Assessment of autism-relevant behaviors in C57BKS/J leptin receptor deficient mice

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ABSTRACT

Gestational diabetes mellitus (GDM) was associated with greater autism risk in epidemiological studies. Disrupted leptin signaling may contribute to their coincidence, as it is found in both disorders. Given this we examined leptin receptor (Lepr) deficient (BKS.Cg-Dock7m +/+ Lepr<sup>−/−</sup> diabetic (db) heterozygous (db/<sup>+</sup>) mice for autism-relevant behaviors. BKS db/<sup>+</sup> females are lean with normal blood glucose, but they develop GDM while pregnant. We hypothesized BKS db/<sup>+</sup> offspring might exhibit physiological and behavior traits consistent with autism. Adolescent body weight, fasting blood glucose, serum corticosterone, social preferences, self-grooming, marble burying, social dominance and cognitive flexibility of BKS db/<sup>+</sup> mice was compared to C57BKS/J (BKS) and C57BL/6J (BL6) mice. Male db/<sup>+</sup> weighed more and had higher blood glucose and corticosterone relative to BL6, but not BKS mice. Also, male db/<sup>+</sup> lacked social interaction preference, explored arenas less, and buried more marbles than BL6, but not BKS males. Male and female db/<sup>+</sup> were more dominant and made more mistakes in water T-mazes locating a sunken platform after its position was reversed than BL6, but not BKS mice. Overall BKS db/<sup>+</sup>, particularly males, exhibited some autism-like social deficits and restrictive-repetitive behaviors relative to BL6, but BKS strain contributions to BKS db/<sup>+</sup> behaviors were evident. Since BKS db/<sup>+</sup> and BKS behavioral and physiological phenotypes are already so similar, it will be difficult to use these models in studies designed to detect contributions of fetal GDM exposures to offspring behaviors.

1. Introduction

Gestational diabetes mellitus (GDM), diagnosed by hyperglycemia arising in pregnancy, is a life-threatening condition impacting 7–17% of pregnancies worldwide (Lavery et al., 2017; Plows et al., 2018). Associations between GDM and increased risk of autism, either independently or along with other risk factors such as maternal obesity or hypertension, were found (Krabowskiak et al., 2012; Ornolly et al., 2015; Xiang et al., 2015; Xiang, 2017; Maher et al., 2018; Wan et al., 2018). Leptin resistance and high plasma leptin are common in obese or GDM pregnancies, and fetal high fat diet exposure can persistently reduce brain leptin receptor (Lepr) expression (Edlow, 2017). In the Boston Birth Cohort, elevated plasma leptin in early childhood was associated with higher autism incidence (Raghavan et al., 2018). On the other hand, leptin deficiency due to perinatal growth restriction produced social and cognitive deficits in mice that were prevented by neonatal leptin treatment (Meyer et al., 2014). Based on these prior findings, we hypothesized constitutive Lepr deficiency would produce impaired social behaviors and restrictive-repetitive behaviors that parallel core symptoms of autism in mice. The “diabetes” or “db” mutation arose in C57BL/KsJ (BKS), an inbred strain with 71% genetic identity to C57BL/6J (Fontaine and Davis, 2016), with alleles from DBA/2J, 129, and C57BL/10 (Mao et al., 2006). A recessive chromosome 4 mutation at the db gene truncates the long isoform of Lepr that is expressed in brain, most richly in hypothalamus (Bahary et al., 1990; Osborn et al., 2010; Valleeau and Sullivan, 2018).
corticosterone occurs after social interactions in adolescent BTBR GDM exposure (Tordjman et al., 2014; Van Dam et al., 2018). Elevated stress responses are adolescent biomarkers shared by autism and fetal equivalent of human cortisol, for which disrupted diurnal cycles and reported. An ancillary goal was to compare corticosterone, the mouse BL6 mice for behaviors relevant to autism. In a prior study, BKS db/db +/+ Leprdb/J dams, as they would be more likely have to GDM, for our study of autism-relevant behaviors. Since db/db mice are sterile, a closely linked coat color gene “misty” (Dock7m+) was introduced into the strain to facilitate distinction of db/+ fertile breeders from non-db carrier wildtype (+/+ mice) (Coleman, 1978). However, use of “misty” (Dock7m+/Dock7m+) non-db carriers as controls can introduce a potential confound for social behavior studies, since other Dock7 hypo-pigmented mutants had low social interaction preference (Blasius et al., 2009). Also Dock 7m+ misty mice exhibit other physiological deficits, including impaired growth (Plows et al., 2017). For these reasons, we compared behaviors of db/+ (BKS.Cg-Dock7m+/+ Leprdb/J) to less well-characterized C57BSJ/J (BKS) mice, and also C57BL/6J (BL6) mice that are commonly used as behavior controls for other strains in autism research.

Our primary goal was to screen db/+ offspring relative to BKS and BL6 mice for behaviors relevant to autism. In a prior study, BKS db/db males exhibited behavioral despair, learning deficits, and less anxiety than BKS controls (Sharma et al., 2010), but db/+ behavior was not reported. An ancillary goal was to compare corticosterone, the mouse equivalent of human cortisol, for which disrupted diurnal cycles and stress responses are adolescent biomarkers shared by autism and fetal GDM exposure (Tordjman et al., 2014; Van Dam et al., 2018). Elevated corticosterone occurs after social interactions in adolescent BTBR T’Tpr3+/J, a popular autism research mouse model (Gould et al., 2014). We hypothesized adolescent db/+ would have higher baseline corticosterone, fasting blood glucose and weights than BKS or BL6 mice. We also hypothesized db/+ would have social behavior deficits and more restrictive-repetitive traits than BKS or BL6 mice. Physiological and behavioral findings from db/+ generally supported our hypothesis, more often for males than females. However most behavioral differences among db/+ and BL6 were also found in BKS versus BL6, indicating a strong BKS background strain influence on them.

2. Materials and methods

2.1. Animals

C57BLKS/J mice (BKS, The Jackson Laboratory (JAX) stock #000662 males and females, 8 each, five weeks old) were purchased for this study. BKS mice acclimated for a week after arrival before use in behavior tests. Founder pairs of db/+ (BKS.Cg-Dock7m+/+ Leprdb/J, JAX stock #000642) and BL6 (C57BL/6J, JAX stock #000664) were also purchased from JAX (Bar Harbor, ME, USA) and were bred in-house. Glucose tolerance tests were performed on day 15 of pregnancy in 3 BL6 and 2 db/+ females that were naive first generation, and 2 BKS females mated after behavior tests. A digital glucose monitor with test strips (In Control True Metrix, H-E-B, San Antonio, TX, USA). Remaining trunk blood was collected into microcentrifuge tubes and stored at 4 °C until use. Serum corticosterone was measured from 5 to 6 randomly selected thawed samples from each strain and sex, using a corticosterone ELISA Kit (ADI-900-097, Enzo Life Sciences, Farmingdale, NY, USA) as directed (small volume protocol for steroid displacement) and microplate reader (SpectraMax 190, Molecular Devices LLC, San Jose, CA, USA).

2.2. Offspring physiological measures

Mice were weighed on the first day of behavior testing and on the day of euthanasia using a digital kitchen scale with bowl (Taylor 3804, Office Depot, USA). Before euthanizing, offspring mice were transferred to clean cages with ad libitum access to water, but no food for 5 h of fasting in the room where they were later euthanized. Fasting started between 0800 and 0900 h, 1–2 h after lights on, and mice were euthanized between 1300 and 1400 h, or 6–7 h after lights on. Mice were taken one by one from their home cage and the filter top was replaced each time. The location of euthanization was 6 ft away from the home cage and was obscured from view of mice in the cage by a counterport. It took 4–5 s to euthanize all mice in a cage. Each mouse was euthanized by cervical dislocation and decapitation by an experienced researcher in under 30 s without use of anesthesia, and blood was immediately collected. Glucose was measured from trunk blood using digital glucose monitors pre-loaded with test strips (In Control True Metrix, H-E-B, San Antonio, TX, USA). Remaining trunk blood was collected into microcentrifuge tubes and stored at 4 °C to clot overnight. Serum was separated from platelets by centrifugation at 2000 ×g for 10 min, and samples were stored at −80 °C until use. Serum corticosterone was measured from 5 to 6 randomly selected thawed samples from each strain and sex, using a corticosterone ELISA Kit (AD1-900-097, Enzo Life Sciences, Farmingdale, NY, USA) as directed (small volume protocol for steroid displacement) and microplate reader (SpectraMax 190, Molecular Devices LLC, San Jose, CA, USA).

2.3. Autism-relevant behavior tests

2.3.1. Sociability preference

Three chamber tests for social interaction and social novelty preference were performed in male and female mice as in prior studies (Moy et al., 2007; Gould et al., 2014). Stranger mice were pre-conditioned for tests by 3 daily confinement sessions (30 min each) under wire mesh cups before use in tests. Tests took place between 1100 and 1600 h, and up to 6 mice were tested simultaneously in different arenas. Under low red light (16 lx, measured by Lux Light Meter Pro App for iOS) each subject mouse was placed in the middle of a three chamber test arena for 10 min to acclimate. Slat doors were opened for 10 min for subjects to explore the full arena. To begin social interaction tests, wire cup cages, one empty (novel object) and the other containing a stranger 129S1/SvImJ mouse the same sex and age as the subject were randomly placed in the left or right end chambers. Mouse behaviors were video recorded for 10 min. To measure social novelty preference, the subject was confined to the center and a second ‘new’ stranger mouse was introduced under the empty cup cage for another 10 min round of video-
recorded behavior testing.

All data from videos was collected by observers who were blind to the strain and sex of subject mice. The cumulative time each mouse spent in each chamber with either strangers (old and new) or objects (cages), was recorded and averaged for db/+ BKS and BL6 males or females to be compared within and between groups. Additionally, sniffing time or time spent by subject mice in focused interactions with strangers (e.g. sniffing) or novel objects (cup cages), with their nose pointed toward the cup cages and with mice being no further than a head length away from the cup cages, was recorded with hand-held timers. Time with the novel object (for interaction tests) or old stranger mouse (for novelty tests) was also subtracted from time with the stranger or new stranger to achieve sociability preference values to compare between groups. During tests the total number of chamber entries and time spent in the middle chamber were measured. Self-grooming behavior was also measured during sociability preference tests, as it is a behavior paralleling the stereotypies seen in autism (Kalueff et al., 2016).

2.3.2. Marble burying

This test is frequently used to assess mouse models of autism for restrictive repetitive traits (Chang et al., 2017). After sociability tests mice were placed individually in 40 × 20 cm sterilized rat cages filled to a depth of 8 cm with wood chip bedding (Teklad, Harlan, Indianapolis, IN), onto which 18 flattened blue glass marbles were placed in a 3 × 6 grid. Room lighting was 16 lx, a lid was put on the cage, and mice were left to bury marbles for 30 min. The total number of marbles >2/3 buried was tallied.

2.3.3. Social dominance

The tube test for social dominance was performed as previously described (Messeri et al., 1975). Tubes were clear acrylic 3 cm ID and 31 cm length with a central slot with a removable mesh divider; room lighting was 300 lx. Male or female offspring were matched against 6 different age and sex matched 129S1/SvImJ mice previously conditioned in tube tests in a transparent acrylic tube divided in the middle by a mesh insert. Mice faced nose to nose and the mesh insert was removed. The first mouse to advance and cause the partner to back out of the tube was the winner of the match, getting 1 point, while the mouse that backed out was considered the loser, and assigned 0 points. All matches were timed and matches exceeding 3 min were a tie and each mouse was given 0.5 points.

2.3.4. Water T-maze for cognitive flexibility

Cognitive flexibility was compared in male and female offspring using a water-T maze test, a modified version of the Morris Water maze for mice (Guariglia and Chadman, 2013). This test was performed in a lit room (300 lx) for both male and female db/+ BKS and BL6 mice. The maze consisted of two aquatic cross mazes, 71 cm × 51 cm (zebrafish cross maze, Noldus, Leesburg, VA, USA), with one inverted to cover the other. The bottom maze was filled 10 cm deep with water made opaque with 10 ml white tempera paint (Sargent, Hazelton, PA, USA) in 5 L deionized room temperature (24 °C) water. The 20 cm each left and right arms and 50 cm runway were used, and the top of the cross was blocked off. The pre-training trial consisted of placing a mouse in the runway of the maze, without a platform, noting the first arm it swam down as its “preferred arm”, removing the mouse from the maze and drying it with a cotton towel. The platform, an inverted plastic container 6 cm deep, was put in the opposite arm from each mouse’s preferred arm for 10 subsequent training trials. After each trial, mice were dried in a pile of cotton towels for about 1–2 min to rest between trials while other mice were tested. The same water (8 L in total volume) was used throughout the testing day, and the platform was shuttled across the top arm for each mouse’s appropriate target arm. A total of 2 mazes were used by up to two operators on any given testing day so males and females could be tested separately. There were 5 acquisition training days,
and 4 reversal training days, with 10 trials each. This was based on acquisition criteria that mice correctly find the platform 80% of the time each day for 3 consecutive days. The cumulative errors/day (total instances an arm was incorrectly entered), % of correct trials/day and average time/day it took mice to find the platform were the main performance measures.

2.3.5. Statistical analyses of data

Pre and post mean weights were compared separately for males and females by 2-way (strain × sex) mixed effect (repeated measures) analysis of variance (ANOVA), while mean blood glucose and corticosterone were compared by 2-way (strain × sex) ANOVA, followed by Sidak’s multiple comparisons for significant outcomes. Social interaction and social novelty test data were analyzed by 3-way mixed effect (repeated measures) ANOVA for chamber time and sniffing time mean comparisons (removing terms from the model if a random effect ≤ 0 to simplify), and by 2-way ANOVA to compare mean chamber time and sniffing preferences, chamber entries, middle dwelling and self-grooming between strain and sex, followed by Sidak’s post-hoc tests. Water T-maze means were compared by 2-way repeated measures ANOVA with Sidak’s post-hoc tests. Mean numbers of buried marbles, tube test wins and times were also compared by 2-way ANOVA and Sidak’s post-hoc tests. Water T-maze means were compared by 2-way repeated measures ANOVA with Sidak’s multiple comparisons to further resolve significant findings. Social interaction means were compared by 2-way repeated measures ANOVA with Sidak’s post-hoc tests. Since sample sizes differed among treatment groups, predicted least squares (LSMD) and 95% confidence intervals (CI) for differences are reported. Effect sizes are reported as $\eta^2$ or $\eta_p^2$ calculated from ANOVA tables (Lakens, 2013). Prism (version 9 for Windows; GraphPad, LaJolla, CA, USA) was used for all statistics and graphs. All figures show mean ± standard error of mean (SEM).

3. Results

3.1. Mid-gestation glucose tolerance tests

Glucose tolerance tests were performed on gestational day 15 in db/+ , BKS and BL6 dams. Mice (2 each BKS and db/+ , and 3 BL6 females) were fasted for 6 h starting at 0800 h and injected (i.p.) at 1400 h with 1 g/kg glucose. Two way ANOVA revealed an interaction (time × strain F (10,20) = 5.5, p < 0.001, $\eta_p^2 = 0.73$) and Sidak’s multiple comparisons indicated at the 15 min post-injection peak blood glucose was higher in db/+ (mean ± SEM = 274 ± 13 mg/dl) **p < 0.001, least squares (LS) mean difference ((LSMD) = 103 mg/dl, 95% CI: 71 to 135 mg/dl) and BKS ((264 ± 25 mg/dl) **p < 0.001, LSM = 93 mg/dl, 95% CI: 61 to 125 mg/dl) than in BL6 (171 ± 9 mg/dl) dams (Fig. 2a). Pregnant db/+ also had higher blood glucose at 30 min (*p < 0.01, LSM = 51 mg/dl and 62 mg/dl, 95% CI: 19 to 82 and 27 to 96) and 60 min (**p < 0.05, LSM = 42 mg/dl and 39 mg/dl, 95% CI:10 to 73 and 4 to 74 mg/dl) time points than BL6 or BKS, respectively. Also the areas under curve (AUC) for BKS (17,160 ± 5,5, p = 0.92, as shown in Fig. 2b. Analysis of just the 0, 30 and 60 min time points revealed significant time (F (2, 8) = 11, *p < 0.001, LSM = −3645, 95%: −6752 to −538.1) and strain (F (2, 15) = 471, *p < 0.01, LSM = −5035, 95% CI: −7871 to −2199) were both less than db/+ (20,805 ± 442, F(2, 4) = 25, p < 0.01, $\eta^2 = 0.92$, as shown in Fig. 2b. Analysis of just the 0, 30 and 60 min time points revealed significant time (F (2, 8) = 70, p < 0.0001, $\eta^2_p = 0.95$) and strain (F (2, 4) = 17, p < 0.05, $\eta^2_p = 0.89$) effects as db/+ blood glucose was higher at 30 min than either BL6 (**p < 0.001, LSM = 51 mg/dl, 95% CI: 22 to 80) or BKS (**p < 0.001, LSM = 62 mg/dl, 95% CI: 30 to 93) dams, and this also occurred at 60 min (**p < 0.05), as Fig. 2c shows. The AUC for just 0, 30 and 60 min time points was greater for db/+ (F(2, 4) = 11, *p < 0.05, $\eta^2 = 0.85$) than BL6 and BKS (**p < 0.05, LSM = −2540 and −2557, 95% CI: 241 to 4839, and 38 to 5076).

Fig. 2. Glucose tolerance tests at gestational day 15 in db/+ , BKS and BL6 dams. Pregnant female mice (2 each BKS or db/+ , and 3 BL6) were fasted for 6 h starting at 0800 h and then injected (i.p.) at 1400 h with 1 g/kg glucose. a) The 15 min post-injection peak blood glucose was higher in db/+ and BKS than in BL6 dams (**p < 0.001), and db/+ dams also had higher blood glucose at 30 and 60 min (*p < 0.05) time points than BL6 or BKS. b) The area under curve (AUC) was greater for db/+ than BKS or BL6 (*p < 0.05). c) Analysis of only the 0, 30 and 60 min, consistent with prior reports (Yamashita et al., 2001, 2003) revealed db/+ blood glucose was higher at 30 and 60 min than either BL6 or BKS dams (*p < 0.05), and d) AUC was greater for db/+ than BKS or BL6 dams (*p < 0.05).
3.2. Offspring weight, fasting blood glucose and corticosterone

Female and male body weights were compared separately by strain in pre-post ANOVAs. Female body weight at the start of behavior tests was lower in BKS (mean ± SEM = 17 ± 0.4 g, strain F (2,22) = 10.4, p < 0.001, $\eta^2_p = 0.49$) and remained there relative to BL6 (22 ± 1 g, $p < 0.005$, LSMD = −5 g, 95% CI: −7.7 to −1.4 g) and db/+ (21 ± 1 g, $p < 0.05$, LSMD = −4 g, 95% CI: −6.9 to −0.5 g) females for the duration of the study, as shown in Fig. 3a. For male body weight, there was an interaction (time × genotype F (2, 25) = 10.7, $p < 0.001$, $\eta^2_p = 0.46$), such that differences between strains were not present at the outset of the study but were evident after behavior tests, 15 days later. Among males, db/+ (28 ± 1.5 g) weighed more than BL6 (22 ± 1 g, $p < 0.0005$, LSMD = 5.7 g, 95% CI: 2 to 9 g) and BKS (22 ± 0.3 g, $p < 0.005$, LSMD = 5.6 g, 95% CI: 2 to 9 g), shown in Fig. 3b. Mean fasting blood glucose measured one day after the 14 days of behavior tests, compared by 2-way ANOVA (strain × sex) revealed no effect of sex or interaction, and was higher in male and female db/+ than in BL6 males or females (strain F (2, 47) = 6, $p < 0.01$, $\eta^2_p = 0.19$, LSMD = 19 mg/dl, 95% CI: 4.6 to 33.1 mg/dl), but not versus BKS male and female mice, as shown in Fig. 3c. Day 15 mean serum corticosterone was compared by 2-way ANOVA (strain × sex), and was higher in male db/+ versus male BL6 (strain F (2, 28) = 13, $p < 0.0001$, $\eta^2_p = 0.48$; sex F (1, 28) = 16, $p < 0.001$, $\eta^2_p = 0.36$; with no interaction, LSMD = 534 ng/ml, 95% CI: 203 to 865 ng/ml) and higher in female db/+ ($p < 0.05$, LSMD = 348 ng/ml 95% CI: 17 to 679 ng/ml) and BKS (p = 0.001, 460 ng/ml, 95% CI: 130 to 791 ng/ml) than in female BL6, as shown in Fig. 3d.

3.3. Autism-relevant behavior tests

3.3.1. Sociability preference

Tests were conducted in three chamber arenas as in Moy et al. (2004, 2007). Social interaction preference, wherein time spent by subject mice in chambers with and sniffing a stranger mouse or empty cage (novel object) was measured for 10 min, were immediately followed by social novelty preference tests. A novel mouse was introduced under the empty cage, while the original “old” stranger mouse remained at the other end of the arena. Time in chambers and sniffing stimulus mice was recorded for 10 min to test social novelty preference.

3.3.1.1. Social interaction preference

3.3.1.1.1. Time in chambers. Male BL6 (strain × sex × chamber F (2, 94) = 4, $p < 0.05$, $\eta^2_p = 0.09$; Sidak’s $p < 0.0001$, LSMD = 201 s, 95% CI: 89 to 313 s) and BKS (p < 0.05, LSMD = 128 s, 95% CI: 3 to 254 s) spent more time in chambers with stranger mice than novel objects, whereas db/+ males and all females did not (Fig. 4a). Between strain and sex comparisons of interaction preferences (stranger time – novel object time) by 2-way ANOVA approached an interaction (strain × sex F (2, 47) = 2.8, $p = 0.07$, $\eta^2_p = 0.11$), and showed male BL6 had greater interaction preference than female BL6 mice (sex F (1, 47) = 4.3, $p < 0.05$, LSMD = 232 s, 95% CI: 37 to 281 s). Due to greater variability than expected, the study was underpowered (1−β = 0.74) to detect strain differences, even just among males. Given the sex difference and $p = 0.07$ for interaction, an exploratory ANOVA of males only revealed db/+ had lower interaction preference than BL6 males (F (2, 25) = 3.9, $p < 0.05$, $\eta^2_p = 0.24$, LSMD = 124 s, 95% CI: 10 to 237 s), but not BKS males,
as shown in Fig. 4b.

3.3.1.1.2. Time sniffing. For sniffing (focused attention on subject mouse or novel object) during social interaction tests there were no interactions, but all main effects were significant (strain F (2, 47) = 35, p < 0.0001, $\eta^2_p = 0.60$; sex F (1, 40) = 4.8, p < 0.05, $\eta^2_p = 0.08$; chamber F (1, 47) = 78, p < 0.0001, $\eta^2_p = 0.63$). Only male BL6 (p < 0.005, LSMD = 74 s, 95% CI: 15 to 133 s) and male BKS (p < 0.0001, LSMD = 94 s, 95% CI: 28 to 160 s) spent more time sniffing stranger mice than novel objects, as Fig. 4c shows. BKS females (p < 0.05, LSMD = 67 s, 95% CI: 5 to 130 s) and BKS males (p < 0.001, LSMD = 117 s, 95% CI: 54 to 157 s) spent more time sniffing strangers than BL6 females. BKS males also spent more time sniffing strangers than BL6 males (p < 0.001; LSMD = 97 s, 95% CI: 36 to 159 s) and db/+ males (p < 0.001; LSMD = 82 s 95% CI: 21 to 143 s), respectively. BKS males spent more time sniffing novel objects than BL6 males (p < 0.005; LSMD = 77, 95% CI: 16 to 139) and BL6 females (p < 0.05; LSMs = 66 s, 95% CI: 5 to 127 s). However, 2-way ANOVA of social sniff preferences (stranger sniff time – novel object sniff time) revealed no differences among strains or sex, and no interaction, as Fig. 4d shows. Supplementary Fig. S1 provides an alternative analysis using preference ratios, instead of differences.

3.3.1.2. Social novelty preference

3.3.1.2.1. Time in chambers. Male db/+ mice were the only group to spend more time with novel stranger mice rather than the “old stranger” mouse they previously encountered in the social novelty preference test (strain × sex × chamber interaction F (2, 94) = 3.8, p < 0.05, $np^2 = 0.07$, Sidak’s p < 0.05, LSMD = 160 s, 95% CI: 8 to 312 s), as shown in Fig. 4e. Two-way ANOVA of social novelty preference (new stranger – old stranger) revealed female BKS mice had lower novelty preference (strain F (2, 47) = 5, p < 0.01, $\eta^2_p = 0.18$; sex × chamber interaction F (2, 94) = 2.5, p = 0.09, $\eta^2_p = 0.1$) than female BL6 (p < 0.05, LSMD = −224 s, 95% CI: −427 to −20.4 s) or female db/+ (p < 0.05, LSMD = −252, 95% CI: −461 to −43 s), as in Fig. 4f.

3.3.1.2.2. Time sniffing. For sniffing time in social novelty tests, there was a strain × chamber interaction (F (2, 94) = 3.5, p < 0.05, $\eta^2_p = 0.07$) and effect of sex (F (1, 94) = 7.8, p < 0.01, $\eta^2_p = 0.07$). This was
because BKS male mice spent more time sniffing “old” social interaction test strangers (Fig. 4g) than any other group (p < 0.05, LSMD and 95% CI: BL6 male – 78 ± 149 to –7 s, BKS female –99 ± –172 to –26 s, db/+ male –102 ± –173 to 31 s, db/+ female: –102 ± –177 to –27 s), except BKS females (p = 0.9). Social novelty sniff preference (new stranger sniff time – old stranger sniff time) comparisons revealed strain differences (F (2, 47) = 3.2, p = 0.05, η² = 0.12). This was due to male db/+ mice trending toward higher social novelty preference than male BKS (Fig. 4g), and consistent with the prior finding that male BKS had greater preference for old versus new strangers (Fig. 4h). There was no effect of sex and no interaction.

3.3.1.3. Other behaviors measured during social preference tests

3.3.1.3.1. Time in middle chambers. Three-way mixed effect ANOVA revealed a significant effect of strain (F (2, 47) = 3.3, p = 0.05, η² = 0.12), but no post-hoc differences, other effects or interactions, as shown in Fig. 5a.

3.3.1.3.2. Chamber entries during sociability tests. In comparing chamber transitions of subject mice during social preference tests, the three-way interaction (strain × sex × test phase) (F (2, 47) = 0.8, p = 0.5), main effect of sex (F(1, 47) = 0.3, p = 0.6) or test phase (F(1, 47) = 0.2, p = 0.68) were not significant. However, there was a significant strain × sex interaction (F (2, 47) = 3.5, p < 0.05, η² = 0.12), and strain effect (F (2, 47) = 17, p < 0.001, η² = 0.41). Male BL6 made more chamber entries than male or female BKS or male db/+ mice during both social interaction (p < 0.01, LSMDs and 95% CIs: BKS female 24: 6 to 43 entries, BKS male 21: 3 to 40, db/+ male 21: 4 to 38 entries) and social novelty (p < 0.001, LSMDs and 95% CIs: BKS female 25: 7 to 44, BKS male 28: 9 to 46, db/+ male 23: 6 to 40 entries) tests, as shown in Fig. 5b. This indicates more extensive arena exploration by BL6 males relative to BKS or db/+ mice. By contrast chamber entries for female BL6, BKS and db/+ did not differ in either interaction or social novelty tests.

3.3.1.3.3. Self-grooming during sociability tests. BKS mice self-groomed more during the social novelty versus social interaction phase of preference tests (test phase F (1, 47) = 21, p < 0.005, η² = 0.31; LSMD = 56 s, 95% CI: 19 to 94 s). There were no significant interactions or other main effects, as shown in Fig. 5c.

3.3.2. Marble burying

BL6 females, male and female BKS mice and db/+ males buried more marbles in 30 min than BL6 males, as Fig. 5d shows. Two-way ANOVA revealed a significant interaction (strain × sex F (2, 47) = 6, p < 0.01, η² = 0.21), with a significant effect of sex (F (1, 47) = 6.4, p < 0.05, η² = 0.12) and of strain (F (2, 47) = 10, p < 0.001, η² = 0.30). Within the BL6 strain, females buried more marbles than males (p < 0.01, LSMD = 6 marbles, 95% CI: 2 to 11). Among males both BKS and db/+ buried more marbles than BL6 (p < 0.05, BKS LSMD = 7 marbles, 95% CI: 2 to 12, db/+ LSMD = 5 marbles, 95% CI: 0.01 to 9). Prior reports of BKS marble burying are lacking, and we expected BL6 and BKS mice to bury marbles similarly. Thus, BKS mice were re-tested 4 h after the last water T maze test. BKS buried less marbles (F (1, 28) = 31, p < 0.001, η² = 0.53 (mean ± SEM = 3 ± 1 marbles, p < 0.005, 95% CI: females, −5.6 marbles, −12 to −3.8, males −5.8, −10 to −1.7) then they had previously buried after their sociability preference tests.

3.3.3. Social dominance in tube test

The tube test reveals relative social dominance in mice (Wang et al., 2014). Many social dominance studies using the tube test do not include female mice. We initially tried BL6 and db/+ females in an exploratory
trial and found they do exhibit social dominance if matched against 129S1/SvImJ females. Due to the timing of this discovery, 3 db/+ females had already arrived to water T-maze and could therefore not be tested for social dominance. For this reason, sample sizes in social dominance tests were 10 male BL6 and db/+ females, 9 female BL6 and only 5 db/+ females. Our 2-way ANOVA of mean % wins found a strain effect (F(2, 44) = 8.3, p < 0.001, $\eta^2_p = 0.27$), with no interaction (F(2, 44) = 0.17, p = 0.84) or sex effect (F(1, 44) = 0.49, p = 0.49). More tube test matches were won by db/+ mice as compared to BL6 mice (p < 0.05, LSMD and 95% CI: females 30%, 3 to 58%; males 28%, 5 to 50%), while BKS mice won an intermediate % of matches not differing from either, as shown in Fig. 6a. The mean duration of tube tests was also compared, and there was a strain effect (F(2, 44) = 6, $p < 0.005$, $\eta^2_p = 0.21$), with no interaction (F(2, 44) = 0.6, $p < 0.55$) or sex effect (F(1, 44) = 0.59, p = 0.45). This was due to longer match durations with BKS females than with BL6 females (p = 0.05, LSMD = 56 s, 95% CI: 3 to 108 s), as shown in Fig. 6b.

3.3.4. Water T maze
The water T maze measures cognitive flexibility, or ability to adapt to situational changes to achieve a desired outcome (escape from water), via spatial learning plasticity (Guariglia and Chadman, 2013).

3.3.4.1. % Error-free trials. For female % correct trials/day, there was a significant effect of time (F(8, 176) = 45, p < 0.0001, $\eta^2_p = 0.67$), and the interaction (strain x time F(16, 176) = 1.7, p = 0.06, $\eta^2_p = 0.13$) approached significance. On acquisition day 1 (A1), db/+ females had lower % correct trials than BL6 (p < 0.05, LSMD = −15%, 95% CI: −56 to −1%), as shown in Fig. 7a. No other days had among strain differences for females.

For male % correct trials/day, there was a significant time effect (F(8, 200) = 33, p < 0.0001, $\eta^2_p = 0.57$), while both strain (F(2, 25) = 3.1, p = 0.06, $\eta^2_p = 0.20$) and strain x time interaction (F(16, 200) = 1.5, p < 0.09, $\eta^2_p = 0.11$) approached significance. On reversal day 1 (R1) db/+ males had lower % correct trials than BL6 males (p < 0.01; LSMD = −28%, 95% CI: −52 to 4), and BKS also nearly did (p = 0.07) as shown in Fig. 7a. No other differences were found on any other days.

3.3.4.2. Trial duration. In the average length of trials for females there was a significant interaction (strain x time F(16, 176) = 6, p < 0.0001, $\eta^2_p = 0.34$) on R1 such that trials took longer for db/+ females (p < 0.05; LSMD = 6 s, 95% CI: 0.0004 to 13 s) and BKS females (p < 0.001, LSMD = 18 s, 95% CI: 12 to 24 s) to complete versus BL6 females. BKS females also took longer than db/+ females to complete matches (p < 0.001; LSMD = 12 s, 95% CI: ≤ 18), as shown in Fig. 7b. For male mice there was a significant interaction (strain x time F(16, 200) = 3.3, p < 0.0001, $\eta^2_p = 0.21$). The average trial time was longest on R1 for BKS versus db/+ (p < 0.0001, LSMD = 17 s, 95% CI: 6.2 to 28) or BL6 males (p < 0.0001; LSMD = 25 s, 95% CI of difference: 13 to 36 s), with no difference among db/+ and BL6 means, as shown in Fig. 7b.

3.3.4.3. Cumulative errors. Among female mice there was a significant interaction (strain x time F(16, 176) = 8, p < 0.0001, $\eta^2_p = 0.42$) in cumulative errors/day in the water T maze test. On acquisition day 1 (A1) db/+ females made more errors than BL6 (p < 0.0005, LSMD = 6 errors, 95% CI:2 to 10.6) and BKS (p < 0.0001, LSMD = 9.5, 95% CI: 5 to 14) than BL6 males, as shown in Fig. 7c. Male BKS and db/+ cumulative errors did not significantly differ (p = 0.14) on R1. There were no other days with cumulative error differences.

Among male mice, the interaction was also significant (strain x time F(16, 200) = 3, p < 0.001, $\eta^2_p = 0.19$) for the cumulative errors/day. On R1 more errors were made by db/+ (strain F(2, 25) = 3.5, p < 0.05, LSMD = 5.4 errors, p < 0.005, 95% CI: 1 to 9.7) and BKS (p < 0.0001, LSMD = 9.5, 95% CI: 5 to 14) than BL6 males, as shown in Fig. 7c. Male BKS and db/+ cumulative errors did not significantly differ (p = 0.14) on R1. There were no other days when cumulative errors differed.

In comparing female to male cumulative errors/day on A1, there was a sex x strain interaction (F(2, 47) = 4.2, p < 0.05, $\eta^2_p = 0.15$), with no significant effect of sex (p = 0.74) or strain (P = 0.14). The only difference in cumulative error was that db/+ females made more errors than BL6 (p < 0.05, LSMD = 6, 95% CI: 0.3 to 12). By contrast on day R1 there was a significant strain effect (F(2, 47) = 25, p < 0.0001), and sex effect (F(1, 47) = 13.8, p < 0.001), with no interaction (F(2, 47) = 12, p < 0.17). This was due to BKS females making more errors than BKS males on R1 (p < 0.005, LSMD = 9, 95% CI: 2.8 to 15.4).

3.3.4.4. R1 perseverative and regressive errors. The number of perseverative errors differed by strain (F(2, 47) = 21, p < 0.0001, $\eta^2_p = 0.47$) and sex (F(1, 47) = 14, p < 0.001, $\eta^2_p = 0.23$) with no interaction. BKS females made more perseverative errors than db/+ (p < 0.005, LSMD = 10, 95% CI: 3.5 to 16), or BL6 (p < 0.001, LSMD = 14, 95% CI: 8 to 20). Male BKS also made more perseverative errors than BL6 (p < 0.05, LSMD = 9, 95% CI: ≥ 3 to 15), as seen in Fig. 7d. There was no difference in incidence of regressive errors (interaction F(2, 47) = 1.8, p = 0.18, strain F(2, 47) = 1.5, p = 0.22, sex F(1, 47) = 0.57, p = 0.45), as shown in Fig. 7e.

4. Discussion
The main autism-relevant behavior finding of this study was lower
Male social interaction preference as measured by time spent in chambers relative to BL6, but not BKS, males. However, when sniffing time, which seems to be more representative of direct interactions with novel mice, was compared there were no differences in social interaction preference among db/+, BKS or BL6 mice. We also observed more marble burying by BKS and db/+ males; and impaired cognitive flexibility in both male and female BKS and db/+ mice as evidenced by their greater execution of platform location errors, and additional time needed to complete day 1 reversal learning trials in the water T maze.

Key physiological discoveries from this study were that BKS db/+ males gained more weight, and BKS db/+ males and females had higher blood glucose and corticosterone levels than BL6 mice, but not BKS mice.

A limitation in our experimental design is that we did not directly compare effects of GDM exposure in offspring, as could be achieved by comparing offspring behaviors from BKS db/+ females crossed with BKS males to those of BKS females crossed with BKS db/+ males, paralleling the elegant approach of a prior study examining physiological and behavioral effects of GDM exposures in C57BL/6 J db/+ mice (Pollock et al., 2015). This would have been a logical next step, had it not been for discovery of elevated 15 min blood glucose in tolerance tests of pregnant BKS females. Furthermore, all findings of autism relevant behaviors were derived from comparisons of db/+ to BL6 mice, and we found that db/+ and BKS behavioral phenotypes rarely differed. This collective outcome of our initial assessment indicates BKS db/+ mice may not be a valid model to use for assessing effects of GDM exposure on offspring behaviors relevant to autism, since the appropriate strain control for db/+ males, BKS, itself shows some autism-like behavior deficits. Another consideration is that the BKS subjects used in this study were purchased for the study rather than being bred in house, which introduces a potential confound due to early life exposure to different environments. The BKS mice for this study were purchased directly because according to the supplier, The Jackson Laboratory, they are a challenging strain to
breed (poor breeders), and this in our hands was also true as only 2 of 8 were pregnant after 6 weeks. A major limitation to drawing solid conclusions from this pilot study is the lack of replicates from each experiment, as the data is derived from a single cohort of mice, and because the study was not repeated.

Time spent in chambers during the standardized three chamber social interaction preference tests we used was initially thought to be the most useful parameter relevant to autism of all the behaviors that could be measured (Moy et al., 2004). Social sniffing time during these tests was subsequently added as a confirmation of chamber dwelling times (Moy et al., 2007). We found that db/+ males exhibited deficits in social interaction preference by chamber dwelling (Fig. 3b), but not by sniffing behavior (Fig. 3d). Furthermore, the difference found was relative to BL6 mice, while the proper control for db/+ is the BKS mouse. Likewise, physiological differences between db/+ and BKS were less clear cut than among db/+ and BL6, indicating that the BKS background strain contributed more than loss of the Lepr to behavioral and physiological properties of BKS db/+ mice. Contributions of genes from other strains such as 129S may mask a substantial mark on BKS and db/+ mouse phenotypes (Mao et al., 2006; Fontaine and Davis, 2016).

Prior studies in a BL6 fetal growth restriction mouse model showed that insufficient leptin levels early in life also result in impaired social interaction in both male and female mice (Meyer et al., 2014). By contrast, in this study db/+ male social preference was attenuated, but BKS and db/+ female offspring exhibited social preferences that were no less than those of BL6, and indistinguishable from BKS females. This outcome was also unexpected, given prior reports of a trend toward social interaction deficits in female Dock7 mutation carriers (Blasius et al., 2009). The reduced chamber entries by db/+ mice that we found in Fig. 4a are consistent with prior reports of hypo-locomotor activity in BKS db/db mice (Sharma et al., 2010). By contrast wild-type offspring of db/+ dams on a BL6 background had increased locomotor activity and exploratory behaviors relative to wild-type offspring of wild-type dams (Pollock et al., 2015). This indicates that the background strain (BKS versus BL6) of db/+ mice shapes how maternal and offspring Lepr deficient genotypes can interact to shape offspring locomotor behaviors.

While among obese patients, complete Lepr deficiency (db/db equivalent) is rare (<3% prevalence), other function-impairing mutations have ≈ 35% prevalence and may contribute to obesity or type 2 diabetes (Baxter et al., 2019). Overexpression of Lepr may also be detrimental to social behaviors, as two overlapping Lepr gene duplications were identified in unrelated autism cases (Prasad et al., 2012). Disruption of leptin signaling via Lepr may also contribute to human GDM, but few studies have directly examined this relationship, and those that have presented a complex picture of extremes. At one extreme, a clinical study of women with GDM had reduced serum leptin and its soluble receptors between 24 and 28 weeks of pregnancy (Mosavat et al., 2018). Genetic variation in Lepr plays a large role in placental leptin expression (Vlahos et al., 2020). Also, epigenetic influences of maternal obesity are associated with placental Lepr downregulation (Nogues et al., 2019).

At the other extreme, serum leptin was significantly higher in women with GDM than in women without it (Chen et al., 2010). Leptin resistance, which can stem from reduced transport to Lepr in brain, may underlie compensatory increases in serum leptin with obesity and/or in pregnancy (Kang et al., 2020). Consistent with this, high childhood leptin levels and rapid weight gain were associated with a greater risk of autism in the Boston Birth Cohort study, suggesting obesity or heritable forms of leptin resistance may be involved (Ragbhavan et al., 2018). A more recent study also found relatively elevated serum leptin in children with autism (Celikkok Sadde et al., 2010). Due to lack of a functional long form of Lepr, db/db and db/+ mice are reported to have elevated leptin (Singer et al., 2009; Osborn et al., 2010). In BL6 mice mid-gestation is a critical stage for sex-specific leptin programming of fetal and postnatal growth (Denisova et al., 2020). Leptin signaling plays many important roles in perinatal development and its disruption can contribute to psychiatric as well as physiological disorders such as high blood pressure (Valleau and Sullivan, 2014; Steinbrekera and Roghair, 2016). Furthermore, it is important to consider Lepr is a cytokine family receptor, closely related to the interleukin IL-6 receptor, a mediator of pro-inflammatory responses. Since other cytokine family receptors are closely related to Lepr in structure, it is plausible that at high levels, leptin may bind to them and trigger a low-grade inflammatory response (Ikuni et al., 2008; Wauman et al., 2017). This may contribute to immune dysfunction marked by elevated IL-6, that is reported to occur in some cases of autism (Vargas et al., 2005; Deverman and Patterson, 2009; Depino, 2013; Siniscalco et al., 2018; Saghazadeh et al., 2019).

Another social behavior outcome from the present study that was rather surprising was greater social dominance of BKS db/+ relative to BL6 in tube tests, where each female or male subject was matched against a common set of 6 age and sex matched female or male 129S1/SvImJ stranger mice for comparison. Other researchers have utilized this test in upregulated serotonin transporter-based mutant mouse models of autism, by matching male controls and mutant mice directly against each other and found the mutant mice won fewer matches (Veenvstra VanderWeele et al., 2012). This outcome is consistent with the understanding that children with autism may perceive social rank of others more strictly, but in a different manner from normal subjects (Ogawa et al., 2019). Since it has been shown that leptin in the brain reduces serotonin transporter density (Charnay et al., 2000) presumably via Lepr, then attenuated Lepr function might be expected to result in higher serotonin transporter expression, and less dominant behavior in tube tests. This outcome would also have been consistent with the discovery of depression-like behaviors in adult mice with induced Lepr deficiency in hippocampus (Guo et al., 2013), given the abundance of neurons co-expressing Lepr and serotonin transporters therein. However, we found db/+ male mice won more rounds than BL6 mice (but not the BKS mice) which was inconsistent with the outcome of an upregulated serotonin transporter.

However, a similar outcome occurred with social dominance in a potassium-chloride cotransporter (KCC2) mutant mouse model of autism, which had disrupted prefrontal cortex signaling (Anacker et al., 2019). Also greater social dominance may not be entirely inconsistent with clinical manifestations of autism. In adolescent peer situations, children with autism are not only victims of bullying (67% of reports) but also become involved as perpetrators (29% of reports) at schools (Park et al., 2020). The tube test measure of social dominance has been validated by other measures, including barbering (removing hair/whiskers of cage mates), as a reliable indicator of social status; and social dominance has been traced to the medial prefrontal cortex (Wang et al., 2014). Differences in how this brain region is neurologically activated by leptin during development may be a key determinant of not only social preference (Meyer et al., 2014) but also dominant tube test behavior outcomes (Veniaminova et al., 2017). Social dominance tube test outcomes were not extensively reported for female mice, although barbering by female mice was characterized in several mouse strains (Kalkeff et al., 2006). Since breeding db/+ females barbered their mates, we decided to examine BL6 and db/+ females in tube tests against age matched 129S1/SvIMJ. We learned females of both strains could indeed perform the tube test within the same timeframe and with equivalent between-strain (db/+ more dominant than BL6) outcomes as the male mice. We hope future studies uncover the mechanisms underlying greater db/+ social dominance.

Restrictive-repetitive behaviors characteristic of autism were paralleled by db/+ increased marble burying and impaired reversal learning in the water T maze relative to BL6, but not if compared to their strain control BKS. Since db/+ males and BKS mice also made fewer chamber entries than BL6 males during sociability tests, reduced locomotor activity in these strains cannot be ruled out as a potential confounding factor. However, based on casual observation of their activities in their home cages, they did not exhibit any major locomotor deficits.
Furhthermore, BKS mice engaged in extensive self-grooming and preference for familiar versus stranger mice, while db/+ mice did not. Also, reversal learning deficits of db/+ and BKS mice relative to BL6 in terms of cumulative errors and % correct trials observed on day 1 were not persistent, as they were not evident on day 2 of reversal training. In this test BTBR mice exhibited more persistent deficits over days of reversal learning and exhibited both regressive and perseverative errors (Guariglia and Chadman, 2013). In contrast, aside from reversal learning day 1 deficits shared by BKS and db/+ mice, subsequent reversal days did not differ indicating the cognitive flexibility deficit was short lived, in comparison to other strains such as BTBR mice.

Regarding how physiological characteristics of db/+ offspring may shape the behaviors relevant to autism that we measured, aside from leptin, serum corticosterone is one potential hormonal mediator. BKS db/+ mice had elevated serum corticosterone relative to BL6 (but again not BKS). This is consistent with prior findings that db/+ male mice on the BL6 background had similar corticosterone levels to db/db mice in prior reports (Burke et al., 2017). Elevated serum corticosterone, and a general state of physiologic arousal has been reported to occur in Fragile X syndrome, but not necessarily in other forms of autism spectrum disorders (Tordjman et al., 2014, Roberts et al., 2018). Prior studies have described an increase in serum cortisol, the human equivalent of corticosterone, occurs as adolescents with autism interact with other children voluntarily or for play as opposed to in anticipation of the social interaction (Taylor et al., 2018; Edmiston et al., 2017, Corbett et al., 2019). Hence in autism, altered timing of the cortisol response appears to be more relevant to shaping social behaviors than an elevated baseline level, as seen in our BKS db/+ . In children exposed in utero to GDM, blunted cortisol stress responses were found (Van Dam et al., 2018). Altered HPA axis function may be the product of serotonin system disruption in fetal development, which is also reported to occur with weight-matched C57BL/6J mice fed a Western diet. J. Diabetes Res., 2017:8503754. doi:10.1155/2017/8503754. PMID:29038790.

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This study is the first we are aware of that assesses autism-like behaviors in male and female offspring from heterozygous db/+ (BKS-Cq-Dock7+/–/J) mouse model of GDM and its BKS background strain. Given recent clinical discoveries of associations between GDM and elevated risk of autism (RR: 1.48: 95% CI: 1.26–1.75, as in Wan et al., 2018), we hoped to find BKS db/+ and its background strain BKS to be a suitable mouse model of GDM in which to study potential mechanisms of action that may contribute to associations with autism. Prior studies suggested the db/+ mouse recapitulated human GDM better than diet or drug-induced diabetes (Pasek and Gannon, 2013), which is why we set out to characterize autism-relevant behaviors of BKA db/+ offspring. We found male social interaction preference deficits, restrictive-repetitive behaviors and increased social dominance in db/+ offspring of db/+ mice, which develop GDM, as compared to BL6 mice that do not. These behavioral phenotypes were accompanied by higher serum corticosterone, weight, and fasting blood glucose. However, BKS mice also exhibited many of the same behaviors as BKS db/+ . This indicates genetic differences between BKS and BL6 strains are prominent contributing factors to BKS db/+ behavior phenotypes that can potentially impair the ability to detect treatment-related changes in such measures.

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