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Gestational Exposure to a Ketogenic Diet Increases Sociability in CD-1 Mice.

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Abstract

Postnatal administration of high-fat, low-carbohydrate ketogenic diets (KDs) is an established and effective treatment option for refractory epilepsy, with more recently identified therapeutic potential across a wide range of preclinical models of neurological and psychiatric disorders. However, the impact of gestational exposure to a KD (GKD) on offspring development remains unclear. Previous work has found that GKD exposure reduces depression- and anxiety-like behaviors in CD-1 mice, whereas postnatal KD improves sociability in several different rodent models of autism. Here we examined how sociability is impacted by GKD. Given that the neuropeptide oxytocin positively regulates affect, anxiety, and sociability, we also examined the effects of GKD on brain oxytocin expression. Male and female CD-1 mice exposed to either a standard diet (SD) or a KD gestationally were cross-fostered with SD dams at birth and remained on a SD from that point onwards. These offspring were then tested for sociability and social novelty (three-chambered test) and depressive-like behaviors (forced swim test) at 10 weeks of age. At the conclusion of testing, brain tissue was collected and immunohistochemically processed for oxytocin expression in hypothalamic and limbic areas. We found that GKD increased sociability and reduced depressive-like symptoms, without affecting oxytocin expression in quantified areas. By expanding the scope of the lasting impact of gestational exposure to a ketogenic diet to include positive effects on sociability, these results indicate that GKDs may have novel therapeutic applications for individuals at risk for developmental disorders of social behavior, including autism and schizophrenia.

Keywords: Ketogenic; Metabolic therapies; Oxytocin; Sociability

Gestational Exposure to a Ketogenic Diet Increases Sociability in CD-1 Mice

Ketogenic diets (KDs) are high fat, low carbohydrate, adequate protein formulations with a history of clinical use in the treatment of drug-resistant (i.e., refractory) epilepsy that spans nearly a century (Wheless, 2008). The specific neural mechanisms underlying the therapeutic effects of KDs in epilepsy are varied and involve processes that are implicated in a wide range of neurological and psychiatric disorders. These effects include stabilized blood glucose levels, increased brain ATP as well as increased levels of ATP-derived neurotransmitters (e.g., adenosine), increased GABAergic (relative to glutamatergic) tone, improved mitochondrial function and antioxidant capacity, and decreased neuroinflammation (Boison, 2017). It is not surprising, then, that researchers have more recently begun to explore the potential of KDs in other clinical disorders. Indeed, postnatal administration of KDs to male mice improves sociability/social communication and decreases repetitive behaviors in rodent models of autism (Castro et al., 2017; Ruskin et al., 2013; Ruskin, Fortin, et al., 2017; Ruskin, Murphy, et al., 2017; Verpeut et al., 2016). Similar improvements are observed in models of schizophrenia; specifically, male mice treated with dizocilpine show improvements in sociability, decreases in repetitive behaviors, and have restored sensorimotor gating (Kraeuter et al., 2015, 2019). Although clinical evidence is quite limited to date, the available data suggest that postnatal administration of KDs to individuals with schizophrenia and autism improves symptomology in these patients (Bostock et al., 2017).

As an increasing number of individuals initiate a KD for therapeutic or other reasons, the likelihood that individuals of childbearing age become pregnant while on these diets also increases. Pregnancy is associated with a suite of metabolic adaptations that are required to support the demands of the developing fetus (Zeng et al., 2017); when this process is disrupted

(e.g., due to maternal undernutrition), there can be lasting negative outcomes for the offspring (Painter et al., 2005). At present it is unclear how maintaining a KD during pregnancy impacts the developing fetus. The limited available data from case studies indicate that women with epilepsy have been able to successfully maintain a KD throughout pregnancy and subsequently give birth to healthy infants (van der Louw et al., 2017). In a series of pre-clinical studies, Sussman and colleagues found that gestational exposure to a ketogenic diet (GKD) in CD-1 mice affects both pre- and postnatal development of offspring. Specifically, they found that overall brain volumes were smaller at embryonic day 13.5 (E13.5) in GKD vs. mice gestationally exposed to a standard diet (GSD), larger at embryonic day 17.5 (E17.5), and equivalent when examined on postnatal days 11.5 (P11.5) and 21.5 (P21.5) (Sussman, Ellegood, et al., 2013; Sussman, van Eede, et al., 2013). The impact of GKD on volumes of specific brain areas is similarly complex and dependent both on brain area and developmental time point examined; for example, the volume of the hypothalamus is relatively smaller in GKD vs. GSD mice at E13.5, equivalent at E17.5, and larger at P11.5 and P21.5 (Sussman, Ellegood, et al., 2013; Sussman, van Eede, et al., 2013). When tested as adults, GKD mice exhibit decreased locomotor activity and increased time spent in the center area in an open field test, as well as decreased immobility time in the forced swim test (Sussman et al., 2015). These findings suggest that GKD has lasting, positive impacts on offspring affect/mood that may be due in part to its effects on early brain development.

When considered in light of the ability of postnatal KD exposure to increase sociability, the changes in pre- and postnatal hypothalamic volumes observed in GKD mice raise the possibility that gestational exposure to these diets may have similar effects via altered development of neurochemical systems within the hypothalamus important for sociability, such

as the neuropeptide oxytocin. Indeed, the hypothalamic oxytocin system in rodents is first detected at approximately E16 (Whitnall et al., 1985), with oxytocin receptors present in various target tissues by E18.5 (Hammock & Levitt, 2013). To begin to test our hypothesis, GKD and GSD mice were examined for social behavior (three-chambered sociability and social novelty test) and affect (forced swim test) at 10 weeks of age. At the conclusion of testing, brains were processed for oxytocin immunohistochemistry. We found that GKD mice spent more time investigating a social stimulus in the sociability test and had decreased immobility time in the forced swim test when compared to GSD mice; in contrast, we found no difference in oxytocin immunoreactivity in the limbic/hypothalamic brain areas examined.

Method

Animals

CD-1 mice were group-housed in polycarbonate cages with wire mesh tops and woodchip bedding; nestlet (Ancare, Bellmore, NY) bedding material was also provided. This specific outbred strain of mice was chosen to allow for the current work to be readily placed within the context of the established literature on gestational exposure to a ketogenic diet (GKD) (Sussman, Ellegood, et al., 2013; Sussman et al., 2015; Sussman, van Eede, et al., 2013). Animals were maintained on a 12:12 hr light:dark cycle with lights on at 7 am; all testing occurred within the light phase of the cycle. Food and water were available *ad libitum*. Animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (8^{th} Ed.) and approved by the Trinity College Institutional Animal Care and Use Committee.

Diet and Breeding

Breeder male and female mice were obtained from Charles River Laboratories at approximately 60 days of age and group-housed by sex upon arrival. After a one-week

acclimation period, mice were separated into groups based on sex and then assigned to either remain on a standard diet (SD; LabDiet 5001, Lab Supply, Fort Worth, TX) or be switched over to a KD (Teklad TD.96355, Envigo, Madison, WI). The macronutrient profiles of these diets are provided in Table 1. This specific KD formulation was chosen based on previous reports confirming that pregnant dams can be maintained on this this diet throughout their pregnancy without adverse health effects for the dam or offspring (Sussman, Ellegood, et al., 2013; Sussman et al., 2015; Sussman, van Eede, et al., 2013). Diet consumption by breeders was not monitored; previous studies in mice have found that animals given *ad libitum* access to a KD self-regulate caloric intake such that it does not differ from that of animals on a SD (Badman et al., 2009). Breeding cages (one male plus three females) were established for each diet condition after animals had been on their assigned diets for one week. Breeders remained on their assigned diets for the duration of their involvement in the study. A separate set of breeders were set up as described above; however, they were maintained on a SD throughout and were utilized strictly for cross-fostering purposes (see below). In all cases, breeding cages were checked daily for the presence of pups.

Cross-fostering

Following parturition, lactating dams maintained on a KD may rapidly (within five days) enter a state of ketoacidosis, which can eventually lead to the death of the dam as well as her offspring (Sussman, Ellegood, et al., 2013; Sussman et al., 2015). Consequently, offspring of experimental (both SD and KD) dams were cross-fostered with foster dams maintained on a SD by postnatal day 1.5 (P1.5). Foster dams were first confirmed to be lactating; in all cases, they had pups of approximately the same age as those of the experimental dams. Litters were culled to

ensure that there were no more than eight pups per foster dam. Experimental dams and sires were euthanized (see below) once all dams in a breeding cage had given birth.

At weaning (P21.5), male and female mice exposed gestationally to a SD (GSD) or GKD were group-housed (three-four animals per cage) by sex and diet condition. No more than four offspring of a given sex from a specific litter were utilized in subsequent behavioral testing; in all cases, offspring from at least four separate litters contributed to the sample sizes for each individual treatment group. It is also important to note that offspring were never exposed (directly or indirectly) to a KD after P1.5, given that they were maintained on a SD from this time point through the conclusion of behavioral testing.

Behavioral Testing

Three-chambered sociability and social novelty test (three-chambered test).

At 10 weeks of age, GSD and GKD mice were tested for their sociability and preference for social novelty using a protocol adapted from that of Crawley and colleagues (Yang et al., 2011). A series of three 10-minute tests were conducted in a Plexiglas arena (22 x 42.5 x 19 cm) divided into three chambers: two outer chambers, each containing a stainless-steel wire cup (10 (h) x 10 (d) cm), and one central chamber. All tests were recorded via a digital video camera and behavioral measures (see below) were scored from video by trained researchers blind to the experimental condition of the subjects. All scoring was done using the JWatcher (v 1.0) software for Windows or Mac.

In the first phase (habituation phase), test subject mice were briefly confined in the central chamber, the doors were opened, and then the subject was allowed to freely explore the apparatus for 10 minutes. In addition to habituating the subject to the apparatus, this phase also allowed for the assessment of locomotor activity (number of chamber crossings) and self-

directed behaviors (time spent grooming) in the absence of any social stimulus. The second phase (sociability phase) began immediately following the conclusion of the habituation phase. Test subjects were again confined within the central chamber, and during this time a novel stimulus mouse was introduced into one of the two wire cups in the outer chambers, with the other cup left empty. The stimulus mice used here and in the subsequent testing phase were all juvenile CD-1 mice that were unrelated to, and of the same sex as, the test subject mice. After the stimulus mouse was secured in the wire cup, the apparatus doors were opened and the test subject mouse was allowed to freely explore the apparatus for 10 minutes. The duration of time spent in each chamber and time spent making direct contact with each wire cup were quantified. For both measures, the preference for sociability was calculated as social time divided by the sum of social and nonsocial time. Finally, mice were tested in a third phase (social novelty phase) 30 minutes following the conclusion of the sociability phase. In this 10-minute phase, the now-familiar mouse from the sociability phase was presented again in the same wire cup, and a novel stimulus mouse was introduced into the second wire cup in the other outer compartment. The same behaviors assessed in the sociability phase were also assessed in this phase; in this case, the preference for social novelty was calculated as novel time divided by the sum of novel plus familiar time. Although it is more common to test the preference for social novelty immediately following the sociability phase when performing the three-chambered test, it has previously been reported that mice are able to show a significant preference for social novelty when the delay is extended to 30 minutes (J.-B. Zhang et al., 2016). Furthermore, extending the intervening time to 30 minutes may help eliminate the ceiling effect in social novelty preference observed when animals are tested for novelty preference immediately following sociability testing.

Experimental and stimulus mice were returned to their home cages following the conclusion of the three-chambered test. The testing apparatus was cleaned with SaniCloth Plus wipes (PDI, Orangeburg, NY), rinsed with water, and dried between subjects.

Forced swim test (FST).

Four days following the completion of the three-chambered test, subjects underwent the FST (Yankelevitch-Yahav et al., 2015). Subjects were placed in a Plexiglas beaker (25 (h) x 15 (d) cm) filled 3/4 with 25°C tap water. At the conclusion of the six-minute test period, the subject was removed, dried with a cotton towel, and placed into a warming cage until completely dry. The beaker was cleaned with SaniCloth Plus wipes, rinsed with water, and refilled between subjects. Tests were recorded with a video camera and scored from video by trained researchers blind to the experimental condition of the subjects. All scoring was done using JWatcher software. The total time spent immobile (floating with a complete lack of voluntary motion, other than minor movements of one-two limbs required to keep the animal's position stable) was quantified during the final four minutes of the six-minute test.

Euthanasia and Blood Collection

Mice were euthanized 24 hours following the FST (experimental offspring) 24 hours after the final dam in a breeding cage had given birth (breeders) via an overdose of isoflurane gas. Blood samples were taken from the lateral tail vein during the euthanasia procedure. Glucose and ketone (β-hydroxybutyrate) levels in blood samples were determined using Precision Xtra meters (Abbott Laboratories, Bedford MA).

Perfusions and Histology

A subset of experimental offspring were overdosed with isoflurane gas and then transcardially perfused with ~50 ml of 0.1 M phosphate buffered saline (PBS) followed by ~50

ml of 4% paraformaldehyde (EMS, Hatfield, PA) in PBS. Brains were removed and post-fixed in 4% paraformaldehyde in PBS overnight followed by 30% sucrose in PBS (1–2 days). Coronal sections (40 μm) were taken using a cryostat and stored in cryoprotectant at −20°C until processed for immunohistochemistry (IHC) for oxytocin.

Free-floating coronal sections were removed from cryoprotectant, rinsed in PBS, and then incubated in 0.3% hydrogen peroxide in PBS for 15 minutes in order to quench any endogenous peroxidase activity. After rinsing in PBS, sections were incubated in 3% normal horse serum (S-2000; Vector Laboratories, Burlingame, CA) in PBS for one hour. Sections were then incubated in a solution containing PBS, 0.4% Triton-X (Sigma, St. Louis, MO), and 1:10,000 polyclonal mouse anti-oxytocin (AB911; Millipore, Temecula, CA) overnight (~18 hours) at room temperature. After rinsing in PBS, tissue sections were incubated in a solution of PBS, 0.4% Triton-X, and 1:600 biotinylated horse anti-rabbit secondary antibody (BA-1100, Vector Laboratories) for one hour at room temperature, rinsed in PBS, and then incubated in a solution of PBS, 0.4% Triton-X, and 1:200 avidin–biotin complex (Vectastain Elite ABC kit; Vector Laboratories) for one hour at room temperature. Tissue sections were rinsed in PBS followed by Tris buffer, and reacted in a 3,3′-diaminobenzidine tetrahydrochloride solution (20 mg 3,3′-diaminobenzidine tetrahydrochloride with 83 μl 30% hydrogen peroxide per 100 ml of Tris buffer; Sigma, St. Louis, MO) for 15 minutes at room temperature, resulting in a brown chromogen reaction product. Tissue sections were rinsed again in Tris buffer followed by PBS in order to terminate the chromogen reaction. At the completion of IHC processing, tissue sections were mounted on gelatin-subbed, positively-charged slides and dried overnight. Slides were then dehydrated through an ethanol series, cleared with Cirtrisolv (Fisher Scientific, Pittsburgh, PA), and coverslipped using Permount (Fisher Scientific).

Imaging and Cell Counting

Tissue sections containing the brain areas of interest were imaged at 10x magnification using a Nikon DS-Ri2 digital camera connected to a Nikon Eclipse E600 light microscope. Photomicrographs were captured by a computer running Nikon NIS Elements software for Windows. Rectangular counting domains (0.53 mm x 0.31 mm) were fitted onto the bed nucleus of stria terminalis (ST) and the paraventricular nucleus (Pa), and each image was then cropped to the boundaries of the fitted counting domain using Inkscape (v 0.92) software for Mac. Cropped images were imported into ImageJ (v 2.0.0) software for Mac, converted to grayscale, and the contrast of each image was enhanced. Darkly stained cell bodies were considered to be positive for oxytocin and were counted using the cell counter plugin for ImageJ. All counting was done by trained experimenters that were blind to treatment condition. Counts of oxytocin-positive cell bodies were averaged across hemispheres (both ST and Pa) and across atlas plates (Pa), and then divided by the area of the counting domain (0.16 mm^2) to generate cell densities.

Statistical Analyses

All data were analyzed using JASP (v 0.10.2) software for Mac. Data were first examined for skewness, kurtosis, and normality (Shapiro-Wilk test) in order to determine if the assumptions of parametric statistical tests were met. When identified, outliers (scores outside the interquartile range (IQR) by more than 1.5*IQR) were removed from the affected data set. No more than one outlier was removed from a single treatment group, per outcome measure. This resulted in analyzed sample sizes that ranged between 7-13 individuals. A power analysis conducted *a priori* determined that a sample size of $n = 7$ is required to observe significant main effects, given $\alpha = 0.5$, $\beta = 0.8$, and $\delta = 0.75$ (medium effect) (Lenth, 2006). Grooming data were square root transformed to address significant positive skewness.

Data from offspring were examined for the effects of diet (GSD vs. GKD) and sex (male vs. female) on each dependent measure using factorial ANOVAs. Data from breeders were examined for the effects of diet (SD vs. KD) on blood glucose and ketone levels using independent samples *t*-tests. In all cases, *p*-values of less than 0.05 were considered *a priori* to be statistically significant. Effect sizes are reported as partial η^2 (ANOVA analyses) or Cohen's *d* (*t*-test analyses).

Results

Behavioral Measures

Starting at 10 weeks of age, male and female GSD and GKD mice were tested for their preference for sociability and social novelty using the three-chambered test. In the absence of any social stimuli (habituation phase), there was no statistically significant effect of gestational diet exposure condition on the duration of time spent grooming, $F(1,43) = 1.16$, $p = 0.29$, $\eta^2 = 0.03$, (Figure 1A). However, GKD mice had significantly fewer chamber crossings compared to GSD mice, $F(1,42) = 24.90$, $p < 0.001$, $\eta^2 = 0.37$ (Figure 1B), during this phase. When subsequently tested with an unfamiliar same-sex individual placed in one of the outer chambers (sociability phase), GKD mice exhibited an increased relative preference to spend time in the same chamber as the social stimulus, $F(1,45) = 4.77$, $p = 0.03$, $\eta^2 = 0.10$, and an increased relative preference to investigate the social stimulus, $F(1,45) = 5.09$, $p = 0.03$, $\eta^2 = 0.10$, compared to GSD mice (Figure 2). This increase in sociability cannot be attributed to alterations in locomotor activity given that there were no statistically significant differences in the total number of chamber entries during this phase, $F(1,45) = 0.81$, $p = 0.37$, $\eta^2 = 0.02$ (Figure 3A), or in the relative preference to enter into the chamber containing the social target, $F(1,44) = 3.11$, $p = 0.09$, $\eta^2 = 0.07$ (Figure 3B). During the final phase (social novelty phase), a novel same-sex

individual was placed in one chamber and the now familiar same-sex individual from the prior phase was placed in the other chamber. There were no statistically significant effects of gestational diet exposure condition on either the relative preference to spend time in the chamber containing the novel social stimulus, $F(1,45) = 3.04$, $p = 0.09$, $\eta^2 = 0.06$, or the relative preference to investigate the novel social stimulus, $F(1,45) = 1.85$, $p = 0.18$, $\eta^2 = 0.04$.

Male and female GSD and GKD mice underwent the FST four days following completion of the three-chambered test. GKD mice spent significantly less time immobile compared to GSD mice, $F(1,45) = 68.08$, $p < 0.001$, $\eta^2 = 0.61$ (Figure 4).

Physiological Measures and Histology

At the completion of behavioral testing, GSD and GKD mice were weighed and blood glucose and ketone levels were assessed. Male mice weighed significantly more than female mice, $F(1,45) = 19.66$, $p < 0.001$, $\eta^2 = 0.30$; however, there was no statistically significant effect of gestational diet exposure condition on weight, $F(1,45) = 0.60$, $p = 0.44$, $\eta^2 = 0.01$ (Figure 5A). Similarly, there was a significant effect of sex (male > female), $F(1,44) = 14.92$, $p < 0.001$, $\eta^2 = 0.25$, but not gestational diet exposure, $F(1,44) = 3.15$, $p = 0.08$, $\eta^2 = 0.07$, on blood glucose levels (Figure 5B). There was no statistically significant effect of sex on blood ketones, $F(1,44) = 0.14$, $p = 0.71$, $\eta^2 = 0.003$, although ketone levels were significantly lower in GKD vs. GSD mice, $F(1,44) = 10.01$, $p = 0.003$, $\eta^2 = 0.19$ (Figure 5C).

Breeders assigned to either a SD or KD were also assessed for blood glucose and ketone levels following removal of litters for cross-fostering. Blood glucose levels did not significantly differ across diet conditions, $t(14) = -1.49$, $p = 0.16$, $d = -0.745$ (Table 2). As expected, blood ketone levels were higher in mice maintained on a KD vs. a SD, $t(14) = -6.73$, $p < 0.001$, $d = -3.37$ (Table 2).

A subset of brains from GSD and GKD mice underwent IHC for oxytocin immunoreactivity, and oxytocin-positive cells were subsequently quantified in the ST and Pa (Figure 6). There were no statistically significant effects of gestational diet condition on the number of oxytocin-positive cells in either the ST, $F(1,31) = 1.29$, $p = 0.26$, $\eta^2 = 0.04$, or the Pa, $F(1,35) = 0.37$, $p = 0.55$, $\eta^2 = 0.01$ (Figure 7).

Discussion

We show here that gestational exposure to a ketogenic diet (GKD) positively impacted markers of sociability and affect in adult CD-1 mice. These effects were not sex-specific and were not associated with any lasting impact on body weight, blood glucose levels, or oxytocin expression in hypothalamic/limbic areas. It is also important to note that although GKD mice were significantly less active in the first (habituation) phase of the sociability and social novelty test (three-chambered test), there were no differences in activity in the second (sociability) phase; as such, the increased sociability observed here cannot be attributed to altered locomotor activity during this phase. The ability of GKD to further increase sociability in a strain of mice that is already highly social suggests that targeting exposure to a KD strictly to the gestational period could have potential as a novel therapy for treating developmental disorders involving social deficits.

The increase in sociability observed in GKD mice is in good agreement with previous studies using postnatal KD exposure protocols. Young adult male rats treated for four weeks with a KD starting at 28 days of age show increased sociability in a social interaction test (Kasprowska-Liśkiewicz et al., 2017). This increase is particularly interesting to note given that both the Kasprowska-Liśkiewicz et al. (2017) study and the present work utilized outbred rodent strains that exhibit normal sociability in the absence of any diet manipulation. KDs are also

effective at improving sociability when administered postnatally to rodent models of deficient social behavior. BTBR mice are an inbred strain that exhibit abnormalities in social interactions, social communication, and repetitive behaviors (McFarlane et al., 2008). When male BTBR mice are tested in the three-chambered test as young adults following at least three weeks on their assigned diets, sociability improved in KD-treated mice to the extent that it was comparable to that of typical mice. Furthermore, these KD-treated BTBR mice also show reductions in stereotyped grooming responses and exhibit improved social transmission of food preferences (Ruskin et al., 2013). Young adult male mice exposed to the anti-epileptic drug VPA during gestation show similar reductions in sociability and increases in repetitive behaviors, which can be reversed following approximately five-six weeks of KD treatment initiated at weaning (Castro et al., 2017). There are widespread alterations in the gut microbiota in both of these models of autism, which to some extent mirror the changes observed in individuals with autism (de Theije et al., 2014; Li et al., 2017; Newell et al., 2016); KD treatment of juvenile male BTBR mice for up to two weeks reverses some of these microbiota changes (Newell et al., 2016). These findings raise the possibility that the effects of postnatal administration of a KD are mediated at least in part by alterations in the gut microbiota. Indeed, the anti-epilepsy effects of KDs are eliminated in germ-free mice or in mice treated with antibiotics (Olson et al., 2018). The extent to which behavioral effects of GKD are dependent upon its impact on the gut microbiota, however, remains to be determined.

The present study included a partial replication of the only other published work to examine behavior in GKD mice, conducted by Sussman and colleagues (2015). They report that GKD mice spend about 50% less time immobile during the forced swim test (FST), indicating that gestational exposure to this diet reduces the expression of depression-like behaviors. We

observed a similar 50% decrease in immobility time in the FST, although the overall time spent immobile in both diet groups was substantially higher than that observed by Sussman and colleagues (2015). This can in part be explained by methodological differences between the studies: we summed immobility time over the course of the final four minutes of the six-minute FST test, as is commonly done when testing for anti-depressive effects of a therapy (Can et al., 2012; Castagné et al., 2011; Yankelevitch-Yahav et al., 2015), whereas Sussman and colleagues limited their quantification to the last three minutes of the test (Sussman et al., 2015). GKD mice also show reduced locomotor activity when tested in a novel arena, as evidenced by the fewer number of chamber entries observed in GKD mice during the habituation phase of the three-chambered test (present study), and the reduced velocity and total distance traveled observed in these mice when tested in an open field (Sussman et al., 2015). This is important to note given that one potential interpretation of the FST results is that GKD mice are simply more active than animals exposed gestationally to a standard diet (GSD), perhaps due to metabolic alterations such as increased accessibility to/utilization of energy stores. If this were indeed the case, then we would expect higher activity across other tests in novel environments; this is not what we and others observe, as described above. Finally, it is worthwhile to note that the present study differs from the results reported by Sussman and colleagues (2015) in that we found a significant decrease in blood ketone levels, but no significant decline in glucose levels, in adult GKD vs. GSD mice. In the absence of more detailed metabolic analyses, it is not possible at present to determine how gestational exposure may reprogram metabolic fuel utilization/availability or the extent to which this may underlie any of the behavioral effects observed in these mice.

The lasting effects of GKD on behavior of adult offspring suggests that there are changes in brain structure/function consequent to this developmental exposure. Previous work has

identified brain volume changes (at both pre- and postnatal timepoints) in GKD mice in several areas implicated in social behaviors, including the hypothalamus, hippocampus, and olfactory bulbs (Sussman, Ellegood, et al., 2013; Sussman, van Eede, et al., 2013). Although the specific links between GKD and changes in brain area volumes remains unclear, two of the mechanisms hypothesized to mediate the therapeutic effects of KDs, i.e., increased availability of ketones and fatty acids (Masino & Rho, 2010), are likely involved. Blood ketone and fatty acid levels rise during pregnancy, and given that the placenta is highly permeable to these metabolites, it is not surprising that their levels rise in the developing fetus as well (Herrera et al., 1987). Ketone bodies can not only be used as a metabolic fuel by the developing brain (Shambaugh, 1985), but can also be used as precursors in lipid biosynthesis, including lipids involved in myelination (Patel et al., 1975; Poduslo & Miller, 1991). Similarly, essential fatty acids (e.g., linoleic acid) and their long-chain polyunsaturated fatty acid derivatives (e.g., docosahexaenoic acid (DHA)) are also critical to normal fetal brain development. DHA is preferentially accumulated within the brain where it is incorporated into the plasma membranes, including synaptosomes (Lagarde et al., 2001; Suzuki et al., 1997). In their unesterified forms, DHA and its derivatives can also bind directly to membrane-bound receptors (e.g., PPARγ) within the CNS, thereby exerting anti-inflammatory effects and promoting cell survival (W. Zhang et al., 2010; Zhao et al., 2011). It is perhaps not surprising, then, that researchers have begun to examine whether maternal DHA supplementation during pregnancy can positively impact social behaviors in offspring. Piglets from sows that received DHA supplementation during pregnancy/lactation engage in more social interactions (e.g., mounting, nosing) with littermates when tested between four-eight weeks of age (Clouard et al., 2015). In the VPA rat model of autism, gestational DHA supplementation decreased the latency to escape in the Morris water maze when male offspring are tested as

juveniles; however, it was unable to improve the deficient sociability evidenced in these rats during the three-chambered test (Gao et al., 2016). But it is important to note that in another animal model of autism (the serotonin transporter knockout/prenatal stress mouse model), combined gestational/preweaning supplementation of DHA was sufficient to restore a preference for social novelty (three-chambered test) and reduce self-directed behaviors (grooming test) in male offspring tested as young adults (Matsui et al., 2018). These conflicting findings may in part be due to the numerous differences in methodological approach between the two studies, which simply highlights the need for additional research into the mechanisms underlying the effects of gestational dietary manipulations on behavior.

Given that markers of both sociability and affect were positively impacted by GKD, it seems likely that neurochemical systems important for these processes may also be affected. Oxytocin is a neuropeptide hormone synthesized by neurosecretory cells within hypothalamic and limbic brain areas, which reaches targeted sites either via direct projections or indirectly via terminal or somatodendritic release in areas adjacent to the cerebral ventricles. Indeed, many oxytocinergic neurons send projections directly to brain areas expressing oxytocin receptors and that have been implicated in the regulation of both social behavior and mood, such as the bed nucleus of the stria terminalis (ST), the amygdala, and the nucleus accumbens (Acb) (Stoop, 2012). In mice and some other rodent species, these projections arise predominantly from populations of oxytocinergic neurons located in two brain areas: the paraventricular nucleus of the hypothalamus (Pa) and the ST (Otero-García et al., 2016). The projections from the Pa to the Acb have been previously implicated in mediating the enhancing effects of oxytocin on social reward in male mice tested using a social conditioned place preference protocol (Dölen et al., 2013), as well as the anti-depressive effects of oxytocin in male voles experiencing depressive-

like symptoms following partner loss (Bosch et al., 2016). When considered in light of the sensitivity of oxytocin-producing cells to perturbations of the developmental environment (e.g., dark rearing (Zheng et al., 2014)), these findings led us to hypothesize that changes in expression of oxytocinergic cells within Pa and ST mediate the increased sociability observed following GKD. The lack of an effect of GKD on oxytocin immunoreactivity in these two brain areas that was observed in the present study by no means precludes the involvement of this system in our behavioral findings. It may be the case that GKD impacts the release of oxytocin and/or the expression of oxytocin receptors in targeted brain areas such as the Acb, rather than peptide expression in the Pa or the ST. Future studies will be required to determine if this is indeed the case.

Modeling GKD and its impact on sociability using mice has several advantages, ranging from the wealth of genetic mouse models of disordered social behavior that this model can be applied to in the future to the well-validated and readily implemented tests of sociability available for use in this species. The extent to which mechanisms of action and impacts on behavior of GKD mice translate to humans, however, remains to be established. One limitation of the GKD mouse model is that the exposure period is restricted to gestation. Developmentally, rodent gestation only encompasses the first two trimesters of human gestation, with the final trimester of human gestation corresponding to the first week of rodent postnatal life (Semple et al., 2013). This limitation could be addressed by cross-fostering GKD offspring with dams on a KD through postnatal day seven. The challenge with this approach is that lactating dams maintained on the KD formulation used here are at risk of developing ketoacidosis (Sussman, Ellegood, et al., 2013; Sussman et al., 2015). Utilizing a KD formulation with a less extreme fat:carbohydrates+protein ratio could potentially overcome this issue. In addition, it’s likely that

at least some of the biochemical effects of KDs differ between rodents and humans. For example, postnatal administration of a commonly used KD formulation to mice results in the development of non-alcoholic fatty liver disease (Schugar et al., 2013) whereas in humans, preliminary evidence suggests that KDs are protective against this disease (Pérez-Guisado & Muñoz-Serrano, 2011; Tendler et al., 2007). This suggests that there may be metabolic adaptations to a KD present in humans that aren't adequately modeled by mice. This point is necessarily tempered by evidence highlighting the conserved properties of KDs across species. For example, postnatal administration of KDs is effective at reducing seizures in both human patients with epilepsy and rodents models of this disease (Lima et al., 2014; Rho, 2017), and there is emerging evidence that the increases in available fatty acids and ketones observed in individuals that are maintained on a KD (Fraser et al., 2003; Kennedy et al., 2007) may represent conserved mechanisms underlying its therapeutic effects in epilepsy (DeGiorgio et al., 2015; Rho et al., 2002; Taha et al., 2008).

In conclusion, we found that markers of sociability and affect were positively impacted by GKD in CD-1 mice. This work agrees with, and substantially extends, the existing literature examining the behavioral effects of developmental exposure to these diets. Additional work in this area is warranted given the growing popularity of these diets amongst women of child-bearing age, as well as the identified potential these diets have across a range of neurological and psychiatric disorders.

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Figure 1. Mean (+/- SEM) time spent grooming (A) and number of chamber entries (B) in male and female mice exposed gestationally to either a standard (GSD) or ketogenic (GKD) diet. Behaviors were measured during the 10-minute habituation phase of the three-chambered sociability and social novelty test. Data are collapsed across sex due to no significant effects of this factor on either measure. n = 22-24 per diet condition. *$p < 0.05$, main effect of diet condition (GSD > GKD) on the number of chamber entries.

Figure 2. Mean (+/- SEM) preference scores for spending time in the chamber containing the social stimulus vs. an empty chamber (Social Chamber panel), and for directly investigating the cup containing the social stimulus vs. an empty cup (Social Stimulus panel), in male and female mice gestationally exposed to either a standard (GSD) or ketogenic (GKD) diet. Behaviors were measured during the 10-minute sociability phase of the three-chambered sociability and social novelty test. Preference scores were calculated as social time divided by the sum of social and nonsocial time and then expressed as a percentage. Data are collapsed across sex due to no significant effects of this factor on either measure. n = 24-25 per diet condition. *$p < 0.05$, main effect of diet condition (GKD > GSD) on preference scores for both the social chamber and the social stimulus.

Figure 3. Mean (+/- SEM) number of chamber entries (A) and preference scores for entering into the chamber containing the social stimulus vs. an empty chamber (B) in male and female mice exposed gestationally to either a standard (GSD) or ketogenic (GKD) diet. Preference scores were calculated as number of entries into the social chamber divided by the sum of social and nonsocial entries and then expressed as a percentage. Data are collapsed across sex due to no significant effects of this factor on either measure. n = 23-25 per diet condition.

Figure 4. Mean (+/- SEM) time spent immobile by male and female mice gestationally exposed to either a standard (GSD) or ketogenic (GKD) diet, during the last four minutes of a six-minute forced swim test. Data are collapsed across sex due to no significant effects of this factor on either measure. $n = 24$ per diet condition. *$p < 0.05$, main effect of diet condition (GSD > GKD) on immobility time.

Figure 5. Mean (+/- SEM) body weight (A), blood glucose levels (B), and blood ketone (β-hydroxybutyrate) levels (C) of male and female mice gestationally exposed to either a standard (GSD) or ketogenic (GKD) diet, taken at the conclusion of behavioral testing. $n = 11$-13 per treatment condition. *$p < 0.05$, main effect of sex (male > female) on body weight and blood glucose levels, and main effect of diet condition (GSD > GKD) on blood ketone levels.

Figure 6. Counting domains for quantifying oxytocin-positive cells. Domains (0.16 mm^2; gray boxes) were fitted to the bed nucleus of the stria terminalis (ST) (A) and the paraventricular nucleus of the hypothalamus (Pa) (B, C). Atlas plates were modified from Franklin and Paxinos (2008), and are organized relative to distance from bregma. 3V, third ventricle; aca, anterior commissure (anterior division); acp, anterior commissure (posterior division); AH, anterior hypothalamic area; AHC, anterior hypothalamic area (central part); AHP, anterior hypothalamic area (posterior part); BAC, bed nucleus of the anterior commissure; Cir, circular nucleus; f, fornix; GP, globus pallidus; ic, internal capsule; LPO, lateral preoptic area; mfb; medial forebrain bundle; MPA, medial preoptic area; MPOL, medial preoptic nucleus (lateral part); MPOM, medial preoptic nucleus (medial part); opt, optic tract; PaDC, paraventricular nucleus of the hypothalamus (dorsal cap); PaLM, paraventricular nucleus of the hypothalamus (lateral magnocellular); PaMP, paraventricular nucleus of the hypothalamus (medial parvicellular); PaV, paraventricular nucleus of the hypothalamus (ventral part); Pe, periventricular nucleus of the

hypothalamus; ; RCh, retrochiasmatic area; RChL, retrochiasmatic area (lateral part); SCh, suprachiasmatic nucleus; SHy, septohypothalamic nucleus; SO, supraoptic nucleus; sox, supraoptic decussation; StHy, striohypothalamic nucleus; STLI, bed nucleus of the stria terminalis (lateral division, intermediate part); STLP, bed nucleus of the stria terminalis (lateral division, posterior part; STLV, bed nucleus of the stria terminalis (lateral division, ventral part); STMAL, bed nucleus of the stria terminalis (medial division, anterolateral part), STMV, bed nucleus of the stria terminalis (medial division, ventral part); VLPO, ventrolateral preoptic nucleus.

Figure 7. Mean (+/- SEM) density of oxytocinergic (OT+) cells in the bed nucleus of the stria terminalis (ST) and the paraventricular nucleus of the hypothalamus (Pa) in male and female mice exposed gestationally to a standard (GSD) or ketogenic (GKD) diet. Data are collapsed across sex due to no significant effects of this factor on either measure. *n* = 15-22 per diet condition.

Table 1

Dietary Profile

Contribution	Macronutrient	LabDiet 5001	Teklad TD.96355
% weight[a]	carbohydrates	48.7	0.5
	protein	23.9	15.3
	fat	5.0	67.4
	vitamins	NS	1.3
	minerals	NS	2.0
% kcal[b]	carbohydrates	58.0	9.2
	protein	28.5	0.3
	fat	13.5	90.5
kcal/g[c]		4.1	6.7

Note that summed percentages do not equal 100% due to unlisted dietary constituents.

[a]Percent contribution of each macronutrient to the total mass (kg) of the diet.

[b]Percent contribution of each macronutrient to the total kcal provided by the diet.

[c]Gross energy per gram of the diet.

NS = not specified.

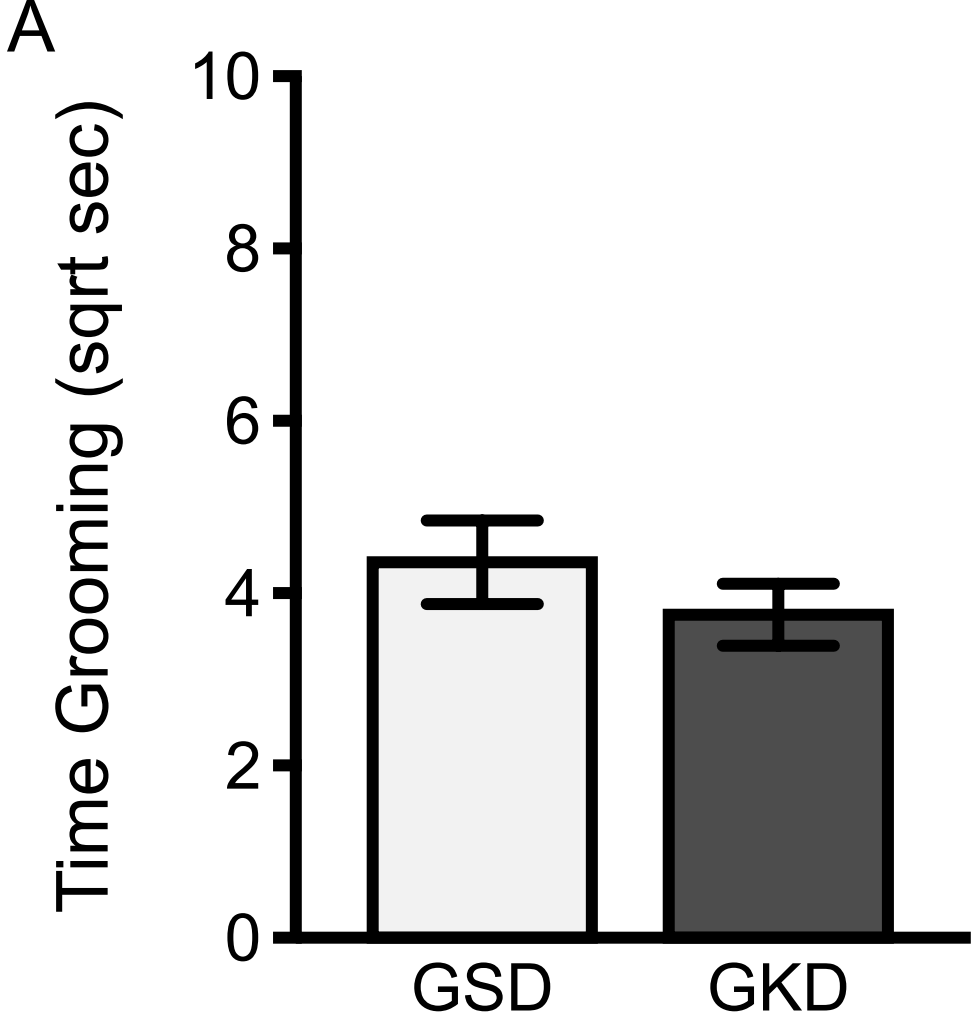
Table 2

Breeder Blood Glucose and Ketone Data

Group	Glucose	Ketones
Control	137.88 ± 11.57	0.59 ± 0.04
Ketogenic	157.25 ± 5.91	1.50 ± 0.13*

Note: Data are presented as mean (± SEM).

*$p < 0.05$, effect of diet condition (ketogenic > control) on blood ketone levels.


A
Time Grooming (sqrt sec)
10
8
6
4
2
0
GSD
GKD

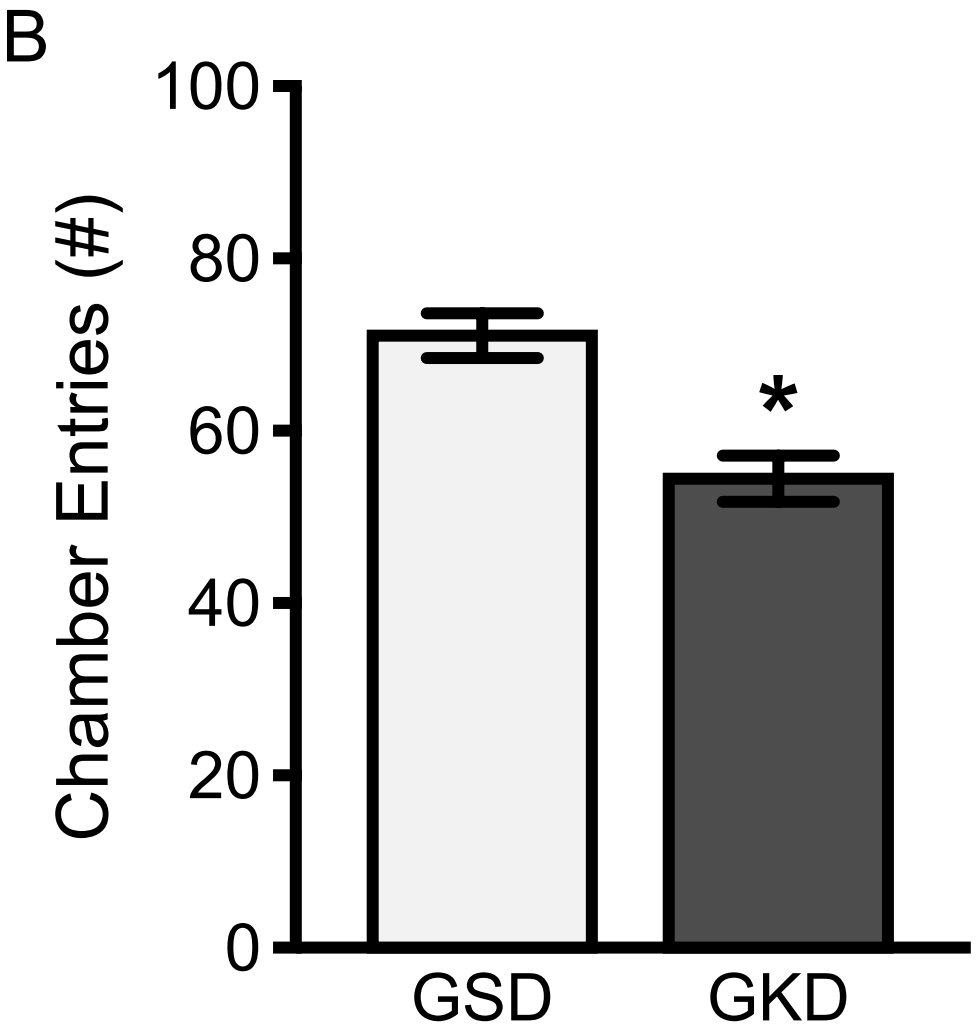
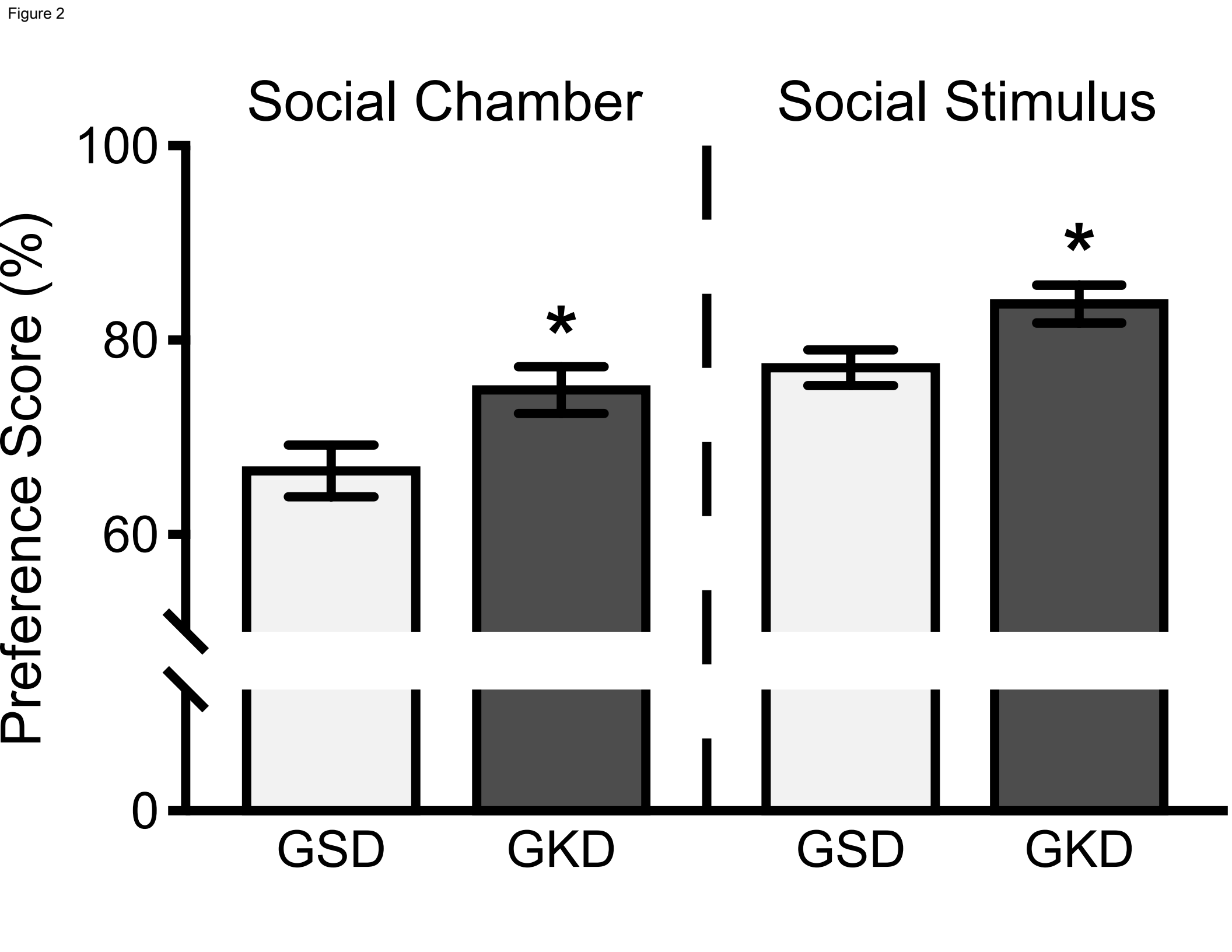
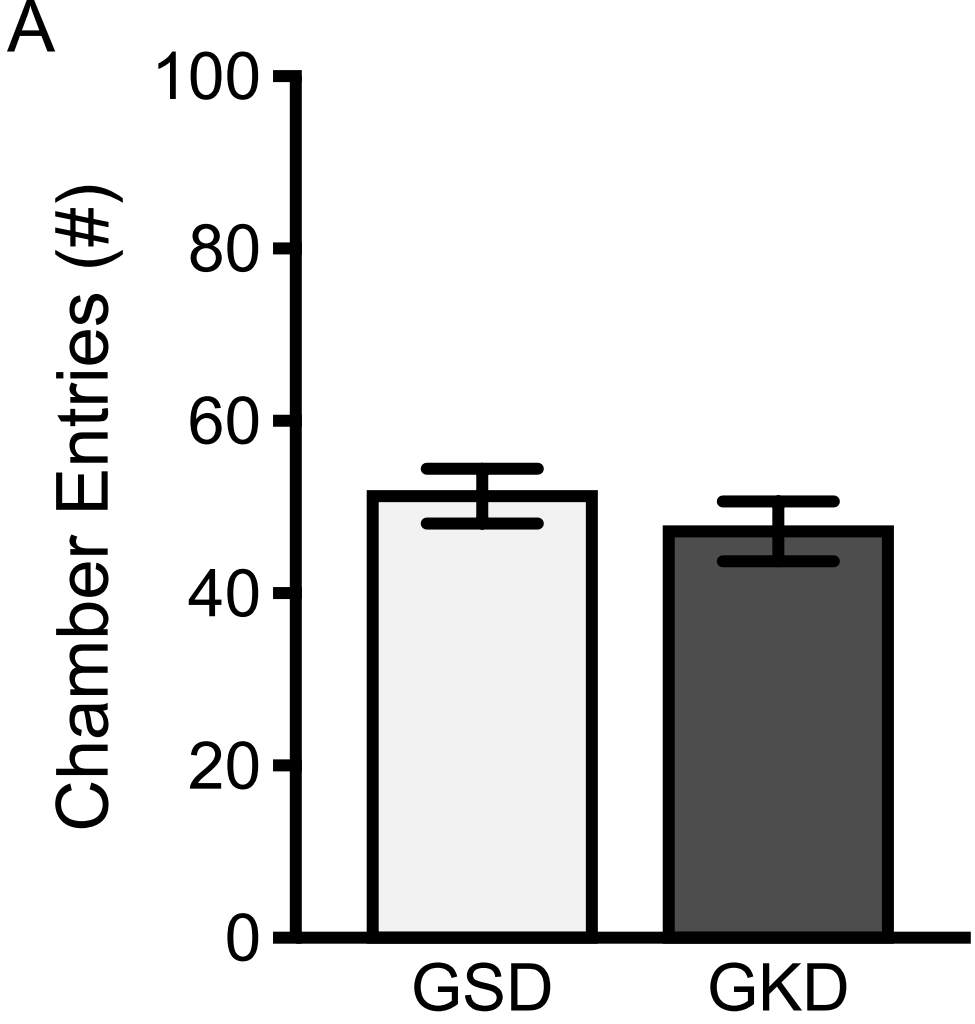

B
Chamber Entries (#)
100
80
60
40
20
0
*
GSD
GKD

Figure 1

Figure 2


Social Chamber
Social Stimulus
Preference Score (%)
100
80
60
0
*
*
GSD
GKD
GSD
GKD


A
Chamber Entries (#)
100
80
60
40
20
0
GSD
GKD

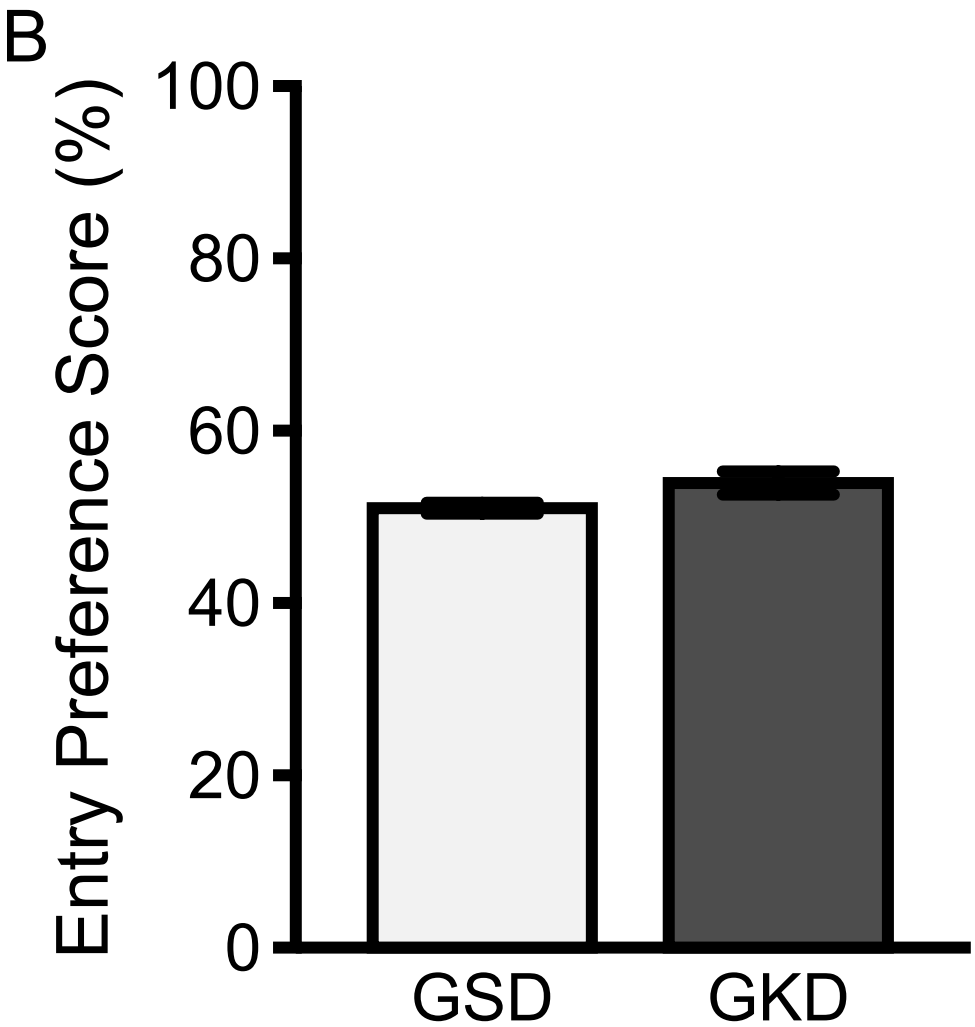

B
Entry Preference Score (%)
100
80
60
40
20
0
GSD
GKD

Figure 3

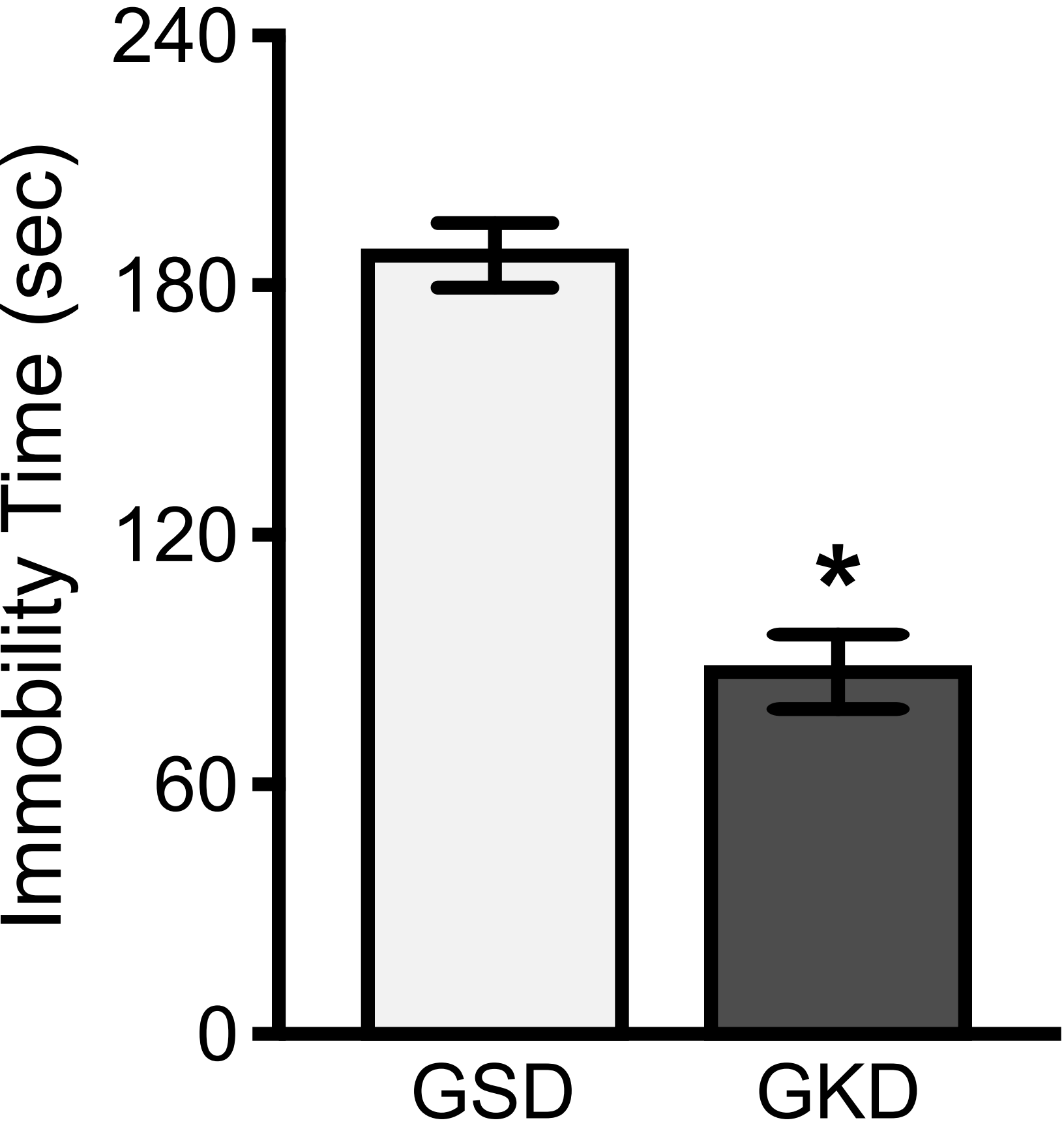


Figure 4

Figure 5

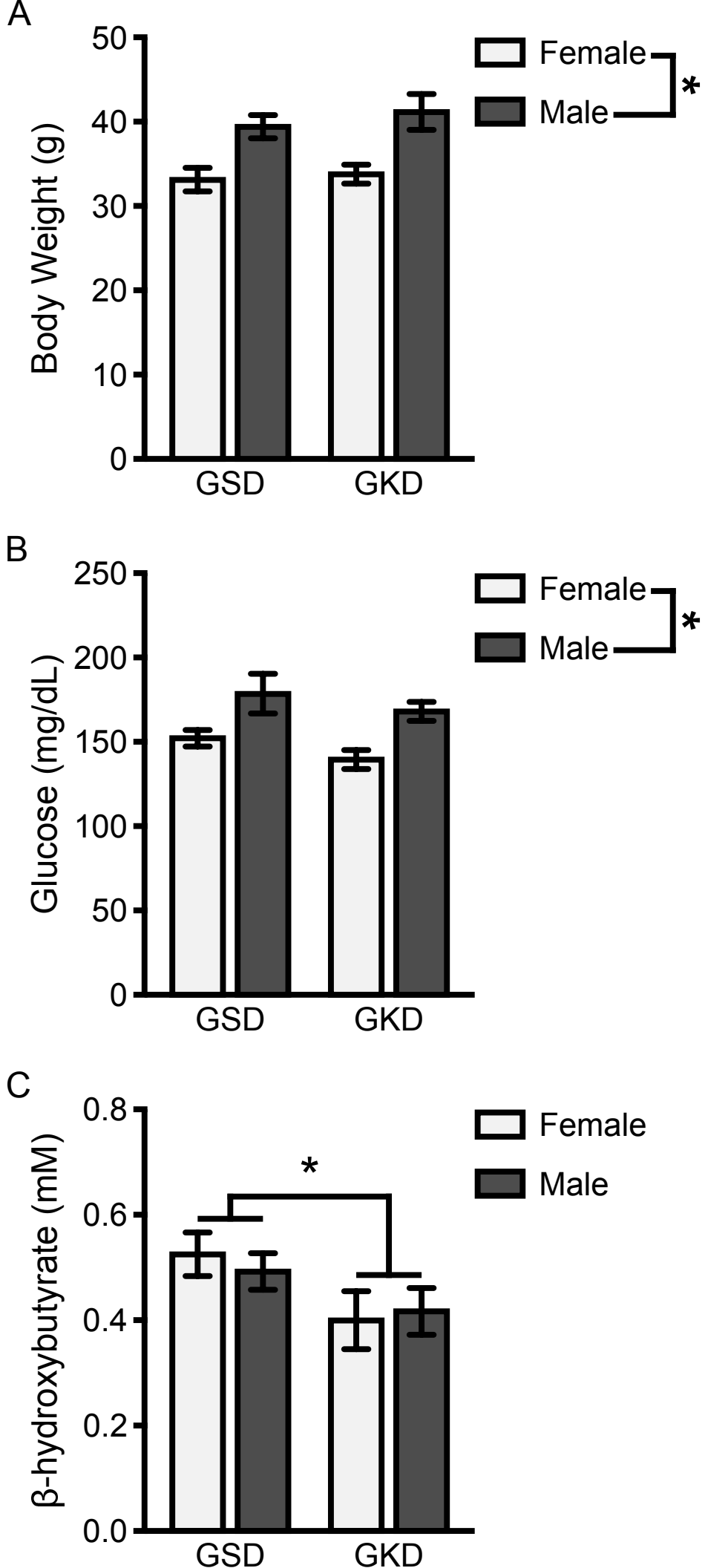

A
Body Weight (g)
50
40
30
20
10
0
GSD
GKD
Female
Male
*
B
Glucose (mg/dL)
250
200
150
100
50
0
GSD
GKD
Female
Male
*
C
β-hydroxybutyrate (mM)
0.8
0.6
0.4
0.2
0.0
GSD
GKD
Female
Male
*

Figure 6

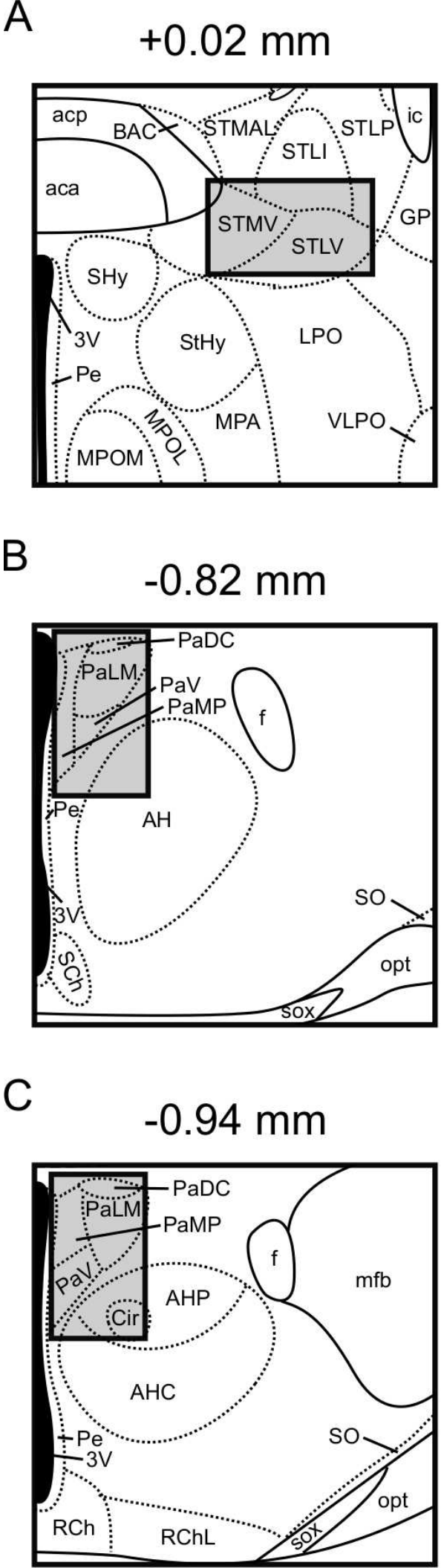

A
+0.02 mm
acp
BAC
STMAL
STLP
ic
STLI
aca
STMV
GP
STLV
SHy
3V
StHy
LPO
Pe
MPOL
MPA
VLPO
MPOM
B
-0.82 mm
PaDC
PaLM
PaV
PaMP
f
Pe
AH
SO
3V
opt
SCh
sox
C
-0.94 mm
PaDC
PaLM
PaMP
f
mfb
PaV
AHP
Cir
AHC
Pe
SO
3V
opt
RCh
RChL
sox

Figure 7