

Department of Pharmacology

# Qualifying Examination (Part I)

July 11-14, 2006

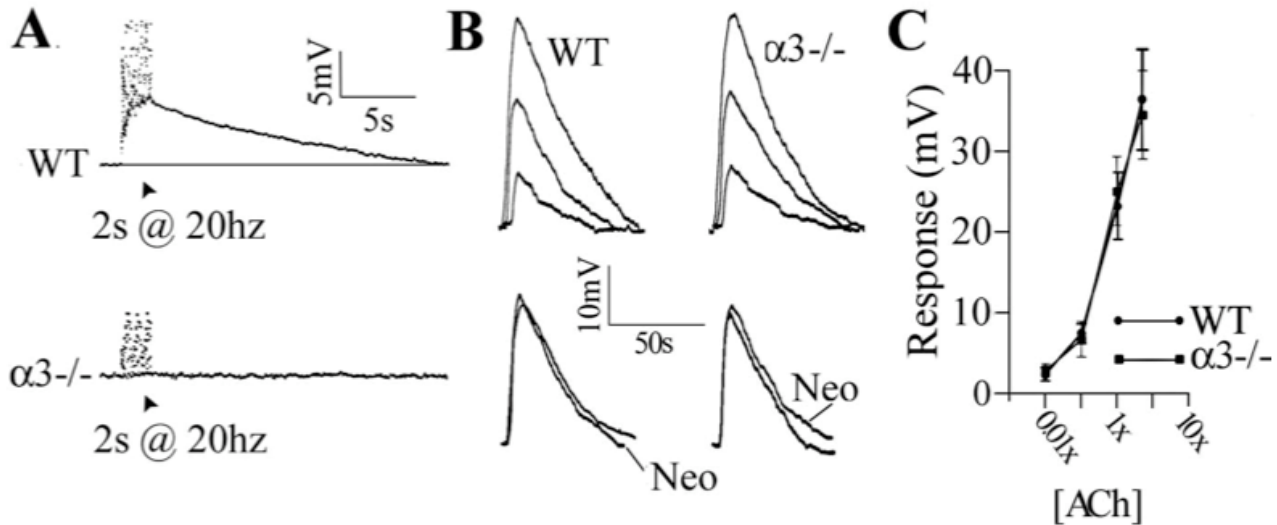
Please remember that this is a closed-book examination. You must be prepared to answer 4 of the 7 questions. Although not necessary, you may prepare written answers, overhead figures, or any type of materials that you think might be useful in the presentation of your answers. You may bring such preparation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

936-1722 (w)  
385-4396 (h)  
300-9569 (c)

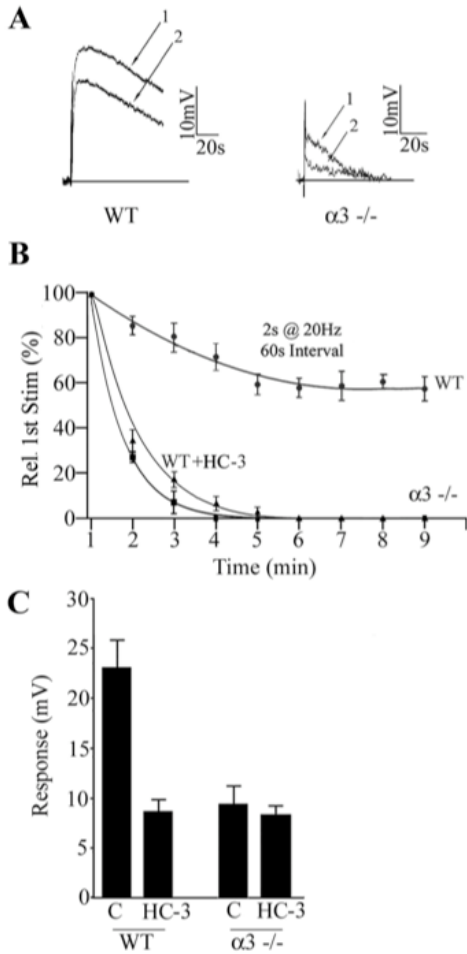
**BEST WISHES FOR YOUR SUCCESSFUL COMPLETION OF THE EXAMINATION!**

Superior cervical ganglia (SCG) receive a cholinergic preganglionic innervation that targets both nicotinic and muscarinic receptors to influence neuronal excitability. Recordings can be made from SCG neurons in the presence of the nicotinic receptor antagonist hexamethonium to reveal the muscarinic receptor-dependent component. When ganglia from an  $\alpha 3$  nicotinic receptor knockout mouse were examined for



responsiveness to preganglionic stimulation (2sec, 20 hz) in the presence of hexamethonium, evoked responses were surprisingly lost (Fig 1A), comparable to what would be observed if the muscarinic receptor antagonist atropine were added to the preparation. Investigators then performed a series of studies to examine why there was no evoked response. In Fig 1B, they examine responses to iontophoretically applied acetylcholine (ACh) at increasing concentrations (top panels) or at a fixed concentration (1X=conc giving same response as 2se, 20hz stimulation) in the presence or absence of the acetylcholinesterase (AChE) inhibitor neostigmine (Neo, 100  $\mu$ M). Neo added to evoked experiments revealed a small, but significant response to evoked stimulation. In Fig 1C, they quantify responses from the dose response study in B in the presence of Neo. All iontophoretic and evoked responses are fully blocked by atropine.

1. Outline the general structure of the different ACh receptors that support cholinergic signaling. If there is molecular heterogeneity that accounts for their signaling potential, describe it. Discuss the different mechanisms by which nicotinic and muscarinic receptors can bring about changes in membrane excitability.
2. From the data in Fig 1, what can you conclude regarding the contributions of altered nicotinic or muscarinic receptor signaling in supporting the loss of evoked responses? Does altered AChE activity appear to play a major role in the loss of responses?



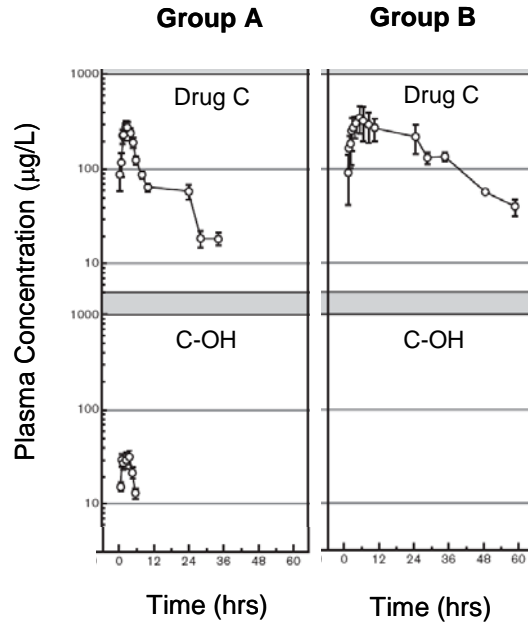
In another series of experiments, investigators explore presynaptic elements that might explain the diminished evoked responses seen in Fig 1. Examination of the density of cholinergic synaptic vesicles as imaged with vesicular acetylcholine transporter (VACHT) antibodies revealed no differences. No differences were seen as well in the morphology of cholinergic terminals, including numbers of docked vesicles, or number of varicosities, as defined by electron microscopy.

In testing the response to repeated preganglionic stimulation in the presence of neostigmine (Fig 2A), the investigators noticed a greater sensitivity to repeated stimulation in the knockout cultures. In A, repeated bursts of 2sec, 20 hz stimulation-1=1<sup>st</sup> response, 2=2<sup>nd</sup> response, show a decline to 65% in wildtype cultures whereas this level fell to 25% in knockout cultures. This change in sensitivity to evoked responses in the knockout cultures was similar to that seen when wildtype cultures are incubated with the presynaptic choline transporter antagonist hemicholinium-3 (HC-3; Fig 2B). Finally, when knockout cultures were pre-incubated for 15 min with HC-3 (20  $\mu$ M) prior to stimulation, wildtype and knockout cultures displayed a very different sensitivity to HC-3, with no change in response evident for knockout cultures.

- Describe the deficits in Fig 2A and indicate several presynaptic mechanisms that could support a change in the response to repetitive stimulation (Also recall that in Fig1A, no neostigmine is present so you see little or no evoked whereas the small response obtained in Fig 2A in the knockout cultures is in the presence of neostigmine).
- How does the data described in Fig 2B and C focus your attention on specific presynaptic deficits? Describe experiments that you could perform to establish the nature of the deficit you propose. Propose a mechanism by which  $\alpha 3$  subunit loss translates into the deficit you believe underlies the changes in evoked responses.

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Question 2

Drug C (100 mg) was orally administered to a group (n=7) of healthy individuals. The plasma concentrations of the parent drug as well as its metabolites C-OH were determined over a 60 hour period. Interestingly, the subjects could be segregated in two distinct pharmacokinetic phenotypes (Group A and Group B). The plasma concentration-time profiles for each group are as follows:



Pharmacokinetic analyses of Drug C and C-OH (mean with range) were performed:

Parameter	Group A (n=4)	Group B (n=3)	p-value
<u>Drug C</u>			
AUC (mg-hr/L)	3 (2.5-4)	9 (7-12)	p<0.01
t <sub>1/2</sub> (hr)	9 (7-10)	14(11-40)	p<0.05
<u>C-OH</u>			
AUC (mg-hr/L)	0.16 (154-163)	ND	p<0.01

Comparison between Group A and Group B, Student's t-test

AUC = area under the plasma concentration-time curve from time = 0 to time = ∞

t<sub>1/2</sub> = half-life

ND = not detectable (below limit of detection)

- Q1. How do you interpret the pharmacokinetic differences between subjects in Group A and Group B?
- Q2. How would you determine the bioavailability, clearance and volume of distribution of drug C in the two groups?
- Q3. What mechanisms are possibly involved in the differences in plasma drug and metabolite concentrations between subjects in Group and Group B?
- Q4. How would you design in vitro and in vivo experiments to test your mechanistic hypotheses?

During your vacation on a remote island in the South Pacific, you come across a small native community where the adult male villagers display a number of characteristics associated with hypothyroidism. You recall that endemic goiter often results from a lack of sufficient iodine in the diet, yet it is unlikely that only adult males would be affected when the entire village subsists on a diet rich in seafood. After spending some time with the village elders, you are invited to a ceremonial rite of passage where teenage, euthyroid boys demonstrate their manhood by hunting for wild boar and begin the daily practice of chewing an exotic orchid root which tribal lore suggests will increase their strength and longevity.

Upon returning to Vanderbilt, you become interested in the biological activities of the orchid root and orally administer a root extract to mice for a two-week period. Treated mice soon demonstrate symptoms of hypothyroidism and you perform radioimmunoassay analyses of thyroid hormone status for these animals (Table 1).

Mouse #	TRH (nM)	TSH (nM)	T <sub>3</sub> (nM)	T <sub>4</sub> (nM)
1	17.6	19.6	0.21	14
2	12.9	23.4	0.43	9
3	14.2	22.1	0.05	6
<b>Normal Range</b>	3.0-5.5	7.3-12.1	1.1-3.1	70-160

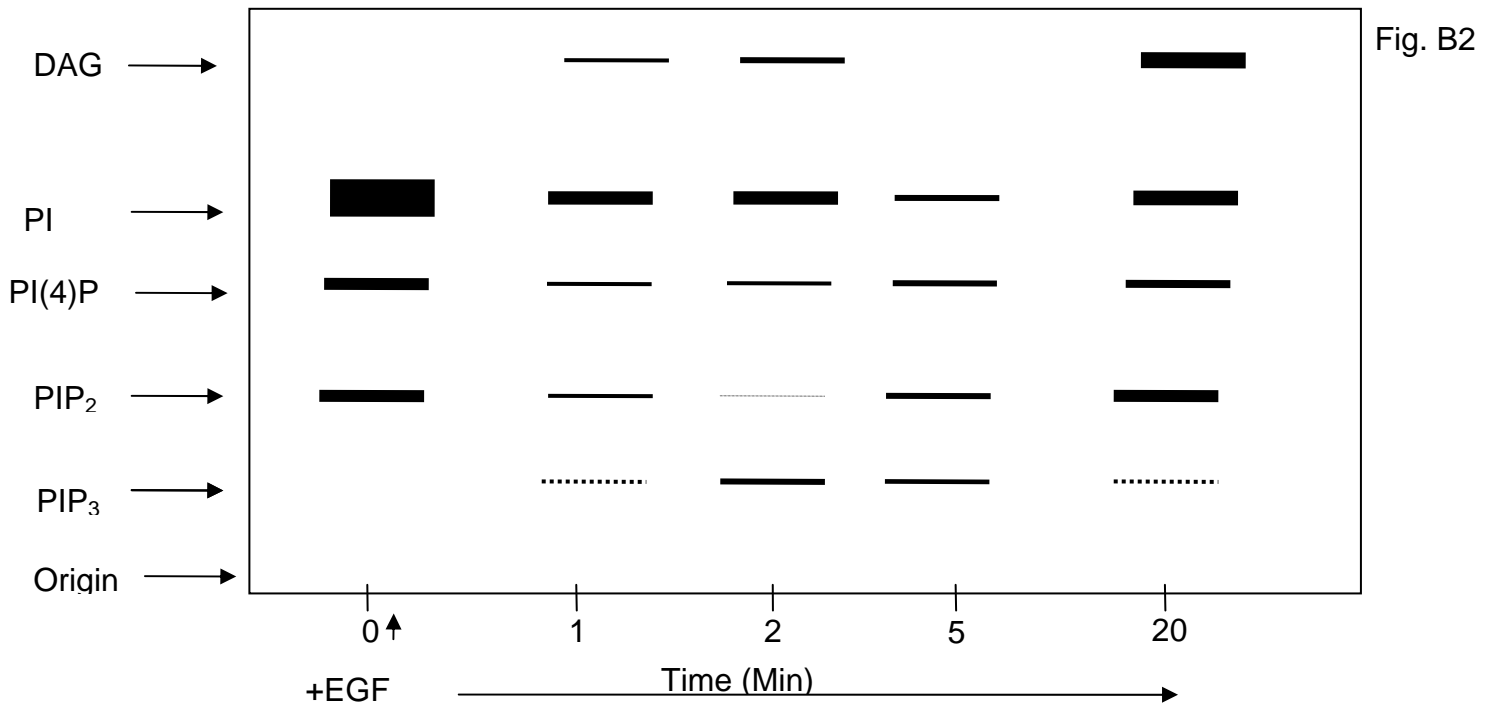
**Table 1.** Portal capillary and serum concentrations for hormones involved in thyroid endocrine status in orchid extract-treated and control mice.

- 1) Describe the characteristics you may have observed in the villagers (and mice) to identify their hypothyroid phenotype.
- 2) Based upon your knowledge of thyroid hormone synthesis and secretion, provide a detailed strategy of how you would experimentally identify the molecular targets through which the active compound(s) in the orchid root act to affect thyroid hormone status. Pay particular attention to describing the model system(s) you would employ as well as the analytical strategies by which you could perform such studies. Provide sufficient experimental detail to convey a thorough understanding of the physiological, cellular and molecular processes underlying the production of thyroid hormone.

**Background/Context:** Stimulation of the Epidermal Growth Factor Receptor (EGF-R) initiates a series of integrated signaling pathways via its receptor tyrosine kinase (RTK) activity. In mammalian cells the basic elements of the biochemical network involve the participation of multiple signal transduction components, including the monomeric GTPase Ras and the lipid kinase PI(3)K (among others). One effector essential to cell growth that is stimulated by both Ras and PI(3)K is mTOR. These pathways regulate cell survival, cell cycle, growth, and glycolysis.

- (A) Describe the **initial signaling events utilized** by growth factor induced EGF-R activation to generate lipid second messengers and mobilize intracellular calcium. Compare to the initial lipid signaling pathways associated with RTKs to those of GPCRs \*(e.g., thrombin).
- (B) Based on your knowledge of cell signaling pathways explain the temporal changes you observe in Fig. B2. (Note the intensities of the various species are indicated by line thickness. The label is in the inositol ring, except for DAG, which was visualized by a fluorescent indicator. The assay is run in the absence of external phosphatase inhibitors).

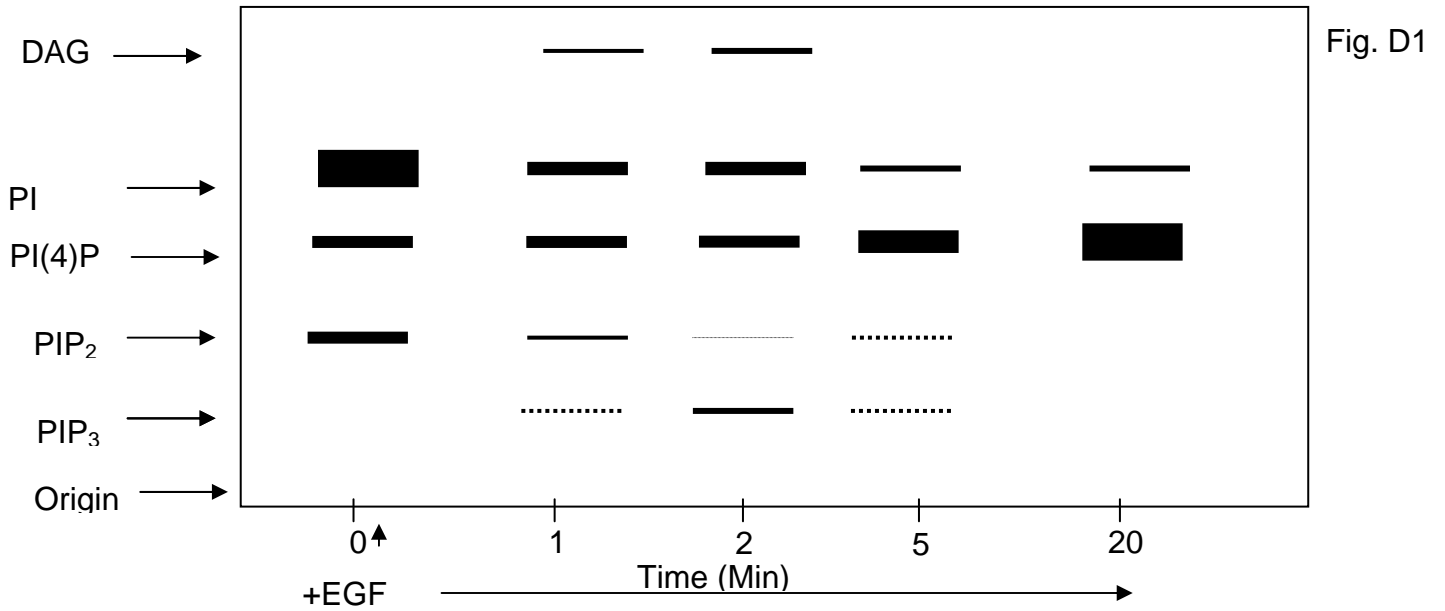
TLC (Rf)



- (C) Like RTKs, GPCRs stimulation (e.g., thrombin) often leads to the activation of phospholipase D (PLD) and cPLA2. Briefly describe the sequence of events that lead to activation of these enzymes and identify the major lipid products generated.

(D) In a screen targeting GTPases and lipid metabolic genes we observe that two knockdowns (PLD and Rac GTPase) resulted in highly similar and reproducible changes in PIP<sub>n</sub> species as illustrated below (**Fig D1**). Knockdowns of either gene product in a tissue-specific inducible system resulted in changes in the response to EGF stimulation. The tlc shown in Fig B2 is the wild type response and the tlc shown in Fig D1 shows the response after knocking down either PLD or Rac GTPase: Explain these changes in the PIP<sub>n</sub> response:

TLC (Rf)



Random mutagenesis in the mouse has inactivated a gene termed X that results in an animal with elevated mean arterial pressure. Characterization of this animal is described below. Figure 1 depicts the actions of losartan administration on mean arterial pressure and angiotensin II, bradykinin, and norepinephrine levels as well as myocardial morphology. Figure 2 presents noradrenaline and angiotensin II levels, mean arterial pressure, heart rate, and myocardial morphology after propranolol administration.

Describe the data outlined below. State a hypothesis as to the role of gene X in the cardiovascular system. State two possible mechanisms for the heart phenotype noted and outline experiments to differentiate between these mechanisms.

Figure 1

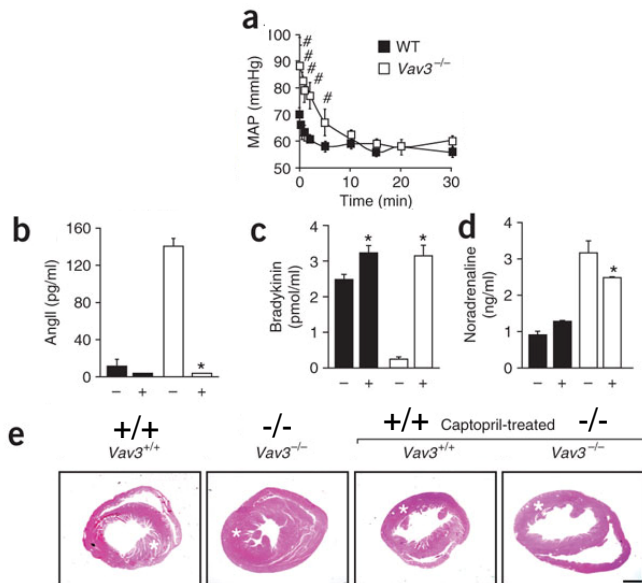
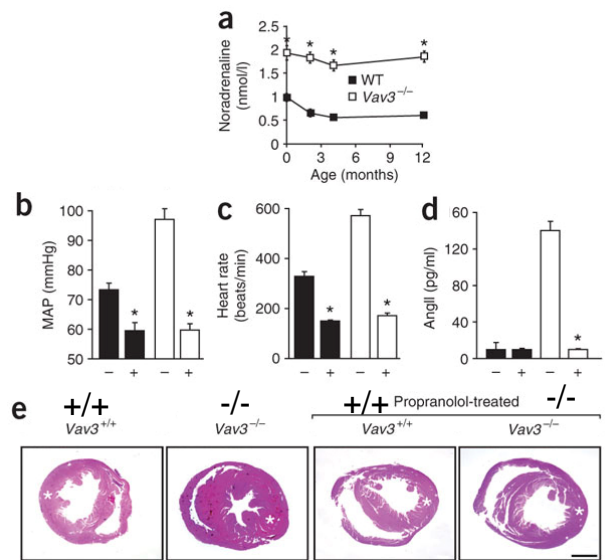


Figure 2



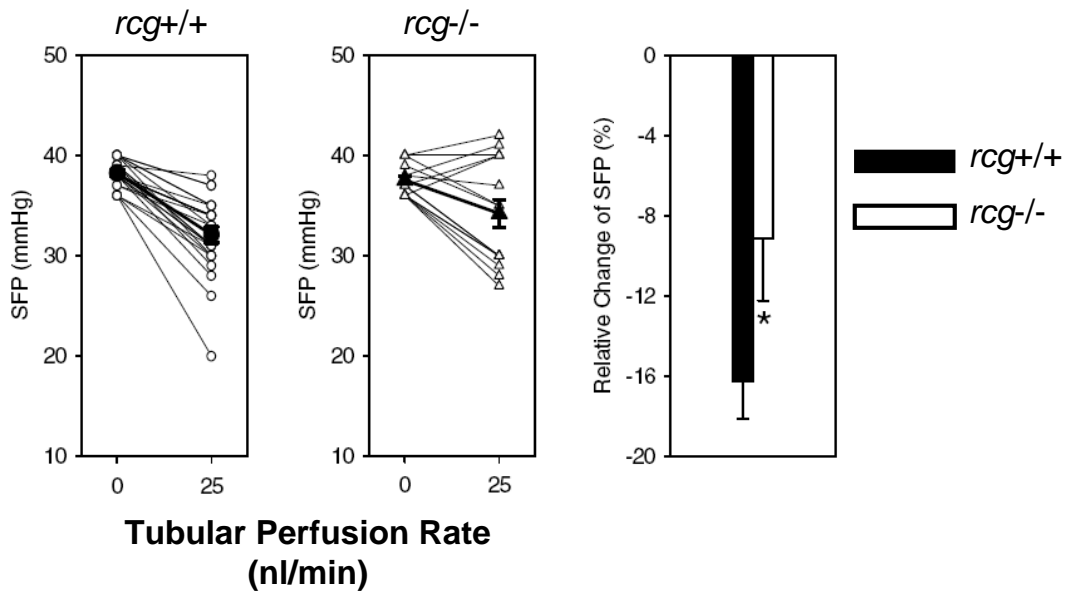
**Figure 1. Renin-Angiotensin System in Vav3<sup>-/-</sup> mice.** (a) Mean arterial blood pressure of either wild type or Vav3<sup>-/-</sup> mice after Losartan (angiotensin receptor blocker) administration at time zero (n=6). (b-d) effect of captopril (angiotensin receptor blocker) on the plasma levels of Angiotensin II, bradykinin, and noradrenaline (n=6 for each) in wild type (black) and vav3<sup>-/-</sup> (white) mice. + = captopril-treated. (e) histological sections of the hearts of 4.25 month-old mice of indicated genotypes, plus and minus captopril. Sections are representative of 5 mice from each group. Scale bars=100 μM. Error bars represent sem \*P<0.01.

**Figure 2. Sympathetic Nervous System in Vav3<sup>-/-</sup> mice.** (a) noradrenaline levels in wildtype and Vav3<sup>-/-</sup> mice (n=5). (b-d) effect of propranolol on the mean arterial pressure, heart rate, and plasma levels of Angiotensin II (n=6 for each) in wild type (black) and vav3<sup>-/-</sup> (white) mice. + = propranolol-treated. (e) histological sections of the hearts of 4.25 month-old mice of indicated genotypes, plus and minus captopril. Sections are representative of 5 mice from each group. Scale bars=100 μM. Error bars represent sem \*P<0.01.

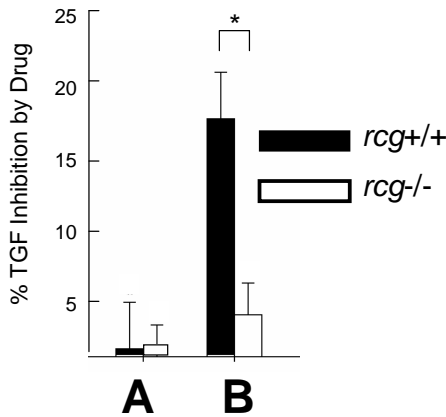


Tubuloglomerular feedback (TGF) describes an intrinsic renal mechanism that modulates glomerular filtration rate (GFR) in response to varying solute delivery to the macula densa. A soluble signal released by cells of the macula densa mediates TGF. The most well studied candidate for this soluble signal is adenosine, but other molecules such as ATP have been proposed. In one model, ATP that is released by MD cells in response to increased tubular NaCl delivery is subsequently metabolized to adenosine by an extracellular enzyme (named *Really Cool Enzyme*) and adenosine is the final mediator of TGF. An alternative model proposes that ATP has direct effects on glomerular vasculature (acting through P<sub>2</sub>X purinergic receptors) and is the TGF signal.

Mice with targeted inactivation of the gene (*rcg*) encoding *Really Cool Enzyme* have been studied. Figure 1 illustrates changes of “stop flow pressure” (SFP), a surrogate measure of single-nephron GFR, in response to different rates of thick ascending limb perfusion in either wildtype mice (*rcg*<sup>+/+</sup>) or *rcg* knockout (-/-) mice. Pharmacological studies illustrated in Figure 2 were also performed.



**Figure 1** – Changes in SFP in response to different rate of perfusion of the thick ascending limb of the loop of Henle in two strains of mice (left panels). The **bold** data symbols represent the mean ± SEM for each condition. Relative change of SFP (%) shown in the far right panel was calculated by comparing measurements between 0 vs 25 nl/min perfusion rates. The asterisk (\*) indicates P < 0.05 comparing *rcg*<sup>-/-</sup> with *rcg*<sup>+/+</sup> mice.



**Figure 2** – Degree of TGF inhibition by a P<sub>2</sub>X purinergic receptor antagonist (A) or adenosine A1 receptor antagonist (B). An asterisk (\*) indicates P < 0.05 comparing *rcg*<sup>-/-</sup> with *rcg*<sup>+/+</sup>.

**Questions:**

1. Provide a complete physiological explanation of the data in Figure 1 with an emphasis on differences between *rcg*<sup>+/+</sup> and *rcg*<sup>-/-</sup> mice.
2. Explain the significance of the data in Figure 2. Which model described in the first paragraph is best supported by these data? Why?

Stimulation of the  $\beta$ 2-adrenergic receptor ( $\beta$ 2AR) is known to induce activation of adenylyl cyclase and ERK. Activation of ERK by the  $\beta$ 2AR can be resolved into two components - an early phase (peaking at 5 min) and a less robust but more sustained phase (10-30 min). To explore the underlying molecular mechanisms that could explain these phenomena, a group of investigators performed the following experiments.

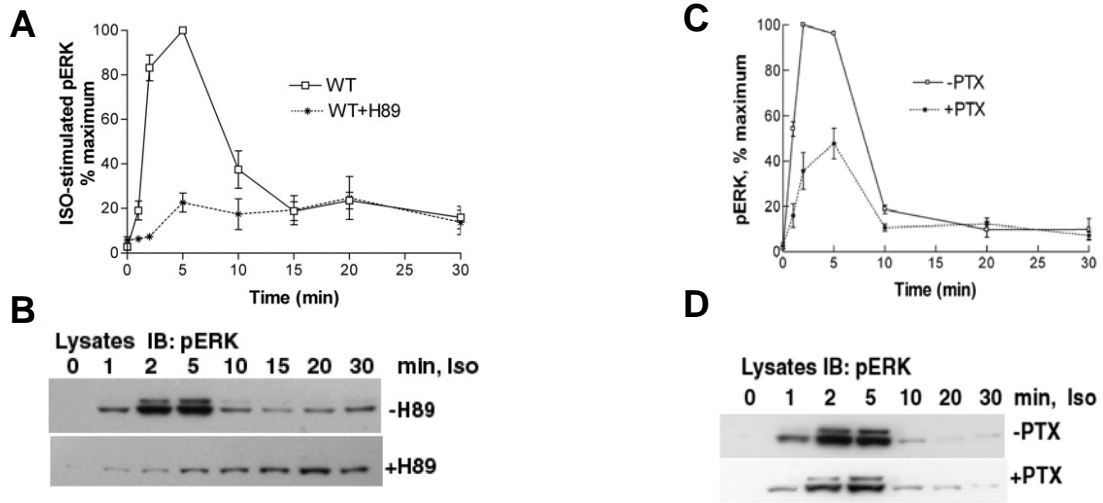


Figure 1. **H-89 and pertussis toxin sensitivity of ERK phosphorylation stimulated by the  $\beta$ 2AR.** HEK-293 cells stably expressing the  $\beta$ 2AR were preincubated with vehicle (*-H89* and *-PTX*), 20  $\mu$ M H-89 (*+H89*; a well defined PKA inhibitor), or 100 ng/ml pertussis toxin (*+PTX*), and then treated with 10  $\mu$ M isoproterenol (*Iso*) for the indicated times. Equal amounts of cell lysate were resolved by SDS-PAGE and analyzed for phospho-ERK (*pERK*) by immunoblotting (*IB*) (*B* and *D*). Signals were quantified by densitometry, normalized for total ERK, and expressed as a percentage of the maximal phosphorylated ERK obtained at 5 min. Each data point represents the mean  $\pm$  S.E. from four experiments (*A* and *C*).

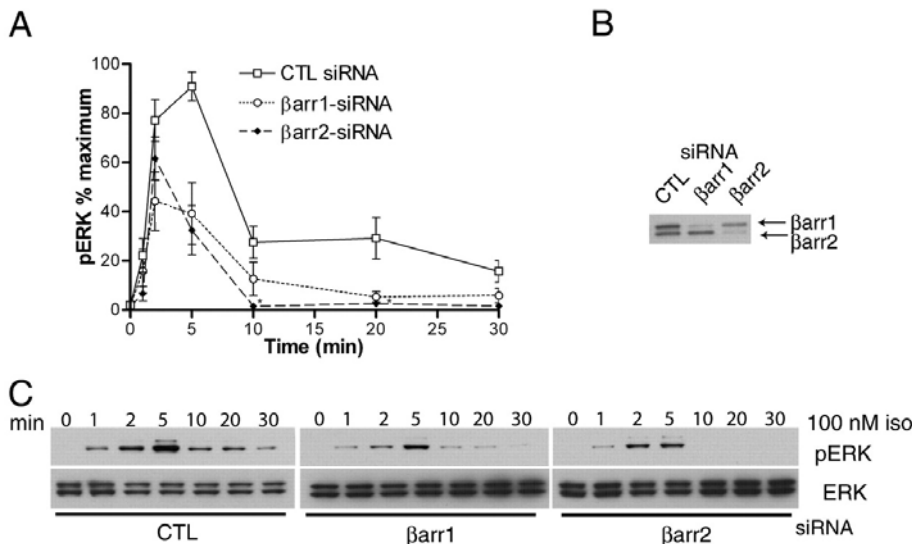


Figure 2. **Effects of  $\beta$ -arrestin siRNA on  $\beta$ 2AR stimulated ERK phosphorylation.** HEK-293 cells stably expressing the  $\beta$ 2AR were transfected with the indicated siRNAs. Serum-starved cells were treated with 10  $\mu$ M isoproterenol (*Iso*) for the indicated times and the resulting cell lysates were analyzed for pERK and total ERK (*A* and *C*) and  $\beta$ -arrestin (*B*) by immunoblotting. pERK bands were quantified, normalized for total ERK levels, and plotted in the *graph* shown in *A*. Signals at each point are expressed as a percentage of the maximal pERK signal with control (*CTL*) siRNA (5 min). The quantification is the mean  $\pm$  S.E. from six separate experiments (*A*).

1. Develop a hypothesis to explain these findings. Describe how the results of these experiments support your hypothesis.
2. Design two independent experiments to test your hypothesis.