Open Access

Physical Properties of Bedding Materials Determine the Marble Burying Behavior of Mice (C57BL/6J)

Chihiro Homma and Kazuyuki Yamada*

Support Unit for Animal Experiments, Research Resources Center, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama, 351-0198, Japan

Abstract: The marble burying test is a simple behavioral psychopharmacology task in which rodents, especially rats and mice, are exposed to glass marbles laying on thick bedding materials. This test has been widely used to assess the effects of a variety of psychoactive drugs. Although the marble burying test clearly reflects drug efficacy, many aspects of the behavior itself remain unclear. In the present study, we assessed the effects of different bedding materials and the number of glass marbles presented on the marble burying behavior of mice (C57BL/6J). Six (bedding materials) × three (number of glass marbles) factorial experiments revealed that the number of marbles buried correlates closely with the quality of the bedding material. Our results provide a basic understanding of certain factors that govern marble burying behavior, which may be applicable to behavioral sciences including psychopharmacology.

Keywords: Marble burying, defensive burying, digging, bedding materials, mice.

INTRODUCTION

Defensive burying is a widely observed behavior in rodents [1-8]. Rats and mice eagerly bury noxious things, such as bad tasting and/or smelling food [1-3], shock prod [4], and unusual objects like glass marbles [2], under their bedding materials. This behavior is thought to be an expression of defensiveness, some forms of anxiety, or compulsiveness [5]. When rats and/or mice are placed in a cage in which glass marbles are laying on sawdust, they immediately and eagerly begin burying the marbles [2]. The marble burying behavior is quite easy to observe and is sensitive to anxiolytic and/or anxiogenic compounds. Therefore, this behavior is often used to assess the properties of psychoactive drugs [6-11].

Certain unique properties of marble burying behavior have been revealed. Animals bury glass marbles even though they are not harmful. Marble burying behavior does not show any habituation after repeated trials [6,12-14], indicating that this behavior is not likely to be affected by learning processes or cognitive function. Furthermore, marble burying behavior does not differ by gender [12]. Although marble burying behavior has been well characterized, the factors governing the burying behavior itself remain unclear. It was recently reported that the marble burying test simply reflects digging and other related behaviors [14-17], calling into question the validity of this behavior for assessing anxiety, defensiveness, compulsiveness, and so on. However, marble burying behavior correlates well with both serotonin (5-HT) transporter (SERT) binding of selective 5-HT reuptake inhibitors (SSRIs) and extracellular 5-HT levels in brains treated with SSRIs [18,19], and thus the behavior may reflect a relation between 5-HT neuronal activity and anxiety, defensiveness, compulsiveness. Therefore, the marble burying test is often used to assess the efficacy of drugs such as SSRIs.

The number of marbles an animal buries is determined by certain factors such as the type of bedding material and the number of marbles used. However, these aspects have not been well studied. For example, many studies have used “saw dust” as the bedding material to study burying behavior because it is lightweight, allowing animals to easily bury the marbles. This feature of “saw dust” might prompt digging, however, leading to a misunderstanding of the emotional state of the animals and/or drug effects. Thus, knowledge of the effects of different bedding materials is required to properly assess burying behavior.

The aim of this study was to assess the effects of bedding material type and number of glass marbles on marble burying test results. Two-factorial within-subject analysis revealed significant effects of bedding materials on the marble burying behavior of C57BL/6J mice.

MATERIALS AND METHODOLOGY

Animals

Seventeen male C57BL/6J mice were used as subjects. Mice were bred in the animal facility of RIKEN BSI (originally purchased from JCL, Inc. Tokyo, Japan, F2 generation) and were about 10 weeks of age at the beginning of the experiment. The breeding room was air-conditioned (22 ± 1°C, 50-60% humidity) with a 12h:12h light-dark cycle (lights on at 0800). Mice were individually housed beginning one week prior to the experiment, and food and water were freely available except during experimentation. All experiments were conducted in the breeding room during the light cycle between 1300 and 1700.

All animal experiments in this study were performed in strict accordance with the guidelines of The Institute of Physical and Chemical Research (RIKEN), Brain Science

*Address correspondence to this author at the Support Unit for Animal Experiments, Research Resources Center, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama, 351-0198, Japan; Tel: +81-48-467-7659; Fax: +81-48-467-6287; E-mail: kaz-yamada@brain.riken.jp

Institute (BSI), and were approved by the Animal Investigation Committee of the Institute.

Apparatus

All experiments were conducted in clear plastic caging with filter tops (Allentown, PA, USA: 29 × 19.5 × 13.5 cm (H)). Glass marbles were clear, green in color and 1.5 cm in diameter. A spontaneous activity monitor (AB System: Neuroscience, Tokyo, Japan) was used to measure activity in the cage during both habituation and test periods.

Bedding Materials

Six types of commercially available bedding materials were used: Soft-chip and Paper-clean (SLC, Shizuoka, Japan), TEK-Fresh (Harlan, IN, USA), Cellu-dri and ALPHA-dri (Shepherd Specialty Papers,, TN, USA), and GreenTru (GREENPRODUCTS, IA, USA) (Fig. **1**). All bedding materials were autoclaved before use.

Spontaneous Activity Monitoring

The ceiling of each compartment of the breeding rack was equipped with a pyroelectric sensor to monitor the movements of mice throughout the experiment. Data were collected and analyzed with a PC equipped with Windows and other commercially available software (AB System). Activity counts represent the number of active time bin (approximately 0.20 - 0.25 sec each) in which spontaneous activity including locomotor activity, rearing and other activities such as stereotypic movements were detected.

Marble Burying Test

A modified procedure based on Yamada *et al.* [20] was employed. Mice were placed individually in plastic cages with the designated bedding material for 30 min (habituation period) and then placed into waiting cages. Ten, twenty or forty glass marbles were then evenly spaced 3 to 7 cm apart on a 4-cm layer of bedding material in the habituation cages. Mice were then reintroduced into the same cage in which they had been habituated. After 30 min, the marble burying period was terminated by removing the mice, and the number of marbles that were more than two-thirds covered with bedding material was counted. Whether a marble was buried was established by two experimenters and was confirmed by manual post-experimental assessment of digital photographs on a PC. After each trial, cages and bedding materials were replaced with fresh ones, and glass marbles were washed with water, dried with a paper towel, and left to return to room temperature.

The marble burying test was repeated twenty times. The first, tenth and the last tests were calibration trials, in which mice were exposed to twenty glass marbles on TEK-Fresh, the bedding material used in our breeding facility. In the second to ninth and eleventh to nineteenth trials each mouse was assigned to one of 17 designated conditions—number of glass marbles (10, 20, or 40) and bedding materials (Soft-chip, TEK-Fresh, Paper-clean, Cellu-dri, ALPHA-dri, or GreenTru). The order of condition for each mouse was counterbalanced using *Latin square design*. Each trial was separated by two to three days. On days between trials, mice were individually housed in a cage with a 2-cm layer of TEK-Fresh.

Statistical Analysis

Statistical analyses were performed by the SPSS™ statistical package (ver 16.0). The repeated measure ANOVA and one-way within-subject ANOVA were used. *Post-hoc* analysis was conducted using Dunnett's *t*-test or Tukey's HSD test. The correlation between the density of the bedding materials and the number of buried marbles was analyzed using Pearson's correlation coefficient. Statistical significance was set at $p < 0.05$.

RESULTS

Effects of Repeated Trials on Spontaneous Activity and Marble Burying Behavior

Because many trials were conducted in this study, we assessed the effects of repeated trials both on spontaneous activity and marble burying behavior (Fig. **2**). Spontaneous activity increased linearly over the trials, and one-way ANOVA revealed a statistically significant main effect of trial number ($F(2,32) = 10.2$, $p < 0.001$, Fig. **2A**). In contrast, the number of buried marbles did not differ significantly with trial number ($F(2,32) = 2.16$, Fig. **2B**), consistent with previous studies [6,12,13].

Fig. (1). Bedding materials used in this study. Numbers under the bedding material names indicate the density (g/L).

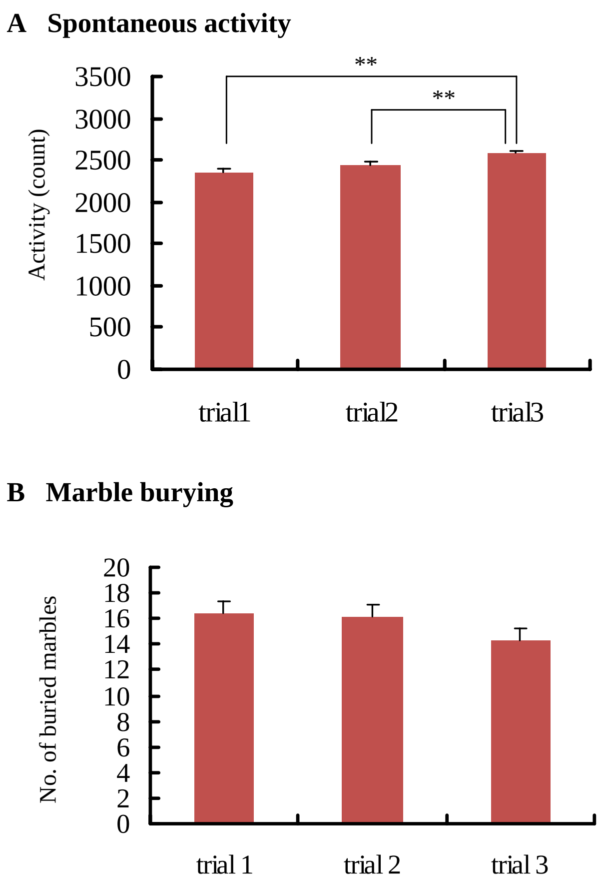

Fig. (2). Spontaneous activity increases with repeated trials, but the number of marbles buried does not. (**A**) spontaneous activity, (**B**) number of marbles buried. Number of marbles used was twenty in each trial. Data represent mean ± SEM. **: $p < 0.01$.

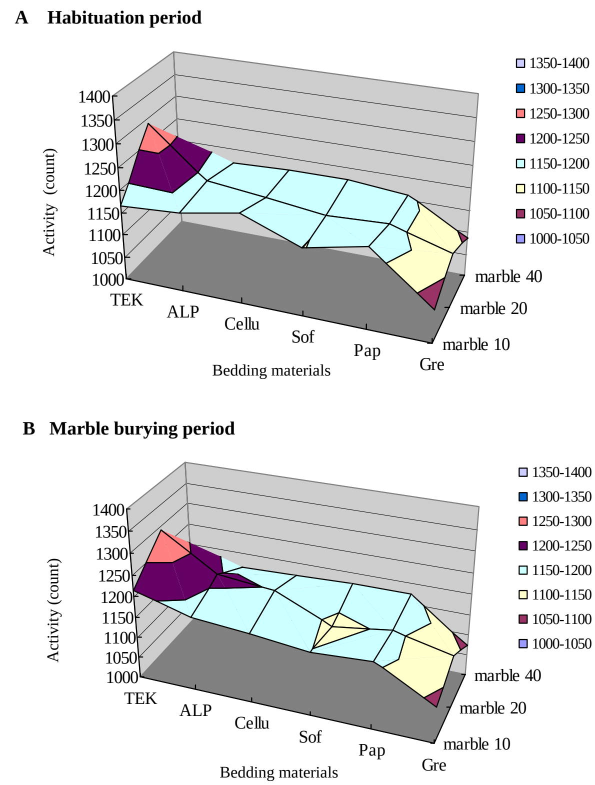

Fig. (3). Mean spontaneous activity during habituation and the marble burying period. (**A**) habituation period; (**B**) marble burying period. The terms 'marble 10', 'marble 20', and 'marble 40' indicate marble burying test trials with 10, 20, and 40 marbles, respectively. TEK: TEK-Fresh, ALP: ALPHA-dri, Cellu: Cellu-dri, Sof: Soft-chip, Pap: Paper-clean, Gre: GreenTru. Statistics are presented in the text.

Spontaneous Activity is Affected by Bedding Material

It is possible that the spontaneous activity level of mice could affect marble burying behavior and might be related to properties of the bedding material. Therefore, we assessed the spontaneous activity of mice during both habituation and the marble burying period (Fig. **3**). Activity was clearly affected by the type of bedding material. During the habituation period (Fig. **3A**), one-way ANOVA revealed a significant main effect of bedding material in 10-, 20-, and 40-marble trials ($F(5,80) = 6.03$, $p < 0.0001$; $F(5,80) = 7.01$, $p < 0.0001$; $F(5,80) = 4.37$, $p < 0.01$, respectively). During the marble burying period (Fig. **3B**), one-way ANOVA revealed a significant main effect of bedding material in the 20- and 40-marble trials ($F(5,80) = 2.52$, $p < 0.05$; $F(5,80) = 3.11$, $p < 0.05$, respectively). Throughout the experiment, mice exhibited the highest activity in the TEK-Fresh trials and showed the lowest activity in GreenTru trials. These results strongly suggest that spontaneous activity of mice is affected by bedding material properties.

Low-Density Bedding Increase Marble Burying Behavior in Mice

Results of the marble burying test are summarized in Fig. (**4**). Mice buried many glass marbles when the density of bedding material was low, for example in Soft-chip (20 g/L) and TEK-Fresh (80.3 g/L) bedding. In contrast, they buried only a few marbles when the density of the bedding material was high, for example in ALPHA-dri (194.7 g/L) and GreenTru (277.4 g/L) bedding. In Paper-clean (124.2 g/L) and Cellu-dri (173.2 g/L) bedding, which are moderately dense, the marble burying behavior was moderate. Furthermore, the effect of the number of glass marbles on the burying behavior was salient. In Soft-chip, TEK-Fresh, Cellu-dri and Paper-clean (partially) trials, the number of buried marbles increased linearly with the number of marbles in the cage. The number of buried marbles did not show a linear increment in the ALPHA-dri and GreenTru trials, however. Fig. (**5**) shows the negative correlation between the number of buried marbles and the density of bedding materials (10 marbles (Fig. **5A**): $r = -0.973$, $p < 0.01$; 20 marbles (Fig. **5B**): $r = -0.962$, $p < 0.01$; 40 marbles (Fig. **5C**): $r = -0.941$, $p < 0.01$, respectively). These results indicate that bedding material density strongly affects the number of marbles buried by mice. In addition, bedding material density may also affect how the mice bury the marbles. Representative photos of burying behaviors in each of the bedding materials are shown in Fig. (**6**). In Soft-chip and TEK-Fresh, mice completely buried the marbles under the low-density bedding material. In Cellu-dri, ALPHA-dri and GreenTru, however, the mice only partially buried the marbles. The texture, as well as density, of the bedding material may also affect burying behavior. Mice buried more marbles in bedding materials with a soft texture (Soft-chip, TEK-Fresh, Paper-clean, Cellu-dri) than in bedding with a hard texture (ALPHA-dri, GreenTru). We replicated experiments several times with a somewhat smaller design and obtained almost identical results (data not shown). These results suggest that bedding material properties may affect how mice bury marbles.

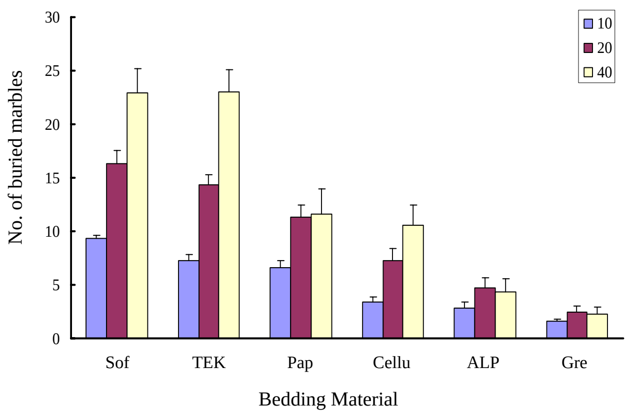

Fig. (4). Marble burying changes with the type of bedding and number of marbles presented. Sof: Soft-chip, TEK: TEK-Fresh, Pap: Paper-clean, Cellu: Cellu-dri, ALP: ALPHA-dri, Gre: GreenTru. 10, 20, and 40 represent trials with 10, 20, and 40 marbles, respectively. Data represent mean ± SEM. Statistics are presented in the text.

Correlation Between Spontaneous Activity and Number of Marbles Buried

As described above, spontaneous activity may affect marble burying behavior. Therefore, we examined the correlation between the spontaneous activity score and the number of marbles buried (Fig. **7**). In both 10- and 20-marble trials, correlation coefficients were not statistically significant, contrary, in the 40-marble trials, the correlation coefficient was statistically significant ($r = 0.20$, $p < 0.05$). Although there is a significant correlation between spontaneous activity and number of marbles, the small effect size for this measure suggests that spontaneous activity does not play a crucial role for marble burying behavior.

DISCUSSION

In this study, we demonstrated that the physical properties of bedding material and the number of glass marbles in the cage significantly affect the marble burying behavior of mice. The number of marbles buried depended on bedding material density and texture. Mice buried more marbles in low-density, soft bedding than in high-density, hard bedding (Figs. **1**, **4**). Furthermore, the number of marbles buried correlated with the number of marbles used for the test when the bedding was not high-density and/or hard in texture (Fig. **4**). Thus, the properties of bedding, rather than the number of marbles used, seems to be the principal determining factor governing marble burying behavior.

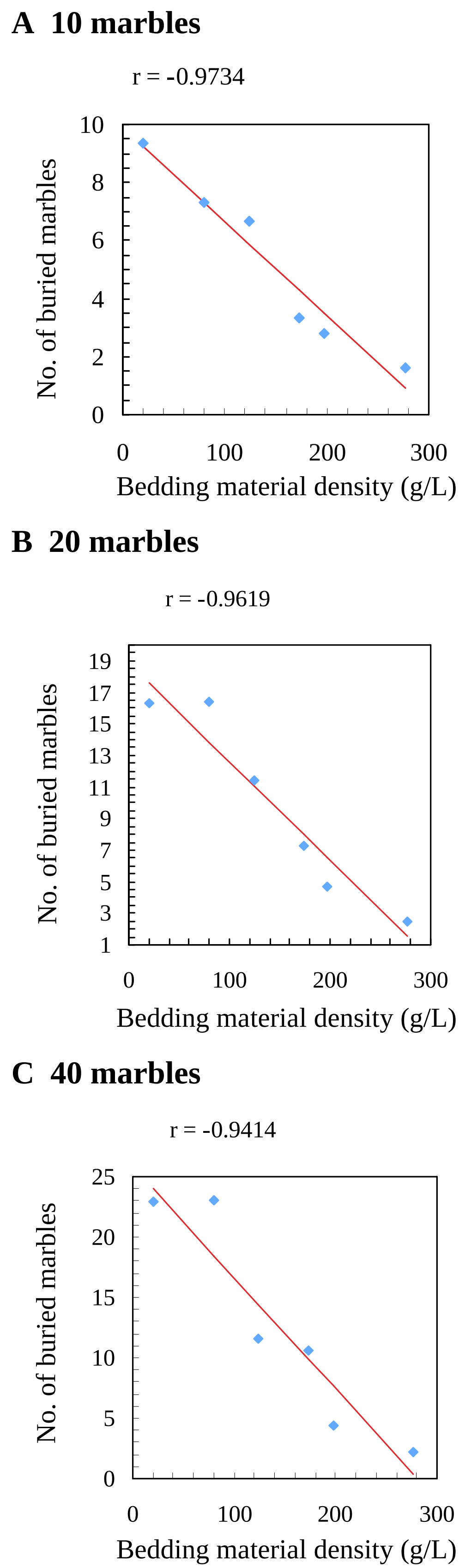

Fig. (5). Correlation between the number of marbles buried and bedding material density. (**A**) trials with 10 marbles; (**B**) trials with 20 marbles; (**C**) trials with 40 marbles. Red lines represent the linear regression.

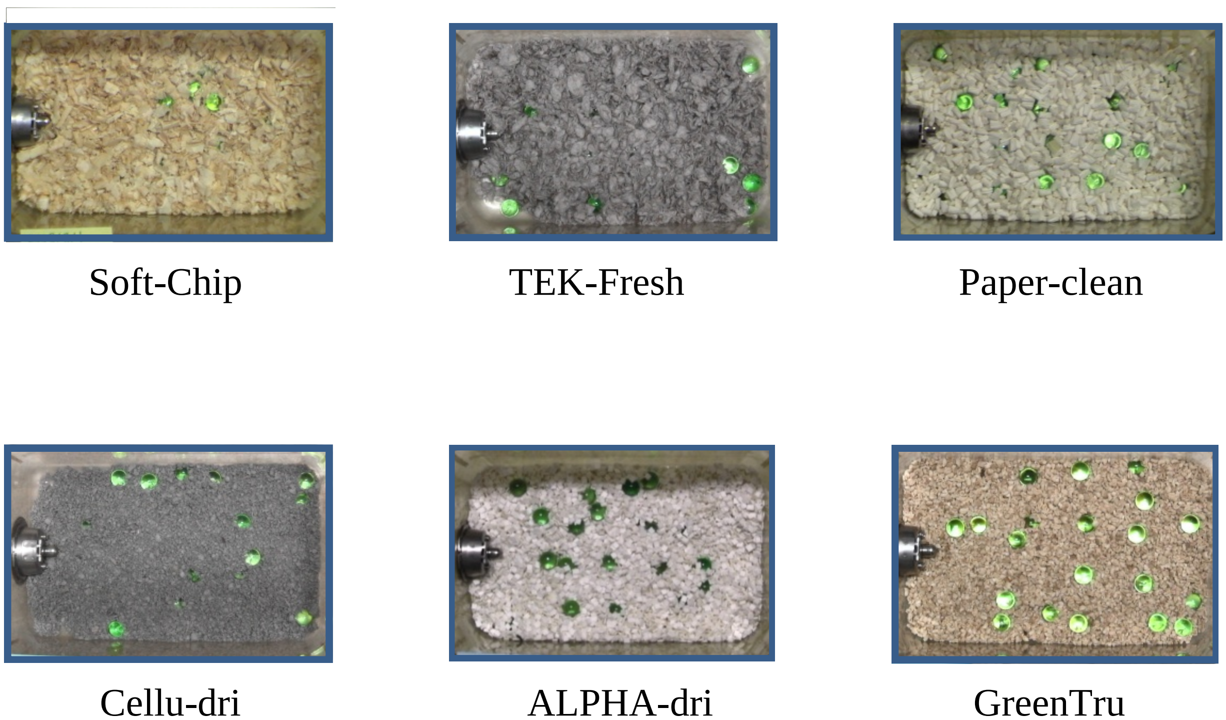

Fig. (6). Representative marble burying behavior in each bedding material.

Bedding material also affected spontaneous activity of mice both quantitatively and qualitatively. Animals were most active in the TEK-Fresh trial and were least active in the GreenTru trial (Fig. **3**). The highest activity in the TEK-Fresh trial might be attributed to the 'familiar environment' not to the physical property of the bedding, because TEK-Fresh is used in our breeding facility. However, this possibility might be excluded in this study because mice show high activity when they are exposed to fresh bedding, showing it is not 'familiar'. Activity also differed qualitatively with bedding type. Mice eagerly dug the layer of bedding in TEK-Fresh and Soft-chip trials, but rarely dug in GreenTru trials (data not shown). Certain studies have suggested that marble burying behavior correlates strongly with digging behavior [14-16], and some marbles might be covered with bedding as an indirect consequence of digging. Thus, marble burying behavior, at least in trials using low-density bedding materials, consists of two qualitatively different behaviors, burying and digging. These mixed behaviors may confound the results of studies assessing drugs effects.

Because animals did not dig much in ALPHA-dri and GreenTru trials, the small number of marbles buried in these trials (Fig. **4**) might reflect "real burying behavior", raising a fundamental question about the marble burying test. In TEK-Fresh and Soft-chip trials, a majority of marbles might have been buried indirectly by the digging behavior. Previous work has suggested that the marble burying test simply measures digging behavior [14-15]. Is marble burying behavior equal to the digging behavior? Or is the contribution of burying to marble burying behavior so small? In this way, marble burying test has some theoretical confusion. Thus, to elucidate this confusion we added some experiments in which the digging behavior in the habituation trial was measured using TEK-Fresh, Soft-chip and GreenTru. The correlation coefficient between the digging duration and the number of buried marbles was statistically significant in Soft-chip experiment ($n=5$, $r=0.897$, $p<0.05$), contrary, not significant in TEK-Fresh and GreenTru experiments ($n=6$, $r= -0.01$, n.s.; $n=7$, $r=0.208$, n.s., respectively). These results suggest that a selection of bedding materials (e.g. TEK-Fresh and/or GreenTru) may be able to dissect out marble burying behavior.

Regardless, the marble burying test is still quite useful for assessing psychotropic drugs [6-11]. For example, in this study, we repeated the marble burying test 20 times for each set of mice, and the number of buried marbles did not change (Fig. **2B**). This lack of habituation provides reliability and validity to experiments having a within-subject design. The marble burying test may have other advantages in psychopharmacological studies, but further study is required to elucidate these advantages.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT): Grants-in-Aid for Scientific Research (C) (19530670, K.Y.).

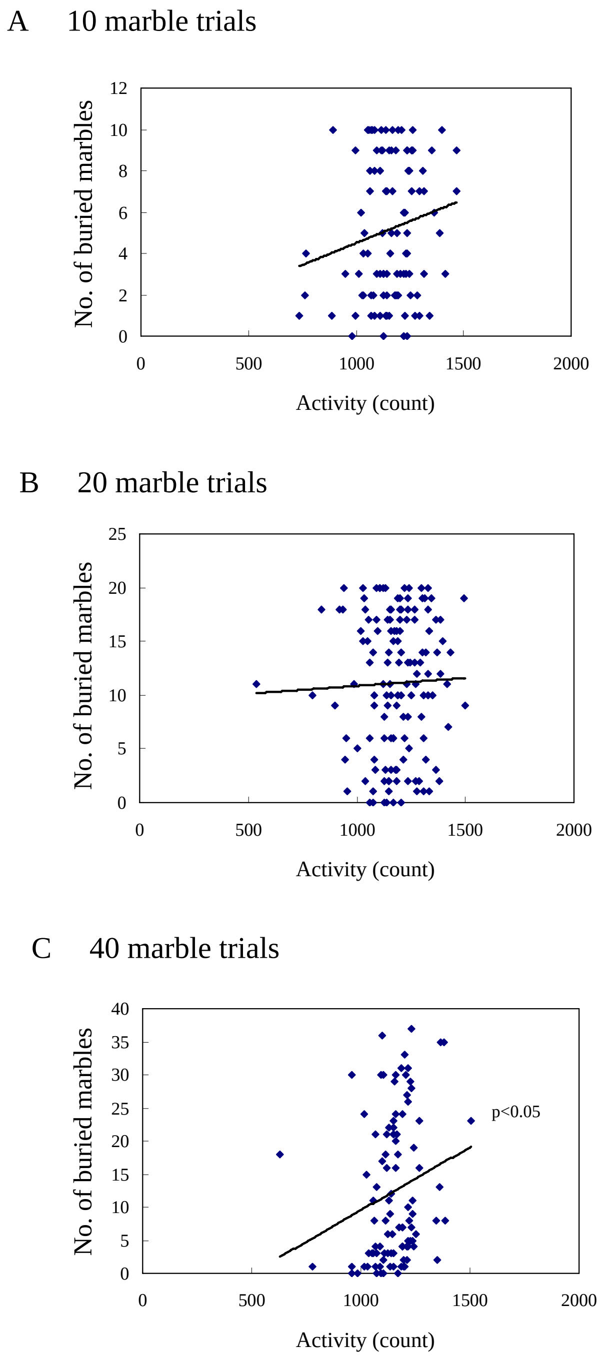

Fig. (7). Spontaneous activity and the number of marbles buried are significantly correlated only for 40-marble trials (r = 0.20, $p < 0.05$).

REFERENCES

[1] Wilkie DM, MacLennan AJ, Pinel JP. Rat defensive behavior: burying noxious food. J Exp Anal Behav 1979; 31: 299-306.

[2] Poling A, Cleary J, Monaghan M. Burying by rats in response to aversive and nonaversive stimuli. J Exp Anal Behav 1981; 35: 31-44.

[3] Parker LA. Defensive burying of flavors paired with lithium but not amphetamine. Psychopharmacology (Berl) 1988; 96: 250-2.

[4] Pinel JPJ, Treit D. Burying as a defensive response in rats. J Comp Physiol Psychiatry 1978; 92: 708-12.

[5] De Boer SF, Koolhaas JM. Defensive burying in rodents: ethology, neurobiology and psychopharmacology. Eur J Pharmacol 2003; 463:145-61.

[6] Broekkamp CL, Rijk HW, Joly-Gelouin D, Lloyd KL. 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 1986; 126: 223-9.

[7] Njung'e K, Handley SL. Effects of 5-HT uptake inhibitors, agonists and antagonists on the burying of harmless objects by mice; a putative test for anxiolytic agents. Br J Pharmacol 1991; 104:105-12.

[8] Nicolas LB, Kolb Y, Prinssen EP. A combined marble burying-locomotor activity test in mice: a practical screening test with sensitivity to different classes of anxiolytics and antidepressants. Eur J Pharmacol 2006; 547: 106-15.

[9] Li X, Morrow D, Witkin JM. Decreases in nestlet shredding of mice by serotonin uptake inhibitors: comparison with marble burying. Life Sci 2006; 78: 1933-9.

[10] Dekeyne A. Behavioral models for the characterization of established and innovative antidepressant agents. Therapie 2005; 60: 477-84.

[11] Bruins Slot LA, Bardin L, Auclair AL, Depoortere R, Newman-Tancredi A. Effects of antipsychotics and reference monoaminergic ligands on marble burying behavior in mice. Behav Pharmacol 2008; 2: 145-52.

[12] Njung'e K, Handley SL. Evaluation of marble-burying behavior as a model of anxiety. Pharmacol Biochem Behav 1991; 38: 63-7.

[13] Archer T, Fredriksson A, Lewander T, Söderberg U. Marble burying and spontaneous motor activity in mice: interactions over days and the effect of diazepam. Scand J Psychol 1987; 28(3): 242-9

[14] Thomas A, Burant A, Bui N, Graham D, Yuva-Paylor LA, Paylor R. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. Psychopharmacology (Berl). 2009. [Epub ahead of print]

[15] Gyertyán I. Analysis of the marble burying response: marbles serve to measure digging rather than evoke burying. Behav Pharmacol 1995; 6: 24-31.

[16] Deacon RM. Digging and marble burying in mice: simple methods for *in vivo* identification of biological impacts. Nat Protoc 2006; 1: 122-4.

[17] Londei T, Valentini AM, Leone VG. Investigative burying by laboratory mice may involve non-functional, compulsive, behaviour. Behav Brain Res 1998; 2: 249-54.

[18] Hirano K, Kimura R, Sugimoto Y, *et al*. Relationship between brain serotonin transporter binding, plasma concentration and behavioural effect of selective serotonin reuptake inhibitors. Br J Pharmacol 2005; 5: 695-702.

[19] Kobayashi T, Hayashi E, Shimamura M, Kinoshita M, Murphy NP. Neurochemical responses to antidepressants in the prefrontal cortex of mice and their efficacy in preclinical models of anxiety-like and depression-like behavior: a comparative and correlational study. Psychopharmacology 2008; 4: 567-80.

[20] Yamada K, Wada E, Yamano M, *et al*. Decreased marble burying behavior in female mice lacking neuromedin-B receptor (NMB-R) implies the involvement of NMB/NMB-R in 5-HT neuron function. Brain Res 2002; 942: 71-8.

Received: April 16, 2008 Revised: September 4, 2009 Accepted: September 4, 2009