ORIGINAL RESEARCH ARTICLE

Title: Early neuroadaptations to an obesogenic diet identify the schizophrenia-related ErbB4 receptor in obesity-induced hippocampal abnormalities

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Contributions: **JDV:** wrote manuscript, performed Western blot experiments and analyses; designed and performed behavioral experiments; **POA:** performed and analyzed ELISA and histological experiments, prepared illustrations, wrote manuscript; **ET, ES:** performed behavior analyses; **SS, ET, MO:** performed Western blot experiments; **IDL:** performed behavioral experiments; **JDV, JDF:** analyzed behavioral data; **AO:** performed MRI experiments and analyses; **BF:** contributed to experimental design and data interpretation, facilitated drugs used in the study; **JDF:** planned experiments, analyzed and interpreted data, wrote manuscript.

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Abstract: Childhood obesity leads to hippocampal atrophy and altered cognition. However, the molecular mechanisms underlying these impairments are poorly understood. The neurotrophic factor neuregulin-1 (NRG1) and its cognate ErbB4 receptor play critical roles in hippocampal maturation and function. This study aimed to determine whether altered NRG1-ErbB4 activities may partly explain hippocampal abnormalities in rats exposed to an obesogenic Western-like diet (WD). Lewis rats were randomly divided into four groups (12 rats/group): **1)** control diet+vehicle (*CDV*); **2)** CD+NRG1 (*CDN*) (daily intraperitoneal injections: 5 µg/kg/day; between postnatal day, PND 21-PND 41); **3)** WD+VEH (*WDV*); **4)** WD+NRG1 (*WDN*). Neurobehavioral assessments were performed at PND 43-49. Brains were harvested for MRI and molecular analyses at PND 49. We found that NRG1 administration reduced hippocampal volume (7%) and attenuated hippocampal-dependent cued fear conditioning in CD rats (56%). NRG1 administration reduced PSD-95 protein expression (30%) and selectively reduced hippocampal cytokine levels (IL-33, GM-CSF, CCL-2, IFN-γ) while significantly impacting microglia morphology (increased span ratio and reduced circularity). WD rats exhibited reduced right hippocampal volume (7%), altered microglia morphology (reduced density and increased lacunarity), and increased levels of cytokines implicated in neuroinflammation (IL-1α, TNF-α, IL-6). Notably, NRG1 synergized with the WD to increase hippocampal ErbB4 phosphorylation and the tumor necrosis alpha converting enzyme (TACE/ADAM17) protein levels. Together, these data suggest a novel interaction between obesogenic diet exposure and TACE/ADAM17-NRG1-ErbB4 signaling during hippocampal maturation. Our results indicate that supraoptimal ErbB4 activities may contribute to the abnormal hippocampal structure and cognitive vulnerabilities observed in obese individuals.

Keywords: Neuroinflammation, Microglia, Obesity, Adolescence, Hippocampus, Behavior, MRI.

Highlights:

- Obesogenic diet consumption during adolescence induces anxiety-like behaviors before the onset of obesity-related changes in metabolism.
- Obesogenic diet-driven abnormal behaviors co-occurred with alterations in hippocampal pro-inflammatory cytokine profiles.
- Obesogenic diet consumption attenuates exogenous NRG1 effects on hippocampal-related behaviors and structure.
- Exogenous NRG1 administration during adolescence resulted in reduced hippocampal volumes and domain-specific cognitive impairments.
- Exogenous NRG1 administration has potent immunomodulatory actions and alters hippocampal microglia morphology.

Introduction

Childhood obesity is a severe medical condition affecting more than 340 million children and adolescents worldwide (World Health Organization, 2016). While obesity is a complex disease, poor diet quality is a significant factor contributing to the global obesity epidemic in children and adolescents (Moreno et al., 2010). Obesity and the consumption of imbalanced obesogenic diets rich in saturated fats and simple sugars have emerged as risk factors for the development of anxiety and other stress-related disorders (Scott et al., 2008; Perkonigg et al., 2009; Johannessen and Berntsen, 2013; Maguen et al., 2013; Mitchell et al., 2013; Kubzansky et al., 2014; Bartoli et al., 2015; Smith et al., 2015; Salari-Moghaddam et al., 2018). Evidence demonstrates that the relationship between obesogenic and anxiogenic phenotypes is bidirectional (Lopresti and Drummond, 2013; Michopoulos et al., 2016; Hruby et al., 2021). In other words, the presence of one increases the risk of developing the other. Thus, it has become crucial to understand better the specific substrates responsible for the intertwined pathophysiology associated with both conditions.

While the neural substrates impacted by obesity are very complex, hippocampal volume deficits have emerged as a prominent anatomical endophenotype (Raji et al., 2010; Cherbuin et al., 2015; Jacka et al., 2015; Mestre et al., 2017). Studies in animals and humans indicate that the hippocampus is essential for reducing energy intake (Kanoski et al., 2011; Stevenson and Francis, 2017; Liu and Kanoski, 2018) and stress reactivity (Jacobson and Sapolsky, 1991; Jimenez et al., 2018). As such, alterations in this brain region may contribute to the broad range of metabolic, cognitive, and emotional disturbances associated with obesity. We reported unique hippocampal vulnerabilities to

the detrimental effects of obesogenic diets (Kalyan-Masih et al., 2016) consistent with human data (Bauer et al., 2015). However, the molecular and cellular basis underlying these vulnerabilities are poorly understood.

The hippocampal volumetric deficits observed in obese individuals could result from dysregulated neurotrophic factors and inflammatory signals. Neuregulin 1 (NRG1) is a trophic factor containing an epidermal growth factor (EGF)-like domain and acting via their cognate ErbB receptor tyrosine kinases. ErbB4, the only autonomous NRG1-specific activated tyrosine kinase, plays a critical role in regulating brain development and homeostasis. NRG1-ErbB4 signaling has been heavily implicated in neuronal migration, synaptic plasticity, and cognition (Mei and Xiong, 2008). NRG1-ErbB4 signaling regulates hippocampal function through increasing neuronal arborization (Gerecke et al., 2004), increasing gamma oscillations in the CA3 region of the hippocampus (Fisahn et al., 2009), modulating long-term potentiation at CA1 hippocampal synapses (Kwon et al., 2008), and modifying stress reactivity and behavior (Dang et al., 2016; Mahar et al., 2017; Clarke et al., 2018). Exogenous NRG1 administration increases hippocampal neurogenesis (Mahar et al., 2011, 2016) and improves learning and memory while rescuing dendritic and synaptic abnormalities in a mouse model of Alzheimer's disease (AD) (Ryu et al., 2016). Similar neurorestorative properties were reported in a rat model of AD (Jalilzad et al., 2019).

Genetic studies have demonstrated strong associations between single-nucleotide polymorphisms in the ErbB4 gene and obesity (Locke et al., 2015; Salinas et al., 2016). Notably, while ErbB4 deletion predisposes mice to metabolic alterations implicated in obesity, NRG1 administration is known to improve metabolic health in obese mice by

lowering blood glucose, improving insulin sensitivity, and reducing caloric intake (Ennequin et al., 2015a; Zeng et al., 2018; Zhang et al., 2018). Recent studies have reported an interplay between NRG1 signaling and high-fat diet (HFD) consumption in mice (Holm-Hansen et al., 2016; Zieba et al., 2019a; b). Zieba et al., demonstrated that HFD consumption during late adolescence has sex-specific effects on open-field (OF) exploration and fear conditioning (Zieba et al., 2019a). Female mice exposed to the HFD during late adolescence exhibited reduced OF exploration and increased cue freezing behaviors (Zieba et al., 2019a). Notably, female heterozygous transmembrane domain NRG1 mutant mice conferred protection against the effects of the HFD (body weight, OF distance traveled, cued freezing) (Zieba et al., 2019a).

Exogenous NRG1 administration confers potent anti-inflammatory effects. We and others have demonstrated that NRG1 anti-inflammatory actions extend to models of neuroinflammation, including cerebral malaria (Liu et al., 2018), nerve agent intoxication (Li et al., 2015), and ischemia (Simmons et al., 2016). Studies demonstrate that ErbB4 inhibition attenuates the beneficial effects of NRG1, supporting an essential role for ErbB4 in NRG1 actions (Tan et al., 2012; Guan et al., 2015; Gao et al., 2016; Zhang et al., 2017).

In this study, we hypothesized that NRG1 administration would confer protection against the detrimental effects of an obesogenic diet on neuroinflammation, hippocampal maturation, and anxiety-like behaviors. We reasoned that examining the responses of the hippocampal NRG1-ErbB4 system to obesogenic diets during adolescence may provide valuable insights into the potential contribution of this pathway to obesity-induced hippocampal deficits and anxiety. This study is the first to investigate the therapeutic

potential of exogenous NRG1 to prevent the early consequences of an obesogenic environment during adolescence.

Materials and Methods

Animals

All the experiments were performed following protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Loma Linda University Health School of Medicine. Female Lewis rats with 8-10 male pups (postnatal day, PND 15) were purchased from Charles River Laboratories (Portage, MI, USA). Upon arrival, female rats were housed with their pups with free access to food and water. At PND 21, adolescent male pups were weaned, housed in groups (2 per cage), and experimental manipulations commenced. Animals were kept in customary housing conditions (21 ± 2 C, relative humidity of 45%, and 12-hour light/dark cycle with lights on at 7:00 AM). The body weights were recorded once a week or daily during the week of behavioral testing. Food consumption was quantified at least twice per week. The rats were never food or water restricted.

Study Design

We used a 2 x 2 design with four groups: **(1)** Control diet, injected with vehicle (CDV); **(2)** Control diet, injected with neuregulin-1 (CDN); **(3)** Western diet, injected with vehicle (WDV); **(4)** Western diet, injected with neuregulin-1 (WDN). **Figure 1** summarizes the timeline of experimental procedures and behavioral tests. The matched low-fat purified control diet (CD, 5-gm% fat, product #F7463) and Western-like high-saturated fat diet

(WD, 20-gm% fat, product #F7462) were obtained from Bio-Serv (Frenchtown, NJ, USA).

The macronutrient composition and fatty acid profiles are detailed in a previous study and summarized in **Supplemental Table 1** (Vega-Torres et al., 2020b). Weight-matched adolescent rats (PND 21) were divided into 4 study groups: CDV, CDN, WDV, and WDN. NRG1 (5 micrograms/kilogram/day) or saline (vehicle) intraperitoneal injections were performed daily between 12:00-1:00 PM. The rationale for injection dosage and route was based on our previous reports describing the neuroprotective effects of this intervention (Li et al., 2012, 2015), NRG1 biodistribution, kinetics, and short half-life in plasma (Rösler et al., 2011; Zhang et al., 2018). Recombinant human NRG1- β 1/HRG1- β 1 EGF domain (NRG1) was provided by Dr. Byron D. Ford (manufactured by R&D Systems, catalog #396-HB-050). **Study 1 (behavioral and anatomical assessment):** All behavior testing sessions involved a 20-30-minute acclimation period to the testing facility. The rats were allowed to consume the custom diets until the completion of the study (PND 21-49). The initial group size was $n = 18$ for each group (divided into four cohorts). Twenty-four rats were randomly selected for MRI studies ($n = 6$ per group). **Study 2 (molecular markers investigation):** To minimize potential carryover effects between behavioral tests and molecular outcomes, we used a separate group of animals *without behavioral manipulations* for all the molecular outcomes reported in this study (RT-PCR, Western blot, and flow cytometry). For this group, an additional injection of NRG1 or saline was administered 1 h before euthanizing the animals at PND 42 (Rösler et al., 2011). The initial group size for Study 2 was $n = 5-6$ for each group (CDV, $n = 5$; CDN, $n = 5$; WDN, $n = 6$; WDV, $n = 6$).

Study 1

Fear-Potentiated Startle (FPS)

We used a *trace* FPS paradigm to evaluate hippocampal-dependent cued fear learning. Each FPS session started with a 5-min acclimation period (background noise = 60 dB). During the first session of the paradigm (fear training), the rats were trained to associate a light stimulus (conditioned stimulus, CS) with a 0.6 mA footshock (unconditioned stimulus, US). The conditioning session involved 10 CS + US pairing presentations. During each CS + US presentation, the onset of the shock (500 ms duration) occurred 3 sec after the offset of the 4 sec light. Light-shock pairings were presented in a quasi-random manner (ITI = 3-5 min). The cued fear acquisition was measured 24 h later. During cued fear acquisition (fear learning testing), the rats were first presented with 15 startle-inducing tones (leaders; 5 each at 90 dB, 95 dB, and 105 dB) delivered alone at 30 sec ITI. Subsequently, the rats were presented with 60 test trials. For half of these test trials, a 20 ms tone was presented alone (tone alone trials; 10 trials for each tone: 90 dB, 95 dB, and 105 dB). For the other half, the onset of the tone (20 ms duration) occurred 3 sec after the offset of the 4 sec light; 10 trials for each pairing: 90 dB, 95 dB, and 105 dB. The 60 trials were divided into 10 blocks of 6 test trials each, which included 3 tone alone trials and 3 light + tone trials. To conclude the testing session, the rats were presented with 15 startle-inducing tones (trailers; 5 each at 90 dB, 95 dB, and 105 dB) delivered at 30 sec ITI. The trials in this session were presented in a quasi-random order (ITI = 30 sec). The startle-eliciting tones had a 20 ms duration. We assessed fear learning by comparing the startle amplitude from light + tone trials (conditioned + unconditioned stimulus, CS + US) relative to tone alone trials (unconditioned stimulus, US). FPS data

was reported as the change (delta) between US and CS + US [FPS = (Light + Tone Startle) – (Tone Alone Startle)].

Open Field Test (OFT)

The OFT was performed in an opaque acrylic open field maze (43.2 cm length × 43.2 cm width × 30.5 cm height). The rats were placed in the center of the field and recorded for 5 min using Ethovision XT (Noldus Information Technology). Behaviors were recorded at normal room lighting conditions (269 lux illumination). We used Ethovision to subdivide the arena into nine zones, which allowed for the evaluation of inner (center) and outer arena exploration. The chamber was cleaned with 70% ethanol between trials. The following equation was used to calculate the anxiety index: Anxiety index = $1 - \frac{[(\text{center cumulative duration} / \text{total test duration}) + (\text{center total entries} / \text{total number of entries to center} + \text{corners})]}{2}$.

Elevated Plus Maze (EPM)

The near-infrared (NIR)-backlit EPM apparatus (catalog #ENV-564A, MedAssociates) consisted of two opposite open arms (50.8 × 10.2 cm) and two enclosed arms (50.8 × 10.2 × 40.6 cm) that were elevated 72.4 cm above the floor. Behaviors were recorded under low light conditions (< 10 lx). The rat was placed on the central platform facing an open arm and allowed to explore the maze for 5 min. The apparatus was thoroughly cleaned after each test session. Behaviors were recorded via a monochrome video camera equipped with a NIR filter. The time spent on both types of arms, the number of entries and duration in both types of arms, the latency to the first entry into any of the

open arms (OA), closed arms (CA) entries, frequency in the head-dipping zone, and stretch attend postures (SAPs) were determined using Ethovision XT tracking software (Noldus Information Technology). From these data, we calculated the anxiety index according to our group and others (Cohen et al., 2012; Contreras et al., 2014; Kalyan-Masih et al., 2016; Vega-Torres et al., 2020b). Anxiety index = $1 - \frac{[(\text{OA cumulative duration} / \text{total test duration}) + (\text{OA entries} / \text{total number of entries to CA} + \text{OA})]}{2}$.

Y-maze Spontaneous Alternation

The Y-maze apparatus was constructed of black Plexiglass with 3 identical arms spaced at an angle of 120 degrees (arms dimensions: 22" L × 4.5" W × 11.5" H). Each rat was placed at the end of the start arm and allowed to move freely through the maze during a 9-min session. The total number of arm entries and alternations from visited to unvisited arms was recorded using a video camera, tracked using Ethovision XT (Noldus Information Technology), and scored manually by two blinded investigators. Entries occur when all four limbs are within the arm. Each arm choice was recorded, and spontaneous alternation behaviors were scored as three successive choices that include one instance of each arm (a triad). The percentage of spontaneous alternations was calculated as the total number of alternations / (total arm entries – 2) × 100. This measure is considered to reflect short-term memory in rats. The alternate arm returns and same arm returns were also scored for each animal to investigate attention within spontaneous working memory. The number of total alternations was calculated as an index of locomotor activity, and rats exhibiting 0 entries were excluded from analyses. Behaviors were recorded at normal room lighting conditions (269 lx). The maze was cleaned with 70% ethanol between trials.

Magnetic Resonance Imaging

All fixed brain tissues underwent *ex vivo* high-resolution imaging using a 9.4 T Bruker Avance Imager (Bruker Biospin, Billerica, MA). All the data were collected with a 2.2-cm field of view, 0.55-mm slice thickness, and a 200x180 acquisition zero-filled to a final 256x256 matrix. T2-weighted RARE (rapid acquisition with relaxation enhancement; T2 RARE) were collected with the following parameters: repetition time (TR) / echo time (TE) = 6482 msec / 49.3 msec, RARE factor of 4, for a final resolution of 110x111x550 microns. The total imaging time was 39 min.

The experimenters were blinded to the group designation during MRI analysis. MRI scans were eddy corrected, and the cranium was removed using MatLab. The Waxholm rat brain atlas was fit to each animal, and regional labels were applied with Advanced Normalization Tools (ANTs). Regional volumes were extracted for 72 regions (144 bilateral regions). All data were extracted and summarized in Excel. Given our interest in hippocampal atrophy, we focused on hippocampal volumes and subfields.

Neurohistology

Tissue Preparation and Immunohistochemistry

Before euthanasia, the animals were deeply anesthetized with an intraperitoneal overdose injection of Euthasol (150 mg/kg; Virbac, Fort Worth, TX) and sacrificed via transcardiac perfusion with 10% sucrose (isotonic at 9.25% in distilled water; prewash) and 4% paraformaldehyde (PFA; fixative) using the Perfusion Two™ Automated Pressure Perfusion System (Leica Biosystems, Buffalo Grove, IL) ($n = 12$; 3 rats per

group). The brains were removed from the cranial vault after MRI imaging and washed with phosphate-buffered saline (PBS) and cryoprotected with sucrose (30%) for 12–16 h at 4°C before embedding in Tissue-Tek® O.C.T.™ compound (Sakura, Torrance, CA, United States). Three (3) brains per group were later sectioned with a cryostat (Cryocut 1800 Cryostat, Leica Biosystems, Vista, CA, USA) at 25 µm thickness in the coronal plane. Regions of interest included dorsal and ventral areas of the CA1 region of the hippocampus of both hemispheres with a distance from Bregma between -4.6 and -5.6. Sections were mounted in glass slides, and immunohistochemistry staining was performed with the microglia/macrophage marker, ionized calcium-binding adaptor protein molecule 1 (Iba-1). First, sections were incubated in a quenching buffer (10% methanol, 1% hydrogen peroxide in PBS) to block endogenous peroxide activity. To block the signal from endogenous avidin, biotin, and biotin-binding proteins in tissue, we used Avidin/Biotin blocking kit (Abcam, USA) followed by incubation with normal serum (5% Donkey and 5% Goat serum) with 1% Triton-X in PBS. Sections were then incubated overnight with a polyclonal rabbit anti-Iba1 primary antibody (1:750, rabbit anti-Iba1, catalog #019-19741, Wako, USA) followed by secondary biotinylated antibody incubation (1:200, goat anti-rabbit IgG, Vector Labs Inc.). The Vectastain Elite ABC HRP kit and DAB peroxidase substrate kit with nickel (Vector Labs Inc., Burlingame, CA, USA) were used to visualize staining according to manufacturer instructions. Following staining, slides were dehydrated in ethanol (79, 95, and 100%), rinsed in Xylene, sealed with Permount Mounting Medium (Fisher Chemical), and coverslips applied for subsequent visualization.

Image Acquisition and Segmentation Processing for Microglial Morphological Characterization

Blinded investigators carried out image acquisition and analyses. Images of tissue sections that were DAB-stained with the Iba-1 antibody were obtained using a Keyence BZ-9000 series microscope (Keyence Corporation, Osaka, Japan) in brightfield at a 40x magnification. To obtain a single high-quality full-focused composite image that showed detailed magnification of ramified processes of the cells and facilitate image segmentation, a multi-plane virtual-Z mode image with 10 images (1 μm thickness) in 20 μm depth of tissue section was captured and later combined using public domain software *ImageJ* (<https://imagej.nih.gov/ij/>). Hippocampal dorsal and ventral CA1 areas were scanned to obtain at least 3 images per region of interest per hemisphere covering a field of view area of 1.2 mm. Each acquired image was saved as a TIFF file and included at least six (6) Iba-1+ cells.

Images were processed in an unbiased and systematic way to obtain a filled image. For this purpose, a series of steps were performed using ImageJ with the Fiji plugin (<https://imagej.net/Fiji>). First, a single high-quality image was obtained by merging all the single images and processed under a minimum threshold feature to soften the background and enhance the contrast. Subsequently, the image was transformed to 8-bit grayscale and binarized to obtain a black and white image by applying a previously established threshold. To obtain a cell image consisting of continuous pixels, manual editing of each image was performed. A set threshold of pixel number addition (no more than 4 pixels) to join processes belonging to a selected cell was established and performed systematically. A 2-pixel set threshold was applied to separate ramification to

neighboring cells when applicable. This step was carefully performed under the view of the original z-stack images with special care to avoid bias. The final filled shape image was saved and later analyzed with *FracLac* V2.5 for *ImageJ* (Karperien, A., *FracLac* for *ImageJ*; <http://rsb.info.nih.gov/ij/plugins/fraclac/FLHelp/Introduction.htm>) (Karperien et al., 2013) to quantify the morphological changes of microglial cells in each experimental group. Foreground pixel quantification of the filled binary images per object (cell) was done with the Box Counting (BC) feature, which counts the number of boxes containing any foreground pixels along with the successively smaller caliber grids. BC box size scale was obtained as a Power Series where the base is raised to the exponent added to it to make successive sizes. Finally, the slope for each object was the average of 12 measurements with different and random placements of the grid. Box Counting and Convex and Hull Area measurements were exported using Excel (Microsoft, Redmond, WA, USA) for record and tabulation. Graphpad Prism version 9.0 (Graphpad Software, La Jolla, CA, USA) was used for statistical analyses.

Study 2

Western Blot

We used a separate group of animals *without behavioral manipulations* for Western blot experiments to minimize potential carryover effects of behavioral tests on molecular outcomes. For this group, an additional injection of NRG1 or saline was administered 1 h before euthanizing the animals to investigate the acute activation of NRG1-ErbB4 signaling pathways. The rats were euthanized with intraperitoneal administration of Euthasol (Virbac) and perfused transcardially with PBS. The rats were rapidly

decapitated, and the brains were isolated, and the hippocampus was dissected out. The tissue was immediately homogenized in 600 μ L MPER Extraction Buffer (Thermo, 78501) (supplemented with protease and phosphatase inhibitors) using Bullet Blender. Homogenates were centrifuged for 10 min at 14,000 x g at 4°C. The supernatant was removed, aliquoted, and stored at -80°C until the day of the experiment. Protein concentration was determined using the Pierce BCA Protein Assay (Thermo, 23225). Homogenates were then mixed with 4x Protein Sample Loading Buffer (LI-COR Biosciences, 928-40004), NuPAGE Sample Reducing Agent (10x) (Thermo, NP0009), and subsequently heated at 95°C for 5 min. Fifty micrograms (50 μ g) of protein were separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen). Proteins were transferred to iBlot Gel Transfer Stacks Nitrocellulose Membranes (Invitrogen) using the iBlot 2 System (Thermo). The blots were washed and blocked for 1 h in 5% dry milk or Intercept (TBS) blocking buffer (LI-COR, 927-60001) and incubated with primary antibodies overnight at 4°C (diluted in 1x TBS with 0.1% Tween® 20). Primary antibody dilutions are described in **Supplemental Table 2**. Blots were subsequently washed in 1x TBS with 0.05% Tween® 20 and incubated with appropriate fluorescent secondary antibodies (LI-COR; diluted at 1:30,000 in 1x TBS with 0.1% Tween® 20) for 90 min at room temperature. Membranes were washed with 1x TBS before imaging. Images were obtained using Odyssey® Fc Imager (LI-COR).

Bead-based Cytokine Detection and Metabolic Status Measurements

To examine neuroinflammation from hippocampal homogenates, we performed a multianalyte bead-based immunoassay (LEGENDplex™ Custom Panel of Rat

Inflammation markers; Biolegend, San Diego, CA, USA) to provide fast and quantitative information on the WD and NRG1 treatment effects in levels of relevant pro and anti-inflammatory cytokines and chemokines. Rats were euthanized with an intraperitoneal administration of Euthasol (Virbac, Fort Worth, TX, USA) and perfused transcardially with ice-cold PBS. Animals were rapidly decapitated, and the brains were isolated. For total protein extraction, hippocampal brain tissue was homogenized in a buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L PMSF, 0.05% Tween-20, and a Protease Inhibitor Cocktail (cOmplete™ Mini Protease Inhibitor Cocktail, Roche Diagnostic, USA). Samples were then agitated for 30 min at 4°C and centrifuged at 12,000 rpm for 20 min at 4°C, the supernatant was then removed. Protein concentration was quantified in triplicates using the DC™ Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions, and absorbance was read at 655 nm using Spectramax i3x detection platform (Molecular Devices, Sunnyvale, CA, USA).

Cytokine evaluation of hippocampal IL-10, IL-1 α , IL-6, IL-1 β , TNF- α , IL-33, GM-CSF, CXCL-1, CCL2, IL-18, IL-12p70, IL-17a, and INF- γ were quantified in triplicates using the bead-based Assay LEGENDplex™ protocol for V bottom plate, according to the manufacturer's instructions. Samples were diluted at 1:4 with Assay Buffer. Cytokine levels were determined using antibodies for each analyte covalently conjugated to a set of microspheres in a carbodiimide crosslinking reaction and detected by a cocktail of biotinylated antibodies. Following binding of streptavidin–phycoerythrin conjugate, the fluorescent reporter signal was measured using a MACSQuant® Analyzer 10 (Miltenyi Biotech, *Bergisch Gladbach*, Germany). FCS files were evaluated with the

LEGENDplex™ Data Analysis Software Suite using the Qognit cloud base platform (Biolegend, San Diego, CA, USA). A five-parameter logistic curve-fitting method was used to calculate concentrations from Median Fluorescence Intensity and normalized to the amount of protein in each sample. The results are reported as pg/mg of protein.

Metabolic and Inflammatory Assessments

Please refer to published work from our lab for detailed protocols on the metabolic biomarker measures used in this study (Kalyan-Masih et al., 2016). Blood glucose: post-prandial blood glucose levels were measured in anesthetized rats by cutting the tail tip right before inducing euthanasia. Glucose levels were measured using a glucometer (OneTouch UltraMini® manufactured by LifeScan Inc, Milpitas, CA, USA) and reported as mg per dL. Study 2 rats were used for the molecular experiments (same rats used for Western blot analyses). Plasma was collected and stored at -80 °C. Corticosterone: Circulating plasma levels of corticosterone (CORT) were measured by ELISA (Enzo Life Sciences, ADI-900-097 Ann Arbor, MI, USA) according to the manufacturer's instructions. Plasma samples were diluted with kit assay buffer (1:5 dilutions) and ran in triplicates. The absorbance was measured at 405 nm with 570 nm correction using the SpectraMax i3X detection platform (Molecular Devices, Sunnyvale, CA). Corticosterone levels were determined as the percentage bound using a standard curve with a detection range between 20,000 - 31.25 pg/mL. Values are reported as picograms per milliliter. Leptin: Plasma samples were used to evaluate leptin levels, samples were diluted with kit assay buffer (1:3 dilutions), ran in triplicates, and measurements were made using an enzyme-linked immunosorbent assay ELISA kit (Leptin Rat ELISA Kit Abcam, ab100773;

Cambridge, MA, USA) according to manufacturer's instructions. Plates were read at 450 nm using the SpectraMax i3X (Molecular Devices, Sunnyvale, CA USA). Specific concentrations for each sample were determined as mean absorbance using a standard curve of samples ranging from 0 to 8,000 pg/mL. Values were then calculated and reported as picograms of leptin per mL. Insulin: Plasma samples were assayed for insulin content using an insulin ELISA kit (Abcam, ab100578, Cambridge, MA, USA) following the manufacturer's instructions. Samples were diluted with Assay Diluent (1:5 dilution) in triplicates, and levels measured against a standard curve from 0-300 uIU. Samples were read at 450 nm using the SpectraMax i3X (Molecular Devices, Sunnyvale, CA, USA). Insulin concentration is reported as uIU per mL. Triglycerides: Plasma samples were used to determine triglycerides levels with the TG assay kit for quantification (Abcam, ab65336; Cambridge, MA, USA) as previously described (Kalyan-Masih et al., 2016). Samples were diluted in with kit assay buffer (1:3 dilution) and ran in triplicates. Triglyceride levels were determined using the SpectraMax i3X plate reader (Molecular Devices, Sunnyvale, CA USA). TG concentration was determined as the mean absorbance at 570 nm using a standard curve of samples ranging from 0 to 10,000 pmol. TG concentration was calculated and reported as milligrams of TG per dL. FGF-21: Plasma samples were assayed for FGF-21 levels using an ELISA kit from R&D Systems (MF2100, R&D Systems, Minneapolis MN, USA) according to the manufacturer's instructions. Samples were diluted with Assay Diluent (1:2 dilution) in triplicates and levels measured against a standard curve from 0-2,000 pg/mL. Samples were read at 450 nm and corrected at 540nm using the SpectraMax i3X (Molecular Devices, Sunnyvale, CA USA). FGF-21 concentration is reported as ng/mL. NRG1: Hippocampal homogenate samples were

used to determine NRG1 levels with the Human NRG1- β 1 ELISA (DY377, R&D Systems, Minneapolis MN, USA). Samples were diluted in kit assay buffer (1:6 dilution) in triplicates. NRG1 levels were determined using the SpectraMax i3X plate reader (Molecular Devices, Sunnyvale, CA USA) as the mean absorbance at 450nm with correction at 540 nm using a standard curve of samples ranging from 0 to 4,000 pg/mL. NRG1 concentration was calculated and reported as nanograms of NRG1 per mL.

Statistics

We analyzed the data using GraphPad *Prism* version 9.0 (Graphpad Software, La Jolla, CA, USA). Shapiro-Wilk statistical analyses were used to determine sample distribution. The Brown-Forsythe test was used to test for the equality of group variances. Two-way analysis of variance (ANOVA) was used to examine the effect of the diet type, treatment, and interaction between factors on outcome measures. Three-way ANOVA was used to investigate the impact of the diet type, treatment, hippocampal regions, and interactions between factors on microglial morphology. Mixed-effects model analyses were used to examine the effect of the diet type, treatment, ErbB4 isoform type, and interactions on hippocampal ErbB4 mRNA levels. Multiple comparisons were made using Sidak's (for one significant main effect; within-group) or Tukey's test (for more than one significant main effect or significant interaction effect; between-group). The ROUT method was used to determine outliers. Principal component analyses (PCA) were performed to reduce the microglia morphological descriptors' dimensionality by using centering/scaling methods. We considered differences significant when $p < .05$. The data is shown as the mean $\pm$ standard error of the mean (SEM).

Images and Figures

Illustrations were prepared using BioRender (www.Biorender.com), OmniGraffle Pro (The Omni Group; Seattle, WA), and Prism (Graphpad Software; La Jolla, CA).

Results

Early impact of macronutrient profile of diet on biometric parameters

Following the reports indicating that exogenous NRG1 improves metabolic outcomes implicated with obesity (Ennequin et al., 2015a; b; Caillaud et al., 2016; López-Soldado et al., 2016; Zhang et al., 2018), we tested whether exogenous NRG1 administration prevents/delays the onset of obesity symptoms. We postulated that exogenous NRG1 administration would attenuate the WD-induced hippocampal structural impairments and alterations in cued learning, spatial working memory, inflammatory signals, and synaptic integrity. **Figure 1** details the experimental timeline and experimental groups (*CDV*, control diet + vehicle injections; *CDN*, control diet + NRG1 injections; *WDN*, Western diet + vehicle injections; *WDN*, Western diet + NRG1 injections). Here, we found that adolescent rats exposed to the Western-like high-fat diet (WD) for three weeks exhibited significantly increased body weight, caloric intake, and post-prandial blood glucose levels relative to the rats that consumed the control diet (CD) ($p < .05$; **Table 1**). However, three weeks of consuming the obesogenic WD was not sufficient to significantly alter critical peripheral obesity biomarkers, including leptin, insulin, triglycerides, and corticosterone ($p > .05$; **Table 1**). Subchronic exogenous NRG1 administration had no significant effects on the biometric parameters assessed in this study ($p > .05$; **Table 1**).

Exogenous NRG1 administration during adolescence reduces trace cued fear learning

Recent work from our lab has focused on identifying early (mal)adaptive responses to obesogenic environments (Kalyan-Masih et al., 2016; Vega-Torres et al., 2018, 2019a, 2020b; Santana et al., 2021). Having demonstrated that exposure to an obesogenic diet during adolescence alters the neural and behavioral substrates implicated with emotional regulation, we asked whether these impairments can be attenuated by administering neuregulin-1 (NRG1), a neurotrophic factor with potent neuroprotective and anti-inflammatory properties (Li et al., 2012; Depboylu et al., 2015; Guan et al., 2015; Simmons et al., 2016).

Trace conditioning is thought to reflect hippocampal-dependent declarative memory. Hippocampal lesions impair acquisition and expression of trace conditioning, while having little effect on the acquisition of amygdalar-dependent delay conditioning (McGlinchey-Berroth et al., 1997; Trivedi and Coover, 2006). Based on our findings reporting structural impairments in hippocampus of rats that consumed an obesogenic diet during adolescence (Kalyan-Masih et al., 2016), we decided to investigate the effect of the WD and exogenous NRG1 on trace conditioning. We found no significant interaction [$F_{(1, 43)} = .79, p = .38$], treatment [$F_{(1, 43)} = .05, p = .82$], or diet [$F_{(1, 43)} = 1.82, p = .19$] effects on foot shock reactivity (**Figure 2A**). We found that daily NRG1 administration (5 µg/kg/day for 21 days between postnatal day, PND21-PND41) led to a significant reduction in fear-potentiated startle (FPS) responses at 24 h following trace cued fear conditioning. Analyses showed a significant interaction [$F_{(1, 43)} = 4.57, p = .038$]

effect in FPS responses, with no significant treatment [$F_{(1, 43)} = 2.81, p = .10$] or diet [$F_{(1, 43)} = .40, p = .53$] (**Figure 2B**). Post hoc testing revealed significant differences between the CDV and CDN rats ($p = .021$). When analyzing each separate tone intensity, we found a stimulus effect (tone alone vs. light + tone) for 90 dB [$F_{(1, 40)} = 64.82, p < .0001$], 95 dB [$F_{(1, 44)} = 27.71, p < .0001$], and 105 dB [$F_{(1, 43)} = 37.61, p < .0001$]. Interestingly, only CDV rats exhibited significant differences between the stimulus type (**Supplemental Figure 1**). This phenotype is consistent with previous reports from our lab proposing that obesity and the consumption of obesogenic diets saturates fear and emotional circuits (Vega-Torres et al., 2018).

WD consumption during adolescence increases indices of anxiety in the EPM

Open-field test (OFT) behavior analyses revealed a significant diet [$F_{(1, 34)} = 9.69, p = .004$], and no significant interaction [$F_{(1, 34)} = .43, p = .52$] or treatment [$F_{(1, 34)} = .27, p = .61$] effects on the OFT anxiety index (**Figure 2C**). Post hoc testing revealed that WDN rats exhibited reduced indices of anxiety in the OFT relative to CDN rats ($p = .019$). These behavioral alterations were accompanied by a significant diet [$F_{(1, 34)} = 5.70, p = .023$], and no significant interaction [$F_{(1, 34)} = .024, p = .88$] or treatment [$F_{(1, 34)} = .0007, p = .98$] effects on the total distance traveled during the 5-min testing session (**Figure 2D**). Interestingly, when the rats were exposed to a more anxiogenic behavioral setting, the elevated-plus maze (EPM), the same groups showed opposite anxiety index results. Analyses revealed a significant diet [$F_{(1, 43)} = 7.27, p = .009$], and no significant interaction [$F_{(1, 43)} = 1.38, p = .25$] or treatment [$F_{(1, 43)} = .066, p = .80$] effects on the EPM anxiety index (**Figure 2E**). WDN rats demonstrated higher anxiety indices in the EPM when

compared to CDN ($p = .017$). We used the number of closed arm entries as a proxy for locomotion in the EPM. We found no significant interaction [$F_{(1, 43)} = .613$, $p = .438$], treatment [$F_{(1, 43)} = .434$, $p = .514$], or diet [$F_{(1, 43)} = .051$, $p = .822$] effects in the number of closed arm entries (**Figure 2F**).

Spontaneous alternations in the Y-maze revealed no significant interaction [$F_{(1, 40)} = .007$, $p = .93$], treatment [$F_{(1, 40)} = .97$, $p = .33$], or diet [$F_{(1, 40)} = .011$, $p = .92$] effects (**Figure 2G**). Analyses for the total number of alternations revealed no significant interaction [$F_{(1, 40)} = 1.00$, $p = .32$], treatment [$F_{(1, 40)} = .32$, $p = .58$], or diet [$F_{(1, 40)} = .22$, $p = .64$] effects (**Figure 2H**).

Exogenous subchronic NRG1 reduces the hippocampal volume in adolescent rats

Exogenous NRG1 confers robust anti-inflammatory (Li et al., 2015; Liu et al., 2018), neuroprotective (Li et al., 2012; Depboylu et al., 2015; Guan et al., 2015), and neurogenic effects (Mahar et al., 2011, 2016). In this respect, we asked whether NRG1 administration prevents hippocampal atrophy in rats that consume obesogenic diets. We examined hippocampal volumetric measurements using magnetic resonance imaging (MRI). We found a significant interaction [$F_{(1, 19)} = 6.61$, $p = .019$] and treatment [$F_{(1, 19)} = 9.48$, $p = .006$] effects on total hippocampal volume (both left and right hippocampi combined), with no diet [$F_{(1, 19)} = .052$, $p = .82$] effects (**Figure 3A-B**). The rats that received prolonged NRG1 administration during early adolescence exhibited smaller hippocampi (CDV vs. CDN, $p = .003$), an effect that was dependent on the diet type. The volumetric differences were not related to changes in total brain volume. Analyses revealed no significant

interaction [$F_{(1, 19)} = .14, p = .71$], treatment [$F_{(1, 19)} = .18, p = .67$], or diet [$F_{(1, 19)} = .25, p = .62$] effects on total brain volume (**Supplemental Figure 2A**).

Hippocampal subfield segmentation revealed unique vulnerabilities to the experimental manipulations in the CA1 region. Analyses revealed a significant interaction [$F_{(1, 19)} = 6.44, p = .020$] and no significant treatment [$F_{(1, 19)} = .11, p = .75$] or diet [$F_{(1, 19)} = 3.16, p = .092$] effects (**Figure 3C-D**), indicating a crossover interaction between the main factors. WDV rats had a significantly smaller CA1 volume than CDV rats ($p = .035$). **Table 2** details hippocampal volumetric data and statistics. The left and right rodent hippocampi exhibit striking anatomical and functional lateralization. We dissected the effects of the WD and hippocampal hemispheric lateralization. We found significant hippocampal hemisphere lateralization [$F_{(1, 19)} = 6.08, p = .023$] and diet type [$F_{(1, 19)} = 4.71, p = .043$] effects, with no significant interaction [$F_{(1, 19)} = .053, p = .82$] effects on hippocampal volumetrics (**Supplemental Figure 2B**). WD rats exhibited a reduced right hippocampal volume relative to the left hippocampal volume in CD rats (post hoc $p = .019$).

Quantitative morphometric analysis shows a diverse morphologic microglia response to an obesogenic diet and exogenous NRG1 treatment in the CA1 region of the hippocampus

Microglia are the innate immune cells of the brain. Notably, microglia have been shown to play a significant role in axonal remodeling and synaptic pruning (Schafer and Stevens, 2013) and hippocampal tissue volume (Nelson et al., 2021). Microglia are highly responsive to obesogenic diets (Hao et al., 2016; Cope et al., 2018) and NRG1 (Simmons et al., 2016; Alizadeh et al., 2017). Given that microglial function is closely related to its

morphology (Fernández-Arjona et al., 2017), we measured an array of 18 key morphometric parameters using fractal analysis (**Supplemental Table 2**). We focused on the CA1 hippocampal subfield based on its role on hippocampal-dependent behavior, abundant ErbB4 expression, and the observed volumetric changes and vulnerabilities of this region to several environmental insults. We performed morphological analyses in 1,981 Iba-1-positive cells (approximately 500 microglia/group). We applied Principal Component (PC) Analysis on the morphometric parameters to trace the possible differences in microglia driving hippocampal structure and behavior changes. PCs with eigenvalues greater than 1 were used (*Kaiser* rule). For all groups, we had three PCs with eigenvalues greater than 1. These PCs explained more than 80% of the accumulated variance between cells (PC1: ~60%; PC2: ~20%; PC3: ~5%; **Supplemental Figure 4**). The analyses allowed for the extraction of presumably independent (non-overlapping) factors reflecting different parameters constituting microglial shape and function in our rat model. We identified several morphometric features with variable contribution values higher than .06 (1/18; 18 total number of features). These morphometric parameters were subjected to PCA. PCA revealed distinctive morphological profiles between groups; PC1 contributed to explaining diet effects, and PC2 helped understand the differences between NRG1-treated groups (PC1 = 58, PC2 = 35%; **Supplemental Figure 5**). **Figure 4A** shows representative microglia morphologies from hippocampal dorsal and ventral CA1 regions in each study group. Evaluation of microglial density (**Figure 4B**) as a measure of cell size based on area demonstrated that the WD significantly decreased microglial density in the right CA1 subfield (diet: [$F_{(1,8)} = 6.82$, $p < .031$]; complete F stats summarized in **Table 5**). This finding indicates an amoeboid and thus phagocytic

microglia phenotype, supporting the reduced CA1 volume and the increased anxiety-like behaviors in the EPM in WD rats. Microglial lacunarity is a measure of heterogeneity that reflects sensitive changes in particular features such as soma size relative to process length (Karperien and Jelinek, 2015). We found that lacunarity was significantly decreased in the right CA1 subfield of rats that consumed an obesogenic WD (**Figure 4C**) (*diet*: [$F_{(1,8)} = 12.09$, $p < .008$]; complete F stats summarized in **Table 5**), suggesting lower complexity with increased microglial transformation (activation).

The span ratio is a measure that describes the cell shape (or form factor) and is based on the ratio of length and height of the Convex Hull area occupied by the cell (Fernández-Arjona et al., 2017). The higher the span ratio, the less difference in area between soma and processes, suggesting a more amoeboid shape. The span ratio was significantly increased in the right CA1 region of rats receiving the exogenous NRG1 (**Figure 4C**) (*treatment*: [$F_{(1,8)} = 15.43$, $p < .004$]; complete F stats summarized in **Table 5**). Circularity is a measure of shape and size. High circularity values are associated with round sphere shapes, suggesting a more amoeboid-like morphology associated with a greater phagocytic activity (Fernández-Arjona et al., 2017). NRG1 administration reduced circularity in the right CA1 region (**Figure 4C**) (*treatment*: [$F_{(1,8)} = 9.67$, $p < .014$]), complete F stats summarized in **Table 5**). Detailed microglial parameter values were reported as mean $\pm$ SEM for each group (**Table 4**). Complete details regarding microglial morphology descriptors statistics can be found in **Table 5**.

WD consumption increases cytokine levels while exogenous NRG1 administration decreases selective cytokine levels in the rat hippocampus

Obesity is associated with a robust neuroinflammatory state via increased inflammatory mediators. Our data revealing diet-induced changes in microglial morphology prompted us to examine hippocampal cytokine levels. Using a bead-based flow cytometry immunoassay, we found that the WD exerted a precise cytokine regulation (**Figure 5**). The rats that consumed the WD exhibited a significant increase in pro-inflammatory cytokines in the hippocampus: IL1- α ($p = .010$), TNF- α ($p = .006$), and IL-6 ($p = .019$). Interestingly, the anti-inflammatory cytokine, IL-10, was also significantly increased in WD rats ($p = .027$). Opposite to the main dietary effects in increasing hippocampal cytokine levels, exogenous NRG1 administration significantly reduced IL-33 ($p = .041$), GM-CSF ($p = .021$), CCL-2 ($p = .037$), and IFN- γ ($p = .031$), confirming the potent immunomodulatory effects of NRG1 (Li et al., 2015; Simmons et al., 2016). **Table 3** includes the results and statistics of the cytokines tested in this study.

WD consumption and exogenous NRG1 synergize to enhance hippocampal ErbB4 phosphorylation and sheddase protein levels

Dysfunctional NRG1-ErbB4 signaling has been recently implicated in obesity and obesity-related complications (Ennequin et al., 2015a; Salinas et al., 2016; Zeng et al., 2018). In support of these reports, we found that the obesogenic WD decreased total ErbB4 protein levels in the hippocampus (**Figure 6A**). Analyses revealed a significant treatment [$F_{(1, 16)} = 7.97$, $p = .012$] and diet [$F_{(1, 16)} = 7.34$, $p = .016$] effect, while showing no significant interaction [$F_{(1, 16)} = .96$, $p = .34$] effects on ErbB4 protein levels. Post hoc testing revealed that WDN rats exhibited significantly lower ErbB4 protein levels when compared to CDV rats ($p = .009$). Notably, exogenous NRG1 administration caused a hyperphosphorylation

of hippocampal ErbB4 (**Figure 6B**). Analyses revealed a significant treatment [$F_{(1, 18)} = 5.82, p = .027$] and diet [$F_{(1, 18)} = 7.36, p = .014$] effect, while no significant interaction [$F_{(1, 18)} = .13, p = .73$] effects on protein levels. ErbB4 hyperphosphorylation was particularly evident in WDN rats relative to the CDV group ($p = .012$). Immunohistological evaluation demonstrated pErbB4 expression in hippocampal Cd11b/c cells from rats treated with exogenous NRG1 (**Supplemental Figure 3**). Supporting the expression and roles for NRG1-ErbB4 signaling in microglia cell function (Gerecke et al., 2001; Mencil et al., 2013; Simmons et al., 2016; Chen et al., 2019).

NRG1 and ErbB4 (trans)activation via proteolytic processing is achieved by the shedding protease TNF- α -Converting Enzyme (*TACE*, also known as *ADAM17*) (Rio et al., 2000; Fleck et al., 2013; Iwakura et al., 2017). We found that the WD increased TACE/ADAM17 protein levels in the hippocampus (**Figure 6C**). We identified a significant treatment [$F_{(1, 17)} = 8.93, p = .008$] and diet [$F_{(1, 17)} = 11.03, p = .004$] effect, while no significant interaction [$F_{(1, 17)} = .099, p = .76$] effects on protein levels. In agreement with the hippocampal pErbB4 protein levels results, we found that WDN rats showed increased TACE/ADAM17 protein levels when compared to CDV rats ($p = .001$).

We measured PSD-95, Akt, and Erk protein levels to investigate the impact of the WD and NRG1 on molecular pathways implicated in ErbB4 signaling. NRG1 administration showed a significant decrease for a postsynaptic regulator of ErbB4 dimerization, PSD-95 (**Figure 6D**). Analyses revealed a significant treatment [$F_{(1, 18)} = 4.47, p = .048$], and no significant diet [$F_{(1, 18)} = 2.83, p = .11$] or interaction [$F_{(1, 18)} = 1.02, p = .33$] effects on PSD-95 protein levels. Analyses for pAkt (normalized to total Akt) showed no significant interaction [$F_{(1, 17)} = .71, p = .41$], treatment [$F_{(1, 17)} = .16, p = .69$], or

diet [$F_{(1, 17)} = 2.80, p = .12$] effects on protein levels (**Supplemental Figure 6A**). Similarly, analyses for pErk1/2 (normalized to total Erk) showed no significant interaction [$F_{(1, 18)} = 1.13, p = .30$], treatment [$F_{(1, 18)} = .88, p = .36$], or diet [$F_{(1, 18)} = .35, p = .56$] effects on protein levels (**Supplemental Figure 6B**).

TACE/ADAM17 protein levels correlate with hippocampal pErbB4

We examined potential mechanisms explaining the reduced ErbB4 expression and increased pErbB4 protein levels in the rats that consumed the obesogenic WD. Twenty-one (21) days of consuming the WD and receiving NRG1 administration was not sufficient to significantly alter NRG1 protein levels in the hippocampus (**Supplemental Figure 7**). Analyses showed no significant interaction [$F_{(1, 18)} = .14, p = .72$], treatment [$F_{(1, 18)} = .031, p = .86$], or diet [$F_{(1, 18)} = .38, p = .54$] effects on hippocampal NRG1 protein levels, as measured by ELISA.

Four (4) structurally and functionally distinct ErbB4 isoforms have been identified. One pair of isoforms differs within their extracellular juxtamembrane domains. These juxtamembrane ErbB4 isoforms are either susceptible (JMa) or resistant (JMb) to proteolytic processing that releases a soluble receptor ectodomain. We used qRT-PCR to examine the effect of the WD and NRG1 on the regulation of hippocampal ErbB4 isoforms. We found lower JMa mRNA levels in the WD rats (relative to CD rats) and higher JMb in the rats that received the exogenous NRG1 (relative to VEH) (**Supplemental Figure 8**). Notably, mixed-effects model analyses revealed a significant interaction between isoform x diet [$F_{(3, 57)} = 3.13, p = .033$] and isoform x treatment [$F_{(3, 57)} = 2.79, p = .049$].

In summary, our data demonstrate that WD consumption reduces hippocampal ErbB4 protein levels while increasing its phosphorylation. TACE/ADAM17 activities may lead to reduced ErbB4 and also to ErbB4 transactivation (Rio et al., 2000). We performed Pearson's correlation analyses to test this idea. ADAM17 hippocampal protein levels showed a robust significant correlation with ErbB4 protein levels ($r = -.59$, $p = .007$; **Figure 7A**). ADAM17 protein levels were positively associated with hippocampal pErbB4 protein levels ($r = .53$, $p = .012$; **Figure 7B**). A significant negative relationship was found between ADAM17 protein levels and PSD-95 protein levels in the hippocampus ($r = -.54$, $p = .012$; **Figure 7C**). **Figure 8** describes our working model. We propose that obesogenic diets rich in saturated fatty acids promote TACE/ADAM17-mediated proteolytic cleavage of critical neurodevelopmental and neuroinflammatory processes, and consequently, lead to hippocampal synaptic/structural impairments implicated with anxiety disorders.

Discussion

This study sought to determine whether altered NRG1-ErbB4 signaling may partly explain hippocampal abnormalities in adolescent rats exposed to an obesogenic Western diet (WD). We demonstrate that both WD consumption and prolonged NRG1 administration during adolescence alter fear and anxiety-like behaviors while reducing hippocampal volume. We also report unique anatomical vulnerabilities to the obesogenic WD, particularly in the CA1 subfield of the hippocampus. These behavioral and anatomical alterations were associated with changes in microglial morphology, unique cytokine profiles, ErbB4 hyperphosphorylation, and increased TACE/ADAM17 protein levels.

The consumption of an obesogenic diet during early adolescence promotes neuroinflammation and anxiety-like behaviors while increasing hemispheric differences in hippocampal volumes.

Obesity and the dietary intake of saturated fats and simple sugars have emerged as risk factors for the development of anxiety and stress-related disorders (Scott et al., 2008; Perkonigg et al., 2009; Johannessen and Berntsen, 2013; Maguen et al., 2013; Mitchell et al., 2013; Kubzansky et al., 2014; Bartoli et al., 2015; Smith et al., 2015). Consistent with studies in humans, we reported that diet-induced obesity (DIO) in rats: **1)** reduces hippocampal volume (Kalyan-Masih et al., 2016), **2)** impairs the maturation of the corticolimbic fear circuits (Vega-Torres et al., 2018), **3)** enhances behavioral vulnerabilities to psychosocial stress (Kalyan-Masih et al., 2016; Vega-Torres et al., 2018, 2019b), **4)** results in profound fear learning and extinction learning deficits (Vega-Torres et al., 2018, 2019b), even in the absence of an obesogenic phenotype (Vega-Torres et al., 2020a), and **5)** leads to alterations in biomarkers implicated in inflammatory responses (Santana et al., 2021).

As valuable as these earlier findings have been in constructing a conceptual framework for the impact of obesogenic environments on hippocampal maturation, our current understanding is primarily based on molecular, cellular, and behavioral analyses of obese versus control animals at the end of dietary manipulations (when rats are considered obese; in our model, approximately at eight weeks after WD exposure). Less is known about the early impact of obesogenic diets on hippocampal structure and function. To address this knowledge gap, we combined volumetric measurements of the hippocampus using magnetic resonance imaging (MRI) with behavioral assays of

hippocampus-based tasks after a 21-day exposure to an obesogenic diet during the early adolescence period (from PND 21-41). Using behavioral tasks dependent on optimal hippocampal function, we demonstrated that three weeks on an obesogenic WD increased anxiety-like indices in the elevated plus maze in adolescent rats. The rats exposed to the obesogenic WD also exhibited increased locomotor activity (exploratory behavior) in the open field test. Interestingly, trace fear conditioning, and spatial working memory tasks were not affected in the animals that consumed the WD. MRI-based volumetrics demonstrated that three weeks on the obesogenic diet was sufficient to enhance hippocampal lateralization effects. These behavioral and anatomical alterations were associated with increased pro-inflammatory cytokines and changes in microglial morphology related to phagocytic phenotypes. Together with our previous studies, the findings reported here indicate that exposure to an obesogenic diet during adolescence selectively affects hippocampal structure and specific cognitive domains. Our investigations demonstrate that early adolescence is a critical window during which obesogenic diets exert anxiogenic effects.

Hyper-NGR1 conditions attenuate trace fear conditioning and reduce hippocampal volume while influencing microglia shape and inflammatory mediators

Neuregulin-1 (NRG1) and its ErbB receptor tyrosine kinases are expressed not only in the developing nervous system but also in the adult brain. ErbB4 is likely to be the primary mediator of NRG1 functions in the brain, and it is mainly expressed in hippocampal GABAergic interneurons, where it is enriched at postsynaptic terminals (Vullhorst et al., 2009). NRG1 signaling participates in several critical neurodevelopmental processes and

is implicated in nerve cell differentiation and synapse formation, radial glia formation and neuronal migration, axon navigation, and neurite outgrowth (Mei and Nave, 2014). Perturbations in NRG1/ErbB4 function have been associated with various neuropsychiatric disorders (Mei and Nave, 2014), resilience to stress (Clarke et al., 2018), and obesity/metabolic syndrome (Ennequin et al., 2015a; Salinas et al., 2016; Zeng et al., 2018; Zhang et al., 2018). Emerging evidence suggests that ErbB4 may also be important in the pathogenesis of obesity in humans (Locke et al., 2015; Salinas et al., 2016).

A key finding of this study is that prolonged NRG1 administration during early adolescence resulted in reduced hippocampal volume and fear conditioning deficits. Evidence suggests that a gain of function of NRG1 may contribute to the pathophysiology of schizophrenia (Hashimoto et al., 2004; Chong et al., 2008; Paterson et al., 2014). In agreement, a mouse model overexpressing NRG1 exhibited marked alterations in hippocampal-dependent function and memory while impacting the expression of several genes implicated in inflammation (Deakin et al., 2012). Interestingly, this study reported increased hippocampal volume in the NRG1 transgenic mice (Deakin et al., 2012). The conflicting result on hippocampal volume may be related to the magnitude in NRG1 levels, the developmental stage of the animals, animal species, or NRG1 peripheral effects (e.g., robust leptin and FGF21 secretagogue). Together, it is clear that NRG1 is a potent modulator of hippocampal volume and cognition.

Microglia cells, the resident immune cells in the brain, are responsible for maintaining a dynamic balance between anti-inflammatory and pro-inflammatory mediators. Microglia cells play a significant role in the behavioral outcomes associated

with obesity (Sobesky et al., 2014; Guillemot-Legrís and Muccioli, 2017; Cope et al., 2018). However, the underlying molecular mechanisms regulating the microglia-dependent inflammatory balance remain poorly understood. Microglia express ErbB2, 3, and 4 receptors (Gerecke et al., 2001; Dimayuga et al., 2003; Calvo et al., 2010) and are highly responsive to NRG1 (Simmons et al., 2016; Alizadeh et al., 2017). Yet, the function of this signaling pathway within microglia is poorly understood. While it is very likely that the observed NRG1 effects are related to changes in local microcircuits (GABAergic; parvalbumin, PV+ interneurons exhibit high ErbB4 expression; (Neddens and Buonanno, 2009)), this study provides additional support to the notion that NRG1-ErbB4 signaling regulates microglial morphology and inflammatory mediators (Calvo et al., 2010; Liew et al., 2016; Simmons et al., 2016; Chen et al., 2019; Shahriary et al., 2019).

An obesogenic diet influences behavioral, anatomical, and molecular responses to exogenous NRG1 administration

Consistent with studies demonstrating the potential involvement of NRG-ErbB signaling in obesity (Ennequin et al., 2015a; Locke et al., 2015; Cai et al., 2016; Salinas et al., 2016; Zeng et al., 2018; Zhang et al., 2018), we found several significant interactions between the obesogenic diet and exogenous NRG1. We present new evidence that an obesogenic diet influences the effect of prolonged NRG1 administration on the fear-potentiated startle, total hippocampal and CA1 subfield volume, and hippocampal cytokine levels (TNF- α , IL-10, IL-6). Interestingly, exogenous NRG1 synergized with the obesogenic diet to enhance ErbB4 activation and its proteolytic processing (Rio et al., 2000). ErbB4 proteolytic cleavage is mediated by the tumor

necrosis factor- α -converting enzyme (TACE) / a Disintegrin And Metalloproteinase 17 (ADAM17)-dependent ectodomain shedding in a protein kinase C (PKC)-dependent manner. Here, we present data suggesting a role for TACE/ADAM17 on ErbB4 activities and synaptic integrity. Future studies will identify the consequences of dysregulated NRG1/ErbB4 ectodomain shedding on hippocampal maturation and cognitive function.

Limitations

Our results demonstrate significant alterations in neurotrophic and neuroinflammatory signals in adolescent rats that consume an obesogenic diet enriched in saturated fats and simple sugars. However, the temporal window in which these effects take place was not established. To better clarify a mechanism, the spatiotemporal expression of hippocampal ErbB4 and associated cytokine profiles must be determined. Interpretation of the study may be confounded by the effect of daily i.p. injections. While this represents one of the best approaches to achieve therapeutic NRG1 levels in circulation (NRG1 has a short half-life), it is possible that the subchronic daily injection regime affected some endpoint measures. Furthermore, it is not clear from our results whether the NRG1 effects on hippocampal structure and behaviors are direct (and whether ErbB4 is responsible for the alterations). The use of inhibitors (e.g., AG1478) or fusion proteins (e.g., ecto-ErbB4) needs to be considered to dissect mechanisms and determine causality. Future experiments are required to dissociate the chronic vs. acute response to the NRG1 injection on molecular targets. Additionally, the study should be replicated in female rats and other rodent strains.

Conclusion

Current pathophysiological models explaining how early exposure to obesogenic diets alters brain and behavior have focused on neurodevelopmental or immunological mechanisms. We report a novel interaction between these factors in the maturing hippocampus. Our results indicate that a short-term (21 days) dietary challenge with an obesogenic WD during the critical neurodevelopmental stage of adolescence disrupts ErbB4 activities in the hippocampus. Most importantly, we continue to provide evidence that WD consumption during adolescence leads to neuroanatomical, inflammatory, and molecular alterations related to depression, anxiety disorders, and cognitive impairments in humans. While extrapolating our data must be made carefully, our results indicate that TACE/ADAM17-ErbB4 hyperfunction may contribute to abnormal hippocampal structural and cognitive vulnerabilities in individuals who consume obesogenic diets.

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Financial Disclosures

All authors report no financial interests or potential conflicts of interest.

Legends

Table 1. Detailed biometric parameters findings and statistics. The adolescent rats that consumed the WD exhibited an increase in body weight, food consumption, circulating FGF-21 levels, and post-prandial blood glucose levels relative to animals consuming the control diet at postnatal day 41. Three weeks of consuming the WD was not sufficient to alter plasma insulin, leptin, corticosterone, and triglyceride levels in adolescent rats. Exogenous NRG-1 administration did not have a significant effect on any of the biometric parameters measured.

Table 2. Detailed hippocampal subfield volumetrics and statistics. (A) Right hippocampal subfield volumes and three-way ANOVA statistics. **(B)** Left hippocampal subfield volumes and three-way ANOVA statistics.

Table 3. Detailed cytokine profiles and related statistics.

Table 4. Detailed microglial morphometric analyses values.

Table 5. Detailed microglial morphology descriptors statistics.

Figure 1. Experimental timeline and procedures. (A) Study 1 timeline and study groups. **(B)** Study 1 timeline of behavioral procedures, MRI, and histology. **(C)** Study 2 timeline of molecular analyses. Abbreviations: **CD**, control diet; **EPM**, elevated plus maze; **FPS**, fear-potentiated startle; **MRI**, magnetic resonance imaging; **NRG1**, neuregulin-1;

OFT, open field test; **PND**, postnatal day; **VEH**, vehicle; **WD**, Western diet.

Figure 2. Behavioral phenotypes associated with early access to an obesogenic diet and prolonged NRG1 administration. (A) Startle reactivity to the foot shocks was not affected by treatment [$F_{(1, 43)} = .05, p = .82$] or diet [$F_{(1, 43)} = 1.82, p = .19$]. **(B)** Fear potentiated-startle (FPS) responses were determined from trace conditioning protocol and showed a decrease in FPS (interaction: [$F_{(1, 43)} = 4.57, p = .038$]). CDN group had a significant decrease in FPS responses when compared to CDV ($p = .021$). **(C)** Open-field test (OFT) anxiety index was affected by the diet [$F_{(1, 34)} = 9.69, p = .004$]. WDN rats exhibited reduced indices of anxiety in the OFT relative to CDN rats ($p = .019$). **(D)** The diet had a significant effect on total distanced traveled in the OFT [$F_{(1, 34)} = 5.70, p = .023$]. **(E)** Elevated-plus maze (EPM) anxiety index was affected by the diet [$F_{(1, 43)} = 7.27, p = .009$]. WDN rats demonstrated higher anxiety indices in the EPM when compared to CDN ($p = .017$). **(F)** Number of closed arms entries in the EPM was not affected by treatment [$F_{(1, 43)} = .434, p = .514$] or diet [$F_{(1, 43)} = .051, p = .822$]. **(G)** Spontaneous alternations in the Y-maze was not affected by treatment [$F_{(1, 40)} = .97, p = .33$] or diet [$F_{(1, 40)} = .011, p = .92$]. **(H)** Treatment [$F_{(1, 40)} = .32, p = .58$] or diet [$F_{(1, 40)} = .22, p = .64$] had no effects on total number of alternations in Y-maze. For all behaviors: sample size = 12 rats / group (before outlier testing).

Figure 3. Obesogenic diet and prolonged NRG1 administration interact to reduce hippocampal volumes. (A-B) Total hippocampal volume (both left and right hippocampi combined) were affected by the treatment [$F_{(1, 19)} = 9.48, p = .006$]. Subchronic NRG1

administration during adolescence resulted in significant total hippocampal volume reduction (CDV vs. CDN, $p = .003$). **(C-D)** Hippocampal subfield segmentation showed a significant interaction [$F_{(1, 19)} = 6.44$, $p = .020$] effects on CA1 region. WDV rats had a significantly smaller CA1 volume than CDV rats ($p = .015$). Sample size = 6 rats / group.

Figure 4. Obesogenic diet and exogenous NRG1 injections alter microglial shape in the hippocampus. Full-focused composite 40x images were acquired from twelve (12) rats. Hippocampal dorsal and ventral CA1 areas were scanned to obtain at least three (3) images per region of interest per hemisphere covering a field of view area of 1.2 mm. Each acquired image was saved as a TIFF file and included at least six (6) Iba-1⁺ cells. **(A)** Representative microglia morphologies from hippocampal dorsal and ventral CA1 regions in each study group. **(B)** Evaluation of microglial density as a measure of cell size based on area demonstrated that the WD significantly decreased microglial density in the right CA1 subfield (diet: [$F_{(1,8)} = 6.82$, $p < .031$]). **(C)** Lacunarity was significantly decreased in the right CA1 subfield of rats that consumed an obesogenic WD (diet: [$F_{(1,8)} = 12.09$, $p < .008$]). The span ratio was significantly increased in the right CA1 region of rats receiving the exogenous NRG1 (treatment: [$F_{(1,8)} = 15.43$, $p < .004$]). NRG1 administration reduced circularity in the right CA1 region (treatment: [$F_{(1,8)} = 9.67$, $p < .014$]). Sample size = 3 rats / group.

Figure 5. Obesogenic diet consumption and prolonged NRG1 injections alter selective inflammatory mediators in the rat hippocampus. Bead-based flow cytometry immunoassay was used to evaluate cytokine levels. **(A-D)** The WD led to a

significant increase in cytokines in the hippocampus: IL1- α ($p = .010$), TNF- α ($p = .006$), IL-10 ($p = .027$), and IL-6 ($p = .019$). **(E-H)** NRG1 administration significantly reduced IL-33 ($p = .041$), GM-CSF ($p = .021$), CCL-2 ($p = .037$), and IFN- γ ($p = .031$). Table 3 includes the detailed statistics of the cytokines listed in Figure 5. Sample size = 5-6 rats / group.

Figure 6. Obesogenic diet intake and subchronic NRG1 administration synergize to promote ErbB4 phosphorylation and TACE/ADAM17 protein levels in the hippocampus. **(A)** Obesogenic WD decreased total ErbB4 protein levels in the hippocampus (diet [$F_{(1, 16)} = 7.34$, $p = .016$]). WDN rats exhibited significantly lower ErbB4 protein levels when compared to CDV rats ($p = .009$). **(B)** Phosphorylation of hippocampal ErbB4 protein levels was affected by the treatment [$F_{(1, 18)} = 5.82$, $p = .027$] and diet [$F_{(1, 18)} = 7.36$, $p = .014$]. ErbB4 hyperphosphorylation was particularly evident in WDN rats relative to the CDV group ($p = .012$). **(C)** TACE/ADAM17 protein levels in the hippocampus were affected by the treatment [$F_{(1, 17)} = 8.93$, $p = .008$] and diet [$F_{(1, 17)} = 11.03$, $p = .004$]. WDN rats showed increased TACE/ADAM17 protein levels when compared to CDV rats ($p = .001$). **(D)** NRG1 administration showed a significant decrease for PSD-95 protein levels in the hippocampus (treatment [$F_{(1, 18)} = 4.47$, $p = .048$]). Sample size = 5-6 rats / group.

Figure 7. TACE/ADAM17 hippocampal protein levels are associated with ErbB4 activities. **(A)** ADAM17 hippocampal protein levels negatively correlates with ErbB4 protein levels ($r = -.59$, $p = .007$). **(B)** ADAM17 protein levels positively correlates with hippocampal pErbB4 protein levels ($r = .53$, $p = .012$). **(C)** ADAM17 protein levels

negatively correlates with PSD-95 protein levels in the hippocampus ($r = -.54$, $p = .012$).

Sample size = 5-6 rats / group.

Figure 8. Working model unifying neurodevelopmental and neuroinflammatory hypotheses implicated in obesity-mediated hippocampal dysfunction. (1) It is plausible that exposure to obesogenic diets rich in saturated fatty acids (SFAs) increase TACE/ADAM17 sheddase expression and activities, **(2)** leading to dysregulation of neuroinflammatory (e.g., tumor necrosis factor alfa, TNF- α) and neurodevelopmental (ErbB4) mediators. **(3)** TNF receptor (TNFR) activation and prolonged ErbB4 cleavage tips the inflammatory balance to a pro-inflammatory state associated with microglial activation. **(4)** Neuroinflammation and microglial activation promote synaptic pruning and remodeling. **(5)** Disruption in microglia-synapse interactions contribute to synapse loss and dysfunction, and consequently hippocampal volume atrophy. **(6)** In summary, we postulate that TACE/ADAM17-mediated inflammation, microglial activation, and hippocampal structural impairments contribute to the obesity-induced cognitive and behavioral deficits implicated in anxiety disorders.

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Table 1

<i>Parameter</i>	CD		WD		<i>F</i>	<i>P</i>
	VEH	NRG1	VEH	NRG1		
Body Weight (g)	146.08 +/- 2.30	146.88 +/- 2.37	155.37 +/- 1.88	157.03 +/- 2.30	Interaction: F (1, 66) = 0.03 Treatment: F (1, 66) = 0.30 Diet: F (1, 66) = 19.20	P=0.85 P=0.58 P<0.0001
Caloric Intake (kcal/d)	52.84 +/- 0.73	53.09 +/- 0.96	63.39 +/- 1.59	66.09 +/- 1.04	Interaction: F (1, 20) = 1.18 Treatment: F (1, 20) = 1.71 Diet: F (1, 20) = 109.20	P=0.30 P=0.21 P<0.0001
PBG (mg/dl)	128.08 +/- 2.77	132.08 +/- 3.08	144.92 +/- 4.32	139.75 +/- 4.04	Interaction: F (1, 44) = 1.61 Treatment: F (1, 44) = 0.026 Diet: F (1, 44) = 11.50	P=0.21 P=0.87 P=0.0015
Insulin (uIU/mL)	38.92 +/- 1.85	41.23 +/- 4.82	34.23 +/- 3.17	37.47 +/- 3.87	Interaction: F (1, 18) = 0.017 Treatment: F (1, 18) = 0.45 Diet: F (1, 18) = 0.26	P=0.90 P=0.45 P=0.26
Corticosterone (ng/mL)	18.01 +/- 1.66	19.46 +/- 3.79	23.40 +/- 3.65	20.94 +/- 3.06	Interaction: F (1, 18) = 0.36 Treatment: F (1, 18) = 0.02 Diet: F (1, 18) = 1.13	P=0.55 P=0.87 P=0.30
Leptin (pg/mL)	597.57 +/- 57.96	515.13 +/- 22.62	640.33 +/- 32.80	532.77 +/- 57.07	Interaction: F (1, 18) = 0.075 Treatment: F (1, 18) = 4.27 Diet: F (1, 18) = 0.43	P=0.79 <i>P=0.05</i> P=0.52
Triglycerides (mg/dL)	118.35 +/- 0.57	119.84 +/- 0.93	119.48 +/- 1.33	114.80 +/- 3.13	Interaction: F (1, 18) = 1.94	P=0.18

Treatment: F (1, 18) = 0.32 P=0.32
Diet: F (1, 18) = 0.43 P=0.43

FGF-21 (ng/mL)	0.72 +/- 0.07	0.59 +/- 0.07	0.91 +/- 0.13	1.12 +/- 0.11
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Interaction: F (1, 18) = 2.75 P=0.11
Treatment: F (1, 18) = 0.70 P=0.70
Diet: F (1, 18) = 11.57 P=0.003

Table 2A. Right hippocampus.

	CD VEH		CD NRG		WD VEH		WD NRG	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total	44.42	0.64	41.39	0.61	43.02	0.50	42.81	0.76
CA1	14.07	0.18	13.11	0.24	13.78	0.27	13.60	0.30
CA2	0.56	0.04	0.49	0.05	0.51	0.04	0.61	0.03
CA3	11.83	0.27	11.06	0.19	11.46	0.21	11.30	0.24
DG	12.56	0.22	11.58	0.28	12.10	0.17	12.17	0.28
SUB	5.40	0.13	5.16	0.11	5.17	0.11	5.14	0.06

Source of Variation	F (DFn, DFd)	P value
Region	F (1.647, 32.93) = 13763	<0.0001
Diet	F (1, 20) = 0.0001678	0.9898
Drug	F (1, 20) = 6.457	0.0194
Region x Diet	F (5, 100) = 0.1115	0.9896
<i>Region x Drug</i>	F (5, 100) = 5.106	0.0003
<i>Diet x Drug</i>	F (1, 20) = 4.937	0.0380
<i>Region x Diet x Drug</i>	F (5, 100) = 3.751	0.0037
Subject		

Table 2B. Left hippocampus.

	CD VEH		CD NRG		WD VEH		WD NRG	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total	46.37	1.09	43.24	0.92	45.30	0.71	44.37	0.72
CA1	13.90	0.30	12.99	0.21	13.48	0.28	13.49	0.43
CA2	0.88	0.06	0.76	0.04	0.84	0.02	0.83	0.07
CA3	11.95	0.48	11.11	0.22	11.71	0.15	11.50	0.25
DG	13.44	0.19	12.38	0.36	13.12	0.28	12.70	0.09
SUB	6.20	0.18	5.99	0.18	6.16	0.20	5.85	0.14

Source of Variation	F (DFn, DFd)	P value
Region	F (1.452, 29.03) = 8810	<0.0001
Diet	F (1, 20) = 0.001380	0.9707
<i>Drug</i>	F (1, 20) = 5.423	0.0305
Region x Diet	F (5, 100) = 0.02894	0.9996
<i>Region x Drug</i>	F (5, 100) = 4.558	0.0009
Diet x Drug	F (1, 20) = 1.597	0.2209
Region x Diet x Drug	F (5, 100) = 1.527	0.1882
Subject		

Table 3. Summary of Measurements for Hippocampal Cytokines

Cytokines	Mean ± Standard Error of the Mean (pg/mg)				F	p
	Control Diet		Western Diet			
	Vehicle	NRG-1	Vehicle	NRG-1		
IL-10	8.44 ± 0.96	19.90 ± 3.38	35.14 ± 9.71	20.56 ± 3.99	Interaction, 4.683	Interaction, 0.0441
					Drug, 0.06692	Drug, 0.7988
					Diet, 5.171	Diet, 0.0354
TNF-α	2.15 ± 0.10	4.61 ± 0.46	8.81 ± 2.04	4.54 ± 0.75	Interaction, 7.620	Interaction, 0.0129
					Drug, 0.5545	Drug, 0.4661
					Diet, 7.285	Diet, 0.0147
IL-1α	1.89 ± 0.23	2.40 ± 0.35	3.94 ± 0.66	2.78 ± 0.42	Interaction, 3.542	Interaction, 0.0771
					Drug, 0.5266	Drug, 0.4779
					Diet, 7.502	Diet, 0.0140
IL-1β	26.04 ± 3.51	33.64 ± 2.17	51.36 ± 11.07	36.74 ± 6.16	Interaction, 2.351	Interaction, 0.1426
					Drug, 0.2340	Drug, 0.6344
					Diet, 3.846	Diet, 0.0655
IL-6	193.63 ± 20.64	301.67 ± 33.35	354.98 ± 41.54	266.54 ± 34.69	Interaction, 7.988	Interaction, 0.0112
					Drug, 0.07946	Drug, 0.7812
					Diet, 3.297	Diet, 0.0861
IFN-γ	66.65 ± 18.03	47.93 ± 7.76	64.12 ± 8.19	31.96 ± 7.76	Interaction, 0.3843	Interaction, 0.5431
					Drug, 5.514	Drug, 0.0305
					Diet, 0.7291	Diet, 0.4044
CXCL1	152.59 ± 3.88	145.80 ± 9.21	165.27 ± 16.81	126.12 ± 11.40	Interaction, 1.788	Interaction, 0.1978
					Drug, 3.603	Drug, 0.0738
					Diet, 0.08352	Diet, 0.7759
CCL-2	174.48 ± 8.05	136.63 ± 13.80	170.40 ± 9.62	153.06 ± 7.60	Interaction, 1.074	Interaction, 0.3137
					Drug, 7.773	Drug, 0.0121
					Diet, 0.3892	Diet, 0.5405
GM-CSF	33.02 ± 4.32	27.02 ± 5.73	53.48 ± 4.13	29.90 ± 7.60	Interaction, 2.209	Interaction, 0.1555

					Drug, 6.250	Drug, 0.0229
					Diet, 3.888	Diet, 0.0651
IL-18	1,056.79 ± 57.36	1,074.40 ± 68.86	1,102.42 ± 36.09	1,106.25 ± 60.97	Interaction, 0.01497	Interaction, 0.9040
					Drug, 0.03624	Drug, 0.8512
					Diet, 0.4736	Diet, 0.5001
IL-12p70	82.11 ± 4.80	91.07 ± 7.73	105.17 ± 10.55	88.31 ± 14.19	Interaction, 1.482	Interaction, 0.2392
					Drug, 0.1385	Drug, 0.7141
					Diet, 0.9161	Diet, 0.3512
IL-17a	18.83 ± 0.54	19.17 ± 1.54	20.69 ± 1.96	15.69 ± 2.39	Interaction, 2.062	Interaction, 0.1682
					Drug, 1.570	Drug, 0.2263
					Diet, 0.1897	Diet, 0.6683
IL-33	357.80 ± 38.71	328.15 ± 33.89	370.01 ± 27.68	255.37 ± 29.44	Interaction, 1.717	Interaction, 0.2074
					Drug, 4.950	Drug, 0.0399
					Diet, 0.8724	Diet, 0.3634

Table 4. Summary of Microglial Parameters Values

Parameters	Mean ± Standard Error of the Mean															
	Control Diet								Western Diet							
	Vehicle				NRG-1				Vehicle				NRG-1			
	Left		Right		Left		Right		Left		Right		Left		Right	
	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
Objects	12.11 ± 0.56	13.22 ± 3.11	12.44 ± 3.18	11.89 ± 4.59	16.00 ± 3.61	15.00 ± 0.51	13.22 ± 1.68	12.22 ± 0.62	14.22 ± 0.29	14.56 ± 1.64	17.22 ± 1.49	15.44 ± 0.87	15.22 ± 2.04	17.22 ± 3.35	10.22 ± 2.25	10.44 ± 0.62
Density	0.57 ± 0.16	0.43 ± 0.14	0.40 ± 0.06	0.50 ± 0.11	0.29 ± 0.02	0.32 ± 0.02	0.36 ± 0.06	0.38 ± 0.06	0.27 ± 0.03	0.27 ± 0.03	0.26 ± 0.01	0.21 ± 0.02	0.34 ± 0.08	0.32 ± 0.07	0.28 ± 0.04	0.34 ± 0.06
Span Ratio	2.10 ± 0.21	1.88 ± 0.03	2.02 ± 0.02	1.77 ± 0.10	1.80 ± 0.07	1.97 ± 0.06	1.95 ± 0.10	2.09 ± 0.01	1.79 ± 0.10	1.80 ± 0.01	1.91 ± 0.06	1.83 ± 0.05	2.04 ± 0.07	2.01 ± 0.08	1.92 ± 0.01	2.29 ± 0.05
Maximum Span Across Hull	86.21 ± 23.79	114.09 ± 33.30	115.05 ± 16.39	94.37 ± 30.57	151.39 ± 20.61	138.34 ± 18.78	130.72 ± 32.07	118.28 ± 23.03	165.78 ± 30.38	159.78 ± 14.18	151.94 ± 3.69	174.55 ± 14.69	131.27 ± 28.21	134.50 ± 22.29	144.76 ± 14.01	130.19 ± 18.35
Convex Hull Area	3,366 ± 1,658	6,443 ± 3,530	5,707 ± 1,530	4,598 ± 2,894	10,482 ± 3,564	8,304 ± 2,481	8,237 ± 4,523	6,366 ± 2,358	13,321 ± 5,130	12,095 ± 2,678	10,262 ± 1,195	13,412 ± 2,377	7,597 ± 3,189	7,566 ± 2,192	8,343 ± 1,304	6,478 ± 1,616
Perimeter	212.76 ± 57.46	286.98 ± 86.04	285.26 ± 41.98	239.02 ± 77.45	384.77 ± 56.46	347.12 ± 47.86	328.20 ± 86.28	294.37 ± 59.18	423.51 ± 81.64	407.88 ± 38.47	385.42 ± 15.07	444.27 ± 42.51	326.49 ± 73.00	335.30 ± 55.93	362.21 ± 35.11	316.88 ± 44.33
Circularity	0.714 ± 0.046	0.729 ± 0.005	0.716 ± 0.003	0.759 ± 0.028	0.732 ± 0.020	0.713 ± 0.005	0.717 ± 0.017	0.709 ± 0.004	0.742 ± 0.025	0.735 ± 0.014	0.724 ± 0.013	0.738 ± 0.015	0.703 ± 0.009	0.710 ± 0.005	0.712 ± 0.004	0.679 ± 0.005
Width of Bounding Rectangle	70.44 ± 19.03	89.10 ± 20.98	92.09 ± 13.91	71.11 ± 24.65	122.23 ± 19.01	105.38 ± 14.93	107.02 ± 28.05	93.41 ± 19.36	137.67 ± 27.18	126.62 ± 12.79	118.88 ± 3.93	139.26 ± 10.98	101.24 ± 22.97	100.18 ± 13.13	110.54 ± 9.17	94.03 ± 10.09
Height of Bounding Rectangle	66.05 ± 17.26	95.11 ± 33.46	91.64 ± 13.17	82.32 ± 24.75	124.28 ± 16.62	116.00 ± 15.29	103.58 ± 27.18	93.51 ± 17.91	133.68 ± 26.34	133.54 ± 12.84	125.03 ± 7.37	144.28 ± 15.30	106.17 ± 23.40	112.77 ± 20.55	120.90 ± 13.66	108.77 ± 17.67
Max Radius from Hull's Centre of Mass	48.56 ± 11.78	62.77 ± 17.67	63.38 ± 9.09	52.45 ± 16.06	82.28 ± 10.78	75.39 ± 9.62	71.70 ± 16.86	64.15 ± 11.97	89.35 ± 15.57	86.65 ± 7.20	82.67 ± 2.05	94.41 ± 7.75	71.73 ± 15.01	73.53 ± 11.23	79.72 ± 7.89	71.11 ± 9.52
Max/Min Radii	2.60 ± 0.21	2.08 ± 0.13	2.20 ± 0.08	1.99 ± 0.09	1.94 ± 0.07	2.01 ± 0.03	2.03 ± 0.13	2.13 ± 0.04	1.88 ± 0.09	1.94 ± 0.07	1.94 ± 0.05	1.92 ± 0.13	2.02 ± 0.11	2.02 ± 0.06	2.05 ± 0.06	2.38 ± 0.09
Mean Radius	37.62 ± 9.97	50.09 ± 14.62	50.04 ± 7.47	41.26 ± 13.33	65.77 ± 9.33	60.49 ± 8.34	57.44 ± 14.70	51.40 ± 10.08	73.03 ± 13.38	70.67 ± 6.31	66.89 ± 2.28	76.49 ± 7.35	56.97 ± 12.48	58.92 ± 9.47	63.11 ± 6.16	56.15 ± 7.88
Diameter of Bounding Circle	86.82 ± 23.97	115.05 ± 33.67	115.75 ± 16.63	95.10 ± 30.98	152.96 ± 20.64	139.48 ± 18.92	131.68 ± 32.30	119.32 ± 23.33	167.14 ± 30.52	161.29 ± 14.16	153.11 ± 3.98	175.89 ± 15.06	132.44 ± 28.45	135.55 ± 22.43	145.73 ± 13.99	131.04 ± 18.30
Maximum Radius	43.41 ± 11.99	57.53 ± 16.84	57.87 ± 8.31	47.55 ± 15.49	76.48 ± 10.32	69.74 ± 9.46	65.84 ± 16.15	59.66 ± 11.66	83.57 ± 15.26	80.65 ± 7.08	76.56 ± 1.99	87.94 ± 7.53	66.22 ± 14.23	67.78 ± 11.21	72.87 ± 7.00	65.52 ± 9.15
CV for all Radii from Circle's Centre	0.17 ± 0.01	0.16 ± 0.01	0.18 ± 0.01	0.16 ± 0.00	0.17 ± 0.01	0.17 ± 0.00	0.16 ± 0.01	0.18 ± 0.00	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.02	0.17 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.19 ± 0.01
CV for all Radii	0.24 ± 0.02	0.21 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.19 ± 0.01	0.19 ± 0.00	0.20 ± 0.02	0.21 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.02	0.21 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.23 ± 0.01
Mean Radii	37.85 ± 10.57	50.35 ± 14.90	50.31 ± 7.59	41.40 ± 13.53	66.43 ± 9.49	61.16 ± 8.40	57.94 ± 14.92	51.80 ± 10.36	73.69 ± 13.39	71.55 ± 6.44	67.83 ± 2.20	77.50 ± 7.46	57.66 ± 12.71	59.75 ± 9.79	64.08 ± 6.62	56.54 ± 8.13
Lacunarity (λ)	0.31 ± 0.07	0.39 ± 0.06	0.40 ± 0.03	0.36 ± 0.05	0.44 ± 0.02	0.41 ± 0.01	0.40 ± 0.03	0.40 ± 0.03	0.51 ± 0.06	0.45 ± 0.01	0.46 ± 0.01	0.55 ± 0.03	0.42 ± 0.04	0.43 ± 0.03	0.44 ± 0.02	0.43 ± 0.04

Table 5. Summary of Statistic Values for Microglial Parameters

Parameters	<i>F</i>		<i>p</i>	
	Left	Right	Left	Right
Objects	ROI, 0.2742 Diet, 0.3921 Drug, 1.429 ROI x Diet, 0.2266 ROI x Drug, 0.009065 Diet x Drug, 0.06563 ROI x Diet x Drug, 0.6550	ROI, 0.5444 Diet, 0.1864 Drug, 1.748 ROI x Diet, 1.000e-013 ROI x Drug, 0.1361 Diet x Drug, 2.534 ROI x Diet x Drug, 0.3361	ROI, 0.6147 Diet, 0.5486 Drug, 0.2661 ROI x Diet, 0.6468 ROI x Drug, 0.9265 Diet x Drug, 0.8043 ROI x Diet x Drug, 0.4417	ROI, 0.4817 Diet, 0.6773 Drug, 0.2227 ROI x Diet, >0.9999 ROI x Drug, 0.7218 Diet x Drug, 0.1501 ROI x Diet x Drug, 0.5780
Density	ROI, 3.696 Diet, 1.474 Drug, 0.6682 ROI x Diet, 1.289 ROI x Drug, 5.310 Diet x Drug, 2.251 ROI x Diet x Drug, 9.357	ROI, 1.061 Diet, 6.824 Drug, 0.08865 ROI x Diet, 0.8167 ROI x Drug, .04138 Diet x Drug, 2.173 ROI x Diet x Drug, 2.706	ROI, 0.0908 Diet, 0.2593 Drug, 0.4374 ROI x Diet, 0.2892 ROI x Drug, 0.0501 Diet x Drug, 0.1720 ROI x Diet x Drug, 0.0156	ROI, 0.3332 Diet, 0.0310 Drug, 0.9273 ROI x Diet, 0.3926 ROI x Drug, 0.8439 Diet x Drug, 0.1787 ROI x Diet x Drug, 0.1386
Span Ratio	ROI, 0.04611 Diet, 0.1785 Drug, 1.007 ROI x Diet, 0.009374 ROI x Drug, 1.260 Diet x Drug, 6.741 ROI x Diet x Drug, 1.952	ROI, 1.429 Diet, 0.4127 Drug, 15.43 ROI x Diet, 6.875 ROI x Drug, 29.31 Diet x Drug, 1.631 ROI x Diet x Drug, 0.1072	ROI, 0.8353 Diet, 0.6838 Drug, 0.3449 ROI x Diet, 0.9252 ROI x Drug, 0.2942 Diet x Drug, 0.0318 ROI x Diet x Drug, 0.1999	ROI, 0.2662 Diet, 0.5386 Drug, 0.0044 ROI x Diet, 0.0306 ROI x Drug, 0.0006 Diet x Drug, 0.2373 ROI x Diet x Drug, 0.7517
Maximum Span Across Hull	ROI, 0.1980 Diet, 1.139 Drug, 0.09756 ROI x Diet, 0.4224 ROI x Drug, 1.372 Diet x Drug, 2.472 ROI x Diet x Drug, 3.431	ROI, 0.3674 Diet, 3.832 Drug, 0.02684 ROI x Diet, 0.9901 ROI x Drug, 0.4896 Diet x Drug, 1.556 ROI x Diet x Drug, 1.207	ROI, 0.6682 Diet, 0.3169 Drug, 0.7628 ROI x Diet, 0.5340 ROI x Drug, 0.2752 Diet x Drug, 0.1545 ROI x Diet x Drug, 0.1011	ROI, 0.5612 Diet, 0.0860 Drug, 0.8739 ROI x Diet, 0.3489 ROI x Drug, 0.5039 Diet x Drug, 0.2476 ROI x Diet x Drug, 0.3039
Convex Hull Area	ROI, 0.006925 Diet, 0.9803 Drug, 0.01110 ROI x Diet, 0.2508 ROI x Drug, 0.8878 Diet x Drug, 2.524 ROI x Diet x Drug, 2.241	ROI, 0.1278 Diet, 2.501 Drug, 0.2810 ROI x Diet, 0.8100 ROI x Drug, 1.486 Diet x Drug, 2.343 ROI x Diet x Drug, 0.8057	ROI, 0.9357 Diet, 0.3511 Drug, 0.9187 ROI x Diet, 0.6300 ROI x Drug, 0.3737 Diet x Drug, 0.1508 ROI x Diet x Drug, 0.1728	ROI, 0.7299 Diet, 0.1524 Drug, 0.6104 ROI x Diet, 0.3944 ROI x Drug, 0.2575 Diet x Drug, 0.1644 ROI x Diet x Drug, 0.3956
Perimeter	ROI, 0.1570 Diet, 1.142 Drug, 0.06528 ROI x Diet, 0.3338 ROI x Drug, 1.356 Diet x Drug, 2.693 ROI x Diet x Drug, 3.294	ROI, 0.4019 Diet, 3.555 Drug, 0.07430 ROI x Diet, 0.7945 ROI x Drug, 0.7639 Diet x Drug, 1.681 ROI x Diet x Drug, 1.233	ROI, 0.7023 Diet, 0.3165 Drug, 0.8048 ROI x Diet, 0.5793 ROI x Drug, 0.2778 Diet x Drug, 0.1394 ROI x Diet x Drug, 0.1071	ROI, 0.5438 Diet, 0.0961 Drug, 0.7921 ROI x Diet, 0.3987 ROI x Drug, 0.4076 Diet x Drug, 0.2309 ROI x Diet x Drug, 0.2991

Circularity	ROI, 0.01093 Diet, 0.0005047 Drug, 0.9625 ROI x Diet, 0.005566 ROI x Drug, 0.1345 Diet x Drug, 1.032 ROI x Diet x Drug, 0.8468	ROI, 0.1408 Diet, 1.621 Drug, 9.667 ROI x Diet, 1.808 ROI x Drug, 6.005 Diet x Drug, 0.3333 ROI x Diet x Drug, 0.007073	ROI, 0.9193 Diet, 0.9826 Drug, 0.3553 ROI x Diet, 0.9424 ROI x Drug, 0.7233 Diet x Drug, 0.3394 ROI x Diet x Drug, 0.3844	ROI, 0.7173 Diet, 0.2386 Drug, 0.0145 ROI x Diet, 0.2156 ROI x Drug, 0.0399 Diet x Drug, 0.5796 ROI x Diet x Drug, 0.9350
Width of Bounding Rectangle	ROI, 0.1934 Diet, 1.136 Drug, 0.004988 ROI x Diet, 0.3551 ROI x Drug, 1.192 Diet x Drug, 3.156 ROI x Diet x Drug, 3.784	ROI, 0.9153 Diet, 2.774 Drug, 0.07531 ROI x Diet, 1.434 ROI x Drug, 0.8459 Diet x Drug, 2.330 ROI x Diet x Drug, 1.900	ROI, 0.6717 Diet, 0.3176 Drug, 0.9454 ROI x Diet, 0.5677 ROI x Drug, 0.3067 Diet x Drug, 0.1135 ROI x Diet x Drug, 0.0876	ROI, 0.3667 Diet, 0.1344 Drug, 0.7907 ROI x Diet, 0.2653 ROI x Drug, 0.3846 Diet x Drug, 0.1654 ROI x Diet x Drug, 0.2054
Height of Bounding Rectangle	ROI, 0.9757 Diet, 1.063 Drug, 0.1408 ROI x Diet, 0.2694 ROI x Drug, 1.232 Diet x Drug, 2.404 ROI x Diet x Drug, 2.554	ROI, 0.1285 Diet, 3.997 Drug, 0.06649 ROI x Diet, 0.6001 ROI x Drug, 0.8813 Diet x Drug, 0.9623 ROI x Diet x Drug, 0.8008	ROI, 0.3522 Diet, 0.3327 Drug, 0.7172 ROI x Diet, 0.6178 ROI x Drug, 0.2993 Diet x Drug, 0.1597 ROI x Diet x Drug, 0.1487	ROI, 0.7293 Diet, 0.0806 Drug, 0.8030 ROI x Diet, 0.4608 ROI x Drug, 0.3753 Diet x Drug, 0.3553 ROI x Diet x Drug, 0.3970
Max Radius from Hull's Centre of Mass	ROI, 0.1926 Diet, 1.139 Drug, 0.1015 ROI x Diet, 0.3153 ROI x Drug, 1.286 Diet x Drug, 2.480 ROI x Diet x Drug, 3.061	ROI, 0.4640 Diet, 3.977 Drug, 0.02660 ROI x Diet, 0.9183 ROI x Drug, 0.5654 Diet x Drug, 1.465 ROI x Diet x Drug, 1.107	ROI, 0.6724 Diet, 0.3170 Drug, 0.7582 ROI x Diet, 0.5898 ROI x Drug, 0.2896 Diet x Drug, 0.1540 ROI x Diet x Drug, 0.1183	ROI, 0.5150 Diet, 0.0812 Drug, 0.8745 ROI x Diet, 0.3660 ROI x Drug, 0.4736 Diet x Drug, 0.2607 ROI x Diet x Drug, 0.3234
Max/Min Radii	ROI, 9.990 Diet, 3.613 Drug, 1.611 ROI x Diet, 16.13 ROI x Drug, 17.35 Diet x Drug, 5.397 ROI x Diet x Drug, 25.86	ROI, 1.230 Diet, 0.04899 Drug, 3.173 ROI x Diet, 5.523 ROI x Drug, 13.04 Diet x Drug, 3.558 ROI x Diet x Drug, 0.03950	ROI, 0.0134 Diet, 0.0938 Drug, 0.2400 ROI x Diet, 0.0039 ROI x Drug, 0.0031 Diet x Drug, 0.0487 ROI x Diet x Drug, 0.0009	ROI, 0.2997 Diet, 0.8304 Drug, 0.1127 ROI x Diet, 0.0467 ROI x Drug, 0.0069 Diet x Drug, 0.0960 ROI x Diet x Drug, 0.8474
Mean Radius	ROI, 0.3036 Diet, 1.212 Drug, 0.06713 ROI x Diet, 0.3811 ROI x Drug, 1.194 Diet x Drug, 2.565 ROI x Diet x Drug, 3.219	ROI, 0.4337 Diet, 3.620 Drug, .04011 ROI x Diet, 0.8918 ROI x Drug, 0.5587 Diet x Drug, 1.608 ROI x Diet x Drug, 1.089	ROI, 0.5967 Diet, 0.3030 Drug, 0.8021 ROI x Diet, 0.5542 ROI x Drug, 0.3064 Diet x Drug, 0.1479 ROI x Diet x Drug, 0.1105	ROI, 0.5287 Diet, 0.0936 Drug, 0.8463 ROI x Diet, 0.3726 ROI x Drug, 0.4762 Diet x Drug, 0.2404 ROI x Diet x Drug, 0.3272
Diameter of Bounding Circle	ROI, 0.1894 Diet, 1.144 Drug, 0.09966 ROI x Diet, 0.4010 ROI x Drug, 1.406	ROI, 0.3576 Diet, 3.796 Drug, 0.02675 ROI x Diet, 0.9714 ROI x Drug, 0.4902	ROI, 0.6749 Diet, 0.3159 Drug, 0.7603 ROI x Diet, 0.5443 ROI x Drug, 0.2698	ROI, 0.5664 Diet, 0.0872 Drug, 0.8741 ROI x Diet, 0.3532 ROI x Drug, 0.5037

	Diet x Drug, 2.502 ROI x Diet x Drug, 3.364	Diet x Drug, 1.564 ROI x Diet x Drug, 1.204	Diet x Drug, 0.1523 ROI x Diet x Drug, 0.1040	Diet x Drug, 0.2464 ROI x Diet x Drug, 0.3044
Maximum Radius	ROI, 0.1894 Diet, 1.144 Drug, 0.09966 ROI x Diet, 0.4010 ROI x Drug, 1.406 Diet x Drug, 2.502 ROI x Diet x Drug, 3.364	ROI, 0.3576 Diet, 3.796 Drug, 0.02675 ROI x Diet, 0.9714 ROI x Drug, 0.4902 Diet x Drug, 1.564 ROI x Diet x Drug, 1.204	ROI, 0.6749 Diet, 0.3159 Drug, 0.7603 ROI x Diet, 0.5443 ROI x Drug, 0.2698 Diet x Drug, 0.1523 ROI x Diet x Drug, 0.1040	ROI, 0.5664 Diet, 0.0872 Drug, 0.8741 ROI x Diet, 0.3532 ROI x Drug, 0.5037 Diet x Drug, 0.2464 ROI x Diet x Drug, 0.3044
CV for all Radii from Cicle's Centre	ROI, 1.051 Diet, 3.613 Drug, 2.735 ROI x Diet, 0.02936 ROI x Drug, 0.09261 Diet x Drug, 0.5020 ROI x Diet x Drug, 1.041	ROI, 2.158 Diet, 0.02939 Drug, 0.8968 ROI x Diet, 3.574 ROI x Drug, 5.949 Diet x Drug, 1.527 ROI x Diet x Drug, 1.163	ROI, 0.3353 Diet, 0.0939 Drug, 0.1368 ROI x Diet, 0.8682 ROI x Drug, 0.7687 Diet x Drug, 0.4987 ROI x Diet x Drug, 0.3375	ROI, 0.1800 Diet, 0.8681 Drug, 0.3714 ROI x Diet, 0.0953 ROI x Drug, 0.0406 Diet x Drug, 0.2517 ROI x Diet x Drug, 0.3124
CV for all Radii	ROI, 6.259 Diet, 2.403 Drug, 0.2050 ROI x Diet, 3.428 ROI x Drug, 2.438 Diet x Drug, 5.630 ROI x Diet x Drug, 16.18	ROI, 1.334 Diet, 0.09211 Drug, 1.762 ROI x Diet, 1.339 ROI x Drug, 4.832 Diet x Drug, 2.111 ROI x Diet x Drug, 0.3416	ROI, 0.0368 Diet, 0.1597 Drug, 0.6628 ROI x Diet, 0.1012 ROI x Drug, 0.1571 Diet x Drug, 0.0450 ROI x Diet x Drug, 0.0038	ROI, 0.2815 Diet, 0.7693 Drug, 0.2210 ROI x Diet, 0.2806 ROI x Drug, 0.0592 Diet x Drug, 0.1843 ROI x Diet x Drug, 0.5750
Mean Radii	ROI, 0.3429 Diet, 1.223 Drug, 0.07455 ROI x Diet, 0.3532 ROI x Drug, 1.219 Diet x Drug, 2.518 ROI x Diet x Drug, 3.221	ROI, 0.4625 Diet, 3.716 Drug, 0.03975 ROI x Diet, 0.8162 ROI x Drug, 0.5777 Diet x Drug, 1.631 ROI x Diet x Drug, 1.103	ROI, 0.5743 Diet, 0.3009 Drug, 0.7917 ROI x Diet, 0.5687 ROI x Drug, 0.3017 Diet x Drug, 0.1512 ROI x Diet x Drug, 0.1104	ROI, 0.5156 Diet, 0.0901 Drug, 0.8470 ROI x Diet, 0.3927 ROI x Drug, 0.4690 Diet x Drug, 0.2374 ROI x Diet x Drug, 0.3243
Lacunarity (Λ)	ROI, 0.03150 Diet, 2.737 Drug, 0.1107 ROI x Diet, 1.871 ROI x Drug, 0.1787 Diet x Drug, 2.863 ROI x Diet x Drug, 5.398	ROI, 0.2166 Diet, 12.09 Drug, 0.9348 ROI x Diet, 2.772 ROI x Drug, 0.7385 Diet x Drug, 3.302 ROI x Diet x Drug, 2.758	ROI, 0.8635 Diet, 0.1367 Drug, 0.7479 ROI x Diet, 0.2085 ROI x Drug, 0.6837 Diet x Drug, 0.1291 ROI x Diet x Drug, 0.0487	ROI, 0.6541 Diet, 0.0084 Drug, 0.3619 ROI x Diet, 0.1345 ROI x Drug, 0.4152 Diet x Drug, 0.1067 ROI x Diet x Drug, 0.1353

Behavioral

Anatomical

Cellular

Molecular

Western-like diet

Increased
anxiety-like behavior Reduced
hippocampal volume Altered microglial
morphometric
parameters:
density and lacunarity Increased hippocampal
cytokine levels (IL-1 α ,
TNF α , IL-6, IL-10).
Disrupted TACE-NRG1-
ErbB4 signaling axis

Exogenous NRG1

Blunted
hippocampal-
dependent fear
learning Reduced
hippocampal volume Altered microglial
morphometric
parameters:
circularity and
span ratio Decreased hippocampal
cytokine/chemokine
levels (IL-33, GM-CSF,
CCL-2, IFN γ).
Disrupted TACE-NRG1-
ErbB4 signaling axis

A

Study 1

Control (CD) or Western Diet (WD) consumption (PND 21-49)

B

C

Study 2

Control or Western Diet

Fig.2

Fig.3

A

B

C

D

A

Fig.5

A

B

C

D

E

F

G

H

Fig.6

A

B

C

D

Fig.7

A

B

C

Fig.8