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# Roles of Gender and Gonadectomy in Pilocarpine and Clonidine Analgesia in Rats

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KIEFEL, J. M. AND R. J. BODNAR. *Roles of gender and gonadectomy in pilocarpine and clonidine analgesia in rats.* PHARMACOL BIOCHEM BEHAV **41**(1) 153–158, 1992.—Central and systemic morphine analgesia as well as both opioid and nonopioid forms of swim analgesia display gender differences with male rats showing greater magnitudes of analgesia than female rats. Since nonopioid swim analgesia is dependent upon muscarinic cholinergic and alpha$_2$-noradrenergic mechanisms, the present study evaluated in rats whether gender, adult gonadectomy or estrous phase altered analgesia induced by either the muscarinic cholinergic receptor agonist, pilocarpine or the alpha$_2$-noradrenergic receptor agonist, clonidine. Pilocarpine (1–10 mg/kg) analgesia was significantly greater in male rats. Female rats displayed 7-fold and 3-fold rightward shifts in peak analgesia on the tail-flick and jump tests respectively. Clonidine (100–500 μg/kg) analgesia was significantly greater on both nociceptive tests in males, but only produced a 2-fold rightward shift in peak analgesia in females on the jump test. Whereas castration failed to shift either dose-response curve, ovariectomy mitigated the gender differences in pilocarpine and clonidine analgesia. Both pilocarpine and clonidine analgesia were not altered by estrous phase changes. These data indicate that gender differences in analgesia are not specific to opioid systems.

Analgesia Gender differences Gonadectomy Estrous phase Pilocarpine Clonidine Rats

THE analgesic responses following morphine (2, 20, 23), opioid-mediated intermittent cold-water swims [ICWS: (31)] and nonopioid-mediated continuous cold-water swims [CCWS: (31)] are significantly higher in male rats relative to female rats. Whereas adult gonadectomy reduces ICWS and CCWS analgesia (33,37) which is reinstated by steroid replacement therapy (32,38), adult gonadectomy reduces morphine analgesia following systemic (8), but not central (23) administration. Further, whereas systemic (3), but not central (23) morphine analgesia is sensitive to changes in estrous phase, CCWS analgesia is impervious to such influences (31). In contrast, analgesia elicited by either the mu-selective opioid agonist, D-Ala$^2$, me-Phe$^4$, gly(ol)$^5$-enkephalin (DAMGO), or the delta-selective opioid agonist, D-Ser$^2$, Leu$^5$ enkephalin-Thr$^6$ (DSLET), were not altered as functions of gender or gonadectomy (24). Moreover, gender differences were not observed for opioid binding on selective mu$_1$, mu$_2$ and delta receptor assays (24).

The neurochemical substrates mediating nonopioid swim analgesia have not been systematically evaluated in terms of gender differences. The muscarinic receptor antagonist, scopolamine, reduces nonopioid CCWS analgesia (35) as well as nonopioid footshock and tailshock analgesia (25, 26, 36). Antagonism of alpha$_2$ noradrenergic receptors alters nonopioid CCWS analgesia (22) and nonopioid footshock analgesia (6, 7, 9). Both the muscarinic receptor agonist, pilocarpine (14,15) and the alpha$_2$ noradrenergic receptor agonist, clonidine (13,28) produce a nonopioid form of analgesia (16, 17, 30, 39). To evaluate the potential for gender differences in cholinergic and noradrenergic analgesia, the present study evaluated whether either pilocarpine or clonidine analgesia were altered on the tail-flick (11) and jump (12) tests as functions of either gender, gonadectomy or estrous phase.

## METHOD

### *Subjects and Surgery*

Eighty-five albino Sprague-Dawley rats (90–100 days of age, Charles River Laboratories) were housed individually in flat-bottomed plastic cages in the Queens College vivarium and were maintained on a 12-h light (0800 h):12-h dark (2000 h) cycle with Purina rat chow and water available ad lib. Male and female rats, matched into sham and gonadectomy groups using preoperative body weights, were anesthetized with Ketamine (100 mg/kg, IM), and castrations and ovariectomies were performed as described previously (33). After testing, each rat was anesthetized (Euthanasia, No. 5, H. Schein) with seminal vesicles of males and fallopian tubes of females removed, blotted dry, and organ weights determined (33).

### *Nociceptive Tests*

Tail-flick latencies were ascertained with a radiant heat source (IITC, Woodland Hills, CA) in which heat was applied to the dorsum of the rat's tail 3–8 cm proximal to the tip. Each session consisted of three latency determinations made at 10-s intertrial intervals. To avoid tissue damage, the determination was terminated if no response occurred after 15 s. Immediately

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thereafter, jump thresholds were ascertained in a chamber (30 × 24 × 26.5 cm) with 16 grid bars spaced 1.9 cm apart. Electric shocks (0.3 s) were delivered through the grids by a shock generator (BRE/LVE) through a shock scrambler (Campden Instruments). An ascending method of limits procedure was employed for each of six trials with footshock initially delivered at 0.10 mA and increased in 0.05 mA steps at 5-s intervals. The jump threshold was defined in mA as the lowest of two consecutive intensities at which the rat simultaneously removed both rear paws from the grids. Previous data (21) have shown that administration of the tail-flick test prior to the jump test failed to alter jump thresholds. Animals were adapted to the testing situations over a minimum 7-day period in which stable baseline latencies and thresholds were observed over the last four days.

*Protocol 1*

Groups of sham (n = 8) and castrated (n = 8) male rats received pilocarpine hydrochloride (Sigma: 1 ml normal saline/kg, IP) at each of the following doses: 0, 1, 2.5 and 5 mg/kg. Groups of sham (n = 8) and ovariectomized (n = 8) female rats received pilocarpine at each of the following doses: 0, 1, 5 and 10 mg/kg. Additional groups of sham (n = 10) and castrated (n = 10) males, and sham (n = 8) and ovariectomized (n = 10) females received clonidine hydrochloride (Boehringer-Ingelheim: 1 ml normal saline /kg, IP) at each of the following doses: 0, 100, 250 and 500 μg/kg. Tail-flick and jump tests occurred 30, 60, 90 and 120 min after each injection condition in each animal in each group. All tests occurred between 2 and 10 h into the light cycle, and treatment conditions were administered in ascending order at weekly intervals to minimize possible tolerance effects. The repeated handling and injections failed to alter latencies and thresholds over time in previous studies in this laboratory [e.g., (4, 22, 35)]. Sham females were tested only during the estrous phase of the cycle which was monitored by daily vaginal smears taken 0–1 h into the light cycle.

*Protocol 2*

Two additional groups of intact female rats received either pilocarpine (10 mg/kg, n = 8) or clonidine (250 μg/kg, n = 8) during the estrous, proestrous and combined met/diestrous phases of the estrous cycle. Tail-flick latencies and jump thresholds were determined prior to, and 30, 60, 90 and 120 min following each injection. Treatment conditions were random and were separated by at least a one-week interval.

*Statistical Analyses*

Split-plot, repeated-measures analyses of variance with Dunnett comparisons ($p<0.05$) evaluated significant differences in tail-flick latencies and jump thresholds between vehicle and each pilocarpine and clonidine dose, among gender and gonadectomy groups, and across test times. Difference score analyses, derived by subtracting each drug score from each corresponding vehicle score, were performed because of significant differences in jump thresholds following vehicle treatment in sham males (0.427 mA) relative to castrated males (0.372 mA), sham females (0.311 mA) and ovariectomized females (0.305 mA). Tail-flick latencies following vehicle failed to differ from each other in sham males (3.27 s), castrated males (3.06 s), sham females (3.18 s) and ovariectomized females (3.25 s). These data are consistent with previous work demonstrating hyperreactivity to shock in female rats [e.g., (23, 24, 31–33)]. The potency of effects was evaluated by constructing log dose-response functions and performing linear regressions for peak (30 min) and total (differences in latencies or thresholds following drug relative to vehicle across the time course) analgesia for each nociceptive measure. The $ED_{50}$ for each measure was calculated as a 50% increase in analgesic magnitude.

## RESULTS

*Pilocarpine Analgesia: Gender and Gonadectomy Effects*

Significant increases in tail-flick latencies were observed following pilocarpine (ANOVAs, $p<0.05$) with gender significantly altering the magnitude of pilocarpine analgesia at the 5 mg/kg dose on the tail-flick test ($p<0.0006$). Pilocarpine (5 mg/kg) analgesia was significantly greater on the tail-flick test in sham males relative to castrated males (30 min), sham females (30–60 min) and ovariectomized females (30 min) (Fig. 1a). Ovariectomized females displayed significantly greater pilocarpine (5 mg/kg) analgesia on the tail-flick test after 30 min than sham females (Fig. 1a). Regression analyses revealed significant differences in the dose-response curves of both peak ($p<0.011$) and total ($p<0.026$) pilocarpine analgesia on the tail-flick test (Fig. 1b, c; Table 1) with sham females displaying rightward shifts of 7.1 for peak effects and 20.2 for total effects on the tail-flick test relative to sham males. Whereas castration failed to alter the dose-response function of pilocarpine analgesia in males on the tail-flick test, ovariectomy appeared to mitigate the rightward shifts in potency observed in sham females (Table 1).

Significant increases were also observed on the jump test following pilocarpine (ANOVAs, $p<0.05$) with gender significantly altering analgesic magnitude following the 1 and 5 mg/kg doses ($p<0.0001$). Pilocarpine (5 mg/kg) analgesia was significantly greater on the jump test in sham males relative to sham females (30–90 min) and ovariectomized females (30–90 min) (Fig. 1d). Ovariectomized females displayed significantly less pilocarpine (5 mg/kg) analgesia on the jump test after 30 min than sham females (Fig. 1d). Regression analyses revealed significant differences in the dose-response curves of both peak ($p<0.0001$) and total ($p<0.0001$) pilocarpine analgesia on the jump test (Fig. 1e, f; Table 1) with sham females displaying rightward shifts of 3.7 for peak effects and 76.9 for total effects on the jump test relative to sham males. Neither castration nor ovariectomy consistently altered pilocarpine analgesia on the jump test relative to intact same-sex controls (Table 1).

*Pilocarpine Analgesia and Estrous Phase*

Pilocarpine significantly increased tail-flick latencies ($p<0.0001$) and jump thresholds ($p<0.0001$) which failed to differ either across estrous phases (tail-flick: $p = 0.71$; jump: $p = 0.38$) or for the interaction between test time and estrous phase (tail-flick: $p = 0.36$; jump: $p = 0.65$).

*Clonidine Analgesia: Gender and Gonadectomy Effects*

Significant increases in tail-flick latencies were observed following clonidine (ANOVAs, $p<0.05$). with analgesic magnitude significantly altered by both gender (250 μg/kg: $p<0.05$) and gonadectomy (500 μg/kg: $p<0.03$). The magnitude of clonidine analgesia (250 μg/kg) was significantly greater on the tail-flick test in sham males relative to castrated males (30 min), sham females (30–60 min) and ovariectomized females (30–60 min) (Fig. 2a). Ovariectomized females displayed significantly greater clonidine (250 μg/kg) analgesia on the tail-flick test after 60 min than sham females (Fig. 2a). Regression analyses failed to reveal significant differences in the dose-response curves of both

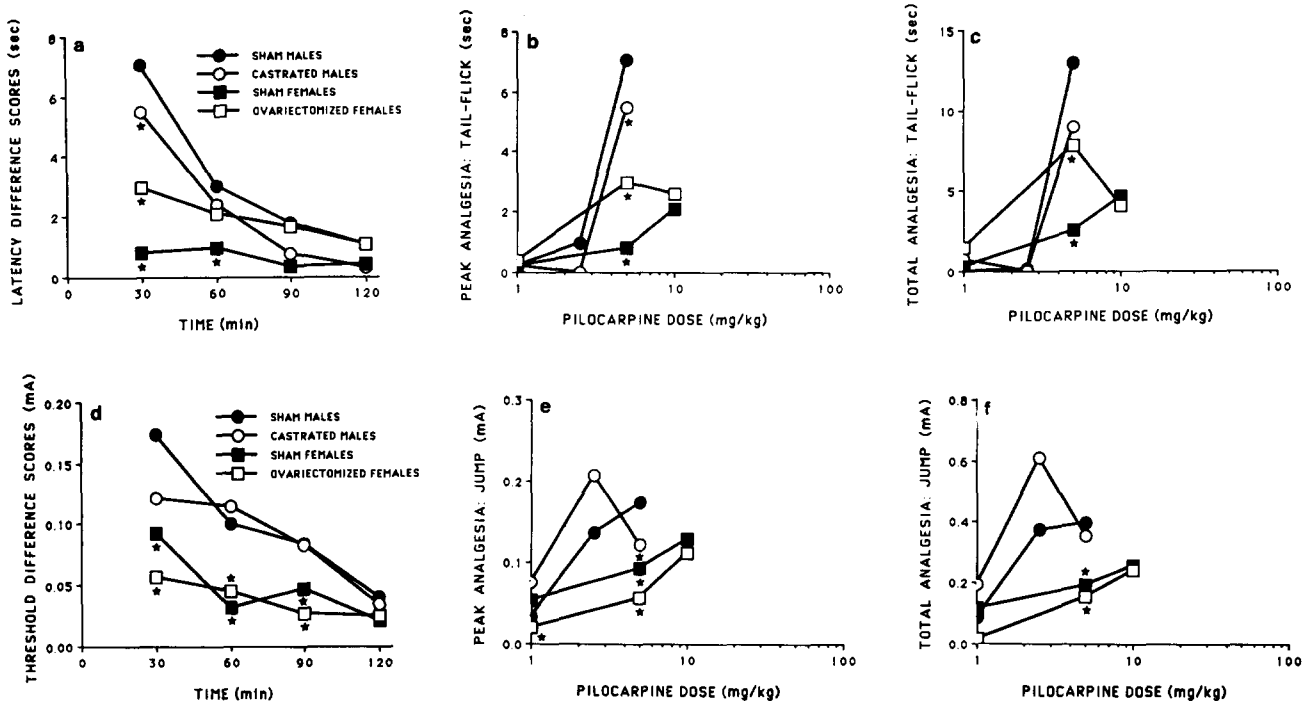


FIG. 1. Alterations in pilocarpine analgesia as measured on the tail-flick (upper panels) and jump (lower panels) tests as functions of gender and adult gonadectomy. Whereas the two left panels (a, d) reflect pilocarpine analgesia across the 120-min time course at a dose of 5 mg/kg, the middle panels (b, e) illustrate peak effects 30 min after pilocarpine administration across doses, and the right panels (c, f) illustrate total analgesia across doses, defined as the sum of pilocarpine effects minus vehicle effects across the 120-min time course. The stars denote significant differences in pilocarpine analgesia relative to sham males (Dunnett comparisons, $p<0.05$).

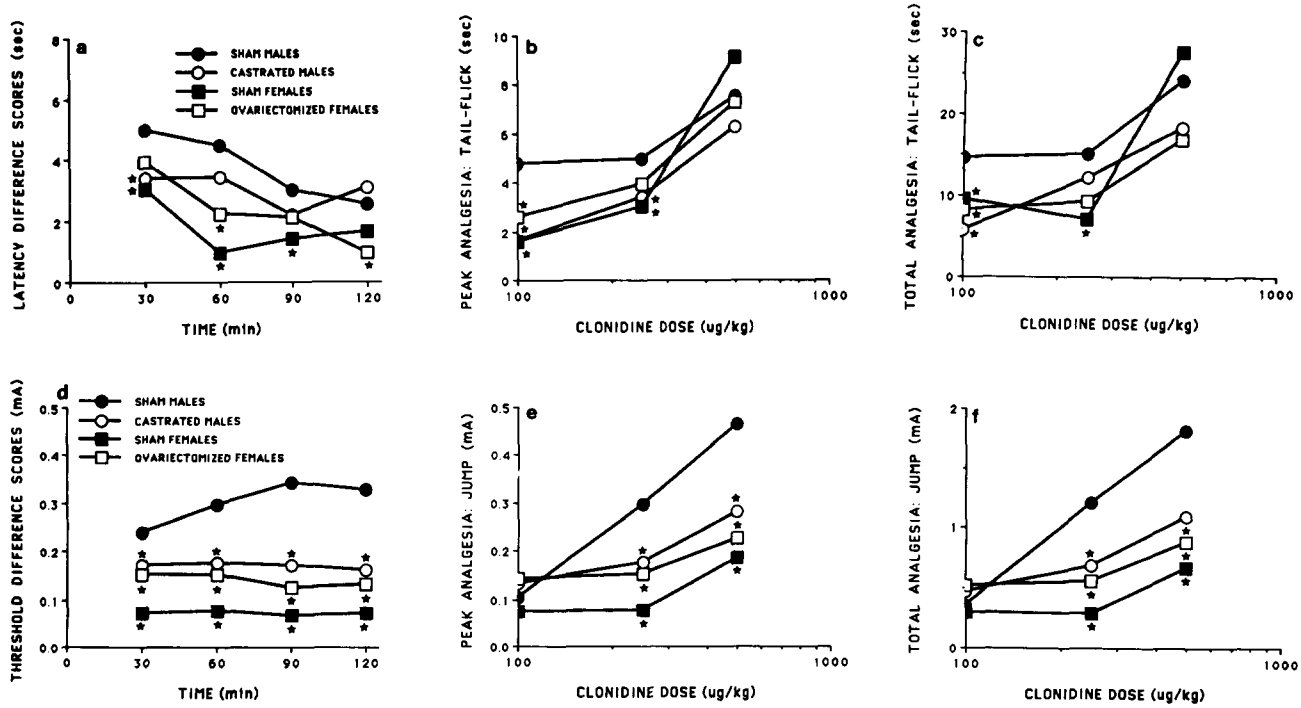


FIG. 2. Alterations in clonidine analgesia as measured on the tail-flick and jump tests as functions of gender and adult gonadectomy. Effects upon clonidine analgesia are reflected in terms of time course (250 μg/kg; left panels: a, d) as well as peak (middle panels: b, e) and total (right panels: c, f) effects across doses. The stars denote significant differences in clonidine analgesia relative to sham males (Dunnett comparisons, $p<0.05$).

TABLE 1

REGRESSION ANALYSES OF THE LOG DOSE-RESPONSE FUNCTIONS OF PILOCARPINE (mg/kg) ANALGESIA IN SHAM AND GONADECTOMIZED MALE AND FEMALE RATS ON THE TAIL-FLICK AND JUMP TESTS

| Condition | Sham Males | Castrated Males | Sham Females | Ovariectomized Females |
|---|---|---|---|---|
| A. Tail-Flick: Peak Effects (30 min) | | | | |
| Slope | 9.38 | 7.00 | 1.68 | 2.46 |
| Intercept | 0.68 | −0.79 | 0.09 | 0.82 |
| SE Est. | 3.10 | 1.88 | 0.82 | 1.75 |
| $ED_{50}$ | 1.93 | 2.50 | 13.71 | 3.74 |
| Ratio-Males | 1.0 | 1.3 | 7.1 | 1.9 |
| B. Tail-Flick: Total Effects | | | | |
| Slope | 17.57 | 10.83 | 4.20 | 3.80 |
| Intercept | −2.09 | −1.39 | 0.11 | 2.29 |
| SE Est. | 4.53 | 3.53 | 1.53 | 3.14 |
| $ED_{50}$ | 3.75 | 7.36 | 75.61 | 31.81 |
| Ratio-Males | 1.0 | 1.9 | 20.2 | 8.5 |
| C. Jump: Peak Effects (30 min) | | | | |
| Slope | .202 | .082 | .073 | .085 |
| Intercept | .040 | .105 | .050 | .016 |
| SE Est. | .042 | .054 | .016 | .025 |
| $ED_{50}$ | 2.49 | 1.52 | 9.10 | 16.73 |
| Ratio-Males | 1.0 | 0.6 | 3.7 | 6.7 |
| D. Jump: Total Effects | | | | |
| Slope | .463 | .277 | .135 | .223 |
| Intercept | .115 | .287 | .118 | .012 |
| SE Est. | .104 | .160 | .053 | .055 |
| $ED_{50}$ | 6.14 | 4.97 | 472.14 | 125.50 |
| Ratio-Males | 1.0 | 0.8 | 76.9 | 20.4 |

peak ($p>0.08$) and total ($p>0.18$) clonidine analgesia on the tail-flick test (Fig. 2b, c, Table 2).

Significant increases were also observed on the jump test following clonidine (ANOVAs, $p<0.05$) with analgesic magnitude significantly altered by both gender (100, 250 and 500 μg/kg: $p<0.0001$) and gonadectomy (250 μg/kg: $p<0.0001$; 500 μg/kg: $p<0.0011$). The magnitude of clonidine analgesia (250 μg/kg) was significantly greater in sham males across the entire time course relative to castrated males, sham females and ovariectomized females (Fig. 2d). Ovariectomized females displayed significantly greater clonidine (250 μg/kg) analgesia than sham females across the time course; indeed the analgesic responses of castrated males and ovariectomized females failed to differ from each other (Fig. 2a). Regression analyses revealed significant differences in the dose-response curves of both peak ($p<0.001$) and total ($p<0.001$) clonidine analgesia on the jump test (Fig. 2e, f, Table 2) with small 2-fold rightward shifts observed in sham females relative to sham males on both tests. Ovariectomy appeared to mitigate the rightward shifts in potency observed in sham females.

*Clonidine Analgesia and Estrous Phase*

Clonidine significantly increased tail-flick latencies ($p<0.0001$). Whereas a significant interaction between test times and estrous phase ($p<0.016$) was observed, the main effect of estrous phase ($p=0.23$) failed to achieve significance. Clonidine (250 μg/kg) analgesia on the tail-flick test was transiently greater during estrous (60 min) and met/diestrous (120 min) relative to the other phases. Whereas significant differences in jump thresholds were observed following clonidine, ($p<0.0001$), they failed to differ across estrous phases ($p=0.70$) or for the interaction between test time and estrous phase ($p=0.83$).

*Gonadectomy Effects*

Significant differences were observed in the weights of the seminal vesicles ($p<0.0001$) of sham ($0.95\pm0.06$ mg) and castrated ($0.06\pm0.01$ mg) males, and in the weights of the fallopian tubes ($p<0.0001$) of sham ($0.71\pm0.04$ mg) and ovariectomized ($0.23\pm0.04$ mg) females, resulting in respective 94% and 68% reductions in accessory gonadal tissue in gonadectomized males and females.

## DISCUSSION

The present study demonstrated that gender profoundly modulates analgesia elicited by the muscarinic receptor agonist, pilocarpine, and exerted lesser effects upon analgesia following the alpha$_2$-noradrenergic receptor agonist, clonidine with males exhibiting larger analgesic effects than females. Significant rightward shifts in the dose-response curve of pilocarpine analgesia were observed in sham females relative to sham males on the tail-flick (peak: 7-fold; total: 20-fold) and the jump (peak: 3-fold; total: 77-fold) tests. The greater shifts in total analgesia on both measures indicated the failure of pilocarpine to maintain analgesic effects across the 2-h time course. The 2-fold rightward shifts in the dose-response curve of clonidine analgesia on the jump test were more modest.

TABLE 2

REGRESSION ANALYSES OF THE LOG DOSE-RESPONSE FUNCTIONS OF CLONIDINE (μg/kg) ANALGESIA IN SHAM AND GONADECTOMIZED MALE AND FEMALE RATS ON THE TAIL-FLICK AND JUMP TESTS

| Condition | Sham Males | Castrated Males | Sham Females | Ovariectomized Females |
|---|---|---|---|---|
| A. Tail-Flick: Peak Effects (30 min) | | | | |
| Slope | 3.83 | 6.52 | 10.57 | 6.54 |
| Intercept | −3.11 | −11.53 | −19.96 | −10.68 |
| SE Est. | 2.83 | 1.54 | 2.07 | 2.04 |
| $ED_{50}$ | 71.8 | 240.9 | 184.3 | 198.3 |
| Ratio-Males | 1.0 | 3.4 | 2.6 | 2.8 |
| B. Tail-Flick: Total Effects | | | | |
| Slope | 13.08 | 17.94 | 28.11 | 12.03 |
| Intercept | −12.35 | −30.28 | −50.76 | −16.59 |
| SE Est. | 8.51 | 5.27 | 9.25 | 5.48 |
| $ED_{50}$ | 147.0 | 379.4 | 237.1 | 502.0 |
| Ratio-Males | 1.0 | 2.6 | 1.6 | 3.4 |
| C. Jump: Peak Effects (30 min) | | | | |
| Slope | .517 | .210 | .153 | .119 |
| Intercept | −.934 | −.293 | −.239 | −.101 |
| SE Est. | .068 | .068 | .104 | .045 |
| $ED_{50}$ | 109.3 | 93.6 | 222.0 | 72.0 |
| Ratio-Males | 1.0 | 0.9 | 2.0 | 0.7 |
| D. Jump: Total Effects | | | | |
| Slope | 2.07 | 0.87 | 0.51 | 0.50 |
| Intercept | −3.78 | −1.28 | −0.75 | −0.51 |
| SE Est. | .248 | .231 | .303 | .134 |
| $ED_{50}$ | 114.2 | 108.3 | 258.1 | 95.0 |
| Ratio-Males | 1.0 | 0.9 | 2.3 | 0.8 |

The main impetus for evaluating gender differences in pilocarpine and clonidine analgesia was that: a) gender differences were observed for nonopioid CCWS analgesia (31), and b) CCWS analgesia is mediated in part by both muscarinic cholinergic (35) and alpha$_2$-noradrenergic (4,22) receptor influences. The analgesic responses following CCWS, pilocarpine and clonidine analgesia are each significantly greater in males than in females. The gender differences observed for these nonopioid forms of analgesia parallel those observed following opioid-mediated ICWS (31), and both systemic (2,20) and central (23) morphine analgesia. In contrast, gender failed to alter the analgesic dose-response curves of the mu-selective agonist, DAMGO or the delta-selective agonist, DSLET (24). Gender differences in pilocarpine and clonidine analgesia may be mediated through gonadal hormone effects upon analgesia-sensitive neurons that contain muscarinic cholinergic or alpha$_2$-noradrenergic receptors. There is evidence of gonadal steroid modulation of noradrenergic and cholinergic function. Hypothalamic guinea pig alpha$_2$-noradrenergic receptors are sexually dimorphic and are differentially modulated by estradiol and progesterone (18,19). Alpha-bungarotoxin binding is also sexually dimorphic and sensitive to gonadal hormone modulation in the mouse amygdala and suprachiasmatic nucleus (1,27). Whereas the ventral medulla mediates muscarinic cholinergic analgesia (5), alpha$_2$-noradrenergic agonists produce analgesia at both spinal and supraspinal sites [see review: (39)]. Estradiol-containing midbrain neurons in the analgesia-sensitive periaqueductal gray project directly to the medulla (10), and may be a source of gonadal interaction.

Any mediation of gender differences in pilocarpine and clonidine analgesia by gonadal hormones would involve either organizational or activational effects (29). Activational roles of gonadal hormones upon pilocarpine and clonidine analgesia were evaluated in the adult gonadectomy and estrous phase studies. Although pilocarpine (5 mg/kg) and clonidine (250 μg/kg) analgesia were significantly reduced in castrated rats, this manipulation failed to alter either analgesic dose-response curve. In contrast, ovariectomy appeared to mitigate the gender differences observed for both pilocarpine and clonidine analgesia, and actually was similar to castrated males for clonidine analgesia on the jump test. Finally, estrous phase failed to alter consistently either pilocarpine or clonidine analgesia on either test in female rats. The main impetus for evaluating adult gonadectomy effects upon pilocarpine and clonidine analgesia was that nonopioid CCWS analgesia is sensitive to gonadectomy differences (33) and steroid replacement therapy (32). In contrast to the modest reductions in pilocarpine and clonidine analgesia following castration, this manipulation reduced CCWS analgesia to levels equal to that of females; this effect was reinstated by pretreatment with testosterone propionate. Further, in contrast to the transient increases in pilocarpine and clonidine analgesia in ovariectomized relative to sham female rats, CCWS analgesia was reduced in ovariectomized rats relative to sham females, but reinstated by testosterone propionate. A similar pattern of more pronounced gonadectomy effects is also observed for opioid-mediated ICWS analgesia (33). These data suggest that either other transmitter/peptide systems more sensitive to adult gonadectomy effects are involved in the mediation of CCWS and ICWS analgesia, and/or adult gonadectomy is also altering in part the coding of the stressful consequences of CCWS and ICWS independent of the analgesic alterations.

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