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Sex differences in the effects of muramyl dipeptide and lipopolysaccharide on locomotor activity and the development of behavioral tolerance in rats

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Abstract

Administration of bacterial agents, such as muramyl dipeptide (MDP) or lipopolysaccharide (LPS), induces a number of illness symptoms including decreased locomotor activity and weight loss. This study provides a detailed multivariate assessment of the effects of repeated exposures of various doses of MDP and LPS, alone and in combination, on various aspects of locomotion in male and female rats. Animals were given a single intraperitoneal injection of either MDP (0.8 or 1.6 mg/kg), LPS (100 or 200 μg/kg), a combination of MDP and LPS (0.8 mg/kg and 100 μg/kg, respectively), or vehicle on Days 1, 4, and 7. Two hours after each injection, locomotor activity was recorded for 30 min in an automated open-field. Both doses of LPS and the high dose of MDP produced significant decrements in locomotor activity in male and female rats, with tolerance becoming evident over repeated administrations, although LPS decreased activity more robustly than MDP. Sex differences were evident in the combined effects of MDP and LPS. Together, MDP and LPS reduced male activity levels in an additive manner but significantly potentiated both horizontal and vertical activity decrements in females. In addition, the rate of behavioral tolerance development to repeated bacterial injections was significantly higher in females than in males. These findings provide evidence for sex differences in the actions of MDP and LPS on various aspects of locomotor activity and in the development of behavioral tolerance to infection.

Keywords: ACTH; Body weight; Corticosterone; Infection; Inflammation; Open-field; Sex hormones; Sickness behavior; Spleen weight; Thigmotaxis

1. Introduction

Concomitant bacterial infections may severely alter and worsen the clinical symptoms and outcome of patients, as has been shown with the combined effects of aerobic and anaerobic bacteria in humans (Brook and Frazier, 1993). The high frequency of occurrence of gram-positive or -negative bacterial infections in humans further underlines the importance of understanding the effects of concurrent bacterial infections. However, to date, few studies have examined the effects of concomitant gram-positive and -negative infections and no consideration has been given to possible sex differences. These were aims of the present study.

Immunogenic compounds, such as muramyl peptides and lipopolysaccharide (LPS), are released during bacterial infection. Muramyl dipeptide (MDP; *N*-acetyl-muramyl-L-alanyl-D-isoglutamine) is a synthetic analogue of the smallest immunogenic component of the cell wall of gram-positive bacteria, whereas LPS composes the smallest immunogenic segment of the outer cell wall of gram-negative bacteria. Both MDP and LPS are capable of producing numerous sickness responses in humans and experimental animals of both sexes. These responses may include fever (Conrad et al., 1997; Meltzer et al., 1989; O'Reilly et al., 1988; Roth et al., 1997a), decreased activity (Avitsur et al., 1997; Yirmiya et al., 1994; Yirmiya, 1996), decreased sexual behavior (Avitsur et al., 1997), increased sleep (Meltzer et al., 1989), decreased food and water intake (Cross-Mellor et al., 2000; Langhans et al., 1991; Langhans, 1996; O'Reilly et al., 1988), hyperalgesia (Wiertelak et al., 1994), and even mortality (He et al., 1992).

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Gram-positive and -negative bacteria share some mechanisms of activation, as CD14 receptors found on circulating macrophages and monocytes are capable of recognizing both MDP and LPS. Although each compound binds to a slightly different CD14 epitope (Teti, 1999; Weidemann et al., 1997), binding in either case results in the increased production and release of pro-inflammatory cytokines (i.e., interleukin [IL]-1β and tumour necrosis factor [TNF]-α), which are involved in immune-to-brain signalling and mediate many of the aforementioned sickness behaviors. The cytokine IL-6 is also released by these immune cells following MDP or LPS administration, but may act in an anti-inflammatory manner (Barton, 1996, 1997; Barton et al., 1996; Tilg et al., 1997).

These sickness behaviors are centrally mediated, as specific receptors for these cytokines exist throughout the brain, and peripheral administration of IL-1 or TNF-α induces these cytokines to be expressed centrally (Hopkins and Rothwell, 1995; Luheshi et al., 1997), which, in turn, can alter both neural activity and behavior (Dantzer, 2001; Hopkins and Rothwell, 1995). Moreover, the central administration of antagonists against these cytokines (e.g., IL-1 receptor antagonist [IL-1ra]) prevents or attenuates many sickness behaviors which normally occur in response to peripheral inflammation, including hypophagia (Pu et al., 2000) and hypoactivity (Linthorst et al., 1995) in male rats. These sickness behaviors have also been disassociated from fever (Konsman et al., 2000; Layé et al., 1995; Maier and Watkins, 1998).

A sexual dimorphism, principally mediated by gonadal hormones, is apparent in immune functioning (Gaillard and Spinedi, 1998; Lahita, 2000). Overall, estradiol has been shown to have immunoenhancing effects on humoral immunity and immunosuppressive effects on cell-mediated immunity, whereas testosterone has immunosuppressive effects on B and T cell proliferation and macrophage activation in rats and mice (Giglio et al., 1994; McCruden and Stimson, 1991; Savita and Rai, 1998; Wichmann et al., 1997). In fact, the enhanced immune functioning typically seen in females is often attributed to the lack of immunosuppressive androgens circulating in their blood plasma (Bilbo and Nelson, 2001). Indeed, the ability to overcome bacterial infection is improved in castrated male mice and testosterone replacement reverses this effect (Schuurs and Verheul, 1990). Moreover, female mice exhibit a greater blastogenic splenocyte response to B cell mitogens (e.g., LPS) than males (Krzych et al., 1981). Despite these dimorphic findings, little is known about sex differences in the expression of sickness behaviors.

In the present study, using rats of both sexes, we examined the effects of MDP and LPS on locomotor activity as decreased activity is a common sickness behavior. It is now well accepted that sickness behaviors are not deleterious side-effects of acute illness, but rather a highly organized and strategic response which allows an organism to better cope with and fight infection (Hart, 1988; Dantzer, 2001). For instance, hypoactivity reduces the animal's chance of being preyed upon and also enables individuals to conserve both energy and body heat which aids in the production of fever and augments immune functioning (Hart, 1988). For these reasons, locomotor activity provides a useful behavioral index of the effects of, and responses to, acute bacterial infection in animals.

Past studies have used automated open-fields (e.g., Digiscan Activity Monitors) for the detailed analyses of spontaneous locomotor activity in rodents and for discriminating between different patterns of activity due to sex, age, or other biological factors (Ossenkopp et al., 1987; Perrot-Sinal et al., 2000; Sanberg et al., 1985), including behavioral responses to LPS in male mice (Engeland et al., 2001c). As different measures of locomotor activity often yield low intercorrelations and can be dissociated (Ossenkopp et al., 1990; Ossenkopp and Mazmanian, 1985), this study quantified various components of both horizontal and vertical locomotor activity. This experimental design allows a behavioral profile to be created which provides greater reliability and validity relative to any single measure of locomotor activity (Ossenkopp et al., 1990; Ossenkopp and Mazmanian, 1985).

Using the tabulation of line crossings and rearings in an open-field, it has been reported that a single peripheral LPS injection (50 or 250 μg/kg ip) reduced locomotor activity equally in male and female rats (Avitsur et al., 1997). In the present study, we carried out a detailed multivariate assessment of various temporal and spatial features of spontaneous locomotor activity in male and female rats given repeated injections of MDP and/or LPS. This allowed us to examine both the developments of tolerance to, and possible interactions between, these compounds. In addition, trunk blood was gathered after the last test session and assayed for both adrenocorticotropin hormone (ACTH) and corticosterone (Cort), as pro-inflammatory cytokines are known to increase the levels of circulating stress hormones (Bethin et al., 2000). Estradiol or testosterone levels were also determined, in females and males respectively, as IL-1β and TNF-α both inhibit the synthesis of sex hormones (Cannon, 1998).

Results of previous studies have found that tolerance to both the physiological and behavioral effects of LPS forms very quickly, often after a single exposure (Langhans et al., 1991; O'Reilly et al., 1988; Porter et al., 1998; Roth et al., 1997a; Tripp et al., 1998). This development of tolerance, which serves to prevent excess inflammation (Ziegler-Heitbrock, 1995), is mediated through decreased macrophage secretions of pro-inflammatory cytokines (Knopf et al., 1994; Mathison et al., 1990; Zeisberger and Roth, 1998) and, in part, by a decreased responsiveness to the cytokines themselves (He et al., 1992). It has also been reported that tolerance to repeated injections of MDP occurs very slowly or not at all (Langhans et al., 1991; Roth et al., 1997a,b; Soszynski et al., 1991; Zeisberger and Roth, 1998), although the only behavioral measure employed was hypo-

phagia (Langhans et al., 1991). Similarly, male rats developed behavioral tolerance to the hypophagic effects of MDP and LPS more slowly than to LPS alone (Langhans et al., 1991; Langhans, 1996).

Based on these previous findings, we expected that rats given an initial injection of MDP or LPS would exhibit significant activity decrements. We further hypothesized that rats injected with MDP and LPS in combination would exhibit significantly greater activity decrements and significantly slower tolerance development when compared to rats injected with either MDP or LPS alone.

2. Material and methods

2.1. Animals

This study used 47 female and 58 male naive Long–Evans rats (Charles River, Canada), 2–3 months of age and weighing 294.0 ± 6.6 and 318.4 ± 7.6 g, respectively. Rats were housed in same-sex pairs in standard polypropylene cages in a temperature-controlled colony room (20 ± 1 °C) maintained on a 12:12-h light–dark cycle (lights on between 0700 and 1900 h). Testing was conducted during the light phase of the light–dark cycle, as we have previously shown that the effects of LPS on locomotor activity levels are greater at this time, regardless of whether testing is conducted in a novel or nonnovel environment (Engeland et al., 2001a; Franklin et al., 2001). Animals were maintained on ad libitum food (Agway lab chow) and tap water. All procedures used were carried out in compliance with the Canadian Council of Animal Care (CCAC) guidelines.

2.2. Apparatus

Behavioral data were collected using six Digiscan Animal Activity Monitors (Accuscan Model RXYZCM-16, Columbus, OH). Each was made of clear Plexiglas, measuring 40 × 40 × 30.5 cm, and was covered with a Plexiglas lid containing airholes. Infrared monitoring sensors were located every 2.54 cm along the perimeter (16 infrared beams along each side) and 4.5 cm above the floor. Two additional sets of 16 sensors were located 15 cm above the floor on opposite sides. Data were collected and analysed by a Digiscan Analyser (Accuscan Model DCM-8, Columbus, OH), which, in turn, sent information to an IBM 486 computer where it was stored for future analysis.

The following five activity measures were calculated directly by the Digiscan Analyser for each given time sample.

Horizontal measures

MT (movement time)—the amount of time (s) spent in horizontal motion

NM (number of movements)—the number of individual horizontal movements made with a minimum stop time of 1 s to separate movements

TD (total distance)—the total horizontal distance travelled (cm)

Vertical measures

VT (vertical time)—the amount of time (s) spent in vertical motion

VM (vertical movements)—the number of individual vertical movements made with a minimum stop time of 1 s to separate movements

AVSP (average horizontal speed) was also assessed (calculated from TD/MT [cm/s]).

2.3. Procedure

The bacterial products used in this study were MDP (adjuvant peptide, no. A-9519; Sigma, St. Louis, MO) and LPS (derived from *E. coli* serotype 0111:B4, no. L-2630; Sigma), which were each dissolved in pyrogen free 0.9% isotonic saline.

Rats were handled for at least 30 min on two separate days before testing. Treatment groups were determined randomly. Three times throughout the experiment (Days 1, 4, and 7) rats were weighed, then treated with MDP and/or LPS and locomotor activity was assessed. On these days, rats were given a single intraperitoneal injection of either MDP (0.8 or 1.6 mg/kg; groups MDP1 [$n = 8$] and MDP2 ($n = 8$), respectively), LPS (0.1 or 0.2 mg/kg; groups LPS1 [$n = 8$] and LPS2 [$n = 10$], respectively), a combined single dose of MDP and LPS (0.8 mg/kg and 0.1 mg/kg, respectively; group COMBINED [$n = 10$]), or 0.9% isotonic saline (equivalent volume; group SAL [$n = 14$]). Results of previous studies have shown that these are appropriate intraperitoneal doses of MDP and LPS for mimicking the clinical features of acute bacterial infection in rats and causing behavioral changes such as hypophagia (Langhans et al., 1991; Langhans, 1996). These doses typically induce lethargy in rats and cause piloerection, shivering and other behaviors related to the conservation of body heat (such as huddling). Given that activity decrements were expected in the treatment groups, it was likely that between group differences would become obscured once the saline-treated rats became habituated to the testing apparatus, which typically occurs within 30 min (Dennison et al., 1992; Mead et al., 1996). For this reason, animals were returned to their home cages for 2 h after injection, and then the locomotor activity of individual rats was recorded in the Digiscan apparatus for 30 min in 5-min time samples. An interval of 2 h between injection and test time was used, as the results of previous studies, using various routes of administration, have demonstrated that MDP- and LPS-induced fevers (Blatteis and Sehic, 1997; Meltzer et al., 1989; Opp and Toth, 1998), as well as LPS-

induced increases in ACTH and Cort (Grota et al., 1997; Takemura et al., 1997), reach early maximal values in rats at this time. In addition, hypophagia in rats commences at this time point after intraperitoneal administration of either MDP or LPS (Langhans, 1996). Body weights were also obtained on Days 2 and 5, 24 h after each injection. In between tests, boxes were thoroughly cleaned with a commercial detergent (Alconox) after which a baking soda solution was used to remove all odors. After each test session, animals were returned to their home cages. On Day 7, rats were decapitated and trunk blood was collected (approximately 150 min postinjection). At this time, the spleen from each animal and testes from the males were removed and wet organ weights were determined.

Prior to injection on test days, vaginal smears were obtained from each female rat using a wet cotton swab, which was then blotted onto a glass slide. Microscopic inspection of the cell types allowed for classification of the cycle on each test day and also revealed that all rats appeared to be cycling normally throughout the study (i.e., none were phase-locked).

2.4. Hormonal assays

Blood collection was completed within 2 min of handling the animals to avoid elevating basal Cort levels (average sampling time was 45 s). Samples were kept on ice for 2–4 h, and then were spun in a centrifuge at 3000

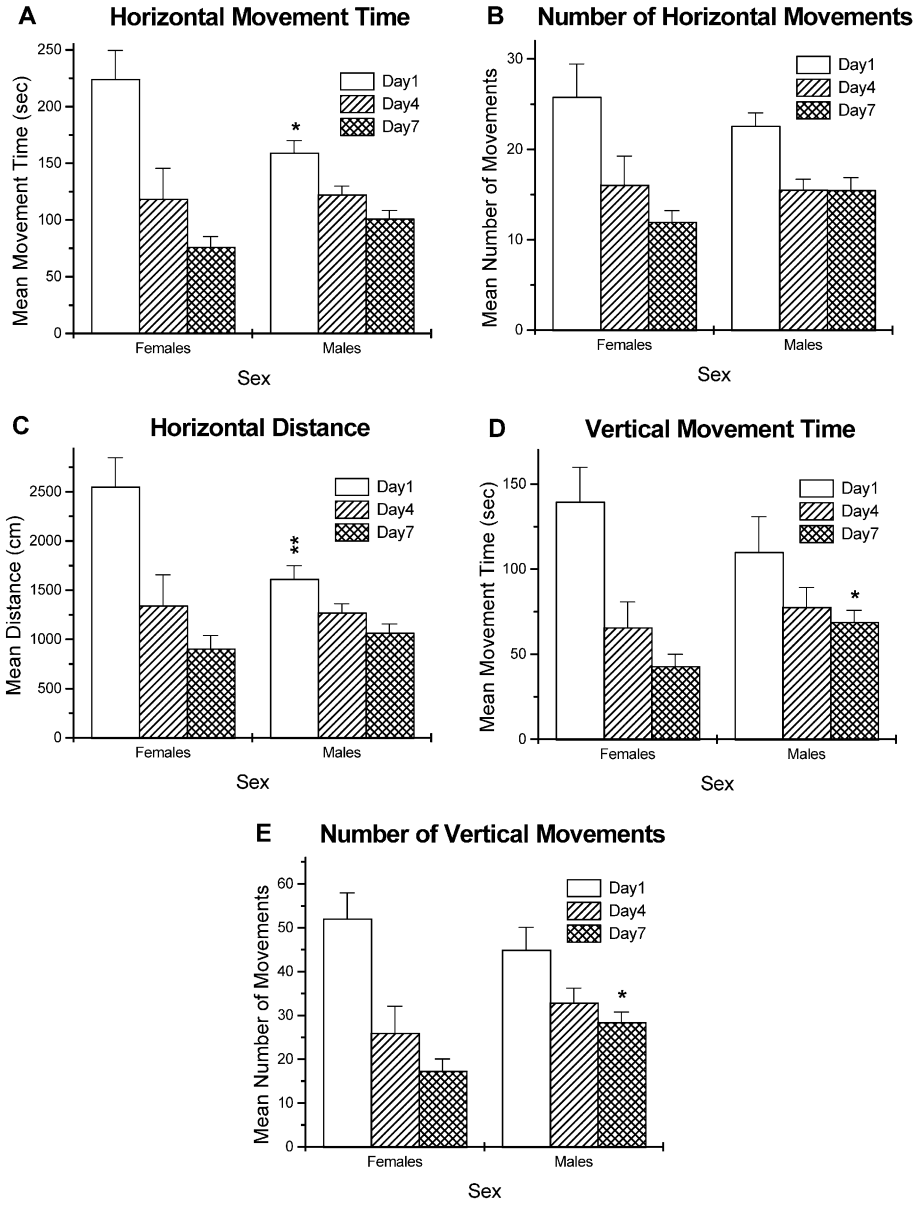

Fig. 1. Group mean (A) horizontal movement times, (B) number of horizontal movements, (C) horizontal distances, (D) vertical movement times, and (E) number of vertical movements for saline treated male and female rats. $*P<.05$ and $**P<.01$ vs. SAL, respectively. Error bars represent standard error of the mean (S.E.M.).

rpm for 20 min. Blood serum was then pipetted off and immediately frozen at −20 °C until later analysis for ACTH, Cort, and either estradiol or testosterone. All assays were performed in duplicate, using commercially available radioimmunoassay (RIA) kits (Coat-A-Count, Diagnostic Products, Los Angeles, CA). The sensitivity of the estradiol assay, as calculated from the standard curve, was 20 pg/ml and the intra-assay coefficient of variation, as measured in triplicate from low, medium and high pools, ranged from 2% to 5%. Similarly, the sensitivity of the testosterone assay was 0.1 ng/ml and the intra-assay coefficient of variation ranged from 11% to 18%. The sensitivity of the Cort assay was 20 ng/ml and the intra-assay coefficient ranged from 2% to 7% (4–12%). For ACTH, the assay sensitivity was 15 pg/ml and the range of the intra-assay coefficient was from 2% to 5%. Due to technical problems, ACTH and estradiol levels were not determined for the MDP1, MDP2, and COM-

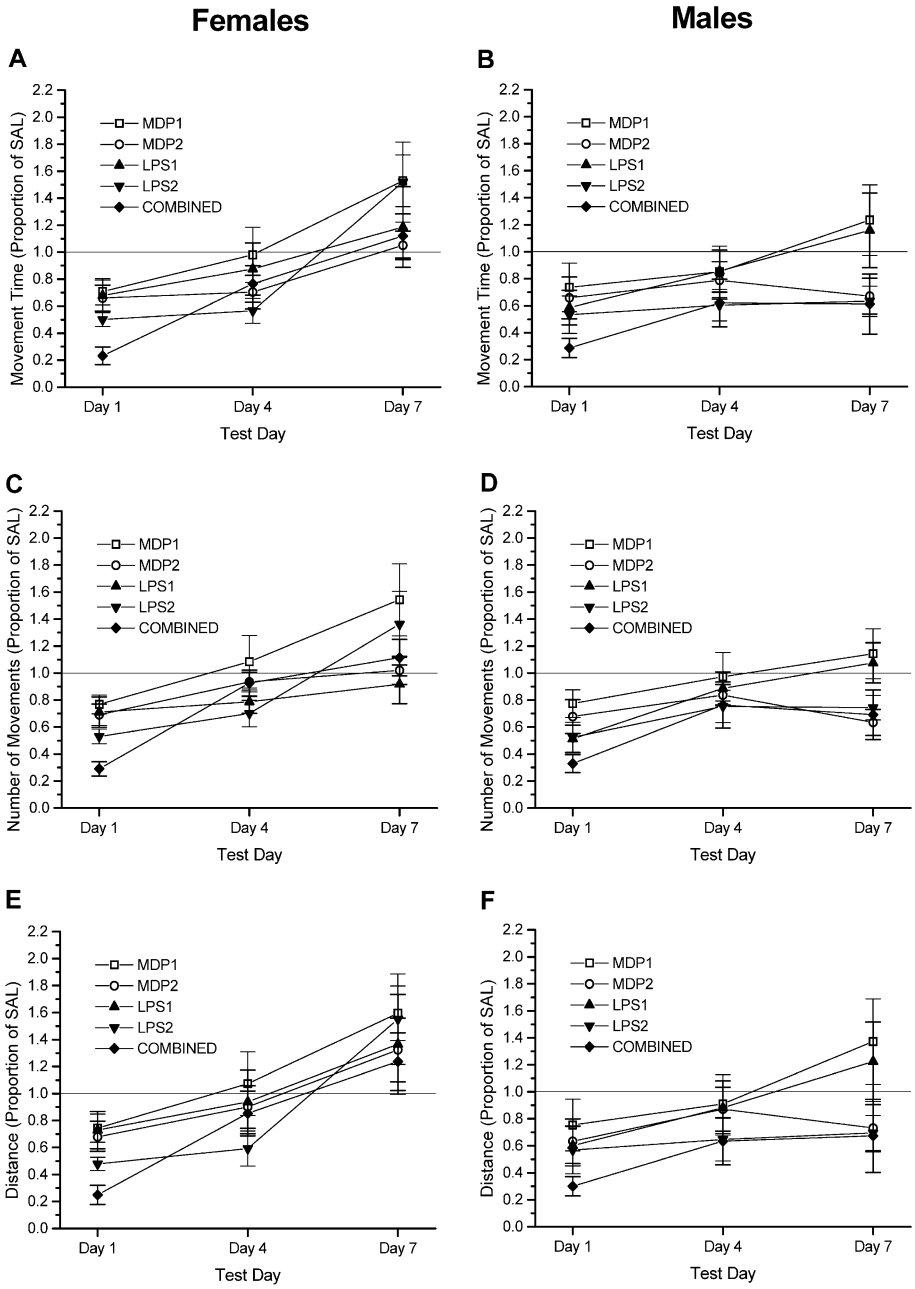

Fig. 2. Group mean horizontal measures of (A–B) movement times, (C–D) number of movements, and (E–F) total distances for treatment groups of both sexes (females: graphs A, C, E; males: graphs B, D, F). Means are expressed as a proportion of same-sex control values (represented by a horizontal line at 1.0 on the *Y*-axis). See Table 1 for a list of significant differences between treatment and control groups and between sexes, and Table 3 for slope comparisons. Error bars represent S.E.M.

BINED female groups, and ACTH values were not determined for the COMBINED male group.

2.5. Statistical analysis

Physiological data were analysed using separate one-way analysis of covariance (ANCOVA) procedures (Packard and Boardman, 1999). Initial or final body weights were used as the covariate where appropriate and adjusted means are represented in the figures where indicated. Behavioral data were analysed using a mixed design analysis of variance (ANOVA) for each separate activity measure. The 5-min time blocks and the three test days were treated as within-subject factors, and drug treatment and sex were treated as between-subjects factors. Post-hoc comparisons were performed using Tukey's HSD method when comparing between sexes and the least significant difference method, using body weight as a covariate, when comparing within a sex. Correlations were determined using Pearson's product moment correlations (r). All hypothesis tests used α=.05 to determine significance. All data were analysed using SPSS 9.0 for Windows.

3. Results

3.1. Activity measures

3.1.1. Sex differences in basal activity

An analysis of the baseline data in saline-treated control animals revealed there was a significant Day × Sex interaction for MT and TD [MT: $F(2,38)=9.74$, P=.001; TD: $F(2,38)=10.87$, $P<.001$], and this interaction approached significance for VT and VM (P=.089 and P=.051, respectively). Post-hoc analyses examining individual test days revealed that, on Day 1, females were significantly more active than males for measures of MT and TD (P=.012 and P=.004, respectively). Furthermore, on Day 7, males were significantly more active than females for measures of VT and VM (P=.031 and P=.011, respectively). There were no between-sex differences on Day 4 (Fig. 1). There was also a main effect of sex for AVSP [$F(1,19)=5.03$, P=.037], as females moved significantly faster than males. Post-hoc analyses revealed that this sex difference was apparent on Day 1 (P=.020) but not on Day 4 or 7 (not shown). There were no differences in basal activity levels during any test

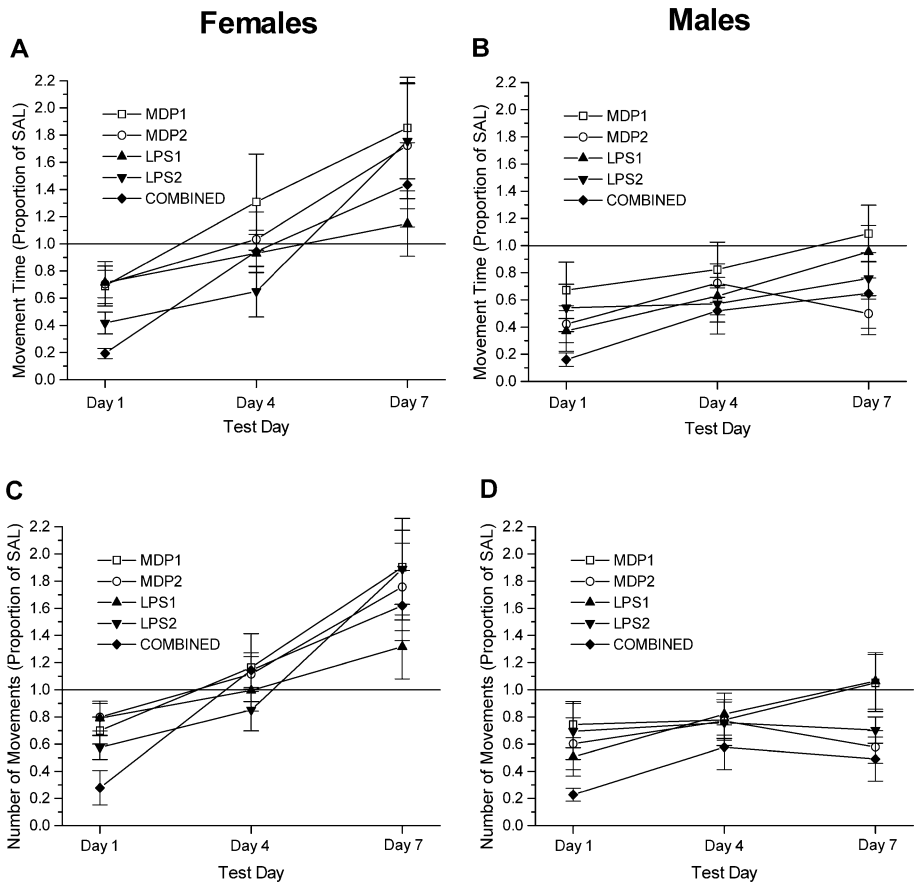

Fig. 3. Group mean vertical measures of (A–B) movement times and (C–D) number of movements for treatment groups of both sexes (females: graphs A and C; males: graphs B and D). Means are expressed as a proportion of same-sex control values (represented by a horizontal line at 1.0 on the Y-axis). See Table 1 for a list of significant differences between treatment and control groups and between sexes, and Table 3 for slope comparisons. Error bars represent S.E.M.

day between proestrus and nonproestrus females (not shown).

3.1.2. Effects of MDP/LPS on activity

As there were sex differences in the baseline activity patterns of the control animals, activity values of MDP/LPS treated animals were transformed into proportions of the same-sex control group (Figs. 2 and 3). This allowed for a more accurate between-sex assessment of relative activity levels compared to controls, as well as of the development of behavioral tolerance across test days.

A series of ANOVAs revealed a significant interaction of Day × Treatment for each of the five main activity measures [MT: $F(10,198)=3.83$, $P<.001$; NM: $F(10,198)=4.49$, $P<.001$; TD: $F(10,198)=3.26$, $P=.001$; VT: $F(10,198)=3.41$, $P=.001$; VM: $F(10,198)=3.40$, $P=.001$]. Animals challenged with bacterial injections typically exhibited lower activity levels compared to controls on Day 1, and these activity decrements were less apparent or did not occur on Days 4 and 7. A significant main effect of treatment was also obtained for each of these five measures across the three test days ($P<.01$ or better for each measure). No main

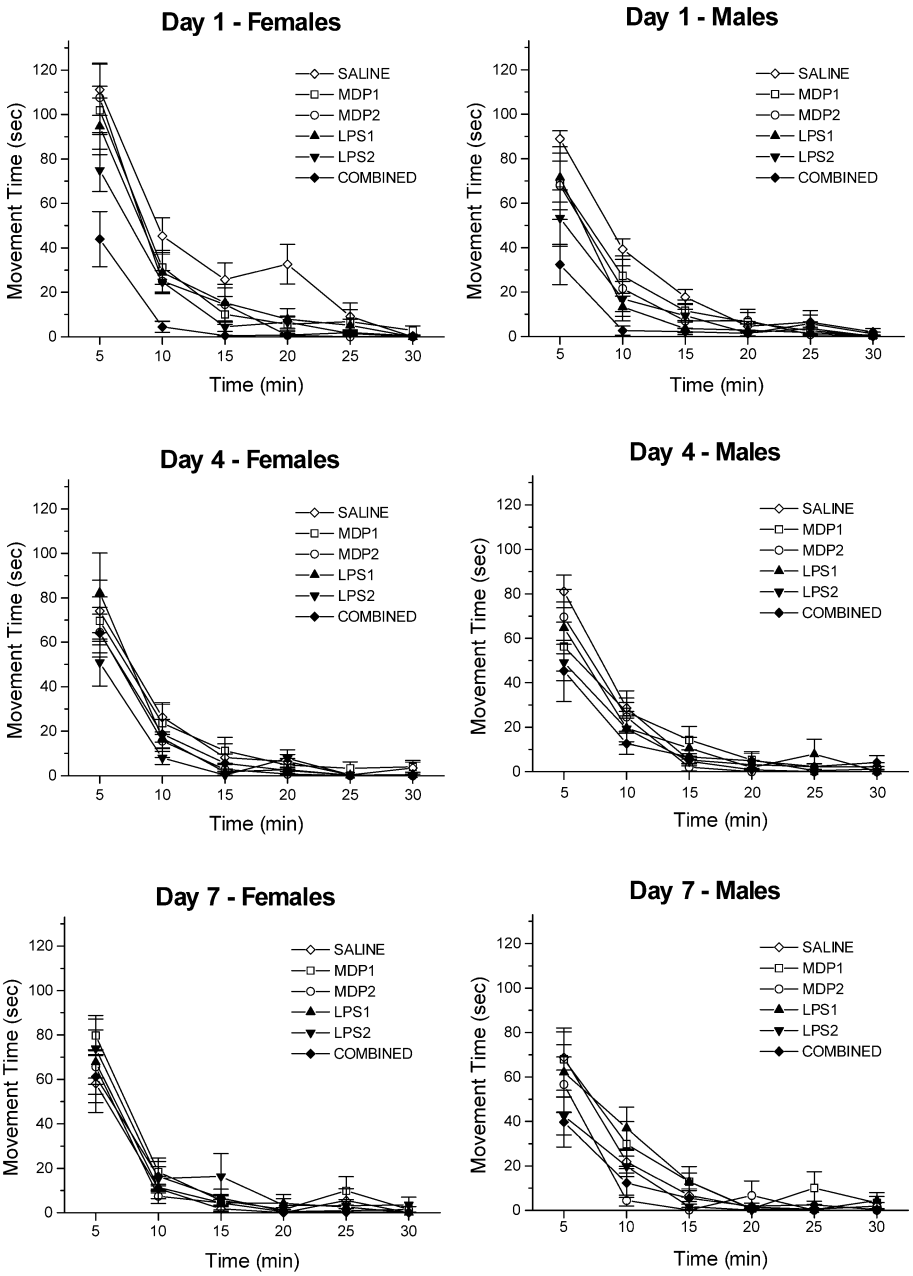

Fig. 4. Group mean horizontal movement time (MT), separated into 5-min time blocks, in both sexes across all test days. Each treatment group exhibited habituation to the open-field on each test day. Similar activity patterns were observed on each of the other main activity measures (NM, TD, VT, and VM). Error bars represent S.E.M.

effects or interactions were apparent for AVSP. Fig. 4 illustrates that typical habituation curves were obtained in the open-field for controls and all treatment groups on each test day.

Post-hoc analyses of Day 1 data revealed that, compared to controls, the activity levels of the MDP1 group did not differ significantly on any activity measure for either sex. In the females, compared to controls, significant activity decrements occurred in the MDP2 and LPS1 groups for measures of MT and NM ($P<.05$ for all measures) and in the LPS2 and COMBINED groups for all five of the activity measures ($P<.01$ and $P<.001$, respectively, for all measures) (Table 1; Figs. 2 and 3). In addition, the COMBINED group was significantly less active for all measures ($P<.01$ or better) than all other treatment groups except for LPS2, and was less active than LPS2 for VM ($P=.045$). In the males, compared to controls, significant activity decrements occurred in the MDP2 group for NM ($P<.05$) and in the LPS1, LPS2, and COMBINED groups for all five measures ($P<.05$ or better) (Table 1; Figs. 2 and 3). In addition, the COMBINED group was significantly less active than the MDP1 group for all measures ($P<.01$ or better) and less active than the MDP2 group for MT and NM ($P<.05$ for each). Further analyses on Day 1 revealed that female rats injected with MDP and LPS in combination were significantly less active on each activity measure than predicted for an additive effect ($P<.05$ or better, Student's *t*-test). Conversely, the activity levels of male rats injected with MDP and LPS in combination did not differ significantly from predicted values (Table 2). Predicted values were based on the summed differences in activity levels between the MDP1 or LPS1 groups and the same-sex control group

Table 1
Activity differences between treatment groups and (a) their respective control group; (b) the same treatment group of the opposite sex

Group	Activity variable	Difference from SAL						Sex difference		
		Females			Males					
		Day			Day			Day		
		1	4	7	1	4	7	1	4	7
MDP1	VM	–	–	–	–	–	–	–	–	*
MDP2	MT	*	–	–	–	–	–	–	–	*
	NM	*	–	–	*	–	–	–	–	*
	VT	–	–	–	–	–	–	–	–	*
	VM	–	–	–	–	–	–	–	–	**
LPS1	MT	*	–	–	**	–	–	–	–	–
	NM	*	–	–	***	–	–	–	–	–
	TD	–	–	–	*	–	–	–	–	–
	VT	–	–	–	**	–	–	–	–	–
	VM	–	–	–	**	–	–	–	–	–
LPS2	MT	**	–	–	**	*	–	–	–	**
	NM	**	–	–	***	–	–	–	–	*
	TD	**	–	–	**	–	–	–	–	*
	VT	**	–	–	*	*	–	–	–	*
	VM	**	–	↑*	*	–	–	–	–	**
COMBINED	MT	***	–	–	***	–	–	–	–	–
	NM	***	–	–	***	–	–	–	–	–
	TD	***	–	–	**	–	–	–	–	–
	VT	***	–	–	**	–	–	–	–	–
	VM	***	–	–	**	–	*	–	*	**

Unless denoted by the symbol ↑, all differences from SAL resulted from treatment groups displaying less activity than control animals. All sex differences resulted from significantly higher activity levels in females compared to males. MT: Movement time, NM: number of movements, TD: total distance, VT: vertical time, VM: vertical movements.

* $P<.05$.

** $P<.01$.

*** $P<.001$.

Table 2
Predicted versus actual activity means following a combined injection of MDP and LPS on Day 1

Activity variable	Measured	Predicted
Females		
MT	52.0 ± 14.6	**86.1** *
NM	45.0 ± 8.2	**73.9****
TD	634.2 ± 179.4	**1203.0** *
VT	14.5 ± 2.6	**25.5****
VM	27.0 ± 5.3	**56.4*****
Males		
MT	45.7 ± 11.3	51.3
NM	44.6 ± 9.1	38.7
TD	484.8 ± 114.6	571.8
VT	10.1 ± 2.1	10.6
VM	17.7 ± 5.3	4.7

Measured values are means ± S.E.M. of female ($n=8$) and male ($n=10$) rats for the 30-min test session on Day 1. The 0.8 mg/kg MDP and 100 μg/kg LPS were injected intraperitoneally in combination. Predicted values were based on the summed differences in activity levels between the MDP1 and LPS1 groups and the same-sex control group. MT: Movement time, NM: number of movements, TD: total distance, VT: vertical time, VM: vertical movements.

* $P<.05$ between measured and predicted values (Student's *t*-test).

** $P<.01$ between measured and predicted values (Student's *t*-test).

*** $P<.001$ between measured and predicted values (Student's *t*-test).

Table 3
Female and male activity slopes, representing the rate of the development of behavioral tolerance after repeated exposure to bacteria, and the probability of between-sex differences

Treatment	Locomotor activity variable				
	MT	NM	TD	VM	VT
MDP1				200.4	
				57.6	
				0.026	
MDP2		55.0	107.6	159.9	
		− 7.2	*16.4*	*− 0.1*	
		0.090	**0.043**	0.093	
LPS1					
LPS2	169.7	138.4	178.7	218.6	223.1
	16.7	*36.5*	*21.3*	*6.2*	*36.0*
	0.014	**0.049**	**0.021**	**0.009**	**0.021**
COMBINED	147.9	137.2		223.5	
	54.2	*60.4*		*46.6*	
	0.093	0.082		**0.002**	

Slopes were calculated using activity levels of all three test days (see Figs. 2 and 3). Females slopes are shown first, male slopes are second and in italics. Data are only shown for probabilities below .10; significant differences are in bold. MT: Movement time, NM: number of movements, TD: total distance, VT: vertical time, VM: vertical movements.

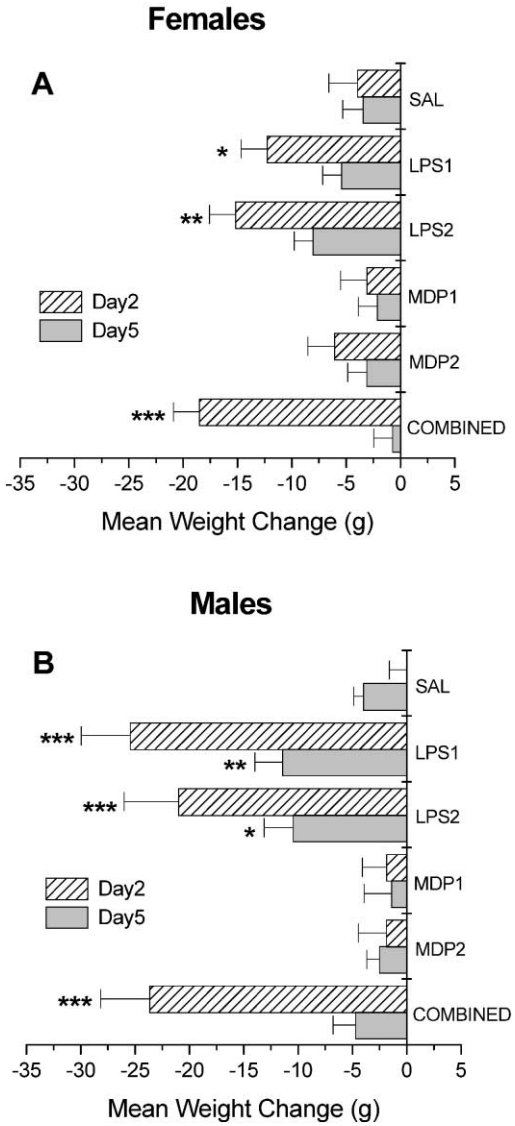

Fig. 5. Group mean changes in body weight 24 h after the first and second intraperitoneal injections (Days 2 and 5, respectively) in (A) females and (B) males. Means are adjusted using initial body weight as a covariate. $*P<.05$, $**P<.01$, and $***P<.001$ vs. SAL, respectively. Error bars represent S.E.M.

(see Langhans et al., 1991 for previous use of this statistical method).

Similar results were also obtained when females that were in proestrus on Day 1 were removed from the analysis (not shown). We have previously found that changes in activity levels following an injection of LPS (200 μg/kg ip) were similar in female Long–Evans rats, regardless of the stage of the estrous cycle (Engeland et al., 2001b).

On Days 4 and 7, female treatment groups exhibited no significant activity decrements compared to controls. In addition, the LPS2 group made significantly more vertical movements than controls on Day 7 (VM: P=.044). In males, the activity levels of the MDP and LPS1 groups also did not differ from controls on Day 4 or 7. However, the LPS2 group exhibited significantly less movement time on Day 4 (MT: P=.020; VT: P=.034), and the COMBINED group made significantly fewer vertical movements on Day 7 (VM: P=.023), than controls.

3.1.3. Development of behavioral tolerance to MDP/LPS

Females developed behavioral tolerance to the bacterial injections faster than males. For instance, on Day 7, when activity was expressed as a proportion of controls, females in all treatment groups except for LPS1 were significantly more active than males on one or more measures ($P<.05$ or better), and the female LPS2 group was significantly more active than males on all five measures ($P<.05$ or better) (Table 1). The acquisition of behavioral tolerance, as determined by the positive slopes created by increasing activity levels across test days in the various treatment groups (Table 3), further supports this contention. Compared to males, the slopes of females were significantly steeper for all activity measures in the LPS2 group ($P<.05$ or better), and on one or more activity measures for all of the other treatment groups except for LPS1 ($P<.05$ or better), further indicating that females became tolerant more quickly than males to repeated intraperitoneal injections of MDP and/or LPS.

3.2. Physiological measures

3.2.1. Body weight changes

In assessing changes in body weight, the ANCOVA for females revealed significant main effects of treatment after the first injection and over the entire test period [$F(5,40)=7.07$, $P<.001$; $F(5,40)=3.19$, P=.016, respectively], whereas males exhibited significant main effects of treatment after both the first and second injections, as well as over the entire test period [$F(5,51)=15.16$, $P<.001$; $F(5,51)=3.94$, P=.004; $F(5,51)=5.38$, $P<.001$, respectively] (Figs. 5 and 6). Post-hoc comparisons revealed that,

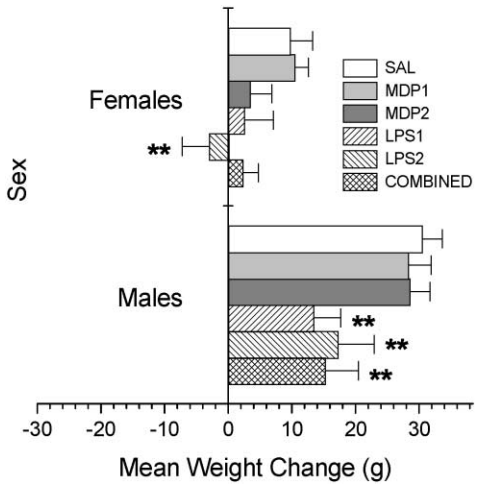

Fig. 6. Group mean changes in body weight from Days 1 to 7. Means are adjusted using initial body weight as a covariate. $**P<.01$ vs. SAL. Error bars represent S.E.M.

Table 4
Mean postmortem organ weights (g) ± S.E.M. after testing on Day 7

Treatment	Spleen weights		Testes weights
	Females	Males	
Saline	0.58 ± 0.02	0.72 ± 0.04	3.12 ± 0.13
MDP1	0.65 ± 0.05	0.84 ± 0.06	3.27 ± 0.12
MDP2	**0.68 ± 0.03***	0.78 ± 0.06	3.15 ± 0.09
LPS1	**0.79 ± 0.04*****	**1.08 ± 0.05*****	3.21 ± 0.12
LPS2	**0.85 ± 0.05*****	**0.98 ± 0.08****	3.22 ± 0.10
COMBINED	**0.77 ± 0.04*****	**0.97 ± 0.07****	**3.38 ± 0.11** *

Means that significantly differ from controls are in bold. All means are adjusted using final body weight as a covariate.
* $P < .05$.
** $P < .01$.
*** $P < .001$.

in both sexes, the LPS and COMBINED groups lost significantly more weight in the 24 h following the first injection than did the SAL or MDP groups ($P < .05$ or better for all comparisons in females; $P < .001$ for all comparisons in males). In the 24 h following the second injection, only the male LPS groups lost more weight than male control animals ($P = .007$ and $P = .01$ for low and high LPS doses, respectively). Furthermore, both male LPS groups lost significantly more weight than did the male MDP groups ($P < .01$ in all cases) or the male COMBINED group ($P < .05$ in both cases). Over the 7-day test period, in females, only the LPS2 group exhibited a significant decrease in body weight gain over the entire test period ($P = .004$), whereas in males the LPS1, LPS2, and COMBINED groups all exhibited reduced body weight gain compared to either controls ($P = .001$, $p = 0.004$, and $P = .001$, respectively) or the MDP groups ($P < .05$ or better in all cases). MDP administration had no significant effect on body weight changes or on overall weight gain in either sex.

3.2.2. Correlational analyses

On Day 1, there was a significant positive correlation between change in body weight and overall activity levels for each measure in males (horizontal: $r = .61 - .71$, $P < .01$; vertical: $r = .53 - .58$, $P < .01$) and in females with the exception of VT (horizontal: $r = .38 - .41$, $P < .01$; VM: $r = .35$, $P = .016$). Therefore, rats which lost the most weight 24 h after treatment were the least active. Similar, though weaker, correlations between loss in body weight and activity levels were also seen on Day 4 of testing in males ($r = .33 - .38$, $P < .05$ or better for each comparison) but not in females.

3.2.3. Organ weights

The ANCOVA revealed that the mean spleen weights, corrected for final body weights, differed significantly among treatment groups for both females [$F(5,40) = 9.44$, $P < .001$] and males [$F(5,51) = 6.03$, $P < .001$]. Post-hoc comparisons revealed that the LPS and COMBINED groups had significantly heavier spleens than the SAL group (females: $P < .001$ for all comparisons; males: $P < .001$, $P = .002$, and $P = .005$, respectively). In addition, the spleens of the female MDP2 group were significantly heavier than spleens of female controls ($P = .039$), but significantly lighter than spleens of female LPS1 and LPS2 groups ($P = .034$ and $P = .001$, respectively). All other groups given MDP displayed no significant spleen weight differences. Mean testicle weights did not differ significantly among treatment groups, although post-hoc comparisons revealed that rats in the COMBINED group had significantly heavier testes than the SAL group ($P = .025$) (Table 4).

3.2.4. Hormonal levels

There were significant main effects of sex for measures of both ACTH [$F(1,38) = 8.02$, $P = .008$] and Cort [$F(1, 100) = 15.10$, $P < .001$], as males had higher ACTH levels

Table 5
Mean blood plasma hormone levels ± S.E.M. after testing on Day 7

	ACTH (pg/ml)	Cort (ng/ml)	Estradiol (pg/ml)	Testosterone (ng/ml)
Females				
Saline	161.6 ± 61.6 (5)	412.9 ± 57.0 (7)	38.5 ± 5.0 (7)	–
LPS1	135.4 ± 33.5 (6)	432.9 ± 80.6 (8)	34.8 ± 4.4 (8)	–
LPS2	74.4 ± 11.4 (6)	392.4 ± 72.1 (8)	41.8 ± 6.8 (8)	–
MDP1	–	340.6 ± 71.4 (8)	–	–
MDP2	–	335.4 ± 42.4 (8)	–	–
COMBINED	–	347.1 ± 47.2 (8)	–	–
Males				
Saline	292.2 ± 69.7 (7)	222.9 ± 37.0 (13)	–	4.25 ± 1.10 (7)
LPS1	195.0 ± 39.8 (8)	284.4 ± 38.3 (7)	–	3.61 ± 1.22 (8)
LPS2	244.1 ± 57.7 (8)	295.3 ± 50.1 (10)	–	2.70 ± 0.74 (8)
MDP1	170.9 ± 99.4 (8)	277.0 ± 59.6 (8)	–	2.69 ± 0.84 (8)
MDP2	188.1 ± 51.8 (8)	204.1 ± 32.2 (8)	–	**1.41 ± 0.18 (8)** *
COMBINED	–	242.1 ± 48.2 (9)	–	**1.75 ± 0.45 (10)** *

Values in parentheses represent the number of samples assayed for each treatment group. Significant differences from SAL are in bold.
* $P < .05$.

and females had higher Cort levels when compared to the opposite sex. There were no significant treatment main effects or interactions for any of the hormones. As levels of these hormones have not, to date, been determined in MDP-treated rats of either sex, a priori comparisons (two-tailed) were performed. Male rats in both the MDP2 and the COMBINED groups had significantly lower circulating testosterone levels than male controls (P=.015 and P=.025, respectively) (Table 5). In females, there were no between-group differences in circulating estradiol levels. Similar results were also obtained when females that were in proestrus on Day 7 were removed from the analysis (not shown).

4. Discussion

The present study provides the first detailed multivariate assessment of the effects of repeated administrations of both single and combined gram-positive and -negative bacterial products on locomotor activity in male and female rats. Examination of the single and interactive effects of repeated injections of MDP and LPS revealed that: (a) both substances decreased activity in male and female rats, although LPS did this more effectively and at a lower dose; (b) MDP and LPS, in combination, produced a potentiated effect in the reduction of locomotor activity in females but not in males; (c) tolerance to the locomotor reducing effects of MDP and/or LPS developed more quickly in females than males.

4.1. Sex differences in basal activity

There were sex differences in the basal activity of the rats on Day 1, with females being significantly more active than males with respect to horizontal measures. These activity differences chiefly occurred because females moved significantly faster than males on Day 1. Both of these findings are in agreement with the results of previous studies on rats, which have reported that such sex differences are most apparent in novel environments when exploratory demands are high and the overall activity levels are exaggerated (Mead et al., 1996; Tropp and Markus, 2001). On Day 4, both sexes exhibited equal levels of horizontal and vertical activity, again supporting previous findings that sex differences in activity dissipate once the open-field is nonnovel (Mead et al., 1996; Tropp and Markus, 2001). However, by Day 7, males exhibited significantly greater levels of vertical activity than females. This novel finding suggests that males may display augmented exploratory interests in a nonnovel environment compared to females. The stage of the estrous cycle did not significantly affect female activity levels on any of the test days, with proestrus and nonproestrus animals being equally active, consistent with previous findings (Engeland et al., 2001b; Tropp and Markus, 2001).

4.2. Effects of MDP/LPS on activity

Although MDP and LPS each reduced locomotor activity levels, the low dose of MDP did not significantly reduce activity in either sex. This observation is in agreement with other studies, which have found that a dose of MDP greater than 1.0 mg/kg was needed to reliably produce hypophagia in rats (Langhans et al., 1991). In addition, this observation is of particular relevance because the low dose of MDP: (a) made up part of the combined treatment and (b) was subthreshold (i.e., it did not significantly reduce locomotor activity on its own). Conversely, on Day 1, the high dose of MDP decreased locomotor activity on one or more measures in both sexes. The effects on activity of the high dose of MDP and the low dose of LPS were comparable in females, but in males the effects of the low dose of LPS were more robust. However, in both sexes, the high dose of LPS produced robust activity decrements on all five measures. Results of previous behavioral studies have reported that the hypophagia-inducing effects of MDP were of a lower magnitude and duration than those of LPS (Gayle et al., 1998; Langhans, 1996; Plata-Salamán et al., 1998). Given that the doses of LPS used in this study were lower than those of MDP by a factor of eight, the current results further suggest that MDP is less effective than LPS in reducing locomotor activity in rats of both sexes, although a more direct comparison using titrated preparations of these bacterial compounds would be useful.

In contrast to the minimal effects of the low dose of MDP by itself, rats that were given the low doses of MDP and LPS in combination were among the least active animals on Day 1. This was most evident in females given MDP and LPS, as this group was significantly less active, on all horizontal and vertical measures, than all of the other female groups except for those given the high dose of LPS. In males, rats given the combined treatment were significantly less active than both controls and rats given MDP.

Despite causing significant decreases in activity compared to controls, neither LPS nor MDP, alone, or in combination, affected the average speed of horizontal activity in either sex. This indicates that the effects of these agents were specific to certain aspects of locomotor behavior and did not induce overall deficits in motor abilities and movements.

4.3. Development of tolerance to MDP/LPS

Tolerance appeared to develop to MDP in both sexes, but it could not be determined how quickly or strongly this developed, as only the high dose produced activity decrements and these were only apparent on Day 1 in both sexes. Results of past studies have found that tolerance to repeated injections of MDP occurs very slowly or not at all (Langhans et al., 1991; Roth et al., 1997a,b; Soszynski et al., 1991; Zeisberger and Roth, 1998). In the future, treating rats

with repeated injections of MDP using a dose of 1.6 mg/kg and a second higher dose should help to clarify the formation of behavioral tolerance to the locomotor-reducing effects of MDP.

Tolerance to LPS developed rapidly in females, as decrements in locomotor activity and body weight were no longer apparent after the second exposure to LPS. In males, tolerance also developed rapidly, although not as quickly as in females, as significant locomotor activity decrements and/or body weight changes were still apparent but diminished by the second exposure to LPS and no behavioral effects were apparent by the third exposure to LPS. These results are consistent with the results of other studies which have found that tolerance to both the physiological and behavioral effects of LPS forms very quickly (Langhans et al., 1991; O'Reilly et al., 1988; Porter et al., 1998; Roth et al., 1997a; Tripp et al., 1998).

Results of previous studies have reported that male rats develop behavioral tolerance to the hypophagic effects of MDP and LPS more slowly than to LPS alone (Langhans et al., 1991; Langhans, 1996). In the current study, neither sex exhibited decrements in activity levels after the second combined injection of MDP and LPS. However, in males, the activity levels remained relatively low compared to controls and were again significantly lower than controls after the third injection for number of vertical movements. Thus, behavioral tolerance did appear to develop at a slower rate in males given both MDP and LPS compared to males given the same dose of LPS alone.

Compared to controls, all female treatment groups exhibited behavioral tolerance by Day 4, whereas male rats in the LPS2 and COMBINED groups still displayed significant activity decrements on Days 4 and 7, respectively. Overall, the rate of tolerance development, as expressed by activity slopes across test days, was also significantly greater in females than in males (Table 3). Moreover, relative to controls, each female treatment group (except for LPS1) was significantly more active than the corresponding male group on one or more measures on Day 7, and in the COMBINED groups females also exhibited significantly greater vertical time than males on Day 4 (Table 1). Taken together these findings indicate there was a robust sex difference (in favour of females) in the pattern and rate of behavioral tolerance development to bacterial infection. Possibly, the immunosuppressive effects of circulating androgens caused male rats to develop tolerance more slowly than the females.

Indeed, it has been shown that sex hormones likely affect tolerance development to endotoxin, as female rats which received two separate injections of LPS (intraperitoneally) both times during proestrus developed tolerance significantly slower than rats injected at other times of the estrous cycle. Furthermore, the sustained sickness behaviors observed in this first group appeared to be related to elevated progesterone levels (Engeland et al., 2001b).

4.4. Body weight changes

On Day 1, the rats which lost the most body weight following drug treatment exhibited the greatest decrements in activity, and the rats which did not undergo a decrease in body weight generally exhibited locomotor levels comparable to controls. Similar correlations have been reported in male mice treated with LPS (Engeland et al., 2001c). By Day 4, this significant correlation between body weight change and activity was no longer apparent in females but was still apparent, although reduced in magnitude, in males. This again reflects a sex difference in the rate of acquired tolerance to bacterial infection.

In this study LPS-injected female rats lost 4.3–5.3% (13–16 g), and male rats lost 6.3–7.5% (20–26 g), of total body weight after the first injection, which is comparable to the LPS-induced weight losses seen in other studies (Yirmiya, 1996). This was likely due to reduced food intake and increased energy expenditure among other factors (e.g., Valles et al., 2000; O'Reilly et al., 1988). Females treated with the high dose of LPS, and all male LPS-treated rats, also displayed significant decrements in total weight gain across the experiment compared to controls. In contrast, injections of MDP did not affect body weight in either sex, consistent with reports from other researchers that used equivalent doses in male rats (Langhans et al., 1991). Since the doses of LPS used were eight times lower than those of MDP, these results suggest that in rats the physiological effects of LPS are much greater than those of MDP. Also, with the exception of the female MDP2 group, LPS-treated but not MDP-treated rats had significantly heavier spleens than controls. As the spleen is a critical immune organ known to increase in size upon immune challenge due to B and/or T cell proliferation (Kumagai et al., 1992), this suggests that in rats LPS is more immunogenic than MDP. Again, this finding that LPS produces a greater effect than MDP in rats should be systematically examined using titrated preparations of these bacterial compounds.

4.5. Hormonal levels

In control animals, females had significantly higher Cort levels than males, despite the finding that males had significantly higher ACTH levels. This suggests that the sensitivity to ACTH at the level of the adrenals was much higher in females than in males, an effect that appears to occur during most stages of the estrous cycle (Atkinson and Waddell, 1997). Previous research with adult rats has shown that both ACTH and Cort levels in females are higher than in males under both basal (Atkinson and Waddell, 1997; Jezova et al., 1996; Lesniewska et al., 1990) and stress conditions (Jezova et al., 1996). Perhaps, the long-term effects of repeated stressors (i.e., injections, open-field exposures) across the seven day test session differentially affected ACTH levels, in males and

females in the current study, relative to what is typically observed after a single stressor. Despite this, there were no significant differences in the levels of ACTH or Cort found circulating in blood plasma after testing on Day 7 between treatment groups. Similarly, it has been reported that newborn rats given daily injections of LPS from postnatal Days 1 to 4 exhibited an absence of rise in Cort, although newborns given IL-1β daily displayed significantly higher Cort levels, when blood was obtained after the last injection and compared to control values (Bumiller et al., 1999). These findings of a lack of elevation in Cort levels after repeated LPS injections are likely due to the rapid formation of endotoxin tolerance, although to confirm this ACTH and Cort levels would have to be determined in naive rats injected with LPS.

Previous studies have found that circulating sex hormones are lowered after infection (Cannon, 1998). In the current study, estradiol levels were similar between treatment groups but testosterone levels were significantly reduced in the MDP2 and COMBINED groups compared to controls. In addition, testicular weight was significantly increased in the COMBINED group, indicating that MDP, and perhaps LPS, had an inhibitory impact on male sex steroid levels. This was likely mediated by IL-1β and TNF-α production, which have each been shown to reduce circulating plasma testosterone concentrations in rats (Cannon, 1998).

4.6. Do MDP and LPS interact?

The activity levels of females given MDP and LPS in combination were significantly lower than predicted by an additive model (see Langhans et al., 1991), indicating that MDP and LPS produced a potentiated behavioral effect in females. This is consistent with the findings of a variety of studies, which have reported that MDP pretreatment can potentiate the effects of LPS (Noso et al., 1988; Parant et al., 1990; Ribi et al., 1979), and also that a combined dose of MDP and LPS potentiated hypophagia in male rats (Langhans et al., 1991). However, in the present study, the activity levels of males given MDP and LPS in combination were similar to levels predicted by an additive model, indicating that the two bacterial products only had an additive effect on spontaneous locomotor activity in males. This absence of MDP/LPS potentiation in males may have been due to the inhibitory effects of circulating androgens on macrophage activation (Wichmann et al., 1997).

It is also possible that locomotor activity levels of males were influenced by a floor effect, and that the LPS dose of 100 μg/kg lowered activity levels to such a point that a combination of MDP and LPS could not significantly decrease activity further. In future studies, the use of a subthreshold dose of LPS would likely avoid any such floor effects and help to clarify this issue of potentiation between MDP and LPS in males.

In males, rats given both MDP and LPS developed tolerance to body weight loss more quickly than rats given either dose of LPS alone. As males appeared to develop behavioral tolerance to MDP and LPS more slowly than to LPS alone, this suggests that the mechanisms underlying tolerance development to weight loss differ from those which underlie hypoactivity.

How might a combined injection of MDP and LPS produce a potentiated effect on sickness behaviors? It is now well accepted that a large family of Toll-like receptors (TLR), which are expressed on the surface of leukocytes and microglial cells, are critical for producing an innate immune response to pathogens (Nguyen et al., 2002). Distinct subtypes of TLRs are capable of recognizing different classes of bacteria. For instance, mice which are TLR4-deficient are unresponsive to LPS whereas TLR2-deficient mice show a normal inflammatory response to LPS but are hyporesponsive to gram-positive bacterial cell walls and peptidoglycan (PGN) (Takeuchi et al., 1999). Taken together, these findings indicate that TLR2 and TLR4 recognize gram-positive and -negative bacteria, respectively. Recently, it was shown in mice that a single systemic injection of LPS caused a transient but robust increase in transcriptional activation of the gene encoding TLR2 throughout the CNS, whereas PGN or lipoteichoic acid from gram-positive bacteria had no effect (Laflamme et al., 2001). This increase in TLR2 expressing cells was widespread and extended from the circumventricular organs to deep brain parenchyma (Laflamme et al., 2001). It is possible that LPS increases the transcriptional activation of TLR2 genes in order to sensitize cells to pathogens such as gram-positive bacteria (Laflamme et al., 2001). This suggests one possible mechanism by which the resulting inflammatory response might be much larger for MDP and LPS in combination than for either bacterial compound alone, as was seen in this study in females.

4.7. Conclusions

In summary, the high dose of MDP and both doses of LPS produced significant locomotor decrements in both male and female rats, although LPS decreased activity more robustly. In addition, LPS caused significantly greater physiological and immunogenic effects than did MDP, based on changes in body weight and spleen sizes, respectively. When combined, MDP and LPS reduced male activity levels in an additive manner but had potentiated effects in females. Female rats also developed tolerance to repeated bacterial injections more quickly than male rats. Despite these interactive effects on behavior, MDP and LPS did not appear to interact with each other on indices of body weight or spleen size in either sex, except that tolerance developed more quickly for body weight changes induced by the combination of MDP and LPS, relative to LPS alone, in male rats.

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