Department of Pharmacology

Qualifying Examination (Part I)

AUGUST 4-8, 2014

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

Date	Time
Tuesday, August 5th	1:00 pm – 3:00 pm
	3:00 pm – 5:00 pm
Wednesday, August 6th	1:00 pm – 3:00 pm
	3:00 pm – 5:00 pm
Thursday, August 7th	1:00 pm – 3:00 pm
	3:00 pm – 5:00 pm
Friday, August 8th	1:00 pm – 3:00 pm
	3:00 pm – 3:45 pm *(Committee Meets to determine results)*
	3:45 pm ***(Results given to students – Pharm South Conf. Rm.)***

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.

Repeated exposure to cannabinoids has been reported to cause increased anxiety. Similarly, enhanced activity of 5-HT2A receptors in the hypothalamic paraventricular nucleus (PVN) has been associated with several mood disorders including anxiety.

The study examined the effect chronic treatment with nonselective CB1/CB2 cannabinoid agonist CP55940 on the level of anxiety in rats and the molecular mechanisms of that effect.

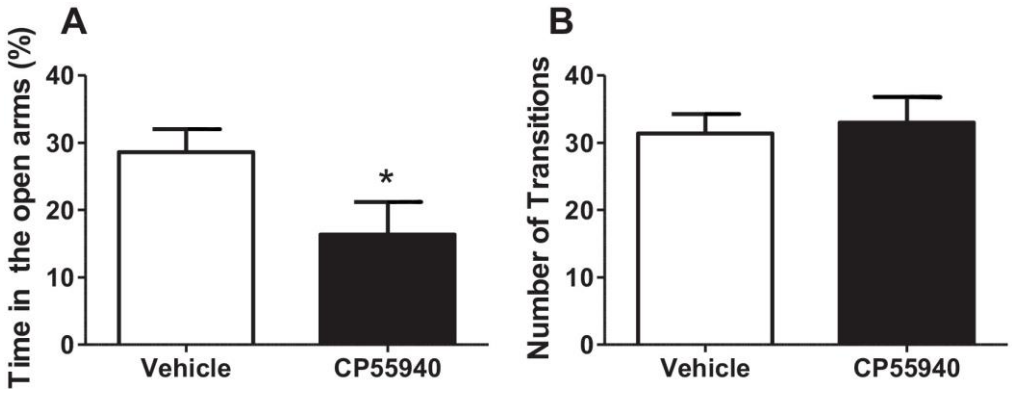


Figure 1. ***Effect of repeated CP55940 treatment on anxiety-like behavior in the elevated plus maze test.*** Rats were treated with CP55940 once a day for 7 days, and the behavior was examined 48 h after the last treatment. (**A**) Percent time spent in the open arms of the elevated plus maze was evaluated for a period of 5 min. (**B**) Transitions between the different arms of the maze was used as an index of locomotor activity. Mean ± SEM, $n = 6–8$ rats. $*p < 0.05$, significant behavioral effect of CP55940 compared to respective vehicle-treated control rats.

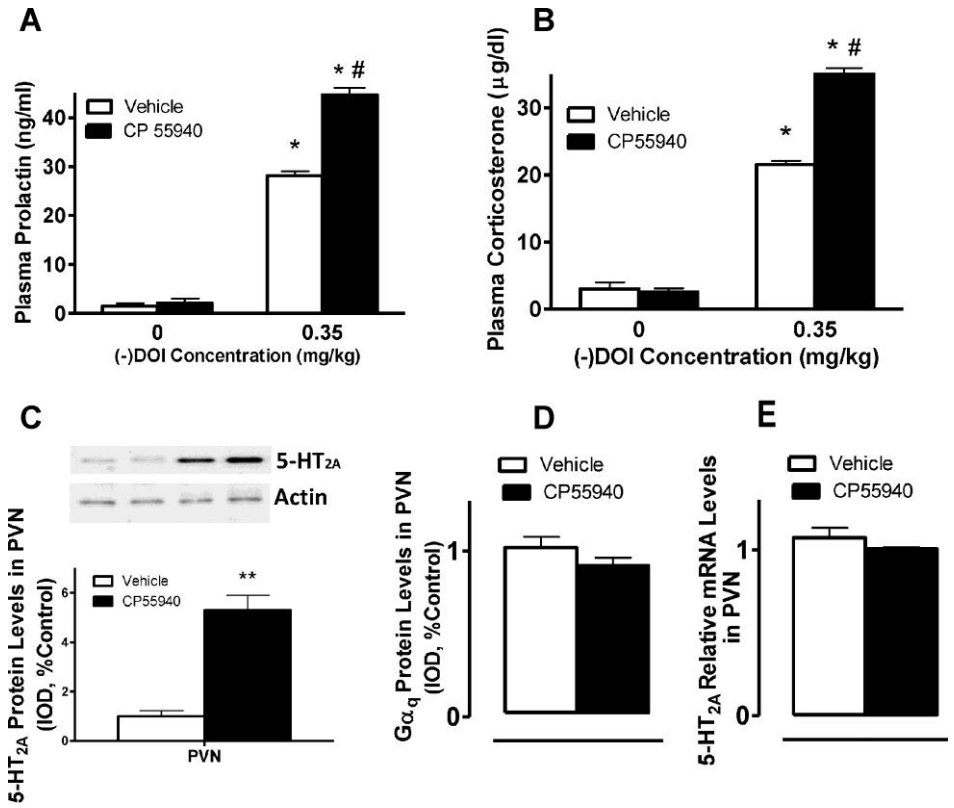


Figure 2. The effect of chronic *CP55940 treatment on the activity and expression of 5-HT2A receptors in the hypothalamic PVN*. Rats were treated with CP55940 once a day for 7 days and challenged with (−)DOI (5-HT2A/2C receptor agonist) 30 min prior to sacrifice. (**A** and **B**) The effect of CP55940 treatment on the plasma prolactin and corticosterone neuroendocrine responses mediated by 5-HT2A receptors in the PVN. *$p < 0.05$, significant effect of (−)DOI challenge in comparison to vehicle-challenged controls. # - $p < 0.05$, significant effect (−)DOI challenge in CP55940 treated rats compared to (−)DOI challenge in vehicle-treated rats. (**C**) The level of the 5-HT2A receptor protein in the PVN of rats treated with CP55940 as compared to vehicle treated controls. β-actin was used as a loading control and similar results were obtained in three separate experiments. **$p < 0.01$, significant effect of CP55940 compared with their respective vehicle-treated controls. (**D**) The expression of the Gαq protein in PVN of CP55940 treated rats compared to vehicle treated controls. (**E**) The level of 5-HT2A receptor mRNA in PVN of CP55940 treated rats compared to vehicle treated controls. Data represent the mean ± SEM of 8 rats per group and were analyzed by one-way ANOVA or *t*-Student test.

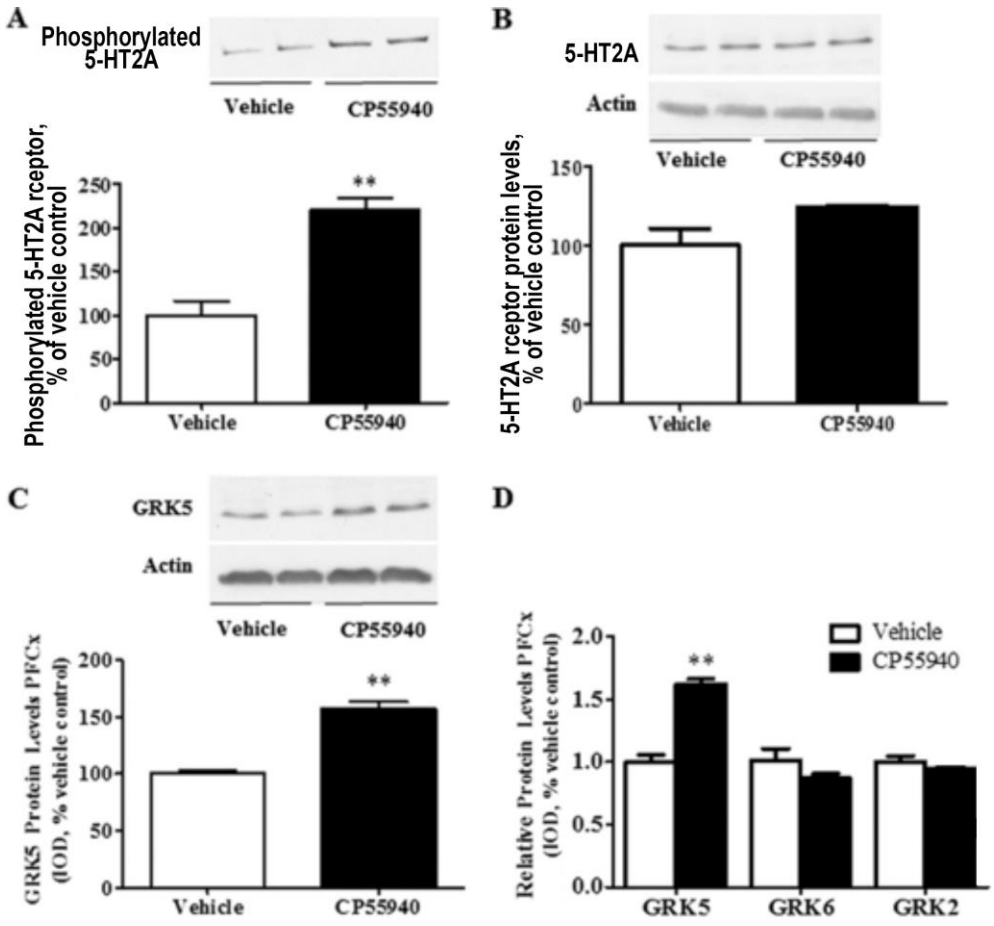


Figure 3. ***The effect of chronic treatment with CP55940 on phosphorylation of 5-HT2A receptors and the expression of indicated GRKs in the hypothalamic PVN.*** Rats were injected with CP55940 once a day for 7 days. After decapitation, the brains were collected, and PVN was dissected. (**A**) Phosphorylated 5-HT2A was detected by Western blot with phosphospecific antibody. (**B**) The total concentration of the 5-HT2A receptor was evaluated by Western blot with specific antibodies. (**C**) The concentration of GRK5 was determined by Western blot with anti-GRK5 antibody. Representative Western blots are shown. β-Actin was used as a loading control. (**D**) The graph shows levels of GRK5, GRK6 and GRK2 following vehicle or CP55940 treatment. **, $p < 0.01$; *, $p < 0.05$, significant effect of CP55940 treatment compared with vehicle-treated controls. The data represent mean ± S.E. (*error bars*) (n = 6–8).

1. **Describe the effect of chronic cannabinoid administration on the anxiety level based on the data Fig. 1.**

2. **Based on the data presented in Fig. 2, how do you explain enhanced responsiveness to the 5-HT2A challenge in cannabinoid-treated rats?**

3. **Based on the presented data, suggest a model linking cannabinoid-induced anxiety, 5-HT2A receptor and GRK5 functions and suggest experiments to test the key point in the model.**

Potential drug-drug interactions of the tyrosine kinase inhibitor, sunitinib, co-administered with therapeutic doses of drug-A or drug-B were investigated in rats; the oral absorption of sunitinib was monitored (Fig. 1). Comparison of the PK parameters following intraintestinal and IV administration are summarized in Table 1. Sunitinib is cleared mainly via biliary excretion, and Fig. 2 shows the biliary excretion of the parent drug in bile duct cannulated rats. Fig 3 shows the brain penetration of sunitinib.

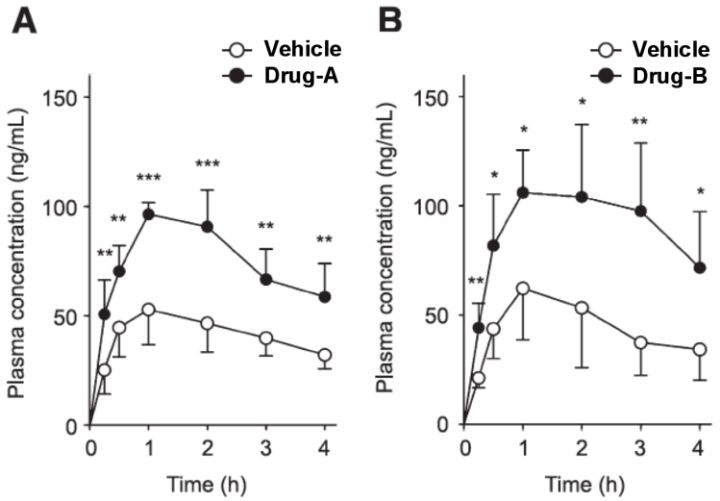


Fig. 1. Plasma concentration-time curve of sunitinib administered intraintestinally in rats pretreated with therapeutic doses of Drug-A (panel A) or Drug-B (panel B). After 15 minutes, the rats were given 3.87 mg/kg of sunitinib. Blood samples were collected at 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0 hours after the dose. Each point represents the mean ± S.D. (n = 4–7). *P <0.05; **P ,<0.01; and ***P < 0.001, significantly different from the vehicle treated group.

Table1: Plasma pharmacokinetics of sunitinib in rats pretreated with Drug-A or Drug-B

	Pretreatment			Pretreatment		
	Vehicle	Drug-A	Fold Increase	Vehicle	Drug-B	Fold Increase
Intraintestinal administration						
Cmax (ng/mL)	59 ±14	100 ± 8b	1.7	5 ± 25	110 ± 23c	1.7
AUC_{0-4} (ng*hr/mL)	165 ± 35	298 ± 37b	1.8	176 ± 70	359 ± 100c	2.0
F	0.27	0.47	1.8	0.26	0.53	2.1
IV administration						
AUC_{0-4} (ng*hr /mL)	175 ± 28	175 ± 40	1.0	168 ± 11	164 ± 13	0.98
Vdss (mL/hr)	3090 ± 484	3320 ± 631	1.1	4070 ± 796	4330 ± 461	1.1
CL (mL/hr)	1410 ± 242	1380 ± 499	0.98	1230 ± 111	1210 ± 272	0.98

a Data for parameters except F are shown as the mean ± S.D. (n = 3–7).
b P , 0.001, statistically significantly different from the vehicle-treated group.
c P , 0.05, statistically significantly different from the vehicle-treated group.

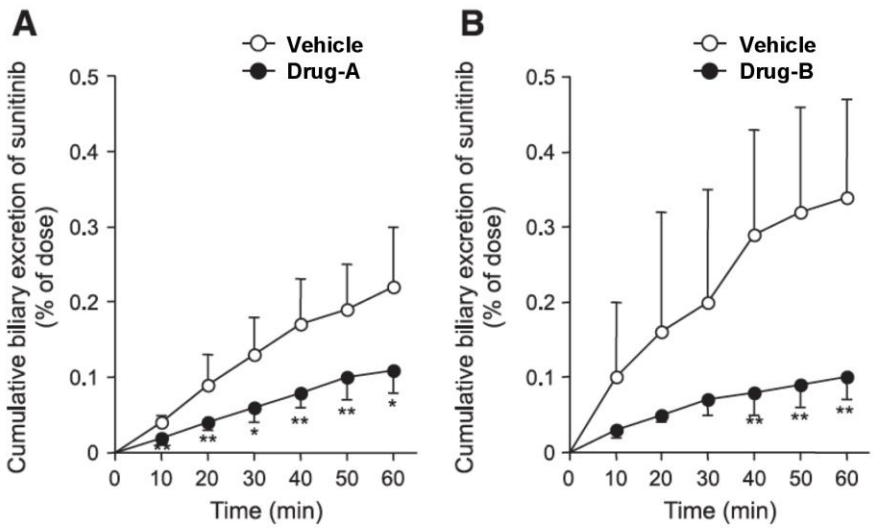


Fig. 2. Cumulative biliary excretion of sunitinib administered intravenously in rats pretreated with therapeutic doses of Drug-A (A) or Drug-B (B). After 15 minutes, the rats were given 0.97 mg/kg of sunitinib. Bile samples were collected for each interval (0–10, 10–20, 20–30, 30–40, 40–50, and 50–60 minutes) after the sunitinib administration. Each point represents the mean ± S.D. (n = 5–7). *P ,<0.05; **P < 0.01, statistically significantly different from the vehicle-treated group.

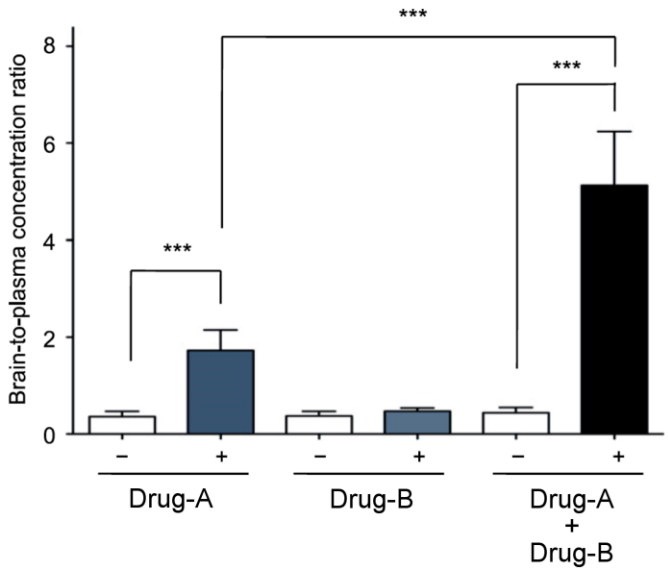


Fig. 3. Brain distribution of sunitinib in rats pretreated with Drug-A, Drug-B, or both Drug-A and Drug-B. After 15 minutes, rats were given 0.97 mg/kg of sunitinib. One hour after the administration of sunitinib, the rats were sacrificed to obtain plasma and brain samples. The brain-to-plasma concentration ratio was obtained at 60 minutes after the intravenous administration of sunitinib. Each column represents the mean ± S.D. (n = 4–7). ***P <0.001, statistically significantly different from the vehicle-treated orPSC833-treated group.

1. **Summarize the observed drug-drug interactions of sunitinib with drug-A and drug-B and propose a mechanistic explanation to account for the observations.**

2. **Outline additional *in vitro* and *in vivo* experiments you would carry out to test or substantiate your proposal.**

3. **Based on data contained within Table 1, what is rate-limiting in the IV disposition of sunitinib?**

You have a mouse line with functional inactivation of a gene called *Pot1*. This gene belongs to a family of genes encoding voltage-gated K+ channels facilitating background potassium flux at the basolateral membrane of non-excitable polarized epithelial cells.

Cardiac function tests reveal arrhythmia and cardiac hypertrophy in *Pot1* KO mice, in line with a role of this gene as a K+ channel, but only in KO mice born from KO dams (KO pups from heterozygote dams have normal echocardiograms and heart size). You also notice additional phenotypes in the mice you breed, including pup mortality, dwarfism (although birth and body weight are normal), osteoporosis, alopecia (hair loss) and low body temperature, but again in mice from KO dams only.

Based on these phenotypes, you suspect the existence of hypothyroidism.

Question 1:
What tests would you run to address this hypothesis further and what do you expect if these mice indeed have hypothyroidism?

Question 2:
What experiment can you think of to prove that these phenotypes in KO pups are caused by a hormone, and lack of T_3/T_4 specifically?

Question 3:
Following injection of ^{124}I by tail vein into WT and KO dams, you obtain the following results by microPET.

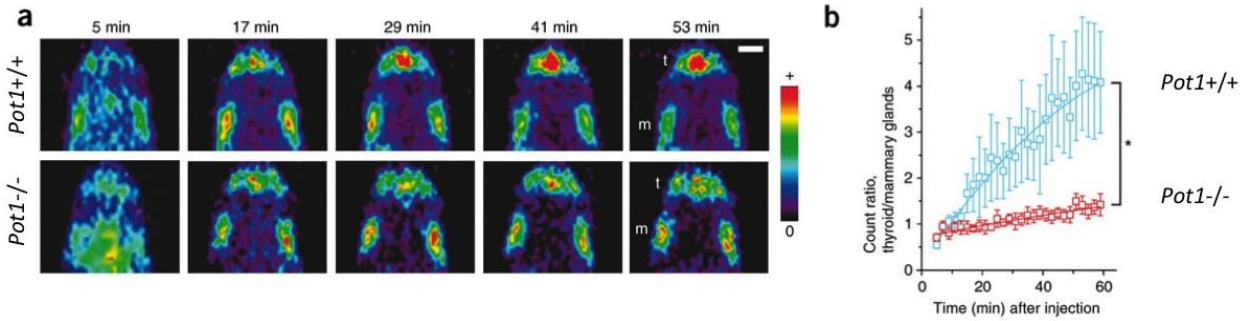


Fig. 1: **a**, Representative microPET images of lactating *Pot1*$^{+/+}$ and *Pot1*$^{-/-}$ dams recorded during the first hour after tail vein injection of ^{124}I. t, thyroid; m, mammary gland. Red indicates highest intensity, as shown by the color intensity scale. Scale bar, 5 mm. **b**, Mean ^{124}I accumulation in thyroid relative to mammary gland from imaging as in **a**, measured as the ratio of maximum radioactivity in each tissue minus mean background count in each mouse.

What do you conclude about the role of *Pot1* in the thyroid? Can you speculate how lack of this K+ channel contributes to the phenotype of these mice?

Extracellular ATP promotes epithelial wound healing response and its mechanism of action was investigated using H292 cells, which are human lung mucoepidermoid cells. Results are shown below.

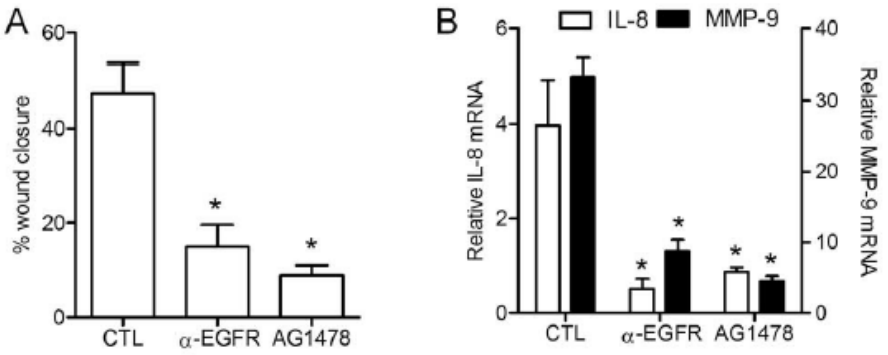


Figure 1. Confluent H292 cells were subjected to scratch wounding (A) or stimulated with exogenous ATP (100 uM, for 8 hours) (B). Wound closure (A) and mRNA levels for interleukin-8 (IL-8) and matrix metalloproteinase 9 (MMP-9) were determined. Both IL-8 and MMP-9 are involved in wound responses. AG1478 is an EGFR antagonist. α–EGFR is a blocking antibody for EGFR.

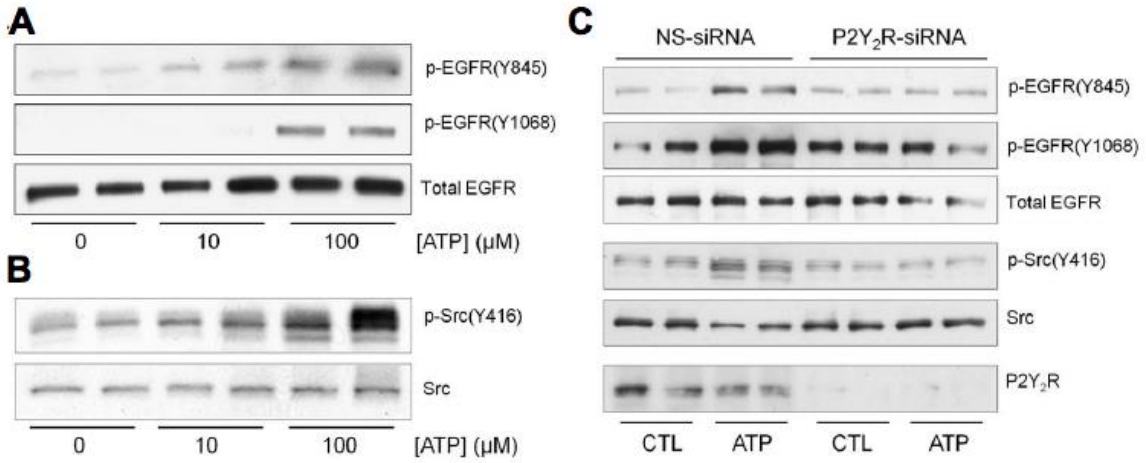


Figure 2. Effect of extracellular ATP on H292 cells. Western blot analyses were carried out using various antibodies as indicated on the right (A-B). (C) Effect of $P2Y_2R$ knockdown on the EGFR phosphorylation. $P2Y_2R$ is a subtype of purinergic P2Y receptor that belongs to GPCR family. CTL, control; NS-siRNA, non-specific siRNA.

1. **Explain the results in Figures 1 and 2.**

2. **Based on the results, propose two independent mechanisms of action by which extracellular ATP activates EGFR.**

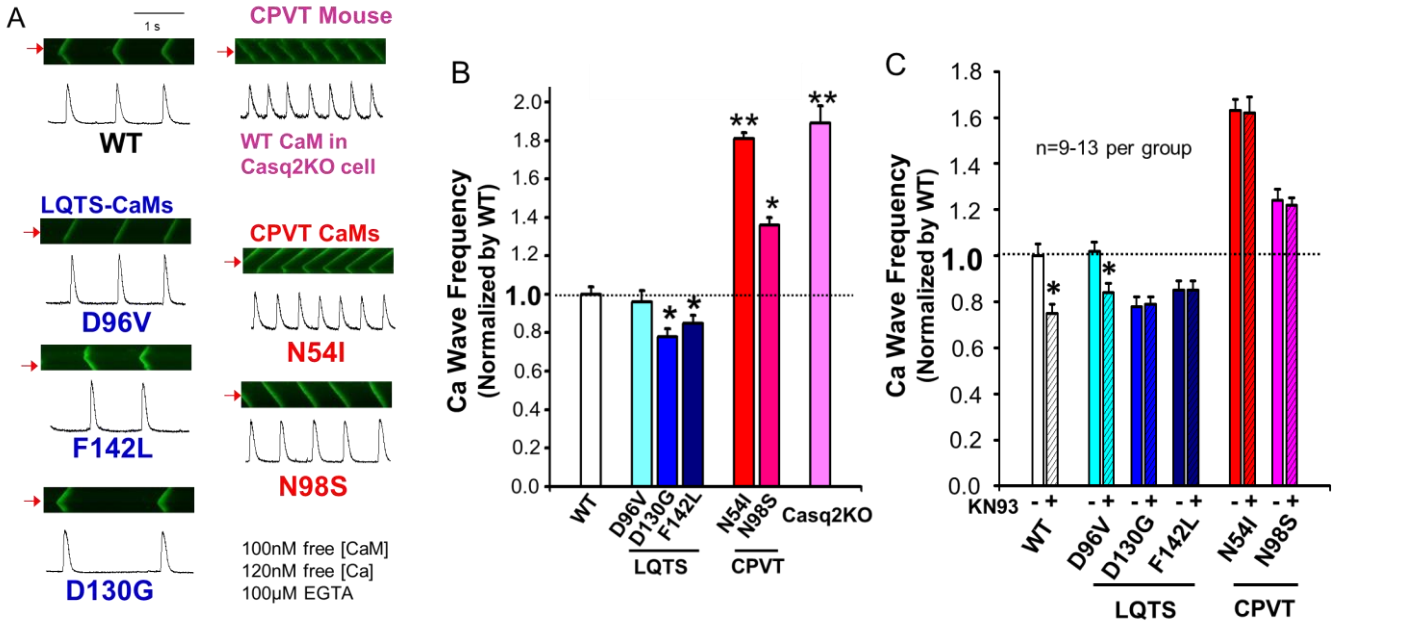
3. **Propose experiments (including results) to investigate the contribution for each of the proposed mechanisms.**

Calmodulin (CaM) is an essential Ca binding protein that transduces Ca signals in a wide range of biological processes. CaM binds to larger proteins and functions as a Ca sensor for decoding Ca signals into downstream responses. In the heart, CaM regulates many ion channels such as the L-type Ca channel (Ca-dependent inhibition), Ca-activated K channels (Ca-dependent activation) and the RyR2 sarcoplasmic reticulum Ca release channel (Ca-independent inhibition). Humans have 3 CaM genes – *CALM1*, *CALM2*, *CALM3* – encoding the identical amino acid sequence that are all expressed in the heart muscle.

Recent genetic studies have identified CaM missense mutations in humans with severe ventricular arrhythmia and sudden cardiac death susceptibility, albeit with distinct clinical presentations: two mutations in *CALM1* (N54I and N98S) were associated with stress-induced polymorphic ventricular tachycardia reminiscent of catecholaminergic polymorphic ventricular tachycardia (CPVT-CaMs), whereas three other mutations in either *CALM1* or *CALM2* (D96V, D130G and F142L) led to recurrent cardiac arrest in infancy associated with severe QT prolongation reminiscent of a long QT syndrome (LQTS-CaMs). CPVT is commonly caused by mutations in sarcoplasmic reticulum genes that increase diastolic Ca leakage through the ryanodine receptor (RyR2) channels, whereas LQTS is usually caused by dysfunctional plasma membrane ion channels that prolong the ventricular action potential.

Nonstandard Abbreviations and Acronyms	
CaM	calmodulin
CaMKII	Ca/CaM kinase II
Casq2	calsequestrin-2
CPVT	catecholaminergic polymorphic ventricular tachycardia
LQTS	long QT syndrome
P_o	single-channel open probability
RyR2	ryanodine receptor 2
SR	sarcoplasmic reticulum

Using recombinant mutant CaM protein, the following experiments were performed to elucidate how mutant CaMs cause CPVT or LQTS



(**A**) Representative confocal line scans from permeabilized mouse ventricular myocytes after 30 min incubation with either WT or mutant CaMs (100nM, physiological free [CaM]). After permeabilization, myocytes were incubated in internal solution composed of 120 nM free [Ca], clamped with EGTA, and 25 µM Fluo.

(**B**) Average data. Bars represent mean+SE of values normalized by WT values on each experimental day. WT (n=45), D96V (n=33), D130G (n=15), F142L (n=20), N54I (n=35), N98S (n=35). Casq2KO: Myocytes isolated from a CPVT mouse model (Casq2 null mice) and incubated with WT-CaM (n=21). **P*<0.05, ***P*<0.01 vs WT CaM.

(**C**) Effect of Ca-CaM dependent kinase II (CaMKII) inhibition with KN93 on Ca wave frequency. Permeabilized myocytes were incubated with 100nM of CaM mutants in presence or absence of KN93 (1µM, 30 min pre-incubation). Bars represent mean+SE. WT (n=40), D96V (n=20), D130G (n=15), F142L (n=20), N54I (n=29), N98S (n=12), *P<0.05 vs + KN93.

1. **Describe the major results of the experiment.**

2. **Formulate a hypothesis on how mutant CaMs regulate RyR2 channels and sarcoplasmic reticulum Ca release and design experiments that will test your hypothesis.**

3. **Below are the results for testing the Ca binding affinity of mutant CaMs in vitro. Based on the results of the Ca binding studies, formulate a hypothesis on how mutant CaMs cause LQTS and design experiments that will test your hypothesis.**

Table. Fold Reduction in Ca-Binding Affinity of LQTS- and CPVT-CaMs

CaM Mutation	CaM-C Domain K_d (Fold Reduction)	CaM-N Domain K_d (Fold Reduction)	Clinical Arrhythmia Syndrome
D130G	53.6	No change	LQTS
D96V	13.6	No change	LQTS
F142L	5.4	No change	LQTS
N98S	3.3	No change	CPVT
N54I	No change	No change	CPVT

The E3 ubiquitin ligase NEDD4-2 (encoded by the *Nedd4L* gene) regulates the epithelial Na^+-channel (ENaC) which is expressed in the collecting duct. Investigators recently generated tetracycline-inducible, nephron specific *Nedd4L* KO (deletion of exons 6-8) mice to determine the role of NEDD4-2 within the renal tubule. Part of their observations are seen in the below figure.

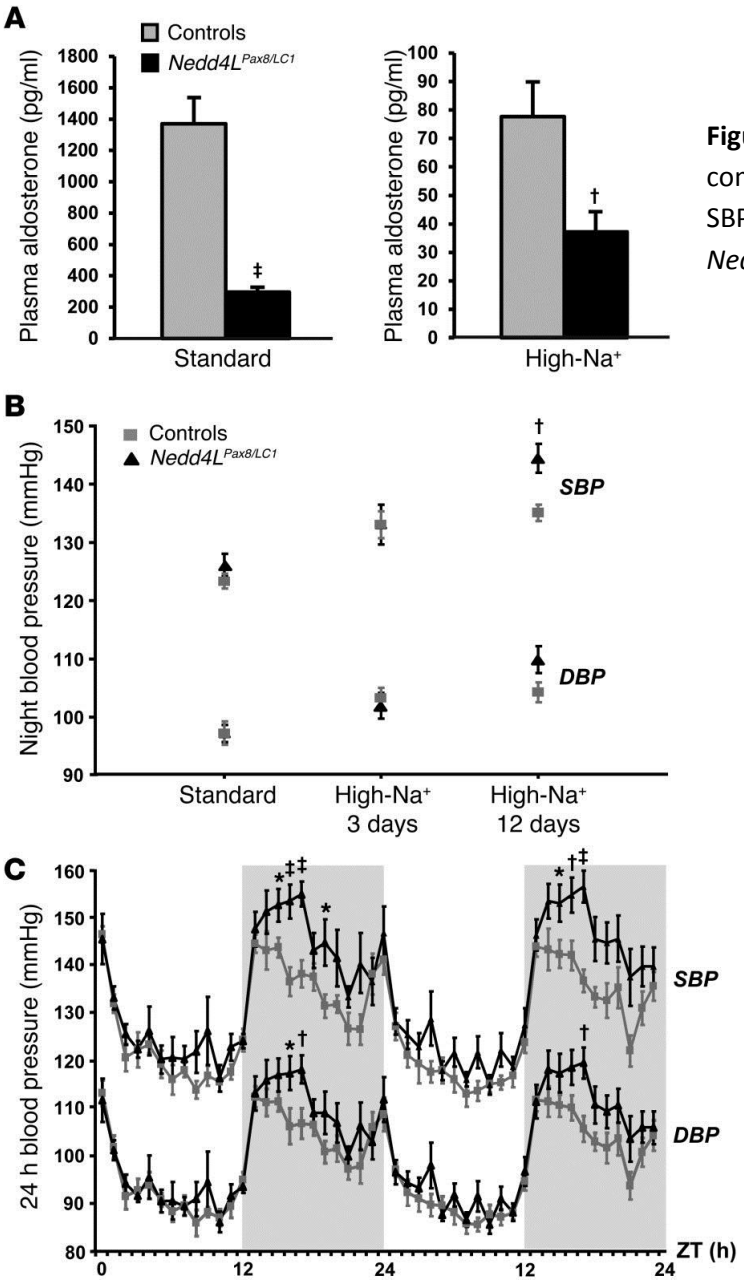


Figure: Plasma aldosterone levels were measured comparing KO mice to controls. Graphs represent SBP and DBP 12-hour night averages using telemetry. *Nedd4L*$^{Pax8/LC1}$ = renal tubular *Nedd4L* KO.

1. **Describe the results in the above figure and develop a hypothesis to explain the results.**

2. **Describe how you would test your hypothesis.**

3. **What would be expected to happen to serum potassium levels, if your hypothesis is correct?**

Agonist-induced endocytosis of the α2 adrenergic receptor (α2AR) is dependent on arrestin3. A recent study implicated a role for spinophilin in this process and showed that i) spinophilin and arrestin3 bind to the α2AR in a mutually exclusive manner and ii) spinophilin antagonizes arrestin3-dependent α2AR activation of ERK. To further explore the molecular mechanism underlying spinophilin's regulation of α2ARs, and to examine potential regulation of α2AR endocytosis by β2 adrenergic receptors (β2ARs), a group of investigators performed the following experiments.

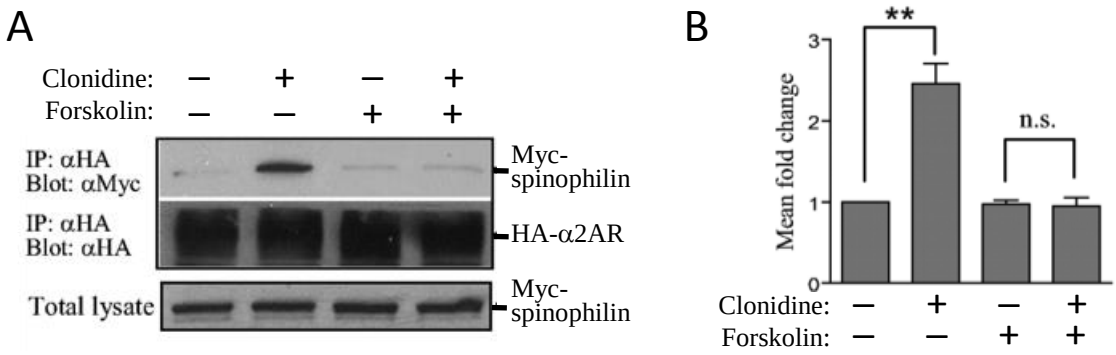


FIGURE 1. *A*) Mouse embryonic fibroblasts (MEFs) stably expressing HA-α2AR and Myc-spinophilin were pretreated with 10 μM forskolin or vehicle for 15 min and then stimulated with 1 μM clonidine (an α2AR-specific agonist) for 5 min. Immunoprecipitations were performed from the cell lysates (*IP: αHA*) and analyzed by Western using antibodies recognizing Myc (*Blot: αMyc*) and HA (*Blot: αHA*). An aliquot of the cell lysate was also analyzed by Western using the Myc antibody. *B*) Quantification of the data in *A*. Shown are the mean fold changes of Myc-spinophilin associated with the α2AR under the indicated conditions. Values represent the mean +/- S.E.; n=3; **, $p<0.01$, *n.s.*, not significant.

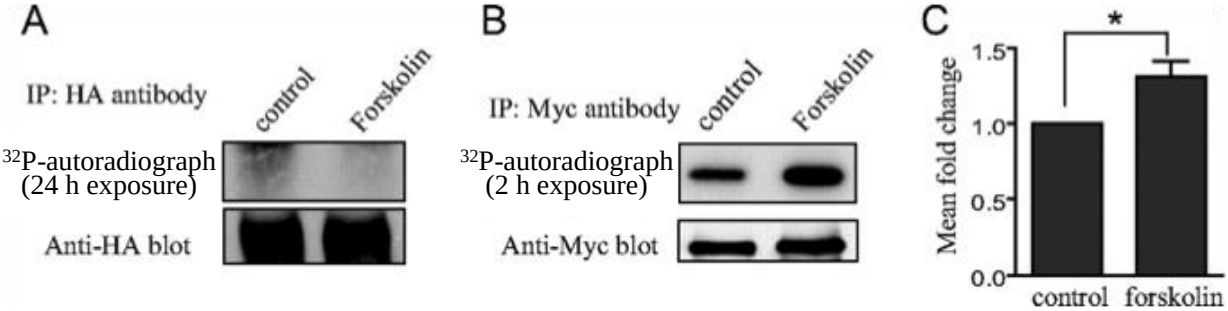


FIGURE 2. MEFs stably expressing HA-α2AR or Myc-spinophilin were labelled with [^{32}P]orthophosphate and then treated with 10 μM forskolin or vehicle for 15 min. HA-α2AR (*A*) and Myc-spinophilin (*B*) were immunoprecipitated from the cell lysates using HA and Myc antibodies, respectively. The immune complexes were subjected to SDS-PAGE followed by autoradiography (top panels) and Western analysis using HA and Myc antibodies (bottom panels). No phosphorylation of HA-α2AR could be detected in response to forskolin treatment. *C*) Quantification of the data in *B*. Shown is the mean fold change of spinophilin phosphorylation in response to forskolin treatment. Values represent mean +/- S.E.; n=7; *, $p<0.05$.

A) What conclusions can be drawn from the data depicted in Figs. 1 and 2?

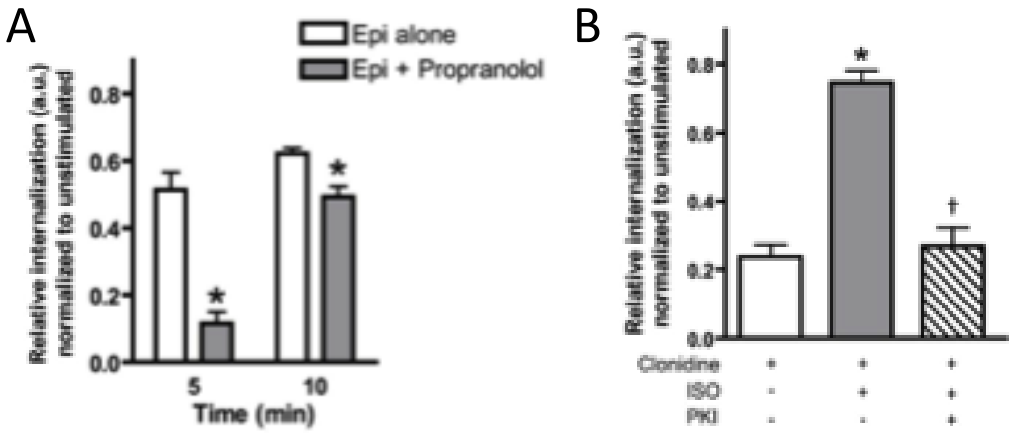


FIGURE 3. *A*) MEFs were stimulated with 100 μM epinephrine alone (for simultaneous activation of α2 and β2 ARs) or in combination with 1 μM of the β2AR antagonist propranolol (for activation of α2ARs only). Shown is the relative internalization of α2ARs in the stimulated cells after normalization to matched unstimulated cells. *, $p<0.01$ versus Epi alone. *B*) MEFs were pretreated with 100 μM ISO (β2AR agonist) and/or 10 μM PKI (PKA inhibitor), and then stimulated with 1 μM clonidine (α2AR agonist) for 10 min. Shown is the relative internalization of α2ARs in the stimulated cells after normalization to matched unstimulated cells. *, $p<0.001$ versus clonidine alone; †, $p<0.001$ versus clonidine plus ISO.

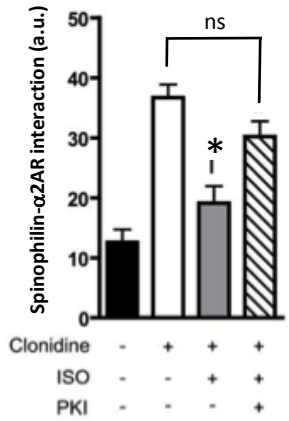


FIGURE 4. MEFs were pretreated with 100 μM ISO alone or in combination with 10 μM PKI, and then stimulated with 1 μM clonidine for 10 min. The amount of spinophilin associated with α2AR was quantified and expressed as arbitrary units (a.u.). Values represent the mean +/- S.E.; n=5; *, p<0.001 versus clonidine alone; ns, not significant.

B) Develop a hypothesis that might explain the the information provided and the collective data shown in Figs. 1-4. Describe how the results support your hypothesis.

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