Digging behavior discrimination test to probe burrowing and exploratory digging in male and female mice

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Abstract

Digging and burying behavior is often used to test anxiety and repetitive behaviors in mice. Different digging paradigms have been developed and have become popular assays for anxiety, obsessive-compulsive disorder (OCD), and repetitive behaviors in mouse models for multiple psychiatric and neurological conditions. However, the interpretation of these tests has been confounded by the difficulty of determining why mice dig. Digging is a naturalistic mouse behavior, that can be focused towards different goals, i.e. foraging for food, burrowing for shelter, burying objects, or even for recreation as has been shown for dogs, ferrets, and human children. Current testing protocols may focus on one type of digging (burrowing, foraging or burying) or allow the animal to dig freely, but interpretation of the results infers the motivation behind the behavior and often assumes that increased digging is a repetitive or compulsive behavior. We asked whether providing a choice between different types of digging activities would increase sensitivity to assess digging motivation. Here, we present a test to make clear determinations between burrowing and exploratory digging in mice. The test was designed to be rapid (less than 30 minutes) and using simple measures, so that it can be easily implemented with or without automated tracking. We found that mice seem to prefer burrowing when the option is available and asked whether food restriction would cause a switch from burrowing to exploration. While males and females displayed subtle behavioral differences at baseline that did not lead to statistically significant results, males readily switched from burrowing to digging outside, while females did not. In addition, when we tested a model of intellectual disability and autism spectrum disorder that had shown inconsistent results in the marble burying test, the *Cc2d1a* conditional knock-out mouse, we found greatly reduced burrowing only in males. Our findings indicate that digging is a nuanced behavior and suggest that male and female rodents may perform it differently. We propose that juxtaposing different kinds of digging will increase sensitivity in detecting deficits and will provide a better insight into behavioral differences.

Introduction

Analysis of digging behavior has been used in rodents to study features of neurological and psychiatric conditions such as anxiety, Autism Spectrum Disorder (ASD), and Obsessive-Compulsive Disorder (OCD) (Bey & Jiang, 2014; de Brouwer et al., 2019; Kazdoba et al., 2016; Thompson et al., 2019). The innate digging and burrowing behaviors displayed by house mouse (*Mus musculus*) strains commonly used in the laboratory are valuable indicators of well-being and motor function (Dudek et al., 1983; Latham & Mason, 2004), and neuroscientists have long measured them as signs of pain, stress, and neurological dysfunction (Deacon, 2006b; Deacon et al., 2001; Jirkof, 2014). However, digging measurements have often been difficult to interpret (de Brouwer et al., 2019; Gyertyán, 1995).

Mice dig for a number of reasons; to avoid noxious stimuli or predators, to seek food, to build shelter for safely raising their young, and possibly for recreation (Arakawa et al., 2007; Blanchard et al., 1995; Latham & Mason, 2004; Powell & Banks, 2004; Sluyter et al., 1996). Deep bedding will induce a mouse to dig into the substrate (Deacon, 2006b), but the motivation behind this behavior remains uncertain. Increased digging behavior is often interpreted as a repetitive response due to anxiety and compulsion (Broekkamp et al., 1986; Thomas et al., 2009). However, a compulsive behavior is defined as excessive and divorced from the consummatory process, i.e. not leading to pleasure or reward (American Psychiatric Association, 2013; Luigjes et al., 2019). Defining whether an activity is pleasurable or excessive is difficult to assess in mice since the motivation for digging is often unknown. Thus, free digging is also used as a measure of a more generic exploratory drive instead (de Brouwer et al., 2019).

One of the most commonly used digging tests is the marble burying test which was designed in an attempt to assign a motive to digging behavior. Marbles are placed on the digging surface and the act of embedding an object in the substrate is studied (Broekkamp et al., 1986). The validity of interpreting marble burying as a sign of anxiety or compulsion has been challenged in multiple studies revealing a need to define the motivation behind digging (Bruins Slot et al., 2008; de Brouwer et al., 2019; Gyertyán, 1995; Hayashi et al., 2010). It remains unclear whether mice actively interact with the marbles as novel or aversive objects or whether burying (and unburying) is simply a side effect of vigorous digging in the vicinity (Gyertyán, 1995; Thomas et al., 2009). This method had its earliest roots in assaying defensive burying in rats (Pinel & Treit, 1978), but burying aversive objects may be a rat-specific behavior not entirely translatable to laboratory mice and digging in the marble burying test may just reflect exploratory activity.

Burrowing, the act of digging for shelter, has been studied in multiple species of rodents and defined as a mandatory behavioral need for laboratory mice by Sherwin et al., (2004). A mandatory behavioral need is a natural behavior whose functional consequences are clearly important to mice and they are strongly motivated to burrow (Deacon, 2006b; Jirkof et al., 2010). While evolutionary changes in underground burrow depth and structure are often studied in the wild or in large apparatuses (Adams & Boice, 1981; Blanchard et al., 1995; Dawson et al., 1988; McEwen et al., 2015; Weber & Hoekstra, 2009), the act of burrowing can be tested in laboratory settings by providing a tube filled with bedding that mice can clear. This protocol was developed by Deacon (2006a) exploring both interaction with food pellets or other non-food related substrates and allowing the mice to burrow for multiple hours.

To develop more naturalistic measures to discern the individual motivation for digging behavior we combined burrowing and free digging assays in a single paradigm. Our approach, the digging behavior discrimination (DBD) task, applies the method described by Deacon (2006a), truncated to 30 minutes and modified to include measurement of free digging as well as other movement parameters. This assay was tested in both male and female mice during baseline, calorie-restricted feeding, and recovery to *ad libitum* feeding conditions to determine whether food seeking may alter the type of digging performed. In addition, we examined a mouse model of intellectual disability and ASD that had shown reduced digging in the marble burying test to ask whether specific types of digging would be affected. Interestingly, we identified multiple differences between male and female mice under food restriction. While previous studies have not reported any sex differences in digging behavior, the DBD test shows there may be differences in digging motivation between males and females and allows for clear differentiation between exploratory digging/foraging and burrowing.

2. Materials and Methods

2.1 Animals

All animal care and use were in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of The George Washington University and Rutgers University. C57BL/6N male and female mice were purchased from Charles River and Taconic and acclimated in house for at least four weeks. The *Cc2d1a* conditional knock-out (cKO) mouse line was generated by crossing *Cc2d1a*-flx mice (Oaks et al., 2017). with a CaMKIIa-cre mouse line driving Cre recombinase expression under the *CaMKIIa*

promoter (Stock 005359, Jackson Laboratories) (Tsien et al., 1996). All animals are fully backcrossed on a C57BL/6N background for at least 6 generations. Genotyping was performed via polymerase chain reaction (PCR) amplification and primers are available upon request.

2.2 Burrowing and exploratory digging discrimination

The test was performed in a clear plastic box 40X24X31.75cm (Lee's Kritter Keeper, X-Large). The box was filled with 5cm of corncob bedding (Bed-o' cobs) to provide ample digging substrate. A "burrow" consisting of a plastic tube (10cm length, 5cm diameter) filled with 17g of white paper bedding (Carefresh) was placed in a corner of the testing arena (**Fig.1A**). To familiarize the mice with the burrow and eliminate the confound of a novel object, a burrowing tube filled with the paper bedding was placed in the home cage of the group-housed test mice the night before testing. Testing was only performed if the tube was empty by the following day. If not, one more night of habituation was granted to assure the mice were able to demonstrate burrowing behavior. On testing day, each mouse was placed in the test apparatus and movement was tracked for 30mins using AnyMaze software (Stoelting) between two testing zones: the burrow area and the rest of the box which was named the "exploration area". Multiple automated testing measures were collected, including time in burrow area, time in exploration area, number of entries in the burrow area, average time per visit, average speed in apparatus and distance traveled in apparatus. Latency to start removing material from the burrow, time spent burrowing or digging, and time to empty the burrow were timed manually from the videos by two independent raters blinded to genotype. Digging in the free area was defined as vigorous digging with spread hind limbs and coordinated use of the forefeet to move substrate

backwards beneath the body or by the sides as previously described (Layne & Ehrhart, 1970; Webster et al., 1981). The weight of the bedding left in the burrow was weighed at the end of the test.

2.3 Food restriction

Food restriction was performed following baseline testing by single housing animals and gradually decreasing daily food rations from 5g to 1g until each animal lost up to 15% of its initial weight in the span of 2-3 weeks. Animals were tested again as described above without the overnight habituation period and returned to *ad libitum* diet for 2 weeks.

2.4 Corticosterone testing

Submandibular blood collection method was used to obtain samples under isofluorane anesthesia. A sterile, stainless steel lancet (MEDI-POINT Stainless Steel Lancet) was used to pierce slightly behind the mandible to collect a 100uL blood sample in an EDTA microtainer blood collection tubes (BD Diagnostics). The collection tubes were then spun at 2000 rpm for 10 minutes to separate the plasma from the blood sample. Corticosterone levels in the plasma were measured using the Arbor Assays Detect X® Corticosterone Enzyme Immunoassay Kit on a Varioskan LUX multimode microplate reader (Thermo Scientific), following the manufacturer's instructions.

2.5 Statistical analysis

All datasets were tested for normality using the Shapiro-Wilk test and appropriate statistical test were applied. One-way ANOVA or the Kruskal-Wallis test were used for baseline cohort measures with respectively Tukey's or Dunn's multiple comparison tests. Two-way ANOVA was used for the food restriction studies (with repeated measures) and to

analyze the *Cc2d1a* cKO cohorts to determine the effect of treatment (food restriction or genotype) and sex with Tukey's multiple comparison test.

3. Results

3.1 Test design for Digging Discrimination test

We sought to develop a novel paradigm to discern the motivation behind different digging behaviors. The test design was based on a combination of existing tests, a burrowing test (Deacon, 2006a), and free digging (Deacon, 2006b). We chose a box larger than the home cage and similar to the one used for marble burying and free digging tests to provide space for movement and exploration. The testing arena was filled with a thick (5 cm) layer of corncob bedding. The "burrow" consisted of a plastic tube as used for burrowing in Deacon (Deacon, 2006a). While the Deacon test packed the tube with food pellets or pea shingles requiring at least 3 hours of testing per animal, we used soft bedding allowing for faster testing times since mice would not burrow readily with higher packing densities or heavier materials. The type of bedding and packing weight of the tube (17g) was determined by testing different packing densities and identifying the optimal amount of bedding that could be completely removed in less than 30 minutes by a wild type mouse. To remove the confound of interacting with a novel object and pre-train the animal for bedding removal, habituation to the tube filled with bedding was performed in the home cage the night before testing.

At the beginning of the test, the burrow tube was placed in a corner of the testing apparatus. For automated video tracking the area surrounding the tube was outlined as the burrowing area to also capture activity close to the tube and the remaining area was

used to monitor movement and exploratory digging activity (**Fig. 1A**). Each mouse was placed in the corner opposite to the burrow and multiple parameters were tracked for 30 minutes. Basal activity levels were monitored by measuring total distance traveled and speed. Interaction with the burrow was quantified by measuring the latency to enter the burrow area, the number of entries in the burrow area, the total time in the burrow area and the average length of visit. Burrowing activity was tested manually by recording the time to empty the burrow and weighing the soft bedding filling remaining in the burrow at the end of the testing time to determine the percent of weight removed. Digging analysis was limited to vigorous digging as defined in the Methods.

3.2 Male and female performance in the DBD test

Performance, test stability, and sex as a biological variable were assessed by testing two separate age-matched cohorts of C56BL/6N males and one cohort of females (**Fig. 1**. M1: N=11; M2 N=13; F: N=10). While females showed increased distance covered in the arena (**Fig. 1B,** M1:33.7±2.4 m, M2:38.7±1.9 m, F: 52.6±3.3 m; M1/F $p<0.0001$, M2/F $p=0.0013$) and speed (**Fig. 1C,** M1: 0.019±0.001 m/s, M2: 0.021±0.001 m/s, F:0.029±0.002 m/s; M1/F $p<0.0001$, M2/F $p=0.0011$), burrowing and exploratory digging performance was comparable among male and female cohorts. Males and females showed similar latency to interact with the substrate in the burrow (**Fig.1D**) and spent similar time in the burrow area (**Fig 1E**). Most animals were able to completely empty the burrow tube within the allotted 30 minutes (1800 s) (**Fig. 1F**). Burrowing efficiency was measured by weighing the substrate remaining in the tube at the end of the test and determining what percentage of the substrate had been removed. Though averages ranged between 94.0±7.8% for the M2

male cohort and 69.5±33.9% for females, no significant differences were observed in burrowing performance (**Fig. 1G**).

Since the automatic tracking of time in the burrow area does not distinguish between time spent interacting with the substrate and time spent inside or outside the tube, two independent raters visually analyzed the videos for burrowing and exploratory digging. Burrowing was measured as time interacting with the substrate and actively pushing substrate outside of the tube. Digging was defined as vigorous digging activity using the front paws with active displacement of substrate. Time spent burrowing was significantly reduced in females when compared to M1, but not M2, nor M1 and M2 were statistically significant from each other (**Fig. 1H,** M1: 172.4±26.9 s, M2: 119.2±11.8 s, F: 105.0±13.4 s; M1/F p=0.04). Since the M2 cohort appeared to remove the most substrate by weight from the tube, time burrowing may not be an accurate measure of efficiency as large amounts of substrate can be removed with limited movement. All cohorts, M and F, showed similar performance in exploratory digging (**Fig. 1I**).

In addition, we noted consistent thin spreading of the soft bedding removed from the burrow on the surface of the cage. Soft bedding was pushed outside of the tube and often methodically distributed around the exploratory area of the arena by spreading it with the nose or front paws in a flicking or wading motion. While the flicking motion was not quantified as we could not determine whether the mice were interacting with the soft bedding or the corncob, we measured how much of the exploration area was covered by soft bedding at the end of the test as a measure of spreading behavior (**Fig. 1J**). There was no significant difference between males and females, but the spreading measure closely resembled the amount burrowed graph suggesting that the animals consistently spread the

material removed from the burrow. Overall, we found that the 30 minute test was sufficient to completely empty the burrow and dispose of the removed material and to discriminate digging within the burrow and exploratory digging in the outside area.

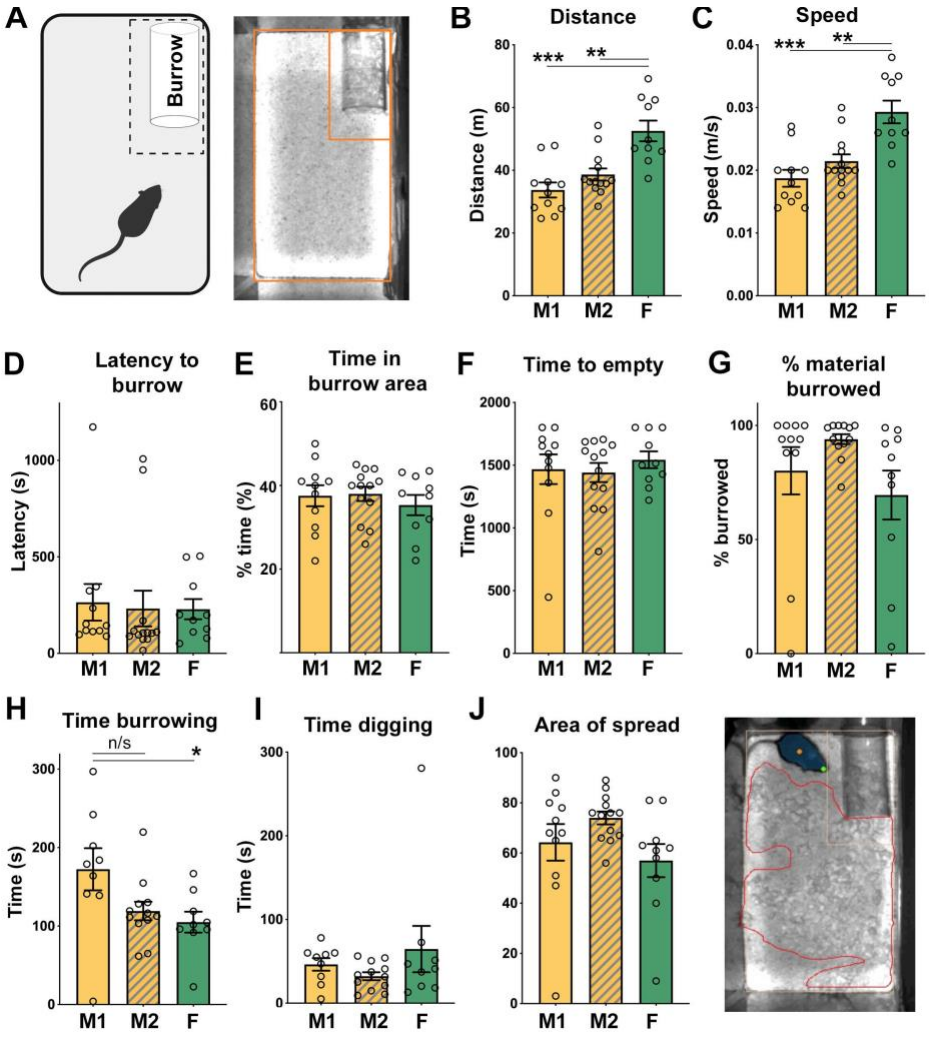

Figure 1. Stability of performance on Digging Behavior Discrimination test. Three cohorts of mice (M1: N=11; M2 N=13; F: N=10) were tested independently to assess stability of burrowing and digging performance and define possible sex differences. (***A***) Test chamber set up and schematic of digging and burrowing zones. (***B-C***) Males (M1, M2) covered similar distances at equal speed, while females showed increased motor activity (**B**) and speed (**C**). (***D-G***) Different cohorts showed similar burrowing performance with similar latency to burrow (**D**.), time spent in the burrow area (**E**.), time to empty the burrow (**F.**), and percentage of material removed (**G.**) (**H.**) Time spent in direct interaction with substrate in the burrow was variable with females significantly different from the M1

group, but not M2. (**I.**) Time spent digging was similar in all groups**.** (**J.**) The substrate was then distributed over the area of the cage (example of spread soft bedding outlined in red on the right). Values are presented as means ± *SEM*. Symbols are individual mouse data points. *$p < 0.05$, **<0.01, or ***<0.001 following multiple comparison tests. All unmarked comparisons were not significant.

3.3 Digging discrimination with food deprivation

We wondered if food restriction would change digging preference and elicit a shift between burrowing behavior and exploratory digging/foraging outside the burrow. We performed the digging discrimination test following a food restriction protocol leading to 10-15% weight loss and after *ad libitum* feeding was restored for 2 weeks (**Fig. 2A**). Males from cohort M2 and female mice were used. During the food restriction condition three mice of each sex escaped in the middle of the trial as soon as they emptied the burrow and were excluded from the analysis. Interestingly, they completed the test following *ad libitum* feeding suggesting that the escape reaction could be due to increased exploratory drive following food restriction. However, results from these animals were excluded from the final analysis in order to only include animals who completed all three trials.

Mice of both sexes covered less distance at lower speed after food restriction and returned to baseline during recovery showing a strong effect of the treatment (**Fig. 2B-C**). In addition, a larger effect was noted in females who displayed a larger reduction in mobility than males (**Fig. 2B-C,** Baseline=Base, Food Restriction=FR, Recovery=Rec. Distance. Base: M, 36.4±1.3m; F, 49.9±3.7 m. FR: M, 26.1±1.9 m; F, 25.4±3.2 m. Rec: M, 37.4±1.3 m; F, 46.3±3.5 m. Speed. Base: M, 0.020±0.001 m/s; F, 0.028±0.002 m/s. FR: M, 0.015±0.001 m/s; F, 0.014±0.02 m/s. Rec: M, 0.021±0.001 m/s; F, 0.026±0.002 m/s. 2-way ANOVA: treatment p= <0.0001 for both distance and speed, sex p=0.008 for distance, sex p=0.006 for speed). Latencies to burrow trended towards faster times, but there was no

significant difference and no effect of treatment or sex (**Fig. 2D**). However, both males and females spent significantly more time outside the burrow area during food restriction and returned to baseline levels after *ad libitum* feeding (**Fig. 2E**, % time outside. Base: M, 61.5±2.0%; F, 65.5±3.2%. FR: M, 73.1±2.5%; F, 81.0±2.8%. Rec: M, 64.0±2.4%; F, 65.8±2.3%. 2-way ANOVA: treatment $p<0.0001$, sex $p=0.051$). In males, this shift resulted in decreased burrowing efficiency (**Fig.2F,** Base: 95.0±2.7%; FR: 76.5±4.7%; Rec: 94.5±1.8%. Base/FR $p=0.0017$, FR/Rec $p=0.024$, 2-way ANOVA: treatment $p=0.0007$) and a substantial increase in time spent in exploratory digging (**Fig. 2G,** Base: 29.8±5.3 s; FR: 109.0±35.7 s; Rec: 20.3±6.1 s. Base/FR $p=0.032$, FR/Rec $p=0.015$, 2-way ANOVA: treatment $p=0.042$). Females had already displayed increased variability in their burrowing performance at baseline and were not affected by food restriction revealing an effect of sex on performance (**Fig.2F,** Base: 60.4±14.2%; FR: 63.3±11.1%; Rec: 92.1±3.5%, 2-way ANOVA: sex $p=0.041$, sex X treatment $p=0.014$). In addition, females did not increase their digging activity, but no effect of sex was noted in the statistical analysis (**Fig. 2G**, Base: 73.5±35.4 s; FR: 87.9±18.8 s; Rec: 60.7±15.3 s, 2-way ANOVA: sex $p=0.28$). Interestingly, only on their third test trial after recovery from food restriction females burrowed as efficiently as males (**Fig. 2F**). When time interacting with the substrate inside the burrow was measured by independent raters, no significant differences were observed, though both males and females showed a trend for spending more time moving substrate in the recovery trial. These results indicate that the weight of material removed is a more sensitive measure of burrowing efficiency as found in the baseline studies.

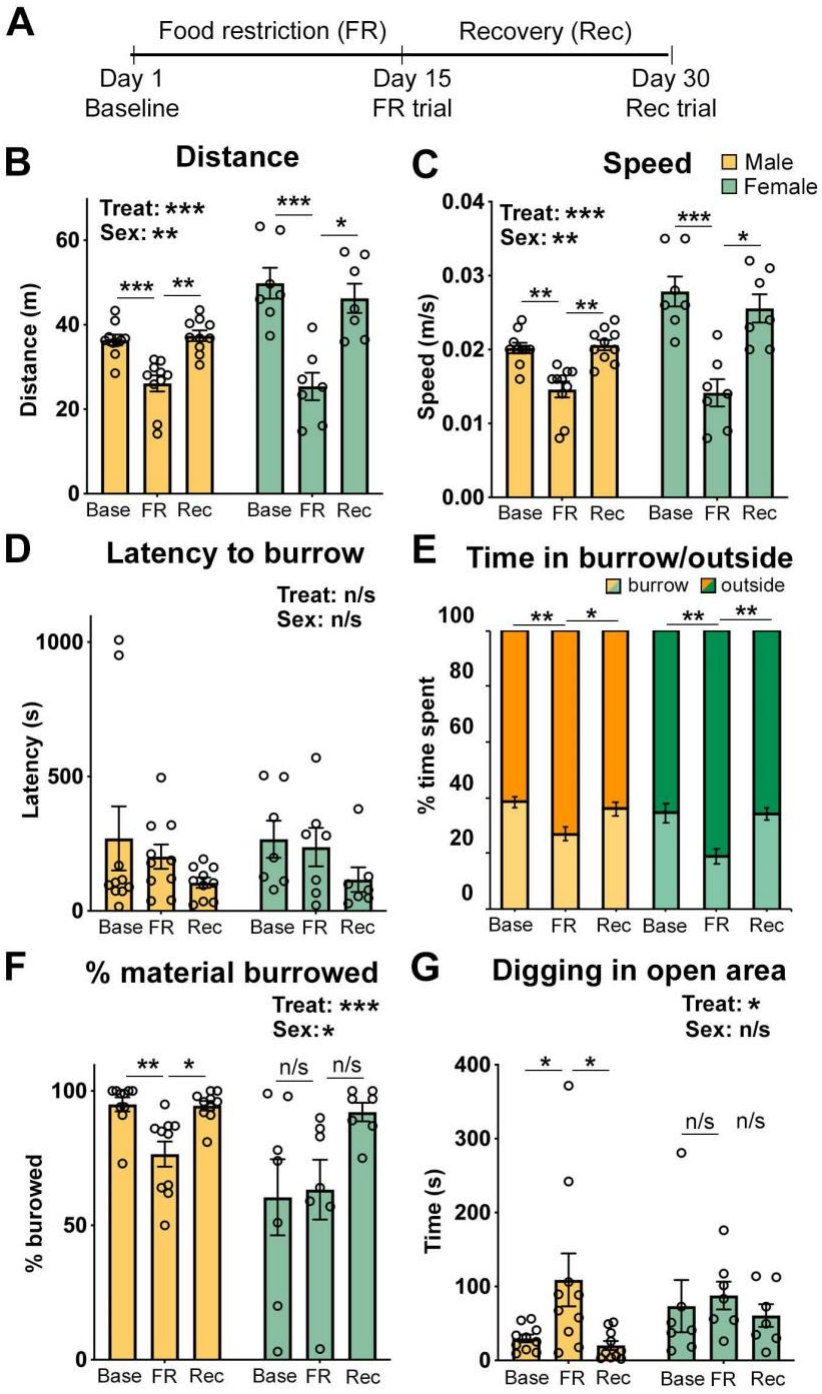

Figure 2. Male and female mice show different free digging and burrowing performance after a food restriction challenge. (A.) Two cohorts of mice (Male: N = 10; Female: N = 7) were assessed using the DBD test at baseline (Base), during food restriction (FR), and once recovered from food restriction (Rec). **(B – C.)** Female mice covered more distance (**B.)** at a faster pace (**C.**) than male counterparts at baseline and showed a more prominent drop to male-like levels of activity during FR. Both sexes recovered to baseline

levels. **(D.)** Latency to burrow did not change significantly, but (**E**.) both males and females spent more time outside of the burrow area during FR. **(F)** Females engage in limited burrowing at baseline and FR, but increase during recovery, whereas males burrow substantially at baseline, reduce during FR, and return to baseline performance during recovery. **(G)** Males spent significantly more time digging during FR, while females maintained constant digging performance. Values are means ± *SD*. Symbols are individual mouse data points. *p < 0.05, **<0.01, or ***<0.001 following multiple comparison tests. All unmarked comparisons were not significant.

Since corticosterone (CORT) levels are elevated by food restriction (Guarnieri et al., 2012; Pankevich et al., 2010), we wondered whether they would correlate with digging performance. CORT levels were measured by ELISA during the baseline testing showing that females had higher baseline CORT levels than males as previously observed (Kitay, 1961; Laviola et al., 2002) (**Fig. 3A**). After food restriction, males followed the expected pattern with an increase in CORT levels and returned back to baseline with *ad libitum* feeding (**Fig. 3B**, Base: 167.5±12.1 ng/µl; FR: 401.4±50.0 ng/µl; Rec: 206.0±13.1 ng/µl. Base/FR p=0.005, FR/Rec p=0.006). Females showed a smaller but significant increase following food restriction, but levels remained elevated in the recovery trial (**Fig.3B**, Base: 336.7±32.1 ng/µl; FR: 480.1±39.3 ng/µl; Rec: 430.9±52.5 ng/µl. Base/FR p=0.009, FR/Rec p=0.765). Thus, no clear correlation was found between CORT levels and burrowing in females.

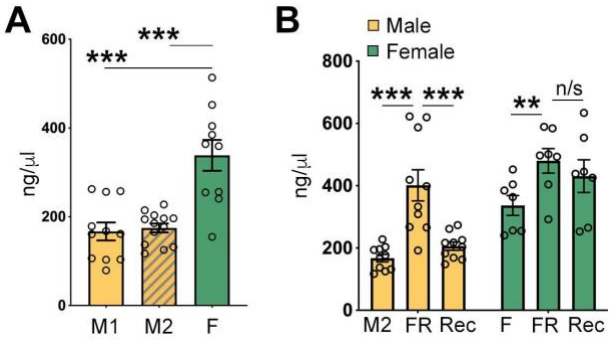

Figure 3. Plasma Corticosterone levels at baseline and with food restriction. (***A***) Female mice exhibited higher concentrations of CORT than males under baseline conditions. (**B**) CORT levels increased in both sexes during FR, but only females retained high levels once recovered from FR. Values are means ± *SD*. Symbols are individual mouse data points. *$p < 0.05$, **<0.01, or ***<0.001

3.4 Digging discrimination in a model of autism and intellectual disability

Our interest in developing a better measure for digging behavior originated from the analysis of a mouse model of autism and intellectual disability, *Cc2d1a* conditional knock-out mice (cKO)(Oaks et al., 2017). *CC2D1A* loss of function leads to a spectrum of psychiatric presentations including severe to moderate intellectual disability, autism spectrum disorder, and aggressive behavior (Basel-Vanagaite et al., 2006; Loviglio et al., 2016; Manzini et al., 2014; Reuter et al., 2017). Mice where *Cc2d1a* is conditionally removed in the forebrain show an array of cognitive and social deficits, hyperactivity, and obsessive grooming, primarily found in males (Oaks et al., 2017; Zamarbide et al., 2019). *Cc2d1a* cKO males buried the same number of marbles as controls, but subsequent analysis of the videos identified a reduction in time spent digging (Oaks et al., 2017). We asked whether the digging discrimination test would be more sensitive in assessing changes in digging behavior.

We generated a cohort of male and female control (cre alone or homozygous floxed) and *Cc2d1a* cKO littermates and performed the DBD test (**Fig 4.** WT M N=8: cKO M N=8: WT F N=10: cKO F N=10). Despite a trend for females being more active, there was no significant difference in distance covered (**Fig. 4A**), speed (**Fig. 4B**), and latency to burrow (**Fig.4C**). Percent of time spent in the burrowing area was also similar with only a trend in reduction for *Cc2d1a* cKO males (**Fig.4D**). As in the previous experiments, the percentage of material removed from the burrow was the most sensitive measure with a 50% reduction

in burrowing efficiency and half the animals barely interacting with the substrate despite hovering in the vicinity of the burrow (**Fig.4E,** WT M: 82.5±3.8%, cKO M: 31.5±16.2%, WT F: 49.7±12.8%, cKO F: 45.6±12.4%. M WT/cKO p=0.027, 2-way ANOVA genotype p=0.023, sex p=0.42). Free digging activity showed a trend for an increase in *Cc2d1a* cKO males compared to WT littermates (**Fig. 4F**).

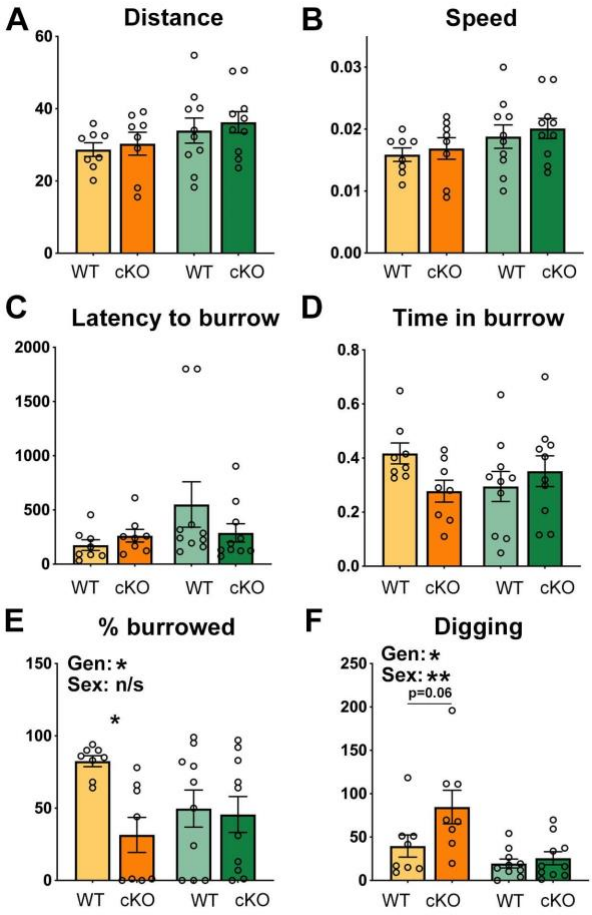

Figure 4. Cc2d1a cKO males show reduced burrowing performance. 4 cohorts of mice (WT M: N=8; cKO M: N=8; WT F: N=10; cKO F: N=10) were tested independently to assess the protocol sensitivity to an animal model of ASD. ***(A-B)*** Between genotype male and females covered similar distances at equal speed, while females showed increased motor activity (**A**) and speed (**B**) when compared to males. (***C***) WT females had a slightly but not statistically significant delay in their first interaction with the substrate in the burrow (latency in **C**.) than all other experimental groups. (**D**) For males, wild type animals spent slightly more time in the burrowing area. (**E**) cKO males burrowed significantly less material than wild type males, a difference not seen between female genotypes. (**F**) cKO males spent more time digging than WT males and spent significantly more time digging

than both female cohorts. Values are means ± SD. Symbols are individual mouse data points. *p < 0.05, **p<0.01, or ***p<0.001 following multiple comparison tests. All unmarked comparisons were not significant.

Discussion

Behavioral tests to measure digging activity as the marble burying and free digging have been used to study features of neuropsychiatric and neurodevelopmental disorders in rodents for decades (Deacon et al., 2001; El-Kordi et al., 2013; Gyertyán, 1995; Jirkof et al., 2010; Jones et al., 2001; Lovegrove, 1989; Njung'e & Handley, 1991; Sungur et al., 2014; Taylor et al., 2017; Thomas et al., 2009). Digging has been used to assess anxiety, compulsions, motor deficits, and exploratory drive (Bey & Jiang, 2014; Deacon et al., 2001; Metz et al., 2017). However, many of the existing protocols are not able to elucidate digging motivation leading to inconsistencies in the interpretation of the experimental measures (de Brouwer et al., 2019; Gyertyán, 1995; Njung'e & Handley, 1991; Thomas et al., 2009). Burrowing is an innate behavior that occurs consistently across mouse strains and throughout generations of lab-bred mice and reflects the intent of building a shelter (Blanchard et al., 1995; Bouchard & Lynch, 1989; Deacon et al., 2001; Dudek et al., 1983; Schmid-Holmes et al., 2001; Sherwin et al., 2004). In this study, we asked whether burrowing could be used in combination with exploratory digging for rapid assessment of motivation of digging behavior. Free digging in relation to burrowing can be used as a model for foraging where mice dig through a substrate to find food (Hörndli et al., 2019; Powell & Banks, 2004; Troxell-Smith et al., 2016). We adapted a protocol developed by Deacon (2006a) that is sensitive to an array of motor and behavioral deficits such as those found in scrapie infections, prion diseases, and Alzheimer's disease (Deacon, 2006a, 2012; Deacon et al., 2001, 2008). We adjusted the amount and texture of the substrate to be

removed from the "burrow" to produce measurable results in a shorter period of time and allow the mouse to decide where to dig. The DBD protocol reliably measured burrowing and exploratory digging behavior in mice that was reproducible in age matched cohorts tested at different times and between sexes. While female mice showed increased mobility in the arena as observed in other digging and marble burying studies (Palanza et al., 2001; Taylor et al., 2017), digging and burrowing performance did not initially appear significantly different as previously reported (Taylor et al., 2017).

We explored different metrics obtained with both automated video-tracking and manual analysis to identify the most sensitive and reliable measures of burrowing and digging behavior. We found that automated tracking of the presence of an animal in the burrowing or exploratory zones did not reflect their digging activity. For burrowing, manual assessment of the time spent interacting with material in the burrow was not as sensitive as the simple measure of weighing the material left inside the tube at the end of the test. While in the marble burying test, marbles can be buried and then unburied with digging (Gyertyán, 1995), mice will remove the soft bedding from the burrow and do not push it back in.

Free digging must be scored manually unless there are appropriate algorithms that will identify specific posture and movement during digging. It is important to note that we used a very conservative measure of digging based on posture and displacement of substrate. Mice can use different additional motions to dig such as flicking substrate with one or both forefeet sometimes wading into the substrate in a forward swim-like motion (de Brouwer et al., 2019; Layne & Ehrhart, 1970). Assessment of these more subtle movements was confounded by the spreading of the soft bedding over the exploration area. Substrate

spreading was an unexpected yet very consistent behavior. Mice would remove large portions of bedding from the burrow and start dispersing it in a thin layer in concentric circles on the surface of the corncob bedding. This behavior appears opposite to nest building behavior where soft substrate is accumulated (Deacon, 2012; Neely et al., 2019). Since we could not determine whether flicking was directed at the soft or hard bedding, we only chose to measure spreading activity by quantifying how much of the arena was covered in soft bedding. The motivation for this additional goal-directed behavior linked with burrowing is unclear. Burrow size and the presence of soil mounds around the burrow entrance vary widely among rodents and often depend on the habitat, so particular attention must be placed in understanding species-specific behavior (Reichman & Smith, 1990; Hu & Hoekstra, 2017). Wild house mice (*Mus musculus*) are known to seasonally clean their burrows of debris and spoiled food by pushing them out of the burrow (Schmid-Holmes et al., 2001). In addition, house mice usually have clear dirt paths or "runways" to the entry of their burrow systems (Avenant & Smith, 2003; Eriksson & Eldridge, 2014). This spreading behavior could reflect an innate response to hide sediment from the excavation or clear the entrance to the burrow.

Both male and female mice would often focus on emptying the burrow first and then spread the soft bedding and explore in the open area. Food restriction is known to alter foraging behavior and eating habits (Dell'Omo et al., 2000; Pankevich et al., 2010). While extended food restriction reduced overall activity and speed as previously shown (Tucci et al., 2006), male mice changed their choice of digging arena and shifted towards spending more time in the exploratory digging area, removing less material from the burrow, and significantly increasing their free digging activity. After *ad libitum* feeding was restored,

digging and burrowing returned to baseline levels. Female mice showed a different response. While burrowing at baseline was not significantly different from males, females showed a much larger standard deviation and inconsistent performance in both the initial study cohort and control littermates for the *Cc2d1a* cKO. Females still tended to spend more time in the free digging area, but digging activity remained constant. Burrowing activity was not altered by food restriction, despite much reduced overall mobility. Interestingly, female burrowing performance improved to levels similar to males in the recovery trial after food restriction.

Mild food restriction lasting over 10 days induces a response in rodents in the hypothalamic-pituitary-adrenal (HPA) axis raising blood levels of CORT (Díaz-Muñoz et al., 2000; Méquinion et al., 2014; Scheurink et al., 1999; Yoshihara et al., 1996). While this CORT increase can be interpreted as a stress response, it is also thought to have an adaptive role leading to increased food anticipatory activity and recreational exercise (Díaz-Muñoz et al., 2000; Pankevich et al., 2010; Scheurink et al., 1999). In our studies, female mice showed higher CORT levels than both male cohorts at baseline as previously established (Kitay, 1961; Laviola et al., 2002). While CORT levels increased in both males and females with food restriction, they only returned to baseline in males and did not appear to correlate with burrowing or digging activity levels in females. Female and male rodents have been shown by multiple groups to have distinct cellular and physiological responses and adaptation to stress and altered feeding regimens (Bale & Epperson, 2015; Massa & Correa, 2020; Rincón-Cortés et al., 2019). Chen et al (2005) in studying the effects of senescence and aging on burrowing also showed that males and females differentially alter their burrowing performance with age and that this change may not be related to

anxiety or novelty. It is possible that males and females assign different value to burrowing and foraging requiring further study. Burrowing has been shown to rely on both the hippocampus (Deacon & Rawlins, 2005) and frontal cortex (Deacon et al., 2003), while food seeking has been linked to the circuitry of the hypothalamus and reward pathways (Massa & Correa, 2020). A modified version of the DBD test where the free digging area is baited with food or where a food patch is provided may help to further define how mice choose between different digging modalities.

The impetus for developing a novel digging paradigm originated from previous studies of a mouse model for ID and ASD, where the *Cc2d1a* gene is conditionally removed in the cortex and hippocampus, the *Cc2d1a* cKO (Oaks et al., 2017; Yang et al., 2019). While these mice showed hyperactivity and obsessive grooming in addition to cognitive and social deficits (Oaks et al., 2017; Yang et al., 2019), reduced digging activity was identified in the marble burying test with no change in marble number (Oaks et al., 2017). We found the DBD test to be more sensitive in defining digging changes with a decrease in burrowing and a trend towards increased exploratory digging in male cKO mice. This finding suggests that the choice to spend more time in exploratory digging than burrowing may reflect hyperactive/repetitive behaviors that are not readily differentiated by the marble burying. Interestingly, there was no difference between wild-type and female cKO mice. *Cc2d1a* cKOs have been shown male-specific behavioral impairments in some behavioral tests linked to sex-specific signaling deficits in the hippocampus (Zamarbide et al., 2019), which may underlie the sex difference in these findings. Interestingly, while no significant difference is found, control females show much more variable performance than males as found in are initial wild-type cohorts.

In summary, the current study underscores the need to consider digging behavior in laboratory house mice as multifaceted and proposes a novel behavioral test to probe digging motivation that can be completed in 30 minutes per animal with simple measures. The ability to distinguish between burrowing and exploratory digging in a single paradigm may be beneficial to provide better data interpretation. For example, it is telling that sex differences in digging performance have started to emerge in this test, as there may be a possibility of sex-specific motivation for different forms of digging requiring further study. Females used in our studies were sexually naïve, but different digging responses may appear when females are building a nest or protecting their young. In addition, variations of this test comparing burrowing and foraging for food may provide insight in food seeking behaviors. By beginning to understand why a mouse performs a complex innate behavior like digging, we may also be able to probe the difference between behavioral need and compulsion.

References

Adams, N., & Boice, R. (1981). Mouse (Mus) burrows: Effects of age, strain, and domestication. *Animal Learning & Behavior*, *9*(1), 140–144.

American Psychiatric Association. (2013). *Diagnostic and Statistical Manual of Mental Disorders* (Fifth Edition). American Psychiatric Association.

Arakawa, H., Blanchard, D. C., & Blanchard, R. J. (2007). Colony formation of C57BL/6J mice in visible burrow system: Identification of eusocial behaviors in a background strain for genetic animal models of autism. *Behavioural Brain Research*, *176*(1), 27–39.

Avenant, N. L., & Smith, V. R. (2003). The microenvironment of house mice on Marion Island (sub-Antarctic). *Polar Biology*, *26*(2), 129–141.

Bale, T. L., & Epperson, C. N. (2015). Sex differences and stress across the lifespan. *Nature Neuroscience*, *18*(10), 1413–1420.

Basel-Vanagaite, L., Attia, R., Yahav, M., Ferland, R. J., Anteki, L., Walsh, C. A., Olender, T., Straussberg, R., Magal, N., Taub, E., Drasinover, V., Alkelai, A., Bercovich, D., Rechavi, G., Simon, A. J., & Shohat, M. (2006). The CC2D1A, a member of a new gene family with C2 domains, is involved in autosomal recessive non-syndromic mental retardation. *Journal of Medical Genetics*, *43*(3), 203–210.

Bey, A. L., & Jiang, Y. (2014). Overview of mouse models of autism spectrum disorders. *Current Protocols in Pharmacology*, *66*, 5.66.1-26.

Blanchard, R. J., Parmigiani, S., Bjornson, C., Masuda, C., Weiss, S. M., & Blanchard, D. C. (1995). Antipredator behavior of Swiss-Webster mice in a visible burrow system. *Aggressive Behavior*, *21*(2), 123–136.

Bouchard, P. R., & Lynch, C. B. (1989). Burrowing behavior in wild house mice: Variation within and between populations. *Behavior Genetics*, *19*(3), 447–456.

Broekkamp, C. L., Rijk, H. W., Joly-Gelouin, D., & Lloyd, K. L. (1986). Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. *European Journal of Pharmacology*, *126*(3), 223–229.

Bruins Slot, L. A., Bardin, L., Auclair, A. L., Depoortere, R., & Newman-Tancredi, A. (2008). Effects of antipsychotics and reference monoaminergic ligands on marble burying behavior in mice. *Behavioural Pharmacology*, *19*(2), 145–152.

Dawson, W. D., Lake, C. E., & Schumpert, S. S. (1988). Inheritance of burrow building inPeromyscus. *Behavior Genetics*, *18*(3), 371–382.

de Brouwer, G., Fick, A., Harvey, B. H., & Wolmarans, D. W. (2019). A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: Mapping the way forward. *Cognitive, Affective & Behavioral Neuroscience*, *19*(1), 1–39.

Deacon, R. M. (2006a). Burrowing in rodents: A sensitive method for detecting behavioral dysfunction. *Nature Protocols*, *1*(1), 118–121.

Deacon, R. M. (2006b). Digging and marble burying in mice: Simple methods for in vivo identification of biological impacts. *Nature Protocols*, *1*(1), 122–124.

Deacon, R. M. (2012). Assessing Burrowing, Nest Construction, and Hoarding in Mice. *JoVE (Journal of Visualized Experiments)*, *59*, e2607.

Deacon, R. M., Cholerton, L. L., Talbot, K., Nair-Roberts, R. G., Sanderson, D. J., Romberg, C., Koros, E., Bornemann, K. D., & Rawlins, J. N. P. (2008). Age-dependent and -independent behavioral deficits in Tg2576 mice. *Behavioural Brain Research*, *189*(1), 126–138.

Deacon, R. M., Penny, C., & Rawlins, J. N. P. (2003). Effects of medial prefrontal cortex cytotoxic lesions in mice. *Behavioural Brain Research*, *139*(1–2), 139–155.

Deacon, R. M., Raley, J. M., Perry, V. H., & Rawlins, J. N. (2001). Burrowing into prion disease. *Neuroreport*, *12*(9), 2053–2057.

Deacon, R. M., & Rawlins, J. N. P. (2005). Hippocampal lesions, species-typical behaviours and anxiety in mice. *Behavioural Brain Research*, *156*(2), 241–249.

Dell'Omo, G., Ricceri, L., Wolfer, D. P., Poletaeva, I. I., & Lipp, H.-P. (2000). Temporal and spatial adaptation to food restriction in mice under naturalistic conditions. *Behavioural Brain Research*, *115*(1), 1–8.

Díaz-Muñoz, M., Vázquez-Martínez, O., Aguilar-Roblero, R., & Escobar, C. (2000). Anticipatory changes in liver metabolism and entrainment of insulin, glucagon, and corticosterone in food-restricted rats. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *279*(6), R2048-2056.

Dudek, B. C., Adams, N., Boice, R., & Abbott, M. E. (1983). Genetic influences on digging behaviors in mice (Mus musculus) in laboratory and seminatural settings. *Journal of Comparative Psychology*, *97*(3), 249–259.

El-Kordi, A., Winkler, D., Hammerschmidt, K., Kästner, A., Krueger, D., Ronnenberg, A., Ritter, C., Jatho, J., Radyushkin, K., Bourgeron, T., Fischer, J., Brose, N., & Ehrenreich, H. (2013). Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism. *Behavioural Brain Research*, *251*, 41–49.

Eriksson, B., & Eldridge, D. (2014). Surface destabilisation by the invasive burrowing engineer Mus musculus on a sub-Antarctic island. *Geomorphology*, *223*, 61–66.

Guarnieri, D. J., Brayton, C. E., Richards, S. M., Maldonado-Aviles, J., Trinko, J. R., Nelson, J., Taylor, J. R., Gourley, S. L., & DiLeone, R. J. (2012). Gene profiling reveals a role for stress hormones in the molecular and behavioral response to food restriction. *Biological Psychiatry*, *71*(4), 358–365.

Gyertyán, I. (1995). Analysis of the marble burying response: Marbles serve to measure digging rather than evoke burying. *Behavioural Pharmacology*, *6*(1), 24–31.

Hayashi, E., Kuratani, K., Kinoshita, M., & Hara, H. (2010). Pharmacologically distinctive behaviors other than burying marbles during the marble burying test in mice. *Pharmacology*, *86*(5–6), 293–296.

Hörndli, C., Wong, E., Ferris, E., Bennett, K., Steinwand, S., Rhodes, A., Fletcher, P., & Gregg, C. (2019). Complex Economic Behavior Patterns Are Constructed from Finite, Genetically Controlled Modules of Behavior. *Cell Reports*, *28*, 1814-1829.e6.

Hu, C. K., Hoekstra, H. E. (2017). Peromyscus burrowing: A model system for behavioral evolution. *Seminars in Cell & Developmental Biology*, *61*, 107-114.

Jirkof, P. (2014). Burrowing and nest building behavior as indicators of well-being in mice. *Journal of Neuroscience Methods*, *234*, 139–146.

Jirkof, P., Cesarovic, N., Rettich, A., Nicholls, F., Seifert, B., & Arras, M. (2010). Burrowing Behavior as an Indicator of Post-Laparotomy Pain in Mice. *Frontiers in Behavioral Neuroscience*, *4*, 165.

Jones, M., Mandelik, Y., & Dayan, T. (2001). Coexistence of Temporally Partitioned Spiny Mice: Roles of Habitat Structure and Foraging Behavior. *Ecology*, *82*(8), 2164–2176.

Kazdoba, T. M., Leach, P. T., Yang, M., Silverman, J. L., Solomon, M., & Crawley, J. N. (2016). Translational Mouse Models of Autism: Advancing Toward Pharmacological Therapeutics. *Current Topics in Behavioral Neurosciences*, *28*, 1–52.

Kitay, J. I. (1961). Sex differences in adrenal cortical secretion in the rat. *Endocrinology*, *68*, 818–824.

Latham, N., & Mason, G. (2004). From house mouse to mouse house: The behavioural biology of free-living Mus musculus and its implications in the laboratory. *Applied Animal Behaviour Science*, *86*(3), 261–289.

Laviola, G., Adriani, W., Morley-Fletcher, S., & Terranova, M. L. (2002). Peculiar response of adolescent mice to acute and chronic stress and to amphetamine: Evidence of sex differences. *Behavioural Brain Research*, *130*(1–2), 117–125.

Layne, J. N., & Ehrhart, L. M. (1970). Digging behavior of four species of deer mice (Peromyscus). *American Museum Novitates, 2429*, 1-16

Lovegrove, B. G. (1989). The Cost of Burrowing by the Social Mole Rats (Bathyergidae) Cryptomys damarensis and Heterocephalus glaber: The Role of Soil Moisture. *Physiological Zoology*, *62*(2), 449–469.

Loviglio, M. N., Beck, C. R., White, J. J., Leleu, M., Harel, T., Guex, N., Niknejad, A., Bi, W., Chen, E. S., Crespo, I., Yan, J., Charng, W.-L., Gu, S., Fang, P., Coban-Akdemir, Z.,

Shaw, C. A., Jhangiani, S. N., Muzny, D. M., Gibbs, R. A., … Reymond, A. (2016). Identification of a RAI1-associated disease network through integration of exome sequencing, transcriptomics, and 3D genomics. *Genome Medicine*, *8*, 105.

Luigjes, J., Lorenzetti, V., de Haan, S., Youssef, G. J., Murawski, C., Sjoerds, Z., van den Brink, W., Denys, D., Fontenelle, L. F., & Yücel, M. (2019). Defining Compulsive Behavior. *Neuropsychology Review*, *29*(1), 4–13.

Manzini, M. C., Xiong, L., Shaheen, R., Tambunan, D. E., Di Costanzo, S., Mitisalis, V., Tischfield, D. J., Cinquino, A., Ghaziuddin, M., Christian, M., Jiang, Q., Laurent, S., Nanjiani, Z. A., Rasheed, S., Hill, R. S., Lizarraga, S. B., Gleason, D., Sabbagh, D., Salih, M. A., … Walsh, C. A. (2014). CC2D1A Regulates Human Intellectual and Social Function as well as NF-κB Signaling Homeostasis. *Cell Reports*, *8*(3), 647–655.

Massa, M. G., & Correa, S. M. (2020). Sexes on the brain: Sex as multiple biological variables in the neuronal control of feeding. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1866*(10), 165840.

McEwen, B. S., McKittrick, C. R., Tamashiro, K. L. K., & Sakai, R. R. (2015). The Brain on Stress: Insight from Studies Using the Visible Burrow System. *Physiology & Behavior*, *146*, 47–56.

Méquinion, M., Caron, E., Zgheib, S., Stievenard, A., Zizzari, P., Tolle, V., Cortet, B., Lucas, S., Prévot, V., Chauveau, C., & Viltart, O. (2014). Physical activity: Benefit or weakness in metabolic adaptations in a mouse model of chronic food restriction? *American Journal of Physiology-Endocrinology and Metabolism*, *308*(3), E241–E255.

Metz, H. C., Bedford, N. L., Pan, Y. L., & Hoekstra, H. E. (2017). Evolution and Genetics of Precocious Burrowing Behavior in Peromyscus Mice. *Current Biology: CB*, *27*(24), 3837-3845.e3.

Neely, C. L. C., Pedemonte, K. A., Boggs, K. N., & Flinn, J. M. (2019). Nest Building Behavior as an Early Indicator of Behavioral Deficits in Mice. *JoVE (Journal of Visualized Experiments)*, *152*, e60139.

Njung'e, K., & Handley, S. L. (1991). Evaluation of marble-burying behavior as a model of anxiety. *Pharmacology, Biochemistry, and Behavior*, *38*(1), 63–67.

Oaks, A. W., Zamarbide, M., Tambunan, D. E., Santini, E., Di Costanzo, S., Pond, H. L., Johnson, M. W., Lin, J., Gonzalez, D. M., Boehler, J. F., Wu, G. K., Klann, E., Walsh, C. A., & Manzini, M. C. (2017). Cc2d1a Loss of Function Disrupts Functional and Morphological Development in Forebrain Neurons Leading to Cognitive and Social Deficits. *Cerebral Cortex*, *27*(2), 1670–1685.

Palanza, P., Morley-Fletcher, S., & Laviola, G. (2001). Novelty seeking in periadolescent mice: Sex differences and influence of intrauterine position. *Physiology & Behavior*, *72*(1–2), 255–262.

Pankevich, D. E., Teegarden, S. L., Hedin, A. D., Jensen, C. L., & Bale, T. L. (2010). Caloric restriction experience reprograms stress and orexigenic pathways and promotes binge eating. *Journal of Neuroscience*, *30*(48), 16399–16407.

Pinel, J. P., & Treit, D. (1978). Burying as a defensive response in rats. *Journal of Comparative and Physiological Psychology*, *92*(4), 708–712.

Powell, F., & Banks, P. (2004). Do house mice modify their foraging behaviour in response to predator odours and habitat? *Animal Behaviour*, *67*, 753-759

Reichman, O. J., Smith S. C. (1990) Burrows and burrowing behavior by mammals. In H.H. Genoways ed., *Current Mammalogy*, Plenum Press, 197-244

Reuter, M. S., Tawamie, H., Buchert, R., Hosny Gebril, O., Froukh, T., Thiel, C., Uebe, S., Ekici, A. B., Krumbiegel, M., Zweier, C., Hoyer, J., Eberlein, K., Bauer, J., Scheller, U., Strom, T. M., Hoffjan, S., Abdelraouf, E. R., Meguid, N. A., Abboud, A., … Abou Jamra, R. (2017). Diagnostic Yield and Novel Candidate Genes by Exome Sequencing in 152 Consanguineous Families With Neurodevelopmental Disorders. *JAMA Psychiatry*, *74*(3), 293–299.

Rincón-Cortés, M., Herman, J. P., Lupien, S., Maguire, J., & Shansky, R. M. (2019). Stress: Influence of sex, reproductive status and gender. *Neurobiology of Stress*, *10*, 100155.

Scheurink, A. J., Ammar, A. A., Benthem, B., van Dijk, G., & Södersten, P. A. (1999). Exercise and the regulation of energy intake. *International Journal of Obesity and Related Metabolic Disorders: Journal of the International Association for the Study of Obesity*, *23 Suppl 3*, S1-6.

Schmid-Holmes, S., Drickamer, L. C., Robinson, A. S., & Gillie, L. L. (2001). Burrows and Burrow-Cleaning Behavior of House Mice (Mus Musculus Domesticus). *The American Midland Naturalist*, *146*(1), 53–62.

Sherwin, C. M., Haug, E., Terkelsen, N., & Vadgama, M. (2004). Studies on the motivation for burrowing by laboratory mice. *Applied Animal Behaviour Science*, *88*(3), 343–358.

Sluyter, F., Korte, S. M., Bohus, B., & Van Oortmerssen, G. A. (1996). Behavioral stress response of genetically selected aggressive and nonaggressive wild house mice in the shock-probe/defensive burying test. *Pharmacology, Biochemistry, and Behavior*, *54*(1), 113–116.

Sungur, A. Ö., Vörckel, K. J., Schwarting, R. K. W., & Wöhr, M. (2014). Repetitive behaviors in the Shank1 knockout mouse model for autism spectrum disorder: Developmental aspects and effects of social context. *Journal of Neuroscience Methods*, *234*, 92–100.

Taylor, G. T., Lerch, S., & Chourbaji, S. (2017). Marble burying as compulsive behaviors in male and female mice. *Acta Neurobiologiae Experimentalis*, *77*(3), 254–260.

Thomas, A., Burant, A., Bui, N., Graham, D., Yuva-Paylor, L. A., & Paylor, R. (2009). Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology*, *204*(2), 361–373.

Thompson, S. L., Welch, A. C., Ho, E. V., Bessa, J. M., Portugal-Nunes, C., Morais, M., Young, J. W., Knowles, J. A., & Dulawa, S. C. (2019). Btbd3 expression regulates compulsive-like and exploratory behaviors in mice. *Translational Psychiatry*, *9*(1), 1–14.

Troxell-Smith, S. M., Tutka, M. J., Albergo, J. M., Balu, D., Brown, J. S., & Leonard, J. P. (2016). Foraging decisions in wild versus domestic Mus musculus: What does life in the lab select for? *Behavioural Processes*, *122*, 43–50.

Tsien, J. Z., Chen, D. F., Gerber, D., Tom, C., Mercer, E. H., Anderson, D. J., Mayford, M., Kandel, E. R., & Tonegawa, S. (1996). Subregion- and cell type-restricted gene knockout in mouse brain. *Cell*, *87*(7), 1317–1326.

Tucci, V., Hardy, A., & Nolan, P. M. (2006). A comparison of physiological and behavioural parameters in C57BL/6J mice undergoing food or water restriction regimes. *Behavioural Brain Research*, *173*(1), 22–29.

Weber, J. N., & Hoekstra, H. E. (2009). The evolution of burrowing behaviour in deer mice (genus Peromyscus). *Animal Behaviour*, *77*(3), 603–609.

Webster, D. G., Williams, M. H., Owens, R. D., Geiger, V. B., & Dewsbury, D. A. (1981). Digging behavior in 12 taxa of muroid rodents. *Animal Learning & Behavior*, *9*(2), 173–177.

Yang, C.-Y., Yu, T.-H., Wen, W.-L., Ling, P., & Hsu, K.-S. (2019). Conditional Deletion of CC2D1A Reduces Hippocampal Synaptic Plasticity and Impairs Cognitive Function through Rac1 Hyperactivation. *Journal of Neuroscience*, *39*(25), 4959–4975.

Yoshihara, T., Honma, S., Katsuno, Y., & Honma, K. (1996). Dissociation of paraventricular NPY release and plasma corticosterone levels in rats under food deprivation. *The American Journal of Physiology*, *271*(2 Pt 1), E239-245.

Zamarbide, M., Mossa, A., Muñoz-Llancao, P., Wilkinson, M. K., Pond, H. L., Oaks, A. W., & Manzini, M. C. (2019). Male-Specific cAMP Signaling in the Hippocampus Controls Spatial Memory Deficits in a Mouse Model of Autism and Intellectual Disability. *Biological Psychiatry*, *85*(9), 760–768.