Department of Pharmacology

# Qualifying Examination (Part I)

DECEMBER 16, 2014

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

| Date | Time |
|---|---|
| Tuesday, December 16th | 11:00 am – 1:00 pm |
| | 2:00 pm – 4:00 pm |
| | 4:00 pm – 4:30 pm *(Committee Meets to determine results)* |
| | **4:30 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.

Variations in the *SLC6A4* gene encoding the 5-HT transporter (5-HTT) have been suggested to impose risk factors for emotional disturbances, and reduced levels of 5-HTT have been associated with increased stress- and anxiety-like behaviors in mice.

The investigators used mouse models with disrupted 5-HTT function to study responses to 5-HT in the prefrontal cortex and its underlying cellular regulation. The models include (1) 5-HTT knockout (KO) and hemyzygous (HET) mice; (2) group- and single-housed (a social isolation stress paradigm) mice; (3) mice treated during the first two weeks postnatal with the SSRI fluoxetine.

Whole cell electrophysiological recordings were performed in coronal slices of the prefrontal cortex of adult mice in response to administration of 5-HT.

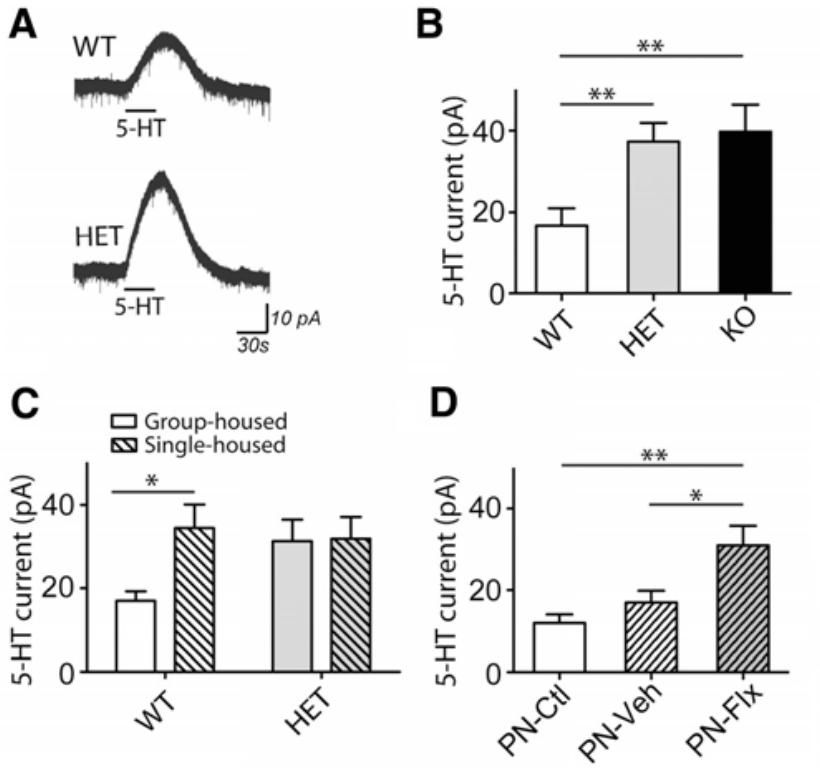



**Figure 1.** Averaged recordings (A) and quantification (B) of the outward currents elicited by 5-HT (10 µM) in neurons from 5-HTT WT, hemyzygous (HET), and 5-HTT knockout (KO) mice. (C) The 5-HT currents in neurons from group-housed WT or HET or single-housed mice. (D) Graph summarizing the 5-HT currents from adult mice treated chronically during the postnatal (PN) period with the SSRI fluoxetine (PN-Flx), vehicle (PN-Veh) or control mice (PN-Ctl).

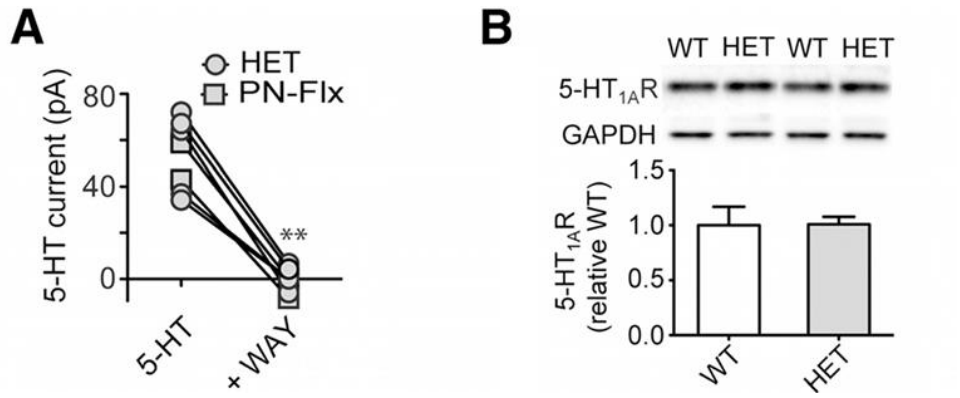



**Figure 2**. (**A**) Scatterplot of the 5-HT response before and after application of the selective 5-HT1A antagonist, WAY100635 (30 nM) in within-cell paired recordings from 5-HTT HET and PN-Flx neurons. (**B**) Representative Western blot (top) and quantification (bottom) showing prefrontal protein levels of 5-HT1A receptor in WT and HET mice.

In another series of experiments, the investigators examined the role of tyrosine phosphorylation in controlling the 5-HT signaling in the prefrontal cortex using pharmacological tools. The results are presented in Fig. 3.

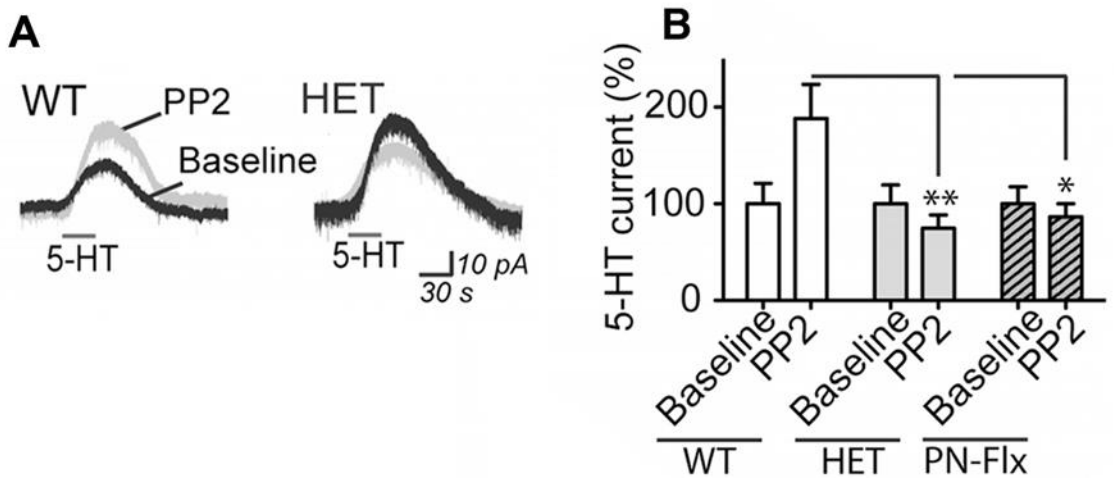



**Figure 3.** (A) The 5-HT responses from WT neurons in the absence or presence of Src family tyrosine kinase inhibitor PP2 (10 µM). (**C**) Quantification of the 5-HT1A responses in the absence or presence of PP2 in neurons from WT, HET, and PN-FLX mice.

**Questions:**

1. **Outline briefly the repertoire of 5-HT receptors that support 5-HT signaling in the brain. Where in the brain do serotonergic neurons reside that provide serotonergic input to the prefrontal cortex?**

2. **From the data in Fig. 1, what can you conclude regarding the alterations in the 5-HT current in the prefrontal cortex in mice with disrupted function of 5-HTT? Do the data in Fig. 2 suggest which 5-HT receptor plays the main role in these responses?**

3. **How do the data described in Fig. 3 focus your attention on specific signaling deficits in mice with disrupted function of 5-HTT? Propose a mechanism by which tyrosine phosphorylation could regulate 5-HT responses in the prefrontal cortex. Describe experiments that you could perform to support your model.**

A new CNS stimulant, Armodafil, was tested in 17 healthy volunteers for its effects on the disposition of midazolam in vivo. First, midazolam was administrated on separate days by oral (5 mg dose) and i.v. (2 mg dose) routes (control). Oral Armodafil was then administered daily for four weeks, during which time a single oral and i.v. administration of midazolam was repeated on separate days during week 3 and the plasma levels of midazolam measured.

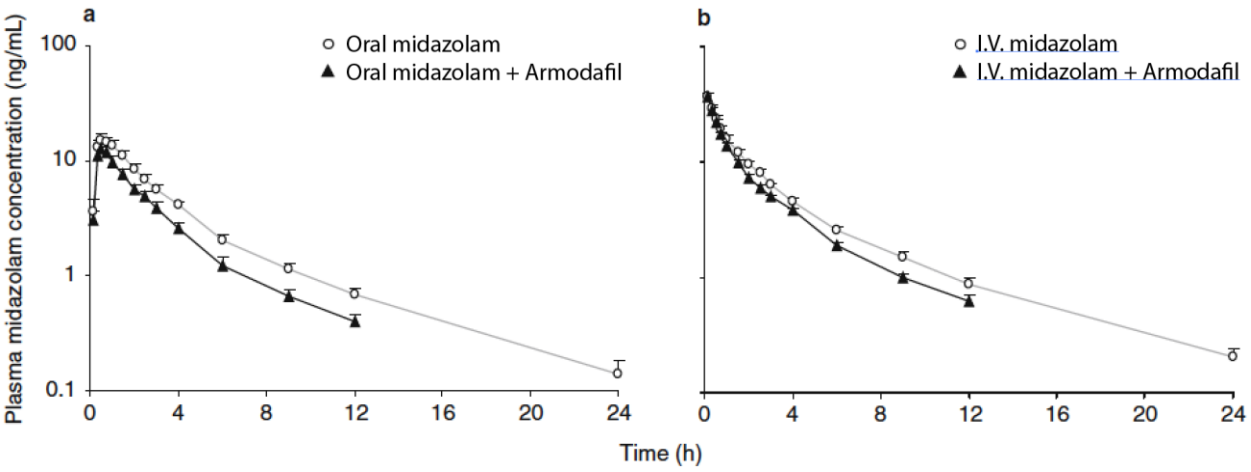



**Figure 1**: Comparison of the midazolam plasma levels, oral and i.v. with and without admonifil
Log-mean (+ SEM) plasma concentration-time curves for (**a**) oral midazolam and (**b**) intravenous midazolam administered alone and following administration of armodafil. **I.V.** = intravenous.

Table 1:
PK parameters of midazolam (oral 5mg, and IV 2mg) alone and during administration of Armodafil

| Parameter | Midazolam (n = 17)[a] | Midazolam + armodafil ratio (n = 17)[a,b] | p-Value |
|---|---|---|---|
| Intravenous | | | |
| AUC∞ (ng • h/mL) | 76.9 ±16.5 | 63.5 ± 12.0 | 0.0270 |
| Oral | | | |
| AUC∞ (ng • h/mL) | 53.8 ± 19.5 | 36.5 ± 16.9 | 0.0093 |
| Cmax (ng/mL) | 18.7 ± 6.3 | 15.2 ± 7.2 | 0.2161 |
| | | | |
| Intravenous | | | |
| $t_{1/2}$ (h) | 5.4 (2.0–7.3)[c] | 4.0 (2.6–6.3)[c] | 0.0128 |
| CL (mL/min) | 432.9 ± 92.1 | 522.2 ± 101.2 | <0.0001 |
| Vz (L) | 196.4 ± 71.9 | 196.8 ± 68.6 | 0.9691 |
| Oral | | | |
| $t_{1/2}$ (h) | 5.0 (2.1–8.6)[c] | 4.0 (1.5–7.7)[c] | 0.2199 |
| tmax (h) | 0.5 (0.3–2.5)[c] | 0.5 (0.3–2.5)[c] | 0.9070 |
| CL(oral) (mL/min) | 1554.6 ± 529.2 | 2283.0 ± 911.8 | <0.0001 |
| F (%) | 27.8 ± 6.4 | 22.9 ± 6.5 | <0.0001 |

[a] Values are expressed as mean ± SD unless specified otherwise.
[b] Following repeated administration of armodafil. [c] Median (range).
**AUC∞** = area under the plasma concentration-time curve from time zero to infinity; **CL** = clearance; **CL(oral)** = total oral clearance; **Cmax** = maximum observed plasma concentration; **F** = absolute bioavailable (relative to the pre-armodafinil intravenous dose); **t1/2** = elimination half-life; **tmax** = time to reach Cmax; **Vz** = volume of distribution during terminal phase after oral administration.

**Questions:**

1. **Briefly summarize and give your interpretation of the pharmacokinetic parameters in Figure 1 and Table 1**

2. **Present a hypothesis on the mechanism underlying the observed drug interaction and outline how you would investigate the mechanism(s) of the effects due to Armodafil.**

   **Bonus question: How would you distinguish between effects on enzyme expression and effects on enzyme activity?**

3. **If Armodafil was a Pgp inhibitor, illustrate how it would be expected to alter the plasma concentration/time curves of a drug that is a good Pgp substrate). (For the purposes of this question you can use the control curves in Figure 1 as the control profiles of the Pgp substrate drug).**

On a low iodine diet, the administration of methimazol (a thyrosine peroxidase blocker) and perchlorate (a Na/iodine symporter blocker) depletes the thyroid of thyroid hormones (TH) and leads to serum T4 levels < 0.2ug/dl (normal: 4ug/dl) and serum T3 levels < 60 ng/dl (normal: 100ng/dl) in WT mice (**Fig. 1A, B**).

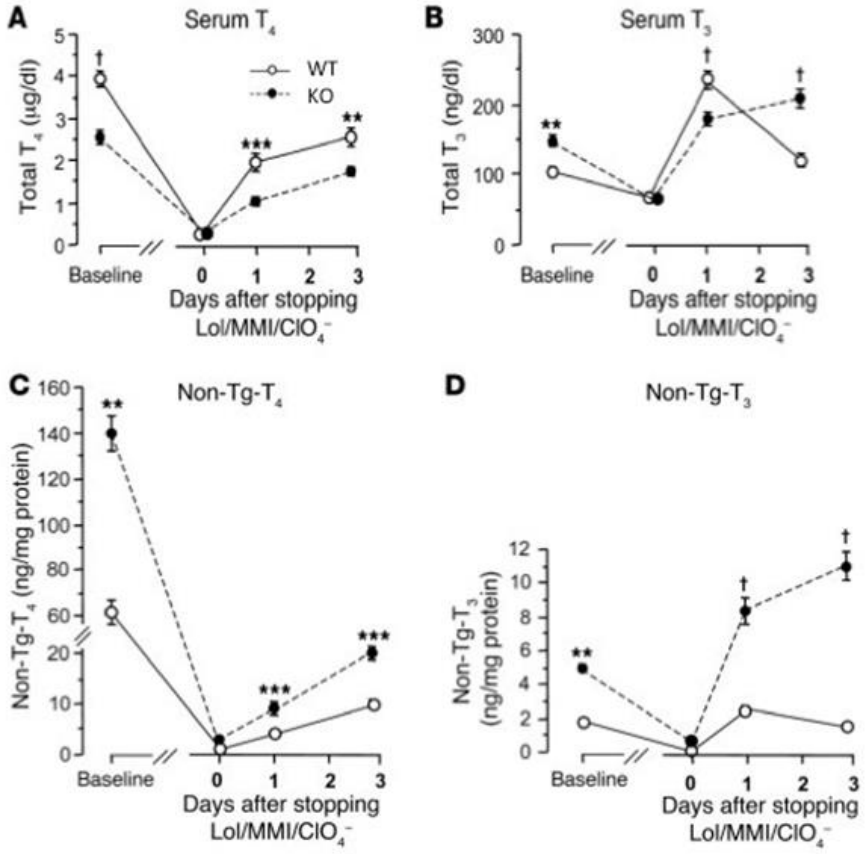



**Figure 1.** Dynamics of rebound of TH synthesis and secretion after chemical suppression was stopped. Shown are serum total T4 (**A**) and total T3 (**B**) concentrations and thyroidal non-thyroglobulin-T4 (**C**) and non-thyroglobulin-T3 (**D**) content (T4 and T3 in the thyroid gland not within the thyroglobulin (Tg) molecule) at baseline and at 0, 1, and 3 days after withdrawal of LoI/MMI/ClO4. ***P* < 0.01, ****P* < 0.001, †*P* < 0.0001.

**Questions:**

1. **Focusing on WT mice only, how do you expect TSH serum levels to change in response to these alterations in T3/T4 levels?**

2. **At day 0, methimazol and perchlorate are withdrawn in WT mice and in a line of mutant mice (KO) generated by random mutagenesis (i.e. the gene mutated is unknown to you). How can you explain the differential response of WT and KO mice with regard to serum T3 and T4 (Fig. 1A, B) and non-thyroglobulin (Tg) T4 and T3 (Fig. 1C, D) a day later?**

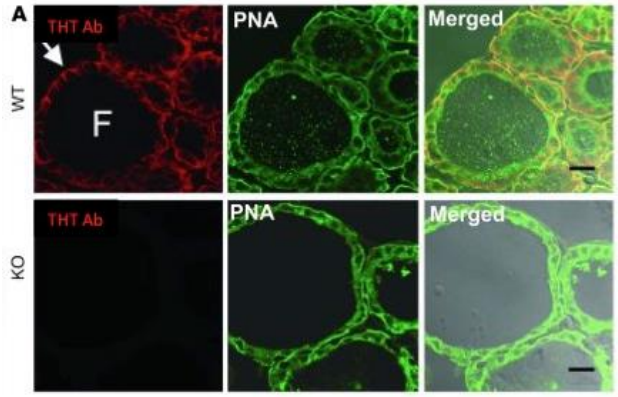



Figure 2: (**A**) Immunoconfocal images from cryosections of thyroid glands prepared from WT and KO mice colabeled with anti-THT antibody (red) and PNA lectin (green). Merged images are shown overlaid on the differential interference contrast image. PNA lectin labeled the thyrocyte plasma membranes. F, follicle. Scale bars: 20 µm.

3. **Describe the thyroid histology in Fig. 2 and discuss what this immunolocalization by fluorescence analysis brings to the understanding of the mutated gene's function in the thyroid.**

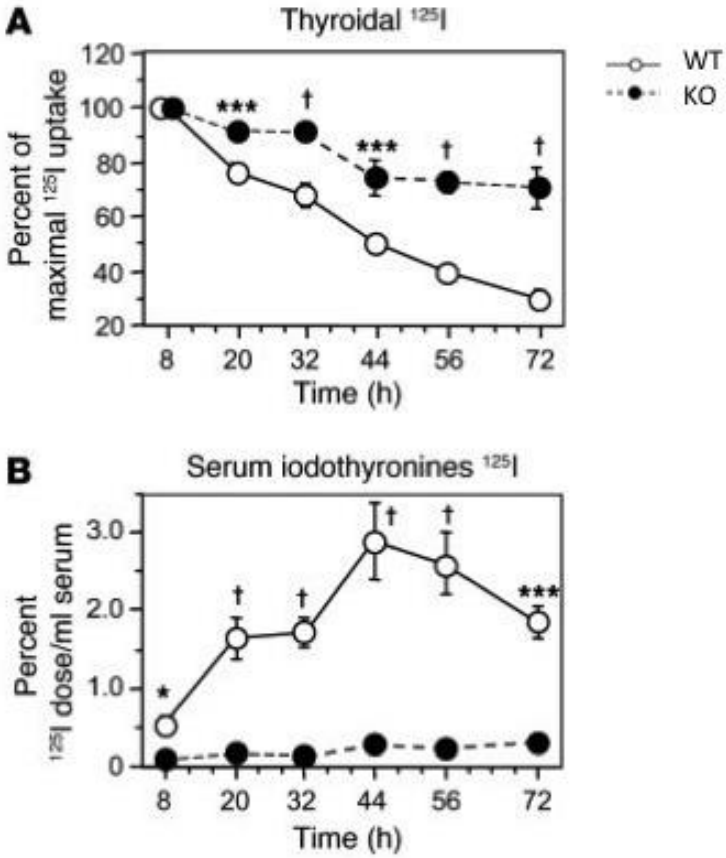



Figure 3: Kinetic TH secretion from the thyroid gland of WT and KO mice. Adult animals were given $^{125}$I, and, at indicated time intervals, the radioactivity was determined in their thyroid glands by counting in vivo and in serum samples. Results of thyroidal radioactivity are expressed as percent of the maximal $^{125}$I uptake, being 100% at 8 hours (**A**). **P* < 0.05, ****P* < 0.001, †*P* < 0.0001.

4. **Eight adult KO mice and 8 WT littermates were fed low-iodine diet for 2 weeks. After the administration of 5 µCi $^{125}$I, the amount of radioactivity in thyroid glands and in serum were determined at different time intervals over the period of 72 hours by counting radioactivity in thyroid and serum samples (Fig. 3A, B). What do the results in Fig. 3 indicate? Based on these independent experiments, what type of protein do you think this gene encodes?**

The TAM family belongs to the RPTK superfamily that has been implicated in diverse human cancers. The TAM family contains three structurally related members, Tyro3, Axl and Mer, and all three members respond to Gas6, a peptide hormone. To explore role of the TAM members in cell proliferation, you have established a cell culture model in Rat2 cells. Rat2 endogenously expresses Axl (Fig 1). To explore how the activity of Axl may be affected by other TAM family members, you transfected the cDNA for Tyro3 into Rat2 cells and isolated two stable clones (Rat2/T3, cl 25 and Rat2/T3, cl 30). In these two clones of the Rat2/T3 cells you observed an increased proliferation when cells were treated with Gas6, compared to Gas6 treated Rat2 cells. Moreover, the increased proliferation of Rat2/T3 is blocked by PD98059, a MAPK kinase inhibitor. To explore the mechanism of increased proliferation in Rat2/T3 cells, the following experiments were carried out and results are shown in Fig 1.

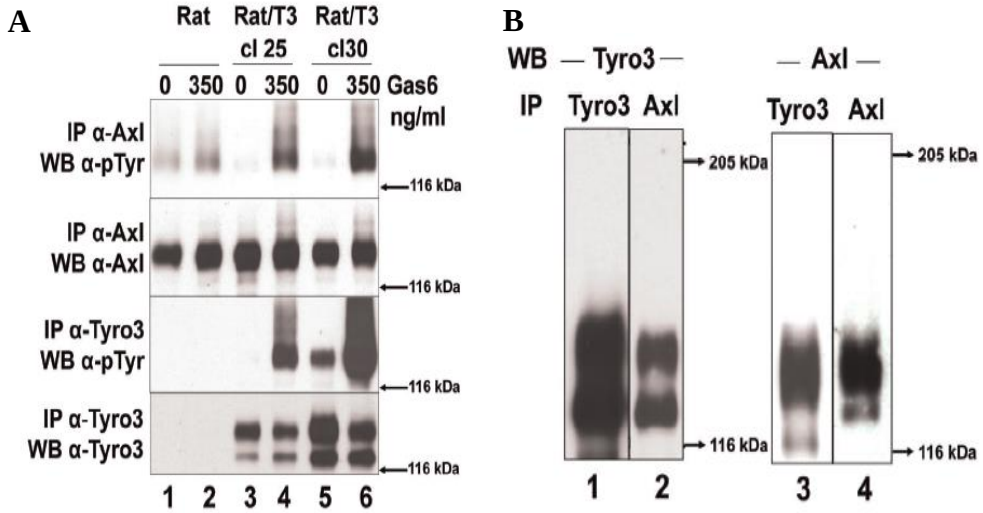



**Fig 1.** Cell lysates of Rat2 or Rat2/T3 that were treated for 10 min with either media (0) or Gas6 (350 ng/ml) were prepared. The lysates were used for immunoprecipitation (IP), and immunoprecipitates were analyzed by Western blotting (WB) using antibodies as indicated. (B) The clone 30 of the Rat2/T3 cells, which were treated with Gas6 (350 ng/ml) for 10 min was used in for IP.

## Questions:

1. **Explain the experiments and results, and provide the explanation for the findings in Fig 1.**

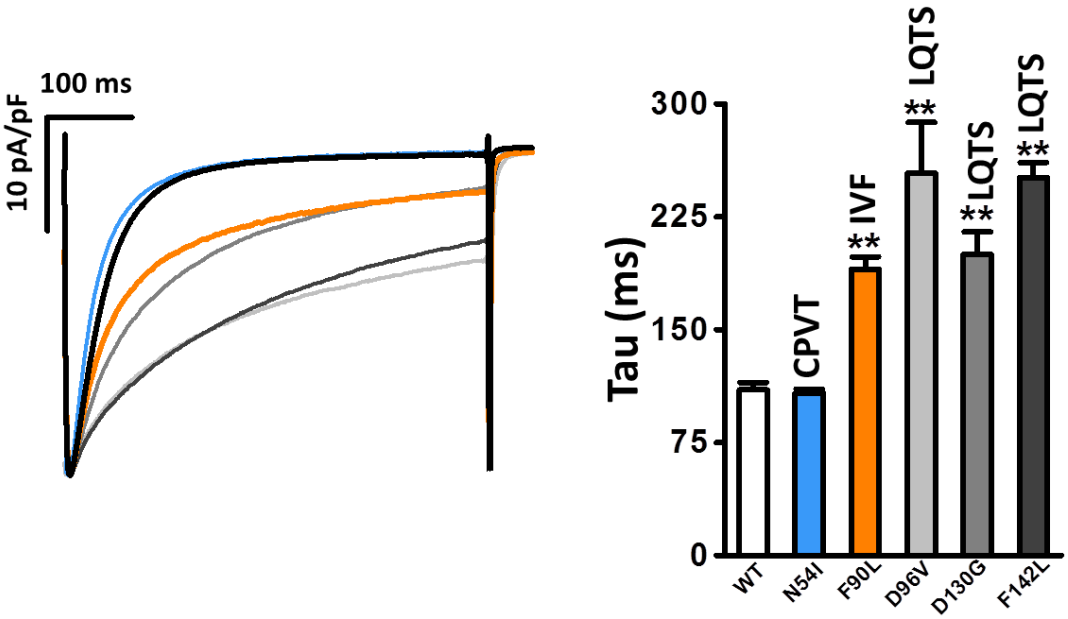
2. **Which signaling pathway do you think is critical for the proliferation phenotype of Rat2/T3 cells?**

3. **Design at least two independent experiments to investigate the involvement of your proposed pathway in the proliferation of Rat2/T3 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 mutations 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.

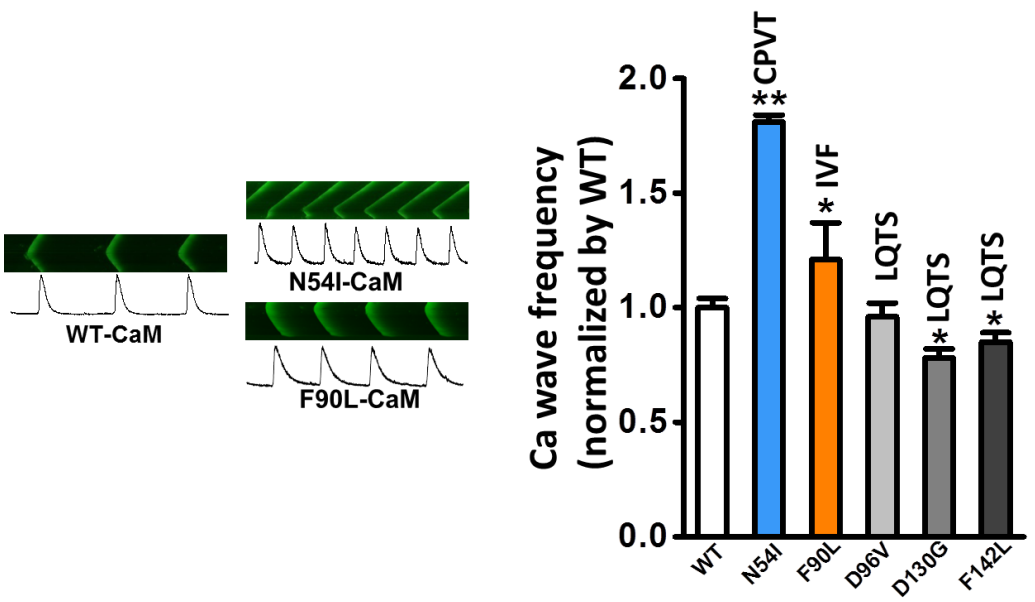
Using recombinant mutant CaM protein, the following experiments were performed to elucidate how mutant CaMs cause LQTS, CPVT and IVF.




(**A**) **Measurement of L-type Ca currents**. Representative Ca current records (top) and average data (bottom) obtained from voltage-clamped mouse ventricular myocytes dialyzed with internal solutions containing either WT or mutant CaM (6 μM). Myocytes were pre-incubated with ryanodine (50 μM) and thapsigargain (1μM ) to prevent SR Ca release. Ca currents were elicited by a voltage step to 0 mV from a holding potential of -70 mV. Bars represent mean+SE of the time-constant (tau) of Ca current inactivation. Peak Ca current amplitude was not different between the groups (data not shown). WT (n=9), D96V (n=8), D130G (n=7), F142L (n=20), N54I (n=7), F90L (n=10), **P<0.01 vs WT

| CaM | $K_d$ (μM) | vs. WT | Disease |
|---|---|---|---|
| WT | 2.8±0.1 | 0 | |
| D130G | 150± 30 | ↓54-fold | LQT |
| D96V | 38± 6 | ↓14-fold | LQT |
| F142L | 15± 0.5 | ↓5-fold | LQT |
| **F90L** | **13±0.2** | **↓4-fold** | **IVF** |
| **N54I** | **3 ±0.2** | **0** | **CPVT** |

**(B) Measurement of Ca binding affinity of mutant CaMs in vitro.** Listed are the Kd values for CaM C-lobe Ca binding (high affinity site). The low affinity N-lobe Ca binding was not affected in any of the mutants tested.




**(C) Measurement of SR Ca release.** Representative confocal line scans (top) and average data (bottom) 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], 100 μM EGTA, and 25 μM Fluo. Bars represent mean+SE of values normalized by WT values on each experimental day. WT (n=45), F90L (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.

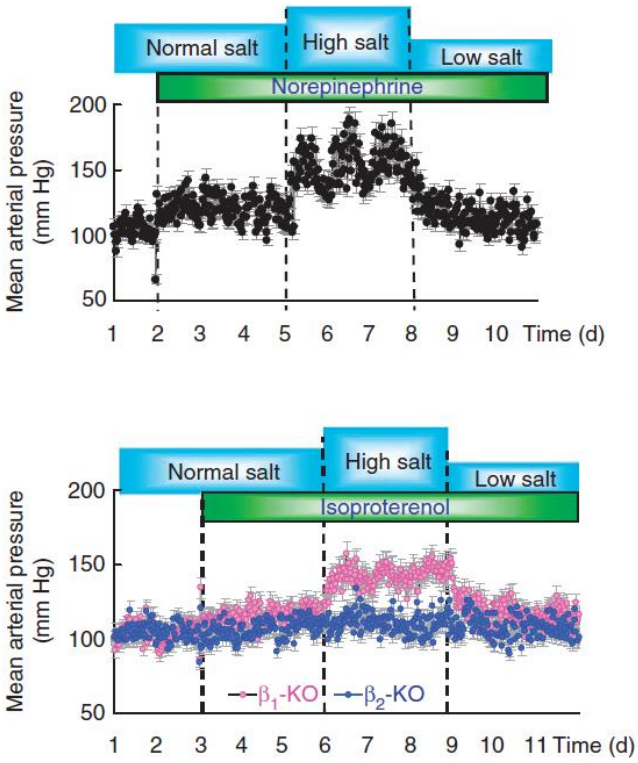
**Questions**

1. **Describe the results of the voltage-clamp (A) and Ca binding studies (B). Formulate a hypothesis on how mutant CaMs cause LQTS and design experiments that will test you hypothesis.**

2. **Describe the results of the Ca release measurements in (C). Formulate a hypothesis on how mutant CaMs regulate RyR2 channels and sarcoplasmic reticulum Ca release and design experiments that will test your hypothesis.**

3. **Based on the differences between the F90L CaM mutations and the other CaM mutants, speculate on the cause of idiopathic VF.**

You are the world-expert (at least you think you are) studying NCC, the Na-Cl cotransporter expressed in the distal convoluted tubule of the kidney. You come across the following data reported in a high-impact publication and wonder if NCC could be contributing.




Top: recordings of mean arterial pressure in NE infused WT mice on normal- or high-salt diet for 3d. Bottom: effects of salt loading on mean arterial pressure in isoproterenol-infused beta-adrenergic receptor KO mice.

**Questions:**

1) **Describe the results and develop a hypothesis.**

2) **How could NCC be involved in the results in the figure?**

3) **How would you test the potential involvement of NCC in this process?**

Appetite-controlling peptides, glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) are secreted from open-type neuroendocrine epithelial cells (L-cells) located in the distal ileum and colon that come in direct contact with gut nutrients. TGR5 is a GPCR that is activated by oleanolic acid (OA) and is expressed on entero-endocrine cells in the gut. Using cultured entero-endocrine cell line, the investigators are trying to elucidate the signaling pathway between TGR5 and PYY/GLP-1 secretion.

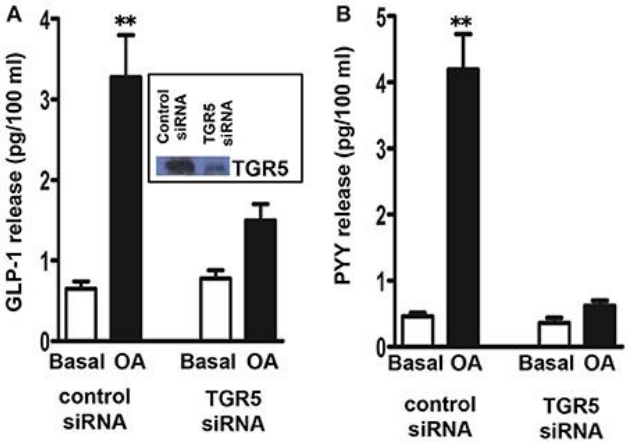



**Fig. 1.** Cells transfected with control siRNA or TGR5-specific siRNA for 48 h and then treated with OA (10 μM) for 30 min. Release of GLP-1 **(A)** and PYY **(B)** into the medium was measured by ELISA. *Inset:* Down regulation of TGR5 expression in cells transfected with TGR5 siRNA was determined by western blot. Results are expressed as pg/100 ml. Values are mean ± s.e.m. of 5 experiments. $^{**}p < 0.001$ vs. basal.

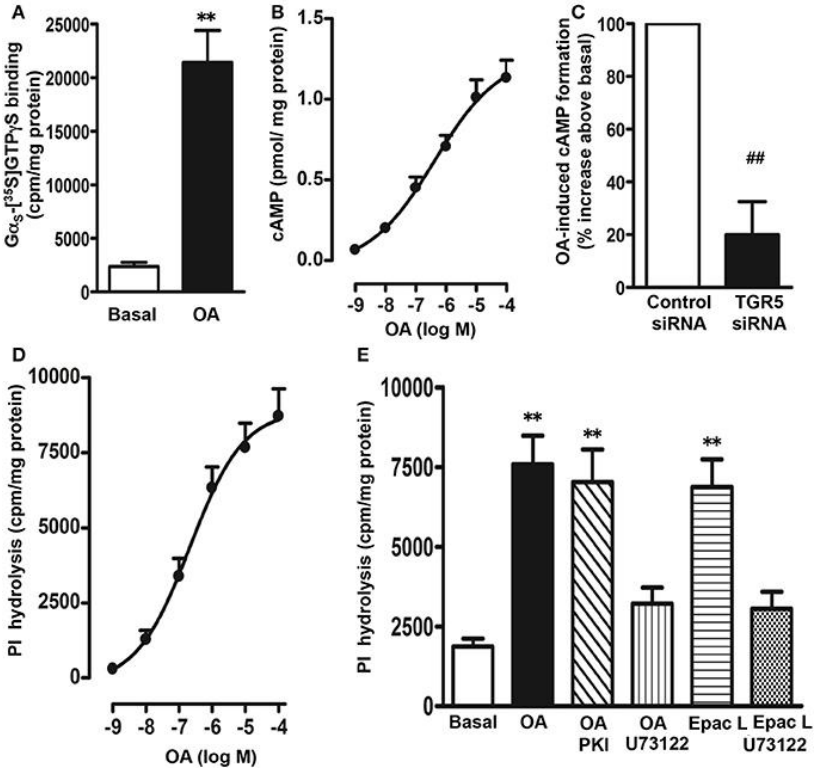



**Fig. 2. Signaling pathways activated by OA**. **(A)** Membranes were incubated with [$^{35}$S]GTPγS in the presence or absence of OA (10 μM) for 20 min. Aliquots were added to wells pre-coated with antibodies to $G\alpha_s$, $G\alpha_q$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ for 2 h and the bound radioactivity was measured. A significant increase in the binding of [$^{35}$S]GTPγS-Gα complexes was obtained to wells coated with $G\alpha_s$ antibody only. $^{**}p < 0.001$ vs. basal. **(B)** Cells were treated with different concentrations of OA for 5 min in the presence of phosphodiesterase inhibitor IBMX (10 μM) and cAMP formation was measured by radioimmunoassay. Results are expressed as pmol/mg protein above basal levels (0.054 ± 0.008 pmol/mg protein). **(C)** Cells transfected with control siRNA or TGR5-specific siRNA were treated with OA (10 μM) for 5 min and cAMP formation was measured by radioimmunoassay. Values are mean ± SEM of 3 experiments. $^{\#\#}p < 0.001$ significant inhibition of cAMP response compared to cells transfected with control siRNA. **(D,E)** Cells were incubated with the cAMP analog that selectively activates non-PKA cAMP signal transducer protein Epac (Epac L; 8-pCPT-2′-*O*-Me-cAMP) (10 μM) or OA (10 μM) in the presence or absence of PLC inhibitor (U73122, 10 μM) or PKA inhibitor (myristoylated PKI, 1 μM). $^{**}p < 0.001$ vs. basal.

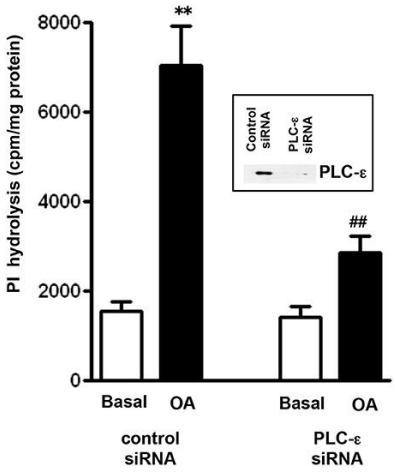



**Fig. 3.** Among other pathways, Epac is thought to activate PLC. Cells transfected with PLC-ε specific siRNA or control siRNA were labeled with myo-[$^3$H]inositol. Cells were treated with OA (10 μM) for 60 s and PI hydrolysis was measured. Results are expressed as cpm/mg protein. Values are mean ± s.e.m. of 4 experiments. **$p < 0.001$ vs. Basal; $^{\#\#}p < 0.001$ significant inhibition of PI hydrolysis compared to response in cells transfected with control siRNA. *Inset*: Expression of PLC-ε in control cells and in cell transfected with PLC-ε siRNA.

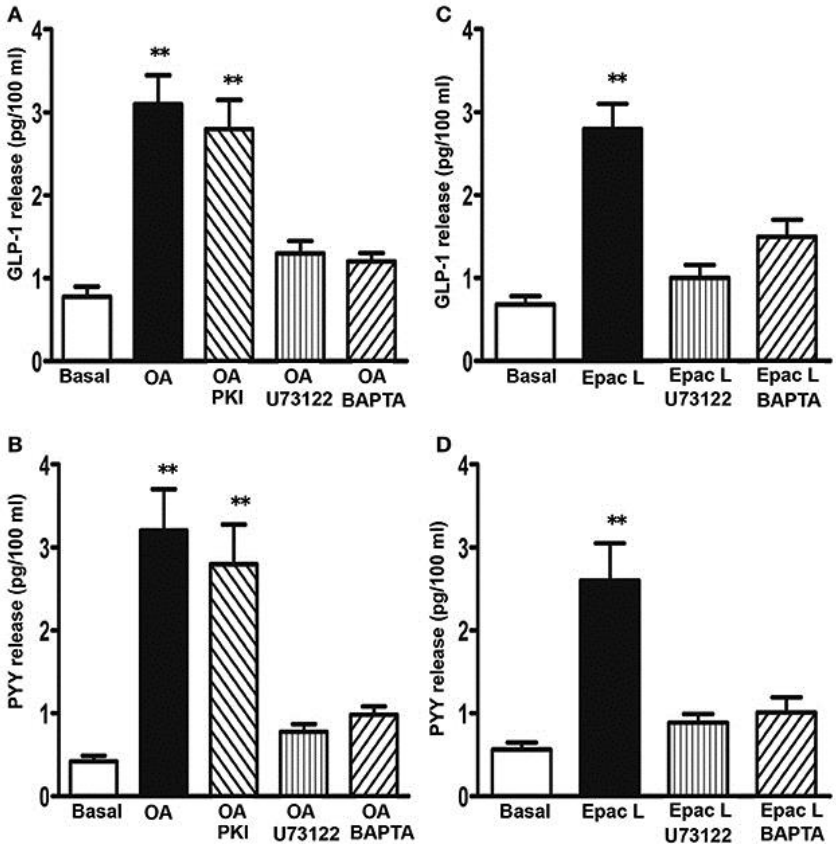



**Fig. 4. Signaling pathways involved in the release of GLP-1 and PYY by OA**. Cells were treated with OA (10 μM) **(A,B)** or 8-pCPT-2′-*O*-Me-cAMP (Epac L, 10 μM) **(C,D)** for 30 min in the presence or absence of PLC inhibitor (U73122, 10 μM), PKA inhibitor (myristoylated PKI, 1 μM), or $Ca^{2+}$ chelator (BAPTA-AM, 10 μM). Release of GLP-1 and PYY into the medium was measured by ELISA and the results are expressed as pg/100 ml. Values are mean ± s.e.m. of 6 experiments. **$p <$ 0.001 vs. basal.

## Questions:

1. **List all components of the signaling pathway between OA and PYY/GLP-1 release. Illustrate proposed signaling pathway by a cartoon.**

2. **Propose experiments to test the involvement of steps in the pathway leading to PYY/GLP-1 secretion that were not tested here.**

3. **How does PLC activity lead to IP3 production? What other signaling molecules are generated? What is their function in the cell and how can you test whether they are necessary for PYY/GLP-1 secretion?**