Department of Pharmacology

Qualifying Examination (Part I)

December 15, 2016

ALL EXAMS TAKE PLACE IN THE BASS CONFERENCE ROOM, 436 RRB

Date	Time	Student Name & Cell Phone Number
Thursday, December 15th	12:00 pm – 2:00 pm	(Exam #1) –
	2:00 pm – 4:00 pm	(Exam #2) –
	4:30 pm (Results given to students – 449 PRB, Pharm South Conf. Rm.) **ALL COMMITTEE MEMBERS TO BE PRESENT**	

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.

BEST WISHES FOR YOUR SUCCESSFUL COMPLETION OF THE EXAMINATION!

Bipolar disorder (BD) is a debilitating life-long illness. Mania is a cardinal feature of BD and is characterized by complex and multifaceted symptoms. Symptoms of BD mania include heightened risk-taking, impaired decision-making, and increased hedonistic (reward-directed) behavior. Increased reward-directed behaviors have not been as readily quantified in patients but are commonly measured in rodents using a progressive ratio breakpoint (PRB) schedule of reinforcement. One of the most common treatments for BD is the mood stabilizer lithium, but its effects on cognitive and motivational aspects of behavior are poorly understood.

The experiments in mice with genetic knockdown of the dopamine (DA) transporter (DAT) (DAT KD) were conducted to assess the role of the DAT dysfunction in the effect of lithium on motivation. DAT KD mice and their wild type (WT) littermates received lithium in drinking water for 8 days (control mice of both genotypes were given plain water) and then were tested in the progressive ratio breakpoint test with food as reward. In addition, measurements of the DA metabolism in the nucleus accumbens were performed.

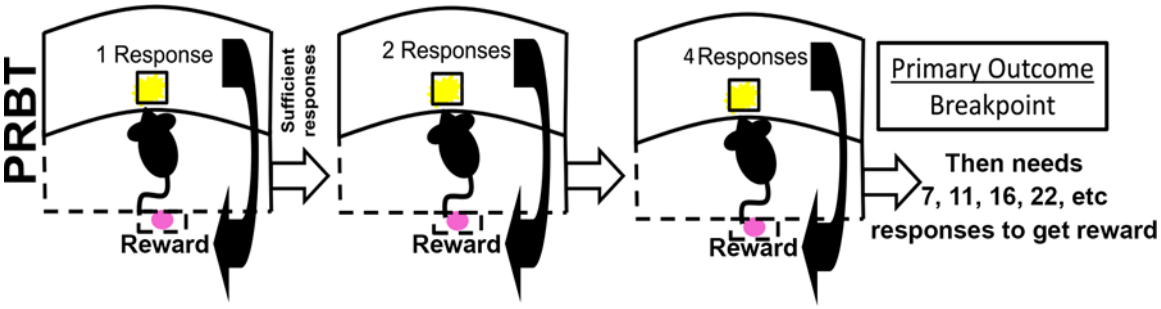


Figure 1. Schematics describing the progressive ratio breakpoint test (PRBT).

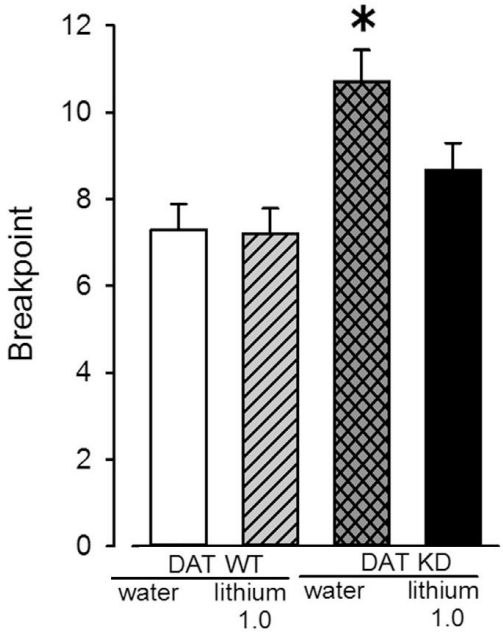


Figure 2. ***Effect of lithium pre-treatment in DAT WT and KD mice in the Progressive Ratio Breakpoint Task.*** DAT KD mice and WT littermates were given lithium in drinking water (1.0 g/l for 8 days) or plain water. Data on breakpoint are presented as mean ± S.E.M. *$p < 0.05$ when compared to DAT WT mice with water or lithium treatment.

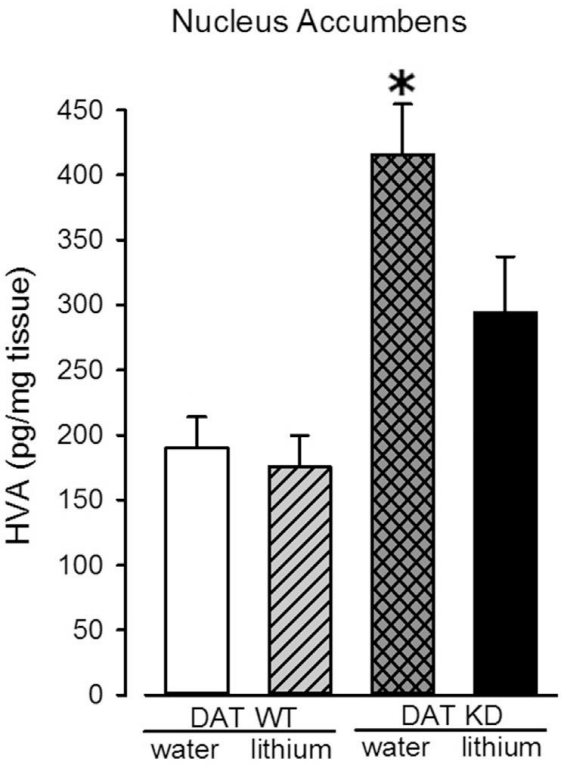


Figure 3. ***The concentration of the dopamine metabolite homovanillic acid (HVA) in the nucleus accumbens of DAT WT and KD mice.*** Data are presented as mean ± S.E.M. **p < 0.01 when compared to water- or lithium-treated DAT WT mice.

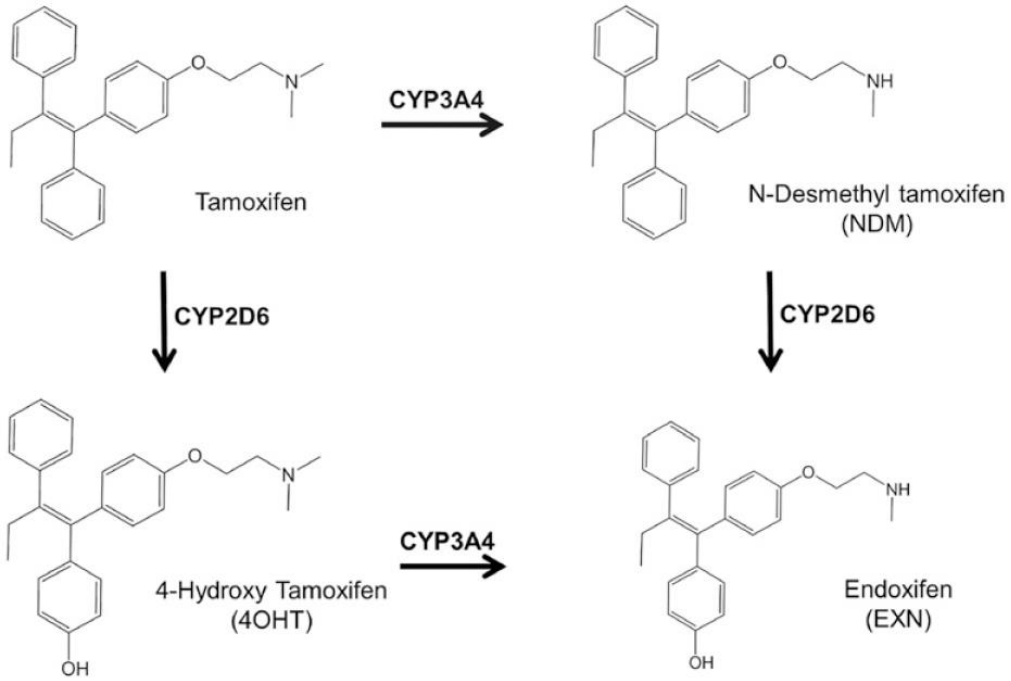
1. **Using the diagram in Fig. 1, describe the progressive ratio breakpoint test procedure. Do you know of a modification of the test used in other experimental setting?**

2. **Describe the data presented in Figs. 2 and 3. What other molecular and behavioral features would you expect to see in DAT KD mice? What method(s) are used to measure neurotransmitter metabolism in living animals?**

3. **Suggest a simple model of the effect of lithium on motivational behavior. Based on the data presented, explain how lithium should affect the motivational symptoms of BD and through what neurochemical mechanism.**

Rifampin has been characterized as a strong activator of human pregnane X receptor (PXR), but not that of rodents, leading investigators to generate a humanized transgenic mouse model expressing human PXR, constitutive androstane receptor (CAR), CYP3A4/CYP3A7, and CYP2D6 (Tg-composite) for investigation of induction of metabolism and disposition. The investigators concluded that the Tg-composite animals gave results that match those already reported in human patient studies.

Formation of active metabolites 4OHT, and Endoxifen and metabolite NDM are illustrated in Figure 1. The effects of rifampin on the disposition of Tamoxifen, NDM, 4OHT and Endoxifen were compared in Tg-composite and wild-type (WT) mice, Figure 2 and Table 1.



Questions

1. **Briefly interpret the data in Fig 2 and Table 1.**

2. **Present an explanation for the reduced levels of metabolites NDM and 4OHT (and relatively unchanged level of endoxifen) after rifampin and how you would experimentally investigate your hypothesis.**

Fig. 2. Summary of blood concentration-time profile of tamoxifen (A), 4OHT (B), NDM (C), and endoxifen (D) following 20-mg/kg oral administration of tamoxifen in transgenic animals expressing human PXR, CAR, CYP3A4/7, and CYP2D6 (Tg-composite). In each panel, solid symbols with solid lines represent transgenic animals treated with vehicle, and open symbols with broken lines represent transgenic animals pretreated with rifampin for 3 days. Concentrations for tamoxifen and its metabolites were below the limit of quantitation at 24 hours. Data are presented as the mean (± S.D.) of four animals.

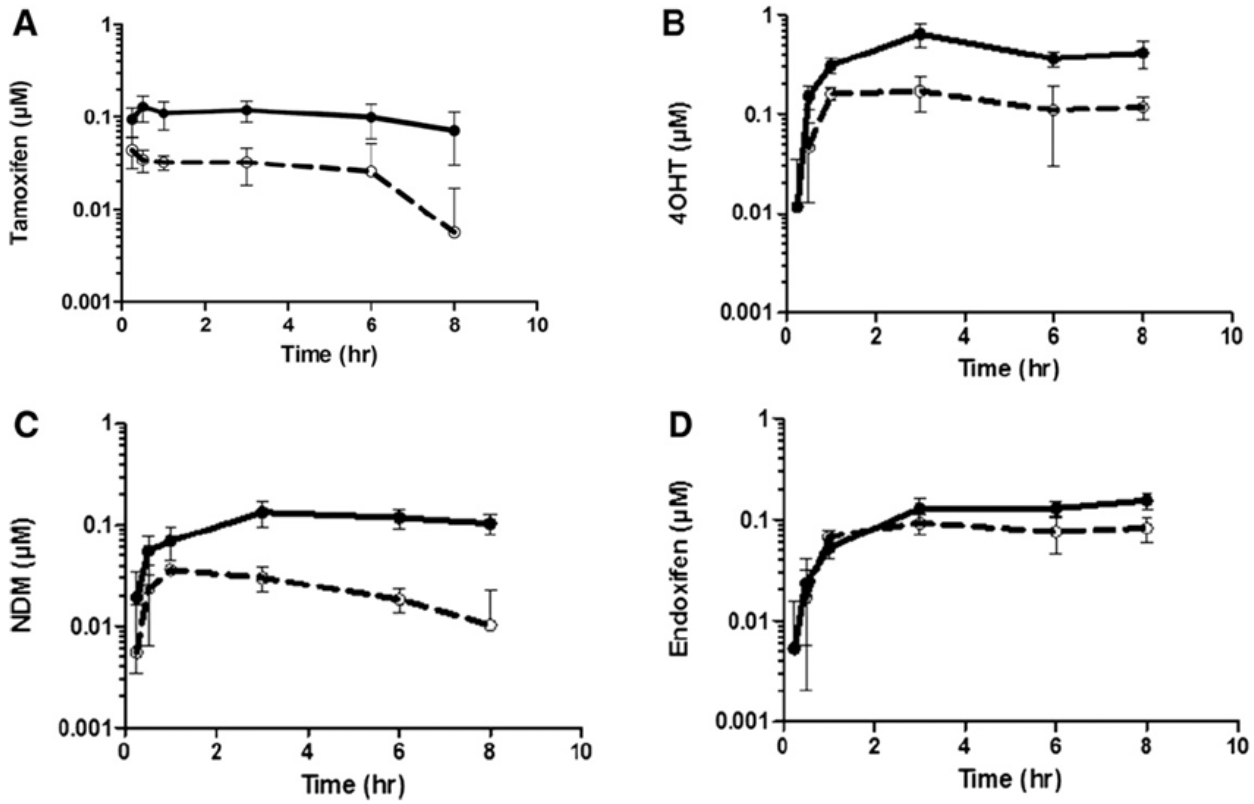


TABLE 1
Summary of mean (± S.D.) $AUC_{0\text{-}8}$ and C_{max} of tamoxifen, NDM, 4OHT, and endoxifen when administered 20 mg/kg tamoxifen orally following vehicle or rifampin treatment for 3 days (N = 4).
* = $p < 0.05$

	Tg-Composite			WT		
	Vehicle	Rifampin	Vehicle/ Rifampin Ratio	Vehicle	Rifampin	Vehicle/ Rifampin Ratio
Tamoxifen						
$AUC_{0\text{-}8}$ (μM*h)	0.82 ± 0.14	0.20 ± 0.12*	4.1	0.92 ± 0.32	1.4 ± 1.0	0.66
C_{max} (μM)	0.15 ± 0.03	0.048 ± 0.013	3.1	0.28 ± 0.15	0.69 ± 0.20	0.40
NDM						
$AUC_{0\text{-}8}$ (μM*h)	0.84 ± 0.22	0.18 ± 0.04*	4.7	0.21 ± 0.10	0.24 ± 0.31	0.88
C_{max} (μM)	0.13 ± 0.04	0.037 ± 0.002	3.5	0.046 ± 0.005	0.094 ± 0.068	0.49
4OHT						
$AUC_{0\text{-}8}$ (μM*h)	3.4 ± 0.5	1.0 ± 0.4*	3.4	0.25 ± 0.07	0.28 ± 0.10	0.89
C_{max} (μM)	0.64 ± 0.17	0.19 ± 0.04	3.4	0.053 ± 0.024	0.056 ± 0.021	0.95
Endoxifen						
$AUC_{0\text{-}8}$ (μM*h)	0.94 ± 0.13	0.74 ± 0.03*	1.3	0.38 ± 0.15	0.26 ± 0.08	1.6
C_{max} (μM)	0.18 ± 0.04	0.11 ± 0.03	1.6	0.080 ± 0.004	0.080 ± 0.016	1.0

Graves Disease is caused by pathogenic autoantibodies that constitutively activate the Thyroid Stimulating Hormone (TSH) Receptor (TSHR). An in vivo drug screen of 100,000 compounds is executed in cells that express the TSHR and a luciferase reporter driven by a cAMP-Response Element (CRE). The following results are obtained from two compounds identified in this screen:

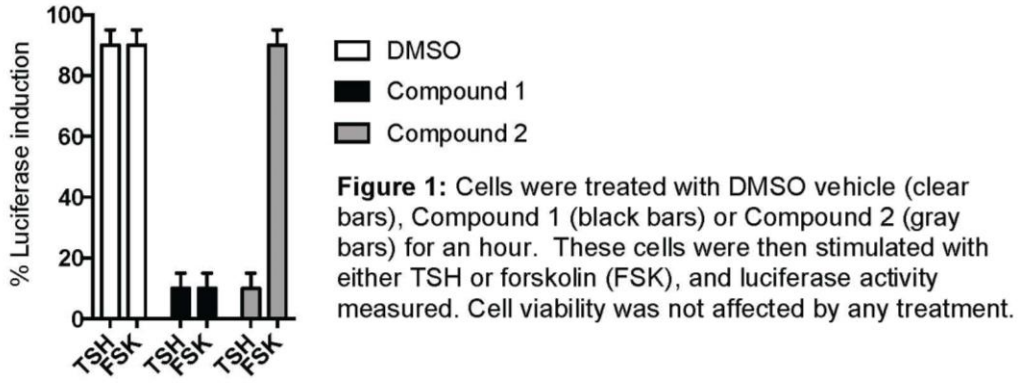


Figure 1: Cells were treated with DMSO vehicle (clear bars), Compound 1 (black bars) or Compound 2 (gray bars) for an hour. These cells were then stimulated with either TSH or forskolin (FSK), and luciferase activity measured. Cell viability was not affected by any treatment.

A. What happens to serum T4 & T3 levels in patients with Graves Disease, and identify 2 symptoms that might be associated with Graves Disease.

B. Explain the logic in using both TSH and forskolin as stimulators in this assay, and explain which of the compounds above represents a better potential drug to treat Graves Disease.

Serum from 14 Graves Disease patients was used to treat the same cells used in the drug screen above. Treatment with the best compound above yielded the following data:

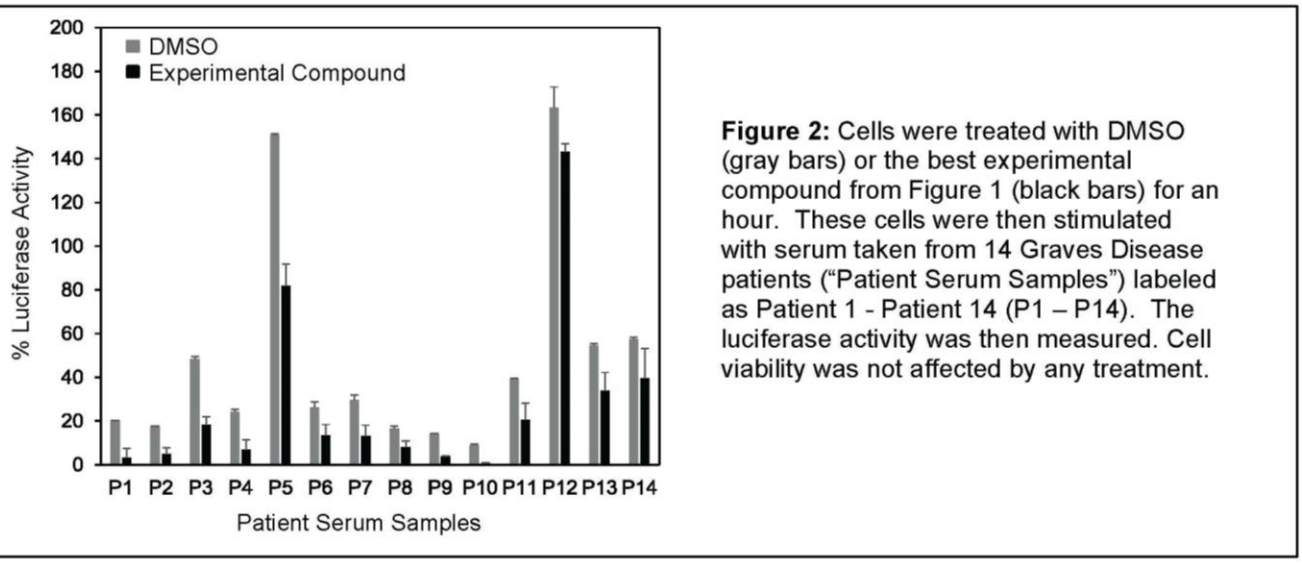


Figure 2: Cells were treated with DMSO (gray bars) or the best experimental compound from Figure 1 (black bars) for an hour. These cells were then stimulated with serum taken from 14 Graves Disease patients ("Patient Serum Samples") labeled as Patient 1 - Patient 14 (P1 – P14). The luciferase activity was then measured. Cell viability was not affected by any treatment.

C. Which patient would you expect to have the lowest serum T4 levels based on the data in Figure 2? Explain your logic.

D. Which patient's symptoms might you expect to be most alleviated by treatment with the experimental compound in Figure 2?

Members of the EGFR family are implicated in various types of the lung cancers. To investigate the contribution of receptors and the critical downstream signaling mechanisms for tumorigenesis, cancer-derived cell lines were analyzed. First, the expression of receptor subtypes in these cell lines was determined (Fig 1, top). These cells were also evaluated for the sensitivity towards Gefitinib, which is tyrosine kinase inhibitor targeting to EGFR (Fig 1, bottom).

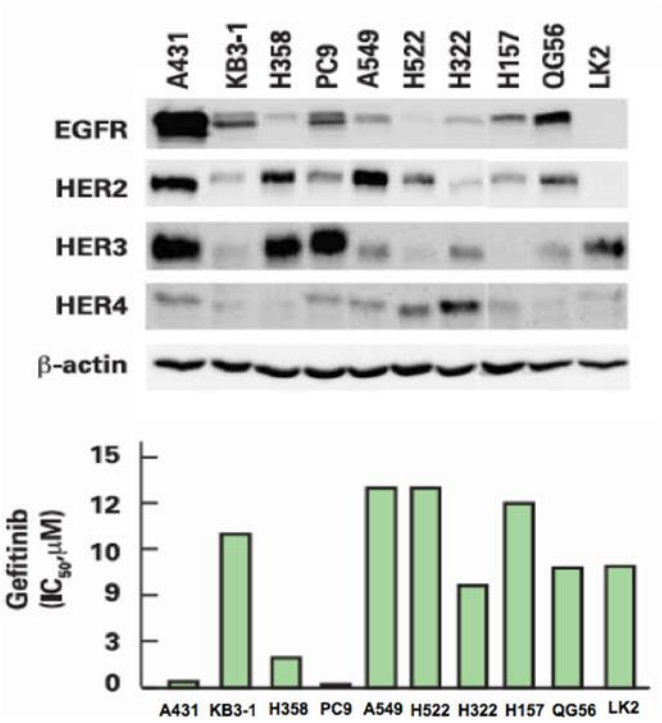


Figure 1: *Expression of EGFR members and drug sensitivity in human cancer cell lines.* Western blotting was performed using isoform-specific antibodies in cell lysates (top). Growth inhibition assays for IC_{50} were performed in the presence of Gefitinib in cultured cells (bottom). **A431** and KB3-1 are epidermoid carcinoma cell lines while the rest were derived from human lung cancers.

Based on the results,

(1) propose experiment to address the molecular basis leading to uncontrolled proliferation focusing on lung cancer cell lines **PC9** and **A549**, respectively. Include in your discussion about the critical receptor isoforms and the known downstream pathways involved.

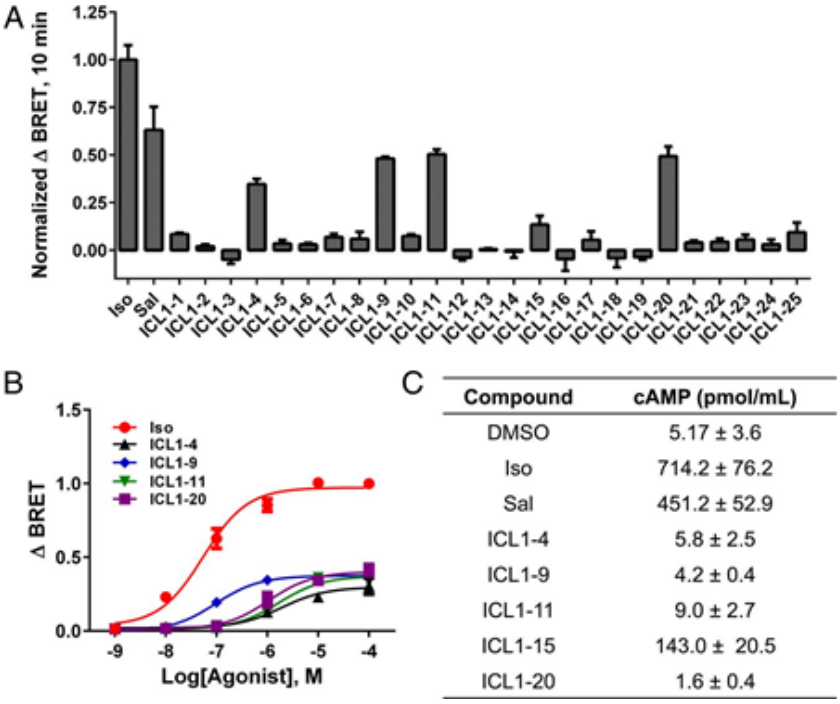
(2) propose a series of experiments to support each of your hypotheses (in the critical receptor isoform and downstream pathways). Elaborate your proposed experimental strategies with anticipated results.

β-adrenergic receptors (βARs) are critical regulators of acute cardiovascular physiology. In response to elevated catecholamine stimulation during development of congestive heart failure (CHF), chronic activation of Gs-dependent β1AR and Gi-dependent β2AR pathways leads to enhanced cardiomyocyte death, reduced β1AR expression, and decreased inotropic reserve. β-blockers act to block excessive catecholamine stimulation of βARs to decrease cellular apoptotic signaling and normalize β1AR expression and inotropy. Whereas these actions reduce cardiac remodeling and mortality outcomes, the effects are not sustained. Converse to G-protein–dependent signaling, β-arrestin–dependent signaling promotes cardiomyocyte survival. Carvedilol, a currently prescribed nonselective β-blocker, has been classified as a β-arrestin–biased agonist that can inhibit basal signaling from βARs and also stimulate cell survival signaling pathways. To understand the relative contribution of β-arrestin bias to the efficacy of select β-blockers, a specific β-arrestin–biased pepducin for the β2AR, intracellular loop (ICL)1–9, was used to decouple β-arrestin–biased signaling from occupation of the orthosteric ligand-binding pocket. Data supporting the activity of ICL1-9 and the effects of ICL1-9 on cardiac contractility are shown below.

1. **Diagram how β1 signaling regulates cardiac contractility.**

2. **Describe the data found in the 3 figures below and state a hypothesis consistent with the data depicted.**

3. **Develop experiments that may include *in vitro* and *in vivo* approaches, to test your hypothesis.**



C

Compound	cAMP (pmol/mL)
DMSO	5.17 ± 3.6
Iso	714.2 ± 76.2
Sal	451.2 ± 52.9
ICL1-4	5.8 ± 2.5
ICL1-9	4.2 ± 0.4
ICL1-11	9.0 ± 2.7
ICL1-15	143.0 ± 20.5
ICL1-20	1.6 ± 0.4

Figure 1. ICL1–9 is a potent β-arrestin–biased pepducin. (*A*) β-Arrestin recruitment was assessed by bioluminescense resonance energy transfer (BRET2) in HEK293 cells transiently transfected with β_2AR–RLucII and GFP10–β-arrestin2. β-Arrestin2 recruitment is reported at 10 min postagonist stimulation with 1 μM isoproterenol (Iso), 5 μM salbutamol (Sal), or 10 μM pepducin. The sequences of these pepducins and initial BRET analysis for isoproterenol, salbutamol, ICL1–4, ICL1–11, ICL1–15, and ICL1–20 have been previously reported, albeit as time courses. Although ICL1–9 exhibited a modest ability to promote β-arrestin recruitment in our previous primary screen, subsequent analysis shows that it has comparable efficacy to ICL1–4, ICL1–11, and ICL1–20. The data are represented by the mean ± SD from three independent experiments. (*B*) ICL1–9 is a high-potency β-arrestin–biased pepducin with an EC_{50} of 96 ± 14 nM with a sequence of LVITAIAKFERLQTVTNY containing an N-terminal palmitate and C-terminal amide (5). ICL1–4 (1.9 ± 0.5 μM), ICL1–11 (1.7 ± 0.5 μM), and ICL1–20 (1.1 ± 0.3 μM) demonstrated comparable efficacy to ICL1–9 but operated with lower potency. The data are represented by the mean ± SD from three independent experiments. (*C*) cAMP production in HEK293 cells using Iso, Sal, and the pepducins that promoted β-arrestin recruitment in *A*. The data represent the mean ± SD from three independent experiments.

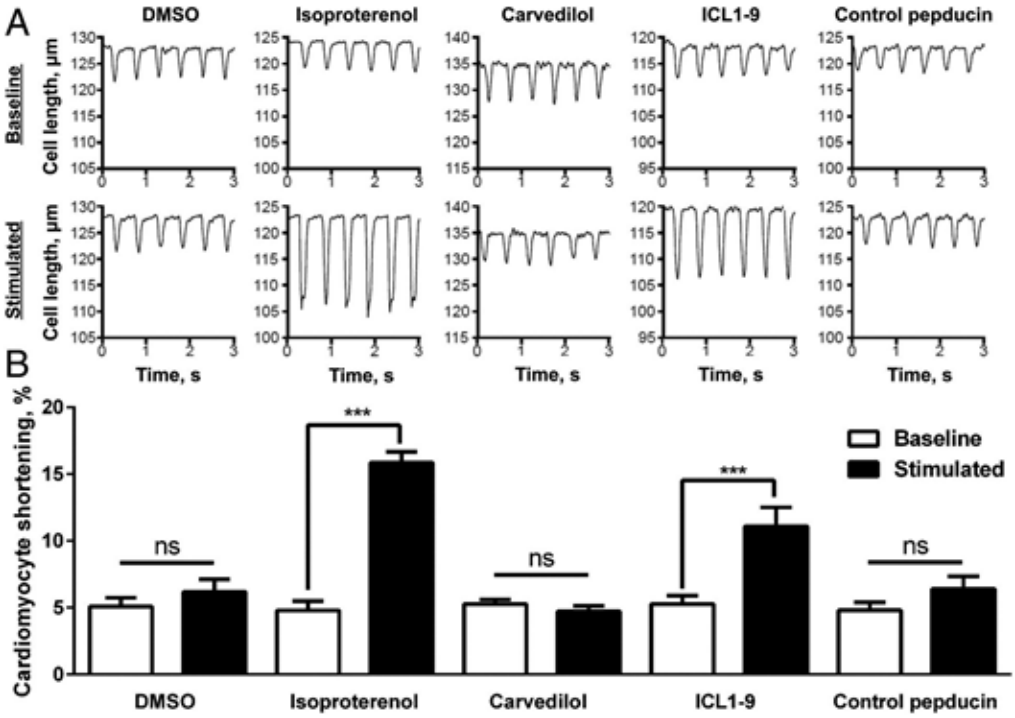


Figure 2. ICL1–9 promotes cardiomyocyte contraction, whereas carvedilol does not demonstrate similar efficacy. (*A*) WT adult murine cardiomyocytes were isolated and assessed for basal and agonist-promoted contractility using a digital videocamera-coupled microscope in the presence or absence of 0.1% DMSO, 0.5 µM isoproterenol, 10 µM carvedilol, 10 µM ICL1–9, or 10 µM control pepducin. Representative cell length (in micrometers) tracings at 2 Hz in the basal or stimulated state for each test condition are reported. (*B*) ICL1–9 was able to promote significant contraction in WT adult murine cardiomyocytes, whereas carvedilol did not stimulate a similar effect. The data are represented by the mean ± SEM from n = 4–8 individual cardiomyocytes from at least three independent primary isolations. ns, not significant, ***P < 0.001 using a one-way ANOVA with Newman–Keuls multiple comparison test.

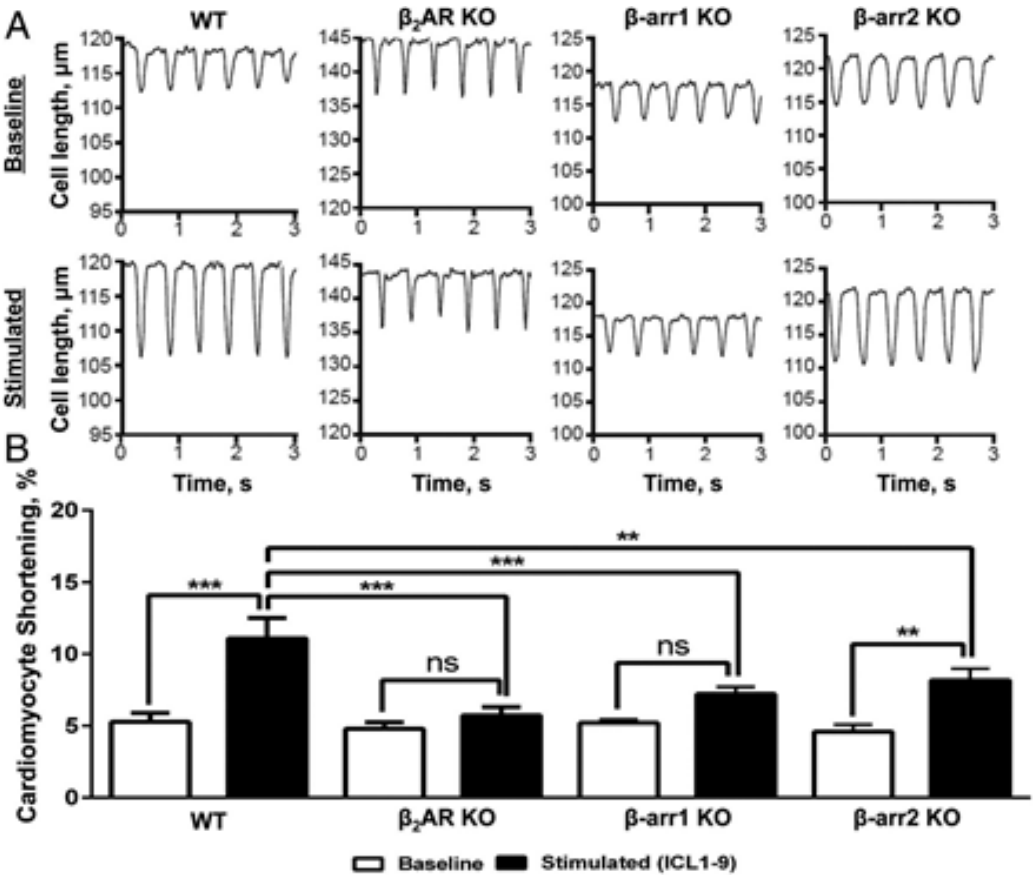


Figure 3. ICL1–9-promoted cardiomyocyte contractility is dependent on the expression of the β_2AR and β-arrestin. (*A*) β_2AR-, β-arrestin1-, or β-arrestin2 knockout adult murine cardiomyocytes were isolated and assessed for basal and agonist-promoted contractility using a digital videocamera-coupled microscope in the presence or absence of 0.1% DMSO or 10 µM ICL1–9. Representative cell length (in micrometers) tracings at 2 Hz in the basal or stimulated state for each test condition are reported. (*B*) ICL1–9 was unable to promote significant contraction in β_2AR- and β-arrestin1 knockout adult murine cardiomyocytes, and its ability to do so in β-arrestin2 knockout cardiomyocytes was significantly reduced. The data are represented by the mean ± SEM from six to seven cardiomyocytes from at least three independent primary isolations. ns, not significant, **P < 0.01, ***P < 0.001 using a one-way ANOVA with Newman–Keuls multiple comparison test.

The tight junction is composed of a complex of multiple proteins, of which the claudins are now believed to form the paracellular pores or channels. Claudin-2 is highly expressed in the proximal tubule, particularly in the late segments and has been shown *in vitro* to function as a high-conductance paracellular Na+ channel. Claudin-2 plays a major role in proximal tubular paracellular Na+ transport. It has been confirmed that claudin-2 KO mice have a 23% reduction in proximal fluid reabsorption. You would predict that claudin-2 KO mice would exhibit urinary NaCl wasting.

You evaluate the role of claudin-2 in salt handling in the kidney by placing mice on a normal and low salt diet and measure urinary sodium. You also evaluate the response to furosemide and observe the following:

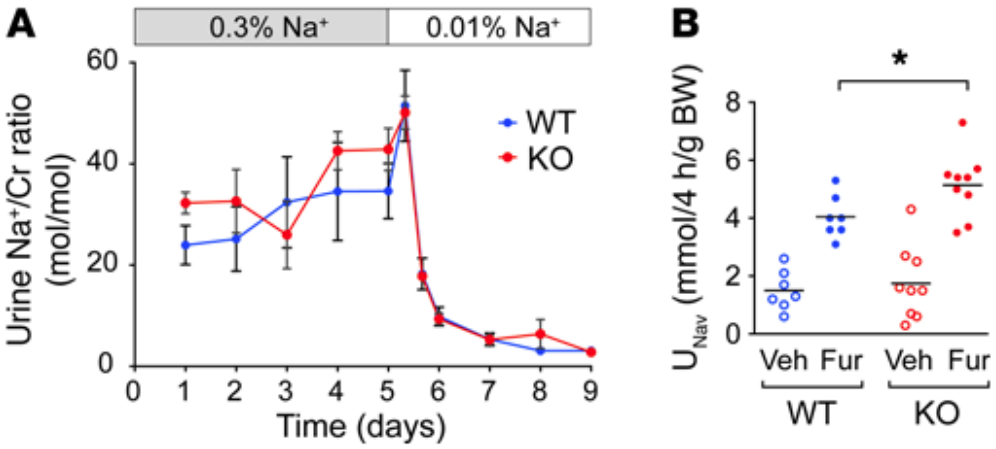


Figure: Effect of claudin-2 KO on renal Na+ handling. (**A**) Effect of dietary Na+ depletion. Urine Na excretion rate, expressed as the ratio of Na to creatinine (Cr) concentration (mean ± SEM, *n* = 4–5 per group), is shown in mice on a normal (0.3%) Na diet (day 1 to day 5), followed by a Na+-deficient (0.01%) diet (day 6 to day 9). (**B**) Furosemide (Fur) challenge test. Mice were administered vehicle (Veh) i.p. on the first day and furosemide 25 mg/kg on the second day. Urine Na+ excretion after furosemide was greater in KO mice than in WT mice. **P* < 0.05, by paired Student's *t* test (*n* = 7–9 per group).

Questions:

1. **Explain the results in Figure A. What do the results suggest about the role of claudin-2 in regulating the level of urine sodium? Would you predict the blood pressure to be different between WT and KO animals?**

2. **Explain the results in Figure B. What is the site of action of furosemide in the kidney? What happens to the amount of sodium in the urine in response to furosemide in the claudin-2 KO animals compared to WT?**

3. **What is your hypothesis based on figures A and B and how would you test it?**

During the past few years, designer receptors exclusively activated by a designer drug (DREADDs) have emerged as powerful novel chemogenetic tools to study the physiological relevance of signaling pathways activated by different functional classes of G protein-coupled receptors (GPCRs). Like endogenous GPCRs, clozapine-N-oxide (CNO)-activated DREADDs not only activate heterotrimeric G proteins but can also trigger β-arrestin-dependent (G protein-independent) signaling. A group of investigators recently developed two mutationally modified versions (M3D-1 and M3D-2) of a non-biased DREADD (M3D) derived from the M3 muscarinic receptor. To characterize these DREADDs and to explore M3 muscarinic receptor signaling, the investigators performed the experiments below.

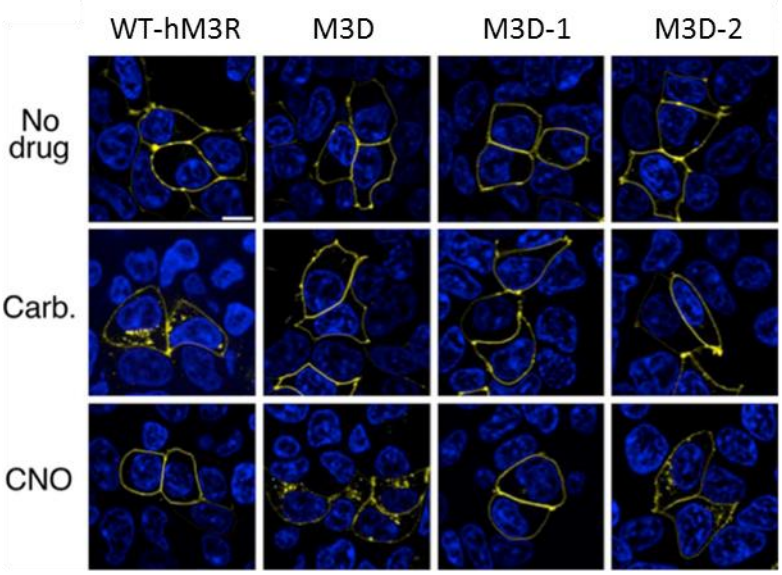


FIGURE 1. **DREADD internalization assays.** HEK293 cells were transfected with the wild type human M3R (WT-hM3R) and the indicated M3R DREADDs (M3D) fused to EYFP (yellow) at their C termini. M3D mimics the WT-hM3R except that it responsive to CNO but not carbachol (Carb.). Live-cell imaging studies were performed 24 h after transfection using a Zeiss Axio Observer microscope. Nuclei were stained with Hoechst 33324 (blue). To study receptor internalization, cells were incubated for 45 min with Carb. or CNO (100 µM each). Images were acquired before (no drug) and after agonist treatment. Scale bar, 10 µM.

TABLE 1. **Ligand binding and functional properties of DREADDs expressed in primary mouse hepatocytes.** Experiments were carried out with primary hepatocytes prepared from WT mice that had been injected with recombinant adeno-associated viruses (AAVs) coding for eGFP (control) or the indicated DREADDs. *N*-[3H]Methylscopolamine ([^{3}H]NMS, an antagonist) saturation binding and CNO-induced Ca^{2+} mobilization experiments were performed. Binding data are presented as means +/- S.E. of three independent experiments, each carried out in duplicate. $[Ca^{2+}]_i$ data are given as means +/- S.E. of three or four independent experiments, each performed in quadruplicate.

		CNO-induced Ca^{2+} mobilization	
Construct	[^{3}H]NMS binding B_{max}	EC_{50}	E_{max}
	pmol/mg protein	*nM*	*-fold over basal*
eGFP	NB[a]	NR[b]	
M3D	0.67 ± 0.19	85 ± 14	7.3 ± 2.3
M3D-1	1.05 ± 0.34	215 ± 58	9.8 ± 1.8
M3D-2	0.88 ± 0.06	NR	

[a] NB means no detectable specific radioligand binding activity.
[b] NR means no significant response up to 50 µM CNO.

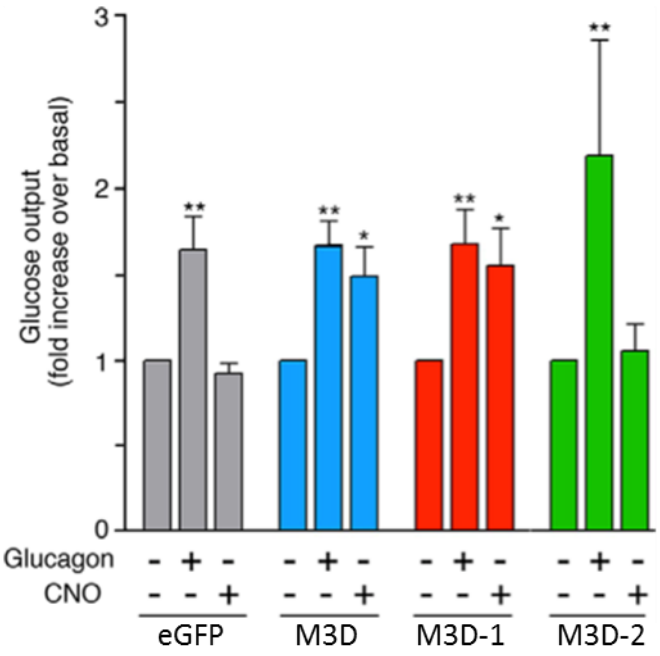


FIGURE 2. **DREADD-mediated increases in glucose output studied with isolated hepatocytes.** Primary hepatocytes were isolated from WT mice (8-week-old males) that had been injected i.v. with recombinant AAV-DREADD viruses coding for the indicated DREADDs or with a control virus (AAV-eGFP) encoding eGFP. Hepatocytes were first maintained in glucose-free medium for 16 h and then incubated with CNO (10 μM) or glucagon (100 nM; positive control) for 4 h at 37 °C. After this time, glucose concentrations were determined in the culture medium. Basal glucose release did not differ significantly among eGFP- and DREADD-expressing hepatocytes (data range: 0.03–0.10 μg of glucose/μg of protein). Data are given as means ± S.E. of three independent experiments.*, $p < 0.05$; **, $p < 0.01$, as compared with non-treated cells.

A) Based on data shown in Fig. 1 and Table 1, what can you say about the functionality of the DREADDs?

B) Develop a hypothesis that might explain the data shown in Fig. 2. Describe how the results support your hypothesis.

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