Department of Pharmacology

Qualifying Examination (Part I)

July 29 – August 1, 2008

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 Joey Barnett at:

936-1722 (w)
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BEST WISHES FOR YOUR SUCCESSFUL COMPLETION OF THE EXAMINATION!

Oolong tea (OT) is widely consumed as a health drink in Japan and China. Recent studies have provided evidence that OT exerts a variety of physiological effects. Lipton, et.al. examined the effects of intraduodenal (ID) injection of OT on renal sympathetic nerve activity (RSNA) and blood pressure (BP) in urethane-anesthetized rats. Figure 1 depicts the effects of OT and decaffeinated oolong tea (DOT) on RSNA, mean arterial pressure (MAP), and heart rate (HR) after ID injection of water, OT, or DOT. In Figure 2 injection of artificial cerebral spinal fluid (aCSF) or the histamine antagonist thioperamide into the intracerebral ventricle (ICV) preceded the ID injection of water or OT by 15 minutes. The effects on RSNA, MAP, and HR are depicted.

Please provide a hypothesis as to the mechanism by which Oolong tea decreases blood pressure.

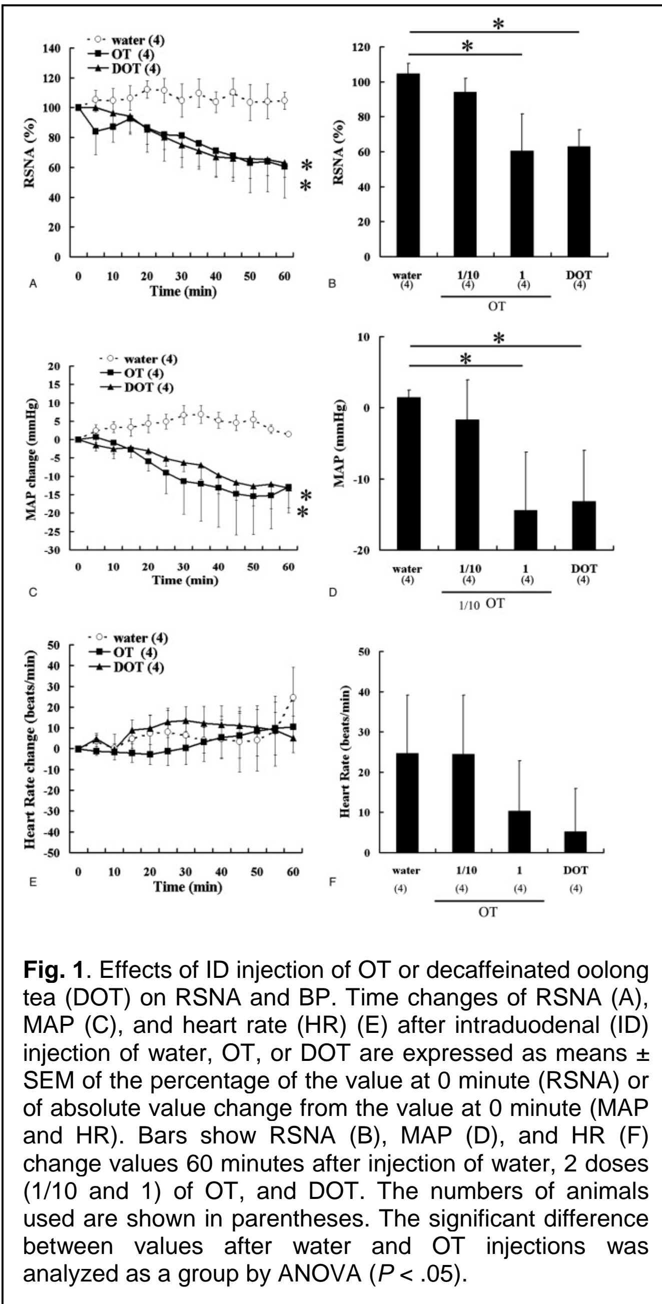


Fig. 1. Effects of ID injection of OT or decaffeinated oolong tea (DOT) on RSNA and BP. Time changes of RSNA (A), MAP (C), and heart rate (HR) (E) after intraduodenal (ID) injection of water, OT, or DOT are expressed as means ± SEM of the percentage of the value at 0 minute (RSNA) or of absolute value change from the value at 0 minute (MAP and HR). Bars show RSNA (B), MAP (D), and HR (F) change values 60 minutes after injection of water, 2 doses (1/10 and 1) of OT, and DOT. The numbers of animals used are shown in parentheses. The significant difference between values after water and OT injections was analyzed as a group by ANOVA (*P* < .05).

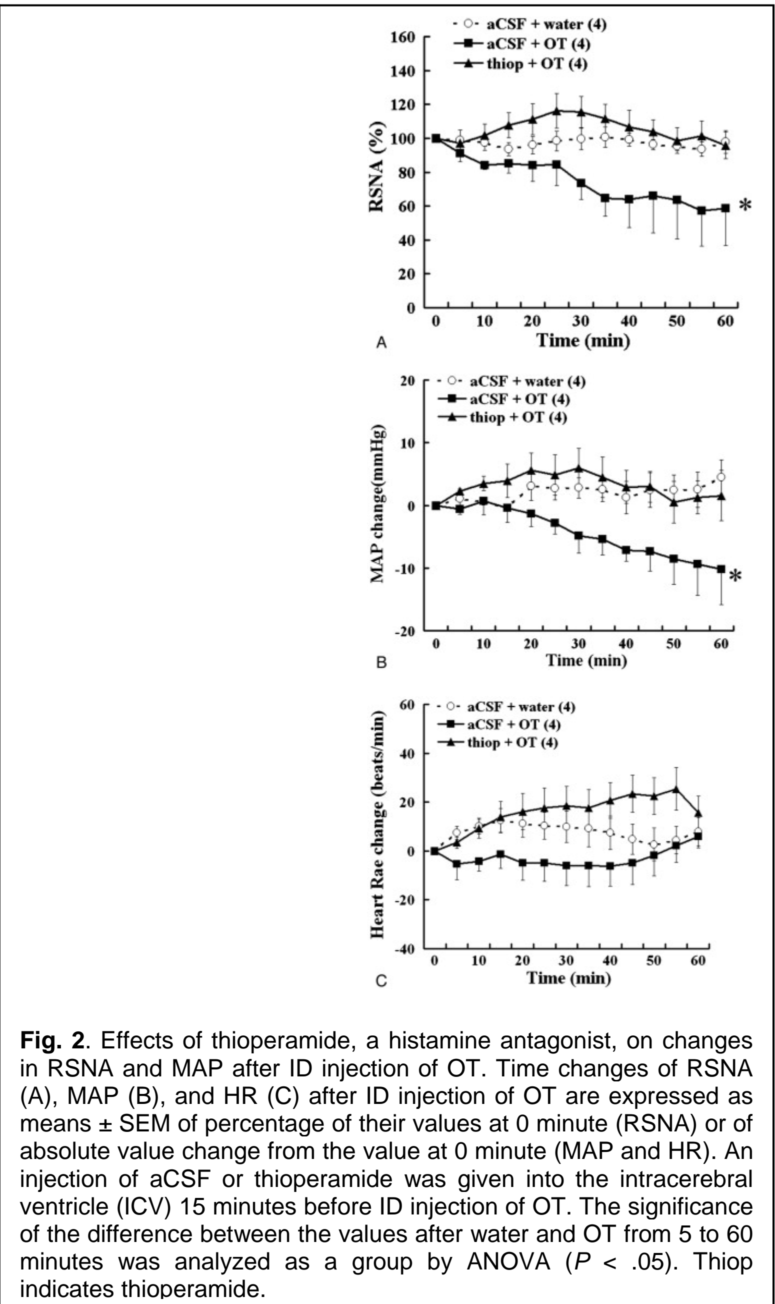


Fig. 2. Effects of thioperamide, a histamine antagonist, on changes in RSNA and MAP after ID injection of OT. Time changes of RSNA (A), MAP (B), and HR (C) after ID injection of OT are expressed as means ± SEM of percentage of their values at 0 minute (RSNA) or of absolute value change from the value at 0 minute (MAP and HR). An injection of aCSF or thioperamide was given into the intracerebral ventricle (ICV) 15 minutes before ID injection of OT. The significance of the difference between the values after water and OT from 5 to 60 minutes was analyzed as a group by ANOVA (*P* < .05). Thiop indicates thioperamide.

Your colleague at MakeMPay Pharmaceuticals isolated a single compound from OT that, when administered to urethane-anesthetized rats, yields data similar to that in Figures 1 & 2. With this compound in hand, propose experiments to test or refine your hypothesis.

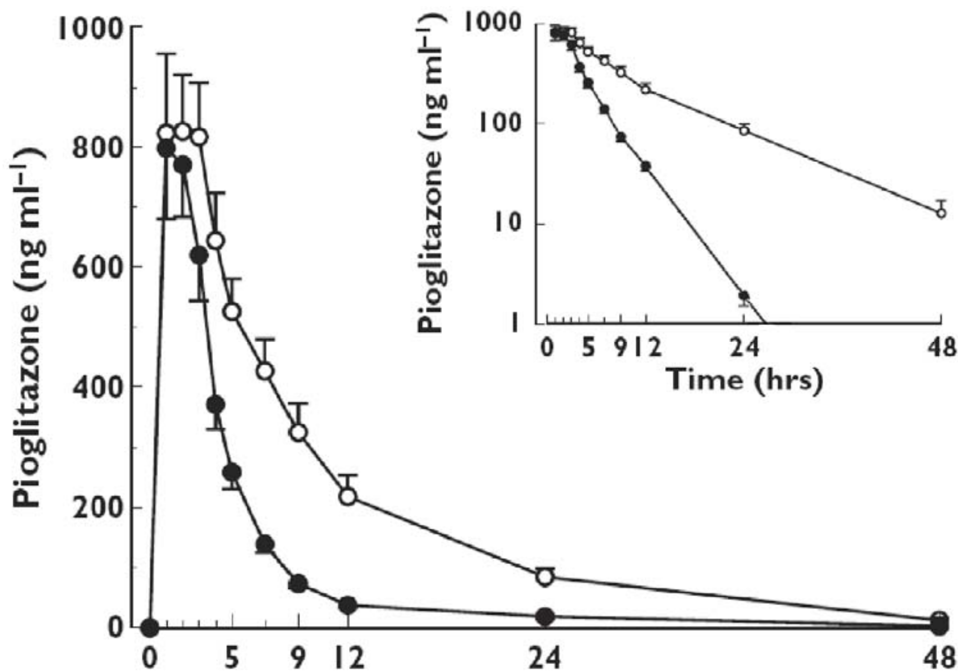
The thiazolidinedione antidiabetic drug pioglitazone was administered to healthy subjects as follows:

In a randomized, two-phase crossover study, ten healthy subjects ingested either 600 mg rifampicin or placebo once daily for 6 days. On the last day, they received a single oral dose of 30 mg pioglitazone. The plasma concentrations and cumulative excretion of pioglitazone and its active metabolite M-IV into urine were measured up to 48 h.

1. Explain the pharmacokinetics of pioglitazone and its main metabolite based on the data given in Figures 1 and 2, and Table 1.

2. How do you explain the effects of rifampicin? Present in vitro and/or in vivo experiments that would test your hypothesis.

Figure 1
Mean ±SEM plasma concentration-time curves for pioglitazone in ten healthy subjects after a single oral dose of 30 mg pioglitazone on the last day of a 6-day treatment with placebo ( ) or 600 mg rifampicin (●) once daily. The inset depicts the same data on a semilogarithmic scale.



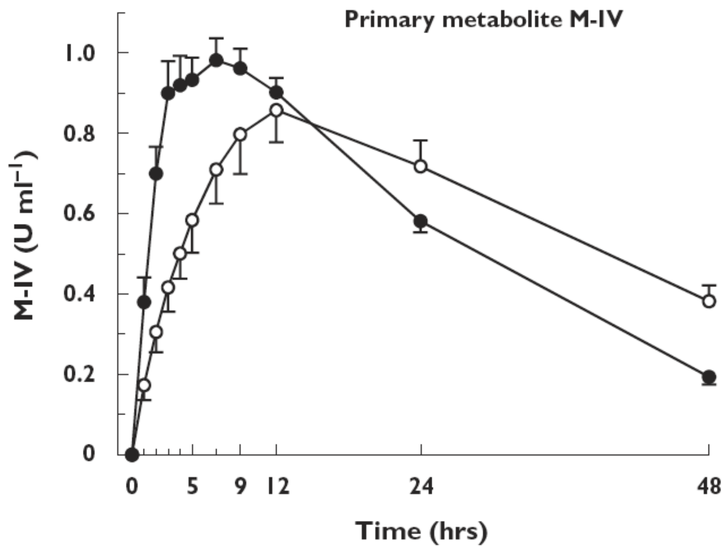


Figure 2 Mean ±SEM plasma concentration-time curves for metabolite M-IV in ten healthy volunteers after a single oral dose of 30 mg pioglitazone on the last day of a 6-day treatment placebo ( ) or 600 mg rifampicin (●) once daily.

Table 1
Pharmacokinetic data for pioglitazone in ten healthy subjects after a single oral dose of 30 mg pioglitazone on the last day of a 6-day treatment with 600 mg rifampicin or placebo given once daily

Variable	Placebo (control)	Rifampicin treatment	Rifampicin phase percentage of control (range)	*P*-value
Pioglitazone				
C_{max} (ng mL−1)	932 ± 335	888 ± 335	95 (52–167)	0.7446
t_{max} (h)	1 (1–3)	1.5 (1–2)		1.0000
$t_{1/2}$ (h)	4.9 ± 1.0	2.3 ± 0.6	47 (29–110)	0.0002
AUC_{0-} (mg L−1 h)	8.61 ± 3.66	3.98 ± 1.20	46 (34–80)	0.0007
Cl_{renal} (ml h−1)	0.12 ± 0.05	0.12 ± 0.06	99 (49–143)	0.9264
Metabolite-IV				
kf (h−1)	0.19 ± 0.05	0.44 ± 0.19	231 (114–475)	0.0041
C_{max} (U mL−1)	0.87 ± 0.28	1.02 ± 0.19	116 (63–219)	0.2479
t_{max} (h)	12 (9–24)	8 (3–12)		0.0072
$t_{1/2}$ (h)	32 ± 10	16 ± 3	50 (32–95)	0.0008
AUC_{0-} (U mL−1 h)	48.2 ± 17.5	32.0 ± 5.1	66 (49–155)	0.0055

Values shown as mean ± SD unless otherwise indicated, tmax data as median (range). Cmax , observed peak plasma concentration; tmax, time to reach Cmax ; t1/2, terminal elimination half-life; AUC0– , area under the concentration versus time curve to infinity; kf , apparent formation rate constant; U, arbitrary units (relative to the ratio of the peak height of the metabolite to the peak height of the internal standard).

Background. You are the director of a Research Division and you get a phone call from an epidemiologist in Ireland that has a perplexing mystery. A small fishing village on the Irish coast has been afflicted with some disorder and they have been baffled as to the cause. They suspect the constellation of symptoms is related to a red tide ("harmful algal bloom") that first appeared a few months ago and all of those experiencing symptoms are consumers of shellfish (e.g., clams, oysters, and scallops). Residents that ate a lot of shellfish generally appear to have worse symptoms than those that ate little. No residents that does not eat shellfish has shown any symptoms. The red tide was analyzed after it first occurred and none of the usual toxins, such as *Alexandrium* toxin (saxitoxin), were detected in levels that should be harmful.

The disorder is unusual in part because of the wide variety of symptoms that have been reported. The most common finding is acute gastic ulceration. A subset of afflicted females reported having less discomfort than usual during their menstruation. There are assorted vascular and pulmonary symptoms, but some report problems associated with vasoconstriction, while others appear to have problems more related to vasodilation.

Within the last few days (well after the symptoms began) an influenza outbreak occurred in the region affecting the fishing village as well as a much wider area. This particular strain of influenza was associated with high fevers. Those residents that had eaten shellfish (referred to as the afflicted group) had less fever than other residents, but otherwise felt the effects of the viral infection. The epidemiologist commented that this is a fairly isolated community composed of interconnected, extended families who generally refrain from taking medications.

Initial Findings. You agree to assist your colleague and utilize your own expertise in pharmacology, including your Synthetic and Analytical Chemistry Facility. You obtain blood samples from the afflicted group as well as other locals that have not eaten shellfish.

(1) In your initial finding you note that platelets from the afflicted group fail to aggregate normally. The control population platelets aggregated at a normal rate. Exposure to influenza alone had no systematic effect on platelets. However, you note that the platelet aggregation abnormality is linearly correlated with the amount of shellfish residents reported consuming. Interestingly, you observe that those residents that stopped eating shellfish more than a week ago had platelet aggregation rates that were approaching normal. They also reported slight improvements in gastric symptoms.

(2) The leukocytes fraction of the blood samples allowed access to a fairly pure preparation of macrophages. One of your routine measurements is an ELISA for measuring Tumor Necrosis Factor (TNFα). You observe that the affected population has significantly highly levels of TNFα than the unaffected population.

(3) A brilliant graduate student in your group has an idea and in the dark of night expresses oncogenic RAS GTPase in the two groups of macrophages. The macrophages from the afflicted group produce high levels of TNFα, whereas the normal macrophages show the anticipated decrease in TNFα levels.

You decide to have the analytical lab measure a number of parameters in their routine screens:

Detailed Analytical Measurements- (a) Genomic array analysis will take several weeks to give you specific information, but the technician tells you a number of genes products are not expressed in the afflicted group as compared to the normal group. (b) Proteomics analysis was somewhat indeterminant. There may be minor differences in individual proteins, but there are certainly no wide spread alterations in the levels of well characterized proteins. (c) Metabolomics- you have a number of extraction protocols that separate different types of metabolites. The standard metabolic parameters were normal, but your newly hired chemist notes that

one of the extraction profiles that specifically isolates linear hydrocarbon compounds showed a difference in profiles after separation on a reverse phase hplc column as well as subsequent analysis by mass spectrometry. Your new chemist is not trained in biology, but her analytical skills are extraordinary. You looked for evidence of differences in phagocytosis (while looking for evidence of an unknown pathogenic organism) by challenging macrophages with lipopolysaccaride (LPS) + ATP. The induced phagocytosis between the afflicted and nonafflicted (normal) groups was the same. Your chemist analyzed these samples using hplc and LC-MS mass spectrometry and results are shown below in **Figs 1 and 2**.

See Figs 1 and 2, next page

Question #1. Your chemist isolated quantities of a previously unknown small molecule that is concentrated in the shellfish from the afflicted area. She notes that this molecule has functional groups that may allow it to form a covalent bond with a protein or protein(s). The details are not important except her comment that the molecule would need to have a high affinity for binding to the protein before it formed the covalent bond (i.e., it would need a high affinity for said protein). From the information given identify the most likely molecular target that could cause this affliction (a target that can potentially explain the constellation of symptoms described). Give the most parsimonious hypothesis to explain the symptoms. Justify your hypothesis by outlining the relevant pathways and explicitly detail how these symptoms can all be related to a single molecular target. A complete answer will include explicitly explaining how you selected the correct target and not closely related targets that fit some (but not all) of the data.

Question #2. Explain the relevance of the finding on Ras GTPase expression. Does this assist you in identifying the molecular target?

Question #3. Based on your conclusion what needs to be done for the afflicted residents? Why?

Fig.1
Reverse Phase HPLC

Table A

Internal Standard

Normal, unstim

Integrated Peak Intensity

0.6
0.5
0.4
0.3
0.2
0.1
0

0 1 3 5 7 9 11 13 15

Time (mins)

Table B

Internal Standard

Normal, stim.

Integrated Peak Intensity

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

0 1 3 5 7 9 11 13 15

Time (mins)

Table C

Internal Standard

Afflicted, stim

Integrated Peak Intensity

0.6
0.5
0.4
0.3
0.2
0.1
0

0 1 3 5 7 9 11 13 15

Time (mins)

Fig 2
LC-MS of individual species within the hplc peaks

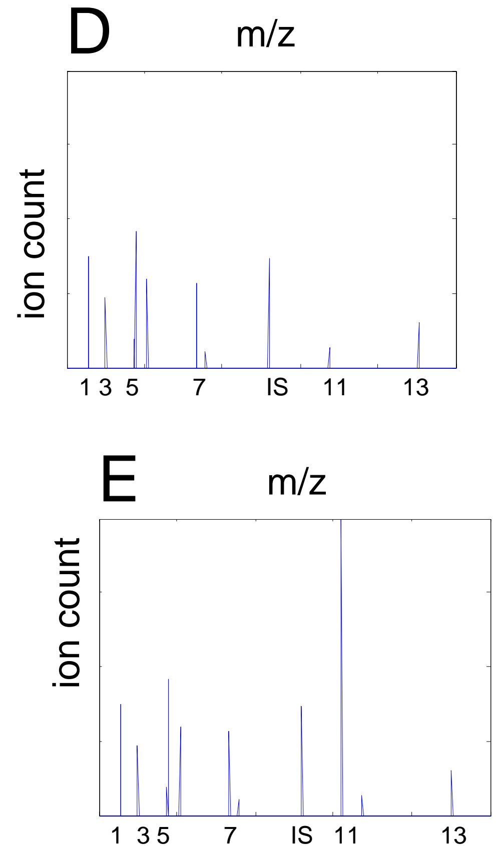


Fig.1. A specialized organic phase extractions was performed on macrophages and extracts chromatographed by reverse phase hplc. The peaks come off in order of hydrocarbon chain length (i.e., shorter followed by longer hydrocarbon chains). All compounds were shown to be linear hydrocarbons that were non-cyclized (i.e., contains no rings). The time of elution is indicated on the x-axis and the peak intensity is shown on the y-axis. Table A is the chromatogram from unstimulated normal subjects (unaffected), Table B is after a short stimulation with 20 uM LPS and 5 mM ATP of normal subjects. Table C is the same stimulation procedure (as B) but in macrophages from the afflicted residents.

Fig 2 shows the ESI-MS negative mode spectra. The major molecular species from each of the indicated peaks (corresponds to the peaks shown on the hplc trace) is indicated in bold (**1**- 227 m/z, 14:0; **3**- 255m/z, 16:0; **5**- 283, 18:0; **7**- 281m/z, 18:1; **IS**- internal standard; **11**- 303m/z, 20:4; and **13**- 327m/z, 22:6) The next number (227-327) indicates the mass/charge (m/z) and numbers following the comma indicate the molecular species (number of carbons, 14-22: number of double bonds, 0-6). The spectra shown above in panel D are from the corresponding peaks from the hplc trace in Table A. This spectra was absolutely identical to the spectra of hplc Table C (data not shown). The spectra shown in panel E is from the hplc peaks shown in Table B. **Notes:** (1) Any unidentified spectral lines in panels D and E may be ignored as background. (2) Hplc traces and ESI-MS spectra from unstimulated samples from afflicted macrophages looked exactly the same as those in hplc trace Table A and panel D (not shown). (3) Differences between the peak heights in the hplc can be directly compared but are not quantitative (i.e., a 2X difference in peak height may be much more than twice as much difference), whereas the differences in the MS peak heights are quantitative.

The "club drug" 3,4-methylenedioxymethamphetamine (MDMA; also known as ecstasy) is a psychostimulant drug of abuse that induces hyperlocomotion in animals.

A recent study shows that pharmacological inhibition and genetic ablation of 5-HT2B receptors abolishes the psychostimulatory effect of MDMA (Figure 1).

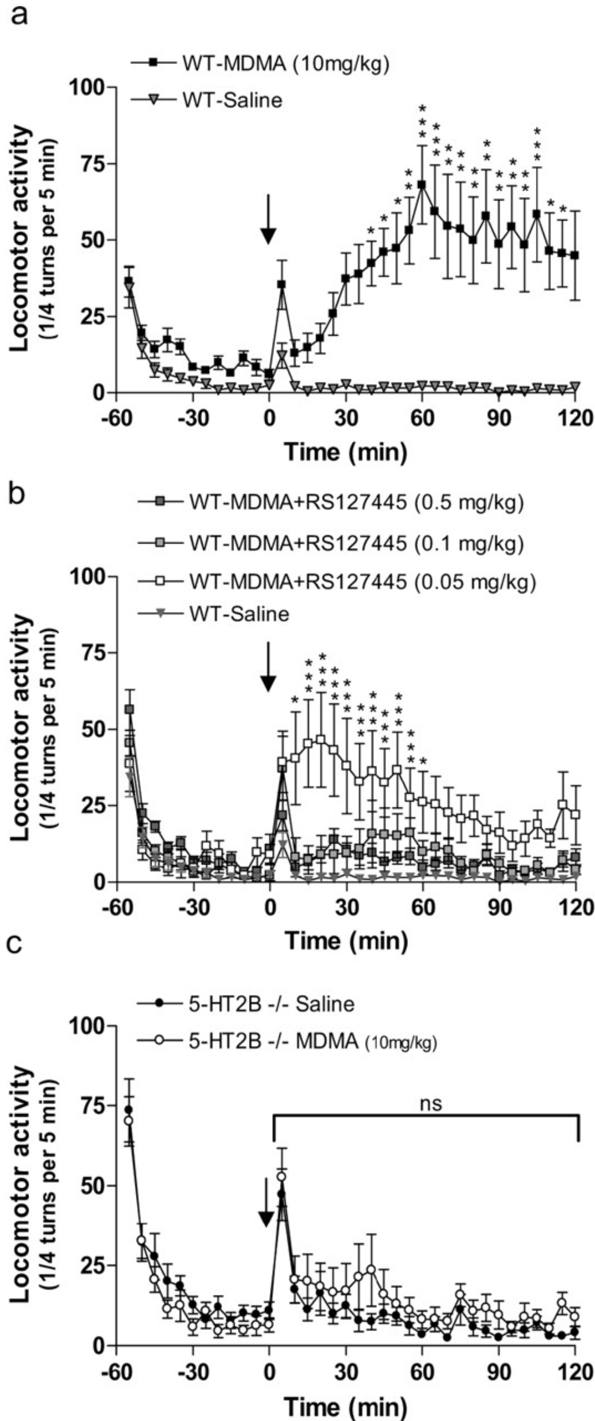


Figure 1. Effect of 5-HT2B receptor inhibition on MDMA-induced hyperlocomotion. **a,** MDMA-induced locomotion in WT mice. WT mice were injected (i.p.) with either MDMA (10 mg/kg; squares) or saline solution (triangles) (arrow) after 1 h of habituation. **b**, RS127445 (selective inhibitor of 5-HT2B receptors) abolishes MDMA-induced locomotion in WT mice. WT mice were injected with RS127445 [0.5 mg/kg (dark squares), 0.1 mg/kg (light gray squares), or 0.05 mg/kg (white squares)] solution 1 h before MDMA (10 mg/kg) injection (arrow). **c**, MDMA-induced locomotion failed in 5-HT2B–/– mice. MDMA (10 mg/kg) or saline solutions were injected after 1 h of habituation (arrow).

A. Describe the molecular mechanism of the MDMA action in the brain. Describe classes of 5-HT receptors. Describe the nuclei that produce serotonin and their location in the brain. Describe the distribution of serotonergic innervation in the brain and main recipient structures.

B. Propose two alternative models of the role of the 5-HT2B receptor in the psychostimulant action of MDMA assuming that 5-HT2B receptors are either pre- or postsynaptic. Propose critical experiments to discriminate between these two models.

C. Describe potential therapeutic uses or compounds targeting the 5-HT2B receptors based on your models.

Researchers at the University of Copenhagen recently created a genetically engineered mouse in which a gene (*Kicr1*) encoding a probable kidney-specific ion channel regulator was deleted. Homozygous 'knockout' mice (*Kicr1*-/-) exhibited significantly increased urinary excretion of water, sodium and potassium as compared with wildtype and heterozygous littermates. In addition, whole kidney glomerular filtration rate was significantly decreased by approximately 50% in *Kicr1*-/- mice as compared with wildtype and heterozygous littermates. Body weight, blood pressure, and plasma concentrations of sodium and potassium were not statistically different between wildtype and *Kicr1*-/- mice, but heart rate was significantly higher in the knockout animals. Specific measurements are illustrated in **Fig. 1**.

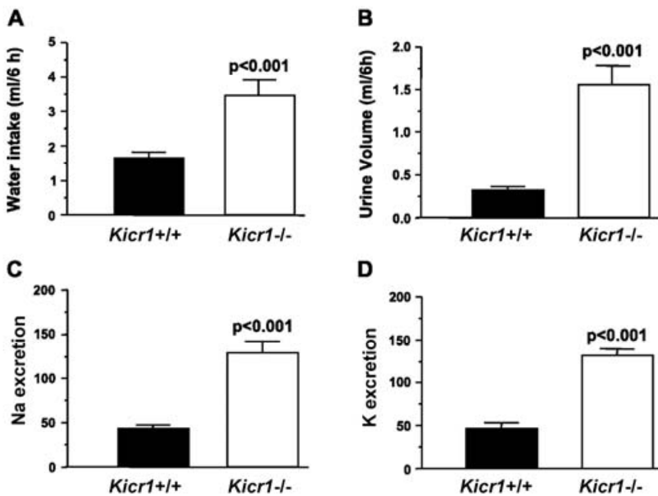


Fig 1 – Baseline renal studies in wildtype (*Kicr1*+/+, filled bars) and knockout (*Kicr1*-/-, open bars) mice determined for a 6 hour period. Data are mean ± SEM. The *p* values compare knockout to wildtype mice.

To identify the location of the renal defect, the investigators used "diuretic pharmacotyping", a strategy examining the effect of specific diuretic agents on the renal phenotype of the animals. **Fig. 2** illustrates their findings.

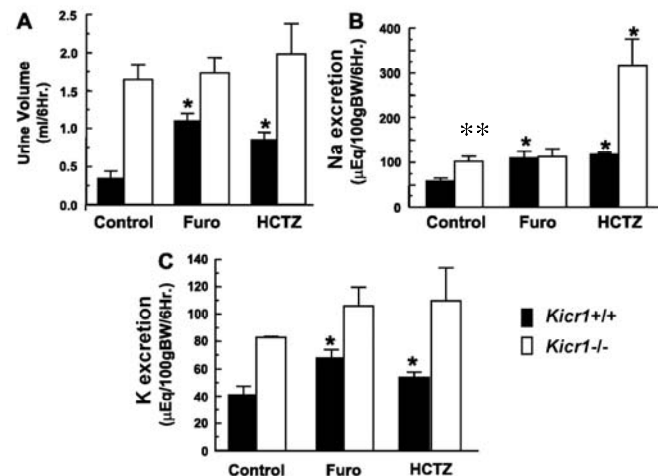


Fig 2 – Effect of diuretics on renal excretion. Drugs were given intravenously at the end of the control period, then urine was collected for 6 hours. Furo, furosemide (loop diuretic); HCTZ, a thiazide diuretic. Statistical significance is indicated by *, $p<0.05$ compared with control period for same genotype; and **, $p<0.05$ compared with wildtype (panel B only).

Questions:

1. Describe the renal phenotype of *Kicr1* knockout mice.
2. Formulate hypotheses to explain the renal phenotype and explain how to test your ideas.
3. Predict the impact of *Kicr1* knockout on tubuloglomerular feedback.

A recently formed pharmaceutical company, Chubby-R-Us, initially focused its research efforts upon the growing problem of human obesity by attempting to identify new compounds that can modulate the central regulation of feeding behavior. Unfortunately, oral administration of one of their newest test compounds significantly increased food intake in rodents. Not deterred by this minor setback, Chubby-R-Us changed it research direction to focus upon that small percentage of Americans that are chronically underweight and have difficulty in maintaining their body mass.

Additional dose-response analyses of their new drug revealed that when administered at levels five-fold higher than those shown to affect food uptake, experimental animals demonstrated weakness, sluggishness and eventually died within about 6 weeks. A more detailed analysis of these mice revealed the fasted, mean blood values indicated below:

Laboratory test	Treated animal value	Normal range
Na^+	112 mM	137-145 mM
Cl^-	81 mM	98-107 mM
K^+	5.4 mM	3.5-5.0 mM
Glucose	90 mg/dL	150-200 mg/dL
Corticosterone	<0.2 µg/dL	AM 6.2-29.1 µg/dL PM 3.0-17.3 µg/dL
ACTH	1745 pg/ml	9-52 pg/ml

ACTH, adrenocorticotropic hormone; Cl^-, chloride; K^+, potassium; Na^+, sodium

1. Based upon all of these observations, develop a hypothesis regarding the physiological changes in response to your compound and propose a mechanism for its action.

2. Describe a series of experiments by which to confirm your proposed mechanism of action.

Stimulation of the AR is known to induce sedation in animal models. To study the *in vivo* relevance of spinophilin and arrestin3 in AR-elicited sedation, the group of investigators examined the ability of α2-agonists to evoke AR-mediated sedation in spinophilin null mice (Sp-/-), arrestin3 null mice (Arr3-/-), and corresponding wild type mice (WT). Sedation was assessed via rotarod latency, which is defined as the time the mice were able to stay on the rotarod treadmill (decreased rotarod latency = increased sedation).

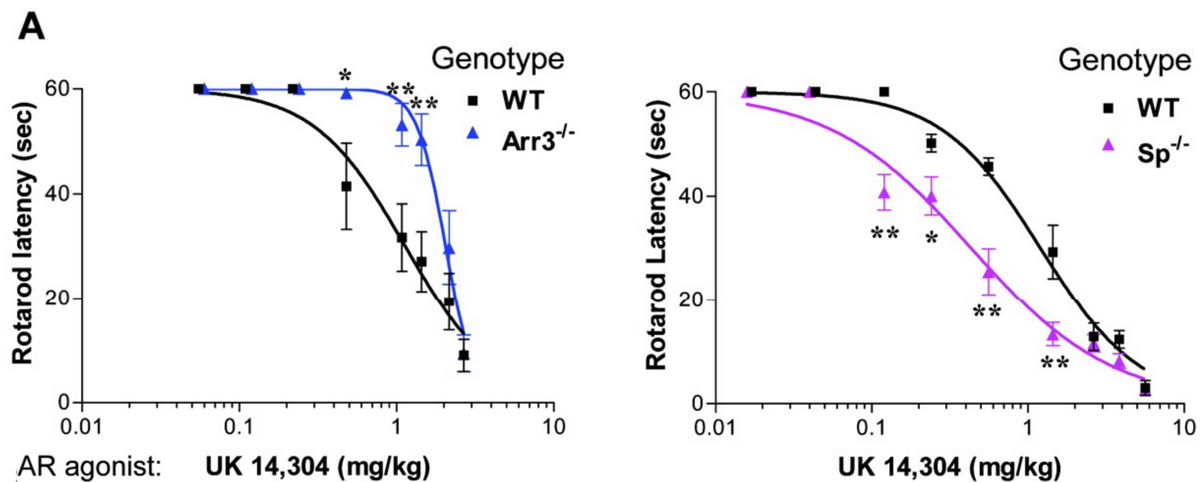


Fig. 3. Sedation in response to a specific AR agonist (*UK14,304*) was assessed via rotarod latency after administration of increasing doses of UK 14,304. The EC50 values for sedation in Arr3–/– and corresponding WT littermates (n = 5 for each genotype) are 2.1 and 1.1 mg/kg, respectively, and the EC50 values for sedation in Sp–/– and corresponding WT mice (n = 11 for each genotype) are 0.4 and 1.2 mg/kg, respectively. *P < 0.01; **P < 0.05.

C) Based on the results of the experiments depicted in Figs. 1 and 2, as well as your knowledge of arrestin's pleiotropic roles in GPCR signaling, how would you explain the results of the experiment depicted in Fig. 3?

D) Other GPCRs, including A1 adenosine receptors, are known to evoke sedation in wild type mice. Design an *in vivo* experiment to test whether spinophilin and arrestin3 are involved in A1 adenosine receptor-elicited sedation.