Sex Difference in κ -Opioid Receptor (KOPR)-Mediated Behaviors, Brain Region KOPR Level and KOPR-Mediated Guanosine 5'-O-(3-[^{35} S]Thiotriphosphate) Binding in the Guinea Pig

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ABSTRACT

We examined whether sex differences in κ -opioid receptor (KOPR) pharmacology exist in guinea pigs, which are more similar to humans in the expression level and distribution of KOPR in the brain than rats and mice. The KOPR agonist *trans*-($\pm$)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]-cyclohexyl)benzeneacetamide methanesulfonate (U50,488H) produced a dose-dependent increase in abnormal postures and immobility with more effects in males than females. Males also showed more U50,488H-induced antinociception in the paw pressure test than females. Pretreatment with the KOPR antagonist norbinaltorphimine blocked U50,488H-induced abnormal body postures and antinociception. In contrast, inhibition of cocaine-induced hyperambulation by U50,488H was more effective in females than males. Thus, sex differences in the effects of U50,488H are endpoint-dependent. We then examined whether sex differences in KOPR levels and KOPR-mediated G protein activation in brain regions may contribute to the observed differences using quantitative in vitro autora-

diography of [^{3} H](5a,7a,8b)-(-)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)1-oxaspiro(4,5)dec-8-yl)benzeacetamide ([^{3} H]U69,593) binding to the KOPR and U50,488H-stimulated guanosine 5'-O-(3-[^{35} S]thiotriphosphate ([^{35} S]GTP γ S) binding. Compared with females, males exhibited more [^{3} H]U69,593 binding in the deep layers of somatosensory and insular cortices, claustrum, endopiriform nucleus, periaqueductal gray, and substantia nigra. Concomitantly, U50,488H-stimulated [^{35} S]GTP γ S binding was greater in males than females in the superficial and deep layers of somatosensory and insular cortices, caudate putamen, claustrum, medial geniculate nucleus, and cerebellum. In contrast, compared with males, females showed more U50,488H-stimulated [^{35} S]GTP γ S binding in the dentate gyrus and a trend of higher [^{35} S]GTP γ S binding in the hypothalamus. These data demonstrate that males and females differ in KOPR expression and KOPR-mediated G protein activation in distinct brain regions, which may contribute to the observed sex differences in KOPR-mediated pharmacology.

Introduction

The κ -opioid receptor (KOPR) is one of three major types of opioid receptors (μ , δ , and κ). Activation of KOPR produces many pharmacological effects including analgesia/antinociception, psychomimesis, dysphoria/aversion, diuresis, antipruritis, and blockade of psychostimulant effects (reviewed

in Liu-Chen, 2004). KOPR is widely distributed in the central nervous system. In the spinal cord KOPRs are found in the dorsal horn, and in the brain KOPRs are enriched in the claustrum, hypothalamus, endopiriform nucleus, neocortex, caudate putamen, and nucleus accumbens (Quirion et al., 1987; Mansour et al., 1988; Quirion and Pilapil, 1991). KOPR expression differs among species, with humans, monkeys, and guinea pigs expressing higher levels of KOPR in the brain than rats and mice (Quirion et al., 1987; Quirion and Pilapil, 1991). The guinea pig is more similar to the human in the distribution of the KOPR in the brain than the rat or mouse. For example, in the guinea pig and human, but not the rat or mouse, KOPR is abundant in the cerebellum and

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ABBREVIATIONS: KOPR, κ -opioid receptor; ANOVA, analysis of variance; AUC, area under the curve; MOPR, μ -opioid receptor; norBNI, norbinaltorphimine dihydrochloride; PAG, periaqueductal gray; U50,488H (U50), *trans*-($\pm$)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]-cyclohexyl)benzeneacetamide methanesulfonate; U69,593, (5a,7a,8b)-(-)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)1-oxaspiro(4,5)dec-8-yl)benzeacetamide; [^{35} S]GTP γ S, guanosine 5'-O-(3-[^{35} S]thiotriphosphate; DA, dopamine; DAT, DA transporter; G/R, [^{35} S]GTP γ S binding/[^{3} H]U69,593 binding.

deep layers (layers V and VI) of the cortex and is found in striosomes (having patchy distribution) within the striatum (Quirion et al., 1987; Quirion and Pilapil, 1991).

In recent years sex differences in KOPR-mediated antinociception have received wide attention. Early studies by Gear et al. (1996a,b) demonstrated that after dental surgery women reported greater analgesic effects than men from the mixed KOPR/ μ -opioid receptor (MOPR) ligands pentazocine, nalbuphine, and butorphanol. However, in experimental human pain models using three types of pain assays (heat, ischemia, and pressure) pentazocine failed to produce any sex difference in analgesia (Fillingim et al., 2004, 2005). To date, no studies have been conducted in humans with selective KOPR agonists because none have been approved for clinical use as analgesics. In most studies in rats, mice, and nonhuman primates, selective KOPR agonists produced more antinociceptive responses in males than females in acute pain assays (Rasakham and Liu-Chen, 2011 and references therein). However, in chronic pain models, the direction of sex differences in the effects of KOPR agonists depends on the pain model used (Binder et al., 2000; Clemente et al., 2004; Elliott et al., 2006a,b).

It has been well documented that KOPR agonists acutely oppose rewarding, locomotor-stimulating, and locomotor-sensitizing effects of drugs of abuse in males (for a review see Shippenberg et al., 2007). Yet, very few studies have been conducted in females (Serhsen et al., 1998; Puig-Ramos et al., 2008). Because the KOPR is significantly involved in regulating the effect of drugs of abuse, it is important to determine whether sex differences in this aspect of KOPR pharmacology exist.

Most preclinical studies of sex differences in KOPR pharmacology have been conducted in the rat and mouse. However, the effects may not completely reflect those in humans because in rats and mice KOPR expression level is lower and the distribution of KOPR in the brain is different from humans. Some studies have been performed in nonhuman primates; however, examining the molecular mechanisms and neural circuitry (e.g., receptor level and distribution) underlying the sex difference is limited in this species (Negus et al., 2002). In this study we examine whether there are sex differences in KOPR-mediated behaviors in guinea pigs. We chose to study guinea pigs for two reasons: 1) this species possesses a similar KOPR expression level and KOPR distribution in the central nervous system to humans and 2) they allow for mechanistic studies to examine molecular, cellular, and circuitry differences between the sexes.

Here, we test the effects of the selective KOPR agonist *trans*-($\pm$)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]-cyclohexyl)benzeneacetamide methanesulfonate (U50,488H) in male and female guinea pigs in three behavioral endpoints: 1) abnormal postures/immobility, 2) antinociception, and 3) inhibition of cocaine-induced hyperactivity. We found sex differences in all three KOPR-mediated behavioral effects in guinea pigs but the direction depended on endpoint of analysis. We therefore tested the hypothesis that the sex differences in KOPR-mediated effects are caused in part by differences in KOPR levels and KOPR-mediated G protein activation in brain regions involved in these behavioral responses. We used quantitative in vitro autoradiography of [^{3} H](5a,7a,8b)-(-)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)1-oxaspiro(4,5)dec-8-yl)benzeneacetamide ([^{3} H]U69,593) binding to the KOPR (Kitchen et al., 1990) and U50,488H-stimulated guanosine 5'-O-(3-[^{35} S]thio-

triphosphate ([^{35} S]GTP γ S) binding (Sim and Childers, 1997) to assess KOPR levels and KOPR-mediated G protein activation, respectively.

Materials and Methods

Animals

Age-matched adult male and female Dunkin-Hartley guinea pigs (3–6 months) were purchased from Elms Hill Laboratory (Chelmsford, MA). The animals were housed in the Temple University School of Medicine animal facility and maintained on a 12-h light/dark cycle with ad libitum access to food and water. Guinea pigs were housed in same-sex groups of three per cage (54 $\times$ 38 $\times$ 28 cm). Animals were allowed to acclimate to animal facilities for at least 1 week before any handling and experiments. Animals were placed into a standard rat cage (40 $\times$ 20 $\times$ 20 cm), two (same sex) per cage, for transport to and from the animal facility on a covered animal transport cart. Animals were allowed to acclimate to the laboratory for at least 90 min before handling and remained in the laboratory for at least 1 h before returning to the central animal facilities. Animals were used in accordance to methods approved by the Institutional Animal Care and Use Committee at Temple University.

Materials

The KOPR agonist U50,488H, the KOPR antagonist norbinaltorphimine (norBNI), naloxone, and cocaine hydrochloride were kindly provided by the National Institute on Drug Abuse (Bethesda, MD). For injection, U50,488H and cocaine were dissolved in saline. NorBNI was dissolved in water. GDP and GTP γ S were obtained from Sigma (St. Louis, MO). [^{3} H]U69,593 (37.4 Ci/mmol), [^{35} S]GTP γ S (1250 Ci/mmol), and phosphor screens were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). All other reagent-grade chemicals were obtained from Sigma or Thermo Fisher Scientific (Waltham, MA).

Behavioral Procedures

Abnormal Postures and Immobility. Guinea pigs (in same-sex pairs of two) were treated with U50,488H (2.5, 5, or 10 mg/kg s.c.) and then placed back into home cages (40 $\times$ 20 $\times$ 20 cm), monitored, and videotaped for 3 h. From videotapes, animals were scored (0–5) every 5 s based on a Likert rating scale we developed from our initial pilot studies and similar to that reported previously by Brent and Bot (1992) (see Table 1).

Under normal conditions, guinea pigs generally display a huddled body posture. After U50,488H treatment animals display marked changes in body postures that include a relaxed elongation of their abdomen, extension of their forelimbs and/or hind limbs (a feature known as splayed limbs), and immobility. The degree and number of changes we observed were used to qualify each score in the rating scale (Table 1). The lowest score of 0 is indicative of no changes

TABLE 1
Abnormal posture/immobility rating scale

Score	Description
0	No body elongation, no leg splayed (control animals)
1	One leg is splayed forward or sideways; body is slightly elongated
2	Two legs are splayed forward or sideways; body is moderately elongated
3	More than two legs are splayed forward or sideways; body is totally elongated; the animal does not move, except for head movement
4	More than two legs are splayed forward or sideways; body is totally elongated; the animal does not move at all
5	More than two legs are splayed; body is totally elongated; the animal does not move at all; hind legs and/or joints are splayed backward

observed (normal body posture), and the highest score of 5 is indicative of the greatest postural changes observed, in which more than two limbs are splayed, hind limbs are extended backward, body is fully elongated, and there is no movement of any body parts including the head. The total (summation) of scores in 1-min intervals (maximum 60) was calculated for each animal.

Data Analysis. Because of the use of an ordinal scale to measure abnormal postures we did not perform parametric statistical analysis on this data set. However, we calculated an arbitrary area under the curve (AUC) value to determine the magnitude of effect of drug treatment over time (Prism 3.0; GraphPad Software, Inc., San Diego, CA). Comparisons between groups were made using a two-way analysis of variance (ANOVA) with sex and drug dose as main factors. Significant effects ($p < 0.05$) were further analyzed using Bonferroni post hoc tests.

Antinociception: Paw Pressure Test. Antinociception was measured using the paw pressure test because this test has been used previously in guinea pigs (Hayes et al., 1987). The nociceptive threshold was determined with an analgesymeter (Ugo Basile, Milan, Italy) using two 75-g loads with a scaling range from 0 to 14. Guinea pigs were habituated to extend one of the hind paws steadily into a pin that applied "pressure" onto the paw. The load capacity that elicited a response from the guinea pig was recorded. Habituation was performed once a day for 3 days. The response of the guinea pig was determined subjectively, and it was essential for each complete test to be performed by a single operator who was unaware of the drug treatment. The typical endpoint response was a marked increase in muscle tone in the whole body, squeak, and/or a withdrawal of the paw. Four baseline readings were taken before drug treatment in 10-min intervals (−30, −20, −10, and 0 min). After drug treatment nociceptive thresholds were determined in 15-min intervals for up to 90 min and then at 120 min post-treatment.

Data Analysis. Repeated-measures three-way ANOVA (SPSS version 19; SPSS Inc., Chicago, IL) was used to determine statistical significant differences between groups, with drug treatment, sex, and time as main factors. Significant differences ($p < 0.05$) were further analyzed using Bonferroni post hoc tests. AUC values of analgesymeter score/time were calculated and analyzed using a two-way ANOVA with sex and drug treatment as main factors. Significant effects were further analyzed by Bonferroni post hoc tests.

Locomotor Activity. Locomotor activity (ambulation and stereotypy) was measured using an automated Digiscan D Micro System (Accuscan Instruments, Inc., Columbus, OH) as described previously (Huang et al., 2009). The chambers were equipped with infrared beams connected to the Digiscan D Micro System software, which recorded successive beam breaks as ambulation counts and bouts of repetitive beam breaks as stereotypy counts. In brief, animals were placed into locomotor chambers and habituated for 30 min and then injected with either saline or 1 mg/kg U50,488H subcutaneously. Thirty minutes later, when U50,488H effects peaked for antinociception and postural changes, animals were treated with saline or 20 mg/kg cocaine intraperitoneally and monitored for an additional 90 min. In experiments to test the effects of U50,488H on habituation (exploratory behavior), animals were injected with saline or 1 mg/kg U50,488H and placed back into their home cages, then 15 min later placed into locomotor chambers, and activity was monitored for an additional 30 min.

Data Analysis: U50,488H Inhibition of Cocaine-Induced Hyperlocomotor Activity. Total ambulation and stereotypy counts measured in the 90-min period after saline or cocaine treatment were analyzed using two-way ANOVA with sex and drug treatment as main factors. Significant effects ($p < 0.05$) were further analyzed using Bonferroni post hoc tests.

Data Analysis: U50,488H Effects on Habituation. Total ambulation and stereotypy counts in the 30-min observation period were analyzed by two-way ANOVA with sex and drug treatment as main factors.

Vaginal Smears

Determination of phases of the estrous cycle by vaginal smears was performed following the methods of Lilley et al. (1997) and Nelson et al. (1982) with modifications. Vaginal smears were prepared daily between 9 AM and noon for 60 days. Smears were collected from the vaginal epithelium by gently inserting a moist, sterile swab less than 1 cm into the vagina and rotating it once or twice against the vaginal wall. The swab was immediately rolled onto a microscope slide, and the dry smears were fixed with methanol for 10 min, rinsed with water, and stained for 1 h or overnight in 2% hematoxylin stain (CS400–1D; Thermo Fisher Scientific) and examined under a light microscope. Images were evaluated according to the cytological findings of Lilley et al. (1997) for determination of stages of the estrous cycle of guinea pigs.

Autoradiographic Experiments

In Vitro Autoradiography of [³H]U69,593 Binding to the KOPR. These experiments were performed as described previously (Kitchen et al., 1990; Slowe et al., 1999). Animals were killed by decapitation, and brains were removed and immediately immersed in isopentane maintained at −35°C on dry ice. Coronal sections (20 μ m) were cut on a cryostat (Leica CM3050 S; Leica, Wetzlar, Germany) maintained at −20°C, thaw-mounted onto gelatin-subbed slides, and stored at −80°C until processed as described below. Sections were incubated with 5 nM [³H]U69,593 in 50 mM Tris-HCl buffer, pH 7.4, for 1 h at 25°C. Nonspecific binding was assessed in the presence of 10 μ M naloxone. Slides were then rinsed three times (2 min each) in cold 50 mM Tris-HCl buffer, pH 7.4, and once (30 s) in deionized H₂O. Slides were dried under a cool stream of air and exposed to tritium-sensitive phosphor screens for 3 weeks in cassettes along with [³H]microscale (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for calibration of results. Brain regions were defined according to the mouse brain atlas of Paxinos and Franklin (1997).

In Vitro Autoradiography of U50,488H-Stimulated [³⁵S]GTP γ S Binding. [³⁵S]GTP γ S binding autoradiography was performed as described previously (Sim et al., 1995; Sim and Childers, 1997). Sections were rinsed in [³⁵S]GTP γ S binding buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4) containing 2 mM GDP for 15 min at 25°C. The sections were then incubated for 2 h at 25°C in [³⁵S]GTP γ S binding buffer with 0.04 nM [³⁵S]GTP γ S and 2 mM GDP, with and without the κ agonist U50,488H (10 μ M). Sections were then rinsed twice (2 min each) in cold 50 mM Tris-HCl buffer, pH 7.4, and once (30 s) in deionized H₂O. Sections were dried thoroughly and exposed to phosphor screens for 48 h in cassettes along with [¹⁴C]microscales (GE Healthcare) for densitometric analysis. Brain regions were defined according to the mouse brain atlas of Paxinos and Franklin (1997).

Analysis of Autoradiography Data. Radioactive images captured on phosphor screens were visualized using a Cyclone Storage Phosphor Scanner (PerkinElmer Life and Analytical Sciences), and data were analyzed using the OptiQuant program. For [³H]U69,593 binding, nonspecific binding was subtracted from total binding, and the resultant values represent specific [³H]U69,593 binding in fmol/mg as determined using [³H]standards. For [³⁵S]GTP γ S autoradiography, images were quantified by densitometric analysis with [¹⁴C] standards. A brain paste standard assay was performed to determine the correction factor necessary to calculate nCi/g for [³⁵S] from nCi/g for [¹⁴C] as described previously (Sim and Childers, 1997). Net agonist-stimulated [³⁵S]GTP γ S binding was calculated by subtracting basal binding (obtained in the absence of agonist) from agonist-stimulated binding. Data are reported as mean values $\pm$ S.E.M of data from six animals, with five sections in each brain. Statistical significance of the data were determined by independent two-tailed Student's *t* test using Prism 3.0.

Results

Sex Difference in Abnormal Postures/Immobility Induced by KOPR Activation. Activation of the KOPR is known to produce sedation and reduce locomotor activity. Because guinea pigs display low levels of baseline activity, we found that assessing reduced activity by U50,488H in locomotor chambers yielded little information. During habituation, exploratory movement scores of guinea pigs are low and the animals habituate very quickly (see Figs. 3 and 4). However, in our pilot studies we observed that administration of U50,488H to guinea pigs produced a peculiar effect on their body posture (e.g., splayed limbs and body elongation) that occurred concomitantly with immobility. Immobility can be viewed as a sign of sedation. Based on the changes in abnormal postures and immobility we observed, we developed a scoring system consisting of a rating scale between 0 and 5 (Table 1), which is similar to that of Brent and Bot (1992). We then compared the effects of U50,488H between male and female guinea pigs. Age-matched adult guinea pigs were injected with saline or U50,488H (2.5, 5, and 10 mg/kg s.c.) at 0 min, placed into home cages, and videotaped. Their posture changes were scored every 5 s, and results are shown as score/min by adding up the scores from each 5-s bin (maximum 60). Figure 1, A and B, shows the time course of effects of three doses of U50,488H. U50,488H caused a dose-dependent increase in posture changes (leg splaying, body elongation) and immobility in both male and female guinea pigs. In males the effect of U50,488H (5 mg/kg) was blocked by pretreatment with the KOPR antagonist norBNI (5 mg/kg i.p.), which was administered 18 h before U50,488H. The time point chosen for norBNI pretreatment is based on reports of its delayed and long-lasting antagonist actions at KOPR (Endoh et al., 1992). These results indicate the effects are mediated by the KOPR.

To determine the magnitude of effect, we calculated the AUC of the score/min data from Fig. 1, A and B, which are shown in Fig. 1C. Two-way ANOVA of AUC values revealed the main

effects of sex ($F_{1,43} = 79.55$; $p < 0.0001$), dose ($F_{2,43} = 132.90$; $p < 0.0001$), and an interaction sex $\times$ dose ($F_{2,43} = 17.01$; $p < 0.001$). Bonferroni post hoc test revealed that the AUC values in males treated with U50,488H at 5 and 10 mg/kg were significantly higher than females at the same dose ($p < 0.05$). norBNI pretreatment significantly reduced the magnitude of effect of U50,488H ($p < 0.05$) (Fig. 1C).

Sex Difference in KOPR-Mediated Antinociceptive Effects. We examined antinociception in the guinea pig using the paw pressure test, a mechanical pain assay previously used by Hayes et al. (1987). We found that males (Fig. 2A) showed more responses to the same dose of U50,488H than females (Fig. 2B). Lower doses of U50,488H (2.5 and 1 mg/kg) were used because at 2.5 mg/kg there was minimal postural change/immobility in both males and females and there was no sex difference. Data analysis by a three-way ANOVA (sex $\times$ dose $\times$ time) with time as a repeated measure revealed a significant difference between sexes ($F_{1,49} = 61.835$; $p < 0.001$), dose ($F_{2,47} = 11.770$; $p < 0.001$), and time ($F_{10,470} = 18.429$; $p < 0.001$) and a significant interaction between sex $\times$ dose ($F_{2,47} = 0.643$; $p = 0.05$). Results of Bonferroni post hoc tests showed significant increases in analgesymeter scores in males at 15 and 30 min after treatment with 1 mg/kg U50,488H and 15, 30, 45, and 60 min after 2.5 mg/kg U50,488H. In females, only 2.5 mg/kg U50,488H produced significant antinociceptive effects at 15 and 45 min after injection. In addition, in males we found that nor-BNI (10 mg/kg) pretreatment blocked the antinociceptive effects of 2.5 mg/kg U50,488H observed at 45 and 60 min.

AUC values of data from Fig. 2, A and B were determined to compare the magnitude of effect. As shown in Fig. 2C, males showed a significant antinociceptive response to U50,488H at 1 and 2.5 mg/kg, whereas females displayed antinociception only at 2.5 mg/kg. Specifically, two-way ANOVA revealed a main effect of sex ($F_{1,48} = 66.71$; $p < 0.0001$) and dose ($F_{2,48} = 15.43$; $p < 0.0001$) and an interaction between sex $\times$ dose ($F_{2,48} = 4.33$; $p < 0.05$). norBNI (10 mg/kg) pretreatment blocked the effects of U50,488H (2.5

Fig. 1. Dose-, time- and sex-dependent effect of U50,488H-induced body posture changes/immobility. After injection with U50,488H (2.5, 5, or 10 mg/kg s.c.), animals were placed back into their home cages, monitored, and videotaped for 3 h. For the norBNI experiments, guinea pigs were injected with norBNI (5 mg/kg i.p.) followed by 5 mg/kg U50,488H (subcutaneously) 18 h later. A and B, their postural changes/immobility were observed and scored in 5-s bins based on the rating scale shown in Table 1, and the sum of scores per min was calculated and shown in the graphs for male (A) and female (B) guinea pigs. C, the magnitude of effect, determined by calculating the AUC, is shown. Data are presented as the mean $\pm$ S.E.M. ($n = 8$ and $8-9$ for males and females, respectively). Males (blue bars) showed a significantly greater change after U50,488H administration compared with females (pink bars). **, $p < 0.01$ male versus female at the same dose. U50,488H effects are dose-dependent. ++, $p < 0.01$, 5 mg/kg or 10 mg/kg versus 2.5 mg/kg of the same sex. ^^, $p < 0.01$, 10 mg/kg versus 5 mg/kg of the same sex. norBNI blocked U50,488H-induced changes as indicated by ##, $p < 0.01$, 5 mg/kg U50,488H + norBNI versus U50,488H 5 mg/kg alone of the same sex.

Fig. 2. U50,488H-induced antinociception in the paw pressure test is dose-, time-, and sex-dependent. The paw pressure test was performed as described under *Materials and Methods*. Four baseline nociceptive threshold responses were recorded in 10-min intervals before drug treatment (−30, −20, −10, and 0 min). Immediately after the last baseline measurement, animals were then treated subcutaneously with saline or U50,488H at the indicated doses at time 0, and nociceptive thresholds were determined in 15-min intervals for 90 min and then at 120 min. A and B, data are plotted as the mean $\pm$ S.E.M. analgesymeter scores for male ($n = 8$ –12) (A) and female ($n = 8$) (B) guinea pigs. In males norBNI (10 mg/kg; Δ , dotted line) was given 18 h before U50,488H (2.5 mg/kg). C, mean AUC values ($\pm$ S.E.M.) are shown. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ versus saline. ^, $p < 0.01$ versus saline same sex. +, $p < 0.05$, 2.5 mg/kg U50,488H versus 2.5 mg/kg U50,488H + norBNI at same time point. ##, $p < 0.01$ versus male same dose.

mg/kg) in males. In addition, baseline values, i.e., absolute weight thresholds, were significantly lower in females. These results clearly indicate that male guinea pigs have greater antinociceptive responses to U50,488H than females.

Sex Difference in KOPR Blockade of Cocaine-Induced Hyperactivity. We assessed the effects of U50,488H (1 mg/kg) on cocaine-induced ambulation and stereotypy. We chose to use this dose because 1) at 2.5 mg/kg animals displayed abnormal body postures and immobility that may confound locomotor responses (Fig. 1) and 2) clear sex differences were found in the antinociceptive assay at 1 mg/kg (males > females; see Fig. 2). Cocaine significantly increased locomotor activity (ambulation and stereotypy) in both males and females (Fig. 3). Two-way ANOVA of total ambulation scores revealed the main effects of sex ($F_{1,42} = 6.65$; $p < 0.05$) and drug treatment ($F_{2,42} = 17.78$; $p < 0.001$) and an interaction sex $\times$ drug treatment ($F_{1,2,42} = 7.78$; $p < 0.005$). Cocaine induced a significantly greater increase in ambulation in females than males (Fig. 3A). In addition, in females, but not males, U50,488H pretreatment significantly reduced cocaine-induced ambulation scores.

For stereotypy, two-way ANOVA revealed the main effects of sex ($F_{1,42} = 7.71$; $p < 0.01$) and drug treatment ($F_{2,42} = 18.80$; $p < 0.01$) but no significant interaction sex $\times$ drug treatment ($F_{2,42} = 1.61$; $p > 0.05$). Overall, regardless of drug treatment females displayed significantly more stereotypic behavior than males. Cocaine treatment increased stereotypy in males and females. In addition, U50,488H blocked cocaine-induced stereotypy in both sexes.

In this experiment, we did not include a separate group treated with U50,488H followed by saline because, as discussed above, guinea pigs exhibit very low baseline activity. However, we performed a separate experiment to assess the effects of a low dose of U50,488H on locomotor activity during the short habituation period. Guinea pigs were injected with 1 mg/kg U50,488H 15 min (time point 0; Fig. 4, A and B)

Fig. 3. Effects of U50,488H on cocaine-induced hyperactivity are sex-dependent. Locomotor activity was monitored for 90 min after animals were treated with saline (1 ml/kg) or 20 mg/kg cocaine (intraperitoneally). Each value represents the total ambulation counts (A) or stereotypy (B) measured over the 90-min period in males (filled bars) and females (open bars). Data are expressed as mean $\pm$ SEM ($n = 8$ /treatment group). A, *, $p < 0.05$ saline-cocaine (S+C) or U50,488H-cocaine versus saline-saline (S+S) treatment; ++, $p < 0.01$ female saline-cocaine versus all other groups. B, **, $p < 0.01$, saline-cocaine or U50,488-cocaine (U+C) versus saline-saline; ##, $p < 0.01$, saline-cocaine versus U50,488-cocaine.

Fig. 4. Effects of U50,488H on exploratory locomotor activity. Male and female guinea pigs were injected with saline or U50,488H (1 mg/kg) and 15 min later placed into locomotor chambers for an additional 30 min. A and B, ambulation counts (A) and stereotypy counts (B) were measured. C, total behavioral counts measured in the 30-min time period are shown. Data are expressed as mean $\pm$ S.E.M. ($n = 4$ /group for males and $n = 7$ – 8 /group for females). There are no significant differences between sex or drug effect observed for each behavioral measure ($p > 0.05$), including the locomotor activity in 15 to 20 min (data shown at 20 min) postinjection.

Fig. 5. Autoradiograms of [^{3} H]U69,593 (~ 5 nM) binding to the κ -opioid receptor in selected coronal sections of the male guinea pig brain at five anatomical levels (A–E). Sections were incubated with ~ 5 nM [^{3} H]U69,593 for 1 h at 25°C , washed, dried, and exposed to tritium-sensitive phosphor screens. Nonspecific binding (N.S.) was measured in the presence of $10 \mu\text{M}$ naloxone at the level of A. Exposure time was 3 weeks. frp, frontoparietal cortex; cg, cingulate cortex; cl, claustrum; en, endopiriform nucleus; cpu, caudate putamen; acb, nucleus accumbens; tu, olfactory tubercle; hpc, hippocampus; thl, thalamus; hyp, hypothalamus; amg, amygdala; dg, dentate gyrus; mg, medial geniculate; pag, periaqueductal gray; sn, substantia nigra; ip, interpeduncular nucleus; cb, cerebellum.

before placement into locomotor chambers, and locomotor activity was measured for 30 min (Fig. 4). The male saline group showed a trend of higher stereotypy than the others, but it did not reach statistical significance (Fig. 4B). As shown in Fig. 4C, there were no significant effects of U50,488H on total ambulation and stereotypy counts in males or females, and there was no significant difference between the sexes. For ambulation two-way ANOVA revealed no main effect of sex ($F_{1,19} = 1.34$; $p > 0.05$) or drug treatment ($F_{1,19} = 0.79$; $p > 0.05$) and no significant interaction ($F_{1,1,19} = 0.27$; $p > 0.05$). For stereotypy two-way

ANOVA revealed no main effect of sex ($F_{1,19} = 0.78$; $p > 0.05$) or drug treatment ($F_{1,19} = 2.31$; $p > 0.05$) and no significant interaction ($F_{1,1,19} = 0.12$; $p > 0.05$).

In Vitro Autoradiography of [^{3} H]U69,593 Binding to the KOPR in the Guinea Pig Brain. [^{3} H]U69,593 binding to the KOPR was examined in coronal sections (from rostral to caudal) of the male guinea pig brain at five anatomical levels. Representative sections of [^{3} H]U69,593 binding are shown in Fig. 5. Very high levels of [^{3} H]U69,593 binding were found in the deep cortical layers, claustrum, and endopiriform nucleus with claustrum having the highest level. Deep layers of cortex correspond to layers V and VI (inner 1/3 neocortex) according to Goodman and Snyder (1982b) and the mouse brain atlas of Paxinos and Franklin (1997). High levels of binding were observed in the cortex, caudate putamen, nucleus accumbens, and substantia nigra (Fig. 5, A and D). Moderate levels of [^{3} H]U69,593 binding were found in the diencephalon, including hippocampus, thalamus, hypothalamus, and amygdala (Fig. 5, B and C), and the mid-brain, including medial geniculate nucleus and periaqueductal gray (PAG) (Fig. 5D). [^{3} H]U69,593 binding was also observed in the molecular layer of the cerebellum (Fig. 5E). Our results are consistent with previous reports (Mansour et al., 1988; Unterwald et al., 1991).

Sex Differences in [^{3} H]U69,593 Binding to the KOPR. We compared the levels and distribution of [^{3} H]U69,593 binding to KOPR between male and female guinea pig brains. Representative pseudo-color autoradiograms are shown in Fig. 6. In general, male and female guinea pigs displayed similar regional distribution (Fig. 6). Quantitation of specific [^{3} H]U69,593 binding was obtained by measuring the density of autoradiograms in the regions of interest. Table 2 shows the analysis of specific [^{3} H]U69,593 binding. Males exhibit significantly higher [^{3} H]U69,593 binding than females in the following brain regions: deep layers of somatosensory cortex and insular cortex, claustrum, endopiriform nucleus, periaqueductal gray, and substantia nigra. However, in any other brain regions we did not observe any significant sex difference in [^{3} H]U69,593 binding.

Fig. 6. Pseudo-color autoradiograms of [³H]U69593 (~5 nM) binding to the κ-opioid receptor in coronal brain sections of male and female guinea pig brain. Experiments were performed as described for Fig. 5. Nonspecific (N.S.) binding was determined in the presence of unlabeled naloxone (10 μM). The color bars are pseudo-color representations of black and white screen images in fmol/mg tissue equivalents. Male and female sections were processed in parallel throughout binding experiments and for development of autoradiograms.

TABLE 2
Quantitative autoradiography of [³H]U69,593 binding to the KOPR in male and female guinea pig brains
Quantitation was made from both the left and right sides of each section. Data are the mean ± S.E.M. of six animals, four or five sections from each brain.

Region	[³ H]U69,593 Receptor Binding	
	Males	Females
	fmol/mg	
Cingulate cortex	94.8 ± 5.5	85.8 ± 1.9
Motor cortex		
Superficial layers	76.8 ± 3.8	77.3 ± 4.5
Deep layers	128.5 ± 10.8	127.7 ± 6.6
Somatosensory cortex		
Superficial layers	70.6 ± 3.1	65.4 ± 3.3
Deep layers	176.5 ± 10.6	148.2 ± 3.2*
Insular cortex		
Superficial layers	97.2 ± 3.8	87.8 ± 1.7
Deep layers	270.9 ± 12.8	238.1 ± 6.0*
Caudate putamen	111.0 ± 3.8	110.2 ± 6.1
Clastrum	262.6 ± 10.0	235.8 ± 6.5*
Endopiriform nucleus	270.9 ± 13.1	238.9 ± 4.6*
Nucleus accumbens		
Core	138.3 ± 3.8	139.3 ± 5.1
Shell	189.2 ± 6.2	184.2 ± 7.9
Septal	47.5 ± 2.7	44.9 ± 5.6
Hippocampus	37.4 ± 1.4	34.5 ± 2.4
Amygdala	39.2 ± 4.0	38.2 ± 1.3
Thalamus	20.9 ± 1.2	21.4 ± 0.9
Hypothalamus	39.8 ± 2.8	39.7 ± 3.5
Dentate gyrus	45.4 ± 1.7	40.8 ± 2.3
Periaqueductal gray	41.6 ± 2.1	35.9 ± 1.2*
Substantia nigra	122.4 ± 6.9	99.8 ± 6.2*
Medial geniculate nucleus	41.1 ± 1.6	40.4 ± 1.7
Cerebellum	71.1 ± 7.5	71.2 ± 3.5

* *P* < 0.05 different from male guinea pigs by the nonpaired two-tailed Student's *t* test.

Autoradiograms of U50,488H-Stimulated [³⁵S]GTPγS Binding. Coronal sections from five brain levels were processed for U50,488H-stimulated [³⁵S]GTPγS binding to examine KOPR-mediated G protein activation. Adjacent sections were used for U50,488H-stimulated [³⁵S]GTPγS binding and [³H]U69,593 binding. This experimental design allows direct qualitative and quantitative comparison between receptor binding and activation of G proteins in a given brain region. It is noteworthy that U50,488H-stimulated [³⁵S]GTPγS binding was found to be blocked by pretreatment with norBNI under

Fig. 7. Autoradiograms of U50,488H-stimulated [³⁵S]GTPγS binding in coronal sections of the male guinea pig brain at five anatomical levels (A–E). Sections were incubated with ~0.04 nM [³⁵S]GTPγS and 2 mM GDP, in the absence (basal) or presence of 10 μM U50,488H. Exposure time was 48 h.

identical experimental conditions, indicating a KOPR-specific effect. The neuroanatomical distribution of [³⁵S]GTPγS binding induced by U50,488H paralleled that of [³H]U69,593 binding. Representative sections of U50,488H-stimulated [³⁵S]GTPγS binding are shown in Fig. 7. Very high levels of U50,488H-stimulated [³⁵S]GTPγS binding were observed in deep cortical layers, claustrum, and endopiriform nucleus, and high levels were found throughout the cortex, caudate putamen, and nucleus accumbens (Fig. 7A). However, in the diencephalon and midbrain, lower levels of U50,488H-stimulated [³⁵S]GTPγS

Fig. 8. Pseudo-color autoradiograms of U50,488H-stimulated [^{35} S]GTP γ S binding in coronal sections of male and female guinea pig brain. Experiments were carried out as described in the Fig. 7 legend. The color bars are pseudo-color representations of black and white screen images in nCi/g tissue equivalents. Male and female sections were processed in parallel throughout [^{35} S]GTP γ S binding experiments and for development of autoradiograms.

TABLE 3

Quantitative autoradiography of U50,488H-stimulated [^{35} S]GTP γ S binding in male and female guinea pig brains

Quantitation was made from both the left and right sides of each section. Data are mean $\pm$ S.E.M. of six animals, five sections from each brain.

Region	Basal		Net U50,488H-Stimulated GTP γ S Binding	
	Male	Female	Male	Female
	<i>nCi/g</i>			
Cingulate cortex	1587.1 $\pm$ 59.4	1484.0 $\pm$ 334.4	411.1 $\pm$ 64.1	334.4 $\pm$ 36.6
Motor cortex				
Superficial layers	1854.3 $\pm$ 55.9	1730.6 $\pm$ 87.2	365.1 $\pm$ 61.6	258.7 $\pm$ 24.6
Deep layers	2090.3 $\pm$ 57.8	2006.7 $\pm$ 139.3	577.1 $\pm$ 86.2	441.8 $\pm$ 55.2
Somatosensory cortex				
Superficial layers	1873.5 $\pm$ 51.1	1724.6 $\pm$ 92.0	304.5 $\pm$ 42.1	168.4 $\pm$ 37.5*
Deep layers	2078.6 $\pm$ 50.5	1980.0 $\pm$ 118.7	720.6 $\pm$ 75.9	434.1 $\pm$ 52.2*
Insular cortex				
Superficial layers	1943.6 $\pm$ 79.3	1819.9 $\pm$ 90.5	417.1 $\pm$ 56.4	237.2 $\pm$ 52.9*
Deep layers	2395.4 $\pm$ 74.2	2262.7 $\pm$ 110.4	846.3 $\pm$ 97.4	465.6 $\pm$ 44.8**
Caudate putamen	927.3 $\pm$ 9.9	862.2 $\pm$ 54.1	309.6 $\pm$ 29.9	219.2 $\pm$ 20.6*
Clastrum	2188.8 $\pm$ 136.9	1904.4 $\pm$ 120.2	548.4 $\pm$ 47.7	306.9 $\pm$ 43.7**
Endopiriform nucleus	1485.6 $\pm$ 44.4	1372.2 $\pm$ 36.9	485.7 $\pm$ 80.7	336.7 $\pm$ 46.5
Nucleus accumbens				
Core	888.2 $\pm$ 19.3	824.9 $\pm$ 89.3	352.1 $\pm$ 33.5	294.1 $\pm$ 56.4
Shell	1027.9 $\pm$ 42.7	861.6 $\pm$ 84.2	323.1 $\pm$ 45.8	390.7 $\pm$ 46.8
Septal	964.4 $\pm$ 46.9	897.1 $\pm$ 84.2	220.6 $\pm$ 72.8	175.9 $\pm$ 33.9
Hippocampus	1222.6 $\pm$ 61.8	1213.1 $\pm$ 104.9	68.1 $\pm$ 22.9	48.7 $\pm$ 19.1
Amygdala	928.0 $\pm$ 29.9	813.0 $\pm$ 77.5	126.4 $\pm$ 33.8	64.5 $\pm$ 26.2
Thalamus	688.9 $\pm$ 54.3	675.1 $\pm$ 68.2	44.6 $\pm$ 12.8	52.1 $\pm$ 13.9
Hypothalamus	661.8 $\pm$ 54.7	560.5 $\pm$ 51.6	178.3 $\pm$ 35.1	258.9 $\pm$ 26.6
Dentate gyrus	1042.3 $\pm$ 48.2	1016.8 $\pm$ 83.0	140.6 $\pm$ 20.3	250.9 $\pm$ 35.9*
Periaqueductal gray	541.7 $\pm$ 25.1	538.2 $\pm$ 12.8	171.2 $\pm$ 22.3	125.8 $\pm$ 4.0
Substantia nigra	683.5 $\pm$ 30.7	654.3 $\pm$ 21.6	343.5 $\pm$ 47.3	332.5 $\pm$ 24.9
Medial geniculate nucleus	562.9 $\pm$ 47.8	630.8 $\pm$ 42.4	131.3 $\pm$ 25.0	60.8 $\pm$ 12.0*
Cerebellum	426.8 $\pm$ 28.5	438.0 $\pm$ 10.7	53.9 $\pm$ 7.9	25.2 $\pm$ 8.4*

* $P < 0.05$; ** $P < 0.01$ different from male guinea pigs by the nonpaired two-tailed Student's t test.

binding were observed (Fig. 7, B–D). In addition, U50,488H-stimulated [^{35} S]GTP γ S binding was seen in the molecular layer of the cerebellum (Fig. 7E).

Sex Differences in U50,488H-Stimulated [^{35} S]GTP γ S Binding. U50,488H-stimulated [^{35} S]GTP γ S binding was compared between male and female guinea pig brains. Representative pseudo-color autoradiograms of U50,488H-stimulated [^{35} S]GTP γ S binding are shown in Fig. 8. Males and females displayed similar neuroanatomical distribution of U50,488H-stimulated [^{35} S]GTP γ S binding. Table 3 shows the analysis of basal and net U50,488H-stimulated [^{35} S]GTP γ S binding. There is no difference between males and females in basal levels of binding, but there are large regional differences. U50,488H-

stimulated [^{35} S]GTP γ S binding was significantly greater in males than females in several brain regions, which include superficial and deep layers of somatosensory cortex and insular cortex, caudate putamen, claustrum, medial geniculate nucleus, and cerebellum. In contrast, in the dentate gyrus of the hippocampus, females exhibited significantly higher U50,488H-stimulated [^{35} S]GTP γ S binding than males. In addition, females exhibited a trend of higher levels in the hypothalamus, but it did not reach statistical significance ($P = 0.09$).

Sex Differences in KOPR-G Protein Coupling Efficiency. To explore the sex difference in KOPR-G protein coupling efficiency in different brain regions, we calculated the ratio between net U50,488H-stimulated [^{35} S]GTP γ S

TABLE 4

Ratios of net U50,488H-stimulated [^{35} S]GTP γ S binding to [^{3} H]U69,593 binding (G/R ratios) in male and female guinea pig brains

Data were obtained from both autoradiographic assays (nCi/g for [^{35} S] and fmol/mg for [^{3} H]). Ratios of [^{35} S] to [^{3} H] were calculated from sections of each animal, and data are expressed as mean values $\pm$ S.E.M. ($n = 6$).

Region	Receptor-G Protein Coupling Efficiency	
	Males	Females
	<i>pCi/fmol</i>	
Cingulate cortex	4.3 $\pm$ 0.7	3.9 $\pm$ 0.4*
Motor cortex		
Superficial layers	4.7 $\pm$ 0.8	3.5 $\pm$ 0.5
Deep layers	4.5 $\pm$ 0.6	3.5 $\pm$ 0.4
Somatosensory cortex		
Superficial layers	4.4 $\pm$ 0.6	2.6 $\pm$ 0.6*
Deep layers	4.2 $\pm$ 0.5	2.9 $\pm$ 0.3
Insular cortex		
Superficial layers	4.4 $\pm$ 0.6	2.7 $\pm$ 0.6
Deep layers	3.2 $\pm$ 0.4	1.9 $\pm$ 0.2*
Caudate putamen	2.8 $\pm$ 0.2	2.0 $\pm$ 0.2
Clastrum	2.1 $\pm$ 0.1	1.3 $\pm$ 0.2*
Endopiriform nucleus	1.8 $\pm$ 0.3	1.4 $\pm$ 0.2
Nucleus accumbens		
Core	2.6 $\pm$ 0.3	2.1 $\pm$ 0.4
Shell	1.7 $\pm$ 0.2	2.1 $\pm$ 0.2
Septal	5.3 $\pm$ 1.6	4.4 $\pm$ 0.7
Hippocampus	1.8 $\pm$ 0.6	1.6 $\pm$ 0.6
Amygdala	2.8 $\pm$ 0.5	1.7 $\pm$ 0.7
Thalamus	2.0 $\pm$ 0.5	2.4 $\pm$ 0.7
Hypothalamus	4.6 $\pm$ 0.9	6.3 $\pm$ 0.9
Dentate gyrus	3.1 $\pm$ 0.4	6.0 $\pm$ 1.0*
Periaqueductal gray	4.3 $\pm$ 0.7	3.5 $\pm$ 0.2
Substantia nigra	2.8 $\pm$ 0.4	3.4 $\pm$ 0.3
Medial geniculate nucleus	3.2 $\pm$ 0.6	1.1 $\pm$ 0.4*
Cerebellum	0.8 $\pm$ 0.1	0.3 $\pm$ 0.1*

* $P < 0.05$, different from male guinea pigs by the nonpaired two-tailed Student's t test.

binding to [^{3} H]U69,593 binding (G/R ratio) for each region of both male and female guinea pig brains (Table 4), according to the method described by Sim-Selley et al. (2000). G/R ratios were significantly higher in males than females in superficial layers of somatosensory cortex, deep layers of insular cortex, claustrum, medial geniculate nucleus, and cerebellum. In contrast, in the dentate gyrus G/R ratios were significantly higher in females than males. These data indicate that males and females differ in KOPR-G protein coupling efficiency in some brain regions.

Discussion

We have found that KOPR activation produces more abnormal body postures/immobility and antinociception in male than female guinea pigs, whereas U50,488H reduces cocaine-induced ambulatory activity more effectively in females than males. Thus, the sex difference in behavioral effects of KOPR activation is endpoint-dependent. Using quantitative in vitro autoradiography, we have observed that male guinea pigs exhibit greater [^{3} H]U69,593 binding and U50,488H-stimulated [^{35} S]GTP γ S binding than females in most brain regions examined, whereas females had higher binding in the dentate gyrus. To the best of our knowledge, this is the first comprehensive examination of sex differences in KOPR expression, distribution, and functional activation (via activation of G proteins) in the brain of animals under nonpathological conditions. The sex differences in KOPR expression and activation in brain are potentially relevant to

the sex differences in KOPR-mediated pharmacological effects, which are discussed below.

U50,488H Produces More Abnormal Postures/Immobility in Males than Females. Our observations that U50,488H produces postural changes and immobility are similar to the previous reports of Brent and Bot (1992) and Bot et al. (1992). They showed that in male guinea pigs U50,488 produced initial increased activity followed by mild sedation and the increase in activity was caused by an increase in dystonic-like abnormal body postures, which leads to animals struggling to stand. U50,488H produced greater changes in body postures/immobility in males than females. To the best of our knowledge, our finding is the first demonstration of sex differences in these KOPR-mediated effects. Although it would be of interest to examine sedation-like behavior using motor tasks such as the rotarod, inverted screen, or wheel running assay, assessing performance on such tasks is difficult for the guinea pig because this species lack the agility and motor coordination required for these tasks.

We observed high levels of [^{3} H]U69,593 binding in the deep layers of cortex, consistent with the reports of Goodman and Snyder (1982a) and Unterwald et al. (1991). It has been suggested that the KOPR localized in layers V and VI of the cerebral cortex contributes to the sedative effects of KOPR agonists. We believe that U50,488H-induced immobility is a manifestation of sedation. The significantly higher [^{3} H]U69,593 binding and U50,488H-stimulated [^{35} S]GTP γ S binding in males than females in the deep layers of cortex are likely to play important roles in the greater posture changes/immobility in males induced by U50,488H. In addition, the cerebellum plays an important role in motor movement and body postures (Parent and Carpenter, 1996). We found that U50,488H-stimulated [^{35} S]GTP γ S binding was significantly greater in males than females in the cerebellum, which correlates with the greater postural effects by U50,488H in males than females.

U50,488H Produces More Antinociception in Males than Females. We observed that KOPR activation produced antinociception in the guinea pig, which is in accord with previous reports using the paw pressure test (Hayes et al., 1987) and hot-plate assays (Tao et al., 1994). Our finding that U50,488H produced greater antinociception in male than female guinea pigs is consistent with many acute pain studies in rats, mice, and nonhuman primates (reviewed in Rasakham and Liu-Chen, 2011). However, it should be noted that the direction of sex differences in KOPR-mediated antinociception is not uniform and depends on the species and type of pain model examined (reviewed in Craft, 2008; Dahan et al., 2008; Rasakham and Liu-Chen, 2011). This discrepancy among species and strains may be caused by differences in genetic factors and/or organization and activation of nociceptive neural circuits (reviewed in Rasakham and Liu-Chen, 2011).

We have found that KOPR is localized in many brain areas involved in pain modulation, including PAG, somatosensory cortex, and thalamus, similar to previous reports (Mansour et al., 1988; Zukin et al., 1988; Unterwald et al., 1991). Males exhibited significantly higher [^{3} H]U69,593 binding and/or U50,488H-stimulated [^{35} S]GTP γ S binding in the somatosensory cortex and PAG than females. These differences are likely to contribute to the sex difference in KOPR-mediated antinociception in guinea pigs. Spinal cord was also exam-

ined, but no significant sex differences were found in [^3H]U69,593 binding and U50,488H-stimulated [^{35}S]GTP γS binding (data not shown).

We found that females have lower thresholds in responding to pressure applied to the paw in the absence of U50,488H, compared with males, indicating that females are more sensitive to painful stimuli. This finding is consistent with those of Barrett et al. (2002), who showed that males have a higher threshold for nociception in the paw pressure test. These findings suggest that there is sexual dimorphism in the endogenous pain modulation systems. Our results that males have higher levels of the KOPR in key brain regions involved in nociception, such as somatosensory cortex and PAG, support this notion. In addition, other systems involved in pain modulation have sex differences. For example, male rats have a higher level of MOPR in the ventrolateral PAG compared with cycling females (Loyd et al., 2008). The higher levels of MOPR and KOPR in the PAG suggest higher endogenous opioid tone in males. In addition, there are sex differences in the organization of the descending pain inhibitory pathway from PAG to rostral ventromedial medulla, which may also contribute to the differences in basal pain threshold (Loyd and Murphy, 2009). Sex differences in nonopioid systems, such as *N*-methyl-D-aspartate and melanocortin-1 receptors, are likely to be contributing factors, too.

Cocaine-Induced Hyperactivity and KOPR Blockade of Cocaine-Induced Hyperactivity Are Greater in Females than Males. Our results that females showed greater cocaine-induced enhancement in locomotor activity are consistent with previous observations in several species that females are more sensitive to the behavioral effects of cocaine (Sell et al., 2000; Schindler and Carmona, 2002). It has been demonstrated that psychostimulant-induced ambulatory behavior and stereotypy are mediated by dopamine (DA) signaling in the nucleus accumbens (Sharp et al., 1987) and the striatum (Asher and Aghajanian, 1974). Thus, sex differences in the DA system in these areas may contribute to the observed effects. For example, Walker et al. (2006) found that electrically evoked striatal DA release and dopamine transporter (DAT) activity were greater in females than males. Cocaine enhanced electrically evoked extracellular dopamine concentrations to a greater extent in female striatum than male (Walker et al., 2006). In addition, females have been shown to have a larger number of DAT binding sites in the striatum than males in humans (Lavalaye et al., 2000) and rats (Rivest et al., 1995).

U50,488H was more effective in blocking cocaine-induced ambulation in female than male guinea pigs, whereas U50,488H inhibition of cocaine-induced stereotypy did not differ between sexes. In addition, no sex differences were found in U50,488H modulation of acute exploratory activity. In several studies using male animals, KOPR agonists have been demonstrated to block psychostimulant reward and psychostimulant-induced locomotor activity (Neumeyer et al., 2000; Shippenberg et al., 2007). This is the first report of a sex difference in KOPR-induced inhibition of cocaine-induced locomotor activity. Our results are different from those of Sershen et al. (1998), who reported that spiradoline potentiated locomotor activity in male mice, whereas it had no effect on female mice. The reason for this discrepancy is unclear; however, different species may be an important factor.

Although females displayed more U50,488H-induced inhi-

bition of hyperlocomotion caused by cocaine than males, we found that there were no significant sex differences in KOPR levels or KOPR-mediated G protein activation in the nucleus accumbens and males showed higher levels of KOPR-mediated G protein binding in the caudate putamen. KOPR has been found to colocalize with DAT on presynaptic terminals (Svingos et al., 2001). Acute activation of the KOPR enhances DAT activity (Thompson et al., 2000) and reduces dopamine signaling (Di Chiara and Imperato, 1988). Thus, it is possible that sexual dimorphism exists in the extent of colocalization of KOPR and DAT, which may consequently affect the functional interaction and hence the effect of KOPR activation on cocaine-induced activity.

Sex Differences Are Endpoint-Dependent. We found that the direction of sex differences in KOPR pharmacology was endpoint-dependent, consistent with previous studies (reviewed in Craft, 2008; Rasakham and Liu-Chen, 2011). Similar endpoint-dependent differences have been observed for MOPR-mediated behaviors. MOPR-mediated analgesia and sedation are greater in males than females, whereas reinforcing and locomotor-stimulating effects of MOPR agonists are greater in females than males (Craft, 2008).

KOPR and Prolactin Release. Our findings that there are significantly higher KOPR expression and a trend of higher levels of U50,488H-stimulated [^{35}S]GTP γS binding in the hypothalamus in females than males may provide an anatomical basis for sex difference in KOPR-induced prolactin release. In Long-Evans rats and humans, KOPR agonists induce more prolactin release in females than males (Manzanares et al., 1993; Kreek et al., 1999). Likewise, in rhesus monkey, salvinorin A increases serum prolactin levels in females via KOPR, but has no effect in males (Butelman et al., 2007). It has to be noted, however, that the expression of KOPR is only one of several mechanisms. Indeed, tuberoinfundibular dopaminergic neuronal activity and prolactin-releasing factors are also important factors (Arita and Porter, 1984; Shin et al., 1988).

KOPR Effects on Emotional States and Addiction. The dynorphin/KOPR system has been shown to regulate emotional states. Activation of KOPR produces dysphoria in humans and aversive effects and stress- and depressive-like behaviors in animals (Mucha and Herz, 1985; Pfeiffer et al., 1986; McLaughlin et al., 2003, 2006; Shippenberg et al., 2007). The endogenous KOPR ligand dynorphin A has been found to induce anxiogenic effects in mice (Narita et al., 2006), whereas KOPR antagonists reduce anxiety (Knoll et al., 2007; Bruchas et al., 2009; Wittmann et al., 2009; Carr and Lucki, 2010) and produce antidepressant-like effects (Mague et al., 2003; McLaughlin et al., 2003, 2006; Carr et al., 2010). However, to date studies of KOPR effects on emotional states have been conducted only in males. Our present studies show that [^3H]U69,593 binding and U50,488H-stimulated [^{35}S]GTP γS binding in the deep layers of insular cortex is greater in males than females, whereas in the amygdala, cingulate, or septum there was no sex difference. It has been shown that the insular cortex is one of most consistently identified regions involved in major depressive disorder (Fitzgerald et al., 2008), and insula plays a crucial part in compulsive drug-seeking/taking behavior (Contreras et al., 2008). Thus, it would be interesting in future studies to investigate whether there are sex differences in KOPR modulation of emotional states and addiction.

KOPR in the Claustrum. The highest levels of [^3H]U69,593 binding and U50,488H-stimulated [^{35}S]GTP γ S binding were found in the claustrum, consistent with previous findings (Goodman and Snyder, 1982a; Unterwald et al., 1991). Previous electrophysiological and functional imaging studies suggest that the claustrum is involved in the processing of multimodal sensory information (Crick and Koch, 2005); however, the precise physiological role of the claustrum is not fully understood.

Route of Injection: Intracerebroventricular versus Subcutaneous. Intracerebroventricular injection will be more congruous for correlation of behavioral effects of U50,488H with levels of the KOPR and U50,488H-stimulated [^{35}S]GTP γ S binding in the brain. However, we chose to do subcutaneous injection because it is the most commonly used route for U50,488H administration and it would be easier for relating it to the existing literature.

Antagonism of U50,488H Effects by norBNI. We found that norBNI inhibited the effects of U50,488H-induced postural changes and antinociception in males, indicating that the effects are KOPR-mediated. We chose males to test antagonistic effects of norBNI because males displayed greater responses to U50,488H. In addition, Craft et al. (2001) showed that norBNI blocked the effect of U69,593 equally well in both male and female rats. Because there is no difference in the structure of the KOPR between males and females, norBNI most likely will block U50 effects in females.

Regional Differences in Basal [^{35}S]GTP γ S Binding. There is a marked variation in basal [^{35}S]GTP γ S binding across different brain regions. Basal [^{35}S]GTP γ S binding is greatly influenced by GDP concentration in the assay (Zhu et al., 1997). We demonstrated previously that cell membranes and brain membranes need different concentrations of GDP to have similar levels of basal binding, for example, 10 μM for CHO-HA-rMOR cells and 80 μM for rat brain caudate putamen (Huang et al., 2007). It is conceivable that there is a brain region-specific requirement for GDP. This difference may be related to variations in the levels of guanine nucleotide binding proteins, including trimeric G proteins and many small G proteins. One practical consideration is that we had to use one concentration of GDP in the experiment for all brain regions, which may contribute to varying levels of basal [^{35}S]GTP γ S binding. As a consequence, the net U50,488H-stimulated [^{35}S]GTP γ S binding as a percentage of basal binding is variable across brain regions and is quite low in some regions, which is consistent with the findings of Sim et al. (1996) and Sim-Selley et al. (2000).

Sex and Region Differences in the G/R Ratio. We determined whether the differences in KOPR expression (R), measured with [^3H]U69,593 binding, correlate with the difference in receptor-G protein coupling (G), measured with agonist-stimulated [^{35}S]GTP γ S binding. We found that the G/R ratio, indicative of the relative efficacy of U50,488H, varied among brain regions and between sexes in some brain regions. There is a large G/R ratio range across regions. For example, in the female brain the G/R ratio ranged from 0.3 pCi/fmol in the cerebellum to 6.3 pCi/fmol in the hypothalamus. In general, the G/R ratios are similar in a given brain region between sexes, indicating that each brain region is unique in its KOPR-G protein relationship regardless of sex. However, there are some regions where we did observe sex

differences. These results indicate that U50,488H-stimulated [^{35}S]GTP γ S binding levels do not correlate with receptor binding levels. A similar lack of a close correspondence has been observed between the MOPR and G protein activation (Vogt et al., 2001). Several possibilities may contribute to this lack of correlation. First, KOPR catalytically activate multiple G proteins of different types, with more than one G protein activated per receptor (Prather et al., 1995; Rottmann et al., 1998). The ratio of G proteins to KOPR may vary between sexes for a given brain region and may differ from region to region for a given brain. Second, it is possible that a significant number of KOPR detected by [^3H]U69,593 binding in the present study may not be coupled functionally. Some of these KOPR binding sites may be localized intracellularly and not available for signal transduction (Wang et al., 2008, 2009).

In some of the brain regions examined females had lower G/R ratios than males. The G/R ratio as we calculated should be interpreted with caution, because we did not determine B_{max} of receptors or agonist-activated G proteins. Therefore, it is not equivalent to the amplification factor (Sim et al., 1996; Maher et al., 2005).

Other Possible Mechanisms Underlying the Sex Difference: Drug Distribution, Pharmacokinetics, and Estrous Cycle. Sex differences in KOPR pharmacology may be caused by differences in drug distribution and pharmacokinetics. However, the observation that the direction of difference was not consistent across the three behavioral endpoints excludes this possibility. In addition, Craft et al. (1998) demonstrated that brain distribution after systemic administration of [^3H]U69,593 did not differ between male and female rats.

Guinea pigs have a much longer estrous cycle than rats or mice. Each cycle lasts 10 to 17 days: proestrus, 2 to 4 days; estrus, 9 to 11 h; metestrus, 2 to 4 days; and diestrus 8 to 10 days (Lilley et al., 1997). In the present studies, we did not examine estrous cycle effects because in our preliminary experiments we did not find that guinea pigs in proestrus/estrus differed from those in diestrus in their responses to U50,488H in all of their behavioral responses. In addition, we found minimal variability within the female subjects in all of the endpoints. Because females were housed together in groups of three per cage it is possible that synchronization of their estrous cycle could be an explanation for low variability within the group. However, in our examination of vaginal smears we did not find synchronization of the estrous cycle, consistent with the previous results of Harned and Casida (1972). Moreover, the finding that estrous cycle phase did not affect the pharmacological effects of U50,488 is similar to those of Craft and Bernal (2001) that phases of the estrous cycle of rats account for only 2.6% of the total variance in their behavioral responses to U50,488H in the hot-plate test. Furthermore, in the four core genotype mice U50,488H produced a slightly greater antinociception at 90 min post-treatment in the hot-plate test in XX neonates in comparison to XY neonates, independent of gonadal status (i.e., male or female) (Gioiosa et al., 2008), indicating that sex chromosomes affect KOPR responses. Lastly, acute treatment with U69,593 decreased acute cocaine-induced locomotor activity in ovariectomized females regardless of estradiol replacement (Puig-Ramos et al., 2008), indicating that ovarian hormones do not influence this KOPR effect.

In summary, we found that male guineas have greater responses to the KOPR agonist U50,488H in antinociception and posture changes/sedation-like effects, but females exhibited stronger U50,488H-induced inhibition of hyperactivity after cocaine. Concomitantly, male guinea pigs generally exhibited greater [³H]U69,593 binding and U50,488H-induced [³⁵S]GTP γ S binding than females in the brain regions involved in pain regulation, sedation, posture changes, and emotion responses. In contrast, females exhibited significantly higher U50,488H-stimulated [³⁵S]GTP γ S binding in the dentate gyrus and a trend of higher KOPR activation in the hypothalamus than males, which are in accord with the report that KOPR agonists elicit greater prolactin release in females. Taken together, the present findings provide some mechanistic insights into the observed sex differences in KOPR pharmacology, which is the first such study. In addition, the observations point toward some potential sex differences in KOPR pharmacology that have not yet been explored, including KOPR-mediated effects on emotional responses and addiction to drugs of abuse.

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Authorship Contributions

Participated in research design:, Wang, Rasakham, Huang, Cowan, and Liu-Chen.

Conducted experiments: Wang, Rasakham, Huang, and Chudnovskaya.

Performed data analysis: Wang, Rasakham, and Huang.

Wrote or contributed to the writing of the manuscript: Wang, Rasakham, Huang, and Liu-Chen.

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