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ELUCIDATING THE INTERACTIONS BETWEEN CHRONIC HIGH-FAT DIET TREATMENT AND GABAERGIC INHIBITION IN THE EMOTIONAL BEHAVIOR OF MICE

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ABSTRACT

Major Depressive Disorder (MDD) is a debilitating condition that affects 6.7% of American adults and poses a serious public health concern. Meta analyses of clinical studies have identified chronic, low-grade inflammation as a predisposing factor in the development of MDD. Obesity, along with a poor diet, contributes to inflammation, which is especially concerning given the high prevalence of obesity in the United States. Longitudinal studies have established a link between obesity and depression, indicating comorbidity among these two disorders. Unhealthy diets with an excessive caloric content, common in modern Western nations, are a risk factor in the development of obesity and may be the source of obesity's increasing pervasiveness. To better study obesity-induced inflammation in the context of mood disorders, high-fat diet (HFD) treatment provides a reliable method for generating animal models of obesity, particularly using rodents. These HFD models, compared to control diet (CD) treated mice, also display the characteristic phenotypes expected of a model of obesity, including chronic metabolic inflammation and insulin resistance.

Neurobiological studies investigating the effects of HFD on the brain have found that rodents treated with HFD exhibit behavioral deficits in anxiety-like behavior, anhedonia, and memory. To further investigate these neurological effects of HFD, and their interactions with genetic and neurophysiological factors, the behavioral consequences of HFD treatment were examined in two different mutant mouse models with an altered ratio of neuronal excitation and inhibition (the E:I ratio). Altered E:I ratio is thought to underlie MDD, and imbalance in the E:I ratio has been shown to produce anxiety- and depression-related behavioral phenotypes in mice.

The first model ($\gamma2^{+/-}$) involves mice that are globally heterozygous for the γ2 subunit of $GABA_A$ receptors. In this model, GABAergic inhibition is reduced, leading to an increased E:I

ratio. The $\gamma2^{+/-}$ model has consistently produced an anxious-depressive-like phenotype in behavioral assessments. This phenotype can be diminished by treatment with antidepressants, thus implicating increased E:I ratio in the onset of mood disorders. A second mouse model (SSTCre:$\gamma2^{f/f}$) involves mice in which somatostatin-positive (SST+) interneurons were disinhibited by selectively deleting the γ2 subunit of $GABA_A$ receptors in these cells. In this model, inhibitory transmission onto hippocampal pyramidal cells was increased by disinhibiting SST+ interneurons, thereby reducing the overall E:I ratio in these mice. Behaviorally, these mice exhibit robust anxiolytic and antidepressant-like phenotypes. Together, results from studies involving $\gamma2^{+/-}$ and SSTCre:$\gamma2^{f/f}$ models suggest that an impaired E:I ratio underlies anxiety- and depression-related behavior.

The hypothesis examined in the present study, which aims to investigate the interactions between HFD and altered E:I ratio on behavior, is that increasing the E:I ratio ($\gamma2^{+/-}$ model) will exacerbate HFD-induced anxiety- and depression-like behavior, including defects in locomotion, grooming, and memory, while reducing the E:I ratio (SSTCre:$\gamma2^{f/f}$ model) will reduce HFD-induced anxiety- and depression-like behavior, including defects in locomotion, grooming, and memory. The results show that HFD treatment led to reductions in locomotion and grooming behavior of both the WT control and $\gamma2^{+/-}$ mutant mice in the OFT and SSPT, respectively. However, the effects of HFD on $\gamma2^{+/-}$ mice were not larger than in WT mice, so only an overall diet effect was present. Results from tests assessing short-term working (Y-maze) and recognition (NOR) memory indicate that the effects of diet and genotype are nonadditive, and that HFD treatment and altered E:I ratio impair behavior via different mechanisms. SSTCre:$\gamma2^{f/f}$ mutant mice presented an anxiolytic phenotype and increased locomotion in the EPM, consistent with studies in the literature. Additionally, HFD treatment of SSTCre:$\gamma2^{f/f}$ mice reduced locomotion in the OFT

and EPM, and also decreased grooming behavior in the SSPT. Overall, the behavioral results observed for CD-treated animals in these experiments did not reproduce all of the phenotypes of $\gamma2^{+/-}$ and SSTCre:$\gamma2^{f/f}$ mice published in the literature, most likely because the genetic backgrounds of the mice analyzed here (C57BL/6J and mixed 129X1/SvJ/C57BL/6J, respectively) were different from the mice described in earlier studies. Due to these unexpected results, additional behavioral experiments will be needed to further assess possible interactions between HFD treatment and altered E:I balance on anxiety- and depression-related behavior.

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CHAPTER 1

Introduction

Major Depressive Disorder (MDD) is a common and debilitating condition that affects 6.7% of American adults and has a lifetime prevalence of 16.2% (Kessler *et al.*, 2003; Center for Behavioral Health Statistics and Quality, 2016). Because MDD affects such a large proportion of the general population, learning more about its molecular etiology is of paramount concern. Bidirectional links between MDD and inflammation have been reported in the literature, with depression altering levels of pro-inflammatory cytokines and the resulting inflammatory response causing depression to persist (Kiecolt-Glaser *et al*., 2015). In fact, meta-analyses have shown that patients with MDD have altered levels of interleukin-1β (IL-1β), IL-6, C-reactive protein (CRP), and tumor necrosis factor α (TNF-α) (Howren *et al*., 2009; Dowlati *et al*., 2010).

Sources contributing to the inflammation seen in depressed patients include physical inactivity, lack of sleep, poor diet, and obesity (Berk *et al*., 2013). Investigating obesity-induced inflammation in the context of MDD is a particularly important concern because obesity has a high prevalence in the United States, with an estimated 17% of children and 35% of adults classified as obese (Ogden *et al*., 2014). Additionally, obesity is comorbid with depression (Luppino *et al*., 2010). As reported by Luppino *et al.* (2010), longitudinal studies in patients have established a reciprocal link between obesity and MDD.

Unhealthy diets, containing a high caloric content, have been identified as a principal risk factor for the onset of obesity (Fock and Khoo, 2013). To aid research efforts in better understanding the links between obesity-induced inflammation and depression, animal models of diet-induced obesity have been established. Particularly, high-fat diet (HFD) treatment can be used

as a reliable method for modeling obesity in rodent studies, and the effects of HFD treatment can be compared to the effects of control diet (CD) treatment. These HFD models show the relevant hallmarks and symptoms expected of an obesity phenotype, including metabolic inflammation, non-alcoholic fatty liver disease, and insulin resistance (Van Der Heijden *et al*., 2015; Waller-Evans *et al*., 2013; Buettner and Bollheimer, 2007; Wang and Liao, 2012).

In previous neurobiological studies, rodents exposed to chronic HFD treatment presented behavioral deficits comparable to those seen in chronic stress models of depression, suggesting that HFD- and stress-induced depressive-like behavior is mediated by overlapping mechanisms (Dutheil *et al*., 2016). In male rats, Dutheil *et al*. (2016) found that chronic treatment with HFD increased anxiety-like behavior as measured by the novelty suppressed feeding test (NSFT) and open field test (OFT), decreased memory performance in the novel object recognition test (NOR), and produced anhedonia in the sucrose preference test (SPT) and female urine sniffing test (FUST). A separate study reported that HFD treatment leads to memory dysfunction; male rats exposed to chronic HFD treatment exhibited impaired memory performance in the Y-maze test of spontaneous alternation and the NOR test (Fu *et al*., 2016). Similarly, evidence from male mice shows that chronic treatment with HFD leads to reduced performance in the Y-maze, increased anxiety-like behavior in the OFT and elevated zero maze test, and increased depression-related behavior in the forced swim test (FST) (Almeida-Suhett *et al*., 2017). The Luscher lab has built upon these findings using additional behavioral assays. Unpublished data by graduate student Mengyang Feng have revealed that HFD-treatment in mice decreased locomotion in the OFT, increased anxiety-like behavior in the OFT and NSFT, caused anhedonia in the sucrose splash test (SSPT) and FUST, and impaired memory performance in the Y-maze. Moreover, Mengyang Feng showed that chronic HFD treatment results in metabolic syndrome and increased expression of

inflammatory cytokines in the brain, consistent with corresponding experiments by others using rats as a model (Dutheil *et al*., 2016). The findings from these studies indicate that HFD-treatment and the resulting inflammation negatively affect emotional behavior, making these results especially important in a clinical context with MDD patients.

One of the mechanisms thought to underlie depression is an increased synaptic excitation-inhibition (E:I) balance in the brain (Luscher *et al*., 2011; Luscher and Fuchs, 2015; Ren *et al*., 2016; Lener *et al*., 2017). To investigate this hypothesis, a $GABA_A$ receptor mutant mouse model with an increased E:I ratio, as previously reported in the literature, was used. This model, referred to as $\gamma2^{+/-}$, was developed by the Luscher lab and consists of mice that are globally heterozygous for the γ2 subunit of $GABA_A$ receptors (Crestani *et al*., 1999). In these mice, one copy of the gene encoding the γ2 subunit (encoded by the Gabrg2 gene) was knocked out, resulting in a loss of the γ2 subunit in approximately 25% of $GABA_A$ receptors, depending on the brain region (Crestani *et al*., 1999; Earnheart *et al*., 2007). As Crestani *et al*. (1999) report, because γ2 subunits are essential for the postsynaptic formation of $GABA_A$ receptors, $\gamma2^{+/-}$ mice suffer from reduced $GABA_A$ receptors mainly at synapses and from a corresponding reduction in synaptic GABAergic inhibitory signaling, especially in the cerebral cortex and hippocampus. Thus, in these mutants, the E:I balance is shifted towards increased excitation.

Crestani *et al*. (1999) also conducted behavior testing on $\gamma2^{+/-}$ mutant mice and revealed a robust anxiety-like phenotype. In the free-choice exploration test, mutants showed a greater number of retractions from novel segments and visited fewer novel compartments than wild-type (WT) control mice (Crestani *et al*., 1999). In the EPM, mutants made fewer entries and spent less time in the open arms than did WT mice (Crestani *et al*., 1999). Finally, in a light-dark choice test, the mutants spent less time in the brightly lit, novel areas compared to WT mice (Crestani *et al*.,

1999). To complement the anxiety-like behavioral phenotype seen by Crestani *et al*. (1999) in the $\gamma2^{+/-}$ mutant model, subsequent studies in the Luscher lab utilized additional behavioral paradigms to further characterize the behavioral defects present in this mouse model. In these studies, compared to WT control mice, $\gamma2^{+/-}$ mutants showed decreased latency to immobility in the FST, and greater time spent immobile in the FST and tail suspension test (TST), both of which assess depression-related behavior (Earnheart *et al*., 2007; Shen *et al*., 2010). Furthermore, Shen *et al*. (2010) also showed that these mice had increased latency to feed in the NSFT, which measures anxiety-like behavior. Lastly, Earnheart *et al*. (2007) used the $\gamma2^{+/-}$ mouse model to show that defects in $GABA_A$ receptors in immature embryonic and adult glutamatergic neurons lead to reduced hippocampal neurogenesis in the adult brain. The outcomes of the Earnheart *et al*. (2007) study suggest that altered $GABA_A$ receptor functioning in immature neurons may be used to predict deficits in adult neurogenesis and the development of anxiety- and depression-like states.

The theory that an increased E:I ratio underlies anxiety and depression was further bolstered by Shen *et al*. (2010), who discovered that treatment with antidepressants can ameliorate the behavioral defects displayed by $\gamma2^{+/-}$ mutant mice. In that study, chronic treatment of $\gamma2^{+/-}$ mutants with the antidepressants fluoxetine and desipramine normalized anxiety-like behavior as measured by the NSFT (Shen *et al*., 2010). However, only desipramine treatment was found to elicit antidepressant-like responses in the FST, TST, and sucrose consumption test (SCT) (Shen *et al*., 2010). Furthermore, administering subanesthetic doses of ketamine to $\gamma2^{+/-}$ mutant mice leads to reduced anxiety- and depression-like behavior in the EPM and FST (Ren *et al*., 2016). Treatment with the antidepressants fluoxetine, desipramine, and ketamine is able to reverse the anxiety- and depression-like phenotypes produced by genetically altering the E:I ratio in $\gamma2^{+/-}$ mutant mice, thus implicating impaired E:I balance in the onset of anxiety and depression mood disorders.

Because the concentration of postsynaptic $GABA_A$ receptors is reduced in $\gamma2^{+/-}$ mutant mice, and because antidepressant treatment (i.e. desipramine) normalizes this GABAergic deficit and reverses the anxious- and depressive-like phenotypes associated with the $\gamma2^{+/-}$ mutant model, the Luscher lab hypothesized that antidepressant drugs ultimately act by increasing GABAergic inhibition and therefore by reducing the E:I ratio (Shen *et al*., 2010; Luscher and Fuchs, 2015). To test this hypothesis, Fuchs *et al*. (2017) developed a mutant mouse model (referred to as SSTCre:$\gamma2^{f/f}$) with genetically enhanced GABAergic synaptic signaling and a decreased E:I ratio. Using the cre-loxP system, somatostatin-positive (SST+) GABAergic interneurons were disinhibited in the forebrain of SSTCre:$\gamma2^{f/f}$ mice by selectively deleting the γ2 subunit of $GABA_A$ receptors and reducing $GABA_A$ receptor concentrations in SST+ interneurons (Fuchs *et al*., 2017). Electrophysiology experiments showed that disinhibition of SST+ interneurons resulted in enhanced inhibitory synaptic inputs to hippocampal pyramidal cells, thus reducing the E:I ratio in SSTCre:$\gamma2^{f/f}$ mutant mice (Fuchs *et al*., 2017). Behaviorally, Fuchs *et al*. (2017) found that SSTCre:$\gamma2^{f/f}$ mutants displayed anxiolytic and antidepressant-like phenotypes compared to $\gamma2^{f/f}$ littermate controls in various behavioral paradigms, as predicted. In anxiety tests, SSTCre:$\gamma2^{f/f}$ mutants spent more time in the open arms of the EPM, while in the NSFT, the mutants showed a decreased latency to feeding (Fuchs *et al*., 2017). In depression-related behavioral assessments, compared to $\gamma2^{f/f}$ littermate controls, SSTCre:$\gamma2^{f/f}$ mutants spent less time immobile and showed an increased latency to immobility in the FST; in the learned helplessness test (LHT), mutants showed fewer escape failures (Fuchs *et al*., 2017). The results reported by Fuchs *et al*. (2017) suggest that a reduced E:I ratio may underlie the anxiolytic- and antidepressant-like behavioral effects brought about by antidepressant drug treatment.

In summary, the results gathered from studies involving $\gamma2^{+/-}$ and SSTCre:$\gamma2^{f/f}$ mutant mice suggest synaptic E:I balance regulates anxiety- and depression-related behavior bidirectionally, with an increased E:I ratio leading to anxious-depressive-like behavior, and a reduced E:I ratio underlying anxiolytic and antidepressant brain states. Increasing the E:I ratio and enhancing excitation in the $\gamma2^{+/-}$ model produced robust anxious- and depressive-like phenotypes, and these behavioral deficits were shown to be reversed following antidepressant treatment. On the other hand, decreasing the E:I ratio and enhancing inhibition in the SSTCre:$\gamma2^{f/f}$ model resulted in anxiolytic- and antidepressant-like phenotypes. Based on this evidence, the purpose of the present study was to investigate whether the behavioral phenotypes associated with a mouse model of HFD-induced MDD were exacerbated or alleviated in mutant mice with increased or reduced synaptic E:I ratios ($\gamma2^{+/-}$ mutants and SSTCre:$\gamma2^{f/f}$ mutants, respectively). It is hypothesized that in the present study, a shift in E:I balance towards increased excitation ($\gamma2^{+/-}$ model) will increase HFD-induced anxiety- and depression-like behavior, including defects in locomotion, grooming behavior, and short-term working and recognition memory, while a shift in E:I balance towards increased inhibition (SSTCre:$\gamma2^{f/f}$ model) will reduce HFD-induced anxiety- and depression-like behavior, including defects in locomotion, grooming behavior, and short-term working and recognition memory.

In the present study, $\gamma2^{+/-}$ and SSTCre:$\gamma2^{f/f}$ mutants, and their respective WT and $\gamma2^{f/f}$ littermate controls were subjected to chronic, 18-week HFD treatment in two cohorts. To allow effective comparisons, normal diet controls were also used in both cohorts. The decision to use $\gamma2^{f/f}$ mice as controls for the SSTCre:$\gamma2^{f/f}$ cohort as opposed to other genotype controls (such as SSTCre and SSTCre:$\gamma2^{f/+}$ mice) was based on findings from previous studies which showed that SSTCre and SSTCre:$\gamma2^{f/+}$ mice were indistinguishable from $\gamma2^{f/f}$ mice in most behavior testing

paradigms (Fuchs *et al*., 2017). Males mice were exclusively tested in the present study because male mice are more susceptible to the detrimental effects of HFD treatment, including weight gain, metabolic alterations, learning deficiencies, and diminished hippocampal synaptic plasticity (Hwang *et al*., 2010).

The results gathered in the present study partially correspond to the evidence presented in the published literature on $\gamma2^{+/-}$ and SSTCre:$\gamma2^{f/f}$ mice since not every behavioral result for the CD-treated mutant animals matched the results reported by previous studies. Overall, HFD treatment reduced locomotion in both WT control and $\gamma2^{+/-}$ mutant mice in the OFT. HFD also reduced grooming behavior of WT and $\gamma2^{+/-}$ mice in the SSPT. In tests assessing working (Y-maze) and recognition (NOR) memory, HFD treatment caused a reduction in memory performance of HFD-treated WT vs. CD-treated WT mice but enhanced performance of HFD-treated $\gamma2^{+/-}$ vs. CD-treated $\gamma2^{+/-}$ mice. These results suggest that the effects of diet and genotype are nonadditive, and that HFD treatment and altered E:I ratio impair behavior via different mechanisms. SSTCre:$\gamma2^{f/f}$ mutant mice presented an anxiolytic phenotype and increased locomotion in the EPM, consistent with previous results of these same mice analyzed on a 129 background (Fuchs *et al*, 2017). HFD treatment of SSTCre:$\gamma2^{f/f}$ mice reduced locomotion in the OFT and EPM, and decreased grooming behavior in the SSPT. However, because of unexpected results that appear to contradict previous results with the same mice on a different genetic background, additional assessments of anxiety-like behavior, grooming, and memory performance will be needed before firm conclusions can be reached regarding the interactions between HFD and altered E:I balance.

CHAPTER 2

Materials and Methods

2.1 Animals

All animal tests and experiments were approved by the Pennsylvania State University Institutional Animal Care and Use Committee (IACUC, #46483), and adhered to the guidelines and policies set forth by the National Institutes of Health (NIH).

A total of 116 male mice were subjected to behavior testing in two cohorts. In the first cohort, SSTCre:$\gamma2^{f/f}$ mutant and $\gamma2^{f/f}$ littermate control mice were originally developed based on a 129X1/SvJ (129) genetic background as previously described (Fuchs *et al*., 2017). These mice were mated with pure WT mice on a C57BL/6J (BL6) genetic background and backcrossed over four to five generations to a BL6 background. For the second cohort, $\gamma2^{+/-}$ mutant and WT littermate control mice were developed on a pure BL6 background. All mice were maintained on a 12-hour light-dark cycle, and given *ad libitum* access to water and food pellets of the appropriate diet (either CD food pellets or HFD food pellets).

Of the 116 total male mice, one cohort consisted of 63 mice, with 17 CD-treated SSTCre:$\gamma2^{f/f}$ mutant mice, 16 HFD-treated SSTCre:$\gamma2^{f/f}$ mutant mice, 14 CD-treated $\gamma2^{f/f}$ control mice, and 16 HFD-treated $\gamma2^{f/f}$ control mice. The second cohort was comprised of 53 mice, with 11 CD-treated $\gamma2^{+/-}$ mutant mice, 11 HFD-treated $\gamma2^{+/-}$ mutant mice, 16 CD-treated WT control mice, and 15 HFD-treated WT control mice.

2.2 Diet Treatments

Rodent chow for both the CD-treated and HFD-treated mice was purchased from Bio-Serv (Flemington, New Jersey, USA). The CD food pellets (product #F4031) contained 3.93 kcal/g (16.3% from fat) and were primarily cornstarch-based. The HFD food pellets (product #F3282) contained 5.49 kcal/g (59.0% from fat) and were primarily lard-based. Diet treatment began once mice reached 5 weeks of age. Mice were subjected to diet treatment for 18 weeks before beginning behavior experiments, and during these 18 weeks, body weights were assessed for all mice on the same day each week. The diet treatment designated for each mouse was continued after the initial 18-week treatment period, and lasted until all behavior testing was concluded.

2.3 Behavior Testing

All control and mutant male mice from both cohorts were subjected to behavior testing once they had received either CD or HFD treatment for 18 weeks. Behavior testing was conducted in the following order: open field test (OFT), elevated plus maze test (EPM), Y-maze test of spontaneous alternation (Y-maze), sucrose splash test (SSPT), and novel object recognition test (NOR). The behavior tests were ordered such that mice were exposed to progressively increasing levels of stress in each test. This specific arrangement of behavior tests was intended to protect the data gathered from more stress-sensitive tests. All mice across both cohorts were subjected to the OFT, EPM, Y-Maze, and SSPT; only WT and $\gamma2^{+/-}$ mice were used for NOR. All testing occurred within the same room, and all tests were carried out in the dark phase under red light of the same intensity (300 lux). All tests began two to four hours after the start of the dark phase of the circadian cycle, and each test was conducted by an investigator blind to genotype.

2.3.1 Open Field Test

The OFT was used to assess locomotion in a novel environment (Prut and Belzung, 2003). The main premise of the OFT is that mice are agoraphobic and experience anxiety when placed in a novel, open space from which they cannot escape. Due to this fear, mice prefer to walk along the periphery of the open field, and increased duration in the center zone of the field and decreased latency to first center zone entry are indications of anxiolytic behavior (Prut and Belzung, 2003). Additionally, the distance travelled by each mouse indicates general locomotion.

The OFT setup was modeled after the setup utilized by Fuchs *et al*. (2017). The open field apparatus consisted of a 50 cm by 50 cm transparent Plexiglas base surrounded by black opaque walls measuring 20 cm in height. White paper was placed underneath the transparent base of the open field to increase illumination. The center zone was defined as a 30 cm by 30 cm space in the center of the open field. At the beginning of each test, mice were placed in the same corner of the apparatus, facing the center, and they were allowed to freely explore the arena for 10 minutes. The first 5-minute interval provides an anxiety-related measure, and the entire 10-minute span was used to measure locomotion. All trials were video recorded by an overhead camera and analyzed using EthoVision XT video tracking software (produced by Noldus Information Technology, Leesburg, Virginia, USA). After each trial, fecal pellets and urine drops were removed, and the test arena was wiped clean before continuing the experiment. The parameters measured in the OFT included total distance travelled in 10 minutes, and duration of time spent in the center zone and latency to first entry into the center zone in the first 5 minutes of the test. A mouse was determined to have entered the center zone when the center point of its body (as determined by the video tracking software) had crossed into the zone.

2.3.2 Elevated Plus Maze Test

The EPM test was conducted as a measure of locomotion and anxiety-like behavior in a novel environment (Lister 1987). The main premise of the EPM is that mice have an aversion to novel, open spaces, as well as to spaces that are elevated. This aversion to open and elevated areas leads mice to spend more time in the closed arms of the EPM (agoraphobia), and increased proportion of time spent in the open arms and increased proportion of entries into the open arms indicate anxiolytic behavior (Lister 1987). Furthermore, the total number of arm entries made by each mouse indicates locomotion within the EPM apparatus.

The EPM setup was modeled after the one utilized by Fuchs *et al*. (2017). The EPM arena consisted of a cross (in the shape of a "+") that was elevated 40 cm above the ground. At the center of the maze was a 5 cm by 5 cm square, and radiating from this central square were two open arms and two closed arms, each measuring 30 cm in length and 5 cm in width. The closed arms were surrounded by transparent Plexiglas walls (20 cm in height), which were higher than in the apparatus (15 cm) used by Lister (1987). The open arms were surrounded by a 3 mm lip to prevent mice from falling off the maze. For each trial, mice were allowed to freely explore the maze for a 5-minute period. All trials were video recorded by an overhead camera and analyzed using EthoVision XT video tracking software. At the conclusion of each trial, fecal pellets and urine drops were removed, and the maze was wiped clean before starting the next trial. The primary data measured by the EPM included proportion of time spent in the open arms, proportion of entries made into the open arms, and total number of arm entries. A mouse was deemed to have entered either a closed or an open arm when the center point of its body (as determined by the video tracking software) had crossed into the arm. In the study by Lister (1987), a mouse was determined

to have entered an arm when all four of its legs were on the arm. Trials in which a mouse fell from the open arms of the maze were excluded from data analysis.

In the original description of the EPM by Lister (1987), trials began by placing mice in the center of the maze, facing an open arm. This was modified in the present study and mice were placed at the end of the same open arm, facing towards the center, to begin each test. In previous EPM trials, mice, regardless of genotype and diet treatment, were unwilling to enter the open arms, and the modification was made in an effort to encourage mice to explore the open arms throughout the 5-minute period.

2.3.3 Y-maze Test of Spontaneous Alternation

Spontaneous alternation in the Y-maze is a test of short-term working memory (Sarnyai *et al.*, 2000). The primary premise of the Y-maze is that mice are inherently curious and if allowed, will choose to explore a novel environment even without any reinforcers. As a result of this innate tendency, mice will investigate a new area rather than reentering a previously visited area. In the Y-maze, a decreased proportion of correct alternations, defined by sequential entries into three different arms (a triad), indicates deficits in working memory (Sarnyai *et al.*, 2000).

The Y-maze apparatus resembles a “Y” shape, with three identical arms emanating from the same point. The maze comprises of black opaque Plexiglas with arms measuring 33 cm in length and 9 cm in width. Each 5-minute trial began by placing a mouse in an arm different from the arms the two previous mice had started in. For example, if the first mouse started in arm A, the next two mice began in arms B and C, respectively. Mice were placed at the end of the designated arm, facing the center of the maze. Mice were allowed to freely explore the arena for the entire 5-minute period, and all trials were video recorded by an overhead camera linked to EthoVision XT

video tracking software. All arm entries in the test were scored manually by an investigator blind to genotype. A mouse was recorded as having entered an arm when its entire body and tail had crossed in to the arm. Following each trial, fecal pellets and urine drops were removed, and the maze was wiped clean to prevent mice from using scent cues to navigate the maze. The primary data collected from this experiment were the percent of correct alternations. A correct alternation was defined when a mouse entered three different arms sequentially. An example of a correct alternation would be if a mouse first entered arm A, then arm B, and finally arm C. The following formula, as described by Sarnyai *et al.* (2000), was used to calculate the percent of correct alternations:

$$\%\ correct\ alternations = \left(\frac{\#\ of\ correct\ alternations}{total\ \#\ of\ arm\ entries\ -2}\right) * 100$$

A modification made to the protocol described by Sarnyai *et al.* (2000) was that the maze used for the present study measured 8 cm higher. Additionally, trials in the present study lasted for 5 minutes, while the trials carried out by Sarnyai *et al.* (2000) lasted for 6 minutes.

2.3.4 Sucrose Splash Test

The SSPT was conducted as a measure of spontaneous self-directed grooming behavior (Nollet *et al.*, 2013). Spraying the dorsal coat of mice with a viscous solution triggers automatic grooming, and decreases in the cumulative grooming duration and in the number of grooming sessions indicate defects in grooming behavior (Nollet *et al.*, 2013).

Prior to starting the SSPT, mice were singly housed for at least 24 hours. The test was carried out in the animals' home cage to prevent any novelty-based alterations in behavior. Mouse cages measured 29 cm in length, 18 cm in width, and 12 cm in height, and comprised of transparent

polycarbonate. Each 5-minute trial began by placing a mouse in a separate cage designated the "spray cage." The dorsal surface of the mouse was sprayed (from approximately 15 cm away) twice using a plastic spray bottle filled with a 10% sucrose solution. Immediately after spraying, the mouse was returned to its home cage, and a stainless-steel wire lid was placed atop the cage. During the trial, an investigator blind to genotype observed mice for cumulative grooming behavior; grooming behavior was categorized as licking any part of the body, such as the paws and fur on the dorsal surface. The data collected included the total number of grooming sessions and cumulative grooming duration over the entire 5-minute period. During the test, mice commonly groomed in bursts, and a grooming session was defined as the period from the start of grooming to a pause in grooming lasting for at least 3 seconds.

2.3.5 Novel Object Recognition Test

The NOR test was conducted as a test of recognition memory (Bevins and Besheer, 2006). The premise behind the NOR test is that mice possess a natural tendency to approach and interact with novelty. Therefore, if presented with both a novel and familiar object, a mouse will spend a greater proportion of time with the novel object (Bevins and Besheer, 2006). The NOR test consists of two phases: in phase one, the animal is familiarized with the testing arena and two copies of the same object, and in phase two, one of the familiar objects is replaced with a novel object. The two phases are separated by either a 1 hour or a 24-hour period. At either of these time points, the inability to distinguish between novel and familiar objects, as defined by the proportion of time spent with each object, indicates deficits in recognition memory (Bevins and Besheer, 2006).

For each trial, two test arenas were used so that two mice could be tested simultaneously. In each trial, mice were paired by diet treatment. The NOR test arenas were composed of

translucent polyethylene and measured 30 cm in length, 26 cm in width, and 14 cm in height. Six total objects were used in the experiment: two glass toothpick dispensers, each measuring 9 cm in height and 5 cm in diameter; two tissue culture flasks filled with blue solution, each measuring 4 cm in length, 2 cm in width, and 11 cm in height; and two 50 mL conical tubes filled with saw dust bedding, each measuring 11 cm in height and 3 cm in diameter. The assignment and placement of novel and familiar objects for each trial were randomly generated. For each trial, two objects were placed in the back left and right corners of the arena, and affixed with tape. At the beginning of each trial, mice were placed at the midpoint of the wall opposite the two objects and facing away from the objects.

Testing occurred over a four-day span. On the first two days, mice were acclimated to the empty arena for 5 minutes each day. On the third day, mice were again acclimated to the empty arena for 5 minutes, and allowed to freely explore the novel environment. Following acclimation, two copies of the same object were placed on opposite sides of the arena (28 cm apart), and mice were allowed to become familiar with the objects for 10 minutes. An hour after the familiarization phase, one of the familiar objects was replaced by a novel object, and mice were allowed to freely interact with both objects for 5 minutes. On the fourth day, 24 hours after the initial familiarization, the previous novel object was replaced by a second novel object, while the original familiar object remained in the same position. Mice were again allowed to freely explore the two objects in the arena for 5 minutes. Trials for both the 1 hour and 24-hour experiments were video recorded by an overhead camera and analyzed using EthoVision XT video tracking software. The primary data gathered from the NOR test was the percent of time spent with novel and familiar objects both 1 hour and 24 hours following familiarization. A mouse was deemed to be interacting with an object whenever its nose was pointed at the object from a distance of 2 cm or less.

2.4 Statistics

All data are presented as the mean ± S.E.M. The collected data were analyzed and graphed using GraphPad Prism 7 software (produced by GraphPad Software Inc., La Jolla, California, USA). Statistical analysis on the results was performed using the two-way analysis of variance (ANOVA) method to compare genotypes ($\gamma2^{+/-}$ vs. WT and SSTCre:$\gamma2^{f/f}$ vs. $\gamma2^{f/f}$) and diets (CD vs. HFD), followed by *post hoc* analysis using Fisher's least significant difference (LSD) test if the interaction between genotype and diet was significant. In the NOR test, unpaired t-tests were used in addition to two-way ANOVA to analyze time spent with two (novel or familiar) objects, and corrected using the Holm-Sidak method. The body weight measurements collected were analyzed using a repeated measures two-way ANOVA with Tukey's multiple comparisons test. Differences in data were considered significant if $P < 0.05$, and data were interpreted as trends if $P < 0.1$.

CHAPTER 3

Results

Graduate student Mengyang Feng contributed to this work by organizing the mouse breeding, collecting weight data for mice in both the $\gamma2^{+/-}$ and SSTCre:$\gamma2^{f/f}$ cohorts, and by performing OFT and EPM tests on mice in the SSTCre:$\gamma2^{f/f}$ cohort. Akshilkumar Patel genotyped the mice, collected data from the remaining behavior tests on the $\gamma2^{+/-}$ and SSTCre:$\gamma2^{f/f}$ cohorts, statistically analyzed all reported data, and graphed all results.

3.1 Chronic HFD treatment increases body weight in both WT control and $\gamma2^{+/-}$ mutant mice

Prior to the start of behavior testing, both WT and $\gamma2^{+/-}$ mice were subjected to either CD or HFD treatment for 18 weeks. Overall, treatment with HFD led mice to gain significantly more weight over the 18-week span compared to treatment with CD, and this difference in weight gain was not attributable to differences in genotype (**Figure 1**). In week 1, when the baseline body weights were established, body weights for each group were clustered at approximately 21 grams (WT CD: 21.4 ± 0.708 g, WT HFD: 21.97 ± 0.328 g, $\gamma2^{+/-}$ CD: 20.943 ± 0.55 g, $\gamma2^{+/-}$ HFD: 21.113 ± 0.394 g; results presented as mean ± S.E.M.), and no significant differences existed between the baseline weights of any groups (WT CD and HFD: $P = 0.26$, $n = 10 – 11$ mice/group; $\gamma2^{+/-}$ CD and HFD: $P = 0.56$, $n = 7 – 8$ mice/group, repeated measures two-way ANOVA). The difference in body weights between WT mice treated with both diets first became significant at week 2 ($P = 0.0073$, $n = 10 – 11$ mice/group, repeated measures two-way ANOVA) and remained highly significant throughout weeks 3 to 18 ($P < 0.0001$).

The difference in body weights between $\gamma2^{+/-}$ mice treated with both diets first became significant at week 4 (P = 0.0002, n = 7 – 8 mice/group, repeated measures two-way ANOVA) and remained highly significant throughout weeks 5 to 18 (P < 0.0001). At week 18, the final body weights were clustered at approximately 51 grams for HFD-treated mice (WT HFD: 51.744 ± 0.611 g, $\gamma2^{+/-}$ HFD: 51.461 ± 0.756 g; mean ± S.E.M.), and 31 grams for CD-treated mice (WT CD: 30.457 ± 0.633 g, $\gamma2^{+/-}$ CD: 31.383 ± 1.459 g; mean ± S.E.M.). The results also point to the absence of a genotype effect on weight gain (P > 0.1, n = 10 – 11 WT mice/group and 7 – 8 $\gamma2^{+/-}$ mice/group, repeated measures two-way ANOVA). Thus, as observed in Figure 1, the increased weight gains of HFD-treated WT and $\gamma2^{+/-}$ mice over an 18-week period are a result of HFD treatment, not genotype.

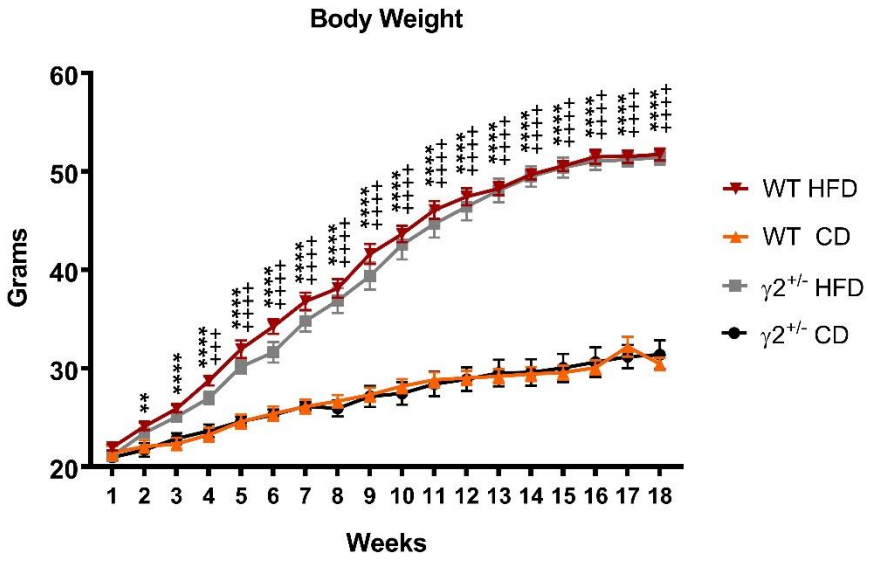

Figure 1. Weight gain in WT and $\gamma2^{+/-}$ mutant mice following chronic diet treatment.

Body weights of WT control and $\gamma2^{+/-}$ mutant mice exposed to either CD or HFD were measured weekly over an 18-week period. Irrespective of genotype, mice that consumed a HFD gained significantly more weight than mice consuming a CD, beginning in week 2 for WT mice (P = 0.0073, n = 10 – 11 mice/group, repeated measures two-way ANOVA) and in week 4 for $\gamma2^{+/-}$ mice (P = 0.0002, n = 7 – 8 mice/group, repeated measures two-way ANOVA). No genotype-based effect indicating differences in body weight between WT and $\gamma2^{+/-}$ mice was observed at any point during the 18 weeks (P > 0.1, n = 10 – 11 WT mice/group and 7 – 8 $\gamma2^{+/-}$ mice/group, repeated measures two-way ANOVA). Asterisk (*) indicates significance between WT mice treated with HFD and CD, and plus sign (+) indicates significance between $\gamma2^{+/-}$ mice treated with HFD and CD. All results are presented as mean ± S.E.M.; **P < 0.01, ****P < 0.0001, +++P < 0.001, ++++P < 0.0001. Body weight data were collected and generously provided by graduate student Mengyang Feng.

3.2 HFD treatment reduces locomotion and latency to first center entry, and $\gamma2^{+/-}$ mutants spend less time in the center zone of the OFT

HFD treatment on mice has been shown to decrease locomotor activity and induce anxiety-like behavior in the OFT (Almeida-Suhett *et al*., 2017). In an EPM test, $\gamma2^{+/-}$ mutant mice displayed an anxiety-like phenotype, indicated by reduced time spent in the open arms and reduced entries into the open arms (Crestani *et al*., 1999). The purpose of the OFT in the present study was to determine whether an increase in E:I ratio in $\gamma2^{+/-}$ mutant mice affects HFD-induced anxiety-like behavior and reduction in locomotion. However, the OFT presented here was conducted under red light (300 lux) as opposed to bright light (7000 lux), making the open field a less aversive environment and compromising the test's ability to measure anxiety-like behavior. Additionally, the $\gamma2^{+/-}$ mice used here were on a BL6 genetic background, which are naturally more anxious than the 129 mice used by Crestani *et al*. (1999). Therefore, the genotype-induced anxiety phenotype of $\gamma2^{+/-}$ mice is more difficult to detect in mutants on the BL6 background than on the 129 background.

The first parameter tested by the OFT was locomotor activity, measured as total distance travelled over a span of 10 minutes. HFD-treated mice of both the WT and $\gamma2^{+/-}$ genotypes showed decreased locomotion as compared to mice of both genotypes exposed to CD (WT: $P = 0.016$, $n = 15 – 16$ mice/group, Fisher's LSD *post hoc* test; $\gamma2^{+/-}$: $P = 0.0002$, $n = 11$ mice/group, Fisher's LSD *post hoc* test; **Figure 2A**). No genotype effect was evident in locomotor activity ($F_{(1, 49)} = 0.417$, $P = 0.52$, two-way ANOVA).

The remaining two parameters tested were center zone duration and latency to first entry into the center zone during the first 5-minute interval of the test. Overall, $\gamma2^{+/-}$ mutant mice spent less time in the center zone than did WT control mice, irrespective of diet treatment ($F_{(1, 49)} = 9.57$,

P = 0.0033, two-way ANOVA; **Figure 2B**). Two-way ANOVA did not show a diet difference in center zone duration ($F_{(1, 49)}$ = 0.509, P = 0.48). Additionally, treatment with HFD was shown to reduce latency to first center entry in both the WT and $\gamma2^{+/-}$ mice ($F_{(1, 47)}$ = 4.63, P = 0.037, two-way ANOVA; **Figure 2C**). No genotype effect was observed in this parameter ($F_{(1, 47)}$ = 0.0512, P = 0.82, two-way ANOVA). In summary, HFD exposure reduces locomotion and decreases latency to first center entry. In general, $\gamma2^{+/-}$ mutants spent less time in the center zone of the open field compared to WT mice.

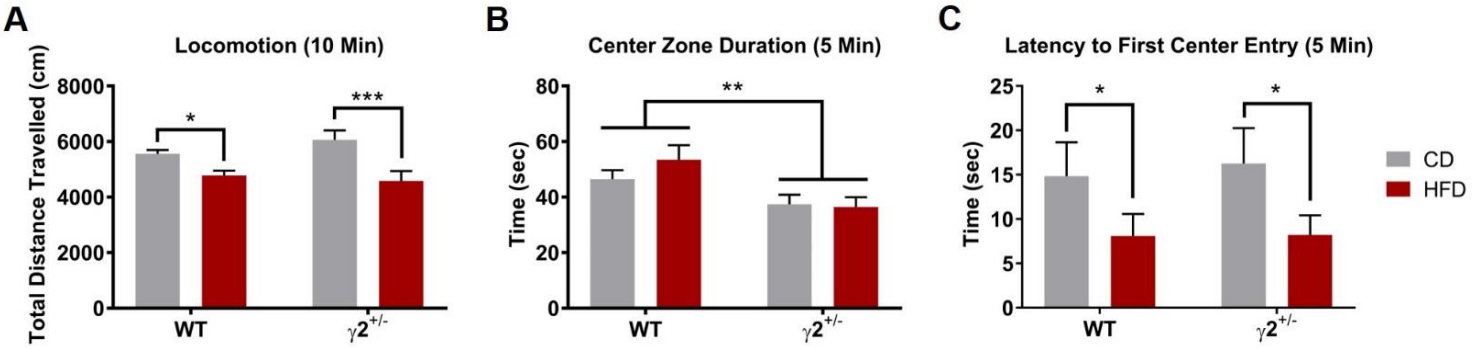

Figure 2. Locomotor activity in WT and $\gamma2^{+/-}$ mice in the open field test.

Panel (A) shows the total distance mice travelled in the open field arena over a period of 10 minutes. HFD treatment reduced locomotor activity in both WT control and $\gamma2^{+/-}$ mutant mice ($F_{(1, 49)}$ = 21.52, P < 0.0001, two-way ANOVA). **Panel (B)** shows the amount of time mice spent in the center zone of the open field during the first 5-minute interval of the test. Overall, $\gamma2^{+/-}$ mutant mice spent less time in the center zone compared to WT mice, regardless of diet treatment ($F_{(1, 49)}$ = 9.57, P = 0.0033, two-way ANOVA). **Panel (C)** shows the latency for mice to make their first entry into the center zone of the open field during the first 5-minute interval of the test. HFD treatment reduced the latency to first center zone entry in both the WT and $\gamma2^{+/-}$ mutant groups ($F_{(1, 47)}$ = 4.63, P = 0.037, two-way ANOVA). All results are presented as mean ± S.E.M.; *P < 0.05, **P < 0.01, ***P < 0.001.

3.3 No diet or genotype effects exist in anxiety-like behavior in WT control and $\gamma2^{+/-}$ mutant mice as measured by EPM

In a previous rodent study, there was a nonsignificant trend toward decreased time spent in the open arms by HFD-treated rats compared to CD-treated rats (Dutheil *et al*., 2016). In terms of genotype effects on mouse performance in the EPM, $\gamma2^{+/-}$ mutant mice have previously been characterized as displaying a significant anxiety-like behavioral phenotype compared to WT control mice (Crestani *et al*., 1999). In the present study, the goal was to determine whether the

anxiety-like phenotype of $\gamma2^{+/-}$ mutant mice is exacerbated by HFD treatment.

The EPM test was used as another measure of locomotion and anxiety-like behavior. The first parameter tested in this test was time spent in the open arms of the maze as a percentage of total time. Two-way ANOVA revealed no significant diet ($F_{(1, 43)} = 1.394$, $P = 0.24$) or genotype ($F_{(1, 43)} = 0.784$, $P = 0.38$) effects on this parameter (**Figure 3A**).

The next parameter of the EPM measured the number of entries made into open arms of the maze as a percentage of total entries. Again, two-way ANOVA revealed no significant diet ($F_{(1, 43)} = 0.183$, $P = 0.67$) or genotype ($F_{(1, 43)} = 1.248$, $P = 0.27$) effects (**Figure 3B**).

The final parameter tested in the EPM test was the total number of entries made into both open and closed arms during the 5-minute trial, which served as a locomotion measure. Overall, a trend was observed in which mice with the $\gamma2^{+/-}$ mutant genotype made more total entries than mice with a WT genotype ($F_{(1, 42)} = 3.496$, $P = 0.069$, two-way ANOVA; **Figure 3C**). No such trend was observed for diet treatment ($F_{(1, 42)} = 0.0168$, $P = 0.9$, two-way ANOVA). In summary, no differences were found between WT and $\gamma2^{+/-}$ mice in the EPM. However, the number of total entries of $\gamma2^{+/-}$ mice showed a trend towards more entries than in WT mice that had not previously been noted, perhaps because it became only evident with the larger group sizes analyzed here.

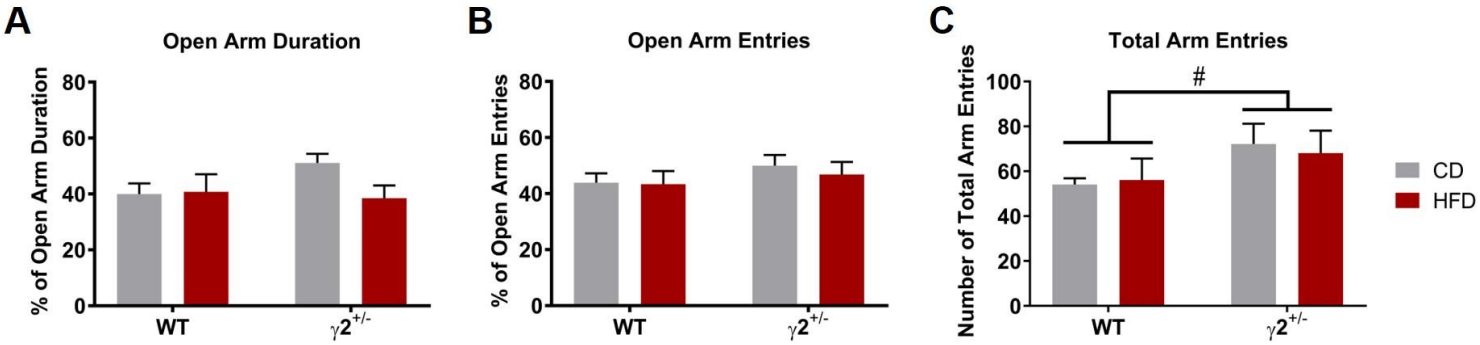

Figure 3. Absence of anxiety-like behavior in WT and $\gamma2^{+/-}$ mice in the elevated plus maze test.

Panel (A) shows the percent of time spent in the open arms of the EPM apparatus. No significant diet ($F_{(1, 43)} = 1.394$, $P = 0.24$, two-way ANOVA) or genotype ($F_{(1, 43)} = 0.784$, $P = 0.38$, two-way ANOVA) effects were observed for this parameter. **Panel (B)** shows the percent of entries made into the open arms of the EPM arena. Again, no significant diet ($F_{(1, 43)} = 0.183$, $P = 0.67$, two-way ANOVA) or genotype ($F_{(1, 43)} = 1.248$, $P = 0.27$, two-way ANOVA) effects were seen in the percent of open arm entries. **Panel (C)** shows the number of total arm entries made during the 5-minute trial, which represents a measure of locomotion in the EPM. A trend in genotype was observed for this parameter, with $\gamma2^{+/-}$ mice making more arm entries than WT mice ($F_{(1, 42)} = 3.496$, $P = 0.069$, two-way ANOVA). All results are presented as mean ± S.E.M.; #$P < 0.1$.

3.4 HFD treatment impairs working memory in WT control mice, but enhances it in $\gamma2^{+/-}$ mutant mice in the Y-maze

Previously, HFD treatment has been shown to decrease short-term working memory performance in the Y-maze test, with HFD-treated mice showing a significantly reduced percent of correct alternations (Almeida-Suhett *et al*., 2017). Additionally, Crestani *et al.* (1999) reported that $\gamma2^{+/-}$ mice did not display significant memory deficits in the Morris water-maze or the Y-maze. The purpose of the Y-maze test presented here was to determine whether HFD treatment-induced defects in working memory would be exacerbated or ameliorated in $\gamma2^{+/-}$ compared to WT mice.

The Y-maze test was utilized as a measure of short-term working memory. The only parameter tested in the Y-maze was the percentage of correct alternations out of total alternations. Because a significant interaction between the diet and genotype groups was identified by two-way ANOVA ($F_{(1, 42)} = 15.86$, $P = 0.0003$), the data were further analyzed using Fisher's LSD *post hoc* test (**Figure 4**). The *post hoc* test showed that HFD-treatment lead to a significant reduction in the percentage of correct alternations in WT mice compared to CD-treatment ($P = 0.0068$, $n = 10 – 15$ mice/group). In contrast, HFD-treatment increased the percentage of correct alternations in $\gamma2^{+/-}$ mice ($P = 0.0079$, $n = 10 – 11$ mice/group, Fisher's LSD *post hoc* test). Of the mice treated with CD, there was a trend toward decreased percentage of correct alternations in $\gamma2^{+/-}$ mice compared to WT mice ($P = 0.059$, $n = 10 – 15$ mice/group, Fisher's LSD *post hoc* test). The opposite outcome was observed in mice treated with HFD: $\gamma2^{+/-}$ mice showed a significant increase in the percentage of correct alternations compared to WT mice ($P = 0.0008$, $n = 10 – 11$ mice/group, Fisher's LSD *post hoc* test). In summary, HFD treatment impaired working memory in WT mice as expected but improved it in $\gamma2^{+/-}$ mice. Furthermore, the genotype effects observed in the Y-maze included

a trend towards impaired working memory in CD $\gamma2^{+/-}$ mice compared to WT mice on the same diet. Conversely, HFD-treated $\gamma2^{+/-}$ mice performed better compared to HFD-treated WT mice.

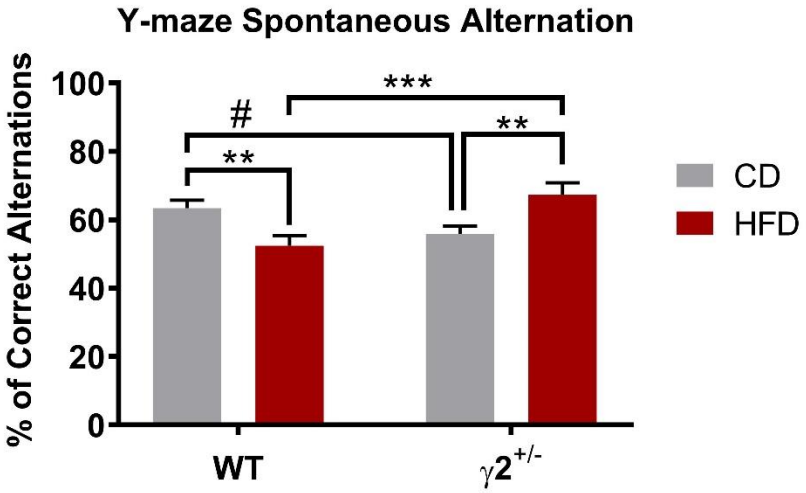

Figure 4. Short-term working memory in WT and $\gamma2^{+/-}$ mice in the Y-maze test.

Results from the Y-maze are displayed as the percent of correct alternations out of total alternations. Two-way ANOVA revealed a significant interaction between diet and genotype ($F_{(1, 42)} = 15.86$, $P = 0.0003$). Analysis using Fisher's LSD *post hoc* test showed a significant HFD-induced reduction in the percent of correct alternations in WT mice ($P = 0.0068$, $n = 10 – 15$ mice/group). Conversely, HFD treatment increased the percent of correct alternation in $\gamma2^{+/-}$ mutant mice ($P = 0.0079$, $n = 10 – 11$ mice/group, Fisher's LSD *post hoc* test). In mice treated with CD, a trend was observed in which $\gamma2^{+/-}$ mutant mice had reduced percent of correct alternations compared to WT mice ($P = 0.059$, $n = 10 – 15$ mice/group, Fisher's LSD *post hoc* test). In mice exposed to HFD treatment, $\gamma2^{+/-}$ mice showed an increase in the percent of correct alternations compared to WT mice ($P = 0.0008$, $n = 10 – 11$ mice/group, Fisher's LSD *post hoc* test). All results are presented as mean ± S.E.M.; #$P < 0.1$, **$P < 0.01$, ***$P < 0.001$.

3.5 HFD treatment impairs grooming behavior in WT control and $\gamma2^{+/-}$ mutant mice in the SSPT

In the study by Dutheil *et al.* (2016), rats exposed to HFD treatment displayed anhedonia in the SPT and FUST, but according to unpublished data by Mengyang Feng, WT mice exposed to HFD treatment show no preference to sucrose in the SPT compared to WT mice exposed to CD treatment. In the SCT, $\gamma2^{+/-}$ mutants displayed a reduction in the volume of sucrose solution consumed, indicating anhedonia (Shen *et al*., 2010). Given these findings, the SSPT presented here was used as a measure of grooming behavior, which may be related to hedonic drive. The goal of this test was to investigate whether HFD-induced reductions in grooming behavior of WT mice would be exacerbated in $\gamma2^{+/-}$ mutant mice.

One of the parameters measured in this test was the cumulative duration of grooming during the 5-minute test. Overall, two-way ANOVA revealed a significant HFD-induced reduction in cumulative grooming duration ($F_{(1, 48)} = 26.79$, $P < 0.000$; **Figure 5A**). *Post hoc* analysis with the Fisher LSD test indicated a HFD-induced reduction in cumulative grooming duration for both WT and $\gamma2^{+/-}$ mice (WT: $P = 0.0016$, $n = 14 – 16$ mice/group; $\gamma2^{+/-}$: $P = 0.0003$, $n = 11$ mice/group). No genotype effect was observed in the cumulative grooming duration ($F_{(1, 48)} = 0.3246$, $P = 0.57$, two-way ANOVA).

The second parameter measured in the SSPT was the total number of grooming sessions undertaken during the 5-minute test. No significant diet ($F_{(1,48)} = 2.111$, $P = 0.15$, two-way ANOVA) or genotype ($F_{(1, 48)} = 0.001034$, $P = 0.98$, two-way ANOVA) effects were seen in this parameter (**Figure 5B**). In summary, all mice treated with HFD in the $\gamma2^{+/-}$ cohort showed reduced grooming behavior compared to CD-treated mice.

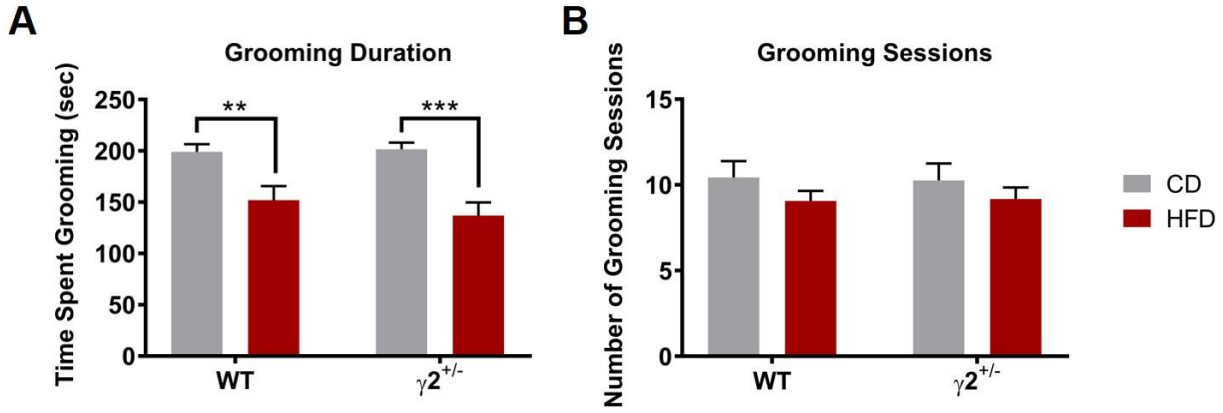

Figure 5. Grooming behavior in WT and $\gamma2^{+/-}$ mice in the sucrose splash test.

Panel (A) presents the cumulative time each mouse spent grooming during the 5-minute test. Overall, there was a significant diet effect on the performance of both the WT and $\gamma2^{+/-}$ mice ($F_{(1, 48)} = 26.79$, $P < 0.0001$, two-way ANOVA). **Panel (B)** presents the number of grooming sessions each mouse participated in. No significant diet ($F_{(1, 48)} = 2.111$, $P = 0.15$, two-way ANOVA) or genotype ($F_{(1, 48)} = 0.001034$, $P = 0.98$, two-way ANOVA) effects were observed. All results are presented as mean ± S.E.M.; $^{**}P < 0.01$, $^{***}P < 0.001$.

3.6 Data suggest successful execution of the NOR test on WT control and $\gamma2^{+/-}$ mutant mice

Experiments in rodents have previously shown that treatment with chronic HFD leads rats to perform worse in the NOR compared to treatment with CD, as shown by a significant reduction in time spent exploring a novel object (Fu *et al*., 2017). However, poor performance in the NOR by HFD-treated WT male mice is not consistently reported in the literature. As mentioned above, Crestani *et al.* (1999) found no difference in memory performance between WT and $\gamma2^{+/-}$ mice in the Morris water-maze test or the Y-maze test. However, because significant diet and genotype effects were seen in the Y-maze test displayed in Figure 4, the NOR test was conducted as a second measure of cognition. The goal of the NOR test was to determine whether the HFD-induced decrease in cognition observed by Fu *et al.* (2016) could be reproduced in HFD-treated $\gamma2^{+/-}$ mice and whether there might be a HFD X genotype interaction.

The NOR test measured the animals' recognition memory. The primary parameter tested in the NOR test was the percentage of time each mouse spent with a novel and a familiar object, and this parameter was measured at two time points: 1 hour and 24 hours following the familiarization phase. When tested 1 hour after familiarization, only WT mice treated with CD were able to distinguish between the novel and familiar objects, as evidenced by the greater proportion of time spent with the novel object (P = 0.0015, n = 14 mice/object, unpaired t-test, **Figure 6A**). However, $\gamma2^{+/-}$ mice treated with HFD did show a trend toward a greater proportion of time spent with the novel object (P = 0.056, n = 10 mice/object, unpaired t-test). The results indicate that WT mice fed with a HFD were unable to distinguish between novel and familiar objects 1 hour after familiarization (P = 0.14, n = 15 mice/object, unpaired t-test), as were $\gamma2^{+/-}$ mice fed with a CD (P = 0.8, n = 10 mice/object, unpaired t-test). Because two-way ANOVA

revealed a trend suggesting an interaction between the diet and genotype groups ($F_{(1, 46)} = 3.419$, $P = 0.071$), the data were further analyzed using Fisher's LSD *post hoc* test. The *post hoc* test revealed a trend in the proportion of time spent with a novel object between $\gamma2^{+/-}$ mice treated with CD and $\gamma2^{+/-}$ mice treated with HFD, with HFD-treated $\gamma2^{+/-}$ mice spending a greater proportion of time with a novel object ($P = 0.078$, $n = 10 – 11$ mice/group).

When the NOR test was repeated 24 hours after familiarization, WT mice treated with CD were once again able to successfully distinguish between the novel and familiar objects by spending a significantly greater proportion of time with the novel object ($P < 0.0001$, $n = 14$ mice/object, unpaired t-test, **Figure 6B**). Additionally, trends were observed towards increased percent of time spent with the novel compared to familiar objects in the three remaining groups, including WT mice treated with HFD ($P = 0.085$, $n = 15$ mice/object, unpaired t-test), $\gamma2^{+/-}$ mice treated with CD ($P = 0.09$, $n = 8$ mice/object, unpaired t-test), and $\gamma2^{+/-}$ mice treated with HFD ($P = 0.09$, $n = 11$ mice/object, unpaired t-test). No significant diet or genotype effects were observed in the proportion of time mice spent with a novel object compared to a familiar object (Diet: $F_{(1, 44)} = 0.3013$, $P = 0.59$, two-way ANOVA; Genotype: $F_{(1, 44)} = 0.0571$, $P = 0.81$, two-way ANOVA). In summary, the results gathered from the NOR test indicate the successful execution of the test. When the test was repeated 24 hours following the familiarization phase, trends showed that each mouse group was able to distinguish between the novel and familiar objects. However, when the test was performed only 1 hour after the familiarization phase, only two groups exhibited trends toward increased time interacting with a novel object (WT CD and $\gamma2^{+/-}$ HFD mice). This result suggests that a consolidation period of 24 hours was necessary for an initially novel object to be recognized subsequently as a familiar object and for it to be less interesting than a new novel object. Moreover, only WT mice treated with CD showed significant differences in time spent

exploring novel vs. familiar objects at both the 1 hour and 24 hour time points. This suggests that genetically increasing the E:I ratio ($\gamma2^{+/-}$ mice) and treating mice with HFD both adversely affect recognition memory and impair performance in the NOR test. However, the detrimental effects of an increased E:I ratio and HFD treatment are not additive since HFD-treated $\gamma2^{+/-}$ mice spent as much time exploring the novel objects as the other groups 24 hours post familiarization.

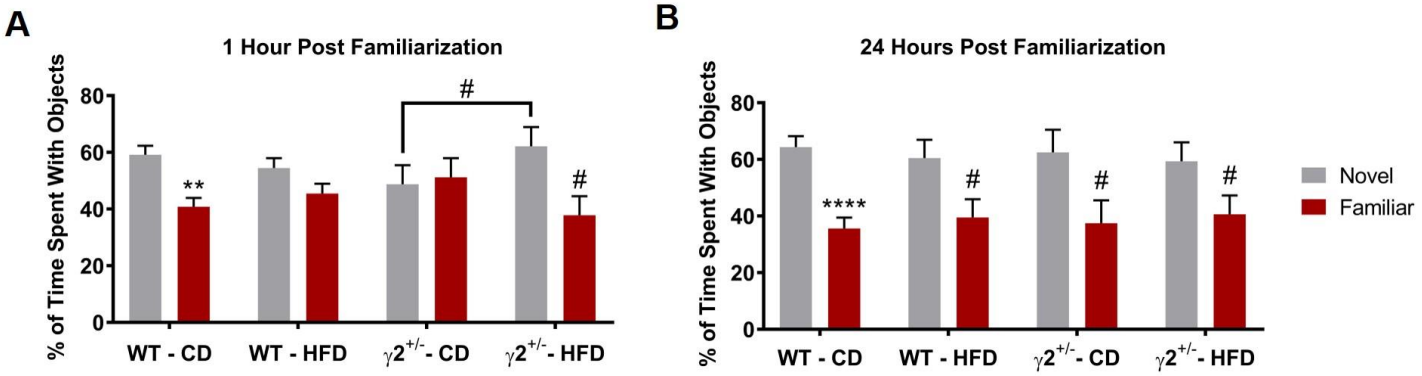

Figure 6. Recognition memory in WT and $\gamma2^{+/-}$ mice in the novel object recognition test.

Panel (A) shows the percentage of time each mouse spent interacting with a novel and a familiar object 1 hour after the familiarization phase. Of the four groups tested, WT mice exposed to CD treatment spent a greater proportion of time with a novel object than a familiar object ($P = 0.0015$, $n = 14$ mice/object, unpaired t-test). A trend was observed for the proportion of time $\gamma2^{+/-}$ mice exposed to HFD treatment spent with a novel object than a familiar object ($P = 0.056$, $n = 10$ mice/object, unpaired t-test). A trend was also observed toward increased time spent with a novel object in HFD-treated $\gamma2^{+/-}$ mice compared to $\gamma2^{+/-}$ mice treated with CD ($P = 0.078$, $n = 10 – 11$ mice/group, Fisher's LSD *post hoc* test). **Panel (B)** displays the percentage of time each mouse spent interacting with a novel and a familiar object 24 hours after the familiarization phase. Akin to panel A, WT mice exposed to CD treatment spent a greater proportion of time with a novel object compared to a familiar object ($P < 0.0001$, $n = 14$ mice/object, unpaired t-test). Similar trends were observed towards a greater proportion of time spent with a novel object compared to a familiar object for WT mice treated with HFD ($P = 0.085$, $n = 15$ mice/object, unpaired t-test), $\gamma2^{+/-}$ mice treated with CD ($P = 0.09$, $n = 8$ mice/object, unpaired t-test), and $\gamma2^{+/-}$ mice treated with HFD ($P = 0.09$, $n = 11$ mice/object, unpaired t-test). All results are presented as mean ± S.E.M.; #$P < 0.1$, **$P < 0.01$, ****$P < 0.0001$.

3.7 Chronic HFD treatment increases body weight in both $\gamma2^{f/f}$ control and SSTCre:$\gamma2^{f/f}$ mutant mice

Similar to WT control and $\gamma2^{+/-}$ mutant mice, $\gamma2^{f/f}$ control and SSTCre:$\gamma2^{f/f}$ mutant mice were also exposed to chronic HFD and CD treatment over 18 weeks before beginning behavior testing. Overall, it was found that treatment with HFD led mice to gain significantly more weight over the 18-week span than did treatment with CD, and this difference in weight gain was not

affected by genotypic differences among the mice tested (**Figure 7**). For this cohort, the baseline weights established in week 1 for each mouse group were all clustered at approximately 23 grams ($\gamma2^{f/f}$ CD: 23.39 ± 0.84 g, $\gamma2^{f/f}$ HFD: 24.19 ± 0.348 g, SSTCre:$\gamma2^{f/f}$ CD: 21.99 ± 0.763 g, SSTCre:$\gamma2^{f/f}$ HFD: 22.77 ± 0.626 g; results presented as mean ± S.E.M.), and no significant differences were found between the baseline weights of any groups ($\gamma2^{f/f}$ CD and HFD: $P = 0.97$, $n = 9$ mice/group; SSTCre:$\gamma2^{f/f}$ CD and HFD: $P = 0.97$, $n = 9$ mice/group, repeated measures two-way ANOVA). The differences in body weights between $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice treated with CD and HFD diets first became significant in week 4 ($\gamma2^{f/f}$: $P = 0.038$, $n = 9$ mice/group; SSTCre:$\gamma2^{f/f}$: $P = 0.026$, $n = 9$ mice/group, repeated measures two-way ANOVA).

These differences became increasingly significant throughout the remaining weeks for both $\gamma2^{f/f}$ mice (week 5: $P = 0.0026$, $n = 9$ mice/group; week 6: $P = 0.0004$, $n = 9$ mice/group; weeks 7-18: $P < 0.0001$, $n = 9$ mice/group; repeated measures two-way ANOVA) and SSTCre:$\gamma2^{f/f}$ mice (week 5: $P = 0.0006$, $n = 9$ mice/group; weeks 6-18: $P < 0.0001$, $n = 9$ mice/group; repeated measures two-way ANOVA). At week 18, the final body weight measurements were clustered at approximately 53 grams for HFD-treated mice ($\gamma2^{f/f}$ HFD: 54.92 ± 1.448 g, SSTCre:$\gamma2^{f/f}$ HFD: 52.12 ± 1.526 g; mean ± S.E.M.), while body weights for CD-treated mice were clustered at approximately 37 grams ($\gamma2^{f/f}$ CD: 37.49 ± 1.531 g, SSTCre:$\gamma2^{f/f}$ CD: 35.94 ± 1.761 g; mean ± S.E.M.). The results presented in Figure 7 also indicate the absence of a genotype effect on weight gain ($P > 0.1$, $n = 9$ $\gamma2^{f/f}$ mice/group and 9 SSTCre:$\gamma2^{f/f}$ mice/group, repeated measures two-way ANOVA). Therefore, the HFD-induced increase in weight gain seen in $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice is not affected by genotype.

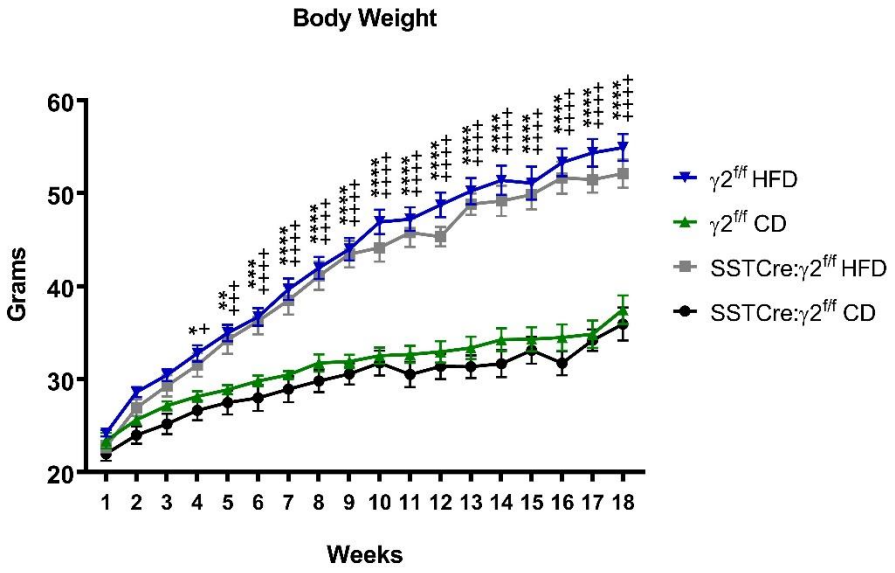

Figure 7. Weight gain in γ2$^{f/f}$ control and SSTCre:γ2$^{f/f}$ mutant mice following chronic diet treatment.

Body weights of γ2$^{f/f}$ control and SSTCre:γ2$^{f/f}$ mutant mice exposed to either CD or HFD were measured weekly over an 18-week period. Overall, mice that were treated with a HFD gained significantly more weight than mice treated with a CD, beginning in week 4 for both γ2$^{f/f}$ ($P = 0.038$, $n = 9$ mice/group, repeated measures two-way ANOVA) and SSTCre:γ2$^{f/f}$ mice ($P = 0.026$, $n = 9$ mice/group, repeated measures two-way ANOVA). No genotype-based effect was seen in relation to weight gain over the 18-week period ($P > 0.1$, $n = 9$ γ2$^{f/f}$ mice/group and 9 SSTCre:γ2$^{f/f}$ mice/group, repeated measures two-way ANOVA). Asterisk (*) indicates significance between γ2$^{f/f}$ control mice treated with HFD and CD, and plus sign (+) indicates significance between SSTCre:γ2$^{f/f}$ mutant mice treated with HFD and CD. All results are presented as mean ± S.E.M.; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, +$P < 0.05$, +++$P < 0.001$, ++++$P < 0.0001$. Body weight data were collected and generously provided by graduate student Mengyang Feng.

3.8 HFD treatment reduces locomotion in SSTCre:γ2$^{f/f}$ mutant mice in the OFT

As reported earlier by Almeida-Suhett *et al.* (2017), mice treated with HFD showed decreased locomotion and increased anxiety-like behavior in the OFT. Additionally, as reported by Fuchs *et al.* (2017), male SSTCre:γ2$^{f/f}$ mutant mice showed increased locomotion in the OFT compared to γ2$^{f/f}$ control mice. In that study, EPM was used to measure anxiety-like behavior, and it showed that SSTCre:γ2$^{f/f}$ mice exhibit an anxiolytic and antidepressant-like phenotype. Given these results, the purpose of the OFT in the present study was to determine whether HFD-induced anxiety and defects in locomotion were ameliorated in SSTCre:γ2$^{f/f}$ mutants compared to γ2$^{f/f}$ controls. However, the OFT presented here was conducted under red light (300 lux) as opposed to bright light (7000 lux), making the open field a less aversive environment and compromising its ability to measure anxiety-like behavior. Also, SSTCre:γ2$^{f/f}$ cohort mice were backcrossed to a

BL6 background, which differs from the pure 129 mice used by Fuchs *et al*. (2017).

The first parameter tested by the OFT was locomotor activity, measured as total distance travelled, over a 10 minute period. Overall, two-way ANOVA revealed a significant diet effect between the distance travelled by $\gamma2^{f/f}$ control and SSTCre:$\gamma2^{f/f}$ mutant mice ($F_{(1, 53)} = 4.738$, $P = 0.034$; **Figure 8A**). Further analysis via Fisher's LSD *post hoc* test revealed that the HFD effect was only significant in SSTCre:$\gamma2^{f/f}$ mutant mice ($P = 0.028$, $n = 11 – 16$ mice/group). Also, no genotype effect was present in locomotor activity ($F_{(1, 53)} = 1.916$, $P = 0.17$, two-way ANOVA).

The second parameter tested in the OFT was the amount of time mice spent in the center zone of the open field arena during the first 5-minute interval of the test. For this parameter, no difference was observed between the CD and HFD treatment groups ($F_{(1, 52)} = 2.043$, $P = 0.16$, two-way ANOVA) or between $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice ($F_{(1, 52)} = 2.781$, $P = 0.1$, two-way ANOVA; **Figure 8B**).

The final parameter examined in the OFT was the latency to first center zone entry during the first 5-minute interval of the test. Similar to the center zone duration parameter, no significant diet ($F_{(1, 50)} = 0.6311$, $P = 0.43$, two-way ANOVA) or genotype ($F_{(1, 50)} = 0.1407$, $P = 0.71$, two-way ANOVA) effects were observed in this parameter (**Figure 8C**). In summary, HFD treatment led to a general reduction in locomotion, and this difference was especially significant in SSTCre:$\gamma2^{f/f}$ mice. No locomotion differences were observed between CD-treated SSTCre:$\gamma2^{f/f}$ mutant and CD-treated $\gamma2^{f/f}$ control mice. Although this result differs from OFT locomotion data reported by Fuchs *et al*. (2017), the difference is likely due to strain differences among the mice tested here and by Fuchs *et al*. (2017). Unexpectedly, SSTCre:$\gamma2^{f/f}$ mutant mice did not show an anxiolytic- and antidepressive-like phenotype in the OFT, and this is likely because the OFT was

conducted under red light as opposed to under more aversive bright light.

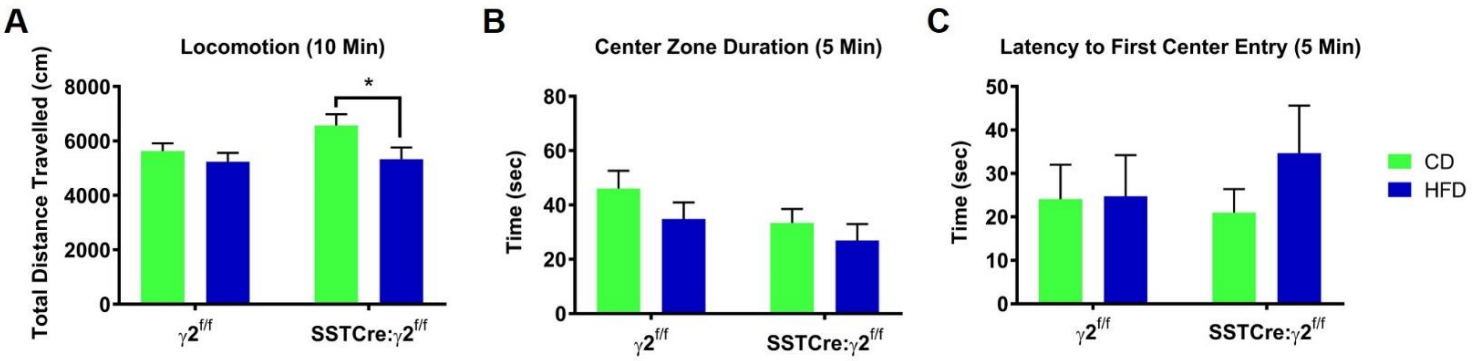

Figure 8. Locomotor activity in $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice in the open field test.

Panel (A) shows the total distance mice travelled in the open field arena over a period of 10 minutes. Overall, a diet effect was present in total distance travelled, with mice exposed to HFD treatment travelling a shorter distance than mice exposed to CD treatment ($F_{(1, 53)} = 4.738$, $P = 0.034$, two-way ANOVA). However, analysis via Fisher's LSD *post hoc* test revealed a significant diet effect only in SSTCre:$\gamma2^{f/f}$ compared to $\gamma2^{f/f}$ mice ($P = 0.028$, $n = 11 – 16$ mice/group). **Panel (B)** shows the amount of time mice spent in the center zone of the open field during the first 5-minute interval of the test. For this parameter, no significant diet ($F_{(1, 52)} = 2.043$, $P = 0.16$, two-way ANOVA) or genotype ($F_{(1, 52)} = 2.781$, $P = 0.1$, two-way ANOVA) effects were observed. **Panel (C)** shows the latency for mice to make their first entry into the center zone of the open field during the first 5-minute interval of the test. Again, no significant diet ($F_{(1, 50)} = 0.6311$, $P = 0.43$, two-way ANOVA) or genotype ($F_{(1, 50)} = 0.1407$, $P = 0.71$, two-way ANOVA) effects were observed for this parameter. All results are presented as mean ± S.E.M.; $*P < 0.05$. Data were collected and generously provided by graduate student Mengyang Feng.

3.9 Weak anxiolytic phenotype observed in SSTCre:$\gamma2^{f/f}$ mutant mice in the EPM

As mentioned above, Dutheil *et al.* (2016) identified a nonsignificant HFD-induced trend of an anxiety-like phenotype of rats in the EPM. Additionally, previous studies have shown that SSTCre:$\gamma2^{f/f}$ mutant mice exhibit an anxiolytic and antidepressant-like behavioral phenotype in this test (Fuchs *et al*., 2017). In the study by Fuchs *et al.* (2017), male SSTCre:$\gamma2^{f/f}$ mice spent a significantly greater proportion of time on the open arms compared to $\gamma2^{f/f}$ mice. Therefore, the aim of the EPM test here was to determine whether the anxiolytic and antidepressant-like phenotype of SSTCre:$\gamma2^{f/f}$ mice would diminish the anxiogenic effect of HFD.

Here, the EPM test provided a second measure of locomotion and anxiety-like behavior, in addition to the OFT. The first parameter of the EPM tested was time spent in the open arms as a

percentage of total time. A trend was observed in which CD-treated SSTCre:$\gamma2^{f/f}$ mice spent more time in the open arms compared to CD-treated $\gamma2^{f/f}$ mice (P = 0.056, n = 15 mice/group, Fisher's LSD *post hoc* test; **Figure 9A**), as predicted based on previous experiments (Fuchs *et al*., 2017). Two-way ANOVA revealed no significant diet ($F_{(1,51)}$ = 1.643, P = 0.21) or genotype ($F_{(1,51)}$ = 2.159, P = 0.15) effects.

The second parameter of the EPM measured the number of entries into open arms of the maze as a percentage of total entries. Again, two-way ANOVA revealed no significant diet ($F_{(1, 55)}$ = 0.4265, P = 0.52) or genotype ($F_{(1, 55)}$ = .9251, P = 0.34) effects (**Figure 9B**).

The final parameter tested using the EPM test was the total number of entries made into both open and closed arms during the 5-minute trial, which serves as a measure of locomotor activity. Of the mice exposed to CD treatment, SSTCre:$\gamma2^{f/f}$ mutant mice made a greater number of total entries compared to $\gamma2^{f/f}$ control mice (P = 0.044, n = 14 – 15 mice/group, Fisher's LSD *post hoc* test; **Figure 9C**), consistent with previous results (Fuchs *et al*., 2017). A diet effect was also present within the SSTCre:$\gamma2^{f/f}$ genotype group, with HFD-treated mice making fewer total entries compared to CD controls (P = 0.024, n = 13 – 14 mice/group, Fisher's LSD *post hoc* test). In summary, no significant genotype or diet effects were observed in anxiety-like behavior in the EPM for $\gamma2^{f/f}$ control and SSTCre:$\gamma2^{f/f}$ mutant mice, possibly because the test was conducted under red light (300 lux). However, a trend was evident in the open arm duration with SSTCre:$\gamma2^{f/f}$ mutant mice spend more time in the open arms compared to $\gamma2^{f/f}$ control mice, indicating a weak anxiolytic effect in SSTCre:$\gamma2^{f/f}$ mutants and matching results by Fuchs *et al*. (2017).

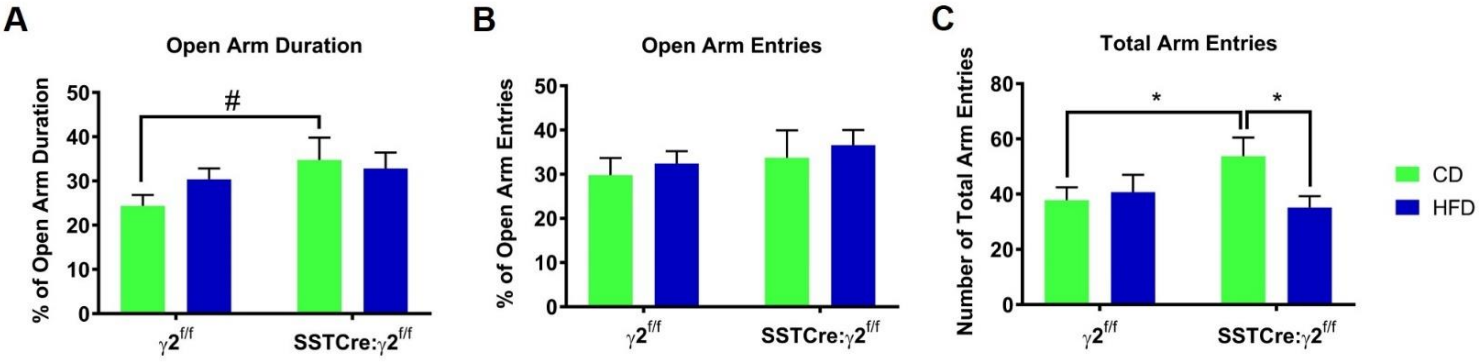

Figure 9. Anxiolytic phenotype in SSTCre:γ2$^{f/f}$ mice in the elevated plus maze.

Panel (A) presents the percent of time spent in the open arms of the EPM apparatus. In this parameter, a trend was observed towards increased time spent in the open arms by CD-treated SSTCre:γ2$^{f/f}$ mutant mice compared to CD-treated γ2$^{f/f}$ control mice (P = 0.056, n = 15 mice/group, Fisher's LSD *post hoc* test). **Panel (B)** presents the percent of entries made into the open arms of the maze. No significant diet ($F_{(1, 55)}$ = 0.4265, P = 0.52, two-way ANOVA) or genotype ($F_{(1, 55)}$ = 0.9251, P = 0.34, two-way ANOVA) was observed in the results of this parameter. **Panel (C)** presents the number of total arm entries made during the 5-minute trial, which represents a measure of locomotion in the EPM. In both CD-treated groups, mice of the SSTCre:γ2$^{f/f}$ genotype made significantly more arm entries than γ2$^{f/f}$ mice (P = 0.044, n = 14 – 15 mice/group, Fisher's LSD *post hoc* test). A diet effect was also present within the SSTCre:γ2$^{f/f}$ genotype group, with HFD-treated mice making fewer total entries than CD-treated mice (P = 0.024, n = 13 – 14 mice/group, Fisher's LSD *post hoc* test). All results are presented as mean ± S.E.M.; #P < 0.1, *P < 0.05. Data were collected and generously provided by graduate student Mengyang Feng.

3.10 No diet- or genotype-induced differences in working memory between γ2$^{f/f}$ control and SSTCre:γ2$^{f/f}$ mutant mice in the Y-maze

As described earlier, Almeida-Suhett *et al.* (2017) found a reduction in the proportion of correct alternations made by HFD-treated mice in the Y-maze, indicating HFD treatment impairs performance in a memory-based test. In the Morris water-maze test conducted by Fuchs *et al*. (2017), no significant differences were identified between γ2$^{f/f}$ and SSTCre:γ2$^{f/f}$ mice in learning and memory in the Morris water-maze. The goal of the Y-maze was to test whether the HFD-induced deficit in short-term working memory was ameliorated in SSTCre:γ2$^{f/f}$ vs. γ2$^{f/f}$ mice.

Behavior in the Y-maze test was used to assess short-term working memory performance. No significant differences were observed in the percent of correct alternations in HFD- vs. CD-treated mice ($F_{(1, 22)}$ = 0.0088, P = 0.93, two-way ANOVA), or between genotypes ($F_{(1, 22)}$ = 1.214,

P = 0.28, two-way ANOVA; **Figure 10**). In summary, no significant trends were identified in working memory between $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice in the Y-maze test.

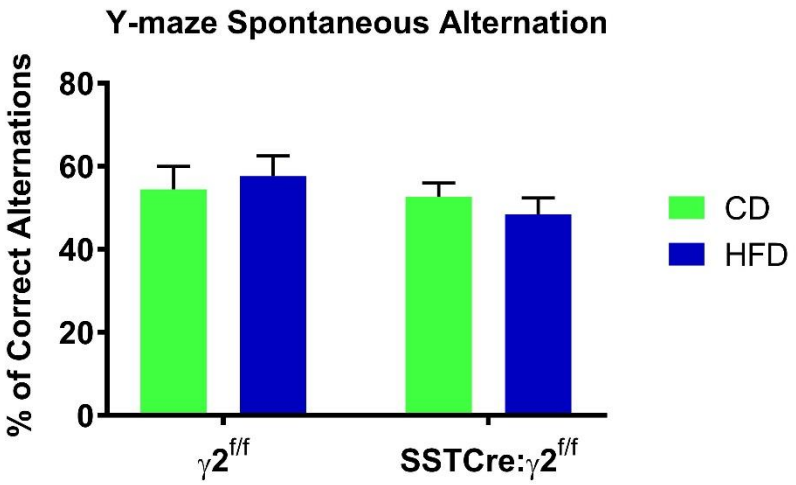

Figure 10. Assessing short-term working memory in $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice in the Y-maze test.

Data from the Y-maze are displayed as the percent of correct alternations out of total alternations. Statistical analysis using two-way ANOVA showed no diet ($F_{(1, 22)}$ = 0.0088, P = 0.93) or genotype ($F_{(1, 22)}$ = 1.214, P = 0.28) effects in the Y-maze. All results are presented as mean ± S.E.M.

3.11 HFD treatment reduces grooming behavior in $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice in the SSPT

HFD-treated rats were reported to display anhedonia in the SPT and FUST in the Dutheil *et al.* (2016) rodent study discussed previously. Using the SPT, Fuchs *et al.* (2017) assessed anhedonia in $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice and reported no difference between male SSTCre:$\gamma2^{f/f}$ mutant vs. $\gamma2^{f/f}$ control mice, with the caveat that the sucrose preference was very high even in control mice (~ 98%), leading to the suspicion of ceiling effect. Here, the SSPT was used as a measure of grooming behavior that may be related to hedonic drive, and to assess whether HFD-induced reductions in grooming of $\gamma2^{f/f}$ mice would be alleviated in SSTCre:$\gamma2^{f/f}$ mutant mice.

The first parameter measured by the SSPT was the cumulative time spent grooming during 5-minutes. A significant interaction was observed between diet and genotype ($F_{(1, 56)}$ = 4.301, P = 0.043, two-way ANOVA; **Figure 11A**). *Post hoc* Fisher's LSD tests indicated a reduction in

grooming duration of HFD-treated SSTCre:$\gamma2^{f/f}$ mice compared to HFD-treated $\gamma2^{f/f}$ mice (P = 0.014, n = 15 – 16 mice/group). Fisher's LSD *post hoc* test also revealed a trend toward reduced grooming duration in HFD SSTCre:$\gamma2^{f/f}$ mice compared to CD SSTCre:$\gamma2^{f/f}$ mice (P = 0.081, n = 15 – 16 mice/group). No significant diet effect was present within the $\gamma2^{f/f}$ group (P = 0.25, n = 13 – 16 mice/group, Fisher's LSD *post hoc* test).

The second parameter measured in the SSPT was the total number of grooming sessions undertaken during the 5-minute test. Two-way ANOVA revealed a highly significant diet effect among both $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice, with HFD-treated mice undertaking fewer grooming sessions compared to CD-treated mice ($F_{(1, 56)}$ = 62.72, P < 0.0001; **Figure 11B**). No genotype effect was found for grooming sessions ($F_{(1, 56)}$ = 1.37, P = 0.25, two-way ANOVA). In summary, HFD treatment led to a defect in grooming behavior in both $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice. However, it should be noted that results in Figure 11 represent preliminary data because the test was performed under nonstandard conditions. No stainless-steel wire lid was placed above the cage, causing mice to groom for a much shorter duration (<50 seconds) than mice in the $\gamma2^{+/-}$ cohort (>150 seconds), seemingly due to heightened anxiety in the absence of the wire lid.

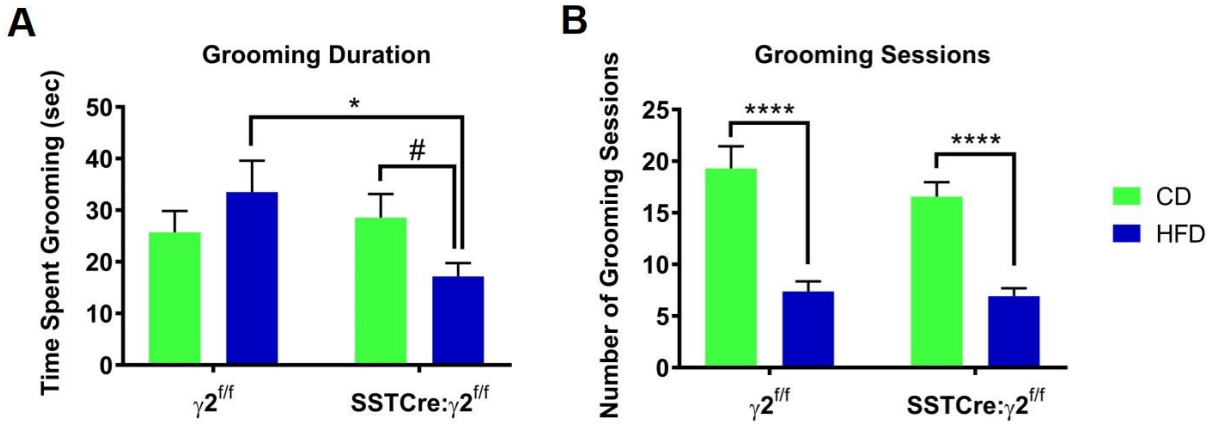

Figure 11. Grooming behavior in $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice in the sucrose splash test.

Panel (A) shows the cumulative time each mouse spent grooming during the 5-minute test. Two-way ANOVA revealed a significant interaction between diet and genotype ($F_{(1, 56)}$ = 4.301, P = 0.043). Further analysis via Fisher's LSD *post hoc* test revealed that SSTCre:$\gamma2^{f/f}$ mice treated with HFD groomed for a significantly shorter duration compared to $\gamma2^{f/f}$ mice treated with HFD (P = 0.014, n = 15 – 16 mice/group). Furthermore, *post hoc* analysis revealed a trend towards decreased grooming duration for SSTCre:$\gamma2^{f/f}$ mice treated with HFD compared to SSTCre:$\gamma2^{f/f}$ mice treated with CD (P = 0.081, n = 15 – 16 mice/group). **Panel (B)** shows the number of grooming sessions each mouse participated in. Overall, a highly significant diet effect was present, with HFD-treated $\gamma2^{f/f}$ and SSTCre:$\gamma2^{f/f}$ mice participating in less grooming sessions compared to their CD-treated counterparts ($F_{(1, 56)}$ = 62.72, P < 0.0001, two-way ANOVA). All data are presented as mean ± S.E.M., #P < 0.1, *P < 0.05, ****P < 0.0001.

CHAPTER 4

Discussion

The purpose of the present study was to investigate whether the behavioral phenotypes associated with a mouse model of HFD-induced MDD were exacerbated or alleviated in mutant mice with increased or reduced synaptic E:I ratios ($\gamma2^{+/-}$ mutants and SSTCre:$\gamma2^{f/f}$ mutants, respectively). At the onset of the study, it was hypothesized that increasing the E:I ratio in the $\gamma2^{+/-}$ model would exacerbate HFD-induced anxious- and depressive-like behavior, including defects in locomotion, grooming behavior, and short-term working and recognition memory. Conversely, it was predicted that reducing the E:I ratio in the SSTCre:$\gamma2^{f/f}$ model would ameliorate HFD-induced anxious- and depressive-like behavior, including defects in locomotion, grooming behavior, and short-term working and recognition memory. The specific phenotypes examined were locomotion in the OFT, anxiety-like behavior in the EPM, grooming behavior in the SSPT, and working and recognition memory in the Y-maze and NOR tests, respectively. Overall, $\gamma2^{+/-}$ mutant mice were indistinguishable from WT mice with respect to HFD-induced reduction of locomotion in the OFT and reduction of grooming behavior in the SSPT. In terms of short-term working memory, HFD treatment induced a reduction in memory performance of WT control mice in the Y-maze but induced an increase in performance of $\gamma2^{+/-}$ mutant mice. Similarly, in the NOR test of recognition memory, HFD treatment increased $\gamma2^{+/-}$ mutants' ability to identify a novel object compared to CD-treated $\gamma2^{+/-}$ mutants in the 1 hour post familiarization test. These data suggest that HFD and $GABA_A$ receptor defects induce impaired memory by very different mechanisms. SSTCre:$\gamma2^{f/f}$ mutant mice presented an anxiolytic phenotype and increased locomotion in the EPM, consistent with previous results of these same mice analyzed on a 129 background (Fuchs *et al*, 2017). HFD

treatment of SSTCre:$\gamma2^{f/f}$ mice reduced locomotion in the OFT and EPM, and decreased grooming behavior in the SSPT.

As displayed in Figures 1 and 7, for both $\gamma2^{+/-}$ and SSTCre:$\gamma2^{f/f}$ cohorts, and their respective WT and $\gamma2^{f/f}$ controls, mice subjected to chronic HFD treatment over an 18-week span gained significantly more weight than mice subjected to chronic CD treatment. Data presented in the weight curves for both the $\gamma2^{+/-}$ and SSTCre:$\gamma2^{f/f}$ cohorts signify the absence of a genotype effect, indicating that the difference in weight gain over 18 weeks was a result of HFD treatment. When baseline weights were measured during Week 1, no significant differences existed among the four diet–genotype groups in either the $\gamma2^{+/-}$ or SSTCre:$\gamma2^{f/f}$ cohorts, thereby excluding differences in baseline weights as an explanation for the differences in weight gain observed between HFD-treated and CD-treated mice.

Compared to the body weight curve of WT and $\gamma2^{+/-}$ mice (Figure 1), control and mutant mice in the SSTCre:$\gamma2^{f/f}$ cohort had higher baseline and final weights for both the CD- and HFD-treated groups (Figure 7). This difference in baseline and final weights may be a result of strain differences between mice in the $\gamma2^{+/-}$ and SSTCre:$\gamma2^{f/f}$ cohorts. All mice in the $\gamma2^{+/-}$ cohort were developed on a pure BL6 genetic background, while mice in the SSTCre:$\gamma2^{f/f}$ cohort were originally based on a 129 genetic background but were backcrossed to BL6 across four to five generations. Strain differences among the mice tested here are nontrivial, especially since strain- and environment-based variability can impact the behavioral performance of mice (Crabbe *et al*., 1999; Abramov *et al*., 2008). In fact, in behavioral studies comparing mice of different strains, including the 129 and BL6 strains, wide variations were shown to exist in several behavioral assessments, including OFT, EPM, water-maze, and forced swim tests (Crabbe *et al*., 1999; Abramov *et al*., 2008). Furthermore, these studies found significant differences in the performance

of each mouse strain depending on the environments the mice were exposed to (Crabbe *et al*., 1999; Abramov *et al*., 2008).

According to the results communicated in the literature, HFD treatment on rodents decreases locomotion, and increases anxiety-like behavior in the OFT and EPM (Dutheil *et al*., 2016; Almeida-Suhett *et al*., 2017). Additionally, $\gamma2^{+/-}$ mutant mice exhibit a robust anxiety-like behavioral phenotype in the EPM (Crestani *et al*., 1999). Conversely, SSTCre:$\gamma2^{f/f}$ mutant mice show increased locomotion in the OFT and an anxiolytic phenotype in the EPM (Fuchs *et al*., 2017). HFD treatment decreased locomotion in both WT control and $\gamma2^{+/-}$ mutant mice in the OFT. For mice in the SSTCre:$\gamma2^{f/f}$ cohort, HFD treatment reduced locomotion only for SSTCre:$\gamma2^{f/f}$ mutants in both the OFT and EPM. However, no difference in locomotion was observed between control and mutant mice in either the $\gamma2^{+/-}$ or SSTCre:$\gamma2^{f/f}$ cohort, indicating HFD treatment produced a similar impairment in locomotion regardless of genotype. Finally, HFD treatment unexpectedly failed to decrease locomotion in $\gamma2^{+/-}$ control mice as it did in WT control mice. This discrepancy in the locomotion data of control mice is likely a result of strain differences between $\gamma2^{f/f}$ and WT mice, since WT mice were on a pure BL6 background while $\gamma2^{f/f}$ mice were on a mixed 129/BL6 background.

In regard to anxiety-like behavior, the OFT conducted in the present study was not designed to detect anxiety, since it was performed under red light (300 lux) as opposed to more aversive bright light (7000 lux). Therefore, the center zone duration and latency to first center entry parameters of the OFT should not be viewed as definitive measures of anxiety. This explains why an absence of an anxiolytic phenotype of SSTCre:$\gamma2^{f/f}$ mutants vs. $\gamma2^{f/f}$ controls was observed in the OFT (Figure 8). Only the EPM was a valid test of anxiety. In the EPM, no diet- or genotype-based effects indicating anxiety-like behavior were observed for mice in the $\gamma2^{+/-}$ cohort, which

contrasts the EPM results reported by Crestani *et al*. (1999) (Figure 3). This discrepancy is likely a result of differences in mouse strain between the animals tested by Crestani *et al*. (1999) and the animals used in the present study. Here, pure BL6 WT and $\gamma2^{+/-}$ mice were used, which are known to be more anxious than the 129 WT and $\gamma2^{+/-}$ mice used by Crestani *et al*. (1999). Thus, the genotype-induced anxiety phenotype of $\gamma2^{+/-}$ mice is more difficult to detect in mutants on the BL6 background than on the 129 background.

For mice in the SSTCre:$\gamma2^{f/f}$ cohort, a trend toward increased duration in the open arms was observed for CD-treated SSTCre:$\gamma2^{f/f}$ mutants compared to CD-treated $\gamma2^{f/f}$ control mice, indicating an anxiolytic phenotype and confirming results by Fuchs *et al*. (2017) (Figure 9). Unexpectedly, unaltered open arm entries of SSTCre:$\gamma2^{f/f}$ mutants vs. $\gamma2^{f/f}$ controls were also observed in the EPM, in contrast to the trend of increased open arm entries for male SSTCre:$\gamma2^{f/f}$ mice reported by Fuchs *et al*. (2017). However, this unexpected result could be due to strain differences between the mice used for the present study and mice used by Fuchs *et al*. (2017). The SSTCre:$\gamma2^{f/f}$ mutant mice analyzed here were on a mixed 129/BL6 genetic background, and they show more pronounced hyperlocomotion in the OFT (~6000 cm) vs. $\gamma2^{f/f}$ control mice than was apparent for SSTCre:$\gamma2^{f/f}$ mutant mice on the 129 background (~4000 cm) in the Fuchs *et al*. (2017) study. This increased locomotion could interfere with the detection of an anxiety-like phenotype in the open arm entries parameter of the EPM.

Previously, HFD treatment was shown to impair short-term working memory in the Y-maze test, with HFD-treated mice showing a significant reduction in the proportion of correct alternations made (Almeida-Suhett *et al*., 2017). As demonstrated in a separate study, no differences were observed between $\gamma2^{+/-}$ mutant and WT control mice in the Morris water-maze test (a test used to assess learning and memory in rodents; the main parameters tested include time

to reach a learned target area and number of entries into that area) or Y-maze test (Crestani *et al*., 1999). In SSTCre:$\gamma2^{f/f}$ mutant mice, no significant differences were identified in learning and memory between the mutants and $\gamma2^{f/f}$ control mice in the Morris water-maze test (Fuchs *et al*., 2017). As expected, in the present study, the Y-maze test showed that HFD treatment impaired working memory in WT mice, as evidenced by a reduction in the proportion of correct alternations, thus matching results by Almeida-Suhett *et al*. (2017) (Figure 4). Additionally, a trend in the data indicated that, among mice treated with CD, $\gamma2^{+/-}$ mice performed worse than WT mice and showed a decreased proportion of correct alternations (Figure 4). Conversely, among HFD-treated mice, $\gamma2^{+/-}$ mice outperformed WT mice and showed a greater proportion of correct alternations. The hypothesis predicted that HFD-induced defects in memory would be exacerbated in $\gamma2^{+/-}$ mutant vs. WT control mice, but the Y-maze results unexpectedly show that HFD treatment improved the short-term working memory of $\gamma2^{+/-}$ mice. These data indicate that genotype and diet effects on memory are nonadditive, and that HFD treatment and an increased E:I ratio impair memory performance by very different mechanisms.

No diet or genotype effects were observed for the SSTCre:$\gamma2^{f/f}$ mouse cohort in the Y-maze (Figure 10). Although this result confirms evidence published by Fuchs *et al.* (2017), who showed that no differences exist between $\gamma2^{f/f}$ control and SSTCre:$\gamma2^{f/f}$ mutant mice in the Morris water-maze, the absence of a HFD effect on the performance of $\gamma2^{f/f}$ control mice is incompatible with data obtained in the Y-maze for WT mice in Figure 4. This discrepancy between WT and $\gamma2^{f/f}$ control mice in their respective Y-maze tests may be due to genetic strain differences among the mice, since $\gamma2^{f/f}$ mice were on a mixed BL6/129 background while the WT mice analyzed in Figure 4 were on a pure BL6 background.

The NOR test serves to assess recognition memory. It was exclusively performed using the $\gamma2^{+/-}$ mouse cohort because in the Y-maze, the HFD-induced defects in working memory were observed in WT mice, with HFD-treated WT mice showing fewer correct alternations compared to CD-treated WT mice. Additionally, significant genotype X diet interactions were also observed for the $\gamma2^{+/-}$ cohort but not the SSTCre:$\gamma2^{f/f}$ cohort in the Y-maze. Previous analyses of rats treated with chronic HFD revealed that they performed worse in the NOR test compared to rats treated with CD, with HFD-treated animals spending less time exploring the novel object (Fu *et al*., 2017). As mentioned above, Crestani *et al.* (1999) observed no differences between $\gamma2^{+/-}$ mutant and WT control mice in the Morris water-maze or Y-maze, which are both memory-based tests (Crestani *et al*., 1999). Data from the NOR test discussed here show that a consolidation phase was necessary before all animals could successfully distinguish between the novel and familiar objects, as evidenced by increased time spent exploring the novel objects (Figure 6). Only two groups (WT-CD and $\gamma2^{+/-}$-HFD) were able to distinguish between novel and familiar objects when recognition memory was assessed within 1 hour of first exposure to the familiarized objects, but when the test was repeated 24 hours after the familiarization phase, all four mouse groups showed preference for the novel object.

Interestingly, the results of the NOR test conducted 1 hour after familiarization recapitulate the results obtained for the $\gamma2^{+/-}$ cohort in the Y-maze. In the NOR test, a trend was observed in which HFD-treated $\gamma2^{+/-}$ mice spent more time exploring the novel objects compared to CD-treated $\gamma2^{+/-}$ mice. HFD-treated $\gamma2^{+/-}$ mice also showed a trend toward increased time spent exploring the novel vs. familiar object, but no such trend was observed for CD-treated $\gamma2^{+/-}$ mice. These results reflect the results obtained for $\gamma2^{+/-}$ mice in the Y-maze, namely the significant increase in percent of correct alternations of HFD-treated $\gamma2^{+/-}$ mice compared to CD-treated $\gamma2^{+/-}$ mice. Similarly, in

WT mice tested in the NOR, CD-treated WT mice were able to distinguish between novel and familiar objects, while HFD-treated WT mice were not. This result also corresponds to the Y-maze, in which CD-treated WT mice showed a significantly greater percent of correct alternations compared to HFD-treated WT mice. Taken together, these corresponding data from both the 1 hour post familiarization NOR test and Y-maze test of short-term working memory suggest that HFD-induced effects on memory performance are more pronounced on short-term vs. long-term memory.

Rodent studies of HFD treatment have reported that male rats exposed to HFD treatment exhibit an anhedonia-like phenotype, as characterized by the SPT and FUST (Dutheil *et al*., 2016). However, unpublished data by the Luscher lab suggest no differences between HFD-treated and CD-treated WT mice in the SPT. In the SCT, $\gamma2^{+/-}$ mutant mice also indicated an anhedonia-like phenotype by consuming less sucrose solution compared to WT mice (Shen *et al*., 2010). Finally, no significant differences were present between SSTCre:$\gamma2^{f/f}$ mutant and $\gamma2^{f/f}$ control mice in the SPT, likely because sucrose preference was very high even in the control mice (~98%), leading to the suspicion of ceiling effect in this test (Fuchs *et al*., 2017). Taken together, these studies from the literature indicate that HFD treatment induces anhedonia, and anhedonia is only affected in a mouse model of increased E:I ratio. Here, the SSPT was conducted as an assessment of grooming, a behavior which is reduced by chronic stress and is reversible by chronic but not acute antidepressant drug treatment (Willner *et al*., 2013). For the $\gamma2^{+/-}$ cohort, results from the SSPT show that HFD-treated WT and $\gamma2^{+/-}$ mice groomed for a shorter duration compared to their CD-treated counterparts, indicating an overall diet-induced reduction in grooming that is independent of differences in sucrose preference (Figure 5). In the SSTCre:$\gamma2^{f/f}$ cohort, the SSPT showed a HFD-induced reduction in the number of grooming sessions, but no genotype-based differences

were observed between $\gamma2^{f/f}$ control and SSTCre:$\gamma2^{f/f}$ mutant mice for that parameter (Figure 11). Furthermore, a trend in the data indicated a HFD-induced reduction in grooming duration in SSTCre:$\gamma2^{f/f}$ mutants vs. CD-treated mutants, as well as a significant reduction in grooming for HFD-treated SSTCre:$\gamma2^{f/f}$ mutants vs. $\gamma2^{f/f}$ control mice. According to the hypothesis, SSTCre:$\gamma2^{f/f}$ mice were expected to show an increase in grooming and resistance to HFD-induced defects in grooming compared to $\gamma2^{f/f}$ controls, based on the robust anxiolytic and antidepressant-like phenotype observed in SSTCre:$\gamma2^{f/f}$ mice (Fuchs *et al*., 2017). However, this was not observed in the SSPT discussed here. Furthermore, as expected, HFD treatment produced the same defect in grooming behavior in both WT control and $\gamma2^{f/f}$ control mice. A major caveat to these results, however, is that SSPT data from the SSTCre:$\gamma2^{f/f}$ mouse cohort should be taken with a grain of salt because nonstandard testing conditions were present for that test. For this cohort's SSPT, no stainless-steel wire lid was placed above the cage, causing control and mutant mice to groom for a much shorter duration (<50 seconds) than mice in the $\gamma2^{+/-}$ cohort (>150 seconds), seemingly due to heightened anxiety in this test environment.

A number of potential steps could be taken to improve the behavioral experiments conducted in the present study. The OFT paradigm used in the present study may not have been as aversive as intended, as evidenced by the fact that mice with a pure 129 genetic background, as tested by Fuchs *et al*. (2017), travelled a shorter distance in the OFT test (<3000 cm) compared to mice with a pure BL6 genetic background (>5000 cm) (Figure 2). In the OFT, modifications could be made in order to better assess for anxiety-like behavior. For example, a bright light (7000 lux) could be placed above the open field arena or fox urine pellets could be placed near the arena to create a more aversive environment. Theoretically, these additions should cause the mice to experience greater stress during the OFT, which should facilitate detection of an anxiolytic

phenotype. In the EPM, the transparent Plexiglas walls surrounding the closed arms could be swapped for black, opaque walls to prevent mice from perceiving the maze's elevation. The intent of this modification would be to increase the relative anxiogenic aversion of the open arms compared to the closed arms, which may facilitate the detection of an anxiolytic phenotype. Furthermore, when conducting the EPM experiment, trials could be started by placing mice in the center of the maze rather than on an open arm, thus preventing mice from being biased towards the open arms.

As a result of the data obtained through the behavioral experiments in the present study, several interesting avenues for further investigation have emerged. Because significant results were not produced from every behavior test, which could be due to inherent variability in how the tests were conducted, new behavioral assays could be used to further probe whether HFD-induced behavioral deficits are exacerbated or ameliorated in mouse models with altered E:I ratios. Tests such as the free-choice exploration and light-dark choice tests could be employed to complement the use of the OFT and EPM as assessments of anxiety-like behavior, and tests such as the SPT and FUST could be employed to assess anhedonia (Dutheil *et al*., 2016; Crestani *et al*., 1999; Fuchs *et al*., 2017). After obtaining clear and robust phenotypes in these tests, mice in the $\gamma2^{+/-}$ cohort could be sacrificed and their brains could be used for analysis in electrophysiological studies; similar electrophysiological analysis could also be done on the brains of SSTCre:$\gamma2^{f/f}$ cohort mice. The proposed parameters that would be examined in these electrophysiological studies would be miniature inhibitory postsynaptic current (mIPSC), spontaneous inhibitory postsynaptic current (sIPSC), and spontaneous excitatory postsynaptic current (sEPSC). The study by Fuchs *et al*. (2017) also analyzed these parameters in SST+ interneurons in the hippocampus and cingulate cortex of SSTCre:$\gamma2^{f/f}$ mutant mice. The study reported reductions in the frequencies

and amplitudes of both sIPSC and mIPSC, but no differences in the frequency and amplitude of EPSCs, and these results were independent of brain region (Fuchs *et al*., 2017). Furthermore, Ren *et al*. (2016) performed electrophysiology experiments to analyze the frequency and amplitude of mIPSC recordings from the hippocampus and anterior cingulate cortex of $\gamma2^{+/-}$ mutant mice. In that study, $\gamma2^{+/-}$ mice exhibited reduced mIPSC amplitude, but not frequency, in both the hippocampus and anterior cingulate cortex (Ren *et al*., 2016). Thus, electrophysiology experiments could build upon the results of the present study by testing whether HFD-induced inflammation alters the neural circuitry of $\gamma2^{+/-}$ mutant mice compared to WT controls, as well as SSTCre:$\gamma2^{f/f}$ mutant mice compared to $\gamma2^{f/f}$ control mice. The overall goal of these electrophysiological studies would be to determine the specific brain regions that contribute to the anxiety-like and anhedonia phenotypes observed in the behavior studies.

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Research Experiences

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