Contribution of GIRK2-mediated postsynaptic signaling to opiate and α_2-adrenergic analgesia and analgesic sex differences

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The analgesia produced by inhibitory G protein-coupled receptor agonists involves coordinated postsynaptic inhibition via G protein-coupled inwardly rectifying potassium channels (GIRKs) and presynaptic inhibition of neurotransmitter release through regulation of voltage-gated Ca^{2+} channels. Here, we used mice lacking the GIRK2 channel subunit to assess the relative contribution of these two effector systems to nociceptive processing in male and female mice. Compared with female WT mice, male WT mice exhibited higher pain thresholds and enhanced opioid (morphine) and α_2-adrenergic (clonidine) receptor-induced antinociception in a spinal reflex test. The GIRK2-null mutation reduced the ''pain'' threshold in male but not in female mice, effectively eliminating the sex differences in pain threshold. In addition, deletion of GIRK2 channels in mutant mice largely eliminated clonidine antinociception and significantly decreased morphine antinociception. Furthermore, the more pronounced morphine and clonidine-induced antinociception in male mice disappeared in the GIRK2 mutants. Based on the almost complete loss of clonidine-induced antinociception in the mutant mice, we conclude that it is primarily mediated by postsynaptic α_2-adrenergic receptors. In contrast, the significant residual morphine effect in the mutant mice points to the presynaptic μ opioid receptor as a major contributor to its analgesic action. Finally, our results suggest that the reduced pain responsiveness of male compared with female mice results in part from GIRK2-coupled postsynaptic receptors that are activated by endogenous antinociceptive systems.

There is considerable evidence for significant sex differences in the response to noxious stimuli. For example, pain threshold is typically lower in women than in men (reviewed in ref. 1), and opioid analgesics exhibit differential efficacy in men and women (2–4). Sex differences in pain threshold and opioid analgesic efficacy have also been observed in rodents (4–6). Although the mechanisms underlying these observations are not fully explained, recent behavioral data (7) suggest that differences in opiate signal-transduction mechanisms are contributors to the observed sex differences.

The signal-transduction cascades activated by opioid and other analgesics culminate in activation of both pre- and postsynaptic effectors: (*i*) inhibition of neurotransmitter release, via inhibition of voltage-gated Ca^{2+} channels (8, 9) and activation of voltage-gated K^+ channels on presynaptic terminals (10), and (*ii*) postsynaptic decrease in excitability, via activation of G protein-coupled inwardly rectifying potassium channels (GIRKs; ref. 11). Although each of these mechanisms contributes to the analgesic effects of both endogenous and exogenous analgesics, the relative contribution of pre- and postsynaptic controls has not been determined. In the present study, we addressed these questions in mice that carry the null mutation for the GIRK2 subunit of the GIRK.

There are four GIRK subunit genes in mammals (GIRK1–4; Kir3.1–4); these form either homo- or heterotetrameric channels (12–14). GIRK2 and GIRK4 homotetramers are found in the brain and heart, respectively, but likely represent a minor fraction of the total GIRK channel pool (15, 16). Whereas GIRK4-containing channels are found in the heart and contribute to parasympathetic slowing of heart rate (16), the GIRK2-containing channels are primary postsynaptic effectors in the brain (15).

GIRK2-null mice have a lower seizure threshold, but otherwise they seem normal (17). Most striking in these mice is the complete disruption of slow inhibitory postsynaptic potentials induced by receptors coupled to $G_{i/o}$ (18). However, inhibition of neurotransmitter release (which reflects activation of the same type of G protein-coupled receptors at presynaptic sites) is not altered (18). This selective impairment of postsynaptic signaling in GIRK2-null mice enabled us to assess the relative contribution of presynaptic and postsynaptic mechanisms to the analgesia mediated not only by opioid- but also by α_2-adrenergic receptors. In addition, we present strong evidence that differences in postsynaptic signaling mechanisms are important contributors to the sex differences in the response to opioid and α_2-adrenergic agonists.

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Methods

Mutant Mice. GIRK2 mutant mice (129/Sv × C57BL/6) were produced as described (17). Heterozygous breeding was maintained to generate GIRK2-null mutant and WT littermates. Genetic identity of the offspring was determined by PCR analysis of the isolated tail DNA as described (17). Animals were separated based on sex and litter and housed four to five per cage. Food and water were available ad libitum and the light/dark cycle ratio was maintained at 12/12 h. Experiments were performed between 11 a.m. and 3 p.m. by an experimenter blind to the mouse genotype. All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at the University of California, San Francisco.

Thermal Stimulation. Withdrawal latency on the hot plate was measured in a Plexiglas container in which the floor was heated to 52.5°C. After a mouse was placed in the container, the latency until hind paw licking or jumping was recorded, and the animal was promptly removed from the container. The cutoff (maximum) latency was set to 60 sec to prevent tissue injury. To assess tail withdrawal latency, radiant heat was applied to the animal's tail until a deflection of the tail was observed. The heat intensity was set so that the baseline latencies were 2.0–4.0 sec (the cutoff latency was 10 sec). Data are reported as percentages of the maximal possible effect (MPE). This percentage of the MPE was calculated by using the following formula: (drug latency − baseline latency)/(cutoff latency − basal latency) × 100.

Abbreviations: GIRK, G protein-coupled inwardly rectifying potassium channel; MPE, maximal possible effect; DRG, dorsal root ganglion; AUC, area under the curve.

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Phase of the Estrus Cycle. After the completion of thermal threshold tests, the genitals of the female animals were inspected to assess the phase of the estrus cycle (19).

Persistent Pain. Intraplantar injections of 5% formalin solution (single 10-μl injection per mouse) were used to study the effects of GIRK2-null mutations on persistent pain. The time spent licking, shaking, or favoring the injected paw over 40 min (2-min periods at 5-min intervals) was recorded.

Blood Flow. Laser Doppler Imager (Moor Instruments, Axminster, U.K.) was used to assess the clonidine effects on blood flow. Mice were anesthetized with ketamine/xylazine (60/8.0 mg/kg), and the entire plantar surface of the paw was scanned. Perfusion was recorded at a resolution of 0.2 mm/pixel twice before drug injection and then at 15 and 30 min after injection.

Drugs. Morphine sulfate (Sigma) and clonidine hydrochloride (Research Biochemicals, Natick, MA) were injected s.c. 30 min before thermal threshold testing in doses of 1.0, 3.0, 10, and 30 mg/kg and 0.03, 0.1, 0.3, and 1.0 mg/kg, respectively. For studies using the formalin test, morphine was administered 10 min before formalin.

Immunocytochemistry. Deeply anesthetized mice (sodium pentobarbital, 100 mg/kg) were perfused with PBS followed by a 10% (vol/vol) PBS formalin fixative. The spinal cords and L4–6 dorsal root ganglia (DRG) were removed, postfixed (4 h, the same fixative), and then cryoprotected. The incubation of spinal cord (30 μm) and DRG (12 μm) sections in 5% (vol/vol) normal goat serum (0.1 M PBS with 0.3% Triton X-100 for 30 min) was followed by an overnight incubation in the rabbit anti-GIRK1 or anti-GIRK2 antiserum (Alomone Labs, Jerusalem, Israel) at 1:1,000 to 1:5,000 dilutions. The sections then were processed by using an avidin-biotin horseradish peroxidase procedure (20). To visualize GIRK1 and GIRK2, a nickel-enhanced diaminobenzidine glucose-oxidase reaction (21) was used.

Statistical Analysis. Data are reported as the mean ± SEM. Student's t test and ANOVA were followed by Bonferroni post tests. Significance level was set at 0.05. All data were analyzed and graphed with PRISM V.3.02 for Windows (GraphPad, San Diego).

Results

Baseline Responses. ***Tail-flick and hot plate tests.*** GIRK channels can be activated by either endogenous or exogenous receptor-agonists. Thus, alteration of baseline nociceptive threshold in the absence of GIRK channels would indicate that endogenous antinociceptive mechanisms use these channels to modulate nociceptive signaling. To explore this possibility, we compared baseline thermal nociceptive threshold in WT and GIRK2-null mutant mice by using the tail-flick and hot plate tests (Fig. 1). The tail-flick test measures a spinal reflex, as even spinalized animals withdraw their tails from a noxious heat stimulus (22). In contrast, the hot plate test has an endpoint (licking of a hind paw or jumping) that is integrated at supraspinal levels (22). We found that WT male mice displayed longer latencies to tail withdrawal compared with female mice (Fig. 1*a*); i.e., females were more sensitive to the noxious heat stimulus. This sex difference, however, disappeared in the GIRK2-null mice. The mutation significantly decreased the threshold for tail withdrawal in male mice, but had no effect on female mice. In fact, WT female mice did not differ from GIRK2-null mutants of either sex (Fig. 1*a*). These results indicate that GIRK2-containing channels are required to establish the elevated thermal nociceptive threshold set point in males, suggesting an increased activity of endogenous antinociceptive systems mediated by postsynaptic receptors at the level of the spinal cord.

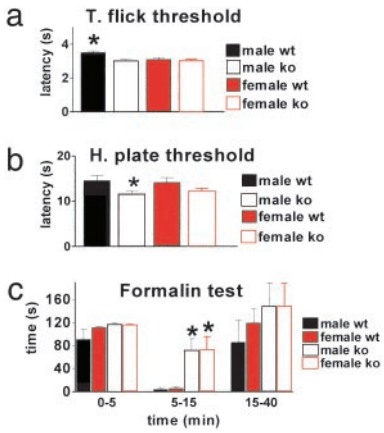

Fig. 1. Baseline threshold tests. (*a*) Tail-flick test responses in male and female WT and GIRK2-null mutant (ko) mice. Between 14 and 19 mice per group were tested. One-way ANOVA ($F_{3,63} = 5.17$) followed by Bonferroni post test indicated that male WT mice exhibited a higher withdrawal threshold than the other three groups of mice (*, $P < 0.05$). (*b*) Hot plate responses in the same mice (one mouse was excluded because of seizures). ANOVA ($F_{3,62} = 2.7$) followed by Bonferroni post test indicated significant differences between male WT and mutant mice (*, $P < 0.05$). (*c*) Pain behaviors elicited by 5% formalin injection into the animals' hind paws. Between four and five mice were tested in each group. One-way ANOVA ($F_{3,16} = 7.131$) analysis of behavior in the normally quiescent interphase (5–15 min after the formalin injection) followed by Bonferroni post test indicated that male and female mutant mice (unlike their WT counterparts) do not exhibit a cessation of pain-related behaviors. (There was no difference in formalin-elicited behaviors between any two groups of mice in either the first or second phase of the response. *, $P < 0.05$ between the WT mice and their respective mutant counterparts.)

In contrast to the tail-flick test, we did not observe any difference in the response latency of WT male and female mice in the hot plate test (Fig. 1*b*.). There was also no significant difference between female WT and mutant mice. However, the response latency of male GIRK2-null mice was significantly shorter compared with their male WT littermates (Fig. 1*b*). Because nociceptive responses in females may vary with their hormonal status (23), we determined the phase of the ovarian cycle in the female mice at the conclusion of each experiment. We did not detect cyclic differences in nociceptive response latencies in either WT or mutant animals; this finding held true for both the tail-flick and hot plate tests of thermal nociception (data not shown). These findings provide evidence for another sex difference in nociceptive processing: GIRK2-containing channel activation by postsynaptic receptors at the supraspinal level may be important for elevation of nociceptive threshold in male but not in female animals.

Formalin test. We used a formalin test to assess the effects of the GIRK2-null mutation on ongoing, persistent pain. WT and mutant animals of both sexes received a 10.0-μl injection of 5% formalin into the left hind paw, and they were then monitored for 40 min for the resulting behavior (favoring, flinching, and shaking of the affected paw). We did not observe any sex differences (Fig. 1*c*) in this test. Interestingly, although both male and female WT mice exhibited the typical two phases of pain behavior [the first phase consisting of an intense period of licking behavior within 5 min of formalin injection followed by a quiescent interphase (between 5 and 15 min after injection), and a second phase consisting of a prolonged (≈40 min) but less intense period of licking behavior], the interphase was abolished in the mutant mice (Fig. 1*c*). We conclude that the interphase of the formalin test results from an active inhibition of pain

behavior, and that it is generated at least in part by GIRK-mediated, endogenous regulatory pathways.

Morphine Antinociception. Next, we investigated the effect of the GIRK2-null mutation on sex differences in the antinociceptive potency of systemic morphine, an opioid that predominantly targets the μ opioid receptor. As Fig. 2*a* shows, in the tail-flick test, the highest dose of morphine (30 mg/kg) elicited the MPE latency (limited to 10 sec to prevent tissue injury) in all mice. At lower doses we observed significant differences among the four groups of mice (male and female animals of the two genotypes). To compare statistically the magnitude of the morphine analgesia, we calculated the area under the dose-response curve (AUC, a measure that integrates the responses across all of the tested doses) for each group (Fig. 2*b*). As expected (based on the sex differences in opioid analgesia reported in ref. 9), WT male mice were significantly more responsive to morphine than were WT female mice. In contrast, we found no difference between the GIRK2-null mutants of either sex, indicating that the mutation more significantly attenuated the response to morphine in male than in female mice. These results suggest that GIRK-mediated postsynaptic signaling mechanisms can completely account for the observed greater efficacy of morphine in male animals. The corollary of this conclusion is that there are prominent presynaptic opiate antinociceptive mechanisms in females and that these mechanisms are completely responsible for the significant residual antinociception observed in the GIRK2-null mutant mice.

The responses to morphine on the hot plate test were qualitatively similar to the responses on the tail-flick test (Fig. 2*c*). We found no difference in the maximal observed responses among groups. However, the comparison of AUCs for all four groups of mice (Fig. 2*d*) revealed that (*i*) morphine produced greater analgesia in male than female WT mice, and (*ii*) the GIRK2-null mutation not only decreased the antinociceptive activity of morphine in both sexes, but also eliminated the sex differences observed with WT animals. Thus, although the GIRK2-mediated postsynaptic effects of morphine contribute to the supraspinally mediated nociceptive responses in both sexes, they seem more relevant in males and can account for the observed sex differences in morphine-mediated antinociception.

Because morphine is a potent inhibitor of pain-related behaviors generated in the formalin test, we used WT and mutant mice to assess the contribution of postsynaptic mechanisms to the antinociception produced by morphine in this test. As we did not observe any sex differences in this test, we studied the effect of morphine only in male WT and mutant mice at doses of 3.0 and 10 mg/kg. These doses were chosen for two reasons: (*i*) they were previously demonstrated to produce antinociception in the formalin test (24), and (*ii*) at these doses, the effect of the GIRK2-null mutation was present in the tail-flick (3.0 and 10 mg/kg) and hot plate (10 mg/kg) tests. Fig. 2*e* illustrates that even the lower dose of morphine inhibited both the first and the second phases of the formalin-elicited responses in both groups of mice (as there was no difference between the sexes, the baseline group includes both male and female mice); the higher dose of morphine resulted in greater inhibition. Because there was no difference in morphine effects between WT and null mutants, we conclude that μ opioid agonists do not depend on postsynaptic signaling for the suppression of persistent pain in this test. Interestingly, both doses of morphine eliminated the quiescent interphase. Thus, it seems that systemically administered morphine, presumably via a presynaptic action, can substitute for the activation of endogenous antinociceptive systems that use GIRKs to generate the interphase in WT mice.

Clonidine Antinociception. To examine whether postsynaptic mechanisms more generally underlie sex differences in antinociception produced by analgesics that activate inhibitory G protein-coupled receptors, we also tested the antinociceptive effectiveness of the systemically applied α_2-adrenergic agonist

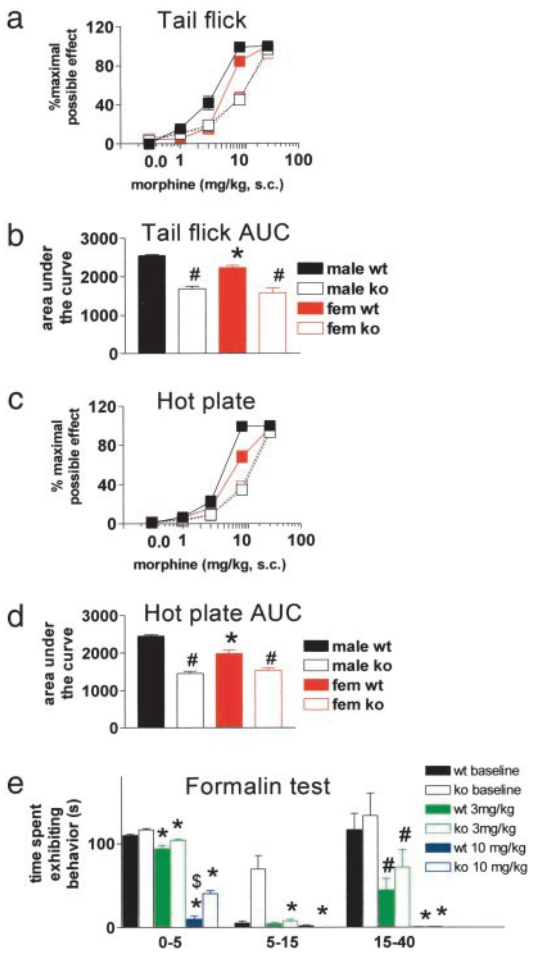

Fig. 2. Effects of the GIRK2-null mutation on morphine antinociception in male and female mice. Between 14 and 19 mice per group were tested. All mice received all of the doses in a randomized order, with a 1-week interval between treatments. (*a*) Morphine dose–response curve generated with tail-flick test (legend is the same as in *b*). (*b*) Comparison of areas under the morphine dose-response curves for WT males, WT females, mutant males, and mutant females. One-way ANOVA ($F_{3,64} = 31.55$) followed by Bonferroni post test indicated a significant difference between male and female WT animals in response to morphine (*, $P < 0.05$). The GIRK2-null mutation decreased antinociceptive effectiveness of morphine in both male and female mice (#, $P < 0.001$, compared with either male or female WT mice). At the same time, the sex difference present in WT mice was eliminated by the null mutation of the GIRK2 gene. (*c*) Morphine dose–response curves generated in the hot plate test. (*d*) Comparison of areas under the dose–response curves for WT males, WT females, mutant males, and mutant females. One-way ANOVA ($F_{3,62} = 35.99$) followed by Bonferroni post test indicated the same morphine response profile as the one in the tail-flick test: (*i*) sex difference in WT animals (*, $P < 0.001$) disappeared in mutant mice; (*ii*) morphine antinociceptive effectiveness in male and female mutant mice was decreased relative to either male or female WT animals (#, $P < 0.001$). (*e*) Effects of morphine on pain-related behaviors elicited by injection of 5% formalin (between five and nine animals per group; because there were no sex differences in the baseline test, male and female animals in the baseline group were combined). Both 3.0 and 10 mg/kg morphine inhibited formalin-elicited first-phase (0–5 min) behaviors (*, $P < 0.001$, compared with the baseline). The 10 mg/kg dose had a greater antinociceptive effect in WT mice ($, $P < 0.001$, compared with the effects observed in mutant mice). Both doses of morphine eliminated the effects of the GIRK2-null mutation in the interphase (5–15 min; *, $P < 0.001$). The 3.0 mg/kg dose of morphine reduced and the 10 mg/kg dose eliminated the second phase (15–40 min) of responses (#, $P < 0.05$ and *, $P < 0.001$, compared with the baseline).

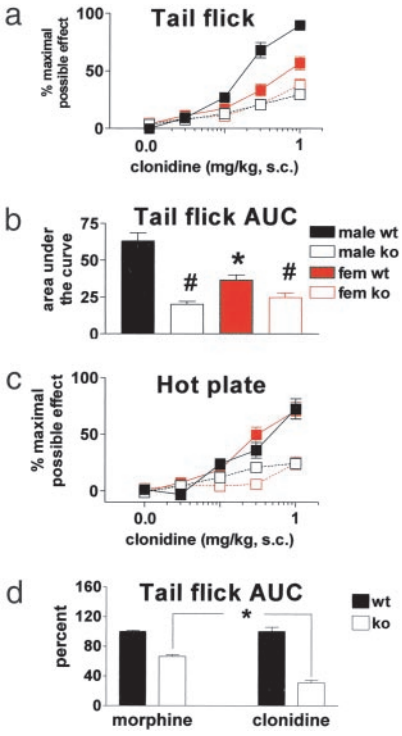

Fig. 3. Effects of the GIRK2-null mutation on clonidine-mediated antinociception. Between 13 and 19 mice per group were tested. (All mice were treated with all of the doses in a randomized order with a 1-week interval between treatments.) (*a*) Clonidine dose–response curve generated by using the tail-flick test. (*b*) Comparison of areas under the dose–response curves for WT males, WT females, mutant males, and mutant females. One-way ANOVA ($F_{3,56} = 24.12$) followed by Bonferroni post test revealed sex differences in clonidine antinociception in WT mice (*, $P < 0.001$ compared with male WT mice). The GIRK2-null mutation decreased clonidine-mediated antinociception (#, $P < 0.05$ compared with WT mice) and eliminated the sex differences. (*c*) Clonidine dose–response curve generated with the hot plate test. (*d*) Comparison of effects of the GIRK2-null mutation on morphine vs. clonidine antinociception. Data are represented as areas under the morphine and clonidine dose–response curves (AUCs) normalized to the MPE observed in WT male animals. Decreases in the antinociceptive effectiveness of each of the two analgesics caused by the GIRK2-null mutation were compared. Two-tailed *t* test indicates significantly greater effect of the mutation on clonidine antinociception (*, $P < 0.001$).

clonidine. Because we did not observe sex differences in clonidine antinociception on the hot plate test (Fig. 3*c*), we concentrated on the tail-flick test (Fig. 3 *a* and *b*). We first determined that clonidine is less efficacious than morphine. This difference is likely caused by the fact that the maximal dose that we could administer was limited by the onset of unacceptable side effects. (At the highest dose administered, 1.0 mg/kg, we observed some degree of piloerection and some decrease in locomotor activity). Despite these limitations, we did observe significant sex differences in clonidine-mediated antinociception. Like morphine, clonidine evoked less total analgesia in female than in male mice (Fig. 3*b*). Furthermore, the GIRK2-null mutation decreased the antinociceptive effectiveness of clonidine in both sexes. Because the effect of the mutation was greater in males, the GIRK2-null mutation, in fact, eliminated the sex differences in clonidine analgesia (Fig. 3*b*). Taken together, these data suggest that, as for morphine analgesia, sex differences in clonidine analgesia arise from sex differences in the postsynaptic inhibitory signaling. In contrast to the results with morphine, the GIRK2-null mutation largely abolished clonidine-mediated antinociception in the tail-flick test in both sexes. Thus, in the mutant animals, the highest dose of clonidine produced only ≈30% of the MPE (compared with 75% of the MPE observed in WT animals). These results suggest that clonidine-mediated antinociception in a spinally mediated pain response (the tail-flick test) results predominantly from activation of postsynaptic signaling mechanisms.

As noted above, the 1.0 mg/kg dose of clonidine produced mild side effects, with no difference between WT and mutant animals. Because increased blood perfusion of a particular body part may contribute to the altered processing of thermal stimuli, and because activation of α_2-adrenergic autoreceptors on the presynaptic terminals of sympathetic postganglionic fibers induces vasodilation via a decrease of sympathetic tone, it was important to assess the effect of the GIRK mutation on blood flow. Thus, we measured peripheral blood flow in the hind paw of anesthetized mice before and after administration of clonidine (1.0 mg/kg). There was no difference between WT and mutant mice under either condition (data not shown), indicating that the GIRK2-null mutation did not affect peripheral blood flow.

Our results demonstrate that the GIRK2-null mutation affected clonidine antinociception to a greater extent than it did morphine antinociception. To compare directly the effects of the mutation on morphine- and clonidine-induced antinociception, we calculated the AUC for each experimental group (morphine WT and null mutants and clonidine WT and null mutants) and expressed it as a percent change from WT animals. Then, we compared the relative decreases in antinociceptive effects of each of the analgesics. Fig. 3*d* illustrates that the GIRK2 gene ablation decreased the effects of clonidine to a greater extent than it did for morphine. In male mice, the antinociceptive effectiveness of clonidine was decreased by 16% and 34% more than the antinociceptive effectiveness of morphine in the hot plate and tail-flick tests, respectively. In female animals, the difference was in the same direction, 8% (tail flick) and 76% (hot plate), indicating that clonidine analgesia, unlike morphine analgesia, primarily depends on postsynaptic signaling mechanisms.

GIRK-Immunoreactivity in Spinal Cord and DRG. The behavioral data suggest that GIRK2-containing channels underlie the observed sex differences in antinociception. Thus, we compared expression levels of the GIRK2 protein in male and female WT mice. As the largest sex differences in antinociception were observed in the tail-flick test, we used an immunohistochemical analysis of spinal cord sections for these comparisons. To confirm the assumption that GIRKs are not expressed in DRG, we also examined lumbar DRG.

Fig. 4*a* shows that GIRK2-like immunostaining was confined largely to the gray matter, with labeling of both neuropil and cell bodies. In both sexes, the most intense staining was in lamina II, at all levels of the spinal cord. This region contains interneurons as well as the terminals of small-diameter nociceptive primary afferents (25). We also observed staining in regions associated with preganglionic autonomic neurons, particularly in the sacral cord (not shown), as well as around the central canal. Most importantly, we found no spinal cord immunoreactivity in the mutant mice, which provides the best evidence for the specificity of the antiserum (Fig. 4*b*). There was no significant difference in the density of immunostaining in male and female mice. In addition, consistent with the absence of opioid-evoked K^+ channel-mediated hyperpolarizing responses in DRG neurons (26, 27), we found no GIRK2-like staining in the DRG (not shown). Finally, although Signorini *et al.* (17) demonstrated that a GIRK2-null mutation can decrease the immunostaining for the GIRK1 subunit of the inwardly rectifying potassium channels in certain brain re-

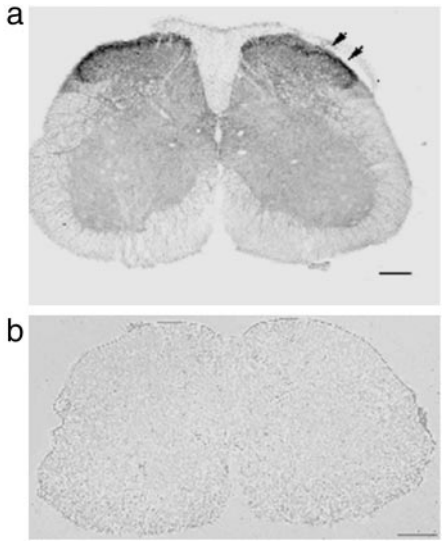

Fig. 4. Transverse section of the spinal cord (from the L5 segment) immunostained with the GIRK2 antiserum (1:2,500), with a nickel-enhanced diaminobenzidine glucose-oxidase reaction. (*a*) Immunostaining in a WT animal. Dark bands of staining (arrowheads) represent GIRK2 immunoreactivity in lamina II. (*b*) Immunostaining for GIRK2 is absent in a mutant animal. (Bar = 0.2 mm.)

gions, we found no difference in GIRK1 staining between the WT and GIRK2 mutant animals.

Discussion

In this study, we report several findings that provide insights into the mechanisms that underlie the analgesia and the analgesic sex differences produced by G protein-coupled receptor system, namely, those targeted by the μ opioid agonist morphine and the α_2-adrenergic agonist clonidine. A major goal of the study was to identify the contribution of postsynaptic effectors to the antinociception elicited by inhibitory G protein-coupled receptor activation. We found that deletion of the major postsynaptic effector, GIRK2 channel, decreases but does not eliminate the analgesic effectiveness of morphine. In fact, the GIRK2-null mutation did not decrease the intrinsic capacity of morphine; higher doses produced maximal analgesic effects, even in mutant animals, regardless of their sex. These data suggest that opioid receptor agonists rely primarily on presynaptic mechanisms (presumably inhibition of transmitter release secondary to reduced Ca^{2+} conductance) for their analgesic effects. In contrast, the GIRK2-null mutation largely abolished the antinociceptive effects of the α_2-agonist, clonidine, indicating that postsynaptic GIRK2-based signaling mechanisms predominate in the analgesia produced by α_2-agonists.

In the present study, we also show that reflex (tail flick) thermal nociceptive thresholds are elevated in male compared with female mice, and that the difference disappears in mice with a deletion of the GIRK2 gene. This finding suggests that there is a tonic, GIRK2-mediated inhibitory control of dorsal horn nociceptive circuitry in male but not in female mice. In addition, we show that the greater efficacy of morphine in males, an observation reported by many laboratories, reflects the GIRK2-mediated component of morphine analgesia. This result likely involves postsynaptic inhibitory control of dorsal horn "pain" transmission neurons. On the other hand, in GIRK2 mutant male and female mice, there is a significant (and comparable) residual antinociceptive effect of morphine, which we suggest is mediated by presynaptic opioid receptors that are not coupled to GIRKs. Finally, as for morphine, we observed a significantly greater antinociceptive effect of clonidine in male mice, which also disappeared in the GIRK2 mutant animals. However, only minimal residual analgesia was observed in the mutant mice. Taken together with the results reported by Blednov *et al.* (28), it seems that this mechanism generalizes to other ligands (ethanol, nicotine, baclofen, oxotremorine, and the WIN cannabinoid agonist) that act via GIRK-coupled receptors to reduce pain behavior.

Luscher *et al.* (18) reported that deletion of the GIRK2 gene eliminates postsynaptic effects of inhibitory G protein-coupled receptor agonists while preserving their presynaptic effects. Based on those results and on the fact that GIRK2 channels seem not to be expressed by primary afferent nociceptors (the present study), we conclude from the behavioral data that GIRK2-based postsynaptic signaling pathways are the major, if not exclusive, contributors to sex differences in morphine antinociception. Those experiments, however, cannot establish whether GIRK2-containing channels themselves are differentially expressed by males and females. To address this possibility, we immunostained tissue from male and female mice but found no difference in GIRK2 immunoreactivity in the spinal cord. It is conceivable that the relevant differences are too small to detect anatomically. Alternatively, GIRK signaling could be profoundly affected by a relatively small decrease in the number of receptors on the cell surface or by a decrease in coupling efficacy upstream to the GIRK channels, because GIRK channel gating requires cooperative binding of multiple (likely four) $\beta\gamma$-G protein subunits (29). Results to date, however, have not found conclusively different levels of opioid receptor expression between male and female animals (30–32).

Although the spinal cord distribution of μ opioid and α_2-adrenergic receptors is comparable and includes dense concentrations in the superficial dorsal horn (33–35) where nociresponsive neurons and the nociceptive afferents that target them are concentrated (25), it seems that clonidine-induced antinociception is primarily mediated by GIRK-based postsynaptic signaling pathways. Thus, it is not surprising that the sex differences were more pronounced for clonidine-induced than for morphine-induced antinociception, as measured by the tail-flick test. Specifically, we found that clonidine was 40% less effective in female than in male mice, compared with a difference of 14% in the morphine-treated mice. The more limited mechanisms through which the α_2-agonist exerts its antinociceptive effect probably underlie clonidine's relatively weak analgesic properties in both animals and humans (in comparison to morphine). Conceivably, a novel therapeutic approach could be developed by combining clonidine with an analgesic that targets primarily presynaptic effectors.

Finally, our results provide an interesting perspective on pain-induced regulatory systems. Specifically, we found that the interphase of the formalin test, a period of inactivity that follows the initially intense period of pain behavior, is lost in the GIRK2-null mutant mice. We conclude that the interphase results from the active inhibition of pain behavior, an idea first proposed by Kaneko and Hammond (36). In effect, this is a feedback inhibition of pain behavior that limits the duration of the intense pain that occurs during the first phase. Our results suggest that the interphase arises from the activation of an endogenous system that uses GIRK-mediated postsynaptic signaling mechanisms to inhibit nociceptive transmission. It is likely that this circuitry is the same one that underlies the GIRK2-mediated tonic regulation of reflex nociceptive processing in male mice.

In conclusion, our results and those of Blednov *et al.* (28) demonstrate that GIRK-mediated postsynaptic signaling pathways are major contributors to the sex differences in the antinociceptive effectiveness of several classes of analgesic agents. Further, our analysis shows that there is a significant difference

in the relative contribution of pre- and postsynaptic signaling to the control exerted by morphine and clonidine. Given that some pain syndromes require multidrug treatments for the control of pain, these studies provide information for rationally based differential treatment approaches in men and women.

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