Department of Pharmacology

Qualifying Examination (Part I)

December 20, 2012

Examination Committee

1. Rich Breyer
2. Chang Chung
3. Al George
4. Ben Spiller

Exam Schedule (all to take place in the Bass Conference Room):

Thursday, December 20, 2012	9:15 am – 11:15 am	EXAM – TK Feaster
	11:15 am – 11:30 am	Qualifying Examination Committee Meeting
	11:30 am	Results to Student

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.

If you have any questions regarding the examination, please contact Seva Gurevich at:

615-322-7070 (w)
615-668-4849 (c)

The molecular mechanisms by which amphetamine (AMPH) elevates the concentration of dopamine (DA) in the brain was examined using striatal slices derived from wild type (WT) mice and mice lacking DA transporter (DAT). Slices were stimulated in the absence and presence of AMPH and DA concentration was measured by amperometry. The results are shown in **Figure 1** below.

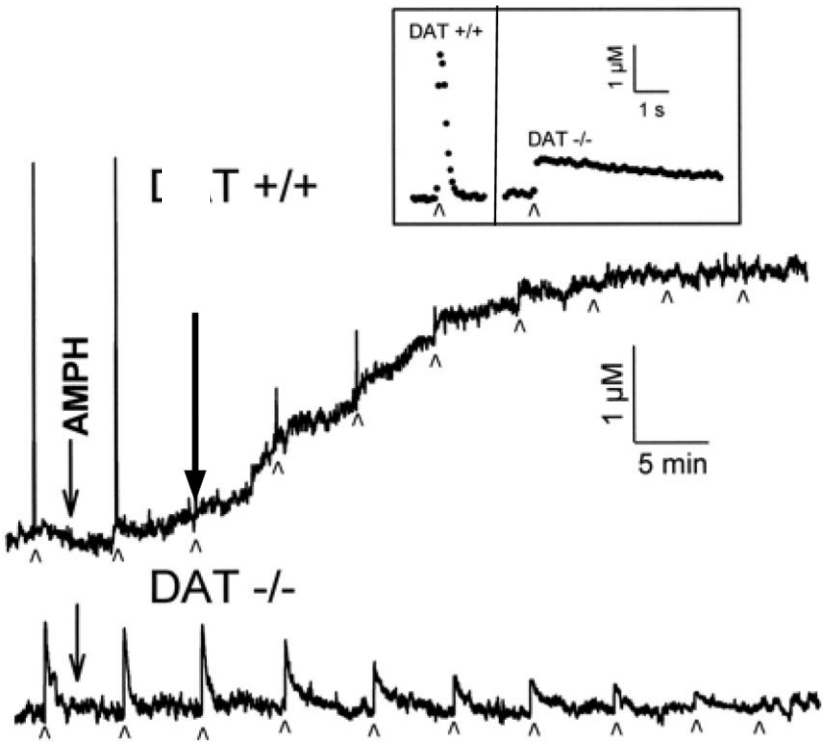


Figure 1. The amperomentric traces showing release of DA from striatal slices of WT (DAT +/+) and DAT knockout (DAT -/-) mice upon electrical stimulation (**^**). Downward arrow indicates the point of AMPH application. The insert box shows a more detailed time-course of DA release following electrical stimulation (^) in slices from WT and DAT knockout mice.

1. **Describe the salient features of the amperometric traces seen after electrical stimulation in slices from WT or DAT knockout (KO) mice, specifically with respect to amplitude and kinetics of the evoked DA response (insert).**

2. **Propose a mechanism that explains the differential response of WT and DAT KO brain slices to amphetamine application.**

3. **Explain the changes in the electrically-evoked DA release following AMPH application. Note that these changes do not occur in WT mice in the absence of DA (data not shown).**

The access of drugs to the brain may be restricted by the blood brain barrier (BBB) and to the CSF by the blood-cerebrospinal fluid barrier (BCSFB). Pgp and another transporter, breast cancer resistance protein (Bcrp), are both expressed on the luminal membranes of the vascular endothelial cells in the brain and choroid plexus.

The roles of Pgp and Bcrp in limiting access to the brain and CSF were investigated in mice using selective substrates, selective and dual transport inhibitors, and knockout animals for each transporter and a double knockout. After intravenous drug administration, animals were sacrificed at different time points for assay of drug levels in blood, CSF, and brain tissue.

1. **The results using the drug dantrolene are shown in Figure 1 and an analysis of the data in Table 1.**
   Table 2 summarizes the results in which the animals were dosed with selective transport inhibitors (Ko143 for Brcp, PSC833 for Pgp), or the dual inhibitor (GF120918) prior to the administration of dantrolene.
   Interpret these data.

2. **A similar protocol was followed using the drug digoxin (Figure 2, Table 3).**
   Interpret these data.

3. **Outline how you would carry out <u>in vitro</u> experiments to analyze the selectivity of dantrolene and digoxin as Pgp and Brcp substrates, and the predicted results.**

4. **A secondary goal of this study was to evaluate whether cerebrospinal fluid (CSF) can be used as a surrogate to assess brain exposures of Bcrp and P-glycoprotein (Pgp) substrates. Interpret the results in terms of this objective.**

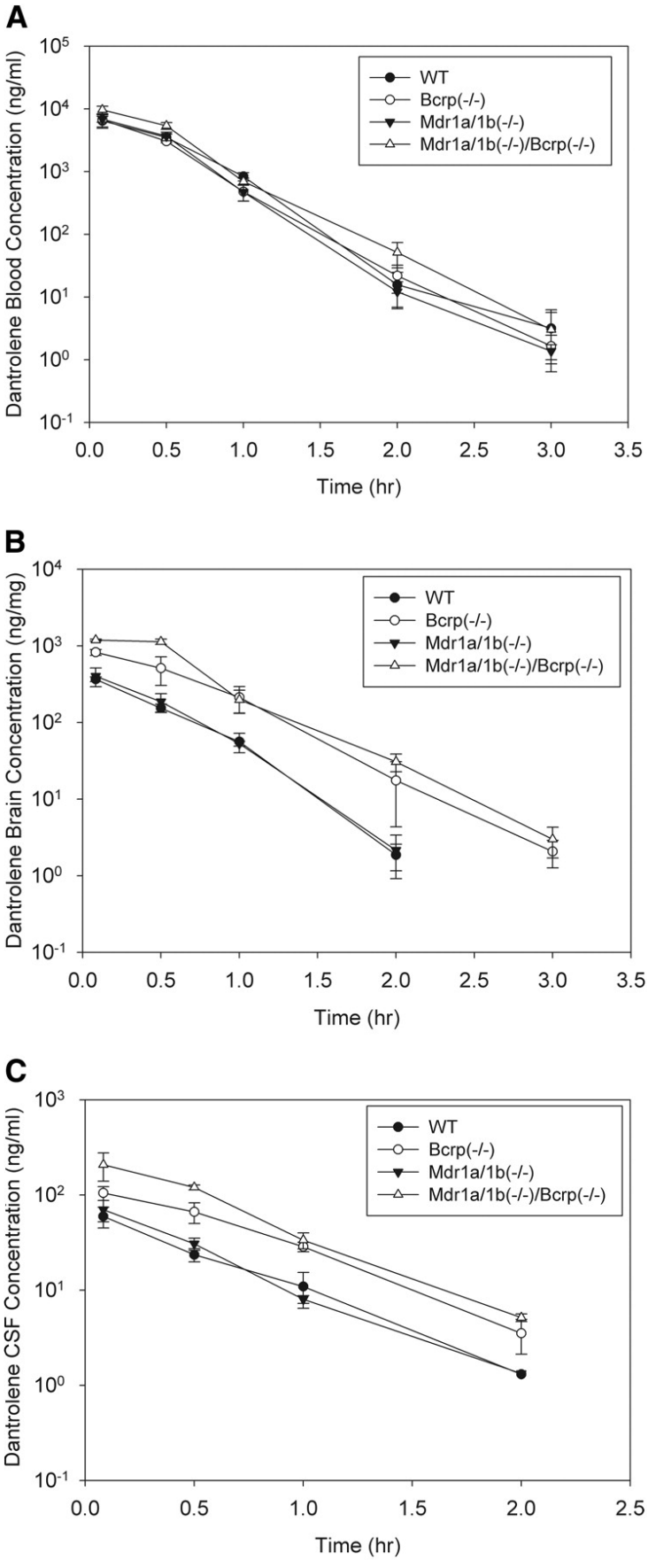


FIG. 1. Dantrolene concentration time profiles of blood (A), brain (B), and CSF (C) in wild-type FVB mice, Bcrp(_/_) knockout mice, Mdr1a/1b(_/_) knockout mice, and Mdr1a/1b(_/_)/Bcrp(_/_) knockout mice after intravenous dosing of dantrolene (2 mg/kg).

Closed circles, concentrations in wild-type FVB mice; open circles, concentrations in Bcrp(_/_) knockout mice; closed triangles, concentrations in Mdr1a/1b(_/_) knockout mice; open triangles, concentrations in Mdr1a/1b(_/_)/Bcrp(_/_) knockout mice.

Data are presented as mean +/- S.D.

[Note: Mdr1a/1b is the Pgp gene in mice]

TABLE 1

Dantrolene AUC changes in wild-type and knockout mice

Dantrolene blood, brain, and CSF AUC in wild-type and Mdr1a/1b and Bcrp knockout mice were determined, and AUC ratios were calculated.

AUC Ratio	Bcrp(−/−)	Mdr1a/1b(−/−)	Mdr1a/1b(−/−)/Bcrp(−/−)
AUC_{blood}, KO/WT	0.91	0.98	1.42
AUC_{brain}, KO/WT	2.94	1.10	4.67
AUC_{CSF}, KO/WT	2.25	1.10	3.81
$AUC_{brain/blood}$, KO/WT	3.24	1.13	3.29
$AUC_{CSF/blood}$, KO/WT	2.48	1.13	2.68
$AUC_{CSF/brain}$, KO/WT	0.77	1.00	0.82

KO, knockout; WT, wild type.

TABLE 2

Dantrolene AUC changes in wild-type mice predosed intravenously with vehicles or inhibitors

Dantrolene blood, brain, and CSF AUC in wild-type mice in the absence and presence of the inhibitors were determined, and AUC ratios were calculated.

AUC Ratio	Ko143	PSC833	GF120918
AUC_{blood}, I/C	1.01	0.84	0.96
AUC_{brain}, I/C	2.19	1.06	2.07
AUC_{CSF}, I/C	2.37	1.15	2.56
$AUC_{brain/blood}$, I/C	2.17	1.26	2.12
$AUC_{CSF/blood}$, I/C	2.35	1.36	2.62
$AUC_{CSF/brain}$, I/C	1.08	1.08	1.24

I, inhibitor; C, control.

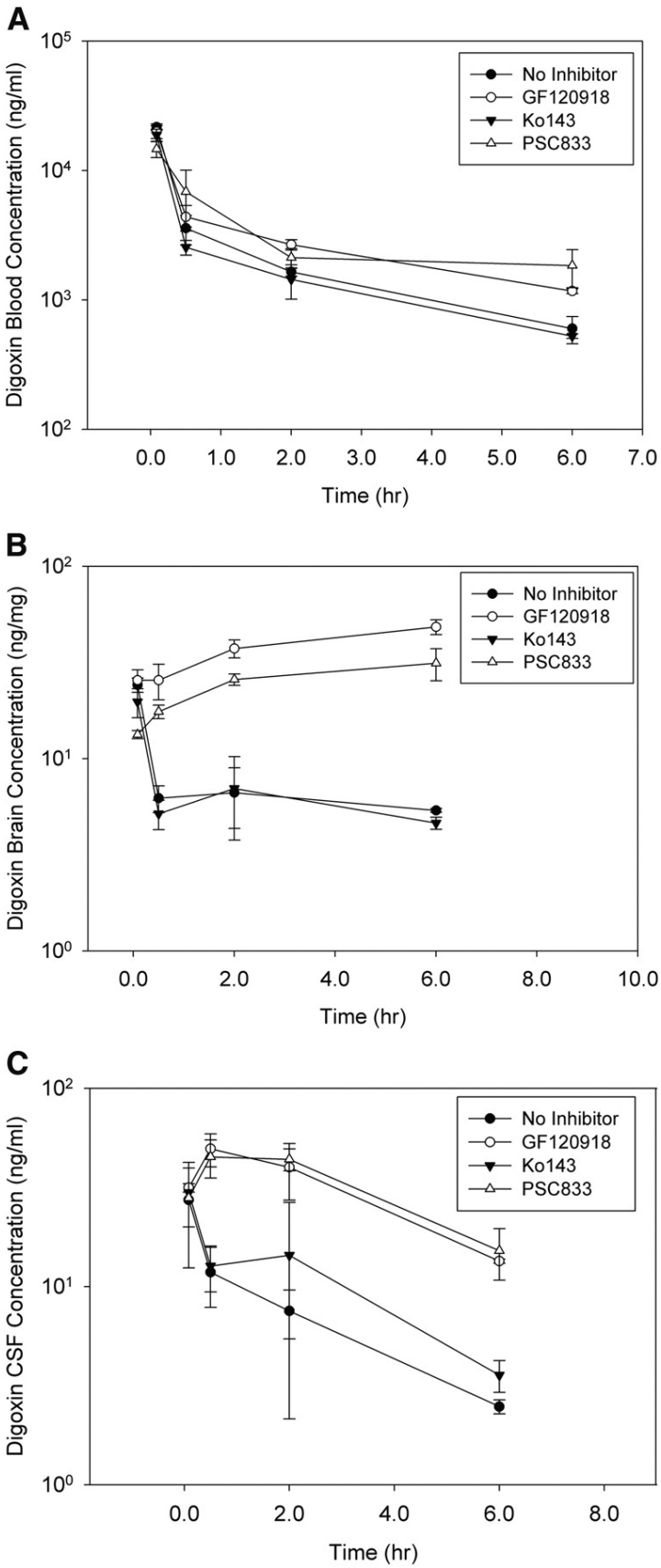


FIG. 2. Digoxin concentration time profiles of blood (A), brain (B), and CSF (C) in wild-type FVB mice after intravenous dosing of digoxin (1 mg/kg).

Closed circles, concentrations in mice that were predosed intravenously with vehicle; open circles, concentrations in mice that were predosed intravenously with GF120918 (10 mg/kg); closed triangles, concentrations in mice that were predosed intravenously with Ko143 (10 mg/kg); open triangles, concentrations in mice that were predosed intravenously with PSC833 (10 mg/kg).

Data are presented as mean +/- S.D.

TABLE 3

Digoxin AUC changes in wild-type mice predosed intravenously with vehicles or inhibitors

Digoxin blood, brain, and CSF AUC in wild-type mice in the absence and presence of the inhibitors were determined, and AUC ratios were calculated.

AUC Ratio	Ko143	PSC833	GF120918
AUC_{blood}, I/C	0.85	1.95	1.82
AUC_{brain}, I/C	0.82	4.65	9.76
AUC_{CSF}, I/C	1.48	5.07	6.56
$AUC_{brain/blood}$, I/C	0.96	2.39	5.35
$AUC_{CSF/blood}$, I/C	1.73	2.60	3.60
$AUC_{CSF/brain}$, I/C	1.80	1.09	0.67

I, inhibitor; C, control.

You have recently identified a strain of mutant mice that demonstrates sluggishness, bradycardia, fatigue, myxedema and cognitive impairment. You hypothesize that these animals have deficits in the hypothalamic-pituitary-thyroid axis and you subsequently quantify hormone levels to obtain the results presented in Table 1:

Mouse #	**TRH (nM)**	**TSH (nM)**	**T_4 (nM)**
1	17.6	19.6	277
2	12.9	23.4	198
3	14.2	22.1	236
Normal Range	3.0-5.5	7.3-12.1	70-160

Table 1. Serum concentrations of hormones involved in thyroid endocrine status in mutant mice.

1. **Describe the hypothalamic-pituitary-thyroid axis and the cellular and biochemical processes involved in the synthesis of thyroid hormone.**

2. **Develop a hypothesis for a precise molecular target that could be defective to explain both the physiologic and hormone changes observed in mutant mice. Provide strategies for testing your hypothesis.**

The TAM RPTK receptor family consists of three structurally related members, Tyro3, Axl, and Mer. Gas6 is a ligand that binds and activates all three receptors, with binding affinities in the nM range. Functional studies have shown that the TAMs play an important role in the immune system by regulating phagocytosis and differentiation of natural killer cells. In the following study the contribution of this RPTK family on cell proliferation was analyzed. Results are shown in Figures 1 and 2.

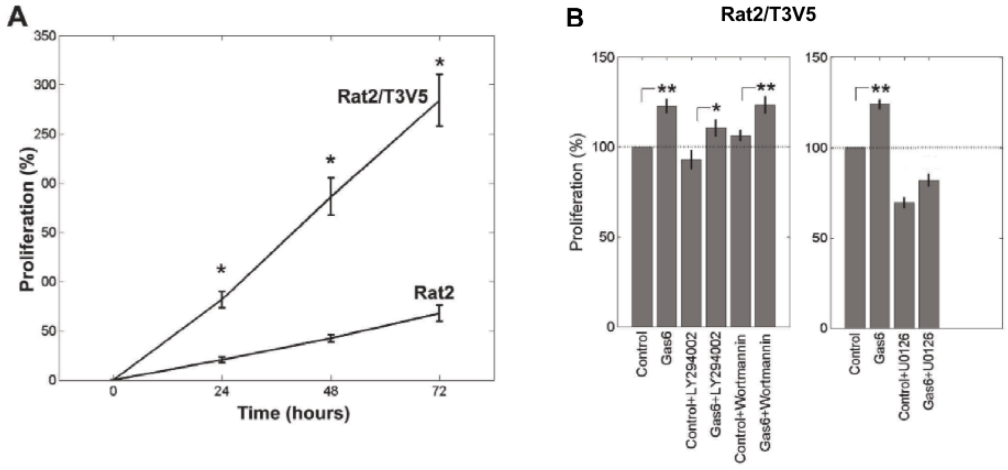


Fig 1. **A**, Enhanced proliferation of Rat2 cells overexpressing Tyro3. Gas6-induced cell proliferation of Rat2 and Rat2/T3V5 cells that overexpress Tyro3 (by transfection). Serum starved cells were stimulated with 250 ng/ml of Gas6 for 0–72 hrs. Proliferative activity is expressed as % increase in optical density obtained at 0 hr. **B**, The effect of the signaling-pathway inhibitors on Gas6 mediated cell proliferation was investigated. Cells were stimulated with DMEM only (control) or DMEM containing 250 ng/ml of Gas6 for 72 hrs in the absence or presence of the indicated inhibitors. Proliferative activity is expressed as % of the control. PI3K inhibitors used were LY294002 and wortmanin. U0126 is a MEK inhibitor.

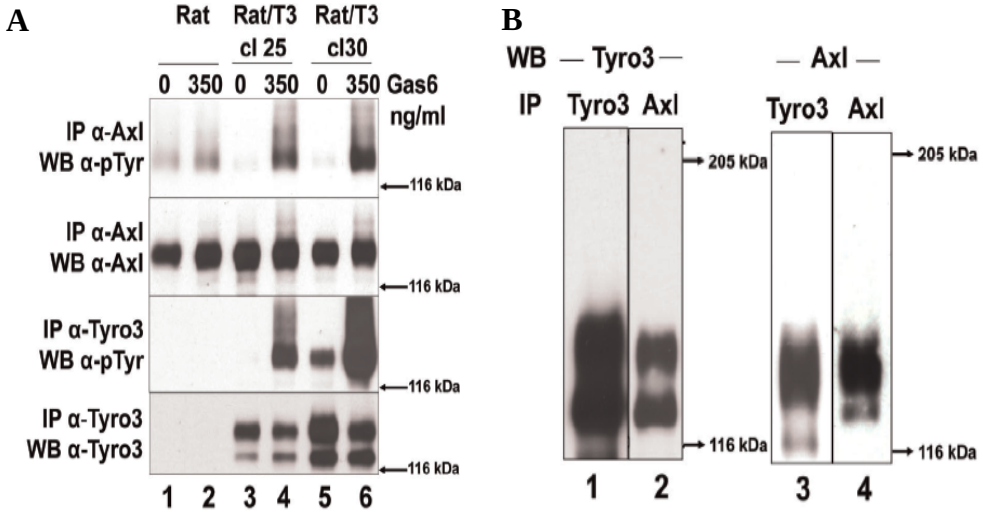


Fig 2. Activation and interaction between Tyro3 and Axl. **A**, Phosphorylation of Axl in Rat2 cells and Rat2/T3V5 cell lines clones 25 and 30. Cells were incubated with DMEM only (0) or 350 ng/ml of Gas6 for 10 min. Cell lysates were prepared and used for immunoprecipitations (IP, α̃Tyro3 or α̃Axl) followed by Western blot analysis. Subsequently, membranes were probed with anti-phosphotyrosine (α-pTyr) antibodies (top and third panels), or with rabbit α-Axl (second panel from the top). The membrane corresponding to Tyro3 IP's was stripped and re-probed with α-Tyro3 (bottom panel). **B,** Tyro3 and Axl co-immunoprecipitate in Rat2/T3V5 cells. Rat2/T3V5 cells were activated with 350 ng/ml Gas6 for 10 min. Detergent extracts were prepared for Tyro3 IP (IP Tyro3, lanes 1 and 3) and for Axl IP (IP Axl, lanes 2 and 4). The immunoprecipitates were analyzed by SDS-PAGE and blotted with α-Tyro3 (lanes 1 and 2) or rabbit α-Axl antibodies (lanes 3 and 4).

1. **Explain the results as shown in Figure 1.**

   A. **Propose a mechanism to explain how overexpression of Tyro3 leads to increased proliferation.**

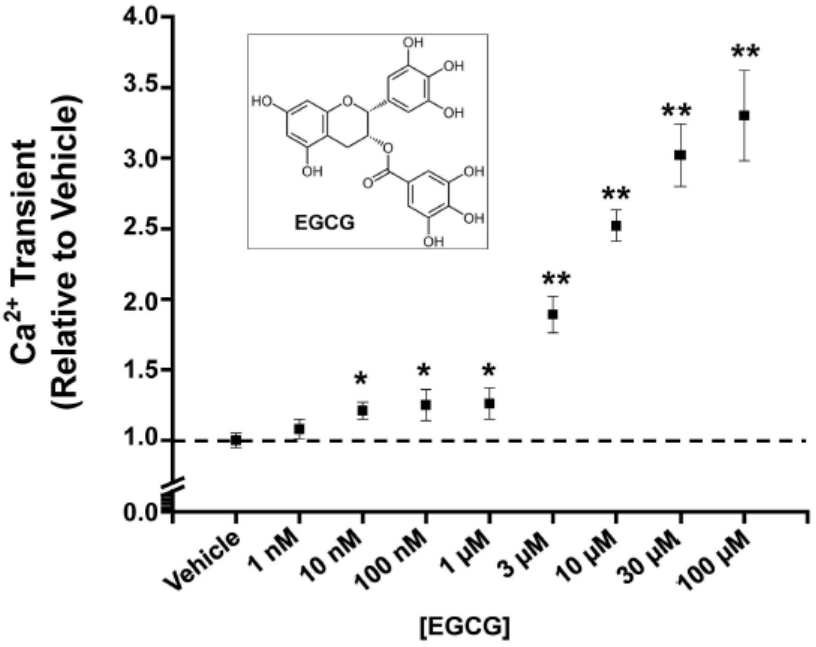
   B. **Design two independent experimental strategies including the predicted outcome of the experiments to support your hypothesis regarding the critical downstream signaling event leading to proliferation.**

2. **Explain the results as shown in Figure 2.**

   A. **Propose the molecular mechanism at the receptor level by which overexpression of Tyro3 leads to increased proliferation.**

   B. **Design two independent experimental strategies to further support your hypothesis**

A recent report investigated the molecular action of (-)-epigallocatechin-3-gallate (EGCG), the major green tea catechin, in cardiac muscle. The figure the EGCG concentration-response relationship of intracellular Ca transients in intact murine myocytes field-stimulated at 1 Hz. n = 8 to 40/group *, p = 0.05 versus vehicle; **, p = 0.0001 versus vehicle.



1. **Describe and interpret the data in Fig. 1**
2. **Generate two hypotheses regarding potential molecular targets of EGCG**
3. **Propose strategies for testing your hypotheses.**

Serum and glucocorticoid-inducible kinase-1 (SGK1) is a ubiquitously expressed enzyme. Investigators in The Netherlands investigated the contributions of SGK1 in renal physiology by studying mice with a kidney-specific inducible knockout of the gene encoding SGK1 in mice. Following induction of the gene deletion, investigators examined the mice under conditions of a normal salt diet (NSD) or low salt diet (LSD) to understand how SGK1 might influence salt and water reabsorption. The following data were generated.

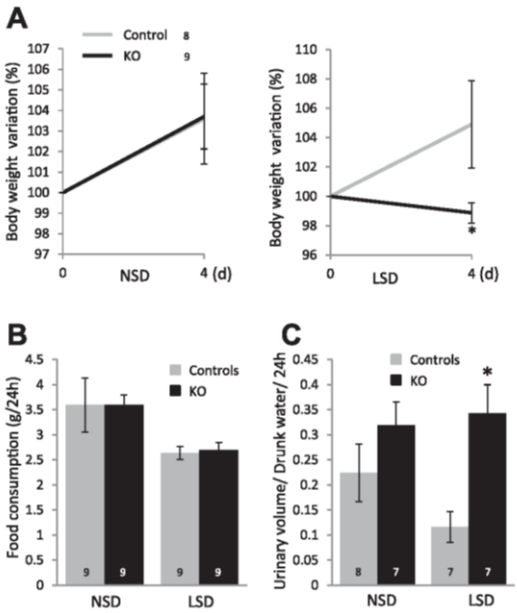


Fig. 1 – Changes in body weight, food intake and urine production in SGK1 knockout mice and littermate controls. Data shown are mean values and SEM; **, p<0.05). (A) change in body weight during 4 days of normal salt diet (NSD; left panel) or low salt diet (LSD; right panel). (B) Food consumption per day on either NSD or LSD. (C) Urine production normalized to water intake for mice on NSD vs LSD.

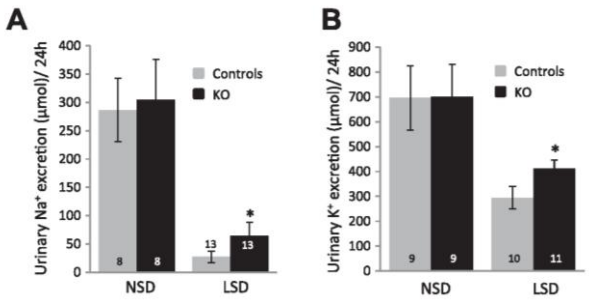


Fig. 2 – Urinary Na (A) and K (B) excretion during NSD vs LSD for SGK1 knockout mice and littermate controls. Data shown are mean and SEM (*, p<0.05).

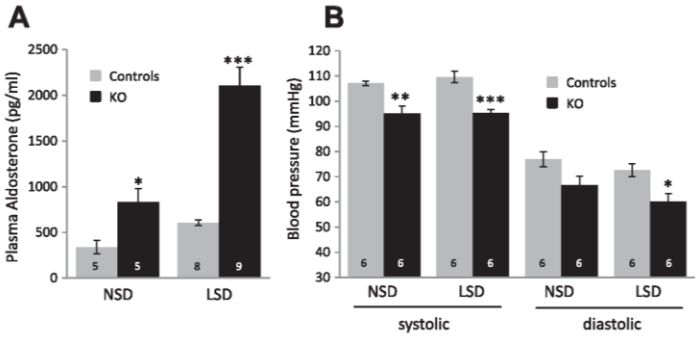


Fig. 3 – Plasma aldosterone (A) and arterial systolic blood pressure (B) during NSD vs LSD for SGK1 knockout mice and littermate controls. Data shown are mean and SEM (*, p<0.05; **, p<0.01; ***, p<0.005).

1. **Explain the observations and formulate hypotheses to explain the responses of SGK1 knockout mice to low salt diet.**

2. **Propose a mechanism to explain the data. How would you experimentally test your hypothesis?**

3. **What other genetic or pharmacological interventions would mimic the effects of SGK1 knockout?**

The investigators study angiotensin II (Ang II)-induced formation of stress fibers in mammalian cells that express endogenous angiotensin receptor AT1A (it's GPCR). It is known that small G protein RhoA mediates stress fiber formation.

In the experiments shown below they used RNAi knockdown of the signaling proteins they expect to be involved in stress fiber formation. The following figures summarize the data on Ang II-induced signaling, including RhoA activation. Note that β-arrestin1 and β-arrestin2 (a.k.a. arrestin-2 and arrestin-3) are two non-visual arrestins with ~70% sequence identity. Panel A shows Western blot data for RhoA. About 90% knockdown of β-arrestin1 with no change in β-arrestin2 expression for panel C was confirmed by Western blot (not shown).

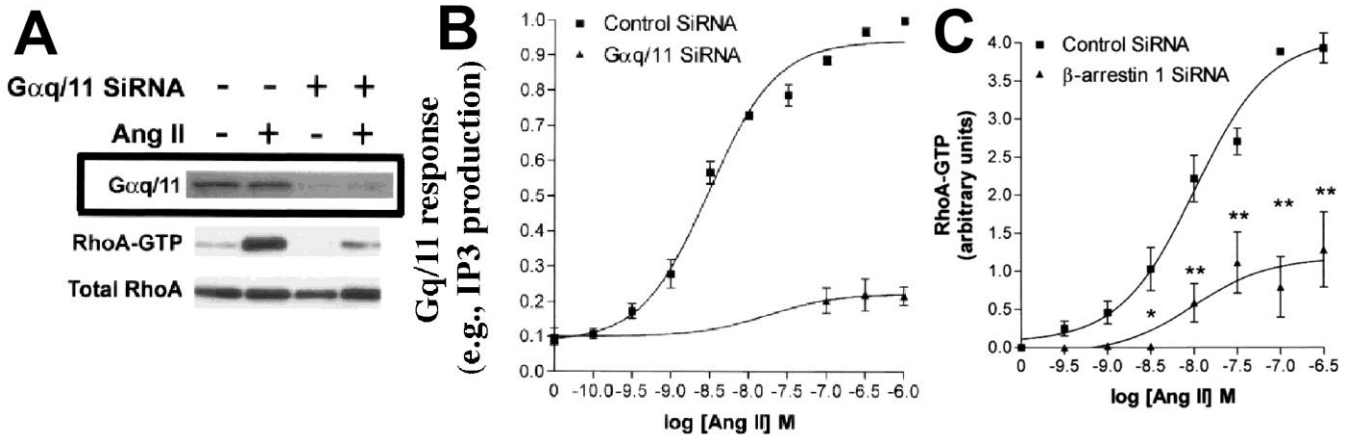


Fig. 1. A. Cells were transfected with control siRNA or siRNA against Gaq/11 and stimulated (+) or not (-) with Ang II. Active RhoA was isolated using pull-down with RhoA effector specifically binding RhoA-GTP. The blots for active and total RhoA are shown. **B.** IP3 production in response to Ang II stimulation in cells transfected with control siRNA or siRNA against Gaq/11. **C.** RhoA activation in response to Ang II stimulation in cells transfected with control siRNA or siRNA against b-arrestin1. In **C**, *, $p<0.05$, **, $p<0.01$ in comparison to the value at the same Ang II concentration obtained in cells transfected with control siRNA (Student's t-test)

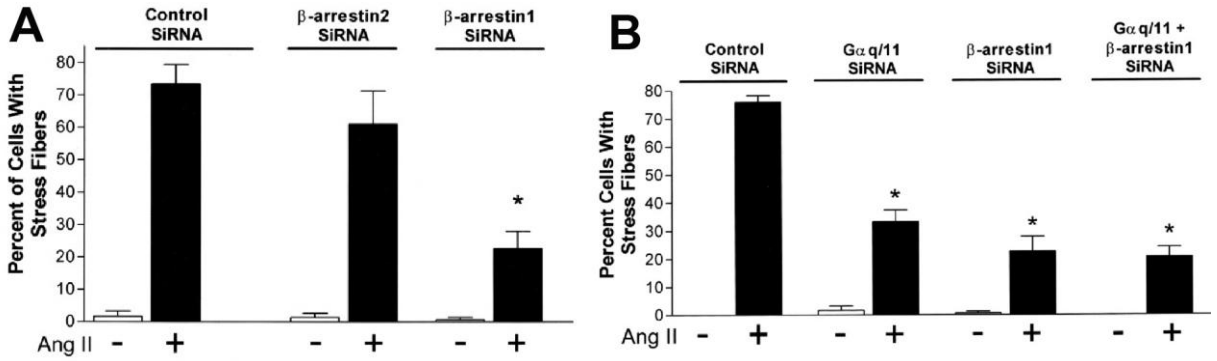


Fig. 2. A. Stress fiber formation in response to **Fig. 1. A.** Ang II stimulation (+) in cells transfected with control siRNA or siRNA against b-arrestin1 or b-arrestin2. **B.** Stress fiber formation in response to Ang II stimulation (+) in cells transfected with control siRNA, siRNA against Gaq/11, siRNA against b-arrestin, or combination of both. In **A** and **B**, *, $p<0.05$ in comparison to control (Student's t-test)

1. **Propose experiments to test whether Ang II promotes stress fiber formation via AT1A receptor:**

    a. **In cells used in the experiments shown above.**

    b. **In a different cell line that does not express endogenous AT1A receptors.**

2. **If you find that AT1A mediates the action of Ang II, propose models consistent with the data shown in Figs. 1 and 2.**

3. **Propose alternative experimental approaches to test the role of β-arrestins in Ang II-induced stress fiber formation:**
    a. **In cells that express endogenous AT1A receptor.**
    b. **In cells that do not express endogenous AT1A receptor.**

4. **Describe signaling cascade downstream of Gq/11. Propose experiments to test the involvement of each of these signaling proteins in stress fiber formation.**