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Evaluation of Marble-Burying Behavior as a Model of Anxiety

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NJUNG'E, K. AND S. L. HANDLEY. *Evaluation of marble-burying behavior as a model of anxiety.* PHARMACOL BIOCHEM BEHAV 38(1) 63–67, 1991.—On individual placement in a cage with 20 evenly spaced glass marbles, female MF1 mice buried 7.8 ± 0.2 marbles. Olfactory stimuli from experimenters hands and sex of mice had no influence on number buried, but most marbles were buried when they were evenly spaced. There was no habituation to these novel objects on serial testing or prehousing with marbles and, in a two-compartment box, mice did not avoid marbles, spending half their time on the marble side. In the pharmacological experiments, locomotor activity was measured separately to indicate the possibility of nonspecific effects. The anxiogenic agents yohimbine and ethyl-beta-carboline-3-carboxylate (β-CCE) did not enhance burying, yohimbine decreased burying at doses also reducing locomotor activity. Diazepam effects depended on dose: 0.1 mg/kg increased burying, 0.25 mg/kg had no effect and 1.0–5.0 mg/kg reduced it. Diazepam increased locomotor activity from 0.1–2.5 mg/kg and had no effect at 5.0 mg/kg. Zimeldine, 10.0 mg/kg, reduced burying but not locomotor activity. Inhibition of marble burying may be a correlational model for detection of anxiolytics rather than an isomorphic model of anxiety.

Anxiety Burying behavior Diazepam Ethyl-beta-carboline-3-carboxylate Marble burying Obsessive-compulsive disorder Yohimbine Zimeldine

RODENTS use bedding material to bury noxious materials. Objects so buried include prods which give them electric shocks (9, 14, 17, 18), rat chow pellets coated with quinine (15), spouts of bottles containing unpleasant tasting liquids such as pepper sauce (15,22), liquids to which they have developed taste aversion after concomitant injection with lithium chloride (22) or amphetamine (15), mouse traps which strike, flashcubes which discharge near them and hoses which direct airbursts into their faces (17). However, rodents also bury harmless objects including rat chow pellets (15), flashcubes which do not flash (17), and glass marbles (1,15).

Selective inhibition of object-burying behavior in rodents has been proposed as test for anxiolytics (1,18). Both the duration and extent of burying of electrified prods by rats were reduced by the anxiolytic agents diazepam, chlordiazepoxide and pentobarbitone (18,19). However, burying 'harmless' objects, i.e., glass marbles, was also reduced by a variety of anxiolytic agents, including benzodiazepines, ethanol and meprobamate, at doses which did not reduce swim-induced grooming; nonanxiolytic centrally active compounds such as neuroleptics reduced marble burying only at doses which did reduce swim-induced grooming (1).

Responsiveness to anxiolytics does not necessarily mean that a test models anxiety (20). Such models require the presence of an aversive stimulus or threat (7, 8, 15) which provokes a response appropriate to minimization of the potential harm (8). This response can be either the emission or the witholding of an activity, depending on the circumstances of the test (8). On this basis, marbles would belong to the innate-fear category of aversive stimulus by virtue of their novelty (7) and the emission of burying behavior would be viewed as an appropriate response because it removes the source of the aversive stimulus. Alternatively, it could be proposed that burying behavior is rewarding or that it is compulsive. The latter proposal is significant because marble burying was reduced by serotonin uptake inhibitors (1,2) which have recently been found to be effective in human obsessive-compulsive disorder (11,13).

In the present experiments, in addition to examining some of the general characteristics of this burying behavior, we have sought to detect a role for novelty by studying habituation to repeated presentation of the marbles. Stimulus properties of marbles have also been sought using a two-compartment box. Mice will avoid an aversive stimulus, such as bright light, presented in one compartment of such a box (4). Alternatively, if burying is rewarding or compulsive, it might be predicted that mice would spend longer on the marble-containing side. Yohimbine and β-CCE (ethyl-beta-carboline-3-carboxylate), anxiogenic agents which have the opposite effects to anxiolytics in many other anxiety models with an appropriate baseline (8), have also been examined together with diazepam. In addition, the serotonin uptake inhibitor zimeldine has been tested, in order to confirm the findings of Broekkamp and Jenck (2) that serotonin uptake inhibitors reduce marble burying without affecting swim-induced grooming. However, since some serotonin agonists have marked effects on grooming behavior [see, e.g., (10)], we have chosen instead to use a

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locomotor activity test to detect whether the drug effects were specific to marble burying.

METHOD

Female MF1 mice (23–35 g) were held in groups of 20 under a 12-h light/12-h dark (lights on 0800 h) cycle, with free access to food (Pillsbury Ltd. diet 41B) and (tap) water in the experimental room for at least 3 days prior to experiment. Experiments took place between 1000 and 1800 h unless otherwise stated. Mice were taken from the stock cages and placed individually in polypropylene cages (42 × 24 × 12 cm) containing 20 clean glass marbles of diameter 1.5 cm evenly spaced on 5 cm deep sawdust without food or water. The ceiling was a metal grid. The number of marbles at least two-thirds buried was counted 30 min later. In some experiments, the mice were observed via closed-circuit television. All drugs were administered IP 30 min before testing. Mice from comparison or control groups were always tested concurrently with mice from test groups within each experimental session.

Two-Compartment Box

The test cage was lined with 5 cm sawdust and was identical to the ones used in testing marble burying except that it was partitioned down the middle by a cardboard wall with a 5.0 × 7.5 cm hole at bottom-center. Ten marbles were evenly distributed on one side of the partition only, the marble side being varied randomly with respect to the cage itself and its orientation within the laboratory. Mice were placed in this box for 30 min, time on each side and number of marbles buried was noted cumulatively at 5, 10 and 30 min.

In the experiments with drug pretreatments, locomotor activity was measured in separate groups of mice during a 5-min placement on a circular runway mounted on an Animex activity meter (LKB Farad; tuning: 40 μA, sensitivity: 25 μA, so that only translocational movements were recorded). These measurements commenced 30 min after injection, equivalent to the time at which mice were placed in the observation cages for measurement of marble burying.

Statistical Analysis

Distribution-free statistical tests were used (16), i.e., Mann-Whitney U for the two-group case and Kruskal-Wallis one-way analysis of variance for the comparisons involving more than two groups. In view of the sample characteristics of 155 untreated female mice (see the Results section), the mean and its standard error (s.e.m.) were, however, used to express central tendency.

Drugs

Diazepam injection (Roche), β-CCE (Roche), yohimbine (Sigma) and zimeldine (Astra) were dissolved in 0.9% sodium chloride (saline).

RESULTS

The following description summarizes the qualitative aspects of marble-burying behavior. On being placed in a cage containing 20 evenly spaced glass marbles, a mouse generally approached a marble within 60 seconds. Typically it would sniff one or more marbles before proceeding to grasp one with its forepaws and push it around with the snout, forepaws or hindlimbs. These episodes alternated with bouts of exploration of the cage floor, walls and roof. When it returned to the marbles, the mouse would repeat the process or include variations such as pushing sawdust towards marbles with the snout or forelimbs, or digging holes in the sawdust and pushing marbles in. Again these bouts alternated with general exploration, or with periods of quiet resting or grooming. Some mice, in addition, walked to a corner of the cage and scattered sawdust all over the cage with their hind limbs. This last activity did not appear to be specifically directed at the marbles though quite a number of marbles were buried in this way. Some marbles were found buried several centimeters below the surface. Sometimes a number of the marbles were found gathered together in the center or to one side of the cage. Since mice also dug sawdust all over the cage, some of the burying was most probably incidental to the digging.

TABLE 1

EFFECTS OF VARIOUS ALTERED CONDITIONS ON NUMBER OF MARBLES BURIED IN 30 MIN

Conditions	No. of Mice	No. of Marbles Buried Mean	sem
Female Mice			
marbles evenly distributed	6	12.4	1.9
marbles grouped in center	6	6.8	2.8*
marbles grouped at one side	6	6.3	1.8*
Handling of Marbles			
handled with bare hands	6	8.0	2.3
handled with gloved hands	6	5.3	1.9 ns
Prehousing With Marbles (20 marbles/4 days)			
no prehousing	6	7.3	1.5
prehousing	6	7.4	1.6 ns
Gender			
male mice	6	6.3	1.6
female	6	8.0	2.3 ns

Unless otherwise stated, marbles were handled with bare hands and evenly distributed, and mice were female. Significance *$2p<0.05$; ns: $2p>0.05$ with respect to concurrent control group.

Mice were found to bury most marbles when the marbles were spread evenly in the cage (Table 1). The number of marbles buried fell almost by half when they were all placed in the center or to one side of the cage. When marbles were evenly spaced, the mean number of marbles buried by 155 untreated female mice was 7.8 ± 0.2, range 0–20. Although passing the symmetry test and having a median (7 marbles buried) coincident with the mean, these data did not conform to either a normal or a log-normal distribution, probably due to truncation of the data at 0 and 20 marbles buried. Male mice buried a similar number of marbles to female mice (Table 1); there was no significant difference in the number of marbles buried by the two sexes. One group of mice was handled with rubber gloves and tested with marbles which had also been handled only with rubber gloves. There was no significant difference in the number of marbles buried by these animals and those buried by control animals tested in the usual way (Table 1).

When a group of 12 mice was tested for marble-burying behavior on 5 consecutive days, there was no significant difference in the overall number of marbles buried each day (Table 2). However, there was considerable, and apparently random, variation in the number of marbles buried by each mouse, since Kendall's

TABLE 2

EFFECT OF REPEATED EXPOSURE TO MARBLES ON NUMBER OF MARBLES BURIED IN 30 MINUTES

Conditions	No. of Mice	No. of Marbles Buried Mean	sem
Same Mice, Consecutive Days:			
day 1	12	9.9	1.5
day 2	12	10.2	1.6 ns
day 3	12	9.4	1.7 ns
day 4	12	9.8	2.4 ns
day 5	12	9.8	1.3 ns
Same Mice, Same Day:			
1300 h	6	6.5	1.3
1500 h	6	5.1	1.1 ns
1700 h	6	5.2	1.5 ns
1900 h	6	7.8	1.6 ns
2100 h	6	4.4	1.4 ns

Female mice, marbles evenly distributed.
Significance ns: $2p>0.05$ (quoted with respect to 1st trial).

coefficient of concordance between days was not significant [W =0.33, $\chi^2(11)=14.92$, $p>0.05$]. A further group of 6 mice were tested for marble burying behavior 5 times at 2-h intervals on the same day, starting at 1300 h (Table 2), but there was no significant change in the number of marbles buried. Mice housed with marbles in their home cage for four days and later tested in the experimental cage also buried the same number of marbles as those which were exposed to marbles for the first time (Table 1).

When mice were tested in a two-compartment box, one of which contained ten marbles and the other none, they spent equal amounts of time in the two compartments. This was true whether the duration of testing was 5, 10 or 30 minutes. The number buried increased with the duration of the test (Table 3).

The effect of diazepam on marble burying (Table 4) depended on dose, progressing from an increase at 0.1 mg/kg, through no effect at 0.25 mg/kg to inhibition at 1.0 mg/kg and above. Locomotor activity, on the other hand, increased from 0.1 to 2.5 mg/kg, but was not significantly changed at 5.0 mg/kg. β-CCE at 1.0 and 5.0 mg/kg had no effects on marble burying. Yohimbine at 1.0 mg/kg did not affect marble burying; 5.0 mg/kg reduced both marble burying and locomotor activity. Zimeldine (10.0 mg/kg) reduced marble burying without affecting locomotor activity.

TABLE 3

MARBLE-BURYING BEHAVIOR IN A TWO-COMPARTMENT BOX

Duration of Test (s)	No. of Marbles Buried Mean	sem	Time of Nonmarble Side (s) Mean	sem	% Time Nonmarble Side
300	4.2	2.0	143	4	47 ns
600	7.3	0.9	292	30	49 ns
1800	8.4	0.4	916	45	51 ns

Data from 10 female mice. One side of the box contained 10 evenly spaced marbles, the other did not contain marbles.
Significance ns: $2p>0.05$ compared with null hypothesis value of 50% of test duration.

DISCUSSION

From the qualitative observations, marble burying appeared to be a mixture of deliberate action on the part of mice and burying incidental to other digging behavior. Since it was found that mice buried most marbles when these were spread evenly in the cage, all the other experiments were done with the marbles so spread. The lower burying rates when the marbles were placed together, to the side or at the center of the cage, may be an indication that the mice then encountered marbles less frequently by chance, combined with the fact that there would be less sawdust surrounding each marble to bury it with. The possibility that fewer marbles were buried because mice had more room to avoid the marbles was addressed with the 2-compartment box (see below). The finding that marble burying was constant across several exposures at different times of day suggests that burying behavior has no clear diurnal pattern.

Since the bulk of this work was done using female mice, the finding that males have the same propensity to bury marbles shows that the behavior is not peculiar to female mice. The lack of consistency in marble burying by individual mice on different days does not accord with previous findings with Swiss CPB:SE mice (1), and may be a strain difference. This lack of consistency was also noted in groups of naive mice from day to day, emphasizing the importance of appropriate concurrent controls.

Gray (7) has identified three categories of aversive stimulus. Secondarily aversive stimuli signal the response-contingency of a specific noxious occurrence and are learned; signals of frustrative nonreward denote the nonavailability of a previously reinforcing event. No evidence was found that burying was a learned response to the presence of marbles: initial approach was rapid and there was no increase in burying rate, either with time in the two-compartment box or on repeated exposure to marbles. Frustrative nonreward might perhaps be indicated if mice bury marbles because they are inedible, however, edible objects are also buried (15). The third category, innate fear stimuli, includes genetically programmed aversion to biologically relevant stimuli and among these, novelty is particularly potent (7). However, in the present experiment, no role could be identified for the novelty of the marbles since no habituation occurred when marbles were presented five times in one day, daily for 5 days or after continuous exposure in the home cage. This confirms and extends previous findings that four daily exposures did not induce habituation and that mice housed with marbles buried them during a test exposure as readily as naive animals (1). Handling washed marbles with gloves did not alter burying, so that olfactory properties acquired from contact with the hands of the experimenter did not appear to have induced aversion.

The possibility that the marbles had unidentified aversive properties was further investigated using a two-compartment box. This procedure has been used in various ways to indicate stimulus properties of an environment. Avoidance of a bright light presented on one side has been used widely as an anxiety model in the mouse (4). Avoidance of marbles would be an alternative response to burying them if the marbles had aversive properties, but mice did not avoid the marbles when given the opportunity to do so at any time during the 30-minute exposure.

Approach behavior has been less studied using a two-compartment technique. Preference for a compartment previously associated with drug treatment is taken as evidence that the drug in question has rewarding, and thus dependence-producing potential (3). In a single compartment, rats remain longer in a heated area after injection of agents which raise hypothalamic set-point (5). Since, in the present experiments, there was no preference for the marble-containing side, the two-compartment technique did not

TABLE 4

EFFECTS OF ANXIOLYTIC AND ANXIOGENIC AGENTS ON MARBLE BURYING AND LOCOMOTOR ACTIVITY

Drug/Dose (mg/kg IP)	No. of Mice	No. of Marbles Buried		Locomotor Activity (counts/5 min)	
		Mean	sem	Mean	sem
Diazepam 0.05	6	8.7	2.3 ns	344	18 ns
Saline	6	6.5	1.4	382	24
Diazepam 0.1	6	11.8	1.5*	476	23*
Saline	6	5.7	1.9	393	29
Diazepam 0.25	6	5.8	2.7 ns		
Saline	6	5.7	1.9		
Diazepam 1.0	6	2.5	1.4*		
Saline	6	5.7	1.9		
Diazepam 2.5	6	0.17	0.17†	512	29*
Saline	6	5.7	1.9	393	29
Diazepam 5.0	6	2.0	0.8*	418	36
Saline	6	6.5	1.4	382	24
β-CCE 1.0	6	7.2	2.9 ns		
β-CCE 5.0	6	5.2	1.6 ns		
Saline	6	6.5	1.4		
Yohimbine 1.0	6	9.3	2.7 ns		
Yohimbine 5.0	6	2.0	0.4*	421	9*
Yohimbine 10.0	6	0.0	—		
Saline	6	8.0	2.1	558	24 ns
Zimeldine 10.0	6	3.2	1.4*	445	23 ns
Saline	6	8.9	1.6	483	25

Female mice, marbles evenly distributed. Locomotor activity measurements performed on separate groups of animals. Significance $*2p<0.05$; $\dagger 2p<0.01$; ns: $2p>0.05$ compared to concurrent saline controls.

provide any evidence that the marbles had reinforcing properties.

Nevertheless, marble-burying behavior was inhibited by the anxiolytic agent diazepam at similar doses to those active in other anxiety models (8), and these doses did not impair motility in the locomotor test. Since both diazepam and zimeldine reduced marble burying at doses which did not affect the high levels of locomotor activity which occur on initial exposure to a circular runway, this latter method may prove as effective as swim-induced grooming (1) as a control for the specificity of drug effects on marble burying (2). For pharmacological studies a separate test of these effects is desirable because of the possibility that locomotor activity and burying behavior might show behavioral competition during the burying test itself. In anxiety models with a suitable baseline, anxiogenic agents such as β-CCE and yohimbine have opposite effects to those of anxiolytics (8). These agents did not increase burying despite the use of doses which are active in other models and a control burying rate which would allow such increases to occur. A significant increase was in fact seen after 0.1 mg/kg of diazepam, a dose which also increased exploratory locomotion in a separate test. Both phenomena might be due to a more general disinhibition of behavior, although at 2.5 mg/kg, diazepam reduced burying while at the same time increasing locomotion.

Many aspects of behavior are suppressed in the presence of a novel environment (7,8) and it is possible that this ability of low doses of diazepam to increase burying rather than the inhibition of burying after higher doses is the expression of its anxiolytic effect in the marble-burying test. In favour of this is the ability of the anxiogenic agent yohimbine to suppress burying. Also zimeldine reduced marble burying, and this could correspond to the finding that serotonin uptake inhibitors are anxiogenic rather that anxiolytic early in treatment (21). However, there are also several arguments against this proposal: in the habituation experiments burying did not increase despite several exposures to the novel environment; unlike diazepam, yohimbine only decreased burying at doses which also decreased activity in the locomotor test; and the anxiogenic agent β-CCE was inactive.

The lack of habituation to marbles suggests the possibility that the burying behavior is compulsive. The ability of serotonin uptake-inhibitors to suppress marble burying has led to the suggestion of a possible relationship of this burying behavior with obsessive-compulsive disorder (OCD) (2). The failure of yohimbine to promote burying would be compatible with its failure to exacerbate OCD (13). However, benzodiazepines are of little benefit in OCD and serotonin uptake inhibitors are not active on first dose [e.g., (11,13)]. OCD sufferers may seek out or avoid the object of their rituals [e.g., (6, 11, 12)], in contrast, diazepam inhibited marble burying, zimeldine was active on first dose, mice neither sought out nor avoided marbles in the two-compartment box and very few buried all marbles presented. Further investigation will be necessary of the extent to which marble burying resembles OCD.

In conclusion, although marble burying was reduced by doses of diazepam and zimeldine which did not affect activity in a locomotor test, we have not been able to find other evidence that marble burying models anxiety, either from its behavioral characteristics or from the actions of anxiogenic agents. Inhibition of marble burying may, therefore, constitute a correlational model for detecting anxiolytics rather than an isomorphic model of anxiety (20). Whether burying is compulsive will need further investigation.

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REFERENCES

1. Broekkamp, C. L.; Rijk, H. W.; Joly-Gelouin, D.; Lloyd, K. L. Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. Eur. J. Pharmacol. 126:223–229; 1986.
2. Broekkamp, C. L.; Jenck, F. The relationship between various animal models of anxiety, fear-related psychiatric symptoms and response to serotonergic drugs. In: Bevan, P.; Cools, R.; Archer. T., eds. Behavioural pharmacology of 5-HT. Hillsdale: Erlbaum; 1989: 321–335.
3. Carr, G. D.; Fibiger, H. C.; Phillips, A. G. Conditioned place preference as a measure of drug reward. In: Liebman, J. M.; Cooper, S. J., eds. The neuropharmacological basis of reward. Oxford: Oxford University Press; 1989:264–319.
4. Costall, B.; Jones, B. J.; Kelly, M. E.; Naylor, R. J.; Tomkins, D. M. Exploration of mice in a black and white test box: Validation as a model of anxiety. Pharmacol. Biochem. Behav. 32:777–785; 1988.
5. Cox, B.; Ary, M.; Lomax, P. Morphine hyperthermia in the rat: an action on the central thermostats. Eur. J. Pharmacol. 36:33–39; 1976.
6. Fyer, A. J.; Mannuzza, S.; Endicott, J. Differential diagnosis and assessment of anxiety: Recent developments. In: Meltzer, H. Y., ed. Psychopharmacology: The third generation of progress. New York: Raven Press; 1987:1177–1191.
7. Gray, J. A. The neuropsychology of anxiety. Oxford: Clarendon Press; 1982.
8. Handley, S. L. Serotonin in animal models of anxiety: the importance of stimulus and response. In: Idzikowski, C.; Cowen, P., eds. Serotonin, sleep and mental disorder. Petersfield: Wrightson Biomedical; 1990:in press.
9. Harder, D. B.; Maggio, J. C. Defensive burying by mice: intraspecific genetic variation and retention. Anim. Learn. Behav. 11:465–473; 1983.
10. Heaton, J. C. P.; Njung'e, K.; Handley, S. L. Behavioural profile of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) a selective 5-HT2 agonist. Br. J. Pharmacol. 91:388P; 1989.
11. Insel, T. R.; Zohar, J. Psychopharmacologic approaches to obsessive-compulsive disorder. In: Meltzer, H. Y., ed. Psychopharmacology: The third generation of progress. New York: Raven Press; 1987: 1205–1210.
12. Mellett, P. G. The clinical problem. In: Beech, H. R., ed. Obsessional states. London: Methuen; 1974:55–94.
13. Murphy, D. L.; Zohar, J.; Benkelfat, C.; Pato, M. T.; Pigott, T. A.; Insel, T. R. Obsessive-compulsive disorder as a 5-HT subsystem-related behavioural disorder. Br. J. Psychiatry 155(Suppl. 8):15–24; 1989.
14. Pinel, J. P. J.; Treit, D. Burying as a defensive response in rats. J. Comp. Physiol. Psychol. 92:708–712; 1978.
15. Poling, A.; Cleary, J.; Monaghan, M. Burying by rats in response to aversive and nonaversive stimuli. J. Exp. Anal. Behav. 35:31–44; 1981.
16. Siegal, S. Nonparametric statistics for the behavioral sciences. New York: McGraw-Hill; 1956.
17. Terlecki, L. J.; Pinel, J. P. J.; Treit, D. Conditioned and unconditioned defensive burying in the rat. Learn. Motiv. 10:337–350; 1979.
18. Treit, D.; Pinel, J. P. J.; Fibiger, H. C. Conditioned defensive burying: A new paradigm for the study of anxiolytic agents. Pharmacol. Biochem. Behav. 15:619–626; 1981.
19. Treit, D. The inhibitory effect of diazepam on defensive burying: Anxiolytic vs. analgesic effects. Pharmacol. Biochem. Behav. 22: 47–52; 1985.
20. Treit, D. Animal models for the study of anti-anxiety agents: A review. Neurosci. Biobehav. Rev. 9:203–222; 1985.
21. Westenberg, H. G. M.; den Boer, J. A. Serotonin-influencing drugs in the treatment of panic disorder. Psychopathology 22(Suppl. 1): 68–77; 1989.
22. Wilkie, D. M.; MacLennan, A. J.; Pinel, J. P. J. Rat defensive behaviour: burying noxious food. J. Exp. Anal. Behav. 31:299–306; 1979.