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Pyridostigmine Bromide Alters Locomotion and Thigmotaxis of Rats: Gender Effects

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HOY, J. B., B. A. CODY, J. L. KARLIX, C. J. SCHMIDT, I. R. TEBBETT, S. TOFFOLLO, F. VAN HAAREN AND D. WIELBO. *Pyridostigmine bromide alters locomotion and thigmotaxis of rats: Gender effects.* PHARMACOL BIOCHEM BEHAV **63**(3) 401–406, 1999.—Male rats and female rats in the proestrous and metestrous stages of estrus were tested to determine the effects of pyridostigmine bromide on locomotion rate and thigmotactic response using doses of 3.0, 10.0, and 30.0 mg/kg. Thirty minutes after administration of the pyridostigmine bromide the rats were videorecorded for 2 h in a 1 m^2 open-field arena. The rats' activities were analyzed for the drug's effect on speed throughout the 2 h and during six 20-min segments. Also, the times that the rats were observed moving through the central 50% of the arena were determined. Locomotion rates decreased significantly, and thigmotaxses increased significantly in all groups of rats as a dose response to pyridostigmine bromide. Habituation occurred over 2 h for both responses, primarily during the first 40 min. Female rats were more affected than males, but metestrous and proestrous females did not differ significantly in their responses. At the 30 mg/kg the effect was persistent throughout the test period. Proestrous females dosed at 30 mg/kg had much higher pyridostigmine bromide serum levels than metestrous females and males. © 1999 Elsevier Science Inc.

Pyridostigmine bromide Locomotion Open field Thigmotaxis Gender effect

PYRIDOSTIGMINE bromide (PB) is an acetylcholinesterase inhibitor that has been used as a treatment for myasthenia gravis for many years (8). A recent study has shown a synergistic effect between DEET and both PB and permethrin when administered to cockroaches (15). Coexposure to PB, *N*,*N*-diethyl-*m*-toluamide (DEET), and permethrin has also been shown to have synergistic behavioral effects in chickens (1). A synergistic effect (LD_{50}) of coexposure to PB, DEET, and permethrin using male rats has been reported (12). In this case, oral administration of PB in propylene glycol resulted in estimation of LD_{50} of 61.6 mg/kg. Combinations of the three drugs at dosages calculated to cause mortality of 48% of the animals caused mortalities of 80 to 90%.

Sublethal effects of neurotoxic compounds may be seen in various measures of locomotor activity (4,9,14). Neurobehavioral screening of pesticide effects on mammals has been reported (13). Low doses of PB (3–12 mg/kg) decreased response frequency during operant tests (17). Gender and estrous cycle were identified as factors in reduced open-field activity produced by interleukin-1b (2). Similarly, gender differences in susceptibility of cockroaches to toxicants has been reported (10). Open-field locomotor activity in rats, using automated data acquisition, can show chemically induced changes in speed and thigmotactic responses (3,4,9,16). Significant changes in the open-field behavior of rats dosed with PB at 5.5% of LD_{50} have been reported (19). In this case, intraperitoneal administration of PB resulted in estimation of LD_{50} at 2699 mg/kg.

The purpose of this study was to determine the effects of PB on locomotor and thigmotactic activity of male rats and female rats in proestrous and metestrous. Furthermore, we sought baseline information for future study of the synergistic effects of PB, DEET, and permethrin on locomotion.

METHOD

Subjects

Sprague–Dawley rats (250 g) were obtained from Harlan–Sprague–Dawley (Indianapolis, IN), and housed same sex, two per cage, under a reversed light cycle of 12 D:12 L (lights

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on 1800 h), and fed rat chow ad lib. The rats were identified by ear-punch code. Each rat was handled about 30 s 5 days/week for at least 7 weeks prior to testing. Treatments were assigned to individuals at random within groups and time of test. Tests were done between 900 and 1700 h. Male, and proestrous, and metestrous female rats were tested two at a time in individual arenas. Male rats were treated first, and whenever possible metestrous females were tested second and proestrous females last. Alternatively, only females of one type were tested if both types were not available on a given day. All dosing and handling of test subjects were done by the same technician.

Estrous Stage Determination

Female subjects were examined 1 to 3 h before testing to determine their estrous cycle status. The criteria for assignment to proestrous or metestrous categories was based on microscopic examination of epithelial cells found in the vaginal fluid of the rats.

Drug and Dosage

PB obtained from Sigma (St. Louis, MO) was orally administered by gavage tube in distilled water at low, medium, and high doses, 3.0, 10.0, and 30.0 mg/kg, respectively, in a volume of 5 ml/kg. Control animals were dosed with matching volumes of distilled water. Test subjects were held 30 min prior to introduction to the test arenas, then placed in the center of the arena about 30 s prior to recording of their activity.

Arenas

The tests for locomotor activity were done in two black ABS plastic arenas that were 100 × 100 × 30-cm high. Each arena was surrounded by a black curtain. The arenas were on opposite sides of a rack that supported lights, video cameras, and video cassette recorders. Indirect low intensity light was provided by three 60-watt red bulbs approximately 2.2 m above each arena, and located so that the center of each arena received about 2 lx and the corners received 1 to 2 lx. Prior to use, feces and urine were removed and each arena was swabbed down with about 10 cc of 80% ethanol solution, and wiped dry with paper toweling. The air-conditioned testing room was maintained at approximately 22°C. The arenas were in a locked room well insulated from outside sounds. Within 1 min of the start of each test the experimenter left the room for the remainder of the automatically recorded 2-h test.

Recording

Horizontal locomotion was recorded using a Topica (model TP-505D/3) CCD video camera and a Sharp (model XA-601) video cassette recorder. Paralax was minimized by mounting the cameras 2 m above the arenas. The 1 m^2 arena was visualized as 240 × 240 pixels. Therefore, a movement over 24 pixels was a move of 10 cm. A speed of 30 pixels/s was about one rat body length/s, or 7.5 m/min. Raw data recorded in pixels/s were converted to m/min before data analysis was completed. All video records were archived following computer analysis.

Locomotor Analysis

Locomotor activity was quantified using Apple Power Macintosh-based software and a Macintosh (model 7100/80 with an AV board installed) (6,7). The software calculates the center of mass of the rat. To avoid including the rat's tail in determining the location, or movement; India ink was applied to the tail prior to PB administration. Each 2-h recording was reduced to an ASCII file of observations at 1-s intervals that represented both the positions of the subject on a 240 × 240 pixel grid (X,Y coordinates) and the running average of locomotion rate over five observations. Sampling at 1-s intervals filtered out recording of short-range stereotypic movement that would otherwise have been scored as locomotion. The raw data were used to calculate speeds for each second of the record, which were then used in lieu of the running average provided by the original analysis.

The number of times that the subject was recorded in the center 50% of the arena was filtered so that only those times that the subject was moving faster than 1.2 m/min (2 cm/s) were counted. That filter excluded observations that might have occurred if a subject had become inactive, thereby avoiding a high center zone score for a subject that had collapsed in midarena.

The ASCII file for each subject was then imported into StatView and further analyzed for locomotion rate and thigmotatic response in six 20-min bins of the 2-h test period.

Blood Serum Analysis

An estimate of the serum level of PB in an individual rat at the beginning of the test period was obtained by waiting at least 5 days after a given rat's locomotion test and taking a blood sample by decapitation 30 min following a second similar dose and anesthesia with methoxyflurane. Female subjects were dosed the second time during the appropriate stage of the estrous cycle. Three milliliter blood samples were kept on ice for 2 h, centrifuged, serum drawn off, and frozen at −70°C. The serum was then analyzed for PB as follows: the serum sample was transferred to a stoppered tube and vortexed with 1 ml of 0.025 M potassium phosphate buffer at pH 3. This mixture was then applied to a Strong Cation Exchange column that have previously been conditioned under vacuum on a Vac Elut manifold (Varian) with methanol, water, and 0.025 phosphate buffer. After application of the sample, the column was air dried for approximately 30 s and then washed with phosphate buffer and 0.1 M acetic acid. The column was again air dried for 30 s before eluting off the adsorbed drugs with 3% ammoniacal methanol. The final extract was evaporated to dryness under nitrogen and the residue reconstituted in 50 μl of methanol. A 20-μl aliquot of the extract was then used for HPLC analysis. This analysis was performed using a Waters 510 pump to deliver solvent at 1 ml/min to a Hypersil 5 μm ODS column. A Waters C18 Guard Pak precolumn was used to protect the analytical column. The detector was a Waters 486 variable wavelength detector set at 272 nm with a Dell 486 data system and Millenium (TM) software. The mobile phase consisted of acetonitrile–0.1% triethylamine in water (adjusted to pH 3.2 with phosphoric acid. 70:30). Quantitative analyses were achieved by comparison of peak areas with unextracted standards. Each determination was taken as the mean of three replicate injections. The calibration graph was produced over the range of 0.05–5 μg/ml.

Experimental Design

The experimental design was three groups of rats × four application rates × 10 subjects for each application rate. Space limitations in the rat colony required that the rats be tested in two batches, 20 males and 40 females each, for a grand total of 120 rats. Each batch was tested over a 15–22-

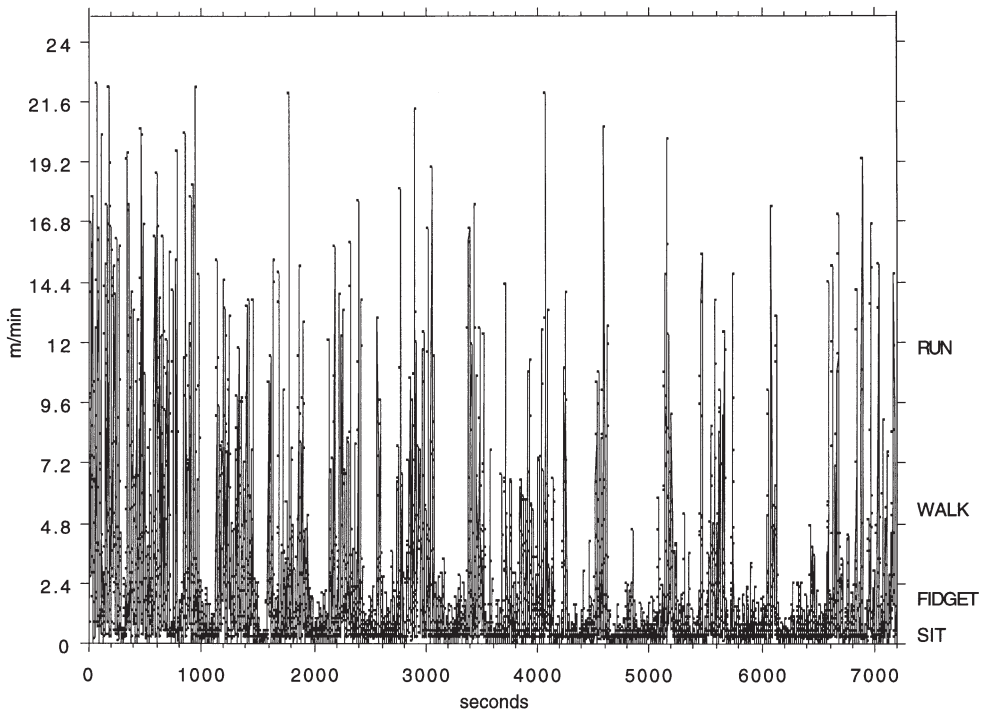

FIG. 1. Typical range and variation in locomotion (m/min) during a 2-h period following administration of vehicle. Note the indications of the type of behavior associated with various speeds at the right margin of the figure.

day period, with 29 days between batches. The data from the two batches were pooled.

Statistical Analysis

Differences between locomotion rates and counts of observations in the center zone of the arena were determined by repeated-measures ANOVA (group × time), for the total 2 h observation time. Comparisons between groups were then done using Duncan's Multiple Range Test ($p < 0.05$). Subsequently, post hoc power calculations were done with assumptions of higher alpha levels using GPOWER (5).

RESULTS

Locomotion rates as high as 30 m/min were observed. Figure 1 illustrates the range and variation of locomotion rate for a typical rat dosed with vehicle. Sitting, fidgeting, walking, and running fall into the progressively higher ranges indicated in the figure. The ranges of speed associated with these activities were subjectively determined, and are provided as a general indication of the alternation of activities over the observation period. Also, Fig. 1 shows a trend toward fewer and shorter peaks throughout the 2-h period as well as the rapid changes in speed.

Locomotion Rate

Habituation of the locomotion rate, as suggested by the reduced number of peaks over time, is more clearly illustrated by the mean speeds found in each successive 20-min period of observation. Figure 2 shows the habituation curves for all groups and treatments of rats. Each group and treatment followed the same pattern, i.e., a rapid decline in mean speed during the first hour, followed by very little change in mean speed during the second hour. The dose effect of PB can also be seen in this figure.

Figure 2 shows locomotor activity (speed in mean m/min) during 20-min segments of the experimental session for male rats and female rats in either proestrous or metestrous phase of the estrous cycle following the administration of vehicle or PB. ANOVA revealed a significant three-way interaction among time of observation, dose, and gender, $F(30, 535) = 1.72$, $p < 0.03$. This figure shows that for subjects given the vehicle speed decreased from an initial high of about 4 m/min to about 1.75 m/min during the final 20 min of the session. ANOVA revealed that the speed decreased as a function of dose, $F(3, 107) = 34.80$, $p < 0.01$. Post hoc analyses showed no significant differences between the administration of vehicle and 3 mg/kg PB, but that the speeds observed after administration of 10 mg/kg PB and after 30 mg/kg PB were significantly lower than vehicle. Planned contrast analyses at each time of observation (Table 1) showed that there were no significant differences between vehicle and 3 mg/kg PB in any of the groups of subjects. Significant differences were observed at all times of observation when the behavioral effects of vehicle were compared to those observed after administration of 10 mg/kg in metestrous and proestrous females. However, in male rats the decrease in speed after 10 mg/kg PB was only significant at time point 2. Planned contrast analyses showed that speed decreased significantly compared to vehicle administration in all groups of subjects after the administration of 30 mg/kg PB.

Center Zone Activity

The distribution of activity within the 1-m^2 arena favored the marginal area in all cases. That bias is illustrated in Fig. 3,

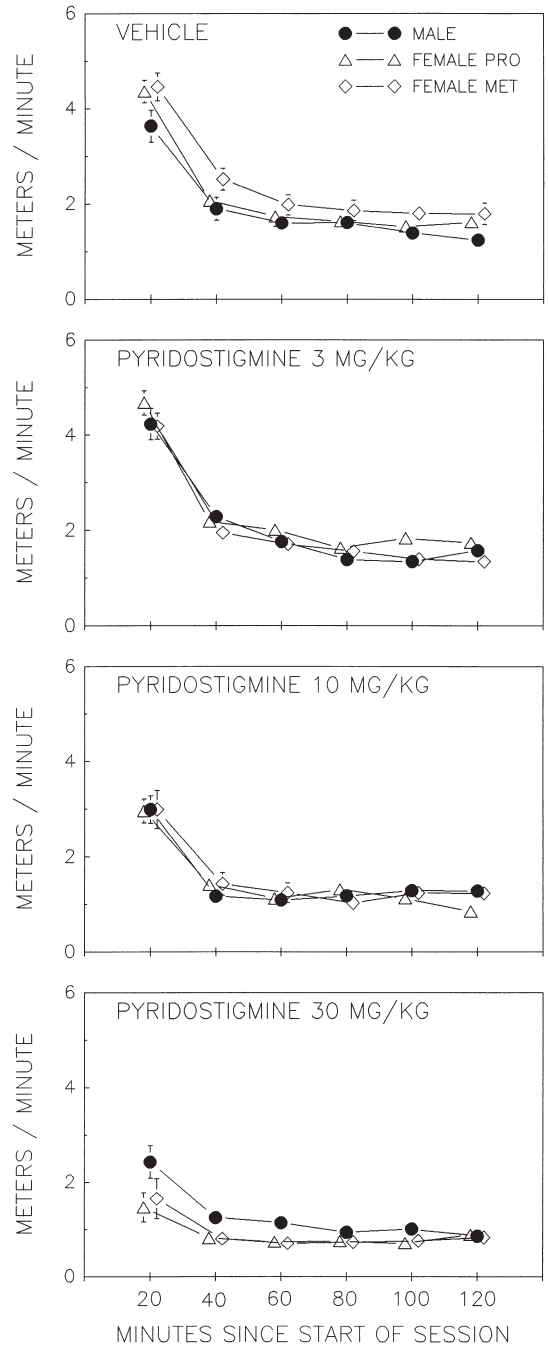

FIG. 2. Mean locomotion rates (m/min) in 20-min segments of 2-h observation periods according to rate of administration of pyridostigmine bromide.

which shows typical traces of the paths of rats given the four treatments used in our study. We quantified the distribution of activity by calculating the percent of the total observations in which the rat was observed moving through the central 50% of the arena. A dose effect was found in all groups of rats.

Figure 4 whose center zone activity for male rats, proestrous female rats, and metestrous female rats following administration of vehicle and 3, 10, or 30 mg/kg PB vs. vehicle.

TABLE 1

EFFECTS OF PYRIDOSTIGMINE BROMIDE (10, 30, AND 10 mg/kg vs. 30 mg/kg) ON LOCOMOTION RATE BY TIME PERIOD OF MALE, AND PROESTROUS AND METESTROUS FEMALE RATS

	Dose (mg/kg)								
	Males			Proestrous Females			Metestrous Females		
Pd.	10	30	10 vs. 30	10	30	10 vs. 30	10	30	10 vs.30
1	NS	33	NS	28	59	*	36	60	*
2	34	34	NS	28	60	*	50	68	NS
3	NS	NS	NS	30	56	*	37	65	*
4	NS	NS	NS	19	48	*	49	62	NS
5	NS	42	NS	26	41	*	28	57	*
6	NS	30	NS	40	35	NS	33	54	NS
Tot	NS	33	NS	30	50	NS	40	62	*

*Significant effects (alpha = 0.05) are indicated by the percent reduction from the control mean for comparisons in the first two columns. An asterisk indicates a significant difference where the effect of 10 mg/kg vs. 30 mg/kg is compared.

This figure shows that subjects tended to spend between 20 and 25% of the session time in the center of the arena following vehicle administration. PB dose dependently decreased the percentage of center zone observations, $F(3, 107) = 28.85$, $p < 0.01$. After the administration of 30 mg/kg PB subjects were in the center zone in less than 10% of the observations. Gender differences or interactions between dose and gender were not found.

Blood Serum Analyses

Postreatment analyses indicated that serum levels of PB for the three test groups were higher, but not proportionately

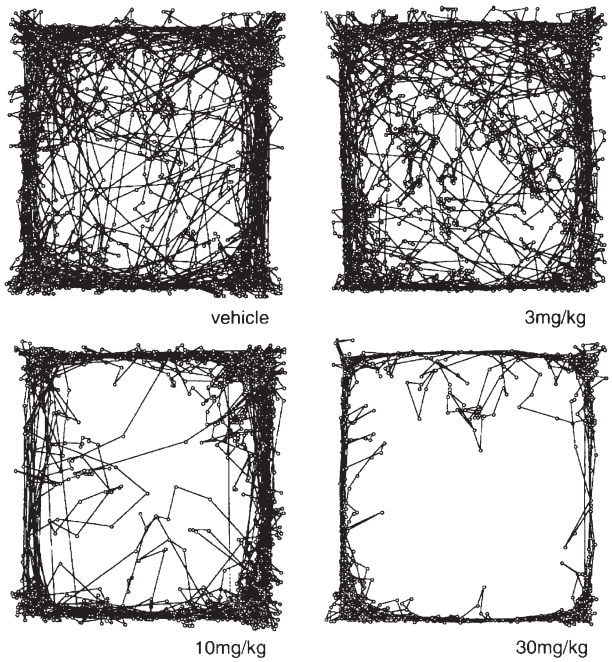

FIG. 3. Typical traces of the paths of male rats during a 2-h observation period, according to the indicated rate of administration of pyridostigmine bromide.

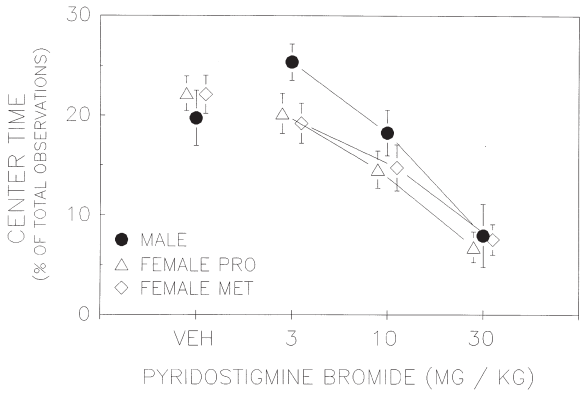

FIG. 4. Center zone time (the average percentage of the total number of observations that the subjects were in the center area (50%) of the arena ±1 SEM) for the groups of male and (proestrous and metestrous) female rats following administration of the vehicle and 3, 10, or 30 mg/kg of pyridostigmine bromide.

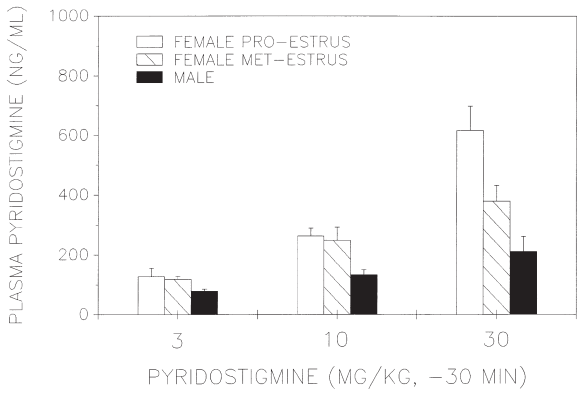

FIG. 5. Serum levels of pyridostigmine bromide (mean nanograms per ml, =1 SEM) observed after the second administration of 3 mg/kg left-most bars), 10 mg/kg (middle bars), or 30 mg/kg (right-most bars).

higher, with increased dose, i.e., a negatively accelerating dose–response curve. Figure 5 shows serum levels of PB observed 30 min after the second administration of 3, 10, or 30 mg/kg. A total of 70 observations figured in this analysis. No PB was found in control animals. The serum levels differed by dose. A significant interaction between PB dose and gender, $F(4, 61) = 5.64$, $p < 0.01$, and subsequent post hoc analyses supported the observation that PB levels in males differed only when compared after 3 and 30 mg/kg PB. In metestrous females, all three doses differed from one another, whereas in proestrous females differences were observed when 3 and 30 mg/kg and 10 and 30 mg/kg were compared, but not when 3 and 10 mg/kg were compared.

DISCUSSION

We have presented our locomotion data in terms of speed in m/min. The observed speeds correspond to the following types of activity, and provide an illustration of the types of behavior seen. A mean rate of less than 1.2 m/min indicated a sluggish rat moving less than 0.2 body length/s. Fidgeting or grooming behavior was recorded as movement less than 2.4 m/min (less than 0.5 body length/s). Walking resulted in a mean speed of less than 7.2 m/min (less than 1.5 body lengths/s). Running resulted in speeds ranging from 7.2 m/min to more than three times that rate.

We found gender differences and PB effects on locomotion rate. A previous study on male rats ($n = 6$) found no effect on the running speed in an open-field test following intraperitoneally administered PB at less than 10% of the LD_{50} (14). Recently, hens ($n = 5$) given 5 mg/kg PB orally for 60 days showed no locomotor effects (1). However, both studies lacked the power needed to find anything less than a catastrophic effect. In another study of PB effects on locomotion, male rats administered pyridostigmine and running on a treadmill became exhausted more rapidly than controls (11). Our findings that female rats were more sensitive than male rats, and the somewhat limited power of our test, suggest that additional tests using female rats in numbers adequate to balance type I and type II error are needed to find or rule out subtle effects. The problem of finding effects on sensitive but rare individuals within a population should also be addressed.

Center zone activity has been found to be a more sensitive measure of intoxication than speed when a stimulant was the toxicant (3,18). We found that PB depressed both measures, but we are not convinced that one measure is more sensitive than the other in our study. Separating the possible interaction of the two is outside the design of this study.

Serum levels of PB were higher in female rats than in male rats. In female rats they were also higher during the proestrous than during the metestrous phase of the cycle. These observations suggest that PB kinetics (liver metabolism and/or urinary excretion) may be modified by circulating gonadal hormones. At present, it is not known what mechanisms might be involved, but such warrants further investigation.

Table 1 shows the percent reduction from control level of locomotion rates for all cases significant at the 0.05 level. The contrast between the sexes is striking, with little effect observed in males. And, although ANOVA failed to show a significant difference between groups of females, the metestrous females quite consistently showed a greater reduction than the proestrous females.

Sublethal behavioral effects, by definition, are more sensitive and more relevant to drug safety than LD_{50}, or even an LD_1. The gender effect in rats is certain, with timing relative to estrous cycle a possible exacerbating factor in the toxicity of PB to females. If humans in general, and females at a crucial point in their menstrual cycle in particular, are more sensitive to PB than rats, the changes in rat locomotor behavior that we have found may be relevant to the clinical use of PB.

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