Department of Pharmacology

Qualifying Examination (Part I)

December 13-15, 2011

EXAM SCHEDULE		
Tuesday, December 13, 2011	12:00 pm – 2:00 pm	
	2:00 pm – 4:00 pm	
Wednesday, December 14, 2011		
	9:00 am – 11:00 am	
	11:00 am – 1:00 pm	
Thursday, December 15, 2011	10:00 am – 12:00 pm	
	1:00 pm – 3:00 pm	
	4:00 pm	Students are given the results in Pharm South

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. <u>You may bring such preparation materials with you to the examination</u>. The oral examination itself will not extend beyond two hours.

Selective serotonin reuptake inhibitors (SSRIs) act as efficacious antidepressant agents by increasing extracellular concentrations of 5-HT in brain. However, clinical efficacy is only achieved after prolonged treatment with an SSRI.

The delay in onset of antidepressant action with an SSRI is thought to be attributed to the time required for desensitization of the somatodendritic 5-HT1A autoreceptors that inhibit the 5-HT release.

The study examined in vivo effects of a novel drug SB-649915-B in comparison to that of an SSRI paroxetine, and the results are presented in Figures below.
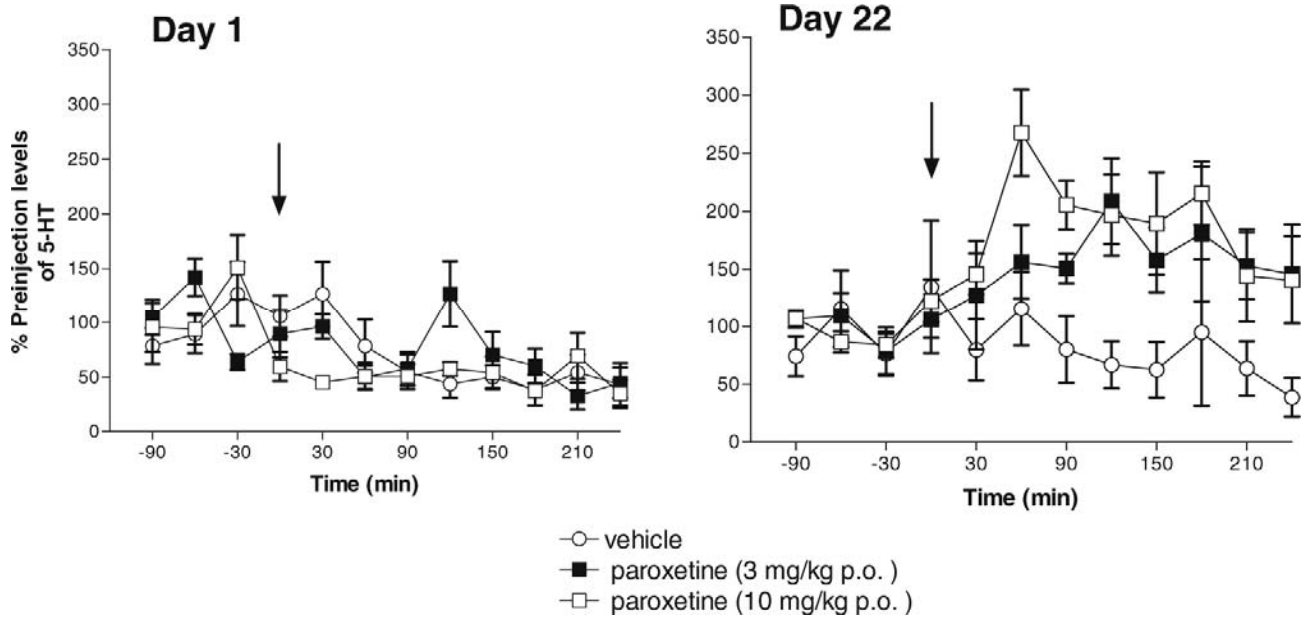

Figure 1. Effects of acute and Day 22 chronic treatment with paroxetine (3 and 10 mg/kg, p.o.) on extracellular 5-HT in rat frontal cortex. Day 1—effects of paroxetine after a single acute challenge; Day 22 shows the effects of a paroxetine challenge following 21 days chronic treatment. Data are expressed as mean percent pre-injection levels ±SEM (n=8–10 per study group). Arrows denote times of administration of vehicle or paroxetine. Increases attained significance after chronic treatment only with 3 ($P<0.05$) and 10 mg/kg ($P<0.01$).
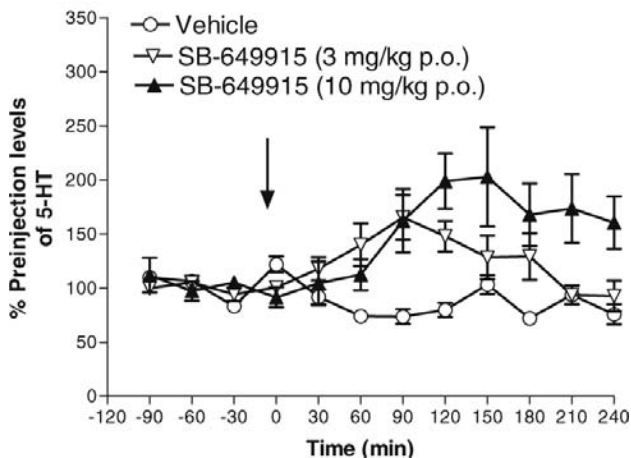

Figure 2. Effect of acute administration of SB-649915-B (3 and 10 mg/kg, p.o.) on extracellular 5-HT levels the frontal cortex of the rat. Data are expressed as mean percent pre-injection levels±SEM (n=9–11 per study group). Arrow denotes time of drug administration. Increases attained significance at 3 and 10 mg/kg ($P<0.0001$) vs vehicle.
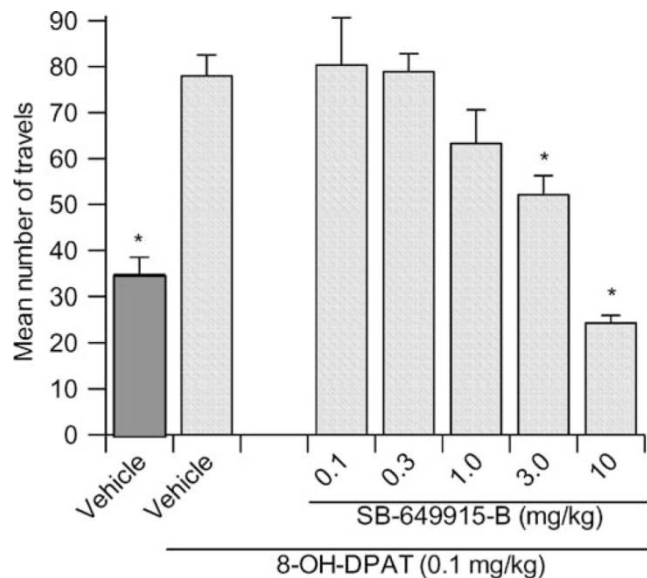
Figure 3. SB-649915-B inhibits hyperlocomotion caused by 8-OH-DPAT, an agonist of 5-HT1A receptors, in the rat. Data are expressed as mean±SEM over a 30-min period (n=8 per study group). *P<0.05 vs 8-OH DPAT alone.

1. What neurotransmitter system(s) and protein(s) are targeted by paroxetine? What disease(s) paroxetine is used to treat? Why does paroxetine induce 5-HT release upon chronic but not upon acute administration?

2. From the data presented in Figs. 1,2,&3, what can you conclude regarding the pharmacological properties and mechanisms of action of SB-649915? What therapeutic action(s) do you think SB-649915 would have?

3. Propose experiments to test you hypothesis about mechanisms of action of SB-649915.

Test drug A was administered to normal volunteers both i.v. and orally at the same time, the oral drug A being labeled with 3 heavy atoms (deuterium, d3) and the i.v. dose being unlabeled (d0), and so that the i.v. and orally administered drug A could be measured separately in the same plasma sample using mass spectrometry. Plasma samples were collected over 12 hours and drug A levels determined by LC-MS.

The experiment was conducted under control conditions (no pretreatment), and on three additional occasions after one week pre-treatment with rifampin, ketoconazole, or grapefruit juice.

The results (for equal doses of A) are summarized in Figure 1 (i.v.) and Figure 2 (oral dose). The PK parameters are given in Table 1 (i.v.) and Table 2 (oral).
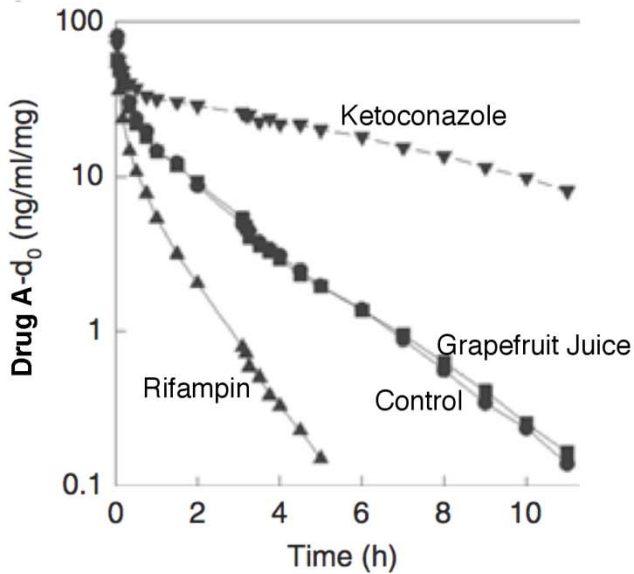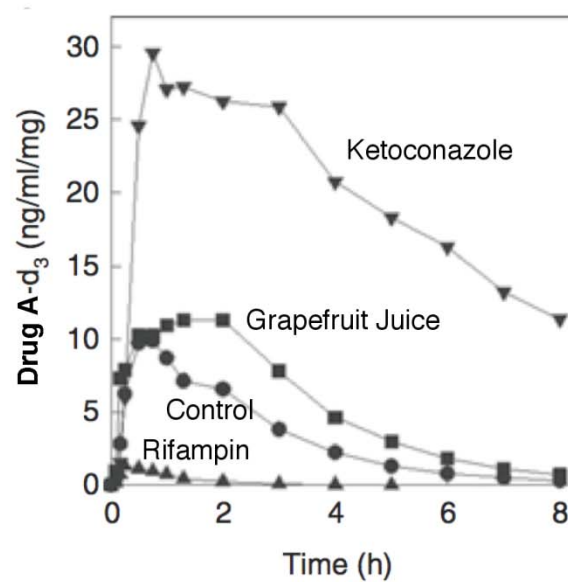
Figure 1

Figure 2

Table 1 Intravenous alfentanil pharmacokinetic parameters

	Control	Rifampin	Grapefruit juice	Ketoconazole
Sequential dosing				
C_{max}/D (ng·ml^{-1}·mg^{-1})	80 ± 40	54 ± 10	59 ± 31	73 ± 19
$AUC_{0-\infty}/D$ (ng·h·ml^{-1}·mg^{-1})	59 ± 27	21 ± 8[a]	57 ± 30	291 ± 159[a]
$CL_{i.v.}$ (ml·kg^{-1}·min^{-1})	4.3 ± 1.8	11.3 ± 3.4[a]	4.5 ± 2.0	0.9 ± 0.4[a]
Elimination $t_{1/2}$ (h)	1.4 ± 0.4	0.9 ± 0.1[a]	1.5 ± 0.4	5.0 ± 1.6[a]
V_{ss} (l/kg)	0.39 ± 0.13	0.48 ± 0.16[a]	0.46 ± 0.14[a]	0.34 ± 0.10
E_H	0.26 ± 0.09	0.71 ± 0.22[a]	0.28 ± 0.11	0.05 ± 0.02[a]

D = dose, V_{SS} = volume of distribution at steady state, and E_H = hepatic extraction (calculated using estimates not required for your answer).
(The term /D in the Table is used simply to normalize [equalize] the dose of A for each condition)

Table 2 Oral alfentanil pharmacokinetic parameters

	Control	Rifampin	Grapefruit juice	Ketoconazole
Sequential dosing				
C_{max}/D (ng·ml^{-1}·mg^{-1})	12±6	1.5±0.9[a]	13±7	33±10[a]
T_{max} (h)	1.0±0.8	0.4±0.2	1.4±1.0	1.3±1.0
$AUC_{0-\infty}/D$ (ng·h·ml^{-1}·mg^{-1})	27±17	1.6±0.8	45±27	253±178[a]
CL/F (ml·kg^{-1}·min^{-1})	11.1±6.8	168±74[a]	6.4±3.8[a]	1.2±0.6[a]
Elimination $t_{1/2}$ (h)	1.2±0.3	0.6±0.1[a]	1.4±0.5	4.7±2.1[a]

D = dose, CL/F = oral clearance, V$_{SS}$ = volume of distribution at steady state.
(The term /D in the Table is used simply to normalize [equalize] the dose of A for each condition)

Questions
How do you interpret and mechanistically explain these data?

Calculate the bioavailability under each condition.

Outline experiments you would perform to check your explanations.

Your laboratory developed a drug (furiosta) that inhibits tumor growth. However, as a side effect, patients placed on furiosta exhibit important alterations in plasma TSH, thyroxine (T4), and 3,5,3_-triiodothyronine (T3) concentrations through mechanisms that have yet to be fully understood.

Experimental animals that have been placed on furiosta for a month display the following plasma profile:

	Plasma T4	Plasma T3	Plasma TSH
control	1.5	25	4
treated	3*	18	20*

*: statistically significant

1) **Draw the homeostatic mechanism whereby TH level are controlled.**

2) **Is a concomitant increase in T4 and TSH plasma level something expected? If not, why?**

3) **What determines the sensitivity of thyrotrophs to TRH, and based on this, how could furiosta then alter T4 and TSH levels?**

4) **What should be the clinical symptoms of the patients taking furiosta?**
   What exam findings might you note?

5) **What if furiosta-treated patients had high TSH, high T3, and high T4, but still had the above symptoms? Where might the defect in thyroid hormone action be?**

Kisspeptins (Kp) are peptide products of the Kisspeptin-1 (KISS1) gene, which are endogenous ligands for the G protein-coupled receptor 54 (GPR54). Recent studies have demonstrated that an increase in GPR54 and KISS1expression in human breast tumors correlates with their metastatic potential. How does GPR54 modify invasiveness of the cancer cells was investigated using one of the potent agonist, Kp10, and an EGFR antagonist, AG1478. In vitro, AG1478 abolishes Kp10 induced invasion of breast epithelial cells (Fig 1). Moreover, Kp10 increases phosphorylation of epidermal growth factor receptor (EGFR) in epithelial cell lines derived from breast tumors (MDA-MB-231 and Hs578T) (Fig 2).
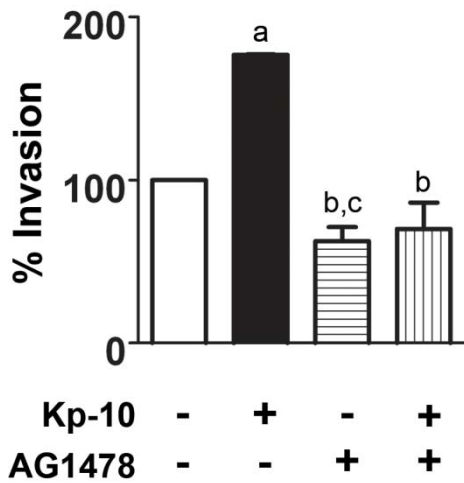

Figure 1. Kp10 increases invasion in MDA-MB-231 cells.
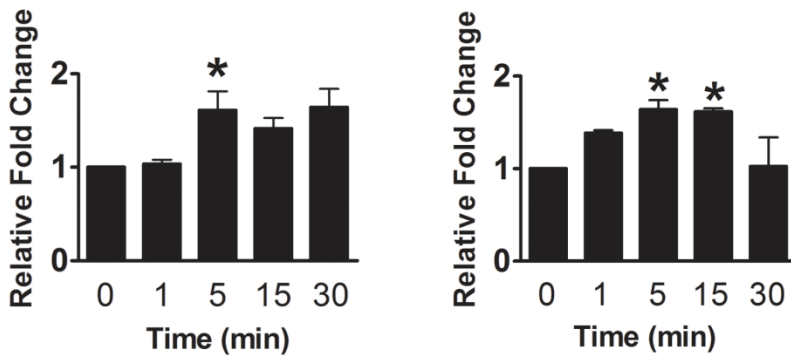a, p<0.05, compared to untreated
b, p<0.05, compared to Kp10 treated

Figure 2. Kp10 stimulates phosphorylation of EGFR in breast cancer cells, MDA-MB-231 (left panel), Hs578T (right panel).

(A), Explain the results in Figures 1 and 2.

(B), (1) Propose potential mechanisms underlying the transactivation of EGFR by GPR54; and (2) Design a series of experiments to support the proposed mechanisms.

(C), (1) What are known signaling pathways that may lead to proliferation of tumor cells? (2) Design at two independent experiments to investigate your hypothesis.

Primary Pulmonary Hypertension (PPH) is accompanied by high mortality and few effective therapies are available. PPH is due to alterations in the smooth muscle of the pulmonary vasculature that greatly increases vascular resistance. Recent studies using a mouse model of PPH yielded the following data. Mice exposed to normoxia in the presence or absence of Imitinib have normal right ventricular systolic pressure. Mice exposed to hypoxia, then placed in a normoxic environment, develop elevated right ventricular systolic pressure indicative of PPH in the presence of vehicle and this response is blunted in mice treated with Imitinib (* $P<0.05$ when compared to vehicle and normoxia; † $P<0.05$ when compared to vehicle and hypoxia). Imitinib is a small molecule kinase inhibitor that is known to inhibit Abl, c-kit, and PDGFR kinase activity.
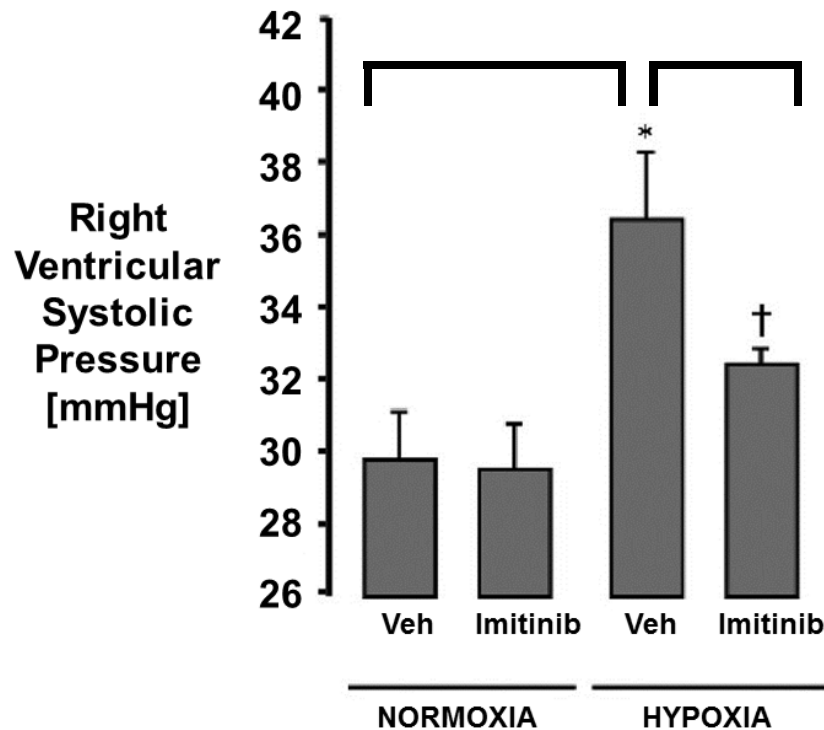
In addition, it was noted that cultured pulmonary smooth muscle cells harvested from mice with PPH had increased expression of Smooth Muscle α Actin (SMαA), Stromal Cell Derived Factor 1 (SDF1), and CXCR4 when compared to pulmonary smooth muscle cells harvested and cultured from unaffected mice. The addition of Imitinib to cells from mice with PPH decreased the expression of SMαA, SDF1, and CXCR4 to normal levels and had no effect on cells from unaffected mice.

Describe the data and state a hypothesis.
Describe experimental approaches *in vitro* and *in vivo* to test your hypothesis.

Researchers in Luxembourg attempted to engineer transgenic mice expressing a fluorescence reporter gene (green fluorescent protein, GFP) driven by a renal epithelial specific promoter. In all transgenic lines, the GFP reporter was consistently expressed in the cortical collecting duct and the connecting tubule. To improve GFP expression, animals were intercrossed to obtain double transgenic mice (two copies of the transgene). Interestingly, one line of animals bred in this manner became unhealthy when placed on a low sodium diet. Specifically, these animals lost body weight despite normal food intake on low sodium chow, and additional investigations revealed abnormalities in urine volume and electrolytes (**Fig 1**). The researchers deduced that the transgene had caused insertional inactivation of a critical gene in the collecting duct.
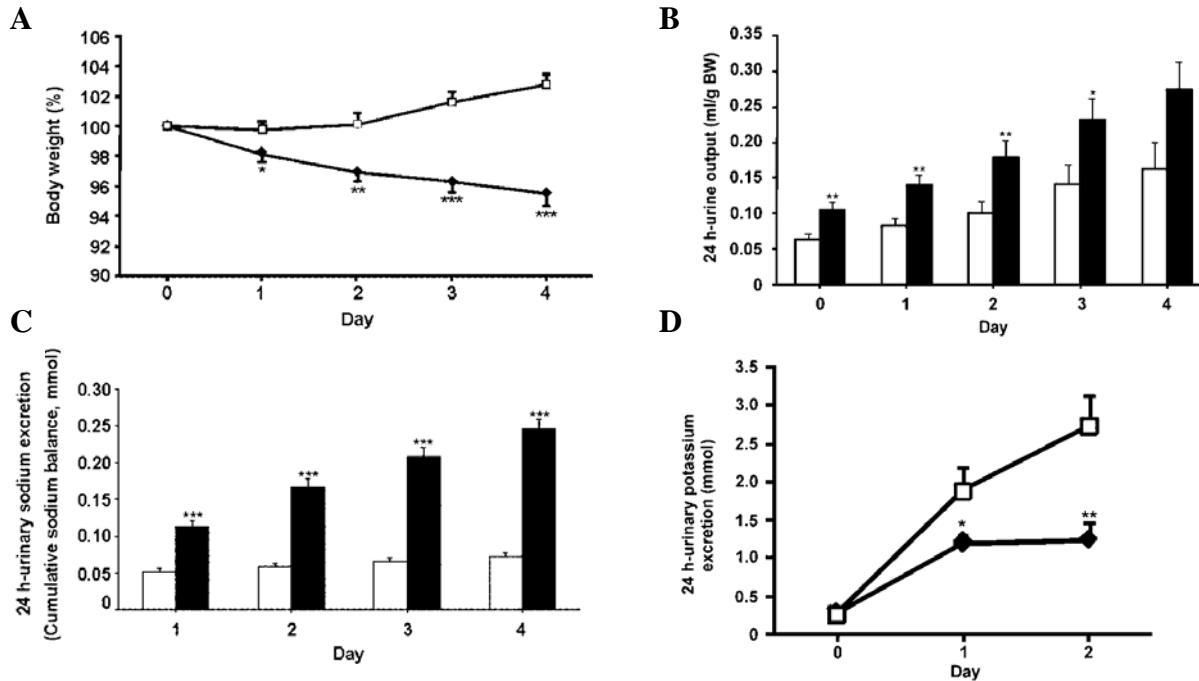

Fig 1 – Phenotype of transgenic mice placed on a low sodium diet. Solid symbols/bars represent the transgenic mice, and open symbols/bars are non-transgenic mice. (A) Body weight of mice placed on an isocaloric low sodium diet beginning on day 0. (B) Daily urine volume. (C) Daily urine sodium excretion. (D) Daily urine potassium excretion. Asterisks indicate level of statistical significance (*, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Questions:

1. Propose at least one hypothesis to explain the phenotype of the transgenic mice.

2. Based upon your hypothesis, predict how the transgenic mice would respond to the major classes of diuretic agents.

3. Explain how you would test your hypothesis.

A group of investigators is studying signaling by Bombyx mori (silkworm) adipokinetic hormone (AKH) receptor (AKHR). In all experiments AKHR was stably heterologously expressed in HEK293 cells and stimulated with indicated concentrations of AKH (a short peptide). The results of these experiments are shown in Figs. 1-4.
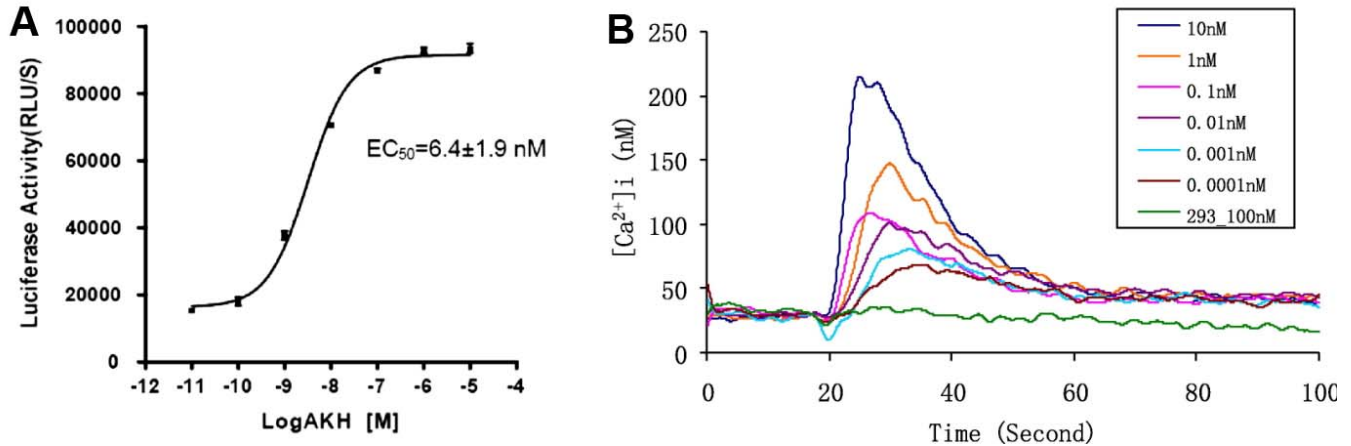

Fig. 1. AKH-induced cAMP accumulation and intracellular Ca2+ mobilization in HEK293 cells expressing Flag-AKHR.
A. cAMP accumulation in HEK293 cells expressing Flag-AKHR and CRE-Luc was assayed in response to different doses of AKH. Data are expressed as the means ± S.E. (n = 3). CRE, cAMP response element; CRE-Luc, the construct expressing luciferase under control of CRE. **B.** Intracellular Ca2+ influx in non-transfected HEK cells (green) or cells expressing Flag-AKHR was measured in response to different concentrations of AKH peptide using the fluorescent Ca2+ indicator fura-2.
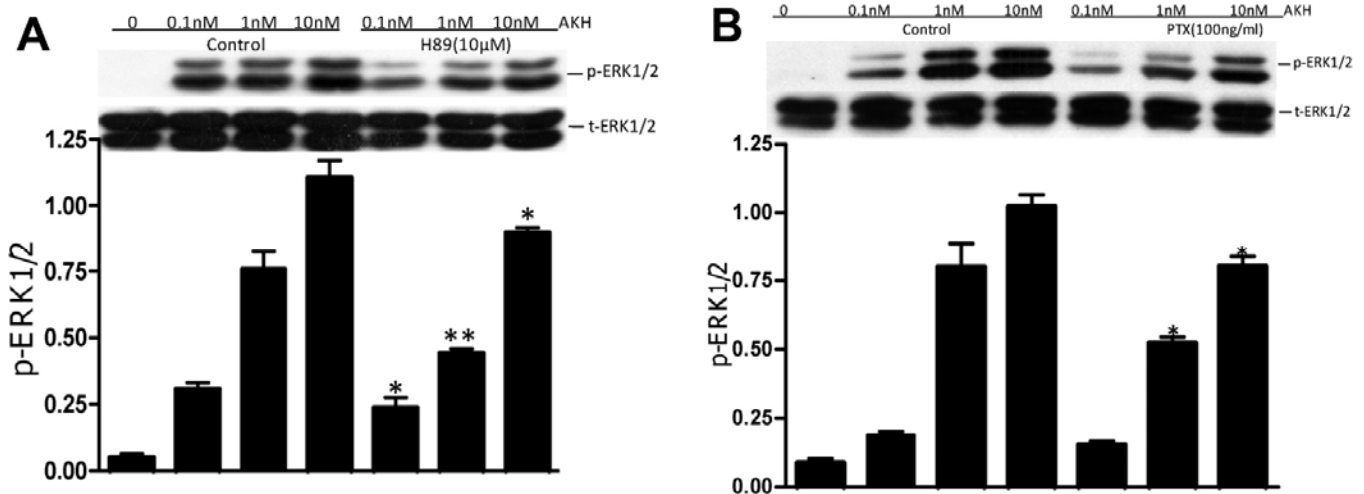

Fig. 2. The effects of PKA inhibitor and pertussis toxin on AKHR-mediated ERK1/2 phosphorylation. A. HEK293 cells expressing AKHR were pre-incubated with vehicle (control) or PKA inhibitor H89 (10 μM) at 37 °C for 1 h followed by stimulation with AKH (0.1−10 nM) for 5 min. **B.** HEK293 cells expressing AKHR were pre-incubated at 37 °C overnight with or without pertussis toxin (PTX, 100 ng/mL) before stimulation for 5 min with AKH at the indicated concentration. Phosphorylated (p-ERK1/2) was measured by Western and normalized to total (t-ERK1/2). The data shown are representative of three independent experiments (mean ± SEM). Statistical analysis: two-tailed Student's t test (*, $P < 0.05$; **, $P < 0.01$, versus corresponding control).
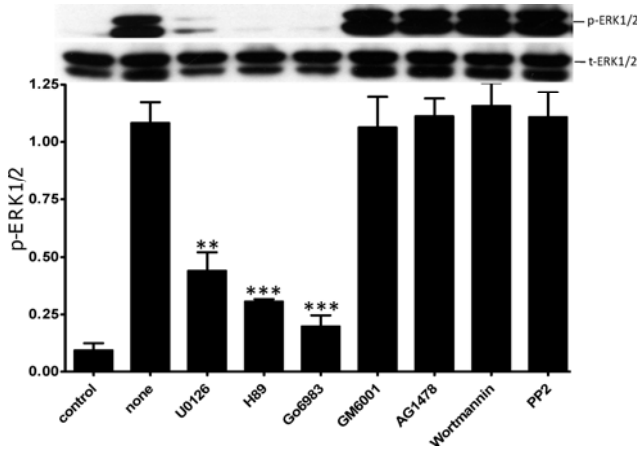
Fig. 3. Effects of EGFR, Src, and PI3K inhibitors on AKHR-mediated ERK1/2 activation. Serum-starved HEK293 cells stably expressing AKHR were pretreated for 1 h at 37 °C with the following: MEK inhibitor (U0126, 100 nM), PKA inhibitor (H89, 20 μM), PKC inhibitor (Go6983, 3 μM), Matrix metalloproteinase inhibitor (GM6001, 5 μM), EGFR tyrosine kinase inhibitor (AG1478, 1 μM), PI3K inhibitor (wortmannin, 1 μM), and Src-family tyrosine kinase inhibitor (PP2, 10 μM). Following pre-treatment the cells were exposed to AKH for 5 min. The data shown are representative of three independent experiments (mean ± SEM). Statistical analysis was performed by a two-tailed Student's t test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus control).
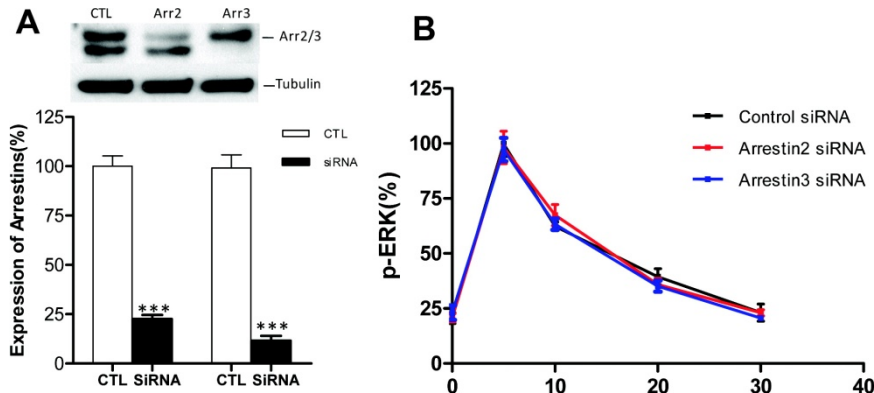

Fig. 4. The role of arrestins in AKHR-mediated ERK1/2 activation. A. Knockdown of arrestins by siRNA. HEK293 cells stably expressing AKHR were transfected with specific arrestin siRNAs or control siRNA. Seventy-two hours after transfection, cells were harvested, and equal amounts of total cell lysate were separated by 12% SDS−PAGE, transferred to nitrocellulose, and incubated with the indicated antibodies. Blots were stripped and reprobed for tubulin to control for loading. **B.** Effect of siRNA-induced knockdown of arrestin expression on AKHR-mediated ERK1/2 activation. Activation of ERK1/2 in HEK293 cells transfected with siRNA for 3 days followed by incubation with 1 μM AKH for different times was assessed by Western blot. Signals were quantified by densitometry and expressed as percentage of phosphorylated ERK1/2 of control obtained at 5 min. Graphs show mean ± SEM from four independent experiments.

Questions:

1. Based on the data, name all types of G proteins AKHR couples to. For each G protein type, indicate the results that suggest its involvement in AKHR signaling. Describe GPCR-initiated signaling pathways that lead to the activation of PKA and PKC.

2. Based on Figs 3 and 4, which signaling proteins and pathways are NOT involved in AKHR-induced ERK activation?

3. Propose experiments that were <u>not</u> performed in this study to test the role of each of the pathways that appear to be involved in ERK activation: a) AKHR -> PKA; b) AKHR -> PKC; c) PTX-sensitive pathways.