infused **orthograde** through the TAL (ie. in the normal physiological direction - will reach the TAL first then pass the macula densa). Fig. 2B illustrates results from an experiment in which L-NAME was perfused **retrograde**.

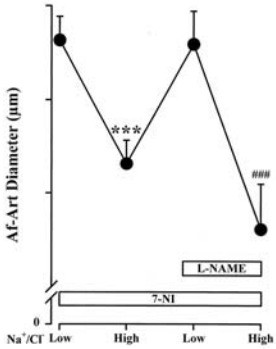


Fig. 2A (orthograde L-NAME)

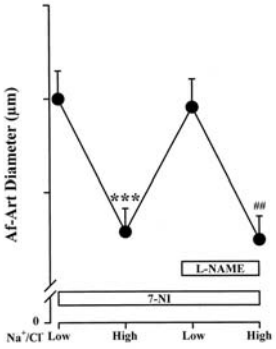


Fig. 2B (retrograde L-NAME)

Part B

Propose a mechanism to explain these findings. Consider where NO is produced, where it might be exerting its effects, and how it might be acting.

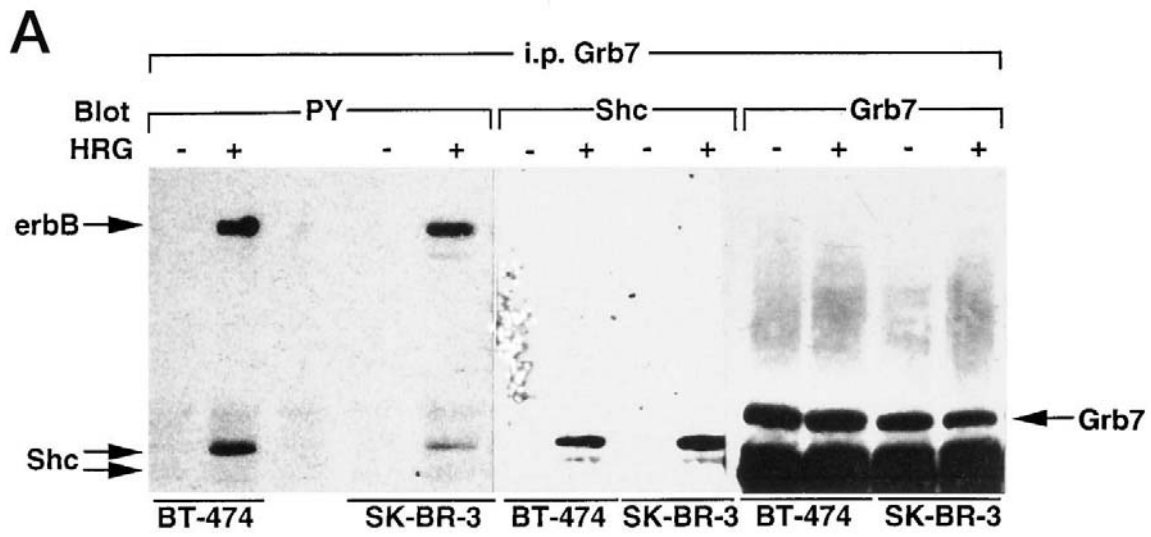
QUESTION 2:

Two cell lines, BT-474 and SK-BR-3 were used to investigate heregulin (HRG)-mediated signaling. Cell lysates from cells treated with either HRG (+, 5nM) or vehicle (-) were prepared and immunoprecipitated with anti-Grb7 antibodies. Immunoprecipitates were analyzed by immunoblotting with the following antibodies (anti-phosphotyrosine, anti-Shc, and Grb7). Results are shown below.

Part A
Explain the results based on your understanding about HRG and signaling

Part B
What is the relationship among ErbB, Shc and Grb7?

Part C
Propose additional experiment(s) to support your hypothesis in (B)



QUESTION 3:

The plasma concentration-time profiles of metyrapone given by intravenous infusion and oral administration to normal healthy subjects are shown below. Also presented are similar curves in the same subjects following 2 weeks oral pretreatment with X985.

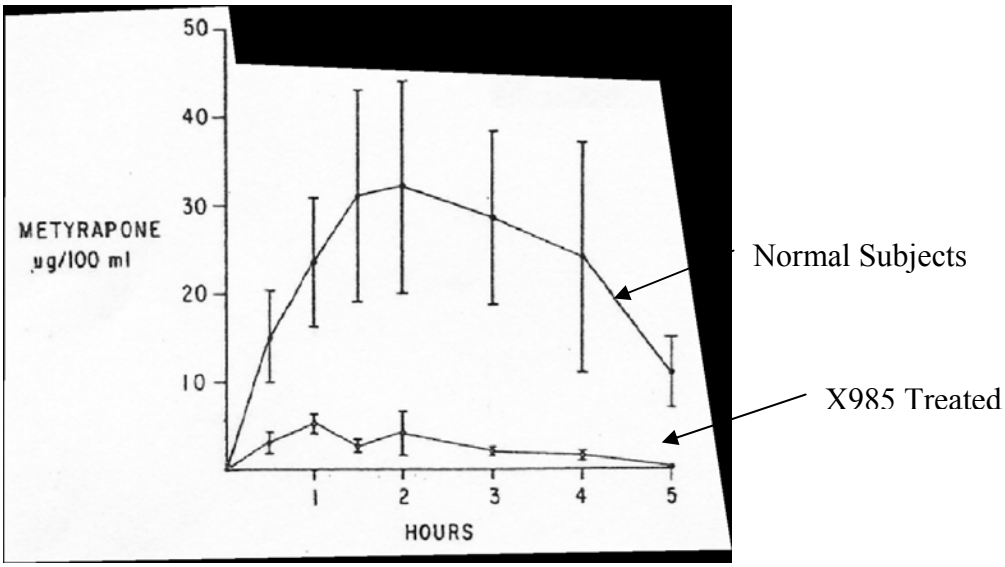


Figure 1. Plasma levels of unconjugated metyrapone after a single 750 mg oral dose in normal and X985-treated subjects. The mean and SEM are given.

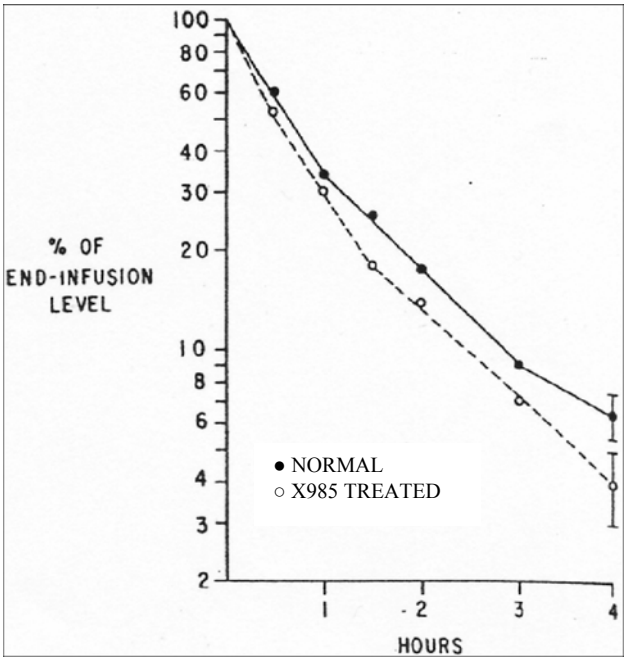


Figure 2. Effect of X985 on the rate of removal of iv metyrapone from plasma. With the exception of the 4 hr values, which give the mean and sem ($p < 0.1 > 0.05$), the mean values are shown. The disappearance of metyrapone was not significantly faster in the X985-treated group at any time during the study.

Present two distinct scenarios, with explanations, that can account for these observations.

QUESTION 4:

For your dissertation project, you have been involved in studies of pro-opiomelanocortin (POMC) gene expression by examining sequence variations in 500 unrelated Tennessee subjects with severe early-onset obesity. In all cases, obesity was manifest before the age of 10 years and the average body mass index (BMI) of the cohort was >4 standard deviations above the age- and gender-adjusted mean. While a total of nine heterozygous POMC sequence variants were identified, two probands (subjects A and B) were heterozygous for the missense amino acid substitution R153G (Figure 1), a highly conserved residue in multiple species including human, pig, mouse and rat.

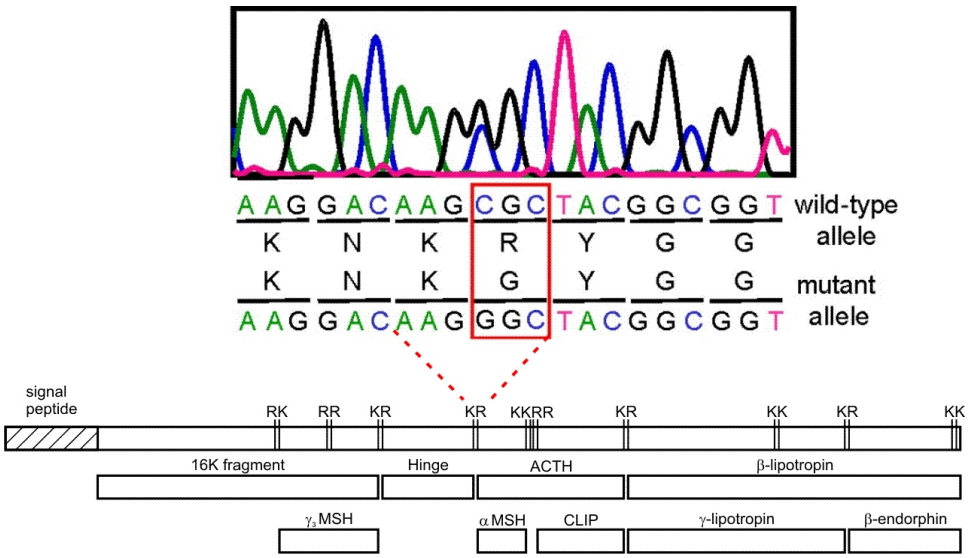


Figure 1. Identification of an obesity-associated sequence variation in POMC. Sequence chromatogram derived from subject B showing a G-to-C substitution resulting in a heterozygous substitution of glycine for arginine at amino acid 153 of POMC. A schematic structure of the POMC prohormone and its peptide products is presented. Amino acids at known proteolytic processing sites are indicated by their single letter code. $_3$MSH, $_3$-melanotropin; ACTH, adrenocorticotropin; CLIP, corticotropin-like intermediate lobe peptide; MSH, -melanotropin.

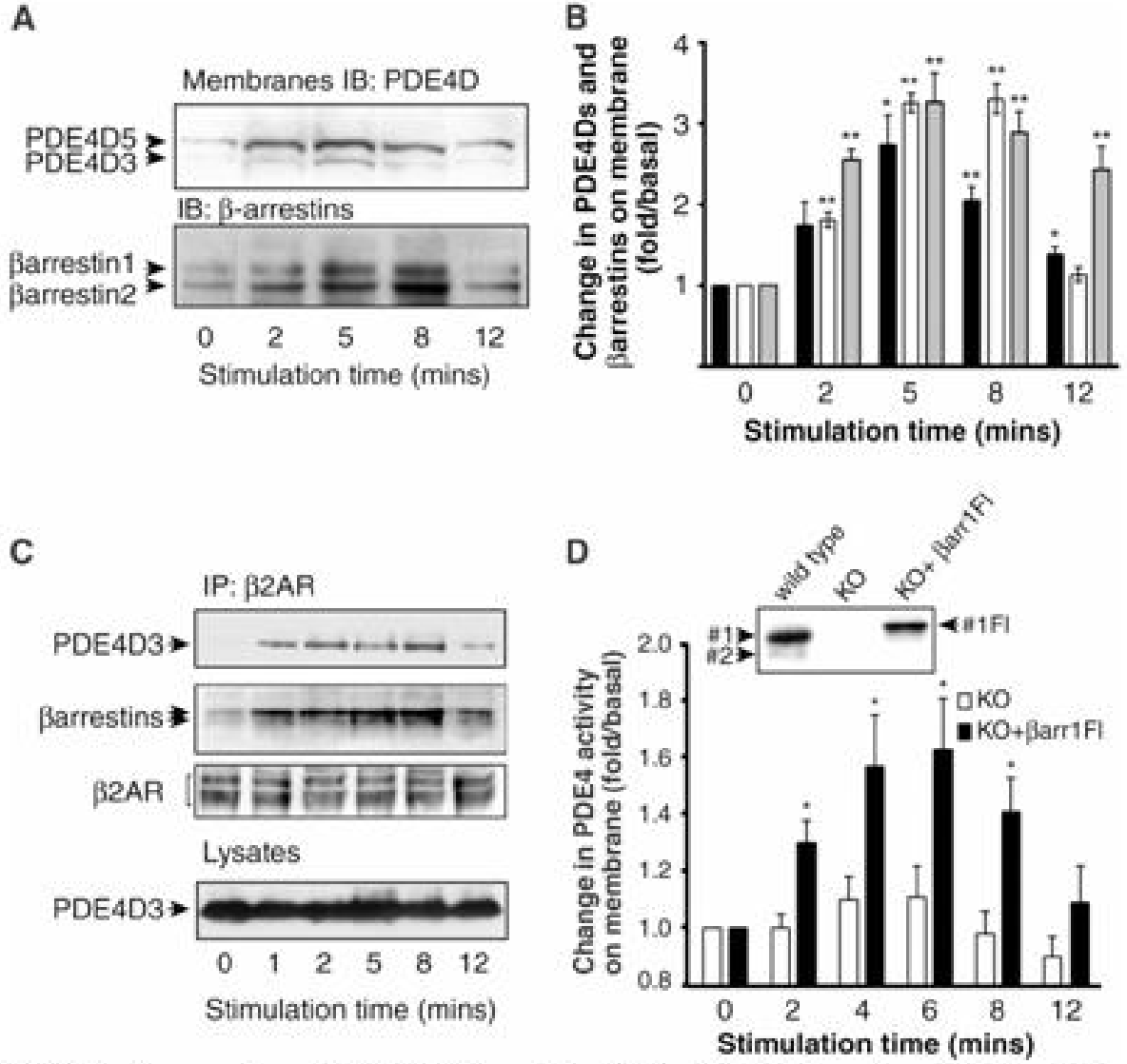
Based upon these observations, you develop a mutant mouse strain via targeted gene modification that bears the same sequence variation identified in the two human probands. Preliminary studies indicate that the mutant animals display an obese phenotype similar to that of their human counterparts.

1) Develop a hypothesis for the molecular basis underlying the observed obese phenotype and design a series of experimental strategies to test it.

2) When heterozygous mutant mice were interbred, the genotype of resultant progeny was as follows: 39 wild-type, 80 heterozygous, 10 homozygous mutant. Your mentor tells you that he wouldn't be surprised if the homozygous mutant mice were hypoglycemic, hyponatremic and died within a few weeks of birth. Explain why your mentor might suggest such phenotypic consequences resulting from the homozygous R153G mutation.

QUESTION 5:

Catecholamines signal through the β2-adrenergic receptor (β2-AR) by promoting production of the second messenger cAMP. The magnitude of this signal is restricted by desensitization of the receptors through their binding to β-arrestins and by cAMP degradation by phosphodiesterase (PDE) enzymes. To investigate the role of β-arrestins in β2-AR signaling, a group of investigators performed the following experiments.



(A) HEK293 cells expressing a FLAG-β2-AR were stimulated with 10 μM isoproterenol for 0 to 12 min and 50 μg of membrane proteins were assayed for the presence of PDE4D3 and PDE4D5 (upper panel) and β-arrestin1 and β-arrestin2 (lower panel) by immunoblotting with antibodies to PDE4D and β-arrestin. **(B)** Quantification of PDE4D (black bars) and β-arrestin (white bars) protein levels and PDE4D activity (grey bars) associating with membranes after isoproterenol stimulation. **(C)** HEK293 cells expressing FLAG-β2-AR and PDE4D3 were stimulated with 10 μM isoproterenol for 0 to 12 min, cell lysates were prepared, and the receptors were immunoprecipitated with an antibody to FLAG conjugated to agarose (IP: β2AR). The immune complexes were analyzed by immunoblotting with antibodies to the indicated proteins (three upper panels); an aliquot of the cell lysates was immunoblotted with antibodies recognizing PDE4D3 (bottom panel). **(D)** Mouse embryonic fibroblasts (MEFs) lacking expression of both β-arrestins (derived from β-arrestin1$^{-/-}$ β-arrestin2$^{-/-}$ knockout mice), stably transfected with empty vector (white bars) or vector containing β-arrestin1-FLAG (black bars), were stimulated with 10 μM isoproterenol, and membrane-associated PDE4 activity was determined. Expression levels of β-arrestin1 (#1), β-arrestin1-FLAG (#1Fl), and β-arrestin2 (#2) were compared by immunoblotting total cellular protein (70 μg) from wild-type MEFs, knockout MEFs transfected with empty vector (KO), and knockout MEFs transfected with vector containing β-arrestin1-FLAG (KO+βarr1Fl) with antibody to β-arrestin.

1) How would you interpret these data? Develop a hypothesis to explain these results and the potential physiological significance of these findings.

2) Design two independent experiments to test your hypothesis.

QUESTION 6:

In response to stretch, the atria release two peptides that appear to mediate vasodilation and a decrease in systolic blood pressure. In an effort to characterize the effects of these two peptides, you have obtained pure preparations of each termed PC and TC. You administer at a single infusion rate PC (Panel A) and TC at three different infusion rates (Panel B) in wild type mice. Next you repeat this experiment using a single infusion of each compound using a strain of mice, termed Unresponsive, that do not decrease blood pressure after PC administration. All drugs are administered by intravenous infusion. Figure 1 (A&B) and 2.

Figure 1

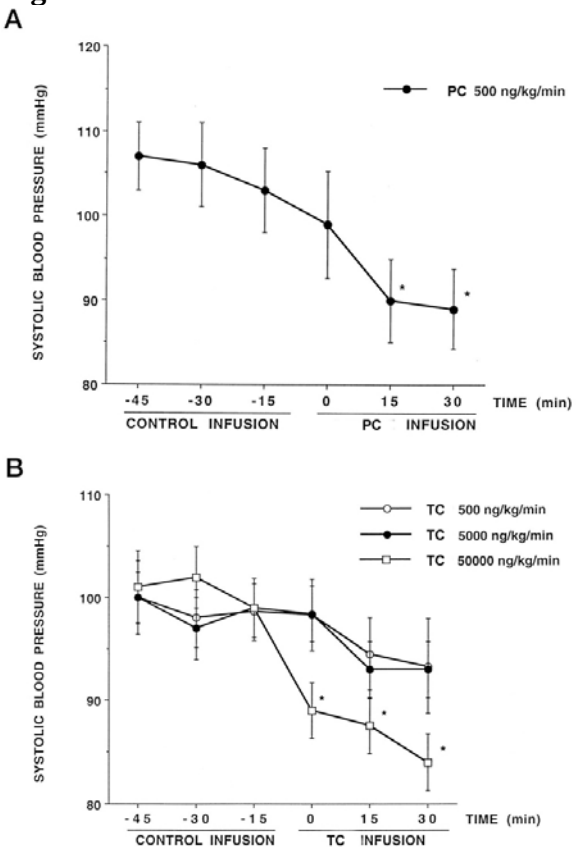


Figure 1 (A, B) Systolic blood pressure in mice responsive to the compound (PC. A-administration of PC. B-administration of 3 doses of TC.

Figure 2

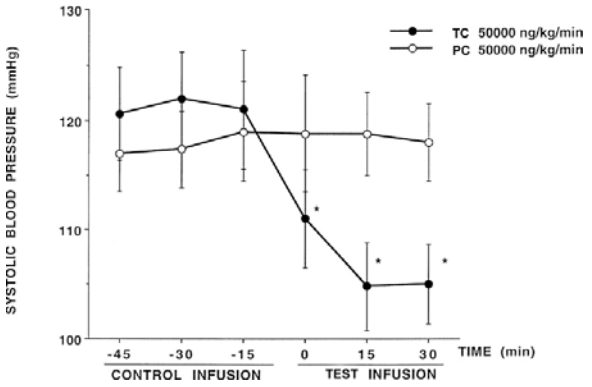
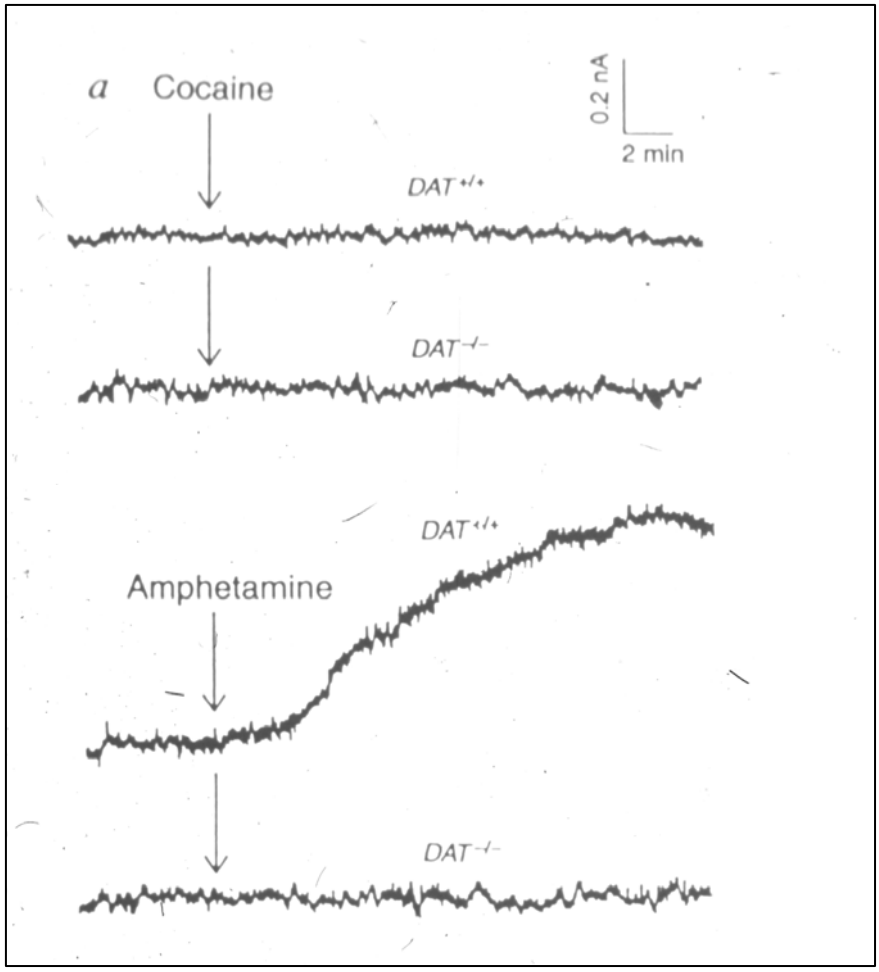


Figure 2 Systolic blood pressure in mice unresponsive to PC. PC and TC administered.

Postulate a testable hypothesis to explain the differences in the effects of PC and TC in these two strains of mice. Outline experiments to test your hypothesis.

QUESTION 7:

Plasma membrane dopamine transporter (DAT) deficient mice have been generated through conventional homologous recombination techniques. When striatal slices are prepared from these mice and exposed to 10 µM cocaine or amphetamine *in vitro*, and dopamine release is monitored with extracellular carbon fiber electrodes, the following data is obtained.



We see that in both the wildtype and the knockout line, cocaine is ineffective in causing a rise in extracellular dopamine whereas amphetamine triggers efflux from the wildtype but not the knockout line. In wildtype mice, intrastriatal amphetamine and cocaine trigger an increase in extracellular dopamine.

A) Describe the mechanistic differences between amphetamine and cocaine in terms of how they trigger enhanced extracellular levels of dopamine *in vivo*. Draw a diagram of a dopaminergic neuronal terminal to illustrate your answer.

B) How does your answer to A) explain the difference seen with cocaine and amphetamine's action on brain slices *in vitro*?

C) Depletol™, structurally similar to reserpine, abolishes the ability of cocaine and amphetamine to enhance extracellular dopamine *in vivo* and *in vitro* if given to animals for 1 week prior to experiments with the latter agents. Describe a mechanism by which Depletol™ is likely to achieve this action. How would you test for your mechanism?