

Sex chromosome complement affects nociception in tests of acute and chronic exposure to morphine in mice

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Abstract

We tested the role of sex chromosome complement and gonadal hormones in sex differences in several different paradigms measuring nociception and opioid analgesia using “four core genotypes” C57BL/6J mice. The genotypes include XX and XY gonadal males, and XX and XY gonadal females. Adult mice were gonadectomized and tested 3–4 weeks later, so that differences between sexes (mice with testes vs. ovaries) were attributable mainly to organizational effects of gonadal hormones, whereas differences between XX and XY mice were attributable to their complement of sex chromosomes. In Experiment 1 (hotplate test of acute morphine analgesia), XX mice of both gonadal sexes had significantly shorter hotplate baseline latencies prior to morphine than XY mice. In Experiment 2 (test of development of tolerance to morphine), mice were injected twice daily with 10 mg/kg morphine or saline for 6 days. Saline or the competitive NMDA antagonist CPP (3-(2-carboxypiperazin-4yl) propyl-1-phosphonic acid) (10 mg/kg) was co-injected. On day 7, mice were tested for hotplate latencies before and after administration of a challenge dose of morphine (10 mg/kg). XX mice showed shorter hotplate latencies than XY mice at baseline, and the XX–XY difference was greater following morphine. In Experiment 3, mice were injected with morphine (10 mg/kg) or saline, 15 min before intraplantar injection of formalin (5%/25 μ l). XX mice licked their hindpaw more than XY mice within 5 min of formalin injection. The results indicate that X- or Y-linked genes have direct effects, not mediated by gonadal secretions, on sex differences in two different types of acute nociception.

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Introduction

Men and women differ in their sensitivity to noxious stimuli and analgesic drugs. When exposed to noxious somatic stimuli, women show lower thresholds, greater ability to discriminate, and higher ratings of pain than men. In the clinic, men and women report endogenous pain of different quality and amount (Berkley, 1997; Mayer et al., 2004). Women show greater

sensitivity than men to some opioid analgesics in post-operative surgery and in laboratory pain models (Fillingim, 2002). Female rodents are generally more sensitive to noxious somatic stimuli than males (Chanda and Mogil, 2006; Kim et al., 1999), and μ -opioid drugs are more effective analgesics in males (Craft, 2003a,b; Craft et al., 2004).

What biological differences lead to these sex differences? Numerous experiments indicate that both activational hormonal effects (sex-specific effects of ovarian and testicular hormones at the time of testing) and organizational effects (permanent differentiating influences of testicular hormones during fetal and neonatal periods) are important (Craft et al., 2004; Craft, 2003a, b; Loyd and Murphy, 2006; Murphy, 2005). For example, estradiol sometimes reduces morphine analgesia (Craft et al., 2004), and testosterone or testicular secretions increase opioid

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analgesia (Mogil et al., 1993; Stoffel et al., 2005). In addition, testosterone, secreted neonatally, organizes brain circuits to produce permanent sex differences in pain and analgesia (Cicero et al., 2002; Craft, 2003a,b; Craft et al., 2004; Krzanowska et al., 2002; Mogil et al., 2000). Sex differences have also been observed in the development of morphine tolerance and its modulation by NMDA antagonists (Bryant et al., 2006; Craft and Lee, 2005; Barrett et al., 2001; Craft et al., 1999), depending on species and experimental procedure (Kest et al., 2000; Craft and Lee, 2005; Bryant et al., 2006). Here we test for the first time the direct effects of sex chromosome genes, which are known to be expressed differently in male and female cells, and contribute to sex differences in neural, behavioral, and other somatic phenotypes (Reisert and Pilgrim, 1991; De Vries et al., 2002; Carruth et al., 2002; Gatewood et al., 2006; Dewing et al., 2003, 2006; Palaszynski et al., 2005; Agate et al., 2003; Arnold, 2002; Arnold and Burgoyne, 2004). We selected three different tests to evaluate the role of sex chromosome complement under different conditions. Using the hotplate test, a well-established model of nociception in rodents, we tested the acute effects of morphine, the opioid drug used most commonly in the clinic. We also studied the development of tolerance to morphine, and its modulation by NMDA circuits, which were previously found to be sexually dimorphic (Bryant et al., 2006). Finally, we tested the effects of morphine on response to intraplantar injection of formalin.

We used the powerful “four core genotypes” (FCG) mouse model to study sex differences in nociception and opioid analgesia (Arnold and Burgoyne, 2004). In FCG mice, sex chromosome complement (XX vs. XY) is independent of gonadal type. The four genotypes include XX and XY⁻ mice lacking the testis-determining gene *Sry* (gonadal females), and XX*Sry* and XY⁻*Sry* mice with *Sry* (gonadal males). Comparisons of gonadal males and females tests for the effects of *Sry* (i.e., direct cellular effects or those mediated by gonadal hormones). Comparing XX and XY mice tests for the effects of sex chromosome complement. In this model we find in three different experimental paradigms that XX mice show greater sensitivity to painful stimuli than XY mice. The results reveal the first evidence for direct effects of sex chromosome complement on nociception.

Materials and methods

Experimental subjects

In FCG mice, the testis-determining gene *Sry* is deleted from the Y chromosome (producing the Y⁻ chromosome), and an *Sry* transgene is inserted on an autosome (Lovell-Badge and Robertson, 1990; Mahadevaiah et al., 1998). The Y⁻ chromosome derives from strain 129. XY⁻*Sry* gonadal males were bred with XX females, producing four genotypes: XX and XY⁻ gonadal females, and XX*Sry* and XY⁻*Sry* gonadal males. XY⁻*Sry* C57BL/6J (B6) breeders were produced by crossing XY⁻*Sry* MF1 males (gift from Paul Burgoyne) to B6 XX females, with successive backcrossing of XY⁻*Sry* progeny to B6 females for 13 or 14 generations. All mice were gonadectomized (GDX) at 55–105 days old (median 75 days), which removes all activational effects, i.e., those stemming from differences in levels of ovarian or testicular secretions at the time of testing. Gonadectomy was performed under isoflurane anesthesia, and mice were injected twice within 24 h of surgery with carprofen, a non-steroidal anti-inflammatory drug. These treatments are not known to interfere with the effects

of morphine. Mice were housed with same-sex siblings and held on LD 12:12 photoperiods with lights on at 07:00. Food and water were provided *ad libitum*. Three to four weeks after gonadectomy, mice underwent testing for Experiments 1 or 2. In all experiments, behavioral observations were made by an observer blind to experimental group of the mice.

All use of animals complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and was approved by the UCLA Chancellor's Animal Research Committee.

Experimental design

Experiment 1: acute effects of morphine in the hotplate test

Following the experimental procedures of Bryant et al. (2006), we placed experimentally naïve mice in a Plexiglas cylinder (8.2 cm diameter × 18.2 cm height) on a hot plate (52.5 °C, Accuscan). The latency to lick the hindpaws or jump was measured with a stopwatch to ±0.1 s accuracy. We adopted a cutoff latency of 60 s to avoid tissue damage. Mice were measured for basal nociception once per day for 3 days before morphine treatment. No significant differences were observed among baselines measured on the three days, so these values were averaged and presented as the hotplate baseline latency. On day three, 30 min after the last mouse had been tested for baseline latency, subjects were injected s.c. with morphine (10 mg/kg, volume 10 ml/kg) or saline ($n=8-10$, 68 mice total), and then tested again 30, 60, and 90 min after injection. Tests were carried out between 09:00 and 12:00.

Experiment 2: development of morphine tolerance

We followed the experimental procedure of Bryant et al. (2006), with some modification, because those authors detected sex differences in the effect of the NMDA antagonist CPP (3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid) on the development of tolerance to morphine. Bryant et al. (2006) reported evidence that the sex difference was not completely explained by circulating levels of gonadal hormones, which provided a rationale for testing for sex chromosome effects. We injected experimentally naïve mice twice daily (at 09:00 and 18:00) s.c. with 10 mg/kg (volume 10 ml/kg) morphine in saline, for 6 days. The competitive NMDA antagonist CPP was injected in saline (10 mg/kg, volume 10 ml/kg, i.p.) 30 min prior to morphine administration on the first 6 days, in an attempt to attenuate the development of morphine tolerance. The control for both morphine and CPP was saline alone (SAL). Thus, there were four drug treatment groups: SAL–SAL, SAL–MOR, CPP–SAL, CPP–MOR ($n=8-10$ per genotype per drug treatment, total 142 mice). On day 7 at 09:00, mice were tested for hotplate baselines, then injected 30 min later with a challenge dose of morphine (10 mg/kg, volume 10 ml/kg, s.c.). The effects of morphine on hotplate latencies were measured at 30, 60, and 90 min after injection.

Experiment 3: acute effects of morphine on formalin test

The formalin test was conducted as described (Sufka et al., 1998). Mice had previously comprised the SAL–SAL group of

Experiment 2, and were tested in the formalin test 4 weeks after Experiment 2 ($n=5-10$ per genotype). The acclimation period (30 min) started when mice were placed in a Plexiglas cylinder (15 cm diameter \times 22 cm height) on a transparent surface. A camera positioned below recorded the experimental session. Morphine (10 mg/kg, volume 10 ml/kg) or saline was injected s.c. 15 min after the start of the acclimation period. The formalin test (50 min session) began at the end of the acclimation period when mice were injected into the footpad of the hindpaw with formalin in saline (5%/25 μ l) and placed back into the observational chambers. Experiments took place between 12:00 and 16:00. The observation period was divided into 10 blocks of 5 min each, and within each block we recorded the time spent in biting/licking of the injected paw, and frequency of rearing behavior.

Statistical analysis

For Experiment 1, we used a 2-way ANOVA on hotplate baselines with factors of sex chromosome (XX vs. XY) and gonadal sex (male vs. female). We used a 4-way ANOVA for repeated measures with between factors of sex chromosome complement (XX vs. XY), gonadal sex (male vs. female), and drug (morphine vs. saline), and the within factor of time (0, 30, 60 and 90 min). When significant differences were detected, we used separate 3-way ANOVAs at each time point.

In Experiment 2, we used a 2-way ANOVA to test the factors of gonadal sex (male vs. female) and sex chromosome complement (XX vs. XY) on hotplate baseline latencies. We also ran a 4-way repeated measures ANOVA to analyze the factors of drug (SAL–SAL, SAL–MOR, CPP–MOR and CPP–SAL), sex (male vs. female), sex chromosome complement (XX vs. XY), and time (within factor, 0, 30, 60 or 90 min). Separate 3-way ANOVAs for XX and XY mice and on each time point mice were also conducted, sometimes followed by Tukey's HSD test of specific effects.

The results of Experiment 3 were analyzed with a 4-way ANOVA for repeated measures (10 time intervals) to test the effects of gonadal sex (male vs. female), sex chromosome complement (XX vs. XY), and drug (saline vs. morphine). When significant differences were detected, we conducted separate 3-way ANOVAs for each time point to clarify the nature of the group differences.

Results

Experiment 1: effects of an acute dose of morphine in the hotplate test

The 2-way ANOVA on hotplate baselines yielded a highly significant effect of sex chromosome complement ($F_{(1,64)}=23.65$; $p<0.00001$) and no significant effect of gonadal sex. XX mice had shorter hotplate baselines than XY mice, regardless of their gonadal sex (Fig. 1). The 4-way repeated measures ANOVA showed a significant analgesic effect of morphine ($F_{(1,60)}=176$; $p<0.000001$, not shown), and a main effect of time ($F_{(3,180)}=41.3$; $p<0.00001$) and the time by morphine interaction ($F_{(3,180)}=69.7$;

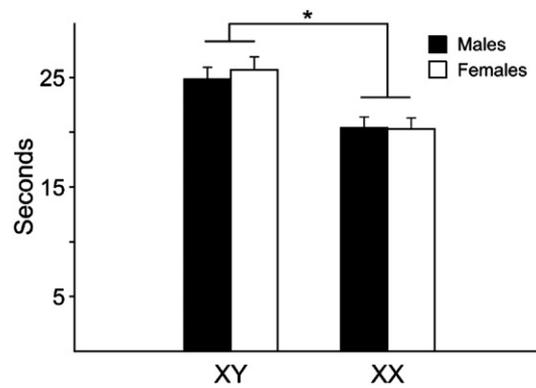


Fig. 1. Hotplate latencies in Experiment 1, in gonadectomized experimentally naïve mice prior to injection of morphine. XX mice showed significantly shorter latencies than XY mice, irrespective of the type of gonad (asterisk, $p<0.00001$).

$p<0.000001$) because of the different effects of morphine at various time points. Other main effects or interactions were not significant. Separate 3-way ANOVAs on each time point also revealed no significant effects of gonadal sex, sex chromosome complement or their interaction. As expected from the 4-way ANOVA, morphine significantly increased hotplate latencies at all time points after time 0 ($F_{(1,60)}=42-687$ $p<0.000001$, data not shown).

Experiment 2: effects of chronic treatment with morphine in the hotplate test

Consistent with the results of Experiment 1, a 2-way ANOVA on hotplate baseline latencies showed a highly significant main effect of sex chromosome complement ($F_{(1,138)}=25.4$, $p=0.00001$) but no effect of gonadal sex or an interaction. XY mice had significantly longer baseline latencies than XX mice regardless of gonadal sex (Fig. 2A). The 4-way repeated measures ANOVA yielded a significant main effect of sex chromosome and significant time by sex chromosome interaction ($F_{(1,126)}=33.03$, $p<0.00001$; $F_{(3,378)}=2.79$, $p<0.05$) (Fig. 2A, Supplementary Figure 1). This result indicates that XX and XY mice differed across the entire experiment, but that the magnitude by which they differed was greater following the administration of the challenge dose of morphine than before the dose.

The 4-way ANOVA also showed a main effect of drug treatment ($F_{(3,126)}=5.55$, $p=0.001$) because treatment with morphine for 6 days produced tolerance, decreasing the hotplate latencies (Fig. 2B, Supplementary Figure 1). The tolerance is shown by differences between the SAL–MOR and SAL–SAL groups ($p=0.01$, Tukey). However, CPP did not attenuate the development of morphine tolerance (no difference CCP–MOR vs. SAL–MOR). There was also a significant main effect of time ($F_{(3,378)}=126$, $p=0.000001$) and a significant time by drug interaction ($F_{(9,378)}=4.15$, $p<0.00005$), because the effects of the challenge dose of morphine on day 7 were greater at shorter latencies than at longer latencies after morphine. The absence of significant interactions of sex chromosome by sex, or sex chromosome by drug, leads to the conservative conclusion that

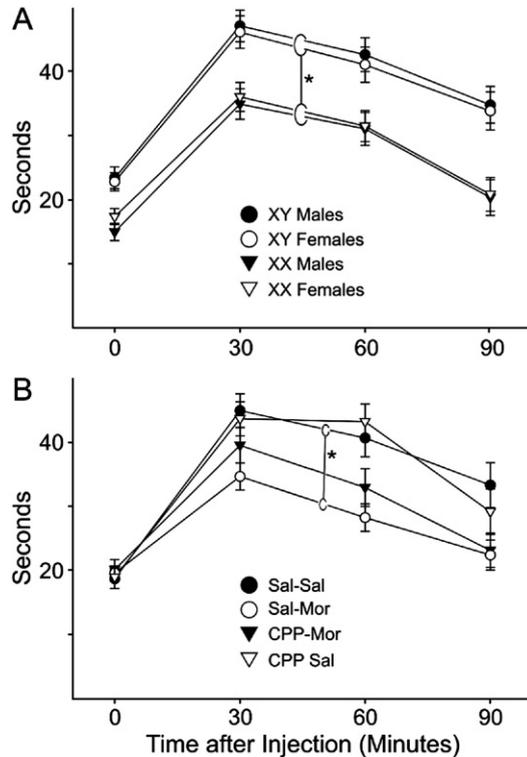


Fig. 2. A, Hotplate latencies of XX mice were consistently shorter than those for XY mice prior to and after injection of the challenge dose of morphine in Experiment 2 (asterisk, $p < 0.00001$). To illustrate the sex chromosome effect clearly, the graphs show XX and XY groups collapsed across drug conditions. The XX–XY difference was larger 30–90 min after the injection of morphine on day 7 than prior to injection (time 0). B, Hotplate latencies of drug groups in Experiment 2. The groups are collapsed across sex and sex chromosome conditions so that the overall drug effects can be observed more clearly. Morphine treatment on days 1–6 induced tolerance (shorter latencies) in response to morphine on day 7 (SAL–MOR shorter latencies than SAL–SAL, asterisk, $p = 0.01$), but CPP did not affect morphine tolerance (no statistically significant difference between CPP–MOR and SAL–MOR).

the effects of the drugs (e.g., the development of tolerance, or effect of CPP) were the same in XX and XY mice, and that the effects of the sex chromosomes were not different in gonadal males and females.

Although the overall ANOVA did not show interactions of the effects of sex chromosome complement and drugs, smaller ANOVAs revealed some evidence that the drug effects were stronger in XX than XY mice. Because the overall ANOVA yielded a significant main effect of sex chromosome and interaction between time and sex chromosome, we conducted two separate 3-way ANOVAs for XX and XY mice. The 3-way ANOVA for XX mice revealed a significant main effect of drug ($F_{(3,68)} = 4.60$, $p = 0.005$) resulting from development of morphine tolerance in XX mice (SAL–SAL vs. SAL–MOR, $p < 0.01$, Tukey)(Supplementary Figure 1). There was also a significant main effect of time ($F_{(3,204)} = 66.4$, $p = 0.000001$) and a significant time by drug interaction ($F_{(9,204)} = 3.99$, $p = 0.0001$) for XX mice. In contrast, a similar 3-way ANOVA in XY mice showed no significant overall main effect of drug or a drug by time interaction, but did show a main effect of time ($F_{(3,174)} = 60.99$, $p < 0.000001$, Supplementary Figure 1).

Experiment 3: effects of an acute dose of morphine in the formalin test

As expected, a general 4-way ANOVA for repeated measures revealed a significant effect of morphine ($F_{(1,53)} = 295$, $p < 0.00001$), time ($F_{(9,477)} = 18.2$, $p < 0.00001$) and time by morphine interaction ($F_{(9,477)} = 16.5$, $p < 0.00001$) on duration of licking the hindpaw injected with formalin. After the first 5 min of the formalin test, morphine eliminated licking behavior, whereas saline-injected mice showed an expected first phase of acute pain followed by a second phase of persistent pain (Fig. 3B). Given the significant main effect of time and interaction of time with morphine, we conducted separate 3-way ANOVAs for each of the 10 time intervals. In the first 5 min, morphine significantly reduced licking behavior ($F_{(1,53)} = 197$, $p < 0.00001$). Interestingly, XX mice licked their hindpaws more than XY mice ($F_{(1,53)} = 5.05$, $p = 0.03$, Fig. 3A), a difference that was apparent for males but not for females, although the interaction between sex and sex chromosome complement was not significant. At other time periods, the sex chromosome effect was no longer significant, whereas morphine was significant in reducing time spent licking ($F_{(1,53)} = 9$ to 193, $p < 0.005$ for each of the last 9 periods).

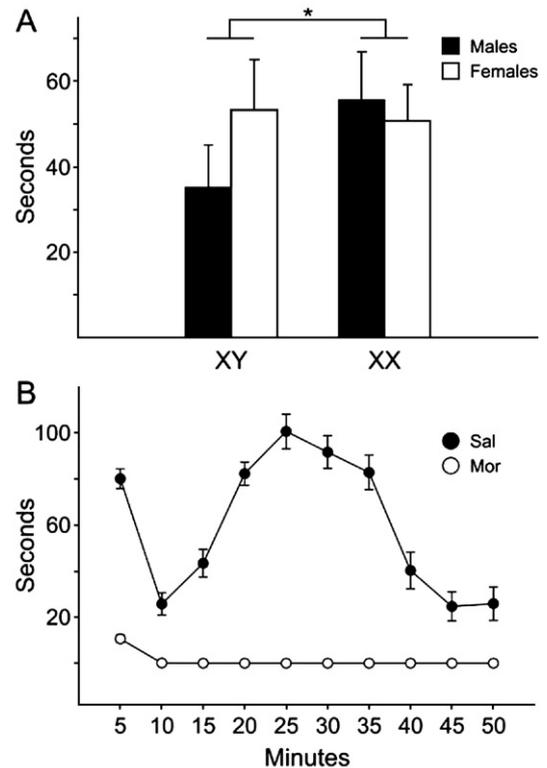


Fig. 3. A, Number of seconds spent licking in the first 5 min of the formalin test in Experiment 3, collapsed across drug treatment groups. XX mice showed significantly more licking than XY mice (asterisk, $p = 0.03$). B, The number of seconds spent licking in each of ten 5-min intervals in Experiment 3, collapsed across sex and sex chromosome groups because of the absence of sex or sex chromosome effects. The saline-injected mice showed the acute phase of licking for about 5 min, followed by a persistent pain phase during the last eight time segments. Morphine abolished all licking.

Rearing behavior was significantly affected by morphine ($F_{(1,53)}=76.6$, $p<0.00001$), time ($F_{(9,477)}=13.3$, $p<0.00001$), and the time by morphine interaction ($F_{(9,477)}=13.2$, $p<0.00001$), but not by sex or sex chromosome complement. Therefore, we ran separate 3-way ANOVAs for each time point, and found that morphine significantly reduced rearing frequency for the first seven periods ($F_{(1,53)}=5.5$ – 31 for each period, $p<0.05$). Some evidence for sex chromosome and sex effects was found for time period VI, when some effects were significant or nearly significant (sex chromosome effect $F_{(1,53)}=3.87$, $p=0.054$; gonadal sex by sex chromosome interaction $F_{(1,53)}=4.54$, $p<0.05$; sex chromosome by morphine interaction $F_{(1,53)}=3.87$, $p=0.054$; sex by sex chromosome by morphine interaction $F_{(1,53)}=4.54$, $p<0.05$). Only XX females administered saline (rather than morphine) displayed rearing behavior in this period (data not shown).

Discussion

The present data indicate that gonadectomized adult C57BL/6J mice with two X chromosomes respond more, or at shorter latencies, to painful stimuli than mice with one X and one Y chromosome. The sex chromosome effect is suggested by tests using two different types of painful stimuli, thermal and chemical, and under experimental hotplate paradigms that involve either no pre-treatment with morphine, or 6 days of pre-treatment with morphine or other drugs. In Experiments 1 and 2, the sex chromosome effect was equivalent in gonadal males and females. In Experiment 2, which assessed development of tolerance to morphine, the analgesic effect of a challenge dose of morphine on the seventh day was greater in XY than in XX mice irrespective of the drug injected for the first 6 days. A similar sex chromosome effect on the effect of morphine was not found in the first experiment, when only one injection of morphine was administered, nor was a sex chromosome effect detected on the effect of morphine in the formalin test (Experiment 3). We did not detect any group differences in the effects of the NMDA antagonist CPP on the development of morphine tolerance in Experiment 2. The results suggest for the first time that sex differences in nociception are caused not only by activational and organizational effects of gonadal steroids as previously reported (Craft et al., 2004), but also by differences in the expression of X or Y genes, perhaps within the brain itself.

The sex chromosome effects found here could be caused by one of several genetic mechanisms (Arnold and Burgoyne, 2004; Arnold, 2004). The XX vs. XY groups differed in the presence vs. absence of non-*Sry* Y genes, which could act within or outside the brain to alter the response to thermal or chemical nociceptive stimuli. Alternatively, the effect could be caused by differences in the expression of X genes. X genes might be expressed higher in XX than XY mice because of the difference in genomic dose. Although some X genes escape inactivation and are expressed higher in female mice than in males (Xu et al., 2002; Carrel and Willard, 2005), in general the process of X inactivation effectively balances the expressed dose of X mRNAs in the two sexes (Itoh et al., 2007). However, females receive a genomic imprint from their fathers, which could affect

gene expression in the cells in which the paternal X chromosome is active, whereas males receive only a maternal imprint. Differences in parental imprint on the X chromosome lead to sex differences in gene expression and influence various behaviors in mice (Davies et al., 2005, 2006; Raefski and O'Neill, 2005), suggesting that X-imprinting is a significant potential source of sex chromosome effects. Moreover, the development of the brain may be different in XX and XY mice because XX cells must undergo X-inactivation, which could make them vulnerable to insults or perturbations of development more than in XY mice (Chen et al., in press). It will be important to determine which X or Y genes contribute to sex differences in nociception, and where they act. Sex differences caused by sex chromosome effects, not mediated by the effects of gonadal hormones, have previously been reported in several neural and non-neural systems (Renfree and Short, 1988; Reisert and Pilgrim, 1991; Carruth et al., 2002; De Vries et al., 2002; Palaszynski et al., 2005; Gatewood et al., 2006; Dewing et al., 2003, 2006; Chen et al., in press).

Because we found no differences between gonadectomized gonadal male vs. gonadal female mice, the pronounced sex differences in levels of gonadal hormones prior to adult gonadectomy (including organizational effects) appear not to have had a differential effect on nociception as measured in the present contexts in B6 mice. The group differences are also not attributable to differences in circulating levels of gonadal steroids (activational effects) at the time of testing, because the mice had no gonads. Because of the lack of organizational and activational effects of gonadal hormones, and because the effects of sex chromosome complement were found in both gonadal males and gonadal females, it is highly unlikely that the XX–XY differences are the result of group differences in gonadal hormones prior to gonadectomy. Why were there no organizational and activational effects in the present study, in contrast to numerous previous reports of the importance of such effects leading to sex differences in nociception and analgesia? Most previous studies have reported sex differences in gonadally intact rats or mice, or have directly manipulated the levels of gonadal hormones in adulthood or at birth (Craft et al., 2004; Mogil et al., 1993; Cicero et al., 2002; Krzanowska et al., 2002). In contrast, we have tested gonadectomized mice, under conditions that are likely to eliminate rather than reveal effects of gonadal hormones. It will be interesting to test whether the sex chromosome effects are also found when mice are tested in the presence of testicular or ovarian hormones in adulthood.

Relatively few studies have studied sex differences in morphine tolerance and the involvement of NMDA systems. Sex differences in tolerance to morphine have been reported in both directions, depending on species and testing conditions (Bryant et al., 2006; Craft et al., 1999). However, gonadally intact female mice develop a greater tolerance to morphine than males (Hopkins et al., 2004; Kest et al., 2000). Here we tested mice 3 to 4 weeks after gonadectomy, and found that both GDX XX and XY mice became tolerant after chronic treatment with morphine regardless of their gonadal sex. CPP injected prior to morphine did not block the development of tolerance in XX or XY mice. This result contrasts with the findings of Bryant et al.

(2006) who reported that a higher dose of CPP (30 mg/kg, i.p.) administered with escalating doses of morphine blocks the development of tolerance in B6 males, but not in intact or GDX female mice. We chose here to use a lower, constant dose of CPP (10 mg/kg) to minimize motor perturbations during the chronic regimen of CPP and morphine. A likely consequence was that we did not observe CPP-induced attenuation of morphine tolerance as in the previous study (Bryant et al., 2006). Moreover, because morphine tolerance was modest under the conditions of the present experiment, the likelihood for observing significant modulation by CPP was small. The two studies agree that GDX females do not show the effect of CPP, which was previously found only in gonadally intact males (Bryant et al., 2006). Thus, one hypothesis is that the effect of CPP on tolerance may require the presence of testicular secretions under the present testing conditions.

Gonadal females have generally been found to be more sensitive to pain and show shorter latencies in response to pain (Fillingim and Maixner, 1995). That finding, however, has depended on species/strain, photoperiod, hormones, behavioral assay and the statistical power of the experiment (Mogil and Chanda, 2005). In studies in which the hotplate was used for pain testing in mice, there is no clear evidence for sex differences on basal nociception (Mogil et al., 2000). In contrast, female mice have shorter baseline latencies than males in other tests of thermal pain such as tail withdrawal (Grisel et al., 1996; Mogil et al., 1996). In the late (persistent pain) phase of the formalin test, gonadally intact females are more sensitive to noxious stimuli than intact males, and castration, ovariectomy or ovarian cycle do not affect the response *per se* (Kim et al., 1999; Chanda and Mogil, 2006). To our knowledge, no previous study reported sex differences in the early acute phase of the formalin test in mice, as we report here. These differences between the present and previous studies could reflect differences in testing or experimental subjects. Alternatively, the sex chromosome effects found in GDX mice could be counteracted or compensated by effects of gonadal hormones and hence not be detected in gonadally intact mice (De Vries, 2004). It will be important to test how XX and XY mice are influenced by different levels of testicular and ovarian hormones in adulthood.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yhbeh.2007.09.003.

References

- Agate, R.J., Grisham, W., Wade, J., Mann, S., Wingfield, J., Schanen, C., Palotie, A., Arnold, A.P., 2003. Neural not gonadal origin of brain sex differences in a gynandromorphic finch. *Proc. Natl. Acad. Sci. U. S. A.* 100, 4873–4878.
- Arnold, A.P., 2002. Concepts of genetic and hormonal induction of vertebrate sexual differentiation in the twentieth century, with special reference to the brain. In: Pfaff, D.W., Arnold, A.P., Etgen, A., Fahrbach, S., Rubin, R. (Eds.), *Hormones, Brain, and Behavior*, vol. 4. Academic Press, San Diego, pp. 105–135.
- Arnold, A.P., 2004. Sex chromosomes and brain gender. *Nat. Rev. Neurosci.* 5, 701–708.
- Arnold, A.P., Burgoyne, P.S., 2004. Are XX and XY brain cells intrinsically different? *Trends Endocrinol. Metab.* 15, 6–11.
- Barrett, A.C., Cook, C.D., Turner, J.M., Craft, R.M., Picker, M.J., 2001. Importance of sex and relative efficacy at the mu opioid receptor in the development of tolerance and cross-tolerance to the antinociceptive effects of opioids. *Psychopharmacology (Berl)* 158, 154–164.
- Berkley, K.J., 1997. Sex differences in pain. *Behav. Brain Sci.* 20 (371+).
- Bryant, C.D., Eitan, S., Sinchak, K., Fanselow, M.S., Evans, C.J., 2006. NMDA receptor antagonism disrupts the development of morphine analgesic tolerance in male, but not female C57BL/6J mice. *Am. J. Physiol., Regul. Integr. Comp. Physiol.* 291, R315–R326.
- Carrel, L., Willard, H.F., 2005. X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature* 434, 400–404.
- Carruth, L.L., Reisert, I., Arnold, A.P., 2002. Sex chromosome genes directly affect brain sexual differentiation. *Nat. Neurosci.* 5, 933–934.
- Chanda, M.L., Mogil, J.S., 2006. Sex differences in the effects of amiloride on formalin test nociception in mice. *Am. J. Physiol., Regul. Integr. Comp. Physiol.* 291, R335–R342.
- Chen, X., Watkins, R., Delot, E., Reliene, R., Schiestl, R.H., Burgoyne, P.S., Arnold, A.P., in press. Sex difference in neural tube closure defects in p53-mice is caused by differences in the complement of X not Y genes. *Dev. Neurobiol.*
- Cicero, T.J., Nock, B., O'Connor, L., Meyer, E.R., 2002. Role of steroids in sex differences in morphine-induced analgesia: activational and organizational effects. *J. Pharmacol. Exp. Ther.* 300, 695–701.
- Craft, R.M., 2003a. Sex differences in drug- and non-drug-induced analgesia. *Life Sci.* 72, 2675–2688.
- Craft, R.M., 2003b. Sex differences in opioid analgesia: “from mouse to man”. *Clin. J. Pain* 19, 175–186.
- Craft, R.M., Lee, D.A., 2005. NMDA antagonist modulation of morphine antinociception in female vs. male rats. *Pharmacol. Biochem. Behav.* 80, 639–649.
- Craft, R.M., Stratmann, J.A., Bartok, R.E., Walpole, T.I., King, S.J., 1999. Sex differences in development of morphine tolerance and dependence in the rat. *Psychopharmacology (Berl)* 143, 1–7.
- Craft, R.M., Mogil, J.S., Aloisi, A.M., 2004. Sex differences in pain and analgesia: the role of gonadal hormones. *Eur. J. Pain* 8, 397–411.
- Davies, W., Isles, A., Smith, R., Karunadasa, D., Burmann, D., Humby, T., Ojarikre, O., Biggin, C., Skuse, D., Burgoyne, P., Wilkinson, L., 2005. Xlr3b is a new imprinted candidate for X-linked parent-of-origin effects on cognitive function in mice. *Nat. Genet.* 37, 625–629.
- Davies, W., Isles, A.R., Burgoyne, P.S., Wilkinson, L.S., 2006. X-linked imprinting: effects on brain and behaviour. *BioEssays* 28, 35–44.
- De Vries, G.J., 2004. Minireview: sex differences in adult and developing brains: compensation, compensation, compensation. *Endocrinology* 145, 1063–1068.
- De Vries, G.J., Rissman, E.F., Simerly, R.B., Yang, L.Y., Scordalakes, E.M., Auger, C.J., Swain, A., Lovell-Badge, R., Burgoyne, P.S., Arnold, A.P., 2002. A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits. *J. Neurosci.* 22, 9005–9014.
- Dewing, P., Shi, T., Horvath, S., Vilain, E., 2003. Sexually dimorphic gene expression in mouse brain precedes gonadal differentiation. *Brain Res. Mol. Brain Res.* 118, 82–90.
- Dewing, P., Chiang, C.W.K., Sinchak, K., Sim, H., Fernagut, P.O., Kelly, S., Chesselet, M.F., Micevych, P.E., Albrecht, K.H., Harley, V.R., Vilain, E., 2006. Direct regulation of adult brain function by the male-specific factor SRY. *Curr. Biol.* 16, 415–420.
- Fillingim, R.B., 2002. Sex differences in analgesic responses: evidence from experimental pain models. *Eur. J. Anaesthesiol., Suppl.* 26, 16–24.
- Fillingim, R.B., Maixner, W., 1995. Gender differences in response to noxious stimuli. *Pain Forum* 4, 209–221.
- Gatewood, J.D., Wills, A., Shetty, S., Xu, J., Arnold, A.P., Burgoyne, P.S., Rissman, E.F., 2006. Sex chromosome complement and gonadal sex

- influence aggressive and parental behaviors in mice. *J. Neurosci.* 26, 2335–2342.
- Grisel, J.E., Mogil, J.S., Belknap, J.K., Grandy, D.K., 1996. Orphanin FQ acts as a supraspinal, but not a spinal, anti-opioid peptide. *Neuroreport* 7, 2125–2129.
- Hopkins, E., Rossi, G., Kest, B., 2004. Sex differences in systemic morphine analgesic tolerance following intrathecal morphine injections. *Brain Res.* 1014, 244–246.
- Itoh, Y., Melamed, E., Yang, X., Kampf, K., Wang, S., Yehya, N., Van Nas, A., Replogle, K., Band, M.R., Clayton, D.F., Scadt, E.E., Lusic, A.J., Arnold, A.P., 2007. Dosage compensation is less effective in birds than in mammals. *J. Biol.* 6, 2.
- Kest, B., Palmese, C., Hopkins, E., 2000. A comparison of morphine analgesic tolerance in male and female mice. *Brain Res.* 879, 17–22.
- Kim, S.J., Calejesan, A.A., Li, P., Wei, F., Zhuo, M., 1999. Sex differences in late behavioral response to subcutaneous formalin injection in mice. *Brain Res.* 829, 185–189.
- Krzanowska, E.K., Ogawa, S., Pfaff, D.W., Bodnar, R.J., 2002. Reversal of sex differences in morphine analgesia elicited from the ventrolateral periaqueductal gray in rats by neonatal hormone manipulations. *Brain Res.* 929, 1–9.
- Lovell-Badge, R., Robertson, E., 1990. XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy. *Development* 109, 635–646.
- Loyd, D.R., Murphy, A.Z., 2006. Sex differences in the anatomical and functional organization of the periaqueductal gray–rostral ventromedial medullary pathway in the rat: A potential circuit mediating the sexually dimorphic actions of morphine. *J. Comp. Neurol.* 496, 723–738.
- Mahadevaiah, S.K., Odorisio, T., Elliott, D.J., Rattigan, A., Szot, M., Laval, S.H., Washburn, L.L., McCarrey, J.R., Cattanaach, B.M., Lovell-Badge, R., Burgoyne, P.S., 1998. Mouse homologues of the human AZF candidate gene RBM are expressed in spermatogonia and spermatids, and map to a Y chromosome deletion interval associated with a high incidence of sperm abnormalities. *Hum. Mol. Genet.* 7, 715–727.
- Mayer, E.A., Berman, S., Lin, C., Naliboff, B.D., 2004. Sex-based differences in gastrointestinal pain. *Eur. J. Pain* 8, 451–463.
- Mogil, J.S., Chanda, M.L., 2005. The case for the inclusion of female subjects in basic science studies of pain. *Pain* 117, 1–5.
- Mogil, J.S., Sternberg, W.F., Kest, B., Marek, P., Liebeskind, J.C., 1993. Sex-differences in the antagonism of swim stress-induced analgesia — effects of gonadectomy and estrogen replacement. *Pain* 53, 17–25.
- Mogil, J.S., Grisel, J.E., Zhangs, G., Belknap, J.K., Grandy, D.K., 1996. Functional antagonism of mu-, delta- and kappa-opioid antinociception by orphanin FQ. *Neurosci. Lett.* 214, 131–134.
- Mogil, J.S., Chesler, E.J., Wilson, S.G., Juraska, J.M., Sternberg, W.F., 2000. Sex differences in thermal nociception and morphine antinociception in rodents depend on genotype. *Neurosci. Biobehav. Rev.* 24, 375–389.
- Murphy, A.Z., 2005. Sex differences in the antihyperalgesic actions of morphine. *Neuropsychopharmacology* 30, S74.
- Palaszynski, K.M., Smith, D.L., Kamrava, S., Burgoyne, P.S., Arnold, A.P., Voskuhl, R.R., 2005. A Yin-Yang effect between sex chromosome complement and sex hormones on the immune response. *Endocrinology* 146, 3280–3285.
- Raefski, A.S., O'Neill, M.J., 2005. Identification of a cluster of X-linked imprinted genes in mice. *Nat. Genet.* 37, 620–624.
- Reisert, I., Pilgrim, C., 1991. Sexual differentiation of monoaminergic neurons — genetic or epigenetic. *Trends Neurosci.* 14, 467–473.
- Renfree, M.B., Short, R.V., 1988. Sex determination in marsupials: evidence for a marsupial–eutherian dichotomy. *Philos. Trans. R. Soc. Lond., B Biol. Sci.* 322, 41–53.
- Stoffel, E.C., Ulibarri, C.M., Folk, J.E., Rice, K.C., Craft, R.M., 2005. Gonadal hormone modulation of mu, kappa, and delta opioid antinociception in male and female rats. *J. Pain* 6, 261–274.
- Sufka, K.J., Watson, G.S., Nothdurft, R.E., Mogil, J.S., 1998. Scoring the mouse formalin test: validation study. *Eur. J. Pain* 2, 351–358.
- Xu, J., Burgoyne, P.S., Arnold, A.P., 2002. Sex differences in sex chromosome gene expression in mouse brain. *Hum. Mol. Genet.* 11, 1409–1419.