High fructose corn syrup consumption in adolescent rats causes bipolar-like behavioural phenotype with hyperexcitability in hippocampal CA3-CA1 synapses

High fructose led to bipolar-like phenotype in adolescent rats

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ABSTRACT

**Background and Purpose.** Children and adolescents are the top consumers of high fructose corn syrup (HFCS) sweetened beverages. Even though the cardiometabolic consequences of HFCS consumption in adolescents are well known, the neuropsychiatric consequences have yet to be determined.

**Experimental Approach.** Adolescent rats were fed for a month with 11% weight/volume carbohydrate containing HFCS solution, which is similar to the sugar sweetened beverages of human consumption. The metabolic, behavioural and electrophysiologic characteristics of HFCS-fed rats were determined. Furthermore, the effects of TDZD-8, a highly specific GSK-3 inhibitor, on the HFCS-induced alterations were further explored.

**Key Results.** HFCS-fed adolescent rats displayed bipolar-like behavioural phenotype with hyperexcitability in hippocampal CA3-CA1 synapses. This hyperexcitability was associated with increased presynaptic release probability and increased density of postsynaptic AMPA receptors, due to decreased expression of the neuron-specific α3-subunit of Na⁺/K⁺-ATPase (NKA) and an increased ser845-phosphorylation of GluR1 subunits of AMPA receptors, respectively. TDZD-8 treatment was found to restore behavioural and electrophysiological disturbances associated with HFCS consumption by inhibition of GSK-3, the most probable mechanism of action of lithium for its mood-stabilizing effects.

**Conclusion and Implications.** This study shows that HFCS consumption in adolescent rats led to bipolar-like behavioural phenotype with neuronal hyperexcitability, which is known to be one of the earliest endophenotypic manifestations of bipolar disorder. Inhibition of GSK-3 with TDZD-8 attenuated hyperexcitability and restored HFCS-induced behavioural alterations.

**Keywords:** high fructose corn syrup, bipolar disorder, hyperexcitability, GluR1, AMPA, GSK-3, TDZD-8

**Abbreviations**

- HFCS – High Fructose Corn Syrup
- SSB – Sugar sweetened beverage
- T2DM – Type 2 diabetes mellitus
- BD – Bipolar Disorder
- NKA - Na⁺/K⁺-ATPase
- OGTT – Oral glucose tolerance test
- OFA – Open Field Arena
- EPM – Elevated Plus Maze
- FST – Forced Swim Test
- MWM – Morris Water Maze
- FUST – Female Urine Sniffing Test
BULLET POINT SUMMARY

What is already known:

- High fructose corn syrup (HFCS) is one of the most important nutritional cause of type 2 diabetes mellitus (T2DM) in children and adolescents.
- T2DM and bipolar disorder are recently recognized comorbidities.

What this study adds:

- HFCS consumption in adolescent rats causes bipolar-like behavioural phenotype with neuronal hyperexcitability.
- Inhibition of GSK-3 with TDZD-8 successfully restored HFCS-induced alterations.

Clinical Significance:

- The relationship between HFCS consumption and emergence of bipolar-like behaviours should be investigated in clinical settings.
- Specific GSK-3 inhibitors may be valuable therapeutics in bipolar patients with comorbid T2DM.
INTRODUCTION

The streamlining of the production process for high fructose corn syrup (HFCS) in 1970s has led this new product to gradually replace sucrose and other commonly used sweeteners. Since then, HFCS consumption has increased rapidly through the increased consumption of sugar sweetened beverages (SSBs), in which HFCS is used as the major caloric sweetener (Marriott et al., 2009). Since the top consumers of SSBs are adolescents rather than adults, adolescents have the highest estimated mean intake of fructose among all age groups (Vos et al., 2008, Marriott et al., 2009). When total caloric intake was accounted for, this continuous rise in the consumption of HFCS is found to be the most important nutritional factor causing increased prevalence of type 2 diabetes mellitus (T2DM) (Gross et al., 2004). Today, it has been very well characterized that the fructose is the reason for the development of HFCS-induced insulin resistance, fatty liver and hypertriglyceridemia (Lim et al., 2010, Baena et al., 2016). Even though the cardiometabolic consequences of HFCS consumption in adolescent period have been relatively studied (Pollock et al., 2012), the neuropsychiatric consequences have yet to be determined.

Rapidly increasing evidence suggests that psychiatric diagnoses, especially bipolar disorder (BD), are significantly more common in patients with T2DM (Wandell et al., 2014, Charles et al., 2016). BD is a genetically heterogeneous, highly heritable and devastating condition with a mean prevalence of 1.8% in children and adolescents (Van Meter et al., 2011). It is the fourth leading cause of disability adjusted life years among the adolescents (Gore et al., 2011). Unfortunately, the pathophysiology of BD and how T2DM is linked to BD remain unknown.

One of the prominent hypotheses for the mechanism of BD is that a primary or secondary dysfunction of Na\(^+\)/K\(^+\)-ATPase (NKA) predisposes or even directly causes the clinical manifestations (Singh, 1970, el-Mallakh and Wyatt, 1995). In the central nervous system (CNS), the catalytic \(\alpha\)-subunit of NKA exists as three isoforms: \(\alpha1\) and \(\alpha2\) are found in both neurons and glia, whereas \(\alpha3\) is exclusively expressed in neurons (Dobretsov and Stimers, 2005). Several single nucleotide polymorphisms across all three \(\alpha\) isoforms have been associated with the BD (Goldstein et al., 2009). Furthermore, intracerebroventricular administration of ouabain, an inhibitor of NKA, is widely recognized as a valid animal model of mania (El-Mallakh et al., 2003). Mice carrying an inactivating mutation in neuron specific...
α3-subunit of NKA showed a **behavioural** phenotype resembling those of patients with BD (Kirshenbaum et al., 2011). It is still not known how this decrease in NKA amount and/or activity translates to the **behavioural** changes evident in patients with BD. Interestingly, diabetes was shown to decrease the amount and/or activity of NKA in different brain regions (Leong and Leung, 1991). The region with the greatest and most significant decrease in NKA activity in the brain was the hippocampus (Leong and Leung, 1991), which is known to be involved in pathophysiology of BD.

Apart from the NKA hypothesis, growing evidence suggests that neuroplasticity plays a central role in the pathophysiology and treatment of BD (Schloesser et al., 2008, Zarate et al., 2006). Specifically, the modulation of AMPA receptor trafficking has gained attention because of being a target for the two most common mood stabilizing agents: lithium and valproate (Du et al., 2004b). Chronic treatments with therapeutically relevant concentrations of lithium or valproate were shown to decrease the synaptic expression of GluR1 subunit of AMPA receptors in the hippocampus by attenuating ser845-phosphorylation of GluR1 (Du et al., 2003, Du et al., 2004a, Du et al., 2008). Increased AMPA receptor density in synapses is thought to be necessary for the emergence of bipolar-like **behavioural** phenotype, as AMPA antagonists attenuates amphetamine-induced hyperactivity (Du et al., 2008). In addition to postsynaptic alterations, presynaptic changes were also linked to BD. One study reported increased glutamate levels in postmortem human bipolar brains (Lan et al., 2009). Magnetic resonance spectroscopy studies further supported the increased glutamate levels in brains of bipolar patients (Yuksel and Ongur, 2010). In addition, both lithium and valproate were shown to decrease the synaptic glutamate levels (Dixon and Hokin, 1998, Hassel et al., 2001). Last but not least, a recent study showed that hippocampal neurons of patients with BD had hyperexcitability, which was reversed by lithium only in neurons derived from patients who also clinically responded to lithium (Mertens et al., 2015). This study clearly demonstrates that hyperexcitability is not only a coincidental feature seen with bipolar disorder, but also a feature contributing to the pathophysiology of it.

In this study, we hypothesized that the consumption of HFCS causes bipolar-like **behavioural** phenotype in adolescent rats. In addition, we have investigated the electrophysiological and molecular effects of HFCS consumption on hippocampal synaptic function as both neuronal hyperexcitability and decreased NKA levels might explain the association between HFCS consumption and bipolar-like **behavioural** phenotype. As previous studies suggested that the
HFCS-induced inflammation might cause the associated neuropsychiatric disturbances (Hsu et al., 2015), we have also investigated the changes in proinflammatory cytokines in serum and hippocampi of HFCS-fed adolescent rats. Previous literature also demonstrated that hippocampal insulin receptors respond to insulin similar to insulin-responsive peripheral tissues (Grillo et al., 2009) and become resistant to insulin in response high fructose consumption (Mielke et al., 2005). Moreover, hippocampal functions were found to be impaired when the expression of insulin receptors and insulin receptor substrate (IRS) in hippocampal neurons were decreased (Grillo et al., 2015, Costello et al., 2012). We investigated whether HFCS-induced alterations are due to impaired insulin pathway in hippocampi of HFCS fed rats.

Inhibition of GSK3 has been shown to be the mechanism of action for lithium and other commonly used mood-stabilizers for their mood-stabilizing effect. (Klein and Melton, 1996, Gould et al., 2004a, Ryves and Harwood, 2001, De Sarno et al., 2002, Chalecka-Franaszek and Chuang, 1999, Li et al., 2004, Gould and Manji, 2005). Recently developed highly specific GSK-3 inhibitors were proven to have mood-stabilizing effects on animal models of BD, suggesting that the primary mechanism of action of lithium is, indeed, inhibition of GSK-3 (Kalinichev and Dawson, 2011, Gould et al., 2004b, Kaidanovich-Beilin et al., 2004, Valvassori et al., 2017). In addition to pharmacological interventions, genetic manipulations of GSK-3 activity further support this hypothesis (O’Brien et al., 2004, Polter et al., 2010, Prickaerts et al., 2006). Because of these indisputable evidences suggesting the critical role of the inhibition of GSK-3 in the treatment of BD, we decided to investigate the effects of TDZD-8, a highly specific inhibitor of GSK-3 (Martinez et al., 2002), on HFCS-induced alterations.
METHODS

Animals. Male Wistar Albino rats (Kobay, Turkey) of 21 days of age, weighing 45-55 g at the time of arrival, were housed as triplets in polycarbonate cages with wood shaving beddings on a 12/12 h light/dark schedule (lights on at 7:00 am) in a temperature (20-22°C) and humidity (40-50%) controlled room. No enrichment in the cages was provided other than what was mentioned above. Rats were given 4 weeks for acclimation prior to any procedure and handled in order to familiarize rats with the experimenter. All experiments and tissue harvesting were done in the light cycle. All procedures were approved by the Hacettepe University Animal Experimentations Ethics Board, necessitating the standards of ARRIVE and NIH (No: 2016/12-08).

Determination of Sample Size. The number of animals for each group in behavioural, electrophysiological, qRT-PCR and ELISA experiments is calculated using two separate methods (Charan and Kantharia, 2013). (I) First, G*Power program (Duesseldorf University, Germany) was used to calculate number of subjects using power analysis. The desired α error (type I error) probability was set as 0.05, and power was set as 0.8 (1-β, 1-type II error probability). These values yielded a total sample size of 30, which equates to 10 animals per group. (II) The second method used for the calculation of the number of animals for each group was the resource equation. The criterion for this method is that total number of animals-total number of groups should be between 10 and 20. According to this method, the maximum number of animals to be used for the experiment is 21, which equals to 7 animals per group. Thus, it was decided to determine a number between these two values that two different methods has given to us. Considering the possible attrition/death and also the 3R policies, we have decided on using 9 animals per group. OGTT protocol used 10 animals per non-drug group and 5 animals for drug group due to the shortage of TDZD-8. 3 rats (1 rat/group) allocated to open field arena and female urine sniffing test on the same day were excluded due to error in system causing loss of data. 12 rats (4 rat/group) were used for intracerebroventricular insulin administration and immunoblotting experiments.

Diets. Control group had ad libitum access to tap water and chow, whereas HFCS group had ad libitum access to a HFCS solution containing 11% weight/volume (w/v) carbohydrate and chow. HFCS group was not presented with tap water in addition to HFCS solution, as SSB consumption in humans is associated with low plain water intake (Park et al., 2012). Diets
were started when rats were on postnatal day 21 and continued for 6 weeks (Figure 1a). Chow and liquid consumptions were tracked twice weekly for the first 4 weeks prior to allocation of rats to vehicle or TDZD-8 treatments.

**Treatments and Experimental Groups.** TDZD-8, synthesized by Dr. Ana Martinez in CIB-CSIC laboratories following described procedures (Martinez et al., 2002), was dissolved (0.4 mg/ml) in 1% DMSO containing normal saline. 2 mg/kg of TDZD-8 was daily given to HFCS-fed rats intraperitoneally for 14 days (TDZD-8 group). The TDZD-8 dose was determined from the previous literature (Collino et al., 2008). The control group (Control group) and a separate set of HFCS-fed rats (HFCS group) were given the vehicle solution in equivalent volume and treatment duration. The rats were allocated to experimental groups randomly and undergone to experiments simultaneously in order to prevent the interference from seasonal variations.

**Oral Glucose Tolerance Test (OGTT).** In order to assess the glucose intolerance and confirm the development of insulin resistance, rats received an OGTT after 14 days of vehicle or TDZD-8 treatment. In order to provide an easy access to tail vein blood, tail tips were cut (2-3 mm in length) using a sterile technique under local anaesthesia (lidocaine pomade 5%), a day before the OGTT. Prior to the OGTT, rats were fasted for 6 h. Blood samples were taken before and after the administration of 2 mg/kg glucose solution by oral gavage at 0 min. The clots on the tail tips were gently removed to obtain each blood sample. The first drop of blood was removed by a sterile gauze, and the second drop was used for the assessment of blood glucose levels with a handheld glucometer (AccuChek Performa Nano, Roche, Switzerland).

**Behavioural Experiments.** Rats were tested in a series of behavioural experiments after a week of treatment with either vehicle or TDZD-8. The first behavioural experiment was carried out 30 min after the vehicle or TDZD-8 administration. The experiments were conducted in an order from the least stressful to the most stressful for the rats. To avoid any scent-induced cues, surfaces and equipments were cleaned between each experiment with 70% ethanol solution. Animal tracking and recording was performed using an in-house developed tracking software (Yucel et al., 2009, Evranos-Aksoz et al., 2017).
Open Field Arena (OFA). OFA was used to assess the locomotion of rats. Rats were placed in the center of a transparent glass open field (45 cm × 45 cm × 45 cm) and allowed to freely explore for 1 h. Total distance traveled was recorded and analyzed.

Elevated Plus Maze (EPM). A plus-shaped maze composed of four perpendicular arms (40 cm × 15 cm) elevated 1 m above the ground was used for the experiment. Two opposing arms were enclosed by 45 cm high opaque walls. Rats were placed at the center of arms, facing an open arm. This test relies on rats’ intrinsic propensity towards the dark and enclosed places (closed arms), and fear of heights/open places (open arms). The time spent and distance travelled in the open and closed arms were recorded and analyzed for 5 min.

Morris Water Maze (MWM). Morris water maze was performed as previously published (Vorhees and Williams, 2006). The water maze apparatus was a black circular pool with the diameter of 180 cm. The pool was filled approximately half-way with warm water (22-23°C). The interior of the pool was featureless as possible, except for 4 visual cues attached. A black cylindrical platform (height of 40 cm, diameter of 12 cm) was placed in the middle of the northwest quadrant. The platform was hidden as it was submerged 1-2 cm below the water surface. Briefly, each rat received 4 acquisition trials per day, for 4 consecutive days. The rats were placed into the pool, facing towards the pool wall. The starting position in each trial was different in order to prevent the rat to memorize a path to platform instead of spatially learning where the platform was. In addition, the order of this various starting positions was also different in different acquisition days. After the rat was placed into the water, it was given 2 min to find the platform. If the rat fails to find it within the allotted time, it was gently guided by the experimenter towards the platform. When the rat found the platform, it was kept there for 15 s in order to appreciate and learn where the platform was located compared to visual cues. The latencies to find the hidden platform were recorded. 48 h after the last acquisition trial, a probe trial was performed. In the probe trial, the hidden platform was removed and the rat was placed into the water from a novel starting point that it had not been used before. This ensured that rats’ quadrant preference was an indication of true spatial memory, rather than a memorized specific swim path. Rats were allowed to swim freely for 60 s. Time spent in the target quadrant (the quadrant where the platform was located in the acquisition trials) was recorded.
**Female Urine Sniffing Test (FUST).** Female urine sniffing test was performed as previously described (Malkesman et al., 2010). Female urine sniffing test is a non-operant test used to assess the sexual reward-seeking behaviour (hedonic behaviour). This test depends on the appetitive response of male rats towards the pheromonal odors found in the urine of female rats. In order to maximize the attractiveness of the urine, it was collected from female rats that are in the estrus phase of their menstrual cycle, as determined by evaluating vaginal smears obtained from several female rats daily. Male rats were presented a cotton swab dipped into distilled water or female urine in two consecutive 3 min sessions separated by an interval of 45 min. The duration of sniffing the tip of the cotton swab was recorded and analyzed.

**Forced Swim Test (FST).** Forced swim test is the experimental paradigm of learned helplessness. The apparatus was a 50 cm tall glass cylinder (diameter of 20 cm) which was filled to a depth of 30 cm with warm water (23-25 °C). Forced swim test was performed in 2 sessions separated by 24 h, as described previously (Castagne et al., 2010). The first 15 min session was to teach the rats that there were no possible ways to escape from the apparatus. In the second session, which lasted 5 min, the duration of immobility was analyzed from the recordings. The immobile behaviour in the swim test is thought to reflect the failure to keep performing escape-directed behaviour due to the behavioural despair learned in the first session.

**In vivo Electrophysiology.** TDZD-8 or vehicle was administered 30 min before the electrophysiology experiments. Rats were anesthetized with intraperitoneal administration of 1.4 g/kg urethane (Sigma-Aldrich, Germany). The depth of anaesthesia was confirmed and tracked by the absence of toe withdrawal reflex, regularity and depth of respirations (gross observation) and heart rate (ECG) throughout the electrophysiology experiment. Afterwards, they were placed into a stereotaxic frame (Stoelting Co., IL, USA) and their body temperatures were kept constant by using a blanket control unit (Harvard Apparatus, MA, USA). A midline incision exposing both lambda and bregma was performed. Burr holes providing access to ventral hippocampal commissure (VHC) (AP -1.2mm, ML 0.1 mm, D 4.5mm) and CA1 (AP -3.9mm, ML 2.2mm, D 2.5mm) areas of the hippocampus were opened. A stimulating bipolar stainless-steel electrode was placed into VHC. A recording borosilicate electrode filled with calcium-free artificial cerebrospinal fluid was placed into CA1. A gold-plated ground electrode was placed through a hole opened on the occipital bone. The Schaffer collaterals passing through the VHC were stimulated every 20 s with 0.1 ms
pulses using a stimulator (S44, Grass Instruments, RI, USA) isolated from the recording system with a stimulus isolation unit (SIU5, Grass Instruments) to evoke CA1 field excitatory postsynaptic potentials (fEPSPs) and field population spikes (pSpikes). The locations of both electrodes were optimized in order to obtain the maximal response possible. The evoked fEPSPs and pSpikes from *stratum radiatum* and *stratum pyramidale* were recorded and amplified with a headstage (Batiray, YSED, Turkey) and an amplifier (Kaldiray EX-2C, YSED) and digitized by a data acquisition system (PowerLab 8/SP, ADInstruments, Australia). Data recordings and analysis were done by using LabChart software (AD Instruments, Australia) and MiniAnalysis (Synaptosoft Inc., GA, USA), respectively. Input-output curves were constructed by applying stimuli with increasing intensities, ranging from 1 V to 15 V. The slopes of fEPSPs and the amplitudes of pSpikes were normalized according to the maximal response obtained from that particular recording. Rats with poor quality recordings indicating the poor location of either of the electrodes, severe bleeding or death during experiment (maximum of 1 rat/group) were excluded from the experiment/analysis. Paired-pulse facilitation and inhibition phenomena were assessed by applying paired-pulses with varying interpulse intervals at a stimulus intensity evoking 50% of maximum response. The rats were sacrificed afterwards and hippocampi were extracted as described below for qRT-PCR and ELISA experiments.

**Intracerebroventricular Insulin Injection.** A different set of 6 h fasted rats were anesthetized with urethane and placed into the stereotaxic frame after the confirmation of the depth of the anaesthesia as described previously. A burr hole was opened to provide access to the third ventricle from the midline using the following coordinates: AP 4.4mm, D 4.3mm. A total volume of 6 µl of 6 mU human recombinant insulin (Sigma-Aldrich, Germany) was infused using a 10 µl Hamilton syringe driven by an in-house developed motorized injector in 10 min. The needle was left in place for additional 20 min, until the rats were decapitated while still under anaesthesia. The dose of insulin and the time between the initiation of insulin infusion and decapitation (30 min) were determined based on previous literature (Grillo et al., 2009). The rats were sacrificed afterwards while they were still under anaesthesia and hippocampi were extracted as described below for immunoblot experiments.

**Decapitation and Tissue Collection.** Rats were killed by decapitation under urethane anaesthesia after the completion of electrophysiology experiments or intracerebroventricular insulin administration, and brains were extracted and immediately put into ice-cold PBS.
solution. Isolation of both hippocampi was performed on an ice-cold plate in less than a minute. Hippocampi were put into either RNAlater (Qiagen, Germany), homogenization buffer supplied by the Plasma Membrane Protein Isolation Kit (ab65400, Abcam, United Kingdom) or frozen immediately for further qRT-PCR, western blot and ELISA studies, respectively. Total trunk blood was collected and centrifuged at 7000×g for 10 min to separate the serum, which was stored at -80°C. Inguinal, epididymal, mesenteric, perirenal, retroperitoneal, and subscapular fat pads were dissected and weighted immediately.

Protein Isolation, Determination of Protein Levels of Lysates and Immunoblotting. A commercially available Plasma Membrane Protein Isolation Kit (ab65400, Abcam) was used and the isolation was performed according to the manufacturer’s protocol. Tablets containing phosphatase inhibitors (ROCHE PhosSTOP, Roche Diagnostics, Germany) were dissolved in the homogenization buffer provided by the protein isolation kit, which already included a cocktail of protease inhibitors. Protein concentrations were determined using commercially available RC DC Protein Assay (Bio-Rad, CA, USA). Extracted protein samples (10 μg) from rat hippocampus were loaded and ran on a 10% acrylamide gel (TGX Stain-Free FastCast Acrylamide Solution, Bio-Rad) at 90 V for 90 min. Proteins were transferred to PVDF membranes using Trans-Blot Turbo Transfer System (Bio-Rad) at 25 V for 7 min. Blots were blocked for 1 h at room temperature with 5% nonfat dry milk in TBS-T Blotting-Grade Blocker (Bio-Rad), and subsequently probed with their relevant antibodies at 1:1000 dilution overnight at 4°C. The following primary antibodies were used in immunoblot experiments: Akt (#4691, CST, MA, USA), pAkt-Thr308 (#13038, CST), pAkt-Ser473 (#4060, CST), GSK-3 (#9336, CST), pGSK-3-Ser9 (#9322, CST), GluR1 (#13185, CST), pGluR1-Ser845 (#8084, CST), NMDAR2A (#M264, Sigma-Aldrich), NMDAR2B (#14544, CST), β-actin (#3700, CST). Anti-Rabbit-ECL secondary (ab97051, Abcam) at a concentration of 1:10000 was applied for 1 h at room temperature, blots were briefly washed in TBS-T and then TBS, then incubated with Immobilon ECL substrate (Merck Millipore, Germany) for 5 min in the dark. Blot images were obtained using Kodak GEL Logic 1500 Transilluminator Integrated Imaging System (Kodak, NY, USA). PageRuler Prestained Protein Ladder 10-180 kDa (Thermo Fisher Scientific) was used as size standards. The intensities of the target bands were normalized according to the intensities of the corresponding beta-actin bands. Relative change of protein levels was reported as fold changes of the control group.
**RNA Isolation, cDNA Synthesis and qRT-PCR.** Gene specific primers (Sentegen, Turkey) were designed to bypass at least one intronic sequence to reduce the possibility of binding to contaminating DNA (Table 1). Total RNA isolation was performed by Trizol-chloroform extraction method (Rio et al., 2010). Total RNA concentration and RNA purity were determined by µDrop plate (Thermo Fisher Scientific, MA, USA). Afterwards, total RNA was reverse transcribed into cDNA using gene specific primers by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). qRT-PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). GAPDH was used as housekeeping control for all samples. Relative expression of the target genes was reported as fold changes of the control group, according to the efficiency corrected ΔΔCt method (Pfaffl, 2001).

**ELISA.** Commercially available ELISA kits (Elabscience, MD, USA) for rat TNF-α (E-EL-R0019), IL-1β (E-EL-R0012), and IL-6 (E-EL-R0015) were performed according to the manufacturer’s instructions. The total protein levels of hippocampal lysates were determined by using RC DC Protein Assay (Bio-Rad, CA, USA). Absorbance measurements were carried out by Multiskan GO spectrophotometer (Thermo Fisher Scientific).

**Statistical Analysis.** Data was presented as mean±standart error. Statistical analyses were performed by GraphPad Prism (GraphPad Software Inc., CA, USA). Student’s t-test or analysis of variance (ANOVA, one-way or two-way) followed by Tukey’s post-hoc test was used when there were 2 or 3 groups to compare, respectively. Post-hoc tests were not performed when F values of ANOVA are not significant. p<0.05 was considered as statistically significant. *, †, and ‡ were used to demonstrate statistical significance between control vs. HFCS, HFCS vs. TDZD-8, and control vs. TDZD-8, respectively.

**Materials.** Suppliers of all commercial kits, drugs and chemicals used in this study were mentioned in their corresponding subsections in the “Methods” section.
RESULTS

HFCS consumption caused a reduction in chow consumption and increased body fat percentage with a slight decrease in total body mass.

After the first week, HFCS group consumed significantly more liquid when compared to the water consumption of control group (Figure 1b). Even though both groups had free access to chow, HFCS intake caused a significant reduction in weekly chow consumption (Figure 1c). After the first 2 weeks, the mean body weight of HFCS-fed rats was less than that of the control group significantly (Figure 1d). However, HFCS rats had a significant increase in the weight ratio of their fat pads, suggesting an increase in fatty body mass and a reduction in lean body mass (Figure 1h).

HFCS consumption increased fasting blood glucose levels and caused glucose intolerance as evident in OGTT.

HFCS consumption caused elevated blood glucose levels after 6 h of fasting as well as at the end of the OGTT (Figure 1e and 1f). Area under the OGTT curve was higher in HFCS group compared to the control group (Figure 1g). High fructose feeding was already shown to induce insulin resistance and T2DM in rodents and non-human primates (Panchal and Brown, 2011, Bremer et al., 2011). Therefore, we have only performed OGTT and did not proceed with further tests.

HFCS consumption caused spontaneous hyperlocomotion, decreased anxiety, increased risk-taking behaviour, hyperhedonnia and susceptibility to behavioural despair.

Inhibition of GSK-3 with TDZD-8 was able to reverse HFCS-induced behavioural disturbances.

In order to assess spontaneous locomotion, individual rats were placed in the OFA. Rats consuming HFCS travelled more compared to the rats of the control group (Figure 2a and 2b). To measure anxiety and risk-taking behaviour, EPM was conducted. HFCS group had an increased time spent in open arms compared to the control group, which suggests decreased anxiety and increased risk-taking behaviour (Figure 2c and 2d). Moreover, HFCS-fed rats were actively exploring the open arms, as evident in increased distance travelled in the open arms (Figure 2e). In order to assess the changes in both social and sexual reward-seeking behaviours, the FUST was carried out. Both control and HFCS groups had significantly increased sniffing durations when presented with female urine after distilled water. When
groups were compared to each other, HFCS group had significantly higher sniffing duration of female urine compared to the control group (Figure 2f). In FST, HFCS group had significantly higher duration of immobility compared to the control group (Figure 2g). To sum up, HFCS consumption caused a spontaneous hyperlocomotion, decreased anxiety, increased risk-taking behaviour, hyperhedonia and susceptibility to behavioural despair.

As expected, treatment with TDZD-8, a GSK-3 inhibitor, reversed the HFCS-induced hyperlocomotion, decreased anxiety, hyperhedonia and susceptibility to behavioural despair back to control levels (Figure 2a-g).

**HFCS consumption impaired hippocampal spatial learning.**

In this study, hippocampus dependent spatial learning and memory was assessed by Morris water maze. The swim speeds of groups were not significantly different from each other (Figure 2h, inlet), suggesting the rats did not suffer from any motor disability. HFCS-fed rats were significantly worse in finding the hidden platform on the first acquisition day, which was not reversed by TDZD-8 treatment (Figure 2h). Even though it is not statistically significant, HFCS group performed worse compared to control and TDZD-8 groups until the last acquisition day. However, the time spent in target quadrant in the probe trial assessing long-term spatial memory was not significantly different between groups (Figure 2i).

**HFCS consumption caused hyperexcitability without altering GABAergic inhibitory activity in rat CA3-CA1 synapses, which was restored by TDZD-8.**

In order to assess the changes in the synaptic strength of HFCS-fed rats, Schaffer collaterals were stimulated and field potentials from the stratum radiatum (synaptic layer) of CA1 region were recorded. The input-output curve of fEPSP slopes shifted significantly towards left in the HFCS group, whereas TDZD-8 caused a rightward-shift, turning HFCS-induced hyperexcitability back to normal (Figure 3a). In paired pulse paradigm, HFCS consumption impaired normal paired pulse facilitation when the interpulse interval was 20 ms. In addition, TDZD-8 treatment was unable to restore this impairment in stratum radiatum paired-pulse facilitation. With greater interpulse intervals, no significant differences were found between groups (Figure 3b).

In addition, we aimed to assess the activity of GABAergic interneurons fine-tuning the cumulative response of the pyramidal neurons. Here, we placed the recording electrode into
the *stratum pyramidale* (soma layer) to record field pSpikes. The input-output curves yielded a similar trend to that observed in *stratum radiatum* (Figure 3c), but there were no significant differences between groups in any of the interpulse intervals applied in paired-pulse paradigm. (Figure 3d).

**Neuronal hyperexcitability was accompanied with increased ser845-phosphorylation of GluR1 subunit of AMPA receptors, maintaining an increased available pool of AMPA receptors to be readily incorporated into the postsynaptic membrane.** Ser845-phosphorylation of GluR1 subunits of AMPA receptors maintains a readily available pool of AMPA receptors to be incorporated to the postsynaptic density. In hippocampi of HFCS fed adolescent rats, the ratio of ser845-phosphorylated GluR1 to total GluR1 was higher compared to control group, which was reversed by TDZD-8 treatment (Figure 4d and 4e). In contrast, NMDAR2A and NMDAR2B subunits of NMDA receptors were found to be unchanged (Figure 4f and 4g).

**HFCS consumption in adolescent rats did not cause local insulin resistance in hippocampus.**

After intracerebroventricular administration of insulin, the ratios of ser473- and thr308-phosphorylated Akt to total Akt protein levels were found to be similar between groups (Figure 4a and 4b). Thr308-phosphorylated Akt cannot be detected by immunoblot unless hippocampi are stimulated by insulin, which confirms the appropriateness of intracerebroventricular insulin administration. In addition, no significant difference was observed for the ratio of ser9-phosphorylated GSK3 to total GSK3 protein levels (Figure 4c).

**HFCS consumption in adolescent rats caused decreased transcription of neuron specific α3-subunit of NKA in hippocampus.**

We tested whether there is a decrease in the levels of α-subunits of NKA in the hippocampi of HFCS-fed adolescent rats. While no significant differences in the mRNA levels of α1- and α2-subunits were observed, a significant reduction in the transcription of the neuron-specific α3-subunit was detected in the hippocampi of HFCS-fed rats (Figure 4h-j). However, TDZD-8 was unable to restore HFCS-associated reduced expression of α3-subunit of NKA.
A systemic inflammatory response was evoked by HFCS consumption, but not locally in the hippocampus.

Here, whether HFCS consumption has caused an increase in serum and hippocampus levels of proinflammatory markers, namely IL-1β, IL-6 and TNF-α, was tested, indicating systemic inflammation and local neuroinflammation, respectively. As expected, the levels of all three proinflammatory markers were found to be increased in serum of HFCS-fed rats, and returned back to normal in TDZD-8-treated rats (Figure 5a-c). However, the differences of hippocampal levels of proinflammatory markers were statistically insignificant with ANOVA, thus no post-hoc tests were performed (Figure 5d-f).
DISCUSSION

HFCS group consumed more liquid when compared to tap water consumption of control group, as HFCS is highly palatable (Ackroff and Sclafani, 2011). In this study, we did not present tap water to HFCS group to mimic the low plain intake of adolescents with SSB consumption (Park et al., 2012). Interestingly, HFCS-fed rats decreased their chow intake significantly, which was also previously observed in a similar study done with mice (Jurgens et al., 2005). Increased HFCS solution with a reduction in chow intake may be the underlying reason of why HFCS-fed rats gained relatively less weight after the second week of tracking period. This is not compatible with human consumption, because humans tend to consume SSBs without reducing their solid food intake, causing calories from SSBs to be “add-on” (Bray, 2013). As a limitation of our design, both lack of tap water intake with forced HFCS consumption and decreased chow intake of HFCS group may be confounding factors effecting the observed behavioural alterations. As the majority of fat pads dissected were visceral, this difference in fatty body mass was due to increased visceral adiposity in HFCS-fed rats. This finding is in parallel with previous studies showing that the increased visceral adiposity mediates the poor cardiometabolic consequences of high fructose feeding in adolescents (Pollock et al., 2012). Interestingly, TDZD-8 treatment failed to restore HFCS-induced visceral adiposity, but recovered HFCS-induced glucose intolerance.

The metabolic consequences of HFCS consumption during childhood and adolescence are relatively well characterized, however the neuropsychiatric consequences are still not recognized enough. We showed for the first time by this study that HFCS consumption in adolescent rats led to spontaneous hyperlocomotion, decreased anxiety, increased risk-taking behaviour, increased social/sexual reward-seeking behaviour and increased sensitivity to behavioural despair paradigm with learning deficits; all of which are characteristically seen in patients with BD. In addition, these behavioural changes associated with HFCS consumption were readily reversible with the inhibition of GSK-3, which is known as the most probable mechanism of action of lithium for its mood-stabilizing effects. Even though there are many limitations to define a rat as being bipolar (Gould and Einat, 2007), spontaneous mania-like behaviour with susceptibility to behavioural despair, combined with rats being responsive to the inhibition of GSK-3 are remarkably suggestive of BD.
The changes in neurotransmission observed in patients with BD are not fully understood (Newberg et al., 2008). In addition, the lack of appropriate animal models of BD to study neurotransmission further limits our understanding in this regard. However, neuronal hyperexcitability is one of the most commonly reported alteration associated with BD. In our study, HFCS consumption resulted in hyperexcitability of CA3-CA1 synapses as evident by a leftward shift of the input-output curve. This can be explained by both presynaptic and postsynaptic mechanisms:

(I) When two stimuli with 20ms of interpulse interval were applied to the control rats in paired-pulse paradigm, the second fEPSP slope was higher than the first, demonstrating the presynaptic Ca$^{2+}$ accumulation caused by two sequential stimuli, causing increased glutamate release after the second stimulus. However, HFCS group showed significantly less facilitation after the second stimulus than the control group, suggesting an increased presynaptic release probability causing depletion of glutamate containing vesicles when no time was given to replenish the stores. This can be explained by the decreased expression of α3-subunit of NKA in HFCS-fed rats, resulting in reduced Ca$^{2+}$ clearance in presynaptic terminals, thus increasing the release probability. TDZD-8 treatment failed to restore both impaired HFCS-induced paired-pulse facilitation and reduced expression of α3-subunit of NKA. Similar to TDZD-8, previous literature showed that lithium did not alter the presynaptic glutamate release probability as suggested by previous paired-pulse facilitation experiments (Du et al., 2008).

(II) HFCS consumption increased ser845-phosphorylation of GluR1 subunit of AMPA receptors, which is required for a readily available pool of AMPA receptors to be incorporated to the postsynaptic density. TDZD-8 restored HFCS-induced excessive phosphorylation of GluR1. This is in concordance with previous studies showing the critical role of AMPA receptors in the pathophysiology of BD (Du et al., 2008, Du et al., 2003, Du et al., 2004a).

In this study, HFCS-induced CA3-CA1 hyperexcitability can be explained by both increased presynaptic release probability and increased pool of AMPA receptors ready to be incorporated to the postsynaptic membrane. However, TDZD-8 treatment only restored the postsynaptic impairment with the attenuation of overall hyperexcitability. In this study, no alterations in GABAergic system, the main inhibitory system of central nervous system, were found.
Evidence supporting NKA hypothesis of BD has been accumulating since early 1950s, including that with Myshkin mice carrying an inactivating mutation in the neuron-specific α3-subunit of NKA, showing bipolar-like behavioural phenotype (Kirshenbaum et al., 2011). It is still not known how this decrease in NKA levels/activity translates to bipolar-like behavioural phenotype. In our study, the α3-subunit is the only subunit whose expression was found to be decreased. Further studies are required to determine whether this alteration in NKA is the primary reason of observed behavioural phenotype or not.

Insulin resistance was reported in whole brain tissue as well as in the hippocampus in high fructose and high fat fed rats (Mielke et al., 2005, Liu et al., 2015). Insulin receptors are coupled to PI3K-Akt-GSK-3 pathway, which is known to be a crucial secondary signaling pathway related to synaptic plasticity, synaptic structure, learning-memory and mood processes. In our study, there were no alterations in phosphorylation levels of Akt and GSK-3 after stimulated with intracerebroventricular insulin, eliminating the presence of insulin resistance locally in the hippocampi of HFCS-fed rats. Even though HFCS-induced hyperexcitability and associated presynaptic and postsynaptic changes were not due to hippocampal insulin resistance, the PI3K-Akt-GSK-3 pathway may still be involved in the process. As NKA was also shown to be linked to PI3K-Akt-GSK-3 pathway (Wu et al., 2013), decreased expression of the α3-subunit of NKA may cause alterations in this pathway leading to observed changes. However, the underlying signaling mechanism couldn’t be unrevealed in this study and requires further focus in the future.

It is known that both western diet consumption and BD are associated with inflammation. Here, we showed that HFCS consumption caused a systemic inflammatory response, which was readily reversible with TDZD-8 treatment. Even though the results of this study showed many functional changes related to the hippocampus, we did not observe a statistically significant local neuroinflammation in the hippocampi of rats fed with HFCS for 6 weeks. This finding does not exclude the existence of inflammation in other brain regions, which might be involved in the pathogenesis of BD, it simply showed that the electrophysiological and molecular changes in the hippocampus cannot be explained by the local inflammation by itself.
In summary, we report for the first time that HFCS fed adolescent rats displayed a bipolar-like behavioural phenotype with associated hyperexcitability of CA3-CA1 synapses. In addition, we demonstrated that both presynaptic and postsynaptic alterations might underlie the HFCS-induced hyperexcitability with little, if any, contribution of inflammation. A decreased expression of neuron-specific α3-subunit of NKA was also evident in hippocampi of HFCS-fed rats, however it should be further studied whether this change causes bipolar-like behavioural phenotype by inducing neuronal hyperexcitability or operates through a different mechanism.
AUTHOR CONTRIBUTIONS

This study is a part of the PhD dissertation of B.A. Conceptualization, B.A. and Y.S.; Designed by B.A., S.T.T., C.K. and Y.S.; Experiments performed by B.A., M.Y., A.B., and A.Y.G.; Writing – Original Draft, B.A.; Writing – Review & Editing, B.A., S.T.T. and Y.S.; Resources, A.M.; Supervision Y.S.


Table 1. Sequences of the primers used in the qRT-PCR experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP1A1</td>
<td>TCCTTAAGCGTGCAATGCG</td>
<td>CTCATCTCCATCACGGAGCC</td>
</tr>
<tr>
<td>ATP1A2</td>
<td>TAGCATACGAAGCGGCTGAG</td>
<td>GATCATGCCGATCTGTCCGT</td>
</tr>
<tr>
<td>ATP1A3</td>
<td>GCCAAGATGGGGGACAAAAA</td>
<td>TGCACGCAGTCGGTATTGTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCCCATTTCTCCACCTTT</td>
<td>TAGCCATATTGTCATACC</td>
</tr>
</tbody>
</table>
FIGURES AND FIGURE LEGENDS

Figure 1. Tracking of the consumption of corresponding diets and the metabolic characterization of the rats. (a) Rats received their corresponding diets for 6 consecutive weeks, beginning from the postnatal day 21. After a month of feeding with their corresponding diets, rats were randomized for either vehicle or TDZD-8 treatments for additional 2 weeks. The last week of the 2 weeks of treatment period consisted of a battery of behavioural tests. At the end of the 6th week, rats were undergone in vivo electrophysiology experiments and sacrificed afterward for tissue collection. OGTT was performed in a different set of rats in order to avoid any interference due to fasting. (b) Weekly liquid consumption was significantly greater in HFCS group compared to the control group, starting from the second week (n=6 cage/group, each cage houses 3 rats). (c) HFCS consumption caused a significant reduction in weekly chow consumption throughout the tracking period of a month (n=6 cage/group, each cage houses 3 rats). (d) The mean body weight of HFCS group was slightly less than that of control group, starting from the day 17. The means of final weights were different significantly between control and HFCS groups (n=9/group, Control 245±6.4 g, HFCS 224±5.0 g, p<0.05). (e) HFCS consumption caused glucose intolerance as evident in OGTT, and TDZD-8 reversed HFCS-induced glucose intolerance (n=10/group for control and HFCS, n=5/group for TDZD-8). (f) HFCS group displayed elevated blood glucose levels after 6 h of fasting, and TDZD-8 partially reversed this elevation (Control 81.2±3.2 mg/dL, HFCS 130±3.4 mg/dL, TDZD-8 109±3.9 mg/dL, F(2,22)=56.5, p<0.05). 2 h after the oral glucose load, the blood glucose levels of HFCS remained high compared to those of control and TDZD-8 rats (Control 101±1.4 mg/dL, HFCS 133±4.7 mg/dL, TDZD-8 106±4.1 mg/dL, F(2,22)=24.9, p<0.05). (g) Area under the OGTT curve (AUC) was higher in HFCS group compared to control group, and TDZD-8 treatment restored towards control levels (Control 16988±328 mg/dL•min⁻¹, HFCS 18500±408 mg/dL•min⁻¹, TDZD-8 16199±361 mg/dL•min⁻¹, F(2,22)=8.46, p<0.05). (h) Total weight of extracted fat pads normalized to total body weight was higher in HFCS group, suggesting a greater fatty body percentage, which was not reversed by TDZD-8 treatment (Control 3.8±0.2%, HFCS 4.5±0.2%, TDZD-8 4.8±0.2%, F(2,26)=7.0, p<0.05).
Figure 2. HFCS consumption caused spontaneous hyperlocomotion, decreased anxiety, increased risk-taking behaviour, hyperhedonia, and susceptibility to behavioural despair with significant impairments in hippocampal learning in adolescent rats. (a) Total distance travelled in the open field arena (n=8/group, Control 18.5±4.5 m, HFCS 34.6±3.8 m, TDZD-8 15.3±3.0 m, ANOVA F_{(2,21)}=7.39, p<0.05). (b) Representative track plots of OFA. (c) Time spent in open arms of the elevated plus maze (n=9/group, Control 6.0±2.5 s, HFCS 42.7±13.7 s, TDZD-8 7.6±3.8 s, ANOVA F_{(2,24)}=6.21, p<0.05). (d) Averaged group heat maps of EPM. (e) Distance travelled in the open arms of the EPM (n=9/group, Control 0.1±0.05 m, HFCS 0.8±0.2 m, TDZD-8 0.2±0.08 m, ANOVA F_{(2,24)}=8.01, p<0.05). (f) Female Urine Sniffing Test. All three groups had significantly increased sniffing durations when presented with female urine after distilled water (n=8/group, repeated measures two-way ANOVA, Stage effect F_{(1,21)}=21.8, p<0.05). When groups were compared to each other, HFCS group had significantly higher sniffing durations of female urine compared to the other groups (n=8/group, Control 8.1±1.1 s, HFCS 14.0±1.5 s, TDZD-8 6.2±2.1 s, repeated measures two-way ANOVA, Group effect F_{(2,21)}=6.24, p<0.05). (g) Immobility duration in the forced swim test (n=9/group, Control 28.9±4.1 s, HFCS 45.9±4.6 s, TDZD-8 29.9±4.3 s, ANOVA F_{(2,24)}=4.84, p<0.05). (h) Latency to find the hidden platform in the acquisition stage of the Morris water maze and mean swim speeds (inlet). The mean swim speeds of groups were not significantly different from each other (n=9/group, Control 0.24±0.005 m/s, HFCS 0.24±0.005 m/s, TDZD-8 0.23±0.004 m/s, ANOVA, F_{(2,456)}=0.474, p>0.05). Repeated measures two-way ANOVA revealed a significant group effect in the acquisition stage of MWM (Acquisition Days × Groups F_{(6,420)}=1.70, p>0.05; Acquisition Days F_{(3,420)}=118, p<0.05; Groups F_{(2,420)}=6.90, p<0.05). In addition, Tukey’s multiple comparisons test detected a difference between HFCS and TDZD-8 groups compared to control group on the first acquisition day. (i) Time spent in the target quadrant in the probe trial of the MWM was not statistically different between groups (n=9/group but one outlier data point was removed from control group as identified by ROUT test, Control 24±0.9 s, HFCS 25±2 s, TDZD-8 25±2.8 s, F_{(2,23)}=0.08, p>0.05).
Figure 3. HFCS consumption caused neuronal hyperexcitability without altering GABAergic inhibitory activity that was restored by TDZD-8 in rat hippocampal CA3-CA1 synapses. (n=9 rats/group, maximum of 1 rat/group was excluded because of either low-quality recording, severe bleeding or death.) (a) Input-output curve of stratum radiatum. HFCS group exhibited hyperexcitability compared to control and TDZD-8 groups (repeated measures two-way ANOVA, Group F(2,23)=4.66, p<0.05). (b) Paired-pulse paradigm in stratum radiatum. HFCS and TDZD-8 groups showed significantly less facilitation compared to control group when interpulse interval was 20ms (Tukey’s multiple comparisons test, control vs. HFCS and control vs. TDZD-8, p<0.05). (c) Representative recordings from stratum radiatum. I/O traces were selected from the responses to stimuli of 7 V. Paired-pulse traces were given for interpulse intervals of 20 ms and 1000 ms. Traces from control group were yellow, whereas traces from HFCS and TDZD-8 groups were red and blue respectively in their corresponding columns. (d) Input-output curve of stratum pyramidale. (e) Paired-pulse paradigm of stratum pyramidale. (f) Representative recordings from stratum pyramidale. I/O traces were selected from the responses to stimuli of 7 V. Paired-pulse traces were given for interpulse intervals of 20 ms and 1000 ms. Traces from control group were yellow, whereas traces from HFCS and TDZD-8 groups were red and blue respectively in their corresponding columns.
Figure 4. HFCS consumption in adolescent rats caused increased ser845-phosphorylation of GluR1 subunit of AMPA receptors and decreased transcription of neuron specific α3-subunit of NKA in hippocampus (n=4/group for immunoblot and n=7/group for qRT-PCR, maximum of 1/group was excluded from the qRT-PCR experiments as the isolation yielded low purity samples). (a) Relative protein levels of ser473-phosphorylated Akt to Akt normalized to β-actin compared to control group. (b) Relative protein levels of thr308-phosphorylated Akt to Akt normalized to β-actin compared to control group. (c) Relative protein levels of ser9-phosphorylated GSK-3 to GSK-3 normalized to β-actin compared to control group. (d) Relative protein levels of ser845-phosphorylated GluR1 subunit of AMPA receptor to GluR1 subunit of AMPA receptor normalized to β-actin compared to control group (Statistical analysis was not performed as n<5/group). (e) Raw immunoblot images of bands of ser845-phosphorylated GluR1, GluR1 and β-actin. (f) Relative protein levels of NMDA2A normalized to β-actin compared to control group. (g) Relative protein levels of NMDA2B normalized to β-actin compared to control group. (h) Relative expression of α1-subunit compared to control group. (i) Relative expression of α2-subunit compared to control group. (j) Relative expression of α3-subunit compared to control group.
Figure 5. A systemic inflammatory response was evoked by HFCS consumption, but not locally in the hippocampi (n=9/group). (a) IL-1β levels in serum. (b) IL-6 levels in serum. (c) TNF-α levels in serum. (d) IL-1β levels normalized to total protein in hippocampus. (e) IL-6 levels normalized to total protein in hippocampus. (f) TNF-α levels normalized to total protein in hippocampus.
For Peer Review

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**Figure a**

Day 1 Day 8 Day 15 Day 22 Day 29 Day 36 Day 42

DIET (WATER or HFCS)

TREATMENT (VEHICLE or TDZD-8)

BEHAVIORAL TESTS

In vivo electrophysiology

OGTT

Decapitation & tissue harvesting

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**Figure b**

Weeks

*

* *

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**Figure c**

Weeks

* 

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**Figure d**

Days

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**Figure e**

Weeks

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**Figure f**

OGTT Blood Glucose (mg/dL)

Fasting 120 min

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**Figure g**

Area Under the OGTT Curve

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**Figure h**

Fat Weight Normalized to Body Weight (%)

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**For Review**

Control HFCS TDZD-8

0 0.5 1.0 1.5

**NMDA2A Fold Change**
(normalized to β-actin)

Control HFCS TDZD-8

0 1 2 3 4

**ATP1A1 Fold Change**

Control HFCS TDZD-8

0 1 2 3 4

**ATP1A2 Fold Change**

Control HFCS TDZD-8

0 1 2 3 4

**ATP1A3 Fold Change**

Control HFCS TDZD-8

0 1 2 3

**pAKT (ser) / AKT**
(normalized to β-actin)

Control HFCS TDZD-8

0 0.5 1.0 1.5

**pGSK / GSK**
(normalized to β-actin)

Control HFCS TDZD-8

0 0.5 1.0 1.5

**ATP1A1**

Control HFCS TDZD-8

5 10 15 20

**ATP1A2**

Control HFCS TDZD-8

0.5 1 1.5 2 2.5 3 3.5 4 4.5

**ATP1A3**

Control HFCS TDZD-8

1 2 3 4

**β-actin**

Control HFCS TDZD-8

0 1 2 3 4

**AMPA**

Control HFCS TDZD-8

1 2 3 4

**pAMPA / AMPA**
(normalized to β-actin)

Control HFCS TDZD-8

0 1 2 3 4

**NMDA2B Fold Change**
(normalized to β-actin)

Control HFCS TDZD-8

0 0.4 0.8 1.2 1.6

**pAKT (thr) / AKT**
(normalized to β-actin)

Control HFCS TDZD-8

0.8 1.2 1.6 2

**pAMPA**

Control HFCS TDZD-8

1 2 3 4

**AMPA**

Control HFCS TDZD-8

1 2 3 4

**β-actin**

Control HFCS TDZD-8

45 kDa 100 kDa 150 kDa

**ATP1A1**

Control HFCS TDZD-8

1 2 3 4

**ATP1A2**

Control HFCS TDZD-8

1 2 3 4

**ATP1A3**

Control HFCS TDZD-8

1 2 3 4

**β-actin**

Control HFCS TDZD-8

0 1 2 3 4