Department of Pharmacology

Qualifying Examination (Part I)

July 17-19, 2012

All Exams take place in the Bass Conference Room (436 RRB)

Tuesday, July 17th	**10:00 am – 12:00 pm (Exam #1)**	**Isaac Zike**
	12:00 pm – 1:00 pm	*Lunch Break for Examination Committee*
	1:00 pm – 3:00 pm (Exam #2)	**Matthew Karolak**
	3:00 pm – 5:00 pm (Exam #3)	**Qiuyan Chen**
Wednesday, July 18th	**10:00 am – 12:00 pm (Exam #4)**	**Michael Nedelcovych**
Thursday, July 19th	**10:00 am – 12:00 pm (Exam #5)**	**Elizabeth Ennis**
	12:00 pm – 1:00 pm	*Lunch Break for Examination Committee*
	1:00 pm – 3:00 pm (Exam #6)	**Michael Grannan**
	3:00 pm – 4:00 pm **(Bass Conf. Room)**	Committee Meets to determine results
	4:00 pm **(Pharm South Conf. Room, 449 PRB)**	Results given to students**

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 Seva Gurevich at:
615-322-7070 (w)
615-668-4849 (c)

** On Thursday at 4:00 pm, students will line up outside the Pharm South Conference Room in the order in which the examinations were taken. You will be called into the Conference Room individually to receive ONLY the results of your exam. There will not be any discussion about the exam at that time. You will then be escorted out the opposite door, into 417 PRB. You may stay or leave, as you wish. If you need discussion of your results with the DGS or members of the committee, instructions on doing so will be provided to you at a later date. Each student will also receive an official letter from the DGS with the results of their examination and instructions on the next step(s) you need to take.

Low-dose psychostimulants, including methylphenidate (MPH; Ritalin) and atomoxetine (ATM, Strattera), are the most effective and widely used form of therapy for attention deficit/hyperactivity disorder (ADHD). Optimal doses of MPH or ATM are known to improve attention and cognitive performance in cognitive tasks dependent on the prefrontal cortex (PFC). However, neurotransmitters and receptors involved in mechanisms underlying the enhancement of attention remain largely unexplored.

The study examined the role of dopamine (DA) and norepinephrine (NE) in the prefrontal cortex (PFC) and striatal regions (nucleus accumbens), as well as the involvement of neurontansmitter receptor subtypes in the behavioral effects of the drugs.

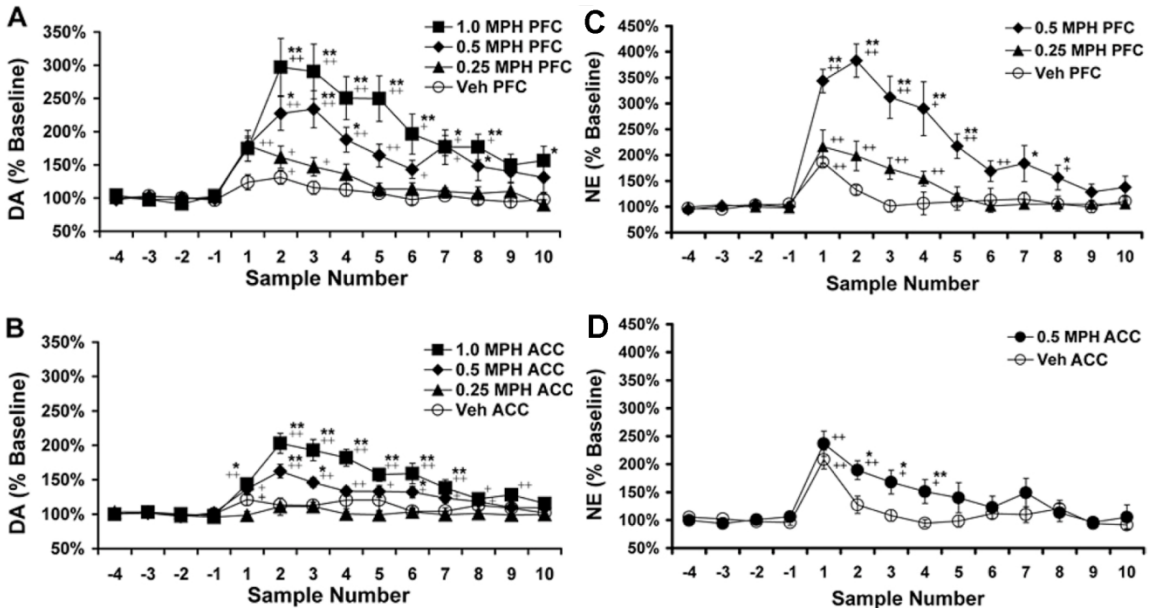


Figure 1. Effects of intraperitoneally administered low-dose methylphenidate (MPH) on extracellular levels of DA (**A,B**) and NE (**C,D**) within the prefrontal cortex (PFC) **(A,C)** and nucleus accumbens (ACC) **(B,D**) in rats. Shown are means ± SEM. The neurotransmitter levels expressed as a percentage of baseline in samples collected by in vivo dialysis in freely moving rats every 16-min before (negative numbers) and after (positive numbers) injection of vehicle or varying doses of MPH (.25, .5, 1.0 mg/kg). In both regions, MPH produced dose-dependent increases in DA and NE levels. At all doses, larger increases were observed within the PFC. At the lowest dose examined (.25 mg/kg), MPH had no noticeable effect on DA or NE efflux within the ACC. +p<.05, ++p <.01 compared with sample immediately preceding drug administration (sample -1); *p<.05, **p<.01 compared with vehicle-treated animals.

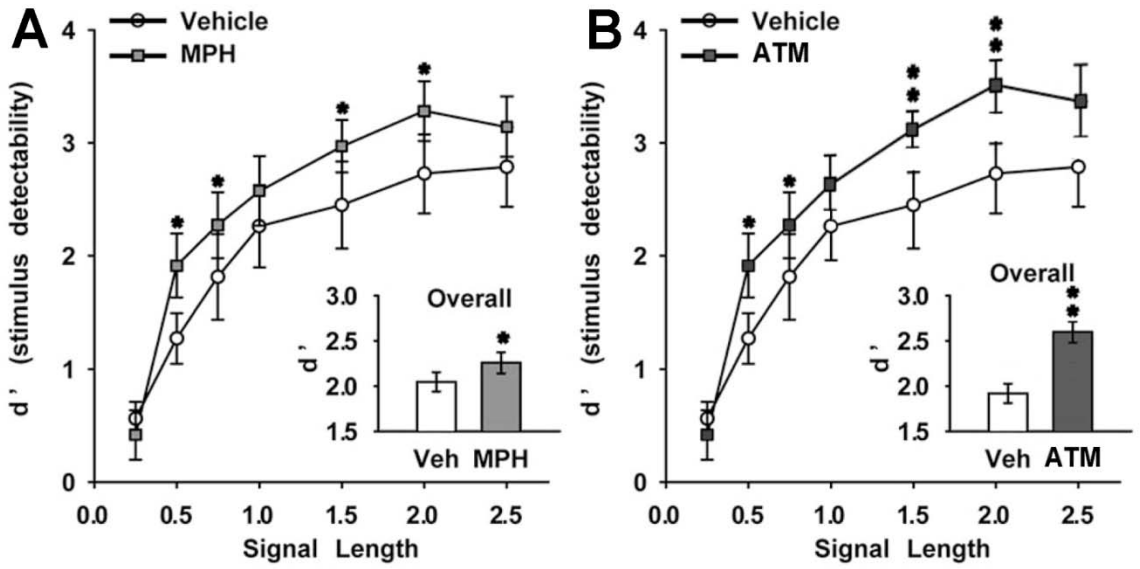


Figure 2. Low-dose methylphenidate (MPH; **A**) and atomoxetine (ATM; **B**) facilitates sustained attention as measured in a visual signal detection task. Effects of vehicle and 0.5 mg/kg intraperitoneal MPH (**A**) or 0.1 mg/kg ATM (**B**) on performance as measured by d' (relative measure of stimulus detectability, which reflects animals attention, i.e., how well the animal detects the stimulus). In this panels, the line graphs displays the effects of vehicle and drugs on d' as a function of signal length; MPH and ATM facilitate performance at all but the shortest signal length. (Insets) Bar graphs indicate d' = calculated across all signal lengths.
*p <.05, **p < .01 compared with vehicle-treated animals.

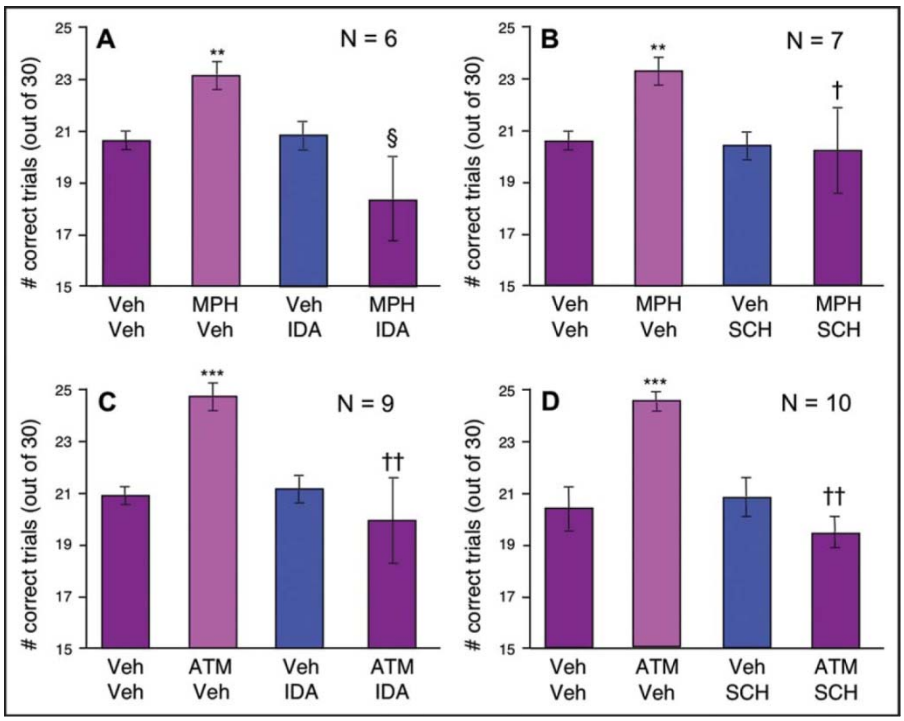


Figure 3. The effect of α2-adrenoceptor antagonist idazoxan (IDA) (**A, C**), or the D1 dopamine receptor antagonist, SCH23390 (SCH) (**B, D**) on enhanced performance in the delayed response task (another measure of sustained attention) induced by optimal doses of methylphenidate (MPH; **A, B**) and atomoxetine (ATM; **C, D**). *p < .05 compared with vehicle; **p < .005 compared with vehicle; ***p < .001 compared with vehicle; § p < .05 compared with IDA + vehicle; †p < .01 compared with MPH+vehicle; ††p < .001 compared with ATM+vehicle. Veh =vehicle.

1. **What neurotransmitter system(s) and protein(s) are targeted by methylphenidate and atomoxetine?**

2. **If you collect the data in an experiment similar to that presented in Fig. 1 with atomoxetine instead of methylphenidate, what would the curves for the PFC and ACC look like?**

3. **From the data presented in Figs. 1,2,&3, what can you conclude regarding the receptor mechanisms mediating the effects of psychostimulants on sustained attention? Which brain region do you think is the site of attention-enhancing action of the drugs and based on what evidence? How can that be tested directly?**

4. **High doses of psychostimulant drugs impair sustained attention. Based on what you know about the role of the dopaminergic and noradrenergic systems in regulating attention, propose a model to explain how low doses of psychostimulants improve attention whereas high doses compromise it (Hint: inverted U-curve).**

D-35 is an antiviral drug used to treat HIV. It has a relatively short plasma $t_{1/2}$ in vivo in humans (2-4 h) and 14 metabolites have been identified. To increase the in vivo exposure to D-35 its co-administration (daily for 7 days) with ritonavir, an antiviral that inhibits P450 metabolism, was evaluated. The results of drug levels measured on day-1 and following the last dose on day-7, are depicted in Fig 1, illustrating the plasma concentrations of D-35 and of a metabolite D-51. Some pharmacokinetic parameters on day 7 of co-administration are summarized in Table 1.

D-51 is not detectable in plasma in the absence of ritonavir co-administration.

Human feces catalyze the enzymatic reduction of D-35 to an intermediate metabolite, D-40 (Fig. 2).

Human liver S9 fraction and cytosol are found to oxidize D-40 to D-51 (Fig. 3).

Figure 1:
Plasma concentration-time profiles of D-35 and D-51 on day 1 and day 7 in humans

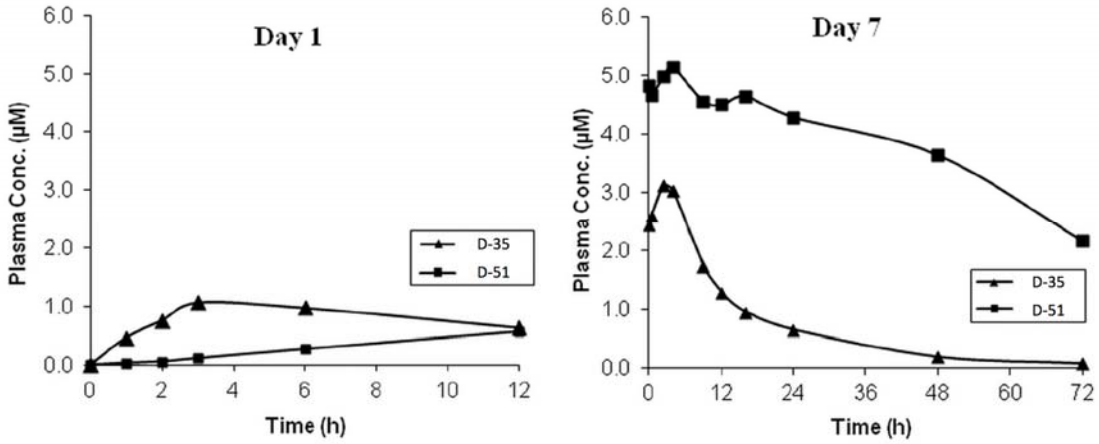


Table 1
Pharmacokinetic parameters of D-35 and D-51 after 7-day dosing of D-35 (150 mg)/RTV (100 mg) (b.i.d.) in humans
Data are means +/- S.D.

Parameters	D-35	D-51
C_{max}, μM	3.40 ± 0.870	5.37 ± 1.27
$AUC_{0-\tau}$, μmol · h/l	28.2 ± 6.46	57.1 ± 14.6
Half-life, h	14.7 ± 0.80	54.5 ± 23.3

Figure 2: Chemical structures of D-35, D-40 and D-51

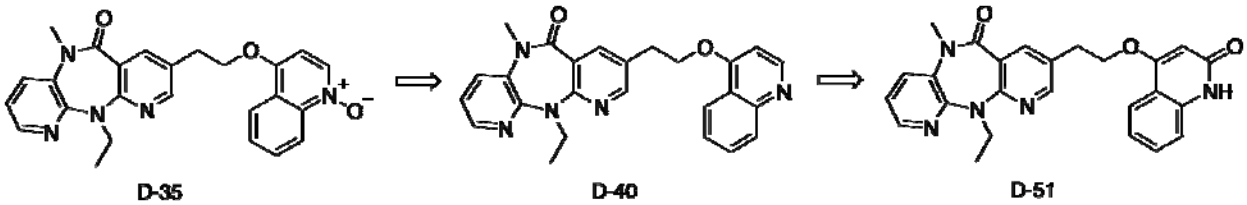
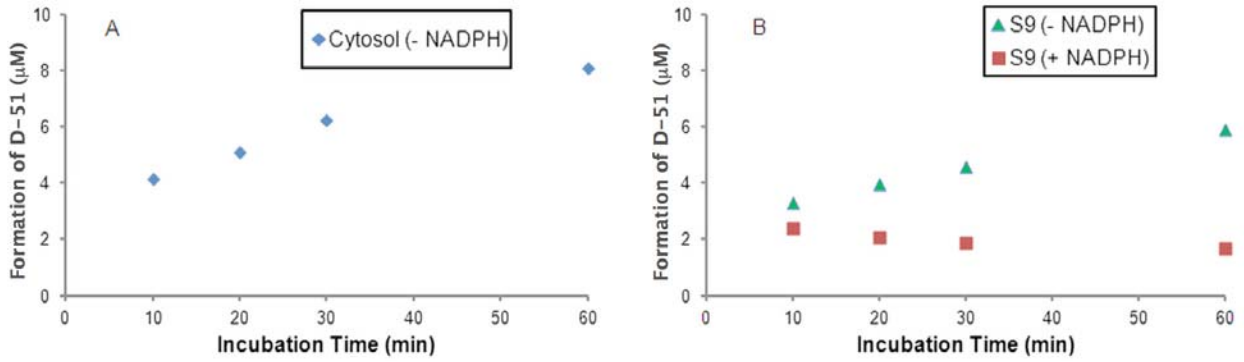


Figure 3:
Formation of D-51 in incubations of D-40 with (A) human liver cytosol in the absence of NADPH and (B) human liver S9 (9,000g supernatant) in the absence and presence of NADPH (*n* = 2)



Questions

1. **Interpret the data in Figure 1 and Table 1. Include in your answer how you account for the changed plasma levels of D-35 and D-51 by day-7 of co-administration.**

2. **Interpret the data in Figure 3.**

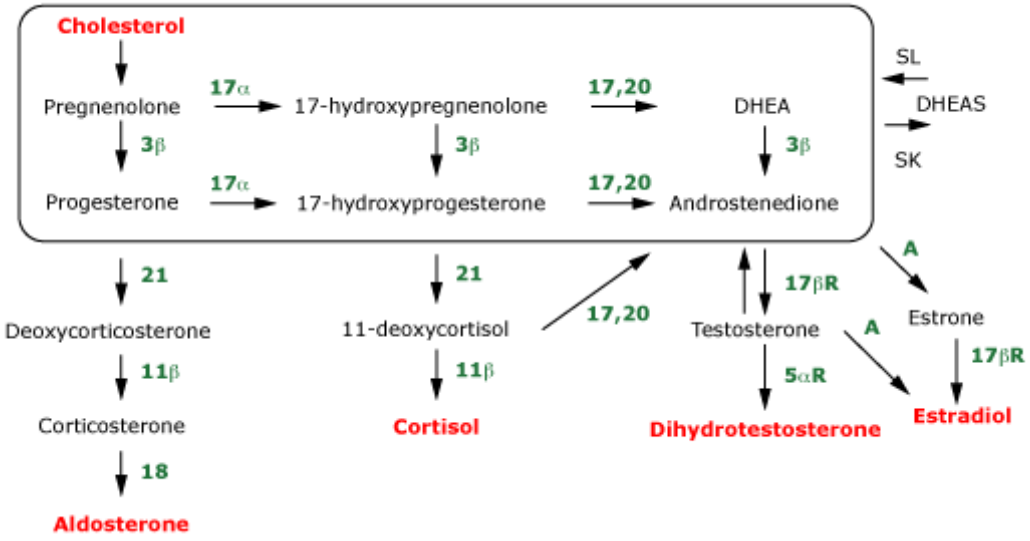
3. **Taking all the available information on metabolism of D-35 into consideration, propose a scheme to account for the metabolic disposition of D-35 during co-administration with ritonavir.**

4. **Propose experiments to further evaluate your hypothesis.**

As scientific director for the rival company for Hair Club For Men, you have been working on drugs that promote hair growth. In your zealous efforts to speed products to this multimillion-dollar market, you have come up with a drug called Viralex. The drug actually causes excessive body hair growth, and subjects receiving the drug reported 1) aggressive behavior and mood swings, 2) weakness, abdominal pain and nausea, 3) darkening of the skin even in areas not exposed to the sun, and 4) low blood pressure.

Unfortunately your colleague who did all of the pre-clinical work has fled the country on unrelated charges, and a warehouse fire destroyed all of the pre-clinical records.

The CEO asks you what went wrong… You dig out your Endocrinology book to find this pathway:



QUESTIONS:

1. **How can Viralex cause all of these symptoms?**
2. **Predict the hypothalamic reaction to Viralex treatment.**
3. **How do steroid hormones regulate gene transcription?**

The dysregulation of receptor protein tyrosine kinase (RPTK) function can result in changes in cell proliferation, cell growth and metastasis leading to malignant transformation. Among RPTKs, the TAM receptor family composed of three members Tyro3, Axl, and Mer has been recognized to have a prominent role in cell transformation. Gas6 is a ligand that binds and activates all three receptors, with binding affinities in the nM range. In the following study the contribution of this RPTK family on cell proliferation and the potential tumorigenic mechanisms were analyzed. Results are shown in Figures 1 and 2.

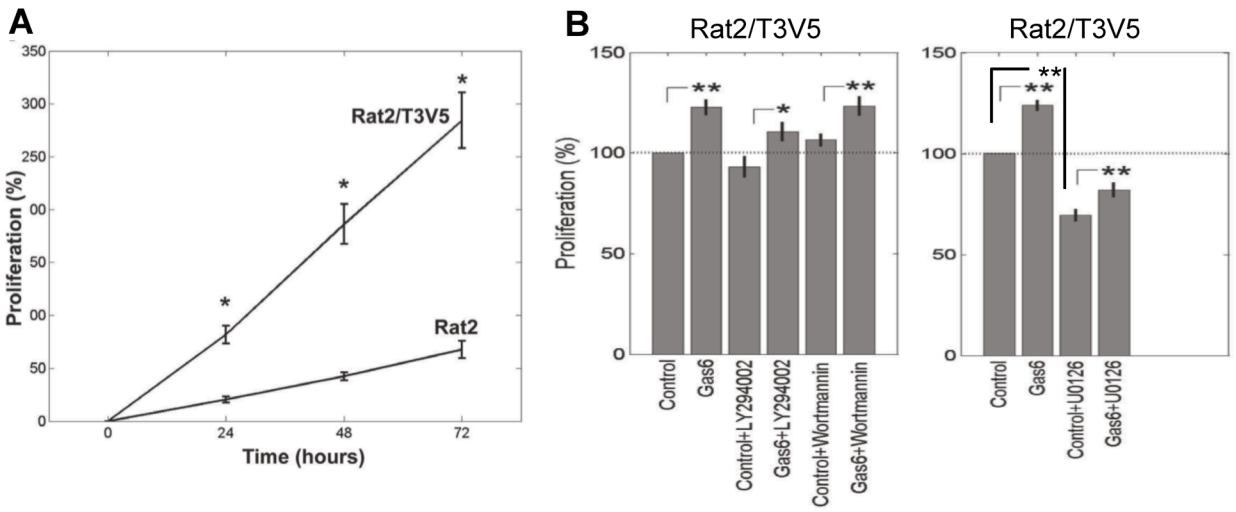


Fig 1. **A**, Enhanced proliferation of Rat2 overexpressing Tyro3. Gas6 induced cell proliferation of Rat2 and Rat2/T3V5 cells that overexpressing Tyro3 (by transfection). Serum starved cells were stimulated with 250 ng/ml of Gas6 for 0–72 hrs. Proliferative activity is expressed as % increase over the optical density (OD) obtained at 0 hrs. **B**, The effects of the signaling-pathway inhibitors on cell proliferation were investigated in Rat2/T3V5 cells. Cells were stimulated with DMEM only (control) or 250 ng/ml of Gas6 for 72 hrs in the absence or presence of the indicated inhibitors. Proliferative activity is expressed as % increase. PI3K inhibitors used were LY294002 and wortmanin. MEK inhibitor used was U0126. * p <0.05, **p<0.01

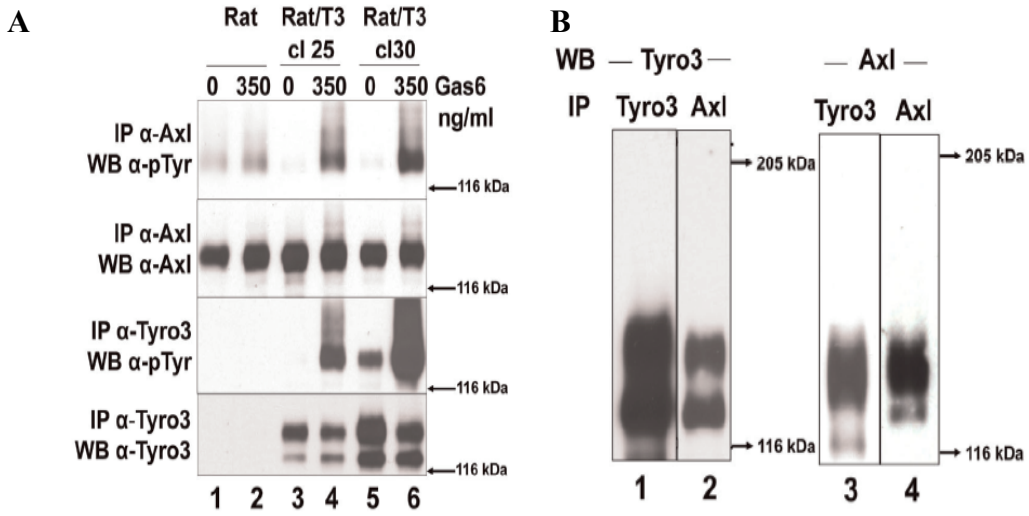


Fig 2. Interaction between Tyro3 and Axl. **A**, Phosphorylation of Axl in Rat2 cells and Rat2/T3V5 cell lines cl25 and cl30. Cells were activated with media only (0) or 350 ng/ml of Gas6 for 10 min. Detergent cell lysates were prepared. The samples were divided in two for Tyro3 and Axl immunoprecipitations (IP) followed by Western blot analysis. The membranes were probed with anti-phosphotyrosine (a-pTyr) antibodies (top and third panels), and with rabbit a-Axl (second panel from the top). The membrane corresponding to Tyro3 IP's was stripped and reprobed with a-Tyro3 (bottom panel). **B,** Tyro3 and Axl co-immunoprecipitate in Rat2/T3V5 cells. Rat2/T3V5 cells were activated with 350 ng/ml Gas6 for 10 min. Detergent extracts prepared for Tyro3 immunoprecipitation (IP) (IP Tyro3, lanes 1 and 3) and for Axl IP (IP Axl, lanes 2 and 4). The samples were separated by SDS-PAGE and blotted with a-Tyro3 (lanes 1 and 2) or rabbit a-Axl antibodies (lanes 3 and 4).

1. Explain the results as shown in Figure 1.
   A), Propose how overexpression of Tyro3 leads to increased proliferation.
   B), Design two independent experimental strategies including the predicted outcome of the experiments to support your hypothesis regarding the critical downstream signaling event leading to proliferation.

2. Explain the results as shown in Figure 2.
   A), Propose the molecular mechanism at the receptor level by which overexpression of Tyro3 leads to increased proliferation.
   B), Design two independent experimental strategies to further support your hypothesis.

In heart failure, chronic catecholamine elevation causes marked dysregulation of β-adrenergic receptors, resulting in various molecular abnormalities, including upregulation of G protein–coupled receptor kinase (GRK), especially GRK2, which is abundant in heart and upregulation of pertussis toxin (PTX)-sensitive G_i proteins. Phosphorylation of β2-adrenergic receptor (β2AR) by GRK2 is a critical determinant of cardiac function. The mechanism by which GRK2 upregulation affects β-adrenergic receptor signaling and leads to heart failure is poorly understood. The following experiments were performed to elucidate these mechanisms.

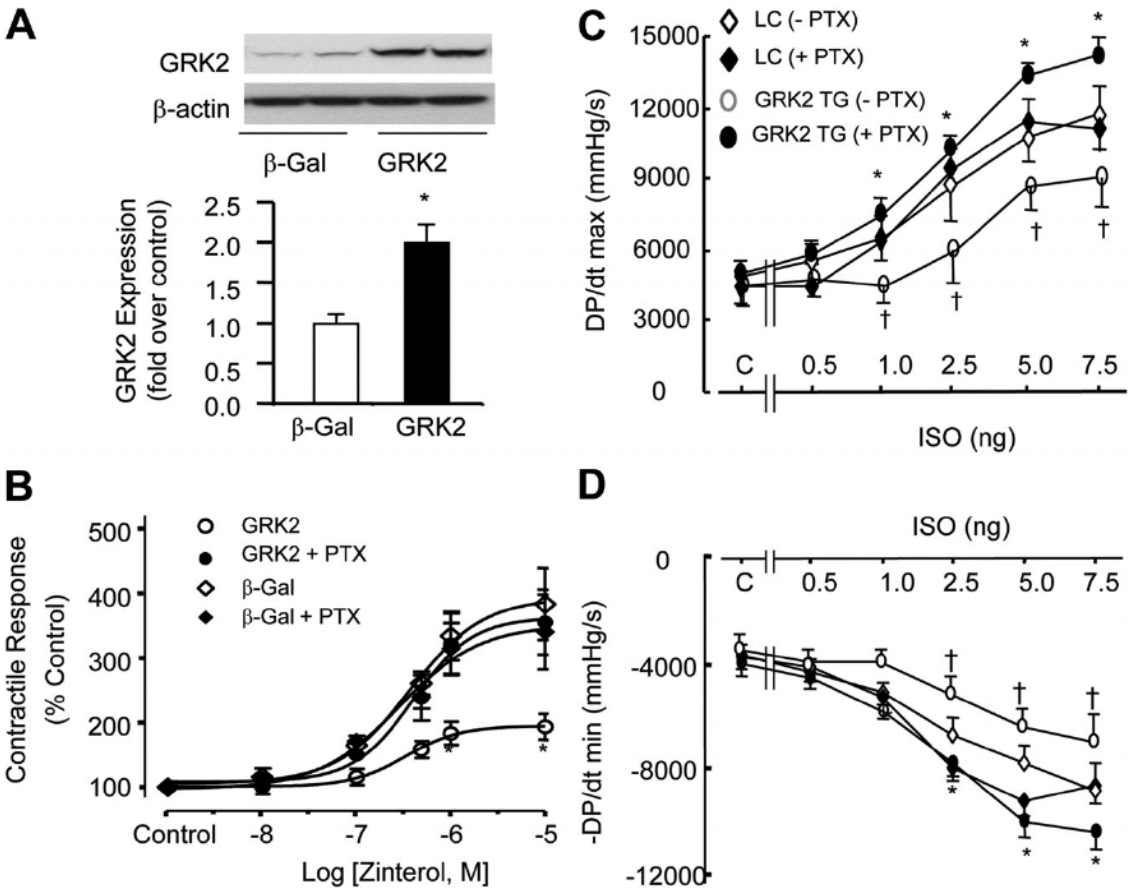


Figure legend: A, Representative Western blot (**top panel**) of GRK2 expression in cultured adult mouse cardiomyocytes infected with adenovirus-GRK2 (Adv-GRK2) or β-Gal (Adv-β-Gal) with quantified data shown in the **bottom panel** (**P*<0.01 versus β-Gal). **B,** Contractile responses of cultured adult mouse cardiomyocytes to zinterol, a β_2AR-selective agonist. Cultured cardiomyocytes were infected with Adv-GRK2 or Adv-β-Gal, **P*<0.001 versus the other 3 groups with 2-way repeated measures ANOVA. **C** and **D,** In vivo assessment of left ventricular contraction (**C**) and relaxation (**D**) in GRK2 overexpressing transgenic (TG) mice and littermate control (LC) mice (**P*<0.001 versus GRK2-TG mice without PTX with 2-way repeated-measures ANOVA; †*P*<0.05 GRK2-TG versus LC in the absence of PTX). ISO indicates isoproterenol.

Question 1: Describe the major results of the experiments and formulate a hypothesis to explain how GRK2 regulates β-AR signaling through modification of G-protein coupling.

Question 2: Design experiments in vitro and in vivo that will test your hypothesis.

The regulation of glomerular filtration rate (GFR) is mediated by several physiological processes including tubuloglomerular feedback (TGF). Recently, investigators at the Boston Institute for Gentility and the Distinguished Educational Academy for Learning (BIGDEAL) examined the effects of metabolites generated by the enzyme heme oxygenase 1 (HO-1) on TGF. HO-1 was demonstrated to be expressed and active in several cell types in the kidney. One metabolite of HO-1 activity is carbon monoxide (CO), which resembles nitric oxide in its ability to readily diffuse through cell membranes and stimulate soluble guanylyl cyclase (sGC). An *in vivo* rat model was employed in which stop-flow pressure (a proxy for GFR) was measured from an occluded proximal tubule while the thick ascending limb (TAL) was perfused at different rates with artificial tubular fluid containing 140 mM NaCl. The following observations were made.

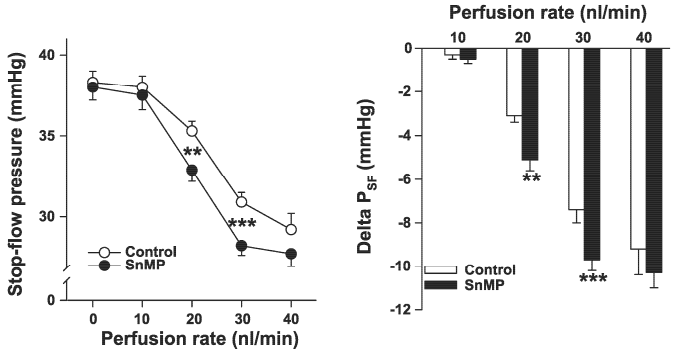


Fig. 1 – *Left panel*: stop-flow pressure (P_{SF}) measured under different TAL perfusion rates in the absence (control) or presence of stannous mesoporphyrin (SnMP), an inhibitor of HO-1. *Right panel*: summary data of net change in P_{SF} under the various conditions. **, $p < 0.01$; ***, $p < 0.001$ for SnMP *vs* control.

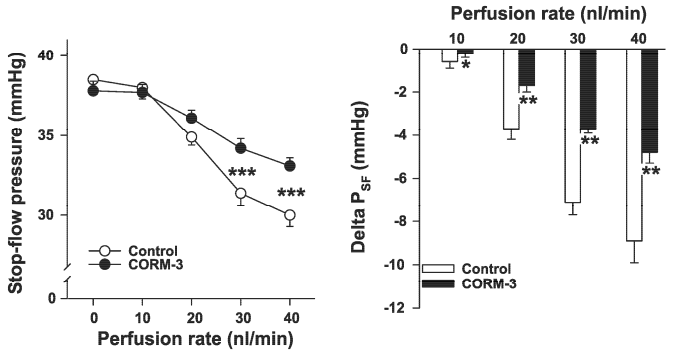


Fig. 2 - *Left panel*: stop-flow pressure (P_{SF}) measured under different TAL perfusion rates in the absence (control) or presence of carbon monoxide-releasing molecule-3 (CORM-3), a CO donor. *Right panel*: Summary data of net change in P_{SF} under the various conditions. **, $p < 0.01$; for CORM-3 *vs* control.

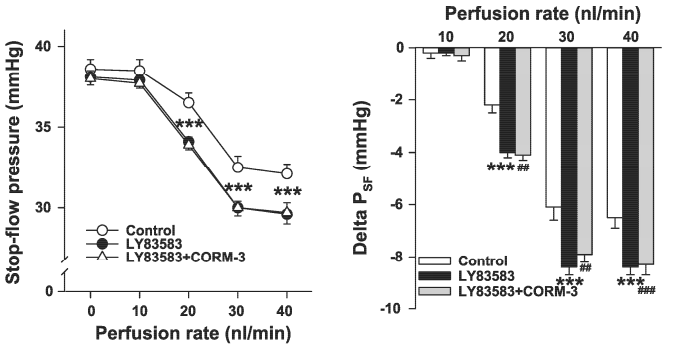


Fig. 3 - *Left panel*: stop-flow pressure (P_{SF}) measured under different TAL perfusion rates in the absence (control) or presence of LY83583, an inhibitor of sGC, with or without CORM-3. *Right panel*: Summary data of net change in P_{SF} under the various conditions. ***, $p < 0.001$ for LY83583 vs control; ##, $p < 0.01$, and ###, $p < 0.001$ for LY83583 + CORM-3 *vs* control.

1. **Propose a hypothesis to explain the effects of HO-1 activity on TGF. Explain how you would test your idea experimentally.**

2. **How could you manipulate other signaling pathways to mimic the effects of CORM-3 on TGF?**

3. **Under what conditions might the effects of HO-1 activity on TGF be most apparent?**

The prostaglandin E_2 (PGE_2) G protein-coupled receptor, EP2, couples to Gs and plays important roles in skin tumor development. Because keratinocyte proliferation is essential for skin tumor development, a group of investigators examined EP2-mediated signaling pathways that contribute to keratinocyte proliferation. These investigators were particularly interested in exploring the contributions of G protein-dependent and G protein-independent pathways in EP2-induced cell proliferation. The results of their experiments are shown below. The following compounds were used in these studies: butaprost, a highly selective agonist for EP2; AG1487 and gefitinib, inhibitors of the epidermal growth factor receptor (EGFR); and H89, a PKA inhibitor.

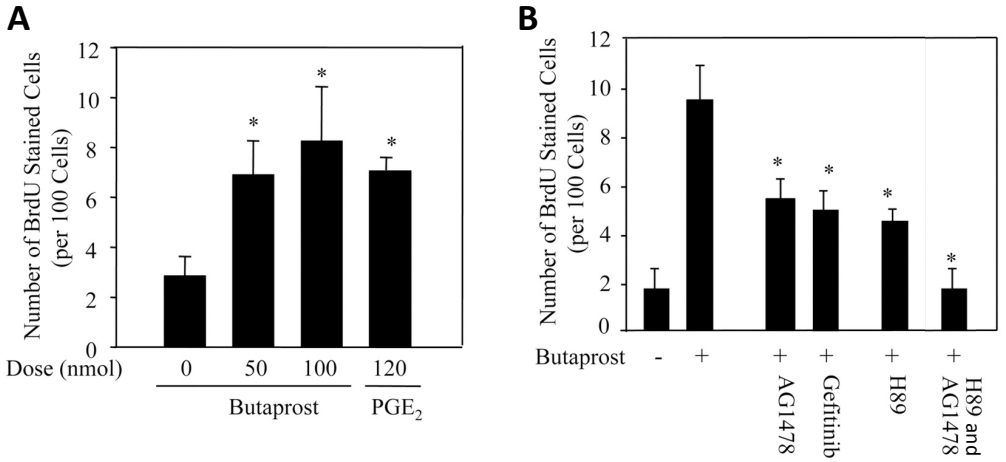


Figure 1. *A*) Mice were treated topically with vehicle, butaprost (50 or 100 nmol), or PGE_2 (100 nmol) for 24 h and euthanized; BrdU was injected i.p. 1 h before sacrifice. The dorsal skin sections were immunostained with an antibody recognizing BrdU. *B*) Mice were pre-treated topically with AG1478 (100 nmol), gefitinib (67 nmol), H89 (500 nmol), or a combination of H89 (500 nmol) and AG1478 (100 nmol) 30 min prior to topical treatment with 100 nmol of butaprost for 24 h; BrdU was injected i.p. 1 h before sacrifice. In *A* and *B*, BrdU-stained cells (i.e. proliferating cells) were counted in five skin sections from each of five mice. Data are expressed as the mean +/- S.D. (n = 5) of the number of stained cells per 100 basal cells in each group.

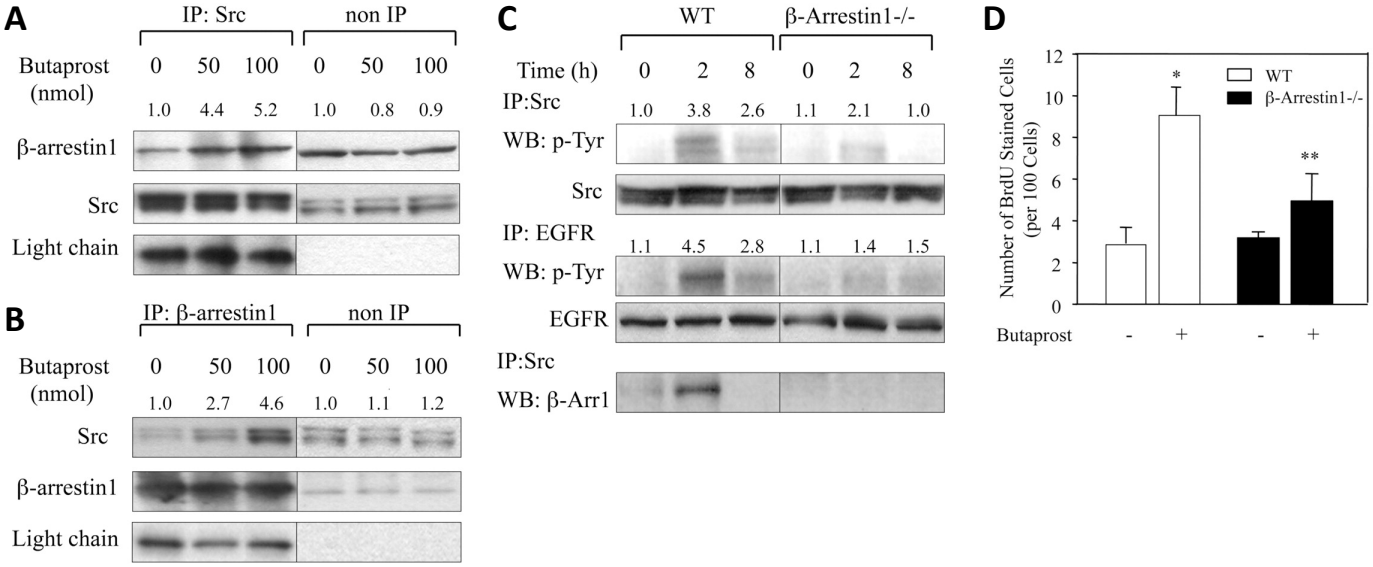


Figure 2. *A,B*) Mice were sacrificed at 2 h after topical treatment with butaprost (0, 50, and 100 nmol). Src (*A*) and β-arrestin1 (*B*) were immunoprecipitated from the skin lysates (200 μg) using Src- and β-arrestin1-specific antibodies. The immune complex (*IP*) and an aliquot of the non-immunoprecipitated lysate (*non IP*) were subjected to Western analysis using β-arrestin1 and Src antibodies. In *A* and *B*, IgG light chain served as a control for protein loading and membrane transfer. The number above each lane shows the relative intensities of the bands to IgG light chain. *C*) WT and β-arrestin1$^{-/-}$ mice were treated for the indicated times with 100 nmol of butaprost. Src and EGFR were immunoprecipitated (*IP:Src* and *IP:EGFR*) from the skin lysates (200 μg) and subjected to Western blot (WB) analysis using p-Tyr, Src, EGFR, and β-arrestin1 antibodies. The number above each lane shows the mean-fold intensity as determined by densitometry. *D*) WT and β-arrestin1$^{-/-}$ mice were treated topically with vehicle (-) or 100 nmol butaprost (+) and sacrificed 24 h later. *, $p < 0.05$ versus mice treated with vehicle alone. **, $p < 0.05$ versus butaprost-treated WT mice. BrdU-stained cells were counted in five skin sections from each of five mice. Data are expressed as the mean ± S.D. (n = 5) of the number of stained cells per 100 basal cells in each group.

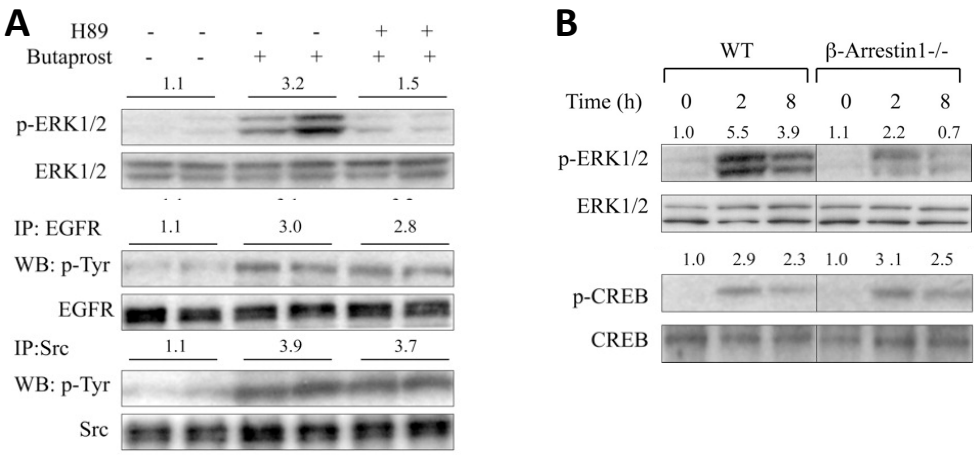


Figure 3. *A*) Mice were pre-treated with H89 (500 nmol) for 30 min and then treated with 100 nmol butaprost for 2 h. Skin lysates were subjected to Western analysis using an antibodies recognizing activated/phospho-ERK1/2 (*p-ERK1/2*) and total ERK1/2. EGFR and Src were immunoprecipitated (*IP:EGFR* and *IP:Src*) from the skin lysates (200 µg) and subjected to Western blot (WB) analysis using p-Tyr, EGFR, and Src antibodies. Total EGFR and Src served as controls for protein loading and membrane transfer. The intensities of the bands were determined by desitometry, and the ratios of p-Tyr signal to total protein are shown above each lane; the number above each lane shows the mean-fold intensity from two mice. *B*) WT and β-arrestin1$^{-/-}$ mice were treated for the indicated times with 100 nmol of butaprost. Skin lysates were immunoblotted for p-ERK1/2, total ERK1/2, p-CREB, and total CREB. Note, CREB is a substrate for PKA. The number above each lane shows the mean-fold intensity as determined by desitometry.

A) What do the data in Fig. 1 tell you about the signaling pathways involved in EP2-stimulated epidermal cell proliferation?

B) Develop a hypothesis that might explain the data shown in Figs. 1-3. Describe how the experimental results support your hypothesis.

C) Design two independent experiments that would allow you to further test your hypothesis.