

Department of Pharmacology

Qualifying Examination (Part I)

December 14 & 15, 2010

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.

Cocaine is an abused psychostimulant that interacts with monoamine (dopamine, norepinephrine, serotonin) transporters blocking the reuptake of these neurotransmitters. Given a chance, animals will self-administer cocaine (press a lever to cause intravenous cocaine infusion). Rodent self-administration is a classic clinically relevant behavioral paradigm to measure reinforcing properties of drugs of abuse.

Experiments were conducted to investigate cocaine self-administration in mice lacking dopamine (DAT^{-/-}) or serotonin (SERT^{-/-}) transporter. Some of the results are presented in Figures 1 and 2 below.

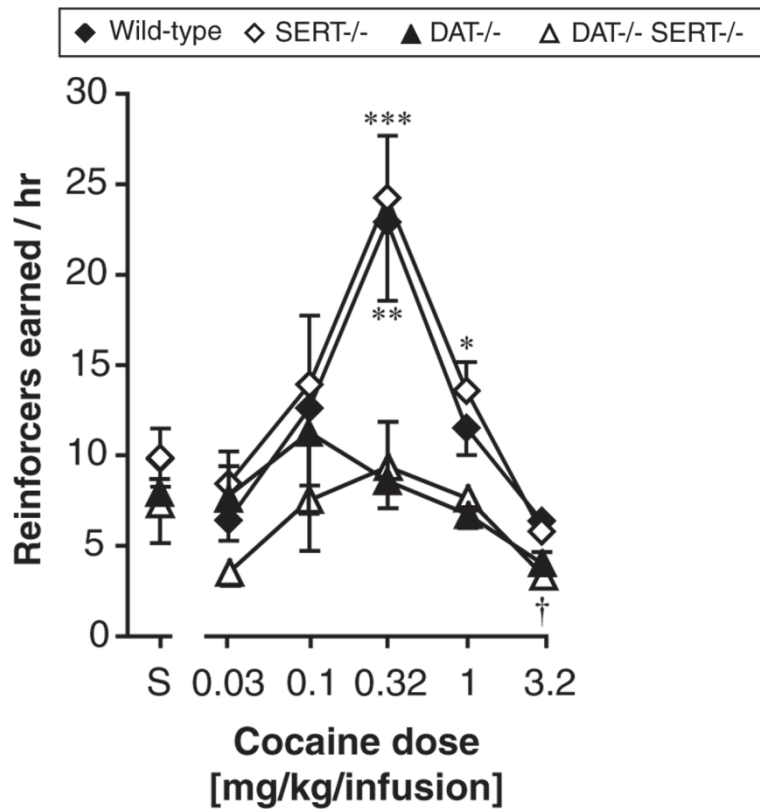


Figure 1. Cocaine self-administration in wild type- DAT^{-/-}, and SERT^{-/-} mice. Cocaine self-administration is expressed as a number of cocaine doses mice self-administer in 1 hour (depending on a size of a single dose). S – saline; * - $p < 0.05$, ** - $p < 0.01$, * - $p < 0.001$ to saline; + - $p < 0.05$ to WT.**

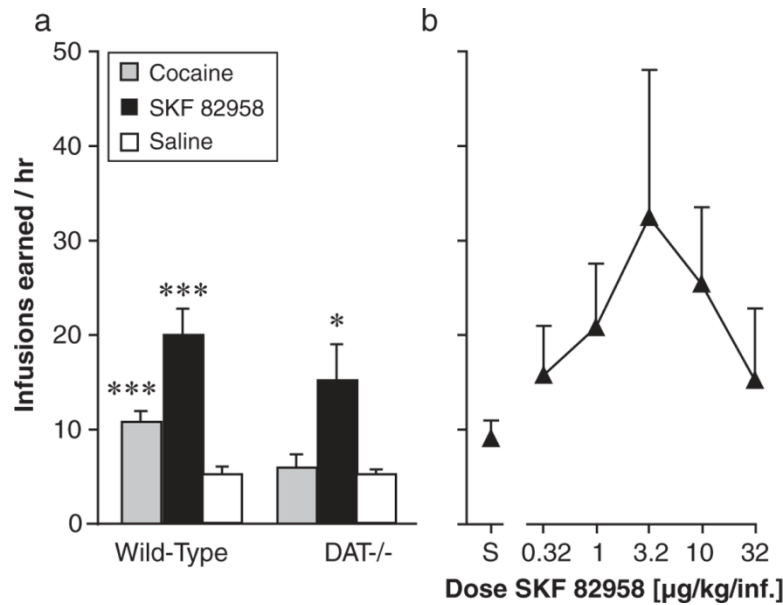
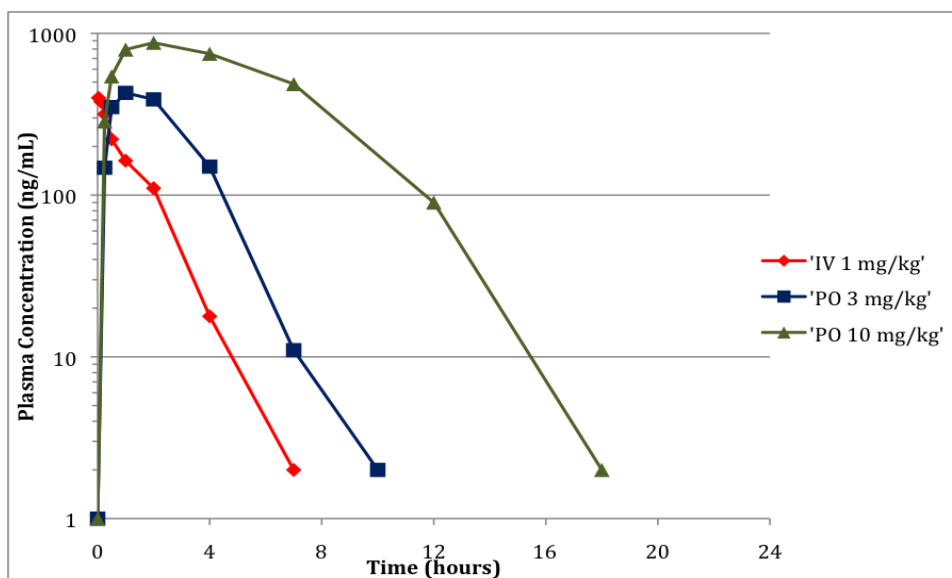


Figure 2. Self-administration of the dopamine D₁-like receptor agonist SKF 82958 in wild type and DAT^{-/-} mice. a) Levels of self-administration in wild-type and DAT^{-/-} mice of cocaine 1.0 mg/kg/infusion, compared with stable responding maintained by 10 μg/kg/infusion SKF 82958, and saline substitution. b) SKF 82958 dose-effect function in DAT^{-/-} mice. **p* < 0.05, ****p* < 0.001 versus saline, paired-sample *t* test.

1. From the data in Figs. 1 and 2, what can you conclude regarding the role of DAT and SERT in reinforcing properties of cocaine? What molecular event(s) caused by cocaine are critical for its reinforcing effect?
2. What other molecular and behavioral features would you expect to see in DAT ^{-/-} mice? How would they respond to amphetamine?

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Question 2

A new drug VU101 under development in the Vanderbilt Drug Discovery program was evaluated for its pharmacokinetic characteristics in the rat. The Figure shows the plasma concentrations and the Table shows pharmacokinetic parameters calculated from these data after administration intravenously (1mg/kg) and orally (3mg/kg and 10mg/kg) to groups of animals.



IV: 1 mg/kg (n=5) Oral: 3 mg/kg (n=3) Oral: 10 mg/kg (n=2)	PK parameters
Cl (mL/min/kg)	27
Vd (L/kg; I.V.)	4.0
T _{1/2} (min; I.V.)	102
AUC (I.V. 1 mg/kg) (hr·ng/mL)	608
Cmax (P.O. 3 mg/kg) (ng/mL)	429
Cmax (P.O. 10 mg/kg) (ng/mL)	875
Tmax (3 mg/kg) (hr)	1
AUC (3 mg/kg) (hr·ng/mL)	1795
AUC (10 mg/kg) (hr·ng/mL)	9418

- 1. Interpret the PK data in the Figure and Table, indicating how the Clearance and Volume of distribution and half-life values were obtained. Estimate the bioavailability.**
- 2. How do you account for the different profiles of the oral plasma concentration/time curves at the two doses? Propose experiments to examine the basis for the difference. Include in your experiments an analysis of routes of elimination of drug VU101.**

Congenital 21-hydroxylase deficiency causing Congenital Adrenal Hyperplasia is accompanied by low cortisol and aldosterone levels and high testosterone level.

- 1- In what tissues are these hormones synthesized?
- 2- To which family of hormones do they belong to? Give at least 3 characteristics for this class of hormones.
- 3- What is the general structure and characteristics of their receptors?
- 4- What homeostatic responses to these hormonal changes (low cortisol, low aldosterone, high testosterone) will lead to in the hypothalamus and pituitary and what will be the effect on target organs?

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Question 4

Aberrant activities of EGFR often lead to tumor formation resulting from uncontrolled proliferation of cells. To investigate the critical receptor subtype and mechanisms underlying persistent signaling from activated receptor in melanoma, primary cultures derived from the tumor were established and employed for investigation.

- By biochemical analysis, you show that all four EGFR's (EGFR1-4) are present in the tumor cells (Figure1).
- To explore which EGFR is responsible for the uncontrolled growth of the cell, you employed two independent methodologies and demonstrate that both EGFR1 and EGFR4, but not EGFR2 and EGFR3, are critical for proliferation of tumor cells in vitro (Figure 2).
- To gain insights into the signaling events that lead to tumorigenesis following the activation of the EGFR1, you demonstrate that activated receptor by tethering to GRB2 activates PLC-gamma (Figure 3).
- Importantly, you show that blocking PLC-gamma greatly reduces the proliferative capability of the tumor-derived cells in vitro (Figure 4).

A. Draw the hypothetical results for Figures 1-4.

B. Provide detailed experimental methodology used to obtain the results in each Figure.

Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant genetic disorder linked to numerous mutations in the sarcomeric proteins. The clinical presentation of FHC is highly variable, but it is a major cause of sudden cardiac death in young adults with no specific treatments. A transgenic (TG) mouse model of FHC with a mutation in tropomyosin at position 180 was used to examine the effects of altered SERCA levels on the development of FHC. Adenoviral-Serca2a (Ad.Ser) was injected into the left ventricle of 1-day-old non-transgenic (NTG) and TG mice. Ad.LacZ was injected as a control. Serca2a protein expression was significantly increased in NTG and TG hearts injected with Ad.Ser for up to 6 weeks. The following data was generated.

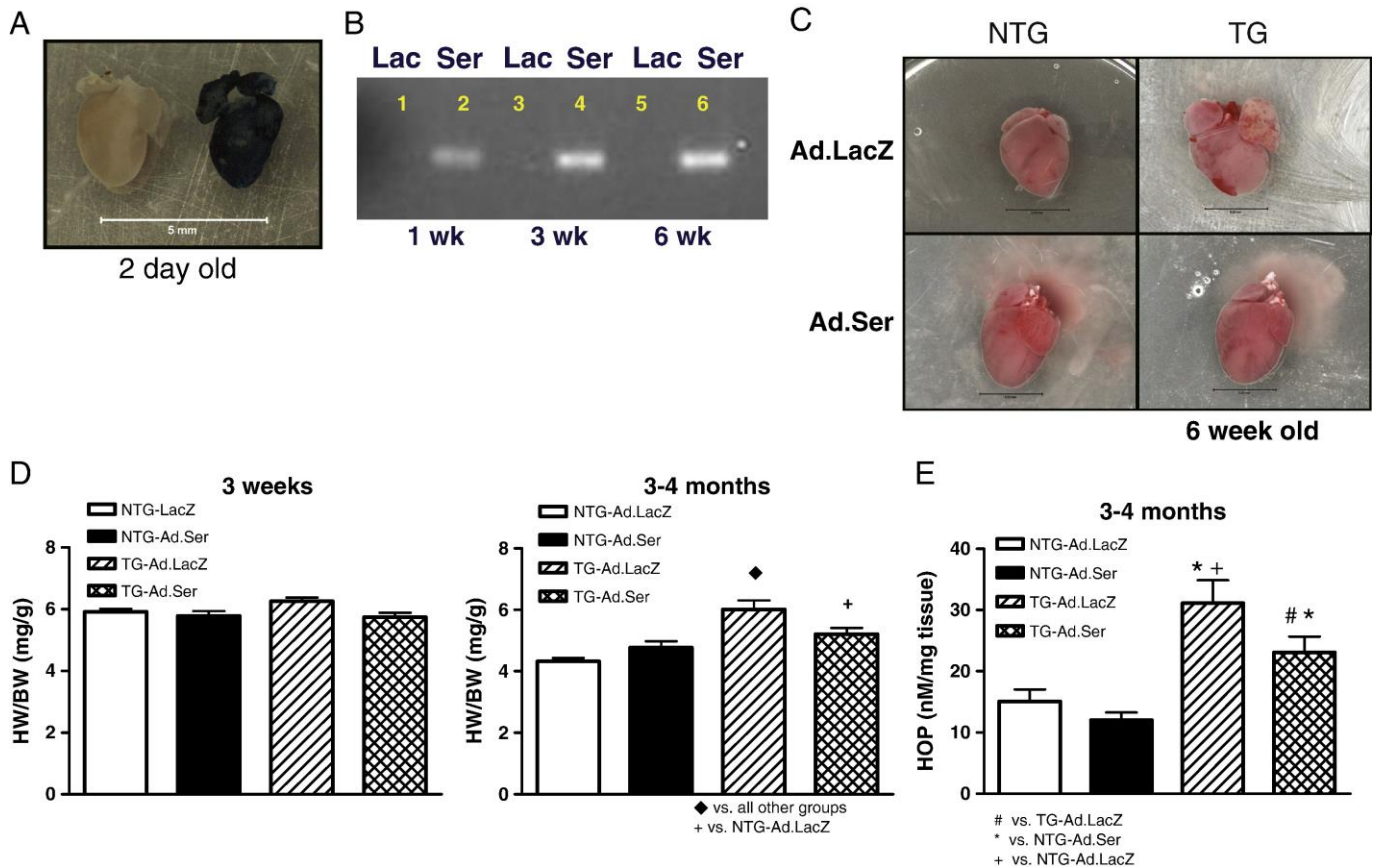


Fig. 1. (A) Representative hearts from a mouse injected with saline (left heart) or Ad.LacZ 1 day after birth and then stained for lacZ. (B) Expression of exogenous Serca2a detected by PCR in samples from 1-week-, 3-week- and 6-week-old mice. (C) Representative hearts from NTG and TG mice injected with Ad.LacZ or Ad.Ser at 6 weeks of age. (D) Heart weight to body weight ratio in NTG and TG mice injected with Ad.LacZ or Ad.Ser at 3 weeks and 3-4 months of age. Data are presented as mean±SEM, n=8-9 per group. (E) Hydroxyproline content in ventricles (used as a measure of fibrosis) from NTG and TG mice injected with Ad.LacZ or Ad.Ser at 3-4 months of age. Data are presented as mean±SEM, n=3-4 per group.

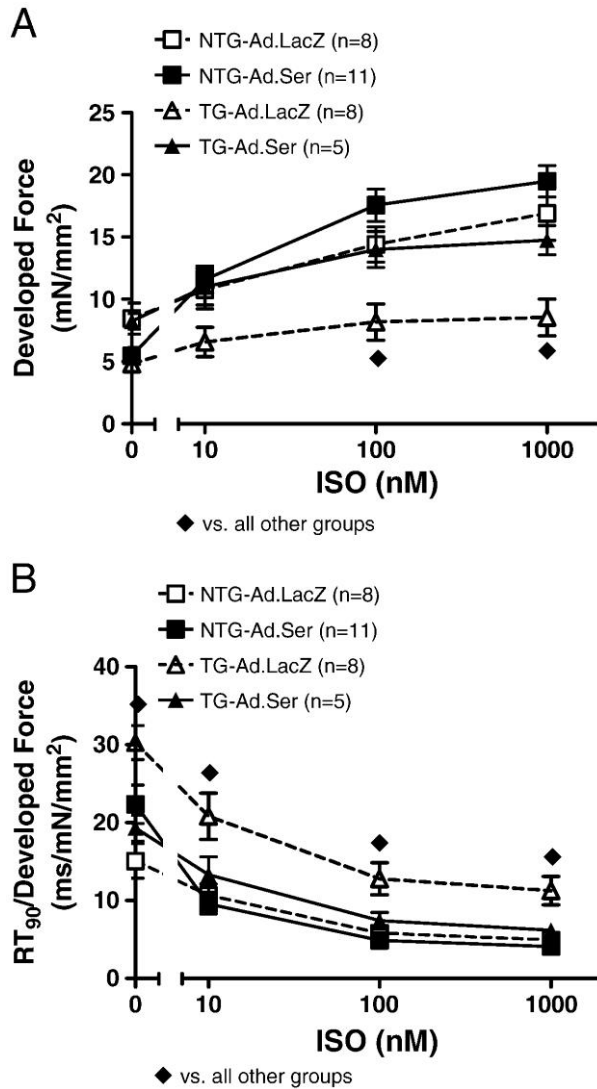


Fig. 2. The effect of isoproterenol (ISO) on developed force and relaxation time in papillary muscles isolated from NTG and TG mice injected with Ad.LacZ or Ad.Ser. (A) Developed force at baseline and during ISO stimulation. (B) Time to 90% of relaxation normalized to developed force at baseline and during ISO stimulation. Data are presented as mean±SEM, n=5–11 per group.

In addition to these data, the investigators noted a decrease in markers of heart failure, such as Atrial Natriuretic Factor, in TG mice injected with Ad.Ser when compared to mice injected with Ad.LacZ.

Describe and interpret the data.

Propose a hypothesis to explain the outcomes presented in Figures 1 and 2.

Based on your knowledge of the role of SERCA in the cardiac myocytes, outline additional experiment to confirm or refute your hypothesis.

The epithelial sodium channel (ENaC) is important for mediating effects of circulating hormones that regulate renal sodium reabsorption. Recent evidence suggests that ENaC is also modulated by local paracrine factors including extracellular nucleotide triphosphates such as ATP. Investigators at the Institute for Salty Science in France examined effects of ATP on ENaC activity in rat cortical collecting duct cells. As the primary index of ENaC activity, the investigators recorded single channel activity and calculated channel open probability (P_o). In these experiments, it was assumed that there were no changes in cell surface expression of ENaC and they observed no changes in single channel conductance. Therefore, higher P_o values correlate with greater ENaC activity. Figure 1 illustrates changes in ENaC P_o that occur within 15 minutes after application of ATP and UTP.

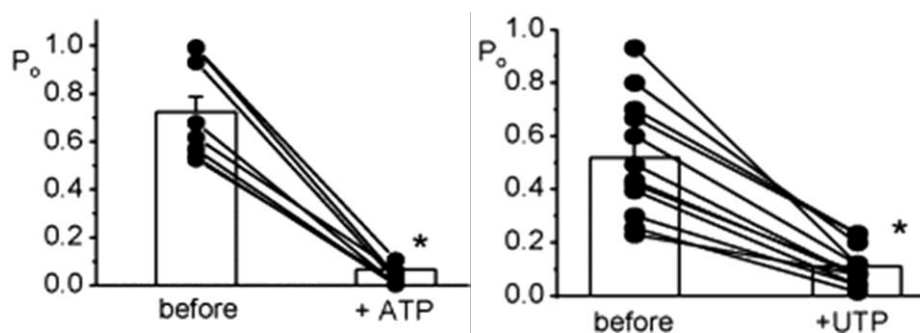


Figure 1 – Open probability (P_o) of ENaC measured before and after extracellular application of ATP or UTP. * $P < 0.01$

Additional experiments were performed to determine if purinergic receptors mediate the effects of ATP. Specifically, mice with homozygous deletion of P2Y2 receptors, a subtype of metabotropic purinergic receptor, were tested (Figure 2).

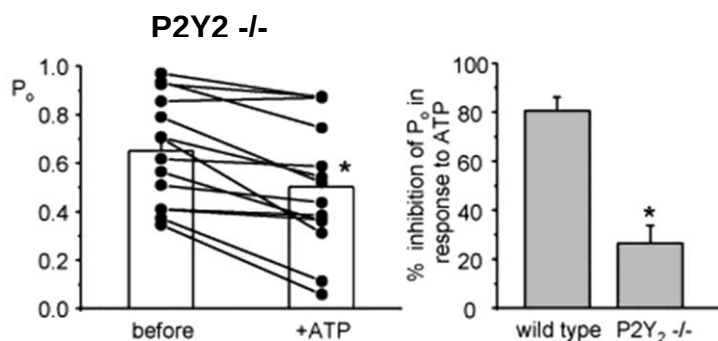


Figure 2 – ENaC P_o before and after extracellular application of ATP in P2Y2 knockout mice ($-/-$) and comparison to the effect in wildtype mice. * $P < 0.01$

1. Summarize the findings of these experiments and propose an alternative to using knockout mice for identifying the receptor(s) mediating the effects of ATP on ENaC.
2. P2Y2 receptors are Gq-coupled. Develop hypotheses to explain how receptor-mediated signaling could affect ENaC and propose experiments to test your ideas.
3. Assuming that the effects of P2Y2 knockout only involve altered ENaC activity, predict the relative level of blood pressure, extracellular volume, renin and aldosterone levels, and urinary excretion of sodium and potassium in these mice as compared to wildtype animals.

D1 dopamine receptors in the striatum are coupled to adenylyl cyclase via $G_{\alpha_{olf}}$, which stimulates it similar to G_{α_s} . A group of scientists is studying the role of $G_{\alpha_{olf}}$ and D1 receptors in drug-induced acute locomotor responses in genetically modified mice. They compare mice carrying both alleles of $G_{\alpha_{olf}}$ ($Gnal^{+/+}$) with hemizygous mice carrying only one allele ($Gnal^{+/-}$), as well as mice with two D1 receptor alleles ($Drd1a^{+/+}$) with hemizygous animals carrying only one D1 receptor allele ($Drd1a^{+/-}$).

Fig. 1 shows effect of the loss of $G_{\alpha_{olf}}$ and D1 receptors on basal and dopamine-stimulated adenylyl cyclase activity in striatal membranes.

Fig. 3 shows locomotor activity in response to direct D1 agonist SKF81259 (D2-specific agonist quinpirole is used as a control), and indirect agonists amphetamine and cocaine.

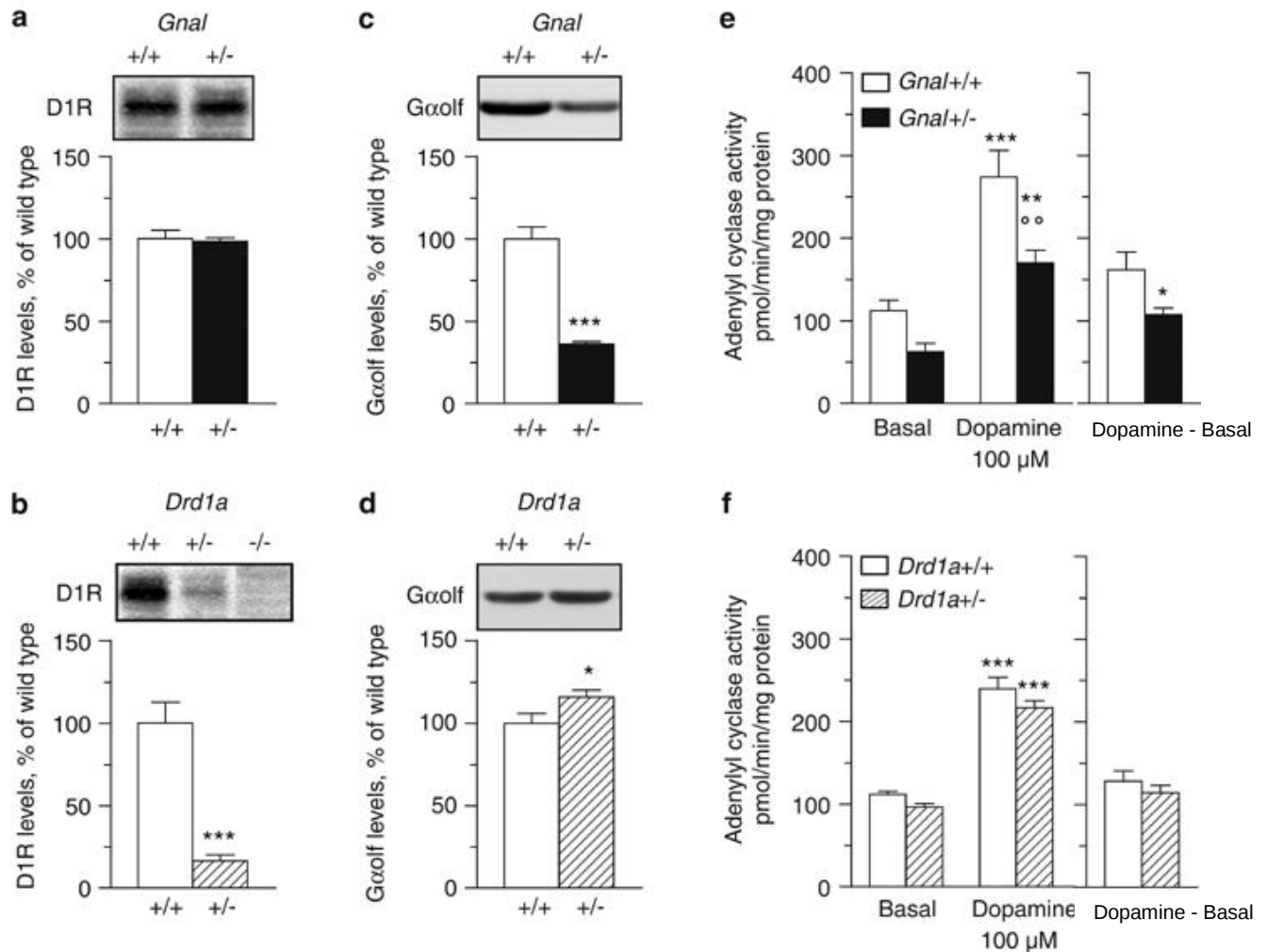


Figure 1. D1R, $G\alpha_{olf}$ levels and AC activity in the striatum of $Gnal^{+/-}$ and $Drd1a^{+/-}$ mice. (a-d) D1R (a, b) and $G\alpha_{olf}$ (c, d) protein levels were measured by immunoblotting in striatal extracts of $Gnal^{+/-}$ ($n=5$, a, c) and $Drd1a^{+/-}$ ($n=6$, b, d) mice and expressed as percentages of the levels found in the striatum of their wild-type littermates ($n=5-6$). Student's t -test: * $p < 0.05$, *** $p < 0.001$. (e, f) AC activity was measured in striatal membranes of $Gnal^{+/-}$ (e) and $Drd1a^{+/-}$ (f) mice and their wild-type littermates in basal condition and with 100 μ M dopamine ($n=5-6$ per group). Two-way ANOVA: $Gnal$ mice, interaction between genotype and treatment $F_{(1, 16)}=1.9$, NS; genotype $F_{(1, 16)}=15.4$, $p=0.001$; treatment $F_{(1, 16)}=47.5$, $p < 0.001$; $Drd1a$ mice, interaction between genotype and treatment $F_{(3, 20)}=0.4$, NS; genotype $F_{(1, 20)}=4.1$, NS; treatment $F_{(3, 20)}=202.0$, $p < 0.001$. Bonferroni post-test: ** $p < 0.01$, *** $p < 0.001$ as compared to saline, ° $p < 0.01$ as compared to wild type. Difference between cAMP formed in basal condition and in the presence of 100 μ M dopamine (delta) was calculated (right panel); Student's t -test: * $p < 0.05$.

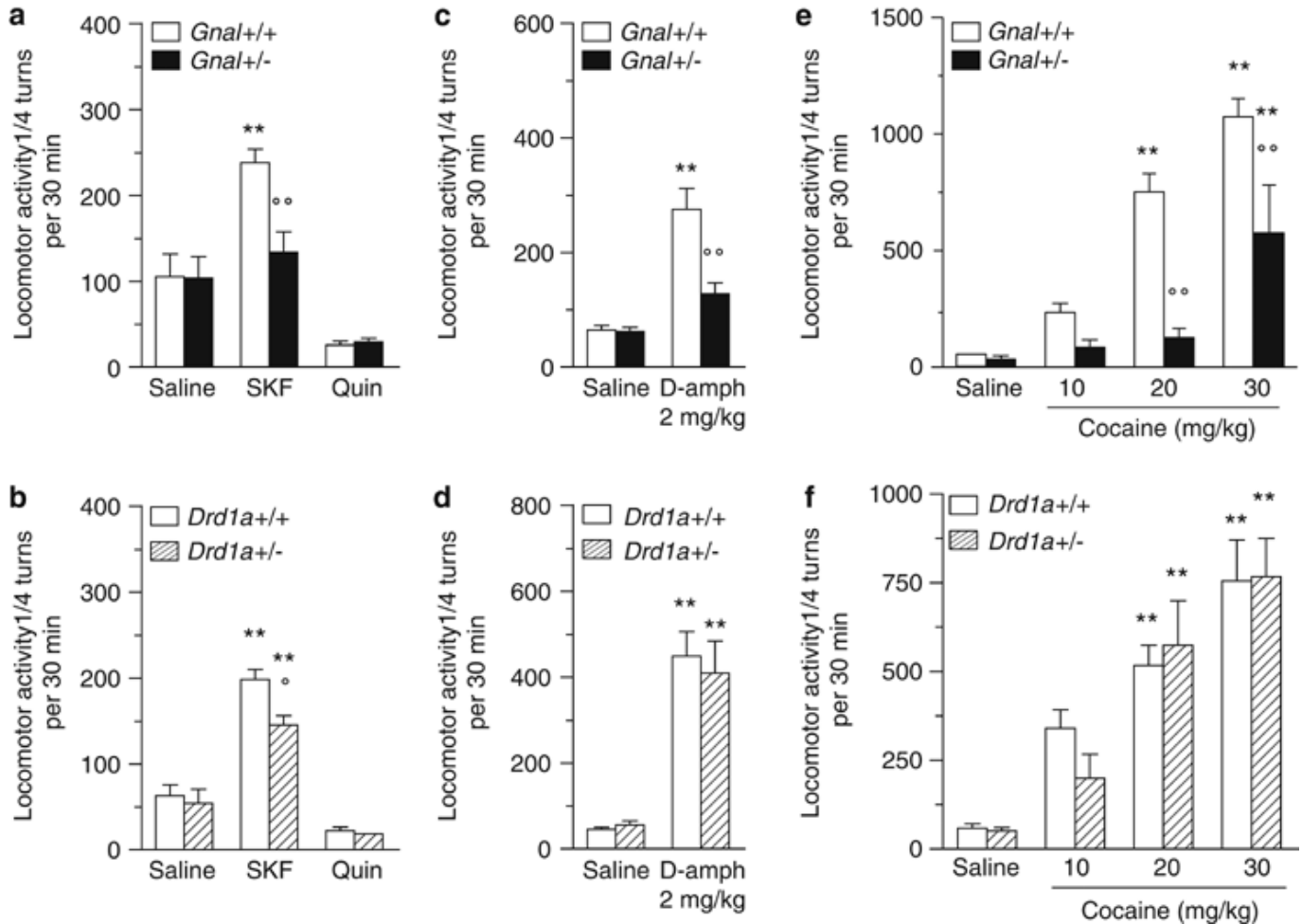


Figure 2. Drug-induced locomotor activity in *Gnal*^{+/-} and *Drd1a*^{+/-} mice. Locomotor activity of *Gnal*^{+/-} (a, c, e) and *Drd1a*^{+/-} (b, d, f) mice and their wild-type littermates were measured after injection of saline, SKF81259 (SKF, 2 mg/kg), or quinpirole (Quin, 2 mg/kg) (a, b), D-amph (2 mg/kg, c, d) and cocaine (10, 20 or 30 mg/kg, e, f). Locomotor activity is expressed as the number of ¼ turns performed by the animals in the circular corridor during the 30 min following injection. Data are means±SEM (*n*=10 per group). Two-way ANOVA: (a and b) interaction between genotype and treatment with dopamine receptor agonists was significant only for *Gnal* mice ($F_{(2, 54)}=5.1$, $p<0.01$); effect of genotype: *Gnal* mice $F_{(2, 54)}=4.8$, $p<0.05$; *Drd1a* mice $F_{(2, 54)}=6.1$, $p<0.05$; effect of treatment: *Gnal* mice $F_{(2, 54)}=34.2$, $p<0.001$; *Drd1a* mice $F_{(2, 54)}=104.1$, $p<0.001$. (c and d) interaction between genotype and D-amph treatment was significant only for *Gnal* mice ($F_{(1, 36)}=11.7$, $p<0.01$); effect of genotype: *Gnal* mice $F_{(1, 36)}=12.7$, $p<0.001$; *Drd1a* mice $F_{(1, 36)}=0.1$, NS; effect of treatment: *Gnal* mice $F_{(1, 36)}=43.0$, $p<0.001$; *Drd1a* mice $F_{(1, 36)}=64.5$, $p<0.001$. (e and f) interaction between genotype and multiple doses of cocaine was significant only for *Gnal* mice ($F_{(3, 74)}=32.0$, $p<0.001$); *Gnal* mice: effect of genotype $F_{(1, 74)}=118.5$, $p<0.001$; effect of cocaine $F_{(3, 74)}=86.24$, $p<0.001$; *Drd1a* mice: effect of genotype $F_{(1, 72)}=0.73$, NS; effect of cocaine $F_{(3, 72)}=30.0$, $p<0.01$. (a-f) Bonferroni post-tests: * $p<0.05$, ** $p<0.01$ as compared to saline, ° $p<0.05$, °° $p<0.01$ as compared to wild type.

1. Reduced expression of which protein reduces dopamine activation of adenylyl cyclase in striatal membranes in vitro?
2. Which protein changes the effects of direct D1 and indirect dopamine agonists on locomotion in vivo?

3. Based on these data, propose signaling pathway from dopamine to locomotion. Can you suggest more than one pathway?
4. Which possible pathway(s) are excluded if you find that D1 (-/-) knockout animals show no dopamine-induced cyclase activation in striatal membranes, and no effects of dopamine agonists on locomotion?
5. Explain why lower expression of one protein in the signaling pathway reduces the response, whereas the reduction of the other does not?