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Marble burying as compulsive behaviors in male and female mice

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Marble burying is considered an, albeit controversial, animal model of the compulsive like behaviors of obsessive-compulsive disorder (OCD). Hallmark features of OCD patients are similarities and, more prominent, differences from anxiety disorders, e.g., the absence of sex differences and resistance to spontaneous remission. We report an experiment on marble burying by male and female C57/BL6/N mice. Animals were administered either the classic anxiolytic drug, diazepam, that targets the GABA receptor or a "pure" inhibitor of the serotonin transporter, escitalopram, that has been reported to be particularly effective in OCD. A burying paradigm that more precisely mimics the human condition was used, e.g., testing in the home environment, chronic drug exposure and acknowledging individual differences by pre-selecting for high marble burying. Results were that there were no sex differences in groups treated with drugs or in control mice. Both diazepam and escitalopram decreased numbers of marbles buried compared to vehicle-only controls in the absence of correlated changes in anxiety. Diazepam, however, was more effective than escitalopram in suppressing MB. The conclusion is that along with serotonin, GABA is involved in regulating compulsive behaviors. The marble burying paradigm may prove more useful for pharmacological drugs tests of impulsivity or attention deficit because of the involvement of serotonin and GABA in both disorders.

Key words: animal model, obsessive-compulsive disorder, anxiety, OCD, sex differences, GABA, serotonin

INTRODUCTION

Obsessive-compulsive disorder, (OCD) is characterized by unwanted, intrusive thoughts and images (obsessions) and repetitive, ritualistic behaviors (behavioral compulsions). The latter presumably serves to reduce anxiety caused by the obsessions. Yet, OCD now is separated from anxiety disorders in DSM-V, largely based on the obsessional component (American Psychiatric Association 2013).

Our research interests are in developing and assessing reliable and valid animal models for psychiatric conditions. OCD has proven to be a particularly difficult condition to model. The task of animal modelers is to develop behavioral measures that isolate compulsions from anxiety measures.

Spontaneous burying of marbles in rodents has been suggested as a compulsive-like behavior (Broekkamp et al. 1986, Deacon 2006, Gyertyan 1995). Marble burying (MB), however, has been criticized on both conceptual and empirical grounds for its ability to serve as a unique benchmark of OCD (Albelda and Joel 2012, Wolmarans de et al. 2016). Indeed, MB has also appeared in the literature as a measure for autism, motivation or general anxiety disorder (GAD) (Ene et al. 2016, Jury et al. 2015, Silverman et al. 2015). Despite the criticisms, MB continues to appear regularly in the literature as a measure of compulsion (Gawali et al. 2016, Kudryashov et al. 2016, Nichols et al. 2016, Satta et al. 2016).

We designed an experiment with mice to assess MB in relation to unique features in OCD patients. 1) In contrast to the notably higher frequencies of most anxiety disorders in women, incidences of OCD have no reliable sex differences (Martin 2003). Our experiment used both males and female mice. 2) There are clearly individual differences in compulsive behaviors among people. Our experiment used pre-tests to select the mice who were most likely to bury marbles. 3) Unlike the anxiety disorders, untreated OCD frequently fails to remit with the passage of time (Taylor et al. 2011). Our experiment tested the selected mice repeatedly to determine spontaneous reductions as marbles became familiar. 4) The consensus is that the neural circuits for OCD and anxiety differ (Burguiere et al. 2015, Hoffman 2011). Neurotransmitters underlying OCD are the monoamines, mostly

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serotonin and, likely, glutamate (Bokor and Anderson 2014, Egashira et al. 2008). GABA remains the primary transmitter thought to underlie most anxieties. Our study compared a classic benzodiazepine, diazepam, and a SSRI, escitalopram, that has proven particularly effective for OCD (Shim et al. 2011, Zohar 2008). Finally, all animals were examined in the open field apparatus as a general measure of anxiety.

Hypotheses for the study included no sex differences in MB, consistency over time for individual MB habits in untreated animals and greatest effectiveness of escitalopram in reducing MB.

METHODS

Animals

A total of 35 male and 35 female C57/BL6/N mice, 40 days of age obtained from Charles River (Sulzfeld,Germany), were acclimatized for 2 weeks before pretests were conducted. After pretesting for spontaneous MB, 42 mice equally divided between sexes were selected as subjects for the experiment. All mice were individually housed in flat bottom plastic Macrolon type II cages measuring 360 cm^2 (Tecniplast, Italy) under SPF conditions. Standard lab diet (Rod16A, LASvendi, Soest) and water were available *ad libitum*. The colony room lighting was a 12:12 h reversed light/dark cycle with lights off at 9am; room temperature (20–22 °C) and relative humidity (50%) are controlled automatically. The Institutional Animal Care and Use Committee and the local authorities (Regierungspräsidium Karlsruhe) approved the experimental protocol (permit number: G-37/15).

Materials

All behavioral sessions were conducted during the nocturnal light cycle and under dim illumination. The open field apparatus (50×50×50 cm) was constructed of black Plexiglas. Movement of the animal in the open field was measured automatically by the Ethovision 4.0 tracking system (Noldus Information Technology, Wageningen, The Netherlands) via an overhead, infrared camera (Ikegami Digital). The tracking system can record locomotor movement in the various quadrants of the open field. The innermost quadrant (25×25cm) was designated as the center arena. After each test, the apparatus was cleaned with 70% ethanol. Marbles used were multi-colored and approximately 16 mm diameter. The home cage of the animal was used for measurement of MB. Bedding consisted of aspen wooden chips (ABEDD LTE-001, Lab & Vet Service, Vienna, Austria) that was approximately 5 cm deep. Bedding was changed weekly but never on the days before a behavioral test.

Diazepam ampules were purchased (Boeringer, Mannheim, Germany). Escitalopram was purchased (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) and solubilized in a 0.9% saline solution for injection.

Experimental Design

Following an initial test to identify the tendency of each mouse to bury marbles, males (N=21) and females (N=21) were selected that had buried at least 6 marbles in the pre-test and randomly assigned to groups. There were 3 groups of each sex (n=7 per grp) that were s.c. injected daily for 11 days with either 0.9% saline (Fresenius Kubi, Bad Homburg) vehicle (Veh only), 2 mg/kg bwt diazepam (Diaz) or 2 mg/kg bwt escitalopram (Esc). These drug dosages are in the low to mid ranges of those employed in the rodent literature (Erfanparast and Tamaddonfard 2015, Nicolas et al. 2006, Pandey et al. 2009, Schneider and Popik 2007).

Procedures

Pre-selection tests of animals for the experiment were completed over 2 days. The pre-selection MB trial was treated as a pre-test, i.e., prior to drug treatments. Using the procedure described below for MB, the 35 females and 35 females were tested for spontaneous MB in their home cages. The 21 mice of each sex burying the most marbles were retained for the experiment, and the other mice were removed to another animal housing room. Drug administrations began at the beginning of behavioral tests of the animals. Injections were done 1 hr before tests.

Behavioral sessions were conducted over 11 days in the open field and in the home cages. Order of tests were counterbalanced between and within groups. In addition to the pretests, experimental subjects were given 2 tests in both apparatus for a total of 4 tests separated by at least 2 days. Test 1 behaviors were conducted during the initial 4 days and Test 2 was conducted during the last 4 days of the 11 days of testing. Animals were injected on all days, including "off" days.

For a session in the open field, a mouse was removed from the colony room to an adjacent experimental room. The animal was placed in the center of the open field apparatus and movement was recorded over the 30 min session. Also recorded was time spent in the center quadrant of the apparatus. The open field is a marker of activity changes under drug influences and time in the central area relative to the

other areas adjacent to the walls serves as a measure of anxiety (Archer et al. 1987, Benatti et al. 2014, Ene et al. 2016).

The procedure used for MB followed the paradigm used commonly in the literature (Deacon 2006, Gawali et al. 2016, Witkin 2008), except that we conducted the tests in the home cages rather than in novel cages. Logic was to mimic human OCD in which compulsive behaviors occur with unsettling disturbances of a familiar environment. For a test, the home cage was moved from the cage rack to a nearby table and the mouse removed from its home cage to a holding cage for approximately 1 min. During that time, 12 marbles were distributed equally around the perimeter of the home cage at least 2 cm from the walls. Marbles were placed on top of the approximately 5 cm-deep wood chip bedding of the home cage. The mouse was returned to its home cage that was placed back into its normal place in the cage rack.

After 30 min, the cage was again moved to the table, and the animal placed in the holding cage while the numbers of marbles buried were counted. Although some marbles were buried out of sight, most often the marbles were buried only partially. We counted the marble buried if it was covered half or more by the bedding.

Statistical Analyses

Assessment of behavioral differences among groups was accomplished with 3-way analyses of variance (ANOVAs). The first 3 x 2 x 3 ANOVA on numbers of marbles buried had main factors of Drug (diazepam, escitalopram or vehicle only) x Sex with Trial (Pre-test, Trial 1 and Trial 2) as a repeated measure. Open field activity used a similar 3×2×2 arrangement, except with 2 trials as repeated measure. Numbers of marbles buried were the primary measure of compulsive behavior. Time in the center arena of the open field served as the measure of anxiety. Distance traveled, in cm, was a measure of general activity. Post-hoc Tukey-Kramer tests were used for pair-wise comparisons of mean group differences. The $p<0.05$ confidence value was used for all analyses.

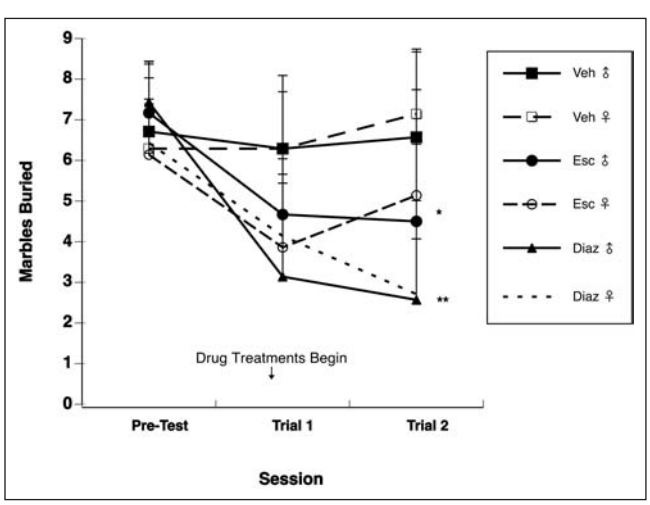

Fig. 1. Numbers of marbles buried by male and female mice during a pre-drug test and over two trials during 11 daily administrations of either vehicle only (Veh), escitalopram (Esc) or diazepam (Diaz). An asterisk (*) indicates significant differences ($p<0.05$) of drug groups from veh controls. The double asterisk (**) indicates Diaz groups differed significantly ($p<0.05$) from the Esc groups.

RESULTS

Numbers of marbles buried in the home cage of the different groups of mice over the three trials (Pre-test and 2 trials with drug exposure) are depicted in Fig. 1. Results of the ANOVA indicated a statistically significant difference for the main effects for drug $F(2,35)=11.45$, $P<0.001$, partial eta squared (η^2)=0.396, and for the repeated factor of trials, $F(2,35)=25.41$, $P<0.001$, $\eta^2=0.417$. The only 2-way interaction that was statistically reliable was trial x drug $F(4,70)=10.59$, $P<0.001$, $\eta^2=0.377$, although trial x sex approached significance, $F(2,70)=2.82$, $P=0.067$, $\eta^2=0.074$. The 3-way interaction failed to achieve significance.

Posthoc group comparisons revealed statistically significant differences ($p<0.05$) between and within groups. The groups did not differ on the pre-test data. However, between group differences showed the mice administered diazepam burying the fewest marbles on

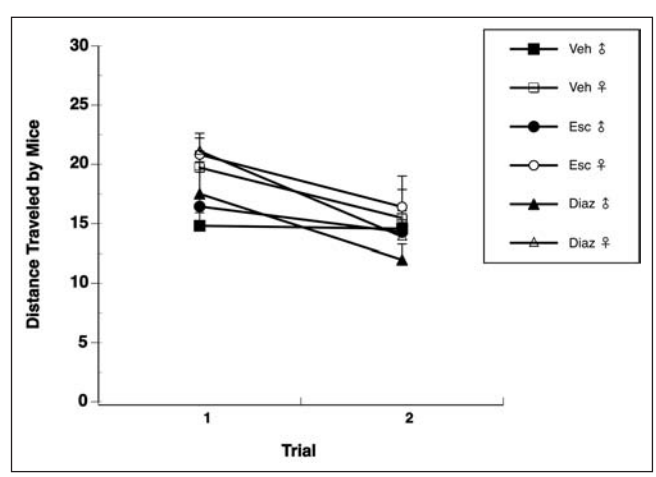

Fig. 2. Distance traveled by male and female mice in the open field during a pre-drug test and over trials during exposure either vehicle only (Veh), escitalopram (Esc) or diazepam (Diaz). There were no differences between groups although the combination of female groups differed significantly ($p<0.05$) the combinations of male groups.

Trials 1 and 2 and the control animals the most, with the escitalopram mice differing from both groups on both trials. Within group differences indicate that the control animals did not change burying over the pre-trial and two drug trials, but the drug groups did change over that period. Diazepam animals reduced burying from Pre-trial to Trial 1 but no further reductions for Trial 2. The escitalopram group had yet a third pattern, decreasing burying from the Pre-trial to Trial 1 and then increasing again for Trial 2. There were no reliable differences between male and female mice in any of the MB comparisons.

Results for the distance traveled measure of general locomotor activity (Fig. 2) revealed no between group differences and only the single main factor of sex was statistically reliable, $F(1,35)=7.02$, $P=0.012$, $\eta^2=0.167$. The females were more active than the male mice, independent of drug treatments. None of the interactions with the sex factor was statistically significant.

Examination of time spent in the center arena of the open field was conducted as a measure of anxiety. Results are in Fig. 3. The 2×3×2 factorial analysis indicated the sex × drug interaction was statistically reliable, $F(1,35)=3.61$, $P=0.037$, partial eta squared (η^2)=0.171. Further analyses revealed that the females in the diazepam group spent the most time in the center arena, and, surprisingly, females of the escitalopram group had the least time in the center area. All other groups were statistically similar. The 3-way interaction nor any of the other 2-way interactions achieved statistical significance.

Within group results were that only the Trials main effect was significant, $F(1,35)=10.30$, $P=0.003$. Overall, both sexes spent more time in the center arena during their second trial than the first trial.

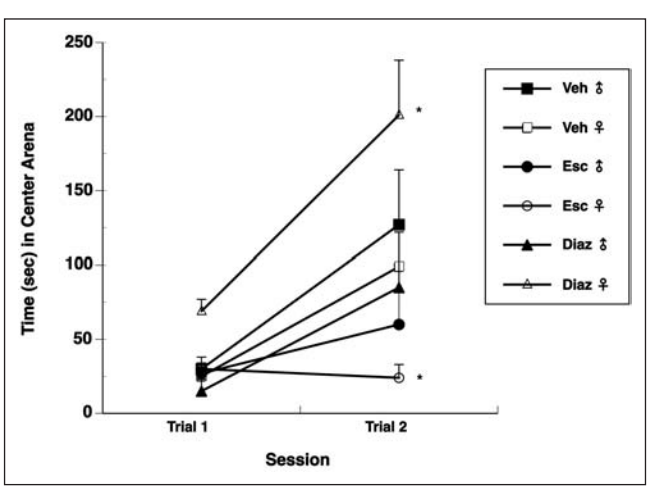

Fig. 3. Time in a central arena of the open field by male and female mice over two trials during 11 daily administrations of either vehicle only (Veh), escitalopram (Esc) or diazepam (Diaz). An asterisk (*) indicates significant differences ($p<0.05$) from the other groups.

DISCUSSION

Results of the experiment included no sex differences in control and drug groups of mice selected for high MB. Males and females administered only vehicle showed consistent levels of burying behaviors while both sexes administered diazepam and escitalopram reduced their MB. Diazepam was more effective in eliminating the behavior than escitalopram. Indeed, the influence of escitalopram on burying appeared to weaken over time. The implication is that, along with the serotonergic system, GABA neurotransmission is critically involved in MB.

The literature places emphasis on serotonin in both patients and animal models of compulsive behaviors. Findings of certain SSRIs being the first line treatment for OCD and that those same SSRIs reduce MB have reinforced the serotonin hypothesis (Egashira et al. 2008). Escitalopram is a notable example (Stein et al. 2008, Wolmarans de et al. 2016). However, longer durations and higher doses of the SSRIs often are needed to treat OCD patients compared to other psychiatric disorders (Bokor and Anderson 2014). More telling, SSRIs fail to reduce symptomology in 40-60% of OCD patients (Pallanti and Quercioli 2006), although benzodiazepines were even less effective (Goddard et al. 2008). The clear implication is that OCD is a complex disorder that involves multiple systems rather the current emphasis on the serotonin receptor (Egashira et al. 2008, Marazziti et al. 2010, Takeuchi et al. 2002).

The value of MB as an animal model for OCD remains an open question (Albelda and Joel 2012). Nonetheless, there are empirical reasons indicating that rodents do not bury objects simply because they are anxious. MB has features not observed in other anxiety paradigms. For example, burying will occur in the safety of the home cage, in the absence of obvious fear or stressful stimuli and burying fails to habituate over test sessions (Chotiwat and Harris 2006, Greene-Schloesser et al. 2011, Thomas et al. 2009).

Our findings provide additional evidence. The most common behaviors in OCD patients are compulsive checking, involving the performance of routines related to security, orderliness, and accuracy but without resolution (Taylor et al. 2011). Our findings confirm that the absence of a reduction in compulsive burying of the control animals. Over time the control animals revealed a similar resistance to extinction of burying despite becoming familiar with the marbles. Finally, there was no obvious relation in our study between MB and time in the center arena of our open field, a measure of anxiety (Benatti et al. 2014). That measure indicated females administered diazepam were least anxious, but escitalopram females were the most anxious. All other group comparisons were not significantly different. The data for general locomo-

tor activity indicated that, over all groups, females were more active. Untreated female rodents often are found to more active than untreated males (Blizard et al. 1975, Palanza et al. 2001, Taylor et al. 2011).

A combination of methodological features makes our experiment a unique contribution to this literature. Animals were tested in their familiar home cages rather than in a novel, neutral apparatus (Witkin 2008). We acknowledged the fact that there are individual differences in compulsive behaviors by pre-selecting mice that buried marbles (Fineberg et al. 2015, Wirth-Dzięciołowska et al. 2005). Whereas we examined burying by both sexes, almost all previous reports have used males, even when testing the influence of ovarian steroids (Gomez et al. 2002, Umathe et al. 2009). We compared diazepam, a classic benzodiazepine and a GABA agonist (Nicolas et al. 2006), with escitalopram, an SSRI that has been described as a pure inhibitor of the serotonin transporter (Stahl 2013). And animals were chronically exposed to the drugs as opposed to acute treatments in most reports in the literature (Jimenez-Gomez et al. 2011).

Yet, diazepam proved more effective (Joel et al. 2004) than escitalopram in decreasing MB. The present experiment, essentially, failed to resolve MB as an animal model capable of dissociating compulsive and anxious behaviors (Albelda and Joel 2012).

This may not be a failing so much as empirical support for OCD and some forms of anxiety being inseparable (Schneier et al. 2008), despite the newest DSM moving OCD from anxiety categories (American Psychiatric Association 2013). Notably, both disorders share neural pathology of the cortico-striatal-thalamic-cortical circuitry (Milad and Rauch 2007, Stahl 2013). Generalized anxiety disorder and OCD both show frontal/striatal hyperactivity. Social anxiety and OCD both show similar anterior cingulate dysfunction (Kim and Gorman 2005).

We remain convinced the MB paradigm has important potential as an animal model for psychiatric disorders. The puzzle is that it is not clear what is actually being measured. Perhaps it is too narrow of a perspective to focus on anxiety or compulsion as the only possibilities. It is entirely possible that there are other dimensions being measured, for example, impulsivity that is neither specifically anxiety or specifically compulsive. Indeed, it has been proposed that individual differences in patients suggest a continuum of compulsivity to impulsivity (Allen et al. 2003, Geller 2006). DSM-V indicates that impulsivity may be performed in patients for pleasure or gratification rather than relief of tension or anxiety. MB could prove useful for innovative pharmacological treatments for impulsive-control, attention deficit and related psychiatric disorders. These are conditions for which both serotonin and GABA, as well as dopamine, glutamate and neurosteroids, have been implicated (Hoffman 2011, Perry et al. 2011, Schule et al. 2011, Yates et al. 2012). We believe a fresh look at the MB paradigm is warranted.

CONCLUSIONS

Our results suggest the conclusion that, independent of sex, marble burying can be suppressed with therapeutic drugs used to treat both anxious and compulsive patients. However, MB cannot clearly distinguish compulsions from anxiety. MB remains an intriguing animal model partly because burying objects appears to be an inherent trait of some, but not all, mice. Moreover, burying has the virtues of ease, reliability and sensitivity to drug treatments,

A broader perspective, thinking "outside the box," may reveal MB as useful for pharmacological drugs tests of impulsivity, attention deficit disorder or other psychiatric disorders that have proven difficult to model in animals.

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CONFLICT OF INTERESTS

The authors report no conflicts of interest.

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