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Studies on U50,488H-induced increases in serum corticosterone level and diuresis in wildtype and mutant mice expressing phosphorylation-deficient kappa opioid receptor

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We previously showed that mice expressing a phosphorylation-deficient kappa opioid receptor mutant (K4A) exhibited reduced U50,488H-induced anti-scratching tolerance in males and reduced conditioned place aversion in females, without changes in acute anti-scratching or hypo-locomotor effects. Here, we examined whether K4A mutations, which markedly diminish β -arrestin-mediated signaling, alter U50,488H-induced increases in serum corticosterone and urine output. K4A and wildtype mice received U50,488H (5 mg/kg, s.c.) or saline. Blood was collected 1 h later and serum corticosterone was measured by ELISA. Urine was collected for 1 h beginning 10 min after injection. U50,488H increased serum corticosterone to similar levels in wildtype and K4A mice of both sexes. U50,488H and nalfurafine enhanced serum corticosterone to similar extents in wildtype mice. Basal corticosterone levels were higher in females than males regardless of genotype. U50,488H significantly increased urine output in both sexes, with no genotype differences. The net increase in urine output was greater in males than females. Thus, KOR phosphorylation and associated β -arrestin-mediated signaling are not required for U50,488H-induced increases in serum corticosterone or diuresis in either sex. These findings also demonstrate, for the first time, that KOR activation produces greater diuresis in male than female mice.

KEYWORDS

diuresis, G protein signaling, kappa opioid receptor, receptor phosphorylation, serum corticosterone

Introduction

Activation of kappa opioid receptor (KOR), coupled to $G_{i/o}$ proteins, produces analgesia, anti-pruritic effect, and water diuresis [1–4]. KOR agonists may be useful for prevention and treatment of substance use disorders [for a review, [5]]. However, KOR agonists also cause sedation, dysphoria, psychotomimesis, and increased corticosteroid levels [1, 2, 6–8], limiting their clinical development.

Activation of G protein-coupled receptors (GPCRs) initiates G proteins-mediated signaling, receptor phosphorylation by GPCR kinases (GRKs), and recruitment of arrestins, leading to receptor regulation and arrestins-mediated signaling. G proteins and arrestins initiate distinct signaling pathways and produce different *in vivo* responses. Biased agonism has attracted

considerable interests, although its validity remains debated [for example, [9–12]]. This concept suggests that G protein- or arrestins-biased agonists may produce therapeutic effects with reduced adverse effects. For KOR, analgesic and anti-scratching effects are generally attributed to G protein signaling, whereas the roles of G protein versus β -arrestin signaling in dysphoria, hypolocomotion, and motor impairment remain controversial [for a review, [13]].

We previously identified U50,488H-induced KOR phosphorylation sites (S356, T357, T363, and S369 in the C-terminal domain) and generated a knock-in mouse line expressing a phosphorylation-deficient KOR mutant (K4A; S356A/T357A/T363A/S369A) [14, 15]. K4A mutations abolished U50,488H-induced KOR phosphorylation in mouse brains, thus eliminating the associated arrestins-mediated signaling. K4A mutations attenuated U50,488H-induced tolerance in anti-scratch effect in males only, and reduced U50,488H-promoted conditioned place aversion (CPA) in females only [15]. In contrast, K4A mutations did not affect acute U50,488H-induced anti-pruritic and hypo-locomotor effects [15]. Whether KOR phosphorylation and related arrestin signaling contribute to U50,488H-induced corticosterone elevation or diuresis remains unknown.

Here, we compared the effects of U50,488H in wildtype and K4A male and female mice on serum corticosteroid and diuresis. A dose of 5 mg/kg (s.c.) was selected for the following reasons. In mice, this dose produces maximal analgesic effects in the second phase of the formalin test and also maximal anti-scratch effects against compound 48/80-induced scratch [16, 17]. In rats, 3 mg and 10 mg/kg U50,488 (s.c.) enhanced diuresis to similar extents [1, 18, 19], and U50,488H elevated plasma corticosteroid levels and at 2.3 mg/kg caused a 3x increase [1]. U50,488H showed similar potencies in wildtype and K4A mice in the anti-scratching test [15].

Materials and methods

Materials

U50,488H was obtained from the NIDA Drug Supply Program (Research Triangle Institute, NC). Filter papers (grade no. 1) were purchased from Advantec MFS (Dublin, CA). A corticosterone ELISA kit (Cat# KGE009) was purchased from R&D Systems (Minneapolis, MN).

Animals

Adult male and female wildtype C57BL/6N mice and homozygous K4A mice on a C57BL/6N background (25–40 g, 5–8 months old) were used. Wildtype mice were purchased from Jackson Laboratory (Bar Harbor, ME). The K4A knockin line was customized generated by homologous recombination (Cyagen, Santa Clara, CA), bred in-house, and genotyped as we described previously [15]. K4A mice were cryopreserved at Jackson Laboratory (#415239, Oprk1tm2.2(K4A) Llh). Mice were housed under standard conditions (22 °C, 50%–60% humidity, 12:12 h light/dark cycle) with food and water *ad libitum*.

Adult male CD-1 mice purchased from Jackson Laboratory were used at about 12 weeks old for comparison of effects of U50,488H and nalfurafine on serum corticosterone levels.

All the procedures were approved by the Temple University Institutional Animal Care and Use Committee (ACUP 4956).

Blood collection and corticosterone determination

Mice were habituated to handling and injection once a day for 3 days. Mice then received U50,488H (5 mg/kg, s.c.), nalfurafine (20 μ g/kg, s.c.) or saline (both 10 μ L/g body weight). One hour later, 50–100 μ L blood was collected from the submandibular vein according to NIH guidelines for survival blood collection.¹ One week later, mice received the alternate treatment in a counterbalanced design. Serum corticosterone was measured using a commercial ELISA kit following published procedures [20, 21]. Serum samples were processed according to the manufacturer's instructions, and absorbance was measured at 450 nm with background subtraction at 550 nm. Concentrations were calculated from standard curves using Prism 10 (GraphPad Software).

Urine output determination

Following ≥ 1 h habituation, mice received U50,488H (5 mg/kg, s.c.) or saline and were returned to home cages for 10 min. Mice were then placed on pre-weighed filter papers in plexiglass cylinders for 1 h. Urine output was determined by filter paper weight gain after removal of feces and normalized to body weight. Treatments were separated by a 72-h washout period.

Data analysis

Data are presented as mean $\pm$ S.E.M. and were analyzed using one-way or two-way ANOVA.

Results

Effects of U50,488H on serum corticosterone levels

Two-way ANOVA revealed a significant main effect of treatment, but no effect of genotype or treatment $\times$ genotype interaction. Thus, U50,488H (5 mg/kg, s.c.) significantly increased corticosterone levels in male and female wildtype and K4A mice, with no genotype differences (Figures 1A,B).

Basal corticosterone levels following saline treatment were compared between sexes (Figure 1C). Two-way ANOVA showed a significant main effect of sex, but no effect of genotype or sex $\times$ genotype interaction. Therefore, female mice exhibited higher corticosterone levels than males. Analysis of U50,488H-induced net increases also revealed no significant effects of genotype or sex.

U50,488H-induced net increases in serum were analyzed (Figure 1D). Two-way ANOVA results showed no significant main effects of sex or genotype, indicating that there are no significant differences between males and females or between wildtype and K4A mice.

We compared effects of nalfurafine and U50,488H on serum corticosterone levels. We found that nalfurafine (20 μ g/kg, s.c.) and

¹ <http://oacu.od.nih.gov/ARAC/survival.pdf>

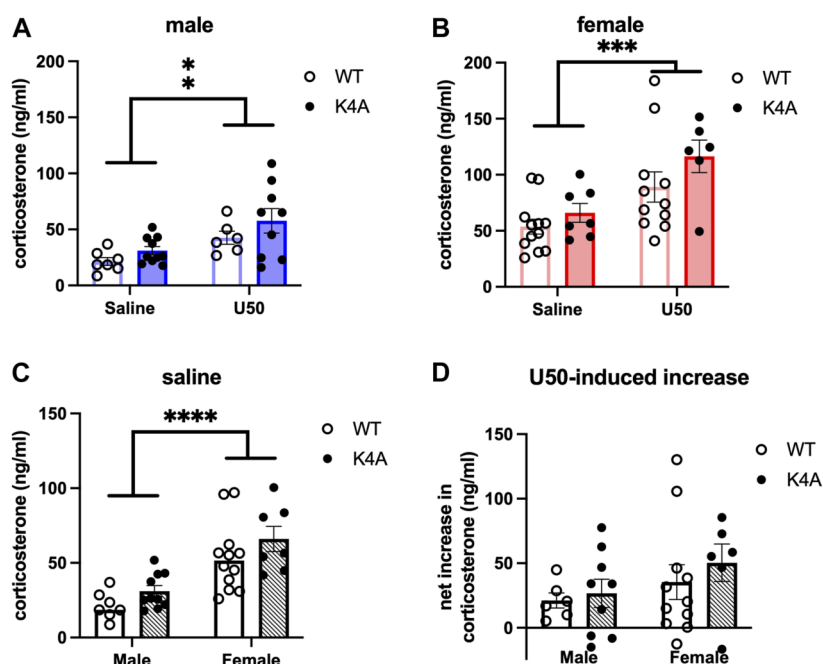


FIGURE 1

U50,488H increased serum corticosterone levels in (A) male and (B) female wildtype and K4A mice and there is no genotype difference. Male and female wildtype and K4A mice were injected with saline or U50,488H (5 mg/kg, s.c.) and blood collected 60 min later. Corticosterone levels in serum were determined with an ELISA kit. Results of 2-way ANOVA analysis showed that there was a significant main effect of treatment in either sex [males, $F(1, 28) = 10.80$, $P = 0.0027$] [females, $F(1, 32) = 13.89$, $P = 0.0007$], but no significant main effect of genotype [males, $F(1, 28) = 2.923$, $P = 0.0984$] [females, $F(1, 32) = 2.964$, $P = 0.0948$] or genotype $\times$ treatment interaction [males, $F(1, 28) = 0.1459$, $P = 0.7053$] [females, $F(1, 32) = 0.4227$, $P = 0.5203$]. Data are expressed as mean $\pm$ S.E.M. ($N = 6$ –12 per group). ** $P < 0.01$, *** $P < 0.001$, compared with saline. (C) Basal serum corticosterone levels were higher in female wildtype and K4A mice than male mice. Data of the saline-treated groups from (A) and (B) were analyzed using 2-way ANOVA, which revealed significant main effects of sex [$F(1, 33) = 29.96$, $P < 0.0001$] but not genotype [$F(1, 33) = 3.247$, $P = 0.0807$]. Thus, female mice had higher basal corticosterone levels than male mice of the same genotype. (D) The U50,488H-induced net increase was similar in both WT and K4A mice, regardless of sex. Data are expressed as mean $\pm$ S.E.M. ($N = 7$ –12 per group). **** $P < 0.0001$, compared with male counterparts.

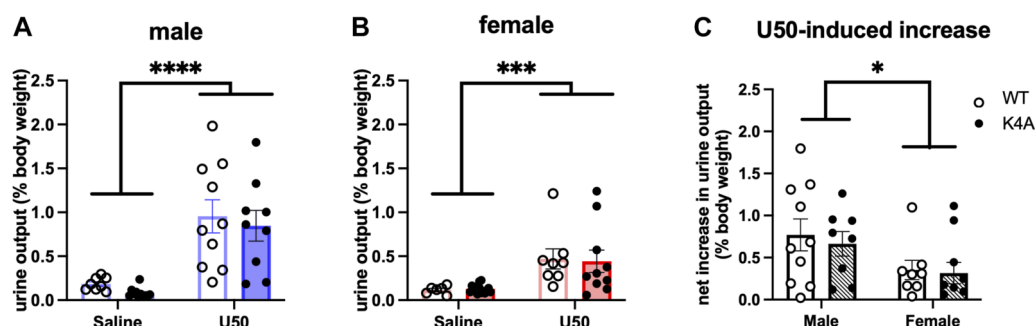


FIGURE 2

In (A) male or (B) female mice U50,488H increased urine output in WT and K4A mice significantly with no genotype differences. Mice were injected with saline or U50,488H (5 mg/kg, s.c.) and measurement of urine output started 10 min after injection for 1 h. Data for males and females were analyzed separately with 2-way ANOVA, which showed significant main effects of treatment [males, $F(1, 34) = 34.16$, $P < 0.0001$] [females, $F(1, 34) = 14.71$, $P = 0.0005$] but no significant main effects of genotype [males, $F(1, 34) = 0.6443$, $P = 0.4277$] [females, $F(1, 34) = 0.006825$, $P = 0.9346$] or treatment $\times$ genotype interaction [males, $F(1, 34) = 0.0002200$, $P = 0.9883$] [females, $F(1, 34) = 0.04719$, $P = 0.8293$]. Data are expressed as mean $\pm$ S.E.M. ($N = 6$ –10 per group). *** $P < 0.001$, **** $P < 0.0001$ compared with saline of the same group. (C) U50,488H-induced net increase in urine output is greater in males than in females. Net increases were calculated by subtracting the mean of each saline group from individual values of the same sex-genotype group. Data were analyzed with 2-way ANOVA, which shows a significant main effect of sex [$F(1, 32) = 6.276$, $P = 0.0175$] but not genotype [$F(1, 32) = 0.2262$, $P = 0.6376$] or sex $\times$ genotype interaction [$F(1, 32) = 0.05151$, $P = 0.8219$]. Data are expressed as mean $\pm$ S.E.M. ($N = 8$ –10/group). * $P < 0.05$ between males and females.

U50,488H (5 mg/kg, s.c.) increased corticosterone to similar extents (Supplementary Figure S1). Nalfurafine was administered at 20 µg/kg (s.c.) as at this dose it produced maximal analgesic and anti-scratch effects (ED₅₀ values of 5.8 mg and 8.0 mg/kg, respectively) [16].

Effects of U50,488H on urine output

Two-way ANOVA revealed a significant main effect of treatment, but no effect of genotype or treatment × genotype interaction. Accordingly, U50,488H (5 mg/kg, s.c.) significantly increased urine output in both sexes regardless of genotype (Figures 2A,B).

Net increases in urine output were calculated by subtracting the mean saline value from each U50,488H-treated data point. Two-way ANOVA showed a significant main effect of sex, but no effect of genotype or sex × genotype interaction (Figure 2C). Thus, U50,488H increased urine output to a greater extent in males than females, independent of genotype.

Discussion

This is the first study to examine the role of KOR phosphorylation in KOR agonist-induced increases in serum corticosterone levels and urine outputs. U50,488H increased serum corticosterone levels and urine outputs similarly in wildtype and K4A mice of both sexes. Females exhibited higher basal corticosterone levels, whereas males showed greater U50,488H-induced net increases in urine output independent of genotype. These findings indicate that KOR phosphorylation and the subsequent β-arrestin-mediated signaling are not involved in U50,488H-induced increases in serum corticosterone or diuresis, which are likely mediated by G protein signaling.

Signaling underlying U50,488H-induced increases in serum corticosterone

We found that nalfurafine (20 µg/kg, s.c.) elevated serum corticosterone to similar extents as U50,488H (5 mg/kg, s.c.). We showed previously that treatment of mice with U50,488H (5 mg/kg, s.c.), but not with nalfurafine (20 µg/kg, s.c.), promotes KOR phosphorylation in the mouse brain and KOR internalization in the mouse ventral tegmental area [22], suggesting that nalfurafine does not activate the associated β-arrestins-mediated signaling. As both compounds produced similar elevation in serum corticosterone, KOR phosphorylation and internalization, proxies of β-arrestin-mediated signaling, are unlikely to be required. This is consistent with the observation that U50,488H increased serum corticosterone similarly in wildtype and K4A mice. It is noteworthy that *in vitro* studies on functional selectivity of nalfurafine have yielded different results. It was demonstrated to be G protein-biased [23–25], un-biased [16] and β-arrestin-biased [26], likely due to differences in assay conditions.

Signaling underlying U50,488H-induced diuresis

Our findings that KOR phosphorylation is not required for U50,488H-promoted increases in urine outputs is consistent with those of Inan, Lee [18] who showed that in the dose ranges producing effective anti-scratch effects U50,488H (1 mg, 3 mg, 10 mg/kg) and nalfurafine (5 µg, 10 µg, 20 µg/kg) increased urine output dose-

dependently in rats, with similar maximal responses. As mentioned above, we have shown that *in vivo* U50,488H (5 mg/kg), but not nalfurafine (20 µg/kg), promotes KOR phosphorylation in the mouse brain and KOR internalization in the mouse ventral tegmental area [22]. Since both compounds produced effective diuresis, KOR phosphorylation and internalization, proxies of β-arrestin-mediated signaling, are unlikely to be involved.

In contrast to our findings, triazole 1.1 and triazole 187, demonstrated to be G protein-biased *in vitro*, produced diuresis with lower maximal effects than U50,488H [27], suggesting possible β-arrestin involvement. Differences in experimental approaches may account for this discrepancy.

Limitations in using G protein inhibitors *in vivo*

As β-arrestins-mediated signaling is not involved, one may infer that G protein-mediated signaling should be required for U50,488H-induced diuresis and elevated serum corticosterone. However, testing the involvement of G proteins *in vivo* using pertussis toxin (PTX) or small molecule inhibitors presents serious methodological issues. Systemic PTX administration causes severe toxicity in animals, including vascular permeability changes [28], disruption of blood-brain barrier [29] and intense systemic endoplasmic reticulum stress [30]. These effects would confound experimental results. Most of small-molecule drugs, including G protein inhibitors, do not readily cross the blood-brain barrier (BBB) [31]. Treatment of mice with PTX or small molecule inhibitors via intracerebroventricular injections or microinjection into specific brain sites to bypass the BBB introduces invasive surgical manipulations that interfere with behavioral and endocrine baselines.

Similar responses to U50,488H in K4A mice and wildtype

If KOR phosphorylation is involved in receptor desensitization, in theory, K4A mice should show higher responses to U50,488H compared with wildtype mice. However, that is not the case in U50,488H-induced increases in urine output and serum corticosterone. This is reminiscent of our previous finding that K4A mutations did not affect acute U50,488H-induced anti-pruritic and hypo-locomotor effects [15]. It may be because the acute exposure to U50,488H (5 mg/kg) is not sufficient to cause KOR desensitization. Rather, higher doses or repeated exposures may be required. We previously found that 5x exposures to 80 mg/kg, but not 5 mg/kg, U50,488H caused tolerance to the anti-scratch effect of U50,488H [15].

Signaling underlying KOR-mediated *in vivo* effects

Previous studies have demonstrated that KOR-mediated analgesic and anti-pruritic effects are mediated by G protein signaling [for reviews, see [13, 32, 33]], based on findings from mutant mouse lines (GRK3^{-/-}, β-arrestin2^{-/-}, p38α MAPK^{-/-}, K4A), distinct KOR agonists, and G protein-biased agonists. Our data indicate that KOR-induced increases in serum corticosterone and urine output are not mediated by β-arrestins-mediated pathway, and likely involved G proteins. In contrast, mechanisms underlying KOR-mediated side effects, especially CPA,

remain unresolved. Studies using genetic and pharmacological approaches indicate distinct mechanisms for CPA, hyperlocomotion, and motor incoordination. CPA involves GRK-, p38 α mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR), cannabinoid receptor 1 (CB1), and protein kinase C (PKC) activation, but not β -arrestin2. KOR phosphorylation is required for U50,488H-induced CPA in female but not male mice. Several G protein-biased KOR agonists produce antinociceptive and anti-scratch effects with reduced side effects, but non-identical side effect profiles, and some still induce CPA. PKC inhibition impairs novelty-induced hyperlocomotion and reduces motor incoordination, whereas mTOR inhibition, β -arrestin2 deletion, or KOR phosphorylation deficiency does not. Motor incoordination is reduced by PKC inhibition or β -arrestin2 deletion, but unchanged by mTOR inhibition.

Possible sex differences in U50,488-induced increases serum corticosteroids and urine output

That U50,488H increases serum corticosterone in mice is consistent with reports in mice, rats, and humans [1, 34–36]. Most previous studies were conducted in males; here we extend these findings to females. Basal corticosterone levels were higher in females, consistent with previous reports [37, 38]. This sex difference likely reflects reduced negative regulation of the hypothalamic-pituitary-adrenal axis [37, 38] and higher corticosteroid-binding globulin concentrations in females [39]. On the other hand, the net enhancements promoted by U50,488H showed no sex differences.

U50,488H and other KOR agonists increase urine output in rats, dogs, monkeys, and humans, and these effects are blocked by KOR antagonists [1, 34, 40–42][reviewed in [43]]. We extended these findings to mice and females, showing greater U50,488H-induced diuresis in males when normalized to body weight. In contrast, Craft, Ulibarri [19] reported similar responses in male and female rats. The reason for this discrepancy is unclear. KOR agonists have been explored as water diuretics for water retention associated with edema, elevated intracranial pressure, and liver cirrhosis [44–47]. Unlike traditional diuretics, they cause little or no loss of K⁺ and Na⁺ in urine. Potential sex differences in humans warrants further investigation.

Choice of U50,488H treatment intervals

For corticosterone measurements, blood samples were collected 1 h after U50,488H administration and urine output was measured from 10 to 70 min post-injection, based on prior studies of corticosterone, diuresis, and KOR phosphorylation. In rats, blood samples for corticosterone measurements were obtained 1 h after U50,488H administration [1, 18]. U50,488H-induced KOR phosphorylation peaks at 30–60 min [22].

Data availability statement

The raw datasets and statistical code supporting the animal behavior conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher upon reasonable request.

Ethics statement

The animal study was approved by Temple University Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

PZ designed research, performed experiments, analyzed data, revised the manuscript. PH designed research, performed experiments, analyzed data, revised the manuscript. KB performed experiments and analyzed data. SK performed experiments. L-YL-C conceived of study, analyzed data, wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontierspartnerships.org/articles/10.3389/adar.2026.17289/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Effects of U50,488H and nalfurafine on serum corticosterone in mice. Adult male CD-1 mice were treated with U50,488H (5 mg/kg, s.c.) or nalfurafine (20 µg/kg, s.c.). One hour later, blood was collected and serum was separated. Corticosterone levels in the serum were

determined with ELISA. Results are expressed as mean $\pm$ SEM (n = 5/ group). Data were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. ****P < 0.0001, ***p < 0.001, compared with the saline group. ns, not significant between the U50,488H and nalfurafine groups.

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