Department of Pharmacology

Qualifying Examination (Part I)

December 15, 2015

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

Date	Time	Student Name
Tuesday, December 15th	9:00 am – 11:00 am	**(EXAM #1)**
	1:00 pm – 3:00 pm	**(EXAM #2)**
	3:00 pm – 3:30 pm (Committee Meets to determine results)	
	3:30 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.

The long-term treatment with opioid drugs is used to control chronic pain. Therefore, it is important to understand how pain affects the rewarding effect of opioids and hence risk of prescription opioid misuse and abuse.

In this study, the rat model of morphine self-administration (MSA) was used to investigate the molecular mechanisms underlying the impact of pain on morphine seeking before and after morphine withdrawal. Chronic pain was induced by injection of complete Freund's adjuvant (CFA) into the left hindpaw.

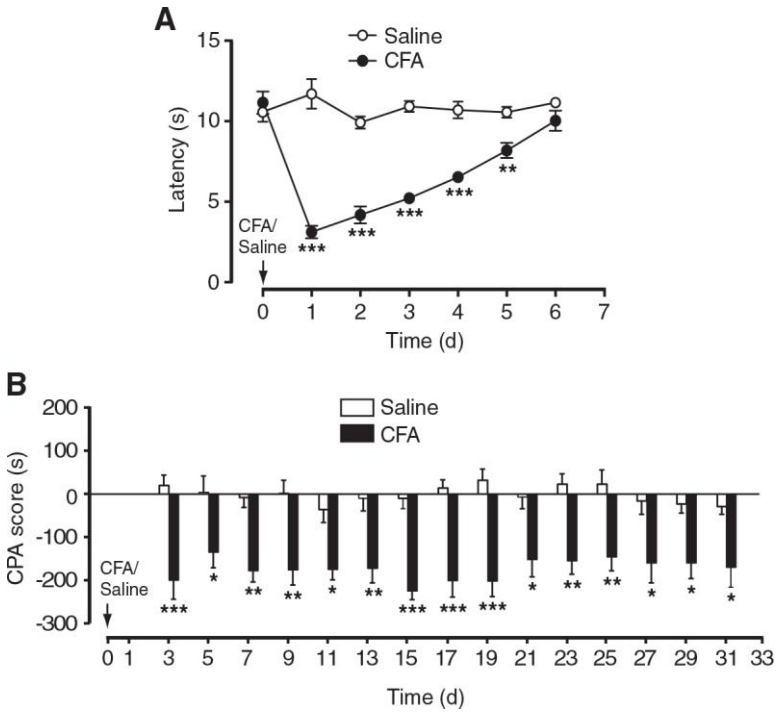


Figure 1. Time courses for the two dimensions of pain. **A**, The sensory dimension (hyperalgesia) of pain was measured by ***paw-withdrawal latencies (thermal stimulus)*** in rats after injection of CFA or saline on day 0 (arrow). **B**, The affective dimension of pain measured by ***conditioned place aversion (CPA)*** in conditioned rats after the same injection of CFA or saline on day 0 (arrow; *p<0.05, **p<0.01, ***p <0.001; two-way ANOVA with Bonferroni post hoc test). The data are shown as the CPA score (the difference in the time spent in the compartment before and after its pairing with the indicated drug).

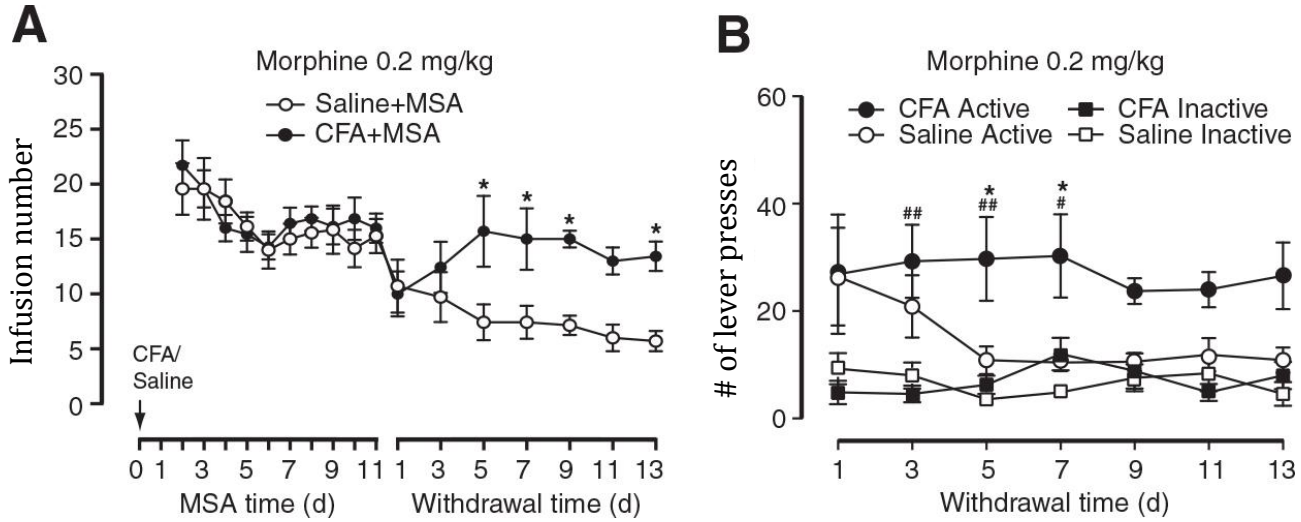


Figure 2. Persistent pain maintains morphine-seeking behavior after withdrawal from self-administered morphine. **A**, The number of daily infusions of morphine in saline- and CFA injected rats before and after withdrawal from morphine dose. CFA or saline was injected on day 0 (arrow), saline or morphine self-administration started on day 2 and morphine withdrawal (morphine substituted with saline) started on day12 (withdrawal day1). **B**, Number of presses of active and inactive levers for data shown in A during morphine withdrawal (** - p<0.01; *** - p<0.001 active lever presses the CFA versus saline group; ###<p0.001 active vs inactive lever presses in the CFA group).

GluA1 subunits of glutamate receptors play a critical role in synaptic plasticity involved in drug reward and in promoting behavioral responses to drugs of abuse and pain. The study next examined the expression of GluR1 in the central nucleus of amygdala, the structure critical for the emotional aspects of pain and generally for the processing of negative emotions.

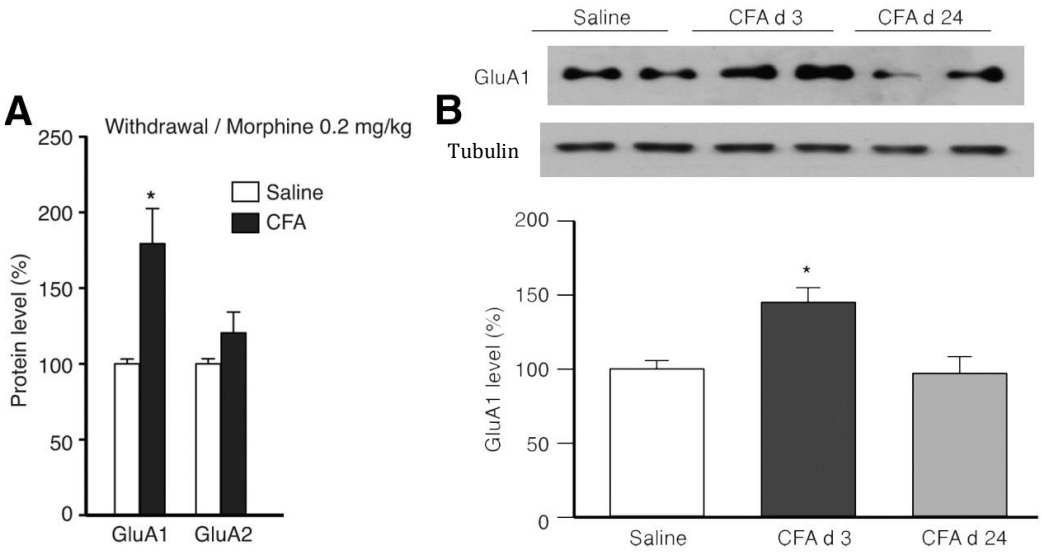


Figure 3. The level of the GluA1 protein in CeA after morphine withdrawal. Normalized protein levels of GluA1 and GluA2 in the central nucleus of amygdala (CeA), a limbic structure critically involved in the affective dimension of pain, in saline- and CFA-injected rats withdrawn from self-administered morphine for 13 d. ***B***, Western blots data for GluA1 in CeA in saline- and in CFA-injected rats that did not undergo morphine self-administration at 3 and 24 d after CFA-injection (Student's *t* test and one-way ANOVA with Bonferroni *post hoc* test).

MeCP2 is a global transcriptional regulator, and genome-wide expression analysis indicates that MeCP2 can activate or repress transcription of many target genes. MeCP2 is increasingly implicated in the epigenetic mechanisms of several neurological diseases including drug addiction and chronic pain. Thus, the study next examined the expression of MeCP2 in the CeA following morphine withdrawal in rats with or without chronic pain.

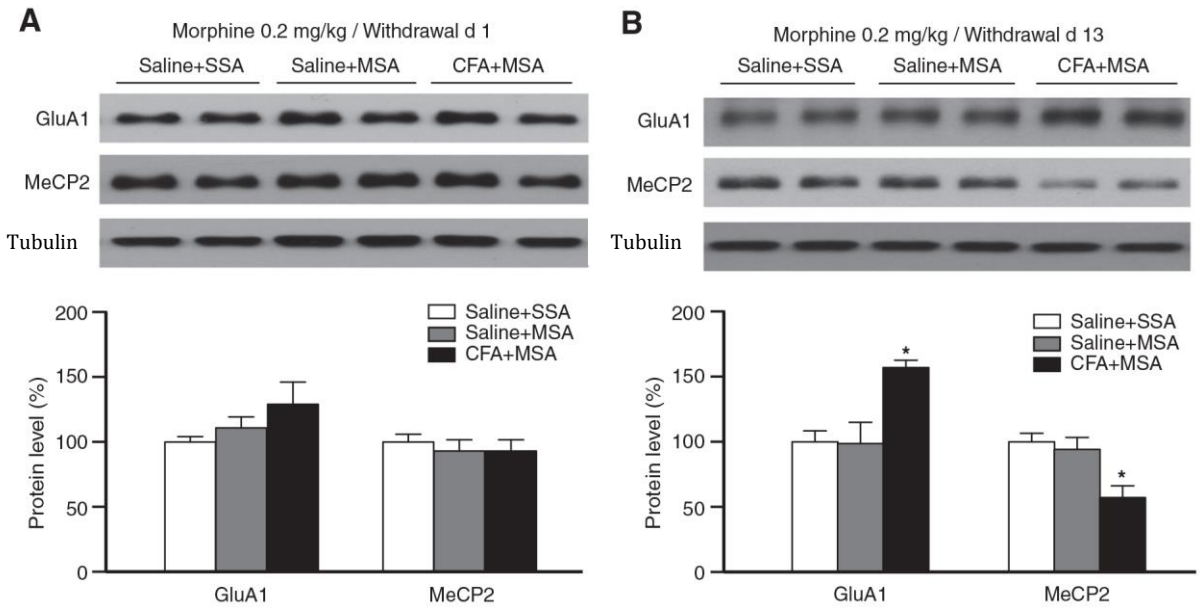


Figure 4. The expression level of MeCP2 after morphine withdrawal. ***A***, ***B***, Western blots data of GluA1 and MeCP2 proteins in the CeA of saline-injected SSA rats (n = 4) and MSA rats injected with saline (n = 4) or CFA (n = 4) on morphine withdrawal day 1 (n = 4, ***A***) and day 13 (n = 4, ***B***; one-way ANOVA with Bonferroni *post hoc* test).

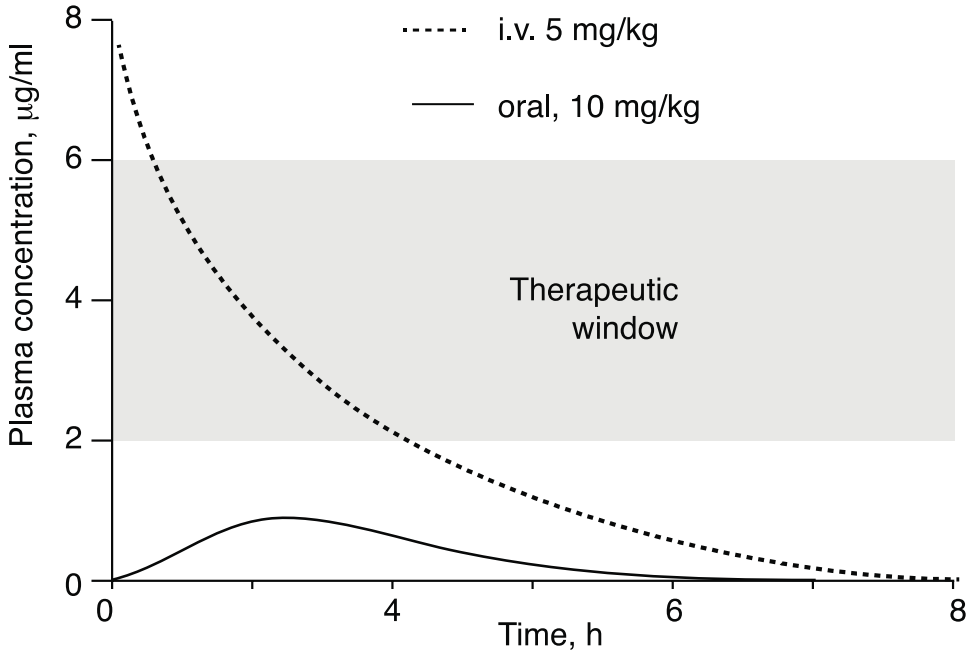
Questions:

1. Briefly describe the behavioral tests used in the study to measure sensory and emotional aspects of pain and the rewarding effect of morphine (and what exactly is measured in each test). What conclusion can you draw based on the data presented in Figs. 1 and 2 about the effect of the chronic pain on the characteristics of pain perception?

2. What conclusions can you reach based on the data presented in Figs. 3 and 4 as to the molecular mechanism of the effect of chronic pain on morphine craving during withdrawal?

3. Propose a simple model regarding the molecular mechanisms of opioid reward explored in this study. Propose experiments to test the model.

Drug **N** under development for hypertension is tested for the first time in vivo in an animal model. You obtain the following data:

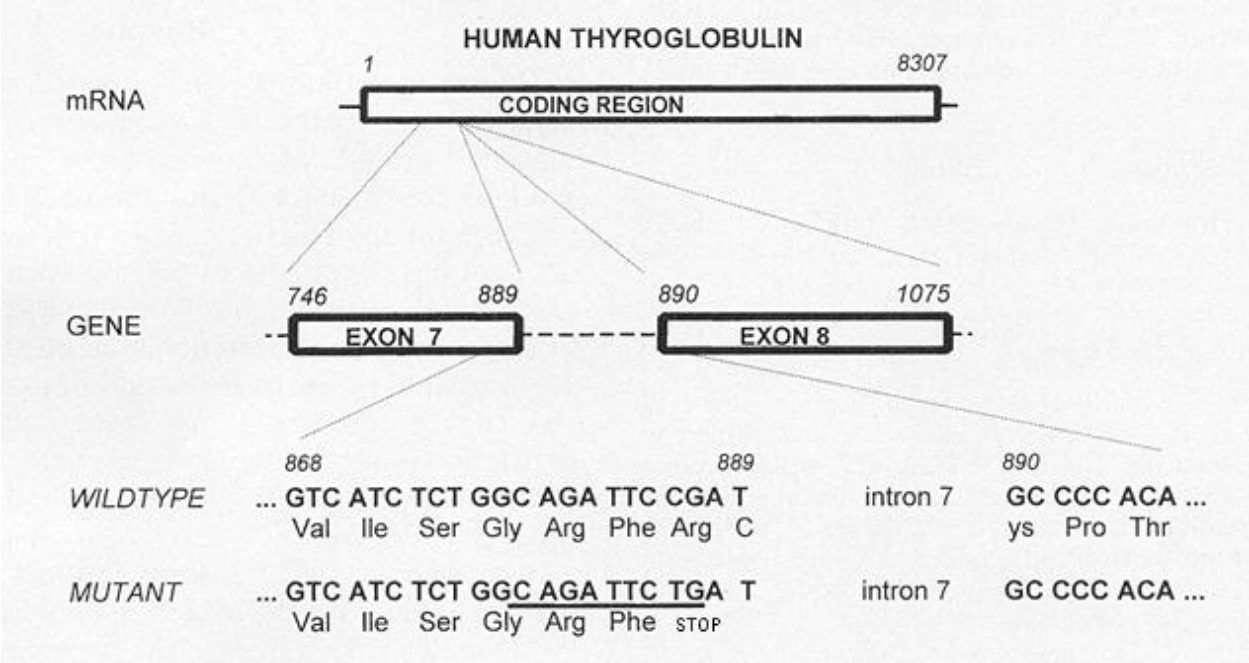


Questions:

1. Interpret these data and provide both *in vivo* and *in vitro* experimental strategies to establish the main factors responsible for the differences observed.

2. Illustrate the expected profiles if the same results were plotted using log plasma concentrations versus time.

You have recently identified a family in which multiple family members have two copies of a mutant thyroglobulin gene containing the C886T transition indicated below:



The presence of this premature stop codon results in the production of a 32 kilodalton thyroglobulin protein rather than the wild-type 330 kD isoform normally produced by the thyroid gland. Blood samples from affected siblings were assessed for total T_4, total T_3, and TSH levels and the results from such measurements are presented in the table below:

Patient #	T_4 (nM)	T_3 (nM)	TSH (mU/L)
1	62.3	2.6	13
2	38.6	1.5	112
3	25.7	1.2	96
Normal Range	70-160	1.1-3.1	<4.5

Questions:

1) Based upon your knowledge of thyroid hormone biosynthesis, how would you explain the levels of T_4, T_3 and TSH in these individuals?

2) All three patients had a diffusely enlarged thyroid gland. Explain this observation.

3) Patients 2 and 3 developed compressive symptoms (e.g., difficulty in breathing and swallowing) due to thyroid enlargement. Suggest an appropriate course of treatment for these individuals.

Administration of NSAIDs (non-steroidal anti-inflammatory drugs) that block the biosynthesis of prostaglandins has beneficial effects for reducing the occurrence of colon cancer. Further studies indicate that prostaglandin E_2 (PGE_2) promotes the growth of colonic polyps in the gastrointestinal tracts of mice. Importantly, EGFR is critical for the proliferation of the intestinal mucosa,, and its expression is up-regulated in colon cancer. To explore the mechanism by which the activity of EGFR may be modulated by PGE_2, several experiments were carried out in human colon cancer cells (Caco-2), and experimental results are shown in Figures 1 and 2.

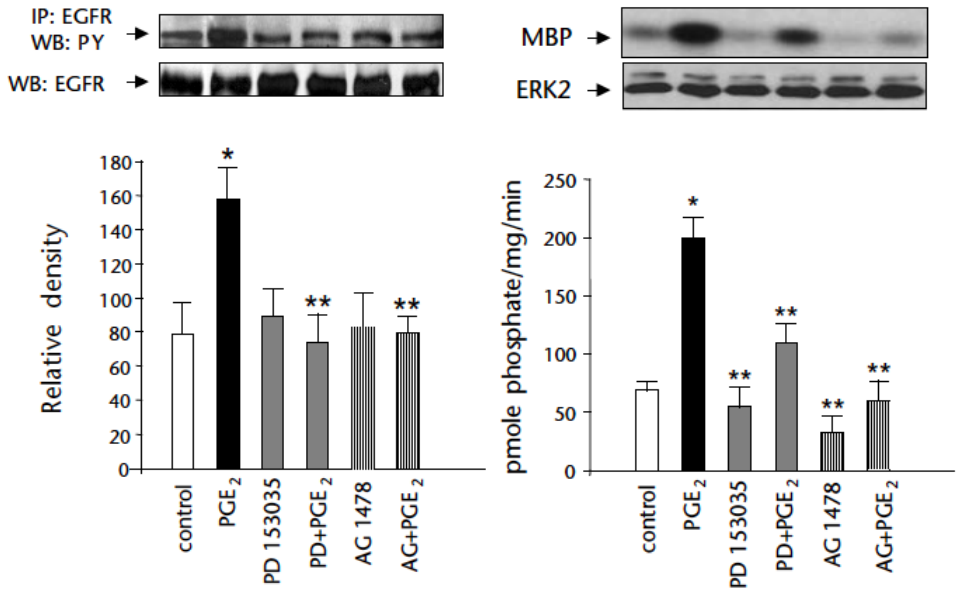


Figure 1: Effects of PGE_2 on the phosphorylation of EGFR (**left panel**). Cells were treated with PGE_2 or/and EGFR inhibitors (PD153035 and AG1478) as indicated below. The levels of EGFR and phosphorylated EGFR were analyzed by immunoprecipitation and Western blotting (top, **left panel**). The ratios between phosphorylated EGFR and EGFR were quantified and shown in a histogram (bottom, **left panel**). PGE_2 activates ERK2/MAPK activity (**right panel**). The ERK2 activity was analyzed by *in vitro* kinase assay following immunoprecipitation from cell lysates using MBP (myosin basic protein) as a substrate in the presence of ^{32}P-ATP (top, **right panel**). Phosphorylation of MBP and ERK2 under different experimental conditions was analyzed by autoradiogram and quantified (bottom, **right panel**). *, $p < 0.05$, versus control; **, $p < 0.001$, versus PGE_2. PY, phosphotyrosine

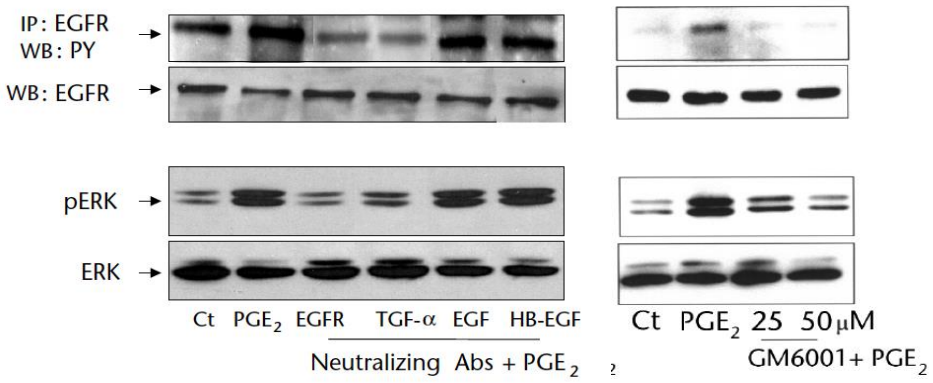


Figure 2: The effect of PGE_2 on the phosphorylation of EGFR (top) and ERK (bottom) in the presence of neutralizing antibodies (against EGFR, TGF-α, EGF and HB-EGF, respectively) (**left panel**) and metalloprotease inhibitor, GM6001 (**right panel**). Ct, control. TGF-α, EGF and HB-EGF are ligands for EGFR.

QUESTIONS:

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

2. Based on these results, propose a hypothesis that links PGE_2 to the phosphorylation of EGFR and ERK (include a diagram).

3. There are four EGF receptors, EGFR1-4. These EGFRs usually form heterodimers. What will be your experimental strategies to identify the critical EGFR for the PGE_2–mediated proliferation in Caco cells.

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.

Genetic studies have identified CaM missense mutations in humans with severe ventricular arrhythmia and sudden cardiac death susceptibility, albeit with distinct clinical presentations: A mutation in *CALM1* (N54I) was 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). **A fifth mutation (F90L) was found in a family with idiopathic ventricular fibrillation and mild QT prolongation.** CPVT is commonly caused by mutations in sarcoplasmic reticulum genes that increase diastolic Ca leakage through the ryanodine receptor (RyR2) channels. LQTS is usually caused by dysfunctional plasma membrane ion channels that prolong the ventricular action potential. The mechanism underlying idiopathic ventricular fibrillation (**IVF**) is not known.

Using recombinant mutant CaM protein, the following experiments were performed to elucidate how mutant CaM-F90L causes IVF:

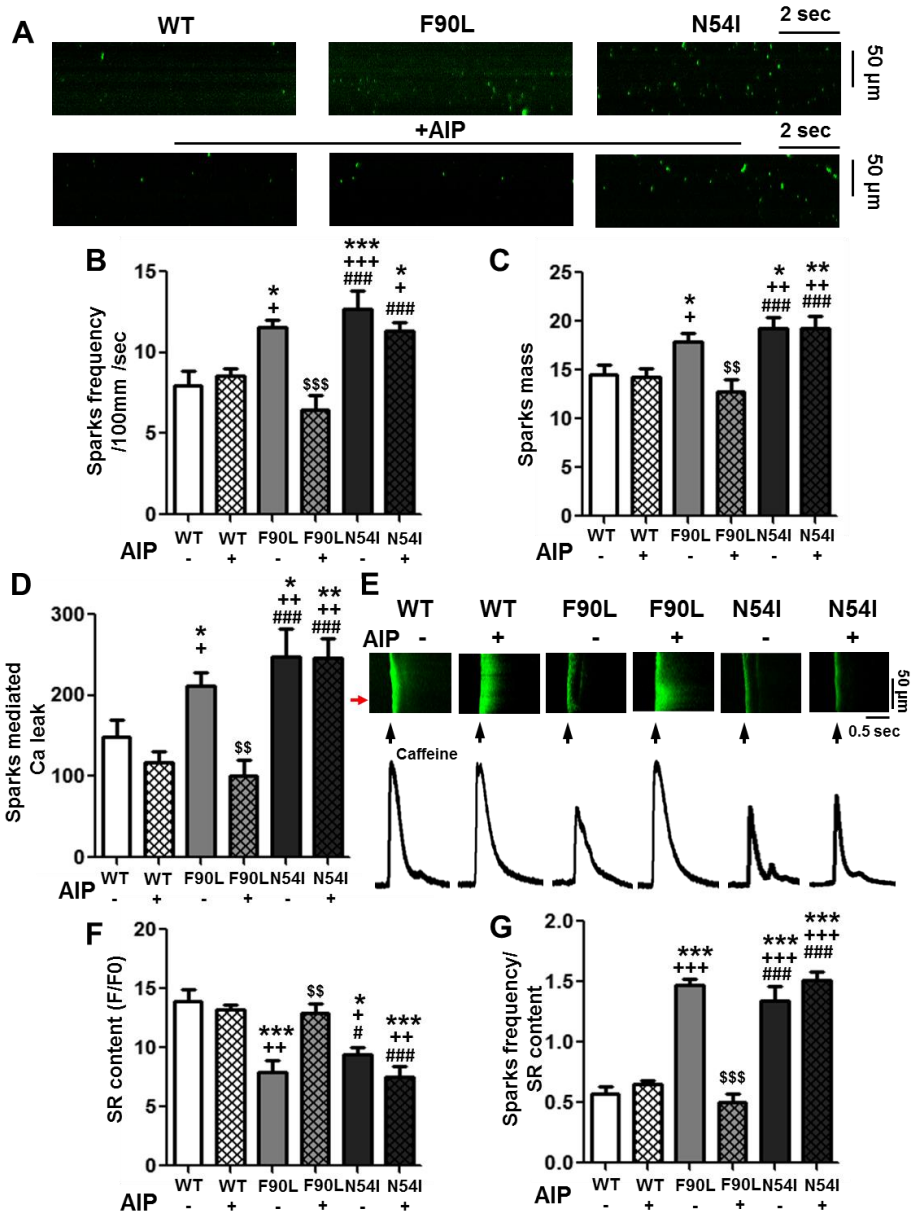


Fig. 1. Measurement of Ca sparks and SR Ca release. Representative confocal line scans (A,E) and average data (**B-D, F-G**) from permeabilized mouse ventricular myocytes after 30 min incubation with either WT or mutant CaMs (100nM, physiological free [CaM]). After permeabilization, myocytes were incubated in internal solution containing 50 nM free [Ca], and 25 µM Fluo. Bars represent mean+SE. WT values on each experimental day. WT (n=45), F90L (n=33), N54I (n=35). **P*<0.05, ***P*<0.01 vs WT CaM.

AIP – Auto-inhibitory peptide (1 µM), a selective inhibitor of Ca-Calmodulin kinase II (CaMKII).

QUESTIONS:

1. Describe the results of the Ca release measurements in Fig. 1. Formulate a hypothesis on how mutant CaM F90L regulate RyR2 channels and sarcoplasmic reticulum Ca release and design experiments that will test your hypothesis.

2. How is F90L different from N54I? What other targets in the cardiomyocytes could be affected?

$P2Y_{12}$ receptor ($P2Y_{12}$-R) signaling is mediated through Gi, ultimately reducing cellular cAMP levels. Because cAMP is a central modulator of arginine vasopressin (AVP)-induced water transport in the renal collecting duct (CD), you want to determine if $P2Y_{12}$-R may play a role in renal handling of water in health and in nephrogenic diabetes insipidus. The most common acquired nephrogenic diabetes insipidus is caused by chronic lithium (Li) administration for the treatment of bipolar disorder, which affects 2% of the general population of the United States. You perform the following experiment using clopidogrel, an irreversible inhibitor of $P2Y_{12}$-R:

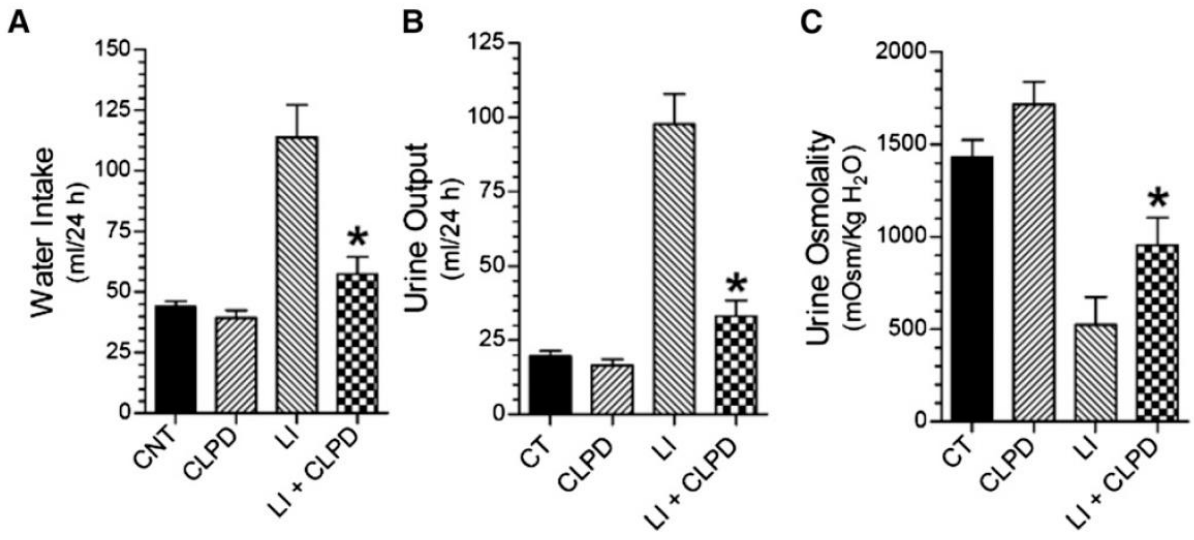


Figure: Effect of clopidogrel administration on Li-induced polyuria and decrease in AQP2 protein abundance in rats. (A) Water intake;(B) urine output; (C) urine osmolality.

Questions:

1) Describe the pathway of the regulation of water reabsorption in the collecting duct.

2) Describe the difference between nephrogenic and central diabetes insipidus.

3) Describe the data. Why does water intake increase concomitantly with urine output? What effect does clopidogrel (CLPD) have on lithium-induced changes in water intake and urine output?

4) Why would $P2Y_{12}$ receptor blockade blunt lithium induced nephrogenic diabetes insipidus based on how the $P2Y_{12}$ receptor signals and the pathway of water regulation in the collecting duct?

MAPKs are activated in response to G protein-coupled receptor (GPCR) stimulation and play essential roles in regulating cellular processes downstream of these receptors. However, very little is known about the reciprocal effect of MAPK activation on GPCRs. To investigate possible crosstalk between MAPK and GPCRs, a group of investigators performed the following experiments.

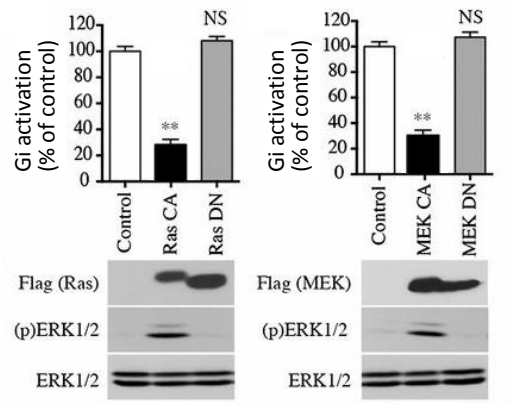


FIGURE 1. HEK293T cells stably expressing HA-tagged CXCR4 (HA-CXCR4; a Gi-coupled GPCR) were transfected with empty vector (*Control*), constitutively active Ras (*Ras CA*), dominant-negative Ras (*Ras DN*), constitutively active MEK (*MEK CA*), or dominant-negative MEK (*MEK DN*). Gi activation was measured 3 min after the addition of CXCL12 (the native agonist for CXCR4) and expressed as a % of the control condition, which was set at 100%. Expression of total ERK1/2, active ERK1/2 [(p)ERK1/2], and the CA and DN forms of Ras and MEK were assessed by Western analysis. **, $P < 0.01$; NS, not significant.

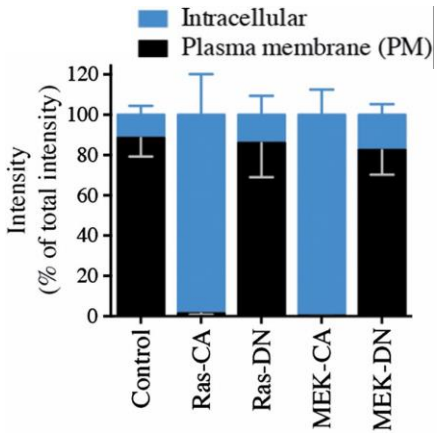


FIGURE 2. CXCR4-YFP localization was assessed by fluorescence confocal microscopy in unstimulated HeLa cells co-transfected with CXCR4-YFP and empty vector (*Control*) or the indicated Ras and MEK mutants. Intensity plots derived from 20 confocal fluorescence microscopy images obtained in three independent experiments were analyzed to quantify CXCR4 localization at the plasma membrane and inside the cell. Data shown represent the mean ± SEM of the 20 images and were normalized to the total fluorescence intensity (plasma membrane + intracellular compartment) set to 100% in each condition.

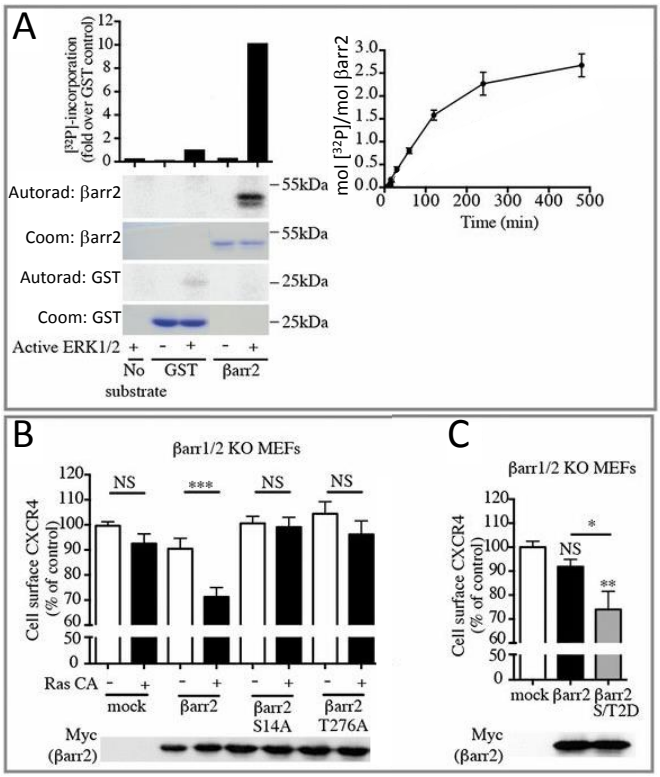


FIGURE 3. (***A***) Phosphorylation of βarr2 was performed with recombinant bovine βarr2 (or GST) as a substrate and recombinant active ERK1 in the presence of [γ^{32}P]-ATP for 15 min. The reaction mixtures were subjected to SDS-PAGE. The gel was stained with Coomassie (Coom) and analyzed by autoradiography (Autorad); ^{32}P incorporation was quantified using a PhosphorImager (*Left*) or during kinetics using scintillation counting (*Right*). The data shown are representative of three independent experiments. (***B*** and ***C***) CXCR4 cell-surface expression was assessed in unstimulated βarr1/2-KO MEFs transiently transfected with HA-CXCR4-YFP, βarr2 WT, or the indicated βarr2 mutants, with or without Ras CA. Data shown represent the mean ± SEM of at least three independent experiments and were normalized to the control condition (100%). Expression of βarr2 WT and mutants were assayed by immunoblotting. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.

QUESTIONS:

A) What major conclusion can be drawn from the data in Fig. 1? Fig. 2? Fig. 3?

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

C) Design a pharmacological experiment that would allow you to further test your hypothesis in non-transfected cells.