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The effects of diazepam dependence and withdrawal on morphine-induced antinociception and changes in locomotion in male and female rats

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Abstract

Male and female rats were exposed for 3 weeks to diazepam (DZ)-filled or empty capsules (CTR) prior to the daily administration of morphine (MOR, 5 mg/kg ip) for 5 days. Thereafter, capsules were removed and 48 h later MOR was injected for the next 5 days. The tail-flick latency (TFL) was measured prior to and 15, 30, and 60 min after MOR assessed analgesia. Locomotion (LOC) was determined before and 15 min after injection. Prior to MOR injection (baseline), male rats were more sensitive to the thermal stimulus and were less active than female rats. Daily MOR injections neither affected the baseline TFL nor LOC. Regardless of gender, MOR produced greater analgesia in DZ-dependent and withdrawn rats than in CTR. MOR analgesia was greater in DZ-dependent male than in female rats. Gender differences in MOR analgesia were not of statistical significance in DZ-withdrawn rats. The first dose of MOR produced more depression of LOC in DZ-dependent female than in male rats. Across the time of MOR injections, female DZ-dependent and withdrawn rats were less active than CTR. LOC increased with repeated administration of MOR in all groups of rats. In summary, DZ dependence and withdrawal enhanced MOR analgesia in rats of both sexes. Regardless of chronic treatment, MOR produced more analgesia and less depression of LOC in male than in female rats. It is suggested that a decrease in the function of the GABAergic system plays a role in alteration of MOR analgesia.

Keywords: Morphine analgesia; Diazepam dependence; Morphine tolerance; Locomotion; Gender

1. Introduction

There is good evidence that benzodiazepines (BZs) interact with opioids. For example, it has been reported that various BZ ligands modify the morphine (MOR) withdrawal syndrome (Gilbert-Rahola et al., 1988; Gray, 1996; Maldonado et al., 1991; Suzuki et al., 1996; Valverde et al., 1992, 1995; Zarrindast and Mousa-Ahmadi, 1999) as well as that the opioid antagonist, naloxone, blocks the antianxiety effect of diazepam (DZ) (Agmo et al., 1995) and antagonizes the locomotor effect of nitrazepam (Nowakowska and Chodera, 1991). Furthermore, the acute administration of BZs increased the pain threshold (Chapman and Feather, 1973; Wuster et al., 1980), the central BZ receptor antagonist, flumazenil, enhanced naloxone-induced analgesia (Cappell et al., 1989), and naloxone reversed the analgesia produced by flumazenil (Davidovich et al., 1988).

The effect of BZs on MOR-induced analgesia has been extensively studied but the data are contradictory and suggest bidirectional modulatory action: antagonism of MOR analgesia by BZs in the brain (Luger et al., 1994; Mantegazza et al., 1982; Zambotti, F., 1986) and potentiation in the spinal cord (Luger et al., 1994; Moreau and Puieri, 1988; Nadeson et al., 1996; Pick, 1997; Rattan et al., 1991; Yanez et al., 1990). Moreover, evidence exists that the BZ and MOR interplay on antinociception depends upon the BZ ligand, dose and mode of administration (single or repeated). With regard to the latter, several papers showed that acute systemic administration of BZs antagonized antinociception induced by MOR (Daghero et al., 1987; Palaoglu and Ayhan, 1986; Rosland and Hole, 1990a,b) while limited and controversial information revealed that chronic pretreatment with DZ or midazolam either antagonized or did not affect the analgesia of acute MOR (Rosland and Hole, 1990b; Tejwani et al., 1993). It is interesting, however, that repeated coadministration of BZs and MOR

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seemed to attenuate development of tolerance to MOR-induced antinociception (Sheu et al., 1995; Sribanditmongkol et al., 1994; Tejwani et al., 1993, 1998).

Several lines of evidence indicate interactions between the opioid and GABAergic systems and suggest that activation of supraspinal $GABA_A$ receptors antagonized MOR-induced analgesia (for reference, see Rady et al., 1993; Sivam and Ho, 1985). It is well established that BZs exert their effect through GABAergic mechanisms. Activation of the central BZ binding site facilitates the inhibitory action of $GABA_A$, whereas chronic BZ treatment is associated with a decrease of function of the GABAergic system and development of physical dependence on and tolerance to some effects of BZ (for reference, see Gallager and Primus, 1993). Thus, assuming that GABAergic mechanisms play a role in modulation of antinociception, it can be speculated that acute BZs antagonize MOR analgesia via stimulation of the GABAergic transmission whereas BZ–MOR antagonism is abolished during chronic BZ treatment due to impairment of function of the GABAergic system.

Accordingly, the present study was conducted to determine the effects of long-term exposure to DZ and discontinuation of treatment on the analgesic effect of MOR in the rat. Since motor impairment may affect the response to thermal stimuli, the total locomotion (LOC) also was assessed after MOR administration in DZ-dependent, DZ-withdrawn, and control (CTR) rats. Furthermore, gender differences were reported both for the antinociceptive effect of MOR (Bartok and Craft, 1997; Boyer et al., 1998; Cicero et al., 1996, 1997; Krzanowska and Bodnar, 1999; Tsang et al., 1999) and the dependence-producing properties of BZ (Pesce et al., 1994; Sloan et al., 1996; Wala et al., 1999; Wilson and Biscardi, 1992) and thus, the DZ and MOR interaction was compared herein in male and female rats.

2. Methods

2.1. Rats

Male and female Sprague–Dawley (body weight $\approx$350 and 250 g, respectively), age-matched ($\approx$90 days old) rats were used in this study. The estrous cycle was not determined in female rats. Rats were housed in accordance with the ''Principles of Laboratory Animal Care'' (NIH publication No. 85-23, revised 1985) in a humidity- and temperature-controlled facility with 12L:12D cycles with a light onset at 0600 h. Each rat was kept separately in a transparent cage with free access to standard laboratory chow and tap water. The experiments were conducted according to a protocol approved by the University of Kentucky Animal Care and Use Committee. The body weights were recorded weekly (prior to implantation of the successive capsule), and thereafter, daily (prior to administration of MOR). Capsules were implanted under sterile conditions and ketamine chloride anesthesia (40 mg/kg ip). At the end of the study, the rats were killed with pentobarbital sodium (120 mg/kg ip).

2.2. Drugs and treatments

Physical dependence on DZ was induced by the capsule implantation technique (Gallager et al., 1985) with minor modifications (Martin et al., 1993). Continuous, slow release of DZ from silastic capsules produced a high level of dependence as indicated by flumazenil-evoked clonic and tonic–clonic convulsions and several other signs and symptoms of abstinence syndrome (Gallager et al., 1985; Martin et al., 1993; Wala et al., 1997; Wilson and Gallager, 1988). Briefly, DZ (a kind gift from Hoffman La Roche) was placed in Medical Grade Silastic tubing (Dow Corning; ID 0.147 cm $\times$ OD 0.195 cm; 7 and 9 cm long in female and male rats, respectively). A capsule contained either 90 mg (female) or 120 mg (male) of crystallized DZ ($\approx$360 mg/kg/week). The rats were subcutaneously implanted in the back with two capsules (loading dose) and thereafter, with an additional capsule at weekly intervals for 3 weeks (maintenance dose). Since a capsule was almost empty a week after its implantation, an average in vivo release of 50 mg/kg/day of DZ can be assumed. This method generated the steady-state plasma levels (μg/ml) equal to: DZ = 2.05 ± 0.37 and 1.44 ± 0.41, nordiazepam = 0.46 ± 0.22 and 0.18 ± 0.07, oxazepam = 0.59 ± 0.45 and 0.05 ± 0.03 in male and female rats, respectively (plasma levels were not significantly different in male and female rats) (Sloan et al., 2000). CTR rats were implanted with empty capsules. After 4 weeks of exposure to DZ or CTR treatment, the capsules were removed under light ketamine anesthesia.

Morphine sulfate (Merck) was administered intraperitoneally (5 mg/kg/day; volume of injection 1 ml/kg): (1) on Day 1 through Day 5 at Week 4 of chronic DZ or empty capsules treatment, and (2) at withdrawal (starting 48 h after removal of DZ or empty capsules) on Day 6 through Day 10 at Week 5 of the study. Time intervals between Injections 1 and 5 as well as between Injections 6 and 10 were equal to 24 h. Time interval between Injection 5 (last MOR administration during exposure to implants) and Injection 6 (first MOR administration after removal of implants) was equal to about 72 h. Table 1 summarizes treatment schedules for implantation of DZ-filled or empty capsules and for administration of MOR in the present study.

Table 1
Schedule of treatments

Weeks 1–3 (Friday)	Implantation of DZ-filled (90 or 120 mg/capsule in female and male rats, respectively) or empty capsules at weekly intervals
Week 4 (Monday–Friday)	Daily injections of MOR (5 mg/kg ip) in rats chronically exposed to DZ-filled or empty capsules
Week 4 (Saturday)	Removal of DZ-filled or empty capsules
Week 5 (Monday–Friday)	Daily injections of MOR (5 mg/kg ip) in rats withdrawn from DZ or control treatment

2.3. Antinociceptive test

Analgesia was measured by the radiant heat tail-flick assay (Analgesia tail-flick apparatus, EMDIE Instrument). The lamp intensity was adjusted to give a baseline (pre-MOR) tail-flick latency (TFL) equal to about 2–3 s. Baseline TFL was measured before injection of MOR and was expressed as the mean of the two trials performed at about 15-min intervals. Analgesia was measured at 15, 30, and 60 min after MOR injection (postMOR TFL). To prevent damage of the tail the cut-off response time was equal to 10 s.

2.4. Motor activity

The Opto-Varimex infrared photocell-based activity monitor (Columbus Instrument) was used to measure motor activity (total LOC) prior to (baseline) and 15 min after injection of MOR. The beam interruptions were monitored along a single axis and all activities (ambulatory counts and stereotypic activity) were scored (5-min session) on a front panel counter. Total activity was determined immediately after the tail-flick test. Everyday rats were tested in the same order. All testing were conducted between 0900 and 1200 h.

2.5. Data analysis

For each rat at each time point, TFL and motor activity (LOC) were normalized for baseline (postMOR − preMOR). Areas under time action curves ($AUC_{0-60\ min}$) were calculated for baseline-normalized TFL. The maximum possible effect (MPE) was defined as $\%MPE = [AUC_{0-60\ min}/AUC_{0-60\ min\ (max)}] \times 100]$ where $AUC_{0-60\ min}$ and $AUC_{0-60\ min\ (max)}$ were calculated for the baseline-normalized actual response time (TFL) and baseline-normalized cut-off time (10 s), respectively. Overall treatment and gender effects were assessed with two-way RM ANOVA (Treatment × Days or Gender × Days). Between-days differences were evaluated with one-way RM AVOVA. Appropriate pairwise comparisons were performed with the Student–Newman–Keuls or Student's *t* tests. Statistical significance was established as $P < .05$.

3. Results

Fig. 1 compares baseline sensitivity to thermally induced pain (preMOR TFL) [Panel A] and baseline total locomotor activity (preMOR LOC) [Panel B]: (1) prior to initiation of study (naive rats); (2) during chronic DZ or CTR (empty capsule) treatments; and (3) at withdrawal in male and female rats. *Baseline TFL*: As can be seen, experimentally naive as well as empty capsule-treated male rats (CTR) were significantly more sensitive to thermally evoked pain than identically treated female rats ($P < .05$, *t* test). The same pattern was also observed in DZ-dependent rats, however, the between-gender differences in preMOR TFL were below the level of statistical significance. The baseline TFL was greater during chronic exposure to DZ and withdrawal than during respective control treatments ($P < .05$, *t* test). Removal of DZ-filled or empty capsules did not significantly affect the baseline TFL. *Baseline LOC*: The data also show that male rats during DZ dependence and withdrawal were significantly less active than identically treated female rats ($P < .5$, *t* test). Naive male rats tended to be less active than naive female rats ($P = .1$, *t* test). DZ-treated male rats were less active than CTR ($P < .0001$, *t* test). As indicated by two-way RM ANOVA (Gender × Time or Treatment × Time), regardless of treatment (DZ or CTR) and gender, the sensitivity to thermally evoked pain (preMOR TFL) and spontaneous motor activity (preMOR LOC) did not change across time of repeated challenges with thermal stimulus (Days 1–10).

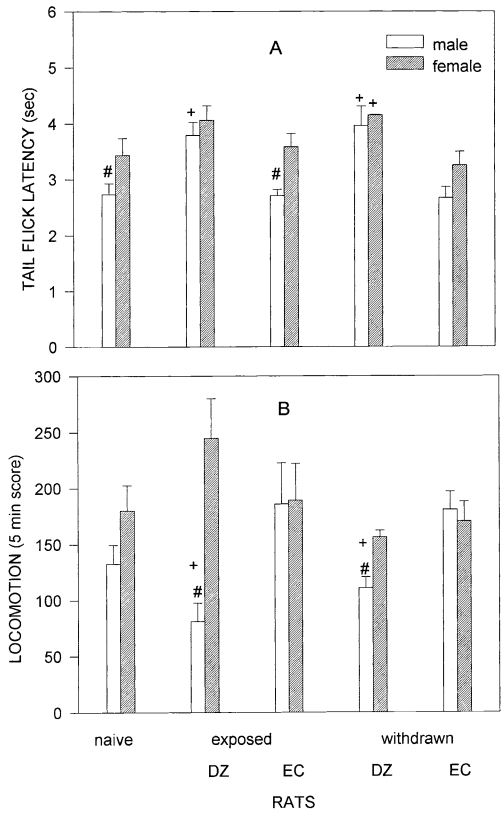

Fig. 1. Responses to thermal stimuli (TFL) [A] and total LOC [B] prior to (naive), during (exposed), and after (withdrawn) chronic exposure to DZ-filled and empty (EC) capsules in male and female rats. The naive values are mean of two baseline observations prior to initiation of DZ or EC treatment (13 rats/sex). The exposed values are mean of preinjection baseline across time (5 days) of daily MOR injections in DZ-exposed (five rats per sex) and EC-exposed (eight rats per sex). The withdrawn values are mean of preinjection baseline across time of daily MOR injections (5 days) in DZ-withdrawn (five rats per sex) and EC-withdrawn (eight rats per sex). Data are mean ± S.E.M. of (*n*) rats. # Significantly different from identically treated female rats ($P < .05$, *t* test); + Significantly different from identically treated control rats (EC) of the same sex ($P < .05$, *t* test).

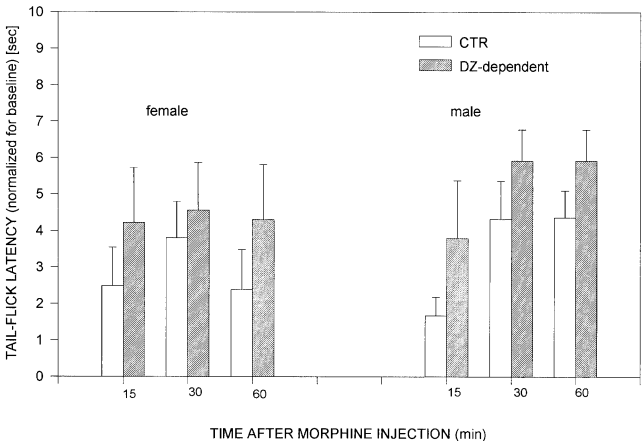

Fig. 2. Responses to thermal stimuli (TFL) after first administration of MOR (5 mg/kg ip, Day 1) in DZ-dependent and CTR male and female rats. Prior to MOR injection, DZ-dependent rats (five rats per sex) were exposed for 3 weeks to DZ-filled implants (120 and 90 mg/week in male and female, respectively); CTR rats (eight rats per sex) were exposed for 3 weeks to empty capsules. Data are normalized for preMOR baseline as defined in text. The data represent mean ± S.E.M. of (n) rats.

Fig. 2 shows analgesia (as indicated by baseline-normalized TFL) produced by a first injection of MOR (5 mg/kg ip, Day 1) in DZ-dependent and CTR male and female rats. As indicated by one-way AVOVA, MOR-induced analgesia did not significantly differ between treatments either in male or female rats [AUC_{0-60} (sec × min) for TFL: 159.3 ± 39.9; 230.4 ± 56.4; 187.3 ± 32.8; 279.0 ± 46.7 for female CTR, female DZ-dependent, male CTR, and male DZ-dependent rats, respectively].

Fig. 3 illustrates responses to thermally evoked pain [percentage of MPE (%MPE)] after daily administration of MOR (5 mg/kg/day ip) in male rats chronically exposed to (Injections 1–5) and withdrawn from (Injections 6–10) DZ-filled or empty capsules (CTR). Fig. 4 illustrates %MPE in female rats. The effects of treatment, gender, and time on MOR-induced analgesia were as follows: (1) *Treatment*: MOR produced significantly greater analgesia in DZ-dependent than in CTR rats [%MPE (Injections 1–5): DZ-exposed>CTR-exposed; $F(1,44) = 16.1$, $P < .0025$ and $F(1,44) = 10.6$, $P < .01$ in male and female rats, respectively; two-way RM ANOVA]. Similar phenomena were observed when MOR was injected during DZ withdrawal [%MPE (Injections 6–10): DZ-withdrawn>CTR-withdrawn; $F(1,36) = 12.5$, $P < .005$ and $F(1,36) = 7.8$, $P < .025$ in male and female rats, respectively; two-way RM ANOVA]. (2) *Gender*: MOR induced significantly greater analgesia in DZ-dependent male than in DZ-dependent female rats while in CTR rats the between-gender differences were below the level of statistical significance [%MPE (Injections 1–5): male>female; $F(1,32) = 40.8$, $P < .00025$ and $F(1,56) = 3.4$, $P = .08$ for DZ-exposed and CTR-exposed rats, respectively; two-way RM ANOVA]. Gender differences in MOR-induced analgesia were not of statistical significance during withdrawal (Injections 6–10). Across the time of MOR administration, the between-gender differences were of statistical significance in male and female rats dependent

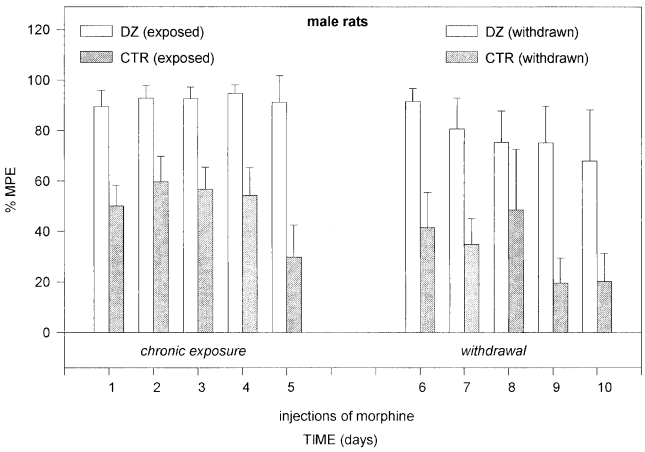

Fig. 3. Responses to thermal stimuli across time of daily administration of MOR (5 mg/kg ip) in male rats dependent on and withdrawn from DZ as well as in control male rats (CTR). *Chronic exposure*: Rats were implanted with DZ-filled (120 mg/week) [DZ (exposed)] or empty capsules [CTR (exposed)] for 3 weeks prior to initiation of MOR treatment (Injections 1–5). *Withdrawal*: Capsules were removed 48 h prior to MOR treatment (Injections 6–10) [DZ (withdrawn) and CTR (withdrawn) rats]. MOR was not administered for 72 h between Injection 5 and 6. Data are expressed as percentage of maximum possible effect (%MPE) as defined in the text. The data represent mean ± S.E.M. of five DZ-treated and eight CTR male rats. ANOVA demonstrated significant between treatments differences in %MPE in male rats: DZ (exposed) > CTR (exposed), $P < .0025$; DZ (withdrawn)>CTR (withdrawn), $P < .005$.

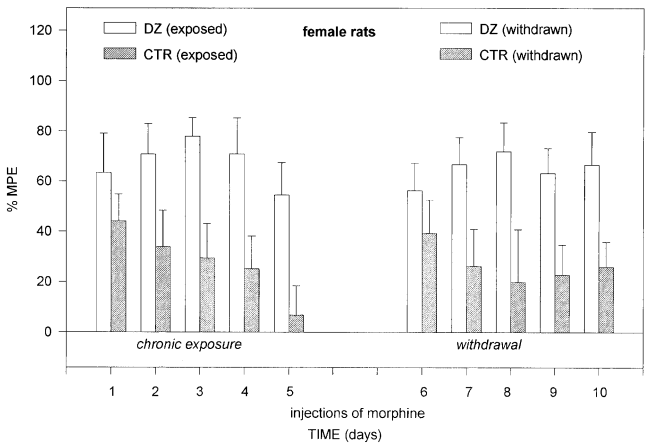

Fig. 4. Responses to thermal stimuli across time of daily administration of MOR (5 mg/kg ip) in female rats dependent on and withdrawn from DZ as well as in CTR. *Chronic exposure*: Rats were implanted with DZ-filled (90 mg/week) [DZ (exposed)] or empty capsules [CTR (exposed)] for 3 weeks prior to initiation of MOR treatment (Injections 1–5). *Withdrawal*: Capsules were removed 48 h prior to MOR treatment (Injections 6–10) [DZ (withdrawn) and CTR (withdrawn) rats]. MOR was not administered for 72 h between Injection 5 and 6. Data are expressed as percentage of maximum possible effect (%MPE) as defined in the text. The data represent mean ± S.E.M. of five DZ-treated and eight CTR female rats. ANOVA demonstrated significant between-treatments differences in %MPE in female rats: DZ (exposed) > CTR (exposed), $P < .01$; DZ (withdrawn) > CTR (withdrawn), $P < .025$.

on and subsequently withdrawn from DZ [%MPE (Injections 1–10): male>female; $F(1,72)=13.8$, $P<.01$; two-way RM ANOVA] but not in CTR male and female rats. (3) *Time*: As indicated by one-way RM ANOVA, MOR analgesia was not significantly different between-days of MOR injections either in DZ-exposed or CTR-exposed male and female rats (Injections 1–5). The same was true for MOR analgesia during withdrawal (Injections 6–10). Furthermore, regardless of gender, MOR analgesia was not significantly different during chronic DZ exposure and at withdrawal (Injections 1–5 vs. Injections 6–10). It should be mentioned, however, that analgesia had a significant negative regression on time [%MPE (Injections 1–5): $F(1,70)=11.3$, $P<.025$] in CTR female, but not in CTR male, DZ-dependent male, and DZ-dependent female rats. On the other hand, the analgesic effect of MOR, as measured during the combined empty capsule exposure and withdrawal (Injections 1–10), showed a significant between-days difference [$F(9,55)=2.9$, $P<.01$; one-way RM ANOVA] as well as a significant negative regression on time [$F(1,70)=11.3$, $P<.0025$)] in CTR male but not in CTR female rats. In DZ-treated and -withdrawn male and female rats, the MOR-induced analgesia did not change across time (Figs. 3 and 4).

Fig. 5 presents changes in total LOC during the daily administration of MOR (5 mg/kg ip) in male rats chronically exposed to and withdrawn from DZ or empty capsules (CTR). Fig. 6 shows the effect of MOR on LOC in female rats. As can be seen, the first dose of MOR (Day 1) produced depression of locomotor activity in all groups of rats. This effect was significantly greater in DZ-dependent female than in DZ-dependent male rats [$F(3,22)=7.7$, $P<.001$), one-way ANOVA and post hoc Student–Newman–Keuls method]. The data show that treatment, gender, and time of administration affected the MOR-induced changes in spontaneous motor activity. (1) *Treatment*: During the time of administration of MOR, DZ-exposed and withdrawn female rats were significantly less active than CTR female rats [LOC (Injections 1–10): DZ<CTR; $F(1,93)=12.4$, $P<.005$; two-way RM ANOVA]. On the contrary, the between-treatments difference in LOC was not of statistical significance in male rats DZ-exposed and withdrawn and in CTR male rats. The female rats were less active when MOR was administered during DZ exposure than at withdrawal [LOC (Injections 1–5)<LOC (Injections 6–10); $F(4,16)=7.3$, $P<.0005$; two-way RM ANOVA]. (2) *Gender*: DZ-dependent female rats were significantly less active than identically treated male rats [LOC (Injections 1–5): female<male; $F(1,32)=7.9$, $P<.025$; two-way RM ANOVA]. DZ-withdrawn female and male rats showed similar activity. Gender differences in LOC were not of statistical significance throughout the daily administration of MOR in CTR rats. (3) *Time*: As indicated by one-way RM ANOVA, regardless of treatment, the effect of MOR on LOC significantly varied between days in female [LOC (Injections 1–10): $F(9,36)=8.1$ and $F(9,57)=5.4$, $P<.0001$ for DZ-treated and CTR female rats, respectively] but not in male rats. Overall, LOC had a significant positive regression on time [Injections 1–10:

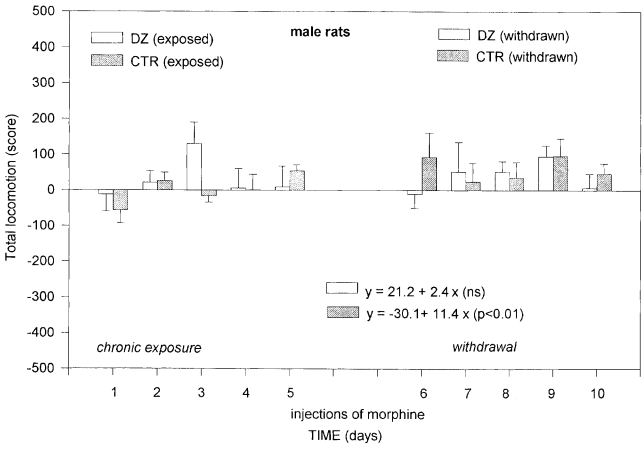

Fig. 5. Total LOC across time of daily administration of MOR (5 mg/kg ip) in DZ-dependent and subsequently withdrawn male rats and in CTR male rats (baseline-normalized scores; 15 min after injection). *Chronic exposure*: Rats were implanted with DZ-filled (120 mg/week) [DZ (exposed)] or empty capsules [CTR (exposed)] for 3 weeks prior to initiation of MOR treatment (Injections 1–5). *Withdrawal*: Capsules were removed 48 h prior to MOR treatment (Injections 6–10) [DZ (withdrawn) and CTR (withdrawn)]. MOR was not administered for 72 h between Injections 5 and 6. The data are mean ± S.E.M. of five DZ-treated and eight CTR male rats.

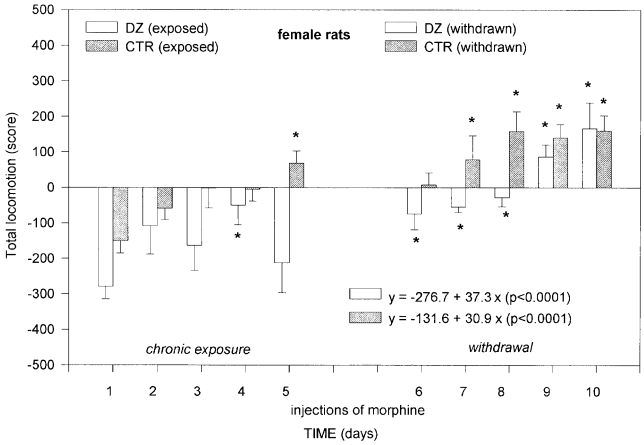

Fig. 6. Total LOC across time of daily administration of MOR (5 mg/kg ip) in DZ-dependent and DZ-withdrawn female rats and CTR female rats (baseline-normalized scores; 15 min after injection). *Chronic exposure*: Rats were implanted with DZ-filled (90 mg/week) [DZ (exposed)] or empty capsules [CTR (exposed)] for 3 weeks prior to initiation of MOR treatment (Injections 1–5). *Withdrawal*: Capsules were removed 48 h prior to MOR treatment (Injections 6–10) [DZ (exposed) and CTR (exposed)]. MOR was not administered for 72 h between Injections 5 and 6. The data are mean ± S.E.M. of five DZ-treated and eight CTR female rats. ANOVA demonstrated significant differences in locomotion in female rats: (1) between treatments: DZ (exposed)<DZ (withdrawn), $P<.005$; DZ (exposed + withdrawn)<CTR (exposed + withdrawn), $P<.005$; (2) between injections (1–10): DZ (exposed + withdrawn), $P<.0001$; CTR (exposed + withdrawn), $P<.0001$. * Significantly different from Day 1 ($P<.05$; Student–Neuman–Keuls).

$F(1,48) = 32.4$ and $F(1,72) = 47.7$, $P < .0001$ for DZ-treated and CTR female rats, respectively, as well as $F(1,72) = 6.9$, $P < .01$ for CTR male rats (Figs. 5 and 6)].

4. Discussion

As indicated herein, chronic pretreatment with DZ-filled capsules did not significantly affect the antinociceptive effect of acute MOR (5 mg/kg ip, Day 1) in male and female rats (AUC_{0-60} for the baseline-normalized TFL). This finding seems to support the results of previous studies showing that pretreatment with DZ or midazolam (daily injections for 8 and 11 days, respectively) did not alter MOR-induced analgesia in the rat (Rosland and Hole, 1990b; Tejwani et al., 1993). However, the present data demonstrate that DZ treatment (3 weeks of continuous exposure to DZ-filled implants) influenced the outcome of analgesia (%MPE) across the time of chronic administration of MOR. Accordingly, when MOR was repeatedly injected (5 mg/kg/day ip, Day 1 through Day 5), a greater antinociception was maintained in DZ-dependent than in CTR rats. Interestingly, increased levels of MOR analgesia also were sustained following removal of DZ-filled implants (Injection 6 through Injection 10). This protracted effect was unlikely due to the residual levels of DZ and its metabolites in plasma and/or brain. Chronic DZ treatment was discontinued 48 h prior to administration of the first dose of MOR while, as has been previously reported, DZ and its major metabolite, nordiazepam, disappeared from plasma and brain with half lives equal to about 1 h in the rat (Friedman et al., 1986).

It has been previously reported that MOR antinociception was stable after repeated coadministration of midazolam (ip) and MOR (10 mg/kg/day sc) for 11 days in rats (Tejwani et al., 1993). Furthermore, MOR pellet-implanted rats repeatedly treated with DZ (ip) showed more antinociception across time in comparison to MOR-dependent rats treated with saline (Sheu et al., 1995; Sribanditmongkol et al., 1994; Tejwani et al., 1993, 1998). Taken together, these data indicate that chronic BZ treatment prevented and/or delayed development of MOR tolerance in male rats. Although in the present study analgesia seems to decline with time in CTR but not in DZ-treated rats, it must be emphasized that there was no evidence of marked MOR tolerance in CTR rats. This was probably due to the dosage regimen employed herein: MOR (5 mg/kg/day) for 5 days, followed by cessation of MOR administration for about 72 h, and the next 5 days of MOR (5 mg/kg/day). For example, significant tolerance developed at Day 7 of daily injections of a higher dose of MOR (10 mg/kg ip) (Tejwani et al., 1993), at Day 5 of MOR (10 mg/kg sc) injections twice daily (Trujillo and Akil, 1991), and at Day 3 after implantation of MOR pellets (two and four pellets on Days 1 and 2, respectively) (Tejwani et al., 1998) in male rats. In all the above cited studies, the analgesic effect of MOR (prior to development of tolerance) was equal to cut-off time (%MPE = 100). Thus, this higher rate of MOR administration was not suitable for the present study. In previous studies, BZs were given in intermittent doses whereas herein, rats were subjected to continuous treatment with DZ-filled capsules (a slow-release technique which circumvents fast metabolism of DZ in the rats). Since physical dependence on DZ, as indicated by flumazenil-induced withdrawal, was more pronounced when rats were continuously exposed to DZ-filled implants than after repeated administration of intraperitoneal doses (Gallager et al., 1985; Martin et al., 1993; Wala et al., 1997; Wilson and Gallager, 1988), determination of the effect of continuous BZ exposure on development of MOR tolerance is in order.

The mechanism by which chronically administered BZ attenuates MOR analgesia is not known nor is it certain whether BZs interact directly with the opioid system or modulate the effects of opioids through an action on the $GABA_A$/BZ/ionophore complex. It appears, however, that the repeated administration of MOR causes changes in the opioid receptors, endogenous opioid peptides, G-proteins or cyclic AMP and that these changes can be altered by chronically administered BZ. For example, MOR and midazolam had opposite effects on the β-endorphinergic system in the rat (stimulation and inhibition, respectively) while concomitant repeated administration of BZ and MOR abolished the stimulatory effect of MOR on β-endorphinergic system (Rattan and Tejwani, 1996). The met-enkephalin brain levels were reduced in rats chronically treated with MOR but not in rats treated with MOR plus DZ or midazolam (Sribanditmongkol et al., 1994; Tejwani and Rattan, 1997). Concomitant administration of midazolam and MOR reversed the MOR-induced decrease in the levels of dynorphins 1–13 (Rattan and Tejwani, 1997). Coadministration of DZ and MOR attenuated an increase in cyclic AMP levels during the naloxone-precipitated abstinence syndrome (Sheu et al., 1995). In addition, DZ antagonized the up-regulation of μ-opioid receptors in MOR-tolerant rats (Tejwani et al., 1998), the GABA-stimulated BZ binding decreased with MOR tolerance in rats (for review, see Sivam and Ho, 1985) while a short-lasting treatment (3 days) with MOR enhanced BZ binding and muscimol-stimulated chloride uptake in mice (Lopez et al., 1990).

Several papers showed that acutely coadministered BZs antagonized the analgesic effect of acute MOR and that this effect was reversed by blockage of the BZ receptor with the specific BZ antagonist, flumazenil (Daghero et al., 1987; Luger et al., 1994; Mantegazza et al., 1982; Palaoglu and Ayhan, 1986; Rady et al., 1993; Rattan et al., 1991; Rosland and Hole, 1990a,b; Zambotti et al., 1986). In contrast, the present data, together with data from other laboratories (mentioned above), revealed that the antagonistic effect of BZs on MOR analgesia did not occur during chronic DZ or midazolam treatments. These observations suggest that acute and chronic occupation of the

BZ site differentially modulate the antinociceptive effect of MOR. It is well-established that activation of the central BZ binding sites ultimately results in a facilitation of the inhibitory action of $GABA_A$ and that at the cellular level, activation of $GABA_A$ receptors inhibits cell firing by activating an ionophore that facilitates Cl^- influx. Furthermore, there is a line of evidence that prolonged stimulation of the $GABA_A$/BZ/ionophore complex results in a decrease of the functional activity of the $GABA_A$ receptor and development of tolerance to some effects of BZ. The mechanism of decrease of GABAergic function is still controversial. Several studies suggest the involvement of down-regulation of BZ receptors, changes in the GABA receptor subunits composition, and/or decrease in allosteric coupling between the BZ binding site and the Cl^- channel (for references, see Miller et al., 1990; Primus et al., 1996; Schoch et al., 1993; Tietz et al., 1999). It is interesting, that GABA subsensitivity was more pronounced after continuous rather than intermittent occupation of the BZ recognition sites (for review, see Gallager et al., 1989, 1991; Gallager and Primus, 1993). Furthermore, decreased function of GABA lasted for up to 5 days after removal of the DZ capsules and for more than 72 h after the last DZ injection (even though brain levels of DZ and its metabolites were below the level of detection) (Gonsalves and Gallager, 1985, 1987). These data suggest that although continuity of occupation of the BZ recognition site(s) plays a key role in attenuation of the functional activity of the $GABA_A$ receptors, the time course of GABA subsensitivity seems to be longer than occupation of receptor(s) by BZ. In view of the above data, it is appealing to speculate that impairment of function of the GABAergic system (due to chronic DZ exposure) prevents desensitization of the endogenous pain modulatory system during chronic administration of MOR. It is important to mention, however, that different BZs produced different types of physical dependence (Martin et al., 1990) and that, due to the diversity of the $GABA_A$ receptor subtypes and heterogeneity of distribution in the CNS, the GABA subsensitivity showed a regional specificity and dependency upon the efficacy of the BZ ligands (for review, see Gallager and Primus, 1993; Primus et al., 1996). Thus, further research is needed in order to establish the effect of chronic administration of BZ with different affinities for the GABA receptor subunits on MOR antinociception.

Despite the existing literature on gender-related differences in opioid-induced antinociception, little is known about the consequences of chronic administration of MOR in male and female subjects. Several papers showed that in comparison to female rats, male rats were more sensitive to the analgesic properties of acutely (systemic and focal brain injections) administered MOR (Bartok and Craft, 1997; Boyer et al., 1998; Candido et al., 1992; Cicero et al., 1996, 1997; Islam et al., 1993; Kepler et al., 1989; Krzanowska and Bodnar, 1999). Interestingly, opposite effects have recently been reported after intrathecal administration of MOR in male and female rats (Tsang et al., 1999). The present data indicate the following: (1) Although chronic MOR induced a greater antinociception in CTR male than in female rats, the differences were not of statistical significance; 2) Sex differences in analgesic effect seemed to be less pronounced across the time of repeated MOR treatment in CTR rats; 3) Gender-related differences in MOR analgesia were of statistical significance during concomitant chronic DZ treatment. Moreover, we found that under the baseline condition (preMOR), sensitivity to thermally evoked pain was greater (shorter TFL) in male than in female rats. The previous reports revealed that baseline latencies on a tail-flick test were shorter in male rats in comparison to female rats (Bartok and Craft, 1997; Forman et al., 1989; Islam et al., 1993) or that there was a lack of gender difference on this measure (Candido et al., 1992; Cicero et al., 1996; Kepler et al., 1991). It can be argued that if male rats are overall more sensitive to thermal stimuli than female rats, then a similar dose of MOR (mg/kg) should produce less antinociception in male than in female rats. This is not the case herein. The mechanism underlying gender-related responsiveness to MOR is not certain. It is thought to relate to several factors such as, differences in number and affinity of opioid receptors, second messengers, gonadal hormones, and/or different mechanism of modulation of nociception, but not to differences in MOR pharmacokinetics in male and female subjects (for references, see Bartok and Craft, 1997; Cicero et al., 1996). As indicated herein, MOR-induced analgesia was significantly greater in male than in female rats during exposure to DZ capsules (Injections 1–5 of MOR), whereas gender differences were less pronounced at withdrawal (Injections 6–10). It has been reported that BZ inhibited the glucuronidation of MOR (Pacifici et al., 1986). The fact that DZ binds to peripheral BZ receptors, which are thought to modulate steroid biosynthesis, suggests that DZ has the potential to influence the hepatic metabolism of MOR differently in males and females. Although only the determination of MOR blood and brain levels can resolve this problem, the following facts seem to contradict the above hypothesis. (1) MOR was eliminated from blood and brain with identical rates in both sexes of rats ($t_{0.5} \approx 43$ min) (Cicero et al., 1997). Since, in the present experiment, MOR was administered in 24-h intervals and antinociception was measured for only 60 min after each injection, it seems unlikely that the sexually dimorphic analgesic effect was due to the marked differences in MOR and/or MOR-6-glucuronide levels in male and female rats. (2) Gender-related differences in MOR antinociception were previously reported both after systemic (Cicero et al., 1996, 1997) and central (circumvention of metabolism factor) (Boyer et al., 1998) routes of administration which argues in favor of an intrinsic difference in responsiveness to MOR in male and female rats. (3) Gender-related differences in antinociception seem to be more obvious after acute (Bartok and Craft, 1997; Boyer et al., 1998; Candido et al., 1992; Cicero et al.,

1996, 1997; Islam et al., 1993; Kepler et al., 1989; Krzanowska and Bodnar, 1999) than after repeated (present as well as unpublished data from our laboratory) administration of MOR. The present data also suggest that across the time of repeated MOR administration, the decline of the antinociceptive effect was more pronounced in CTR female than in male rats. A similar finding was reported for CD-1 mice where the rightward shift of the dose–response curve for MOR was significantly greater in female than in male mice treated with MOR for 3 or 7 days (Hopkins et al., 1999). On the contrary, the present data show that during DZ dependence, MOR antinociception was stable in both sexes. Thus, it can be speculated that the significant sexual dimorphism of MOR analgesia in DZ-dependent rats and less pronounced gender differences in CTR rats were due to the unchanged and diminished analgesic potencies of chronic MOR during DZ dependency and CTR treatment, respectively. It is noteworthy, that during DZ withdrawal there was a small trend towards a decrease in MOR analgesia with time (Injections 6–10) in male but not in female rats. However, due to the study design, it is not known whether the time course of MOR analgesia was different in both sexes during long-term withdrawal from DZ treatment (beyond the time of the present study). We are not aware of published data on gender differences in the time course of spontaneous BZ withdrawal, however, in comparison to DZ-dependent female rats, DZ-dependent male rats showed a more intense flumazenil-precipitated withdrawal (Sloan et al., 1996).

Finally, it has been demonstrated that naive, DZ-dependent, and DZ-withdrawn female rats expressed more activity (preMOR LOC) than male rats exposed to the same treatments. This is in agreement with reports on greater general activity in females than in males both in basal conditions and after aversive stimuli (for review, see Cecceralli et al., 1999). DZ dependency and withdrawal did not significantly affect baseline LOC in males and females. The present data also show that, regardless of treatment, the depressant effect of first dose of MOR on LOC (Day1), as well as the increase in activity produced by further doses of MOR, were more pronounced in female than in male rats. Tolerance to the acute locomotor depressant effect of MOR developed more rapidly in CTR than in DZ-treated (dependent and subsequently withdrawn) female rats while the differences were less pronounced in male rats. Thus, these data show that the chronic effects of MOR on locomotor activity were substantially dependent upon gender. Taken together, the above comparisons indicate concordance between higher analgesic and lower motor effects of systemic MOR in male than in female rats. Similarly, greater antinociception and a lesser effect on motor activity were observed in male than in female rats following the central administration of MOR into the ventromedial medulla (Boyer et al., 1998). As indicated herein, the between-gender differences in analgesic and motor effects of MOR were not abolished by chronic DZ treatment.

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