Altered Calcium Permeability of AMPA Receptor Drives NMDA Receptor Inhibition in the Hippocampus of Murine Obesity Models

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Abstract

Evidence has accumulated that higher consumption of high-fat diets (HFDs) during the juvenile/adolescent period induces altered hippocampal function and morphology; however, the mechanism behind this phenomenon remains elusive. Using high-resolution structural imaging combined with molecular and functional interrogation, a murine model of obesity treated with HFDs for 12 weeks after weaning mice was shown to change in the glutamate-mediated intracellular calcium signaling and activity, including further selective reduction of gray matter volume in the hippocampus associated with memory recall disturbance. Dysregulation of intracellular calcium concentrations was restored by a non-competitive α -3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA) antagonist, followed by normalization of hippocampal volume and memory recall ability, indicating that AMPARs may serve as an attractive therapeutic target for obesity-associated cognitive decline.

Introduction

The global obesity epidemic has put forth evidence that high-fat diets (HFDs) (40-65% kcal derived from fat) or simple sugars (sucrose or high fructose corn syrup) promote obesity, which has a negative effect on neurocognition [1,2]. Early life exposure to obesogenic diets like simple sugars and saturated fatty acids induce learning and memory disturbance, which is presumed to be based on increased neuroinflammation and reduced neurotrophin mediated regulation of neurogenesis and synaptic plasticity [1]. Furthermore, a western diet with dense energy including saturated fat and simple sugar intake could invoke a vicious cycle of hippocampal dysfunction and impaired feeding behavior, stimulates excess intake, obesity, and resulting in hippocampal dysfunction, which further accelerates obesity, followed by cognitive dysfunction [2]. To our great concern, HFDs during the juvenile/adolescent period induce alterations in hippocampal morphology and function [3-6]. Several evidence accumulated various hippocampal dysfunction such as synaptic plasticity of long-term potentiation (LTP) and long-term depression (LTD) with obese HFD-treated adult male-but not female-mice [3], and spatial learning in the radial-arm maze [4] by HFDs. The impairment of LTD was identified as the most relevant change in synaptic plasticity due to HFD consumed during the juvenile period [4]. These apparent alterations of hippocampus synaptic plasticity associated with spatial and memory disturbance strongly suggest an influence of HFDs on glutamatergic neurotransmission [6].

AMPA receptors as well as NMDARs play a crucial role for these forms of synaptic plasticity [7, 8]. We identified Ca^{2+} -permeable AMPAR (CP-AMPA) is related to the pathophysiology in neurological disorders such as brain tumor [9], and also relevant to amyotrophic lateral sclerosis (ALS) [10], cocaine addiction [11], neuropathic pain [12], and epilepsy [13]. Changes in the expression of CP-AMPA receptors can alter synaptic properties, lead to damage of selectively vulnerable neurons [7, 8] CP-AMPA receptors are more widespread than originally thought and play a role in synaptic plasticity and neuronal death, though Ca^{2+} influx through AMPARs is modest compared with that through NMDARs [8, 14]. An issue of functional consequences of AMPA receptor mediated Ca^{2+} signaling remains elusive in post synaptic membrane. Although

accumulated evidence indicated that AMPA receptor mediated Ca^{2+} -influx modulates efficacy of fast synaptic transmission and can trigger LTP, relatively little is known about the postsynaptic target of Ca^{2+} [8]. Fast interaction between AMPA and NMDA receptors by intracellular calcium was experimentally observed by introduction of recombinant CP-AMPA and Ca^{2+} -impermeable NMDAR mutant channels into HEK 293 [15]. Ca^{2+} -entry through AMPARs inactivates co-localized NMDARs in the time range of excitatory postsynaptic currents, but the significance of this phenomenon in neurological disorders is unknown. In this study, we show here role of AMPAR-mediated Ca^{2+} -entry in obesity mice. NMDAR blocked by this CP-APMAR induces spatial and social memory disturbance in these mice.

Hippocampal atrophy was affected by obesity, and type II diabetes mellitus as well as Alzheimer disease, traumatic brain injury, obstructive sleep apnea, and depression [5]. Using high-resolution structural imaging combined with molecular and functional interrogation, in a male murine model of obesity treated with HFDs for 12 weeks after weaning, we examined changes in the glutamate-mediated intracellular calcium signaling and activity, volumetric analysis of hippocampal subregions as well as the rest of brain, and behavioral estimation. Both murine models of obesity showed similar changes in the glutamate-mediated intracellular calcium signaling and activity associated with memory recall disturbance. In the quantitative volumetric analysis of the brain by 11.7 T magnetic resonance imaging (MRI) (Bruker BioSpec 117/11, Bruker BioSpin GmbH, Germany), we found a notable sign of brain atrophy caused by the reduction of brain volume. We discovered for the first time that altered calcium permeability of AMPA receptor drives NMDA receptor inhibition in the acute slice of hippocampus in these disease models. Furthermore, dysregulation of intracellular calcium concentrations was restored by a non-competitive AMPAR antagonist [16], followed by normalization of hippocampal volume and memory recall ability, indicating that AMPARs may serve as an attractive therapeutic target for obesity-associated cognitive decline.

Lastly, we examined whether similar findings in murine obese models were adopted in humans, we conducted volumetric analysis using MRI (Discovery MR750; General Electric, Milwaukee, WI) T2 data for overweight and obese people compared with that of normal-weight volunteers. BMI impacts negatively on human gray matter volume, interaction with default mode network(DMN) and anti- DMN dynamics, and network connectivity of hippocampal memory circuits [17, 18].

Materials And Methods

Animals

Male C57BL/6J background mice were obtained from CLEA Japan Inc. and Japan SLC, respectively, because male mice are reported to be more vulnerable to the effects of HFD on weight gain, metabolic alterations and deficits of learning, and hippocampal synaptic plasticity. These animals were housed three or four per cage in a standard 12 h dark-light cycle room at 25°C with freely available food and water. All animal experiments were performed in accordance with the guidelines of the Animal Experiment

Ethics Committee of the University of the Ryukyus (approval number: A2019239). Time series changes in average food intake and average body weight in each condition were monitored.

Feed for control and high fat diet

In the case of the control diet (CD) for mice, we used CE-2 feed (Japan CLEA, Tokyo, Japan). The composition table of CE-2 feed is shown in **Table S1**; the total energy was 339.1 kcal/100 g, and crude fat was 4.61%. In contrast, in the case of high-fat diets (HFDs), we used F2HFD2 feed (Oriental Yeast Co., Ltd., Tokyo, Japan). The total energy of F2HFD2 was 640 kcal, consisting of 58% lard (wt/wt), 30% fish powder, 10% skim milk, and a 2% vitamin and mineral mixture (equivalent to 7.5% carbohydrate, 24.5% protein, and 60% fat)[19]. The other components of F2HFD were similar to those of CE-2. At 4 weeks of age, groups of mice were subjected to HFDs (F2HFD2 feed). Control mice were fed a low-fat diet (CE-2 feed).

Oral delivery of perampanel (PER)

HFD mice were orally administered with HydroGel (Clear H₂O, Portland, ME 04101, USA) at a dose of 5 mg/kg body weight (HFD-fed mice with PER treatment group). In every HFD mice, one in one cage, PER was orally administered at a dose of 5 mg/kg body weight (HFD mice with PER treatment group), and both groups were monitored for body weight and intake of HydroGel twice a week to adjust the administration dose of PER to 5 mg/kg per body weight.

Wheel-running activity

Wheel cages (MELQUEST, Japan, Model RWC-15) were used to monitor individual mouse activity. Wheel rotation was monitored and recorded every 10 min for 14 days, as reported previously [20].

Open field test and elevated plus maze

Open field test: In a space surrounded by a 50 cm square and 40 cm high wall (Muromachi Kikai, Japan), mice were allowed to act freely for 5 min, and the trajectory was analyzed using a CompACT VAS/DV video-tracking system (Muromachi Kikai, Japan) [21]. Elevated plus maze: A space was elevated to a height of 50 cm from the floor, consisting of two open arms, two closed arms (30 × 6 cm each), and a neutral zone. Mice were placed in the center of the neutral zone, facing a closed arm, and allowed to move freely for 3 min. The time spent in the open and closed arms and the frequency of visits to the different arms were recorded and scored using the CompACT VAS/DV video-tracking system (Muromachi Kikai, Japan).

Novel object recognition test

Mice with CD, HFDs, and HFDs with PER treatment were handled for 5 min daily for 5 days prior to the start of the novel object recognition test. The mice were habituated to a 35 cm square and 25 cm high box for 10 min on day 1 and were provided with two identical objects (familiar object) for 10 min on day 2. On day 3, one of the familiar objects was exchanged with a novel object of different shape and color

(Fig. 2f). The behavior of the mice was monitored for 5 min using a video camera, and the videos were digitized and stored on a personal computer. The search time for each object was measured via offline analysis [22].

Morris water maze

Memory impairment was assessed using the Morris water maze test, as previously described [23]. In summary, the water maze pool (Muromachi Kikai, Tokyo, Japan), with a diameter of 120 cm, contained opaque water (room temperature) with a platform (10 cm in diameter) submerged 2 cm below the surface. The hidden platform task took 4–7 days (two sessions per day, 3 hours apart), during which two trials were performed each day (15 min apart). The platform location remained constant, and the entry points were changed semi-randomly between the trials. Twenty-four hours after the last day of the hidden platform task, a 1 min probe trial was performed without the platform. The entry point for the probe trials was in the quadrant opposite to the target quadrant. Memory retention was evaluated by the amount of time spent in the correct quadrant where the escape platform was located in the hidden platform trial. The performance was monitored using the CompACT VAS/DV video-tracking system.

Pattern completion test mediated contextual and spatial recall

The Morris water maze task was conducted in mice with CD, HFDs, and HFDs with PER treatment as described previously [24]. All experiments were conducted at approximately the same time of day. The mice were transported from the colony to a holding area, where they were undisturbed for 30 min prior to the experiment. The test was performed in a rectangular dimly lit room with a circular pool (Muromachi Kikai, Tokyo, Japan), with a diameter of 120 cm, filled with opaque water made with skim milk (Morinaga, Japan) maintained at room temperature. Four large objects illuminated with floor lamps were hung on black curtains surrounding the pool as extramaze cues. A hidden circular platform (10 cm in diameter) was placed 1 cm below the water surface, and the mice were trained to find the platform with four trials per day for 12 days, with an inter-trial interval of approximately 60 min. During training, the mice were released from four pseudorandomly assigned start locations (N, S, E, and W) and were allowed to swim for 300 s. If a mouse did not find the platform within 300 s, it was manually guided to the platform and allowed to rest on the platform for 15 s.

A probe trial was conducted on day 13 under the full-cue condition (P1). The mice were released at the center of the pool and allowed to swim for 300 s in the absence of the platform. Following the probe trial, the mice received four training trials in the presence of the platform to avoid memory extinction that may have occurred during the probe trial. Subsequently, the mice received four probe trials with extra maze cue manipulations, one probe trial per day, without retraining between probe trials. For the one-cue probe trial (P2), one cue located more distally from the platform was kept, and the other three cues were removed from the surrounding curtains. For the two-cue probe trial (P3), one cue located close to the platform and the cue used in the one-cue probe trial remained, but the other two cues were removed from the surrounding curtains. For the no-cue probe trial (P5), all four extra maze cues were removed. Data for

training and probe trials were collected and analyzed using the CompACT VAS/DV video-tracking system software. The escape latency to the hidden platform (goal arrival time) was measured.

Five-trial social memory assay

The five-trial social test was performed as described previously [25]. In summary, subject mice with CD, HFDs, and HFDs with PER treatment were individually housed for 7 days before testing to establish territorial dominance. On the day of testing, a female mouse was presented to the subject male mouse's cage (Fig. 2g) for four successive 5 min trials with a 10 min inter-trial interval. In the fifth trial, a novel female mouse was presented (Fig. 2g), and the duration of the social investigation was recorded.

Contextual fear conditioning

Contextual fear conditioning was applied according to published protocols with slight modifications [26]. The mice were transported to an animal experimental room and allowed to acclimatize for at least 30 min prior to contextual fear conditioning training. The mice were then placed in the foot shock system model MK-450MSQ (Muromachi Kikai Co. Ltd., Japan) and allowed to explore for 2 min followed by three electric foot shocks (0.8 mA, 2 s, and 2 min intervals). Animals were left in the apparatus for a further minute before removal.

Acquisition of MRI data for mice

Anatomical brain images of 8 ex vivo mice with CD, HFDs, and HFDs with PER treatment were obtained using a Bruker BioSpec 117/11 11.75 Tesla MRI scanner (Bruker BioSpin GmbH, Ettlingen, Germany). A three-dimensional (3D)-prepared rapid gradient-echo (MPRAGE) sequence was acquired as high-resolution 100 μ m isovoxel image of voxel-based morphometry (matrix size: 280 $\times$ 220 $\times$ 220; field of view: 28 $\times$ 22 $\times$ 22 mm; repetition time: 2000 ms; echo time: 1.78 ms; flip angle: 12 degrees; inversion time: 800 ms; echo train length: 13; numbers of average: 2). A rapid acquisition protocol by relaxation enhancement (RARE) sequence was used to draw the region of interest in the acquired data image (matrix size: 280 $\times$ 220 $\times$ 220; field of view: 28 $\times$ 22 $\times$ 22 mm; repetition time: 1500 ms; echo time: 25 ms; flip angle: 180°). We obtained MPRAGE and RARE images simultaneously in 1 single scan. MRI data acquisition of ex vivo mice was obtained from the heads of four mice that were fixed with PBS into the MRI coil simultaneously. Brains of the four mice were confirmed using raw MRI data. Rodent MRI was performed at the University of the Ryukyus under the OIST Sign Cooperation Agreement (Research theme: Medical impacts of brain volume control effect for reserve capacity of brain atrophy and delay the onset of symptoms of cognitive impairment - By using high resolution MRI analysis system-).

Image preprocessing and estimations for voxel-based morphometry analysis

Voxel-based morphometry (VBM) analysis and preprocessing of MPRAGE and RARE images were performed using the SPM8 analysis tool (Wellcome Department of Clinical Neurology, London; <http://www.fil.ion.ucl.ac.uk>) and SPMMouse toolbox (<http://www.spmmouse.org/>). The raw data were divided into four mice head MRI images and stored separately. The 3D (x-y-z-) coordinates of the

divided images were transformed to the SPM standard coordinate system, and we defined the origin of the 3D coordinates as the bregma point. Then, the brain images were segmented into gray matter (GM), white matter, and cerebrospinal fluid using a segmentation tool (installed in the SPM8 system). The image was divided into GM images using tissue probability maps in the SPMMouse toolbox. These divided GM images were improved by contrast and normalized to the deformation of images. Finally, these images were smoothed using a 200 μm isotropic Gaussian kernel method (installed in the SPM8 system). A smoothed image was applied to the VBM analysis. The volume of hippocampal subfields (CA1, CA2, CA3, DG, and EC) was calculated using the ROI files [18, 27-29]. The differences among the mean values of the whole brain volumes from CD, HFDs, and HFDs with PER treatment were tested by one-way analysis of variance (ANOVA). When there was a significant difference between the three groups by ANOVA ($p < 0.05$), a Scheffe post hoc analysis (Scheffe) was performed between the CD vs. HFDs groups; CD vs. HFDs with PER treatment groups; and HFDs vs. HFDs with PER treatment groups.

Human subjects

The participants of this study were 117 healthy volunteers (mean age 37.8 ± 19.6 years; 65 females, 52 males) and five patients with benign tumors (mean age 55.5 ± 9.6 years; two females, three males), and all participants agreed in writing to participate in this study, in event-related memory tasks and T1-weighted imaging by 3-TMRI. The participants were divided into three groups based on body mass index (BMI) according to the WHO criteria: normal weight ($\text{BMI} < 25$), overweight ($\text{BMI} \geq 25$ and < 30), and obese ($\text{BMI} \geq 30$). There were 84 patients in the normal weight group (mean BMI 20 ± 1.8), 27 in the overweight group (mean BMI 26 ± 1.2), and 11 in the obese group (mean BMI 32 ± 2.3). All experiments were approved by the ethical committee of the University of the Ryukyus for medical and health research involving human subjects and were performed in accordance with guidelines of human experiment regulations at University of the Ryukyus (approval number:111).

Behavioral task paradigm

Details of the fMRI experiment of the event-related memory task used in this study are described in a previous report [17]. The memory task consisted of 108 photographs of 16 lure sets, 16 repeat sets, and 44 novel items. Participants were instructed to respond with buttons whether the photo stimulus shown on the display was a novel item (new), repeated photograph (same), or similar to but not the same as the previous photograph (lure). The button responses of the participants during the memory task were recorded on a personal computer, and the correct answer rates for new, same, and lure were calculated. The correct answer rate for each task (new task, lure task, and same task) was calculated using the following formula: *The correct answer rate (%) = number of correct answers to the presented task / total number of presented tasks $\times 100$* . In our experiments, the total number of presented tasks for the new stimulus was set to 76, and the total number of tasks for the same and lure stimuli were both set to 16.

Functional MRI data acquisitions for behavioral task

Functional and structural images of the brain were obtained using 3T-MRI (Discovery MR750; General Electric, Milwaukee, WI). A sequence of echo planar imaging (EPI, repetition time: 1500 ms, echo time: 25

ms, flip angle: 70°, matrix size: 128 × 128, field of view: 192 × 192, in-plane resolution: 1.5 × 1.5 mm², 23 slices, 3 mm thickness, 0 mm space) was used for the functional images for measuring BOLD contrast. The anatomical brain image was obtained using a three-dimensional (3D) spoiled gradient recalled echo (SPGR) sequence (1 mm slice thickness in sagittal section, matrix size: 256 × 256, field of view: 256 × 256 mm, repetition time: 6.9 ms, echo time: 3 ms, flip angle: 15°). A high-resolution T2-weighted fast spin echo sequence (matrix size: 512 × 512, field of view: 192 × 192 mm, repetition time: 4300 ms, echo time: 92 ms, in-plane resolution: 0.375 × 0.375 mm², 23 slices, 3 mm thickness, 0 mm space) was obtained for visualizing the hippocampal structure and co-registration of 3D SPGR images and EPI functional images.

Imaging processing for behavioral task fMRI

Realignment, temporal correlation, spatial normalization, and spatial smoothing of the functional images were preprocessed and analyzed using SPM12. After preprocessing, the BOLD contrast images of new, same, and lure conditions of the individual subject were calculated from the functional image data: the hippocampal subregions as the CA3, CA1 and DG, and the perihippocampus region as the parahippocampus gyrus, perirhinal cortex, and entorhinal cortex were drawn manually with a pen tablet on the high-resolution coronal T2-weighted image based on the hippocampus atlas [30]. The percentages of signal changes in the BOLD response in the hippocampal regions of each subject were extracted using the MarsBar toolbox. 3D-SPGR images were used for the analysis of voxel-based morphometry. The T1-weighted images were segmented into GM, white matter, and cerebrospinal fluid images using the SPM 12 segmentation tool. The volume of GM in the whole brain was calculated after spatial normalization and modulation of the GM image.

Image acquisition for functional connectivity analysis

MRI data were acquired using a GE Medical Discovery MR 750 3T scanner with a 32-channel head coil. Participants who lay on the scanner bed in the supine position were fixed to the head and neck by form pads and a Philadelphia neck collar to minimize head movement. Resting-state fMRI images were acquired using a single-shot EPI sequence covering the whole brain (42 axial slices, 4 mm thickness with no inter-slice gap; repetition time, 2000 ms; echo time, 30 ms; flip angle, 70°; matrix size, 64 × 64; field of view, 256 × 256). A total of 150 volumes were imaged over a single session. The anatomical brain images were acquired using T1-weighted, sagittal 3D SPGR sequences.

Data preprocessing and analysis for functional network

Image preprocessing and functional network analysis were performed using SPM12 and CONN toolbox 18.b (www.nitrc.org/projects/conn, RRID: SCR_009550) [31]. The details were described in a previous report [17]. Images were preprocessed in order of realignment, slice-timing correction, coregistration, normalization, smoothing, and segmentation. A noise of BOLD signal was removed by linear regression of potential confounding effects in the BOLD signal, and temporal band-pass filtering (temporal frequencies below 0.008 Hz or above 0.09 Hz).

We analyzed the default mode network (DMN) and functional connectivity in functional network analysis using the CONN toolbox. First as an individual analysis, regions with a positive correlation to BOLD fluctuations of a posterior cingulate cortex (PCC) and a precuneus were calculated as a DMN map. Next, regions with a negative correlation to the DMN map were calculated as anticorrelation DMN maps. Mean images of DMN map and anticorrelation DMN map of the normal weight, overweight, and obese groups were calculated using a one-sample t-test (voxel-level threshold of $p < 0.001$ uncorrected, and a cluster-level threshold of false discovery rate [FDR] corrected $p < 0.05$). In the functional combination analysis, we computed the nodes and edges in the graph theory parameters. The correlation coefficient of the fluctuation of BOLD signal between each ROI was calculated for seeds using the 132×132 ROI map (default ROI atlas of CONN toolbox)-based seed-based connectivity measure [32]. The ROI-to-ROI degree (number of edges that any node has between each node) and betweenness centrality (the number of shortest paths of a vertex that passes to any two pairs (j, i) of nodes within a graph) were calculated by an associated ROI-to-ROI correlation 132×132 matrix, established using our 132×132 ROI map. The degree is defined at each node as the number of edges from/to each node. Degree (d_i) is defined as the following formula: $d_i = \sum_j A_{i,j}$, where $A_{i,j}$ is the associated ROI-to-ROI correlation of the 132×132 matrix. $i = 1, 2, 3, \dots, 132$; $j = 1, 2, 3, \dots, 132$. Betweenness centrality (BC_i) represents a hub that connects other functional modules and nodes [31], and BC_i is defined as follows: $BC_i = \frac{\sum_{j, k \neq i} [i \in P_{j,k}]}{(N-1)(N-2)}$, where $P_{j,k}$ is the number of nodes in the shortest path (shortest path when passing through any node defined with i as a variable) between each pair (j, k) of the nodes, and N is the total number of nodes in a graph of our ROI map. $j, k \neq i$; $N = i \times j = 132^2$. We calculated the mean value of each node and the number of edges in the normal-weight, overweight, and obese groups, respectively. In addition to the PCC and the precuneus in the DMN, the anterior cingulate cortex, cerebellar lobules *CrusI*, and hippocampus related to hippocampal memory function were set as regions of interest as reported previously [17], and the functional connectivity of the three groups was analyzed. A functional connectivity map of each group was illustrated using the BrainNet viewer (<https://www.nitrc.org/projects/bnv/>) [33].

Brain slices Ca imaging

Hippocampal brain slices were prepared from 17–18-week-old male C57BL/6J background mice, which divided the mice into CD, HFDs, and HFDs with PER treatment groups according to previous reports [34]. After cutting the slices (thickness, 300–400 μm) using the Linear Slicer (PRO 7N, Dosaka EM, Japan), hippocampal brain slices were incubated in the normal Krebs Ringer (125 mM NaCl, 2.5 mM KCl, 10 mM D-glucose, 1.25 mM NaH_2PO_4 , 26 mM NaHCO_3 , 2 mM CaCl_2 , 1 mM MgCl_2 , continuously bubbled by mixture gas [95% O_2 ; 5% CO_2]) for 60 min at room temperature to recover the cutting damage. The Ca indicator Fluo-3 AM (excitation wavelength: 508 nm, emission: 525 nm, K_d , 0.4 $\mu\text{mol/L}$) (5 μM) (Dojindo, Kumamoto, Japan) was loaded into the brain slice preparations for 90 min. To monitor the $[\text{Ca}^{2+}]_i$, Fluo-3 fluorescence intensity (F525) was monitored using a photomultiplier under a confocal microscope (excitation wavelength 488 nm; LSM5 PASCAL, Carl Zeiss, Germany). In our experiment, a low-magnification ($\times 2.5$) objective lens (FLUAR 2.5 $\times$, NA=0.12, Carl Zeiss, Germany) was used to perform Ca imaging of the entire hippocampal slice (Fig. 1). Fluo-3 fluorescent images (512×512 pixels) were

digitized and stored every 10 s for 50 min on a personal computer. Each F525 of CA1, CA2, CA3, DG, and EC regions was normalized (NF525) by the average value of F525 (from 0 to 5 min before AMPA or NMDA application) under offline analysis (Microsoft Excel, Microsoft Corporation, WA). During the Ca imaging, brain slices were continuously perfused (2 mL/min) by extracellular solution containing 125 mM NaCl, 2.5 mM KCl, 10 mM D-glucose, 1.25 mM NaH_2PO_4 , 26 mM NaHCO_3 , 2 mM CaCl_2 , 1 mM MgCl_2 , and 1 μM tetrodotoxin continuously bubbled by mixture gas (95% O_2 ; 5% CO_2) in the perfusion chamber (35 mm μ -dish, Ibidi GMBH, Gräfelfing, Germany). In the case of the NMDA application, concentration of MgCl_2 was set to 0 mM to prevent the Mg-dependent inhibition of NMDAR. Maximal value of NF525 was estimated as the maximal value of NF525 within 5 min after AMPA (Tocris, Bristol, UK) (100 μM) or NMDA (Tocris, Bristol, UK) (50 μM) application. We used the cyclothiazide (CTZ) (100 μM) or glycine (Tocris, Bristol, UK) (10 μM) to prevent the desensitization of AMPAR or to activate the NMDAR, respectively, and used the PER (Eisai Co., Ltd., Tokyo, Japan) (100 μM), GYKI (Tocris, Bristol, UK) (100 μM), or (2R)-amino-5-phosphonovaleric acid (APV) (Tocris, Bristol, UK) (50 μM) as an antagonist of AMPAR or NMDAR respectively. We also used 1-naphthyl acetyl spermine (NASPM) (Tocris, Bristol, UK) (20 μM) as an antagonist of Ca^{2+} -permeable AMPAR [35].

Surface AMPAR subunit of cross-linking with BS³

Hippocampal slices from 17–18-week-old male C57BL/6J background mice (CD, HFDs, and HFDs with PER treatment) were prepared as reported previously [34]. After preparing and recovering the hippocampal slice, we separated the CA1, CA2, CA3, DG, and EC regions from the hippocampal slice using an anatomical knife. CA1, CA2, CA3, DG, and EC regions were added to Eppendorf tubes (Eppendorf, Hamburg, Germany) containing ice-cold artificial cerebrospinal fluid (ARTCEREB; Otsuka Pharmaceutical Co., Tokyo, Japan) with 2 mM BS³ (Thermo Fisher Scientific, Wilmington, DE, USA). Incubation was performed for 30 min on ice. Cross-linking was terminated by quenching the reaction with 100 mM glycine (10 min at 4°C). Hippocampal subregions were resuspended in ice-cold lysis buffer containing protease and phosphatase inhibitors (25 mM HEPES, pH 7.4, 500 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM phenylmethyl sulfonyl fluoride, 20 mM NaF, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1× protease inhibitor mixture [Sigma-Aldrich, St Louis, MO], and 0.1% Nonidet P-40 [v/v]) and homogenized rapidly by sonicating for 5 s. The total protein concentration of lysates was determined using the Lowry method. Samples were aliquoted (~15 aliquots per mouse) and stored at -80°C for future analysis. BS³ samples were analyzed directly by SDS-PAGE without purification, and surface and intracellular bands were measured in the same lane, avoiding the need for normalization and increasing sample throughput. Total protein lysates (40 μg) were loaded and electrophoresed on a 5–10% Tris-HCl gel (Thermo Fisher Scientific, Wilmington, DE) under reducing conditions, and proteins were transferred to nitrocellulose membranes (Thermo Fisher Scientific, Wilmington, DE) for immunoblotting. Membranes were blocked with a blocking reagent, Blocking One (Nacalai Tesque, Tokyo, Japan), for 30 min at room temperature. Membranes were then incubated with anti-GluA1 (1:1000; Merck Millipore, Burlington, MA) and actin (1:5000, Protein Teck, Chicago, IL, USA) overnight at 4°C. Membranes were incubated for 30 min with HRP-conjugated anti-rabbit IgG (1:3,000; Cell Signaling Technology, Danvers, MA, USA) and

washed extensively again in TBS-T. Membranes were immersed in chemiluminescence detecting substrate Chemi-Lumi One (Nacalai Tesque, Tokyo, Japan) for 1 min, and then detected using a LuminoGraph1 imaging system (Atto, Tokyo, Japan). The surface and intracellular bands in each lane were analyzed using a CS Analyzer (Atto, Tokyo, Japan).

RT-PCR

Hippocampal slices of 17–18-week-old male C57BL/6J background mice (CD, HFDs, and HFDs with PER treatment) were prepared as reported previously [34]. After preparing and recovering the hippocampal slices, we separated the CA1, CA2, CA3, DG, and EC regions from the hippocampal slices using an anatomical knife from C57BL/6J background mice. The CA1, CA2, CA3, DG, and EC regions of mice were isolated and immediately lysed with 0.5 mL TRIzol RNA isolation reagent (Thermo Fisher Scientific, Wilmington, DE) in a disposable homogenize tube BioMasher2® (Nippi, Tokyo, Japan). Total RNA was extracted individually from each region according to the rest of the protocol for the TRIzol RNA isolation reagent provided by the manufacturer (Thermo Fisher Scientific, Wilmington, DE). Using the PrimeScript RT Reagent Kit (Takara, Shiga, Japan), 1 µg of total RNA was reverse transcribed. An aliquot of the resultant cDNA was diluted 1:10 and added to a master mix of TB Green Premix ExTaq (Takara, Shiga, Japan), and real-time PCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The real-time PCR conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 34 s. The fluorescence intensity at every annealing step was captured, and threshold cycle time values were determined using the 7500 software. The relative beta-actin expression levels were further normalized to the respective control (vehicle group). The relative expression level of a target gene in each sample was determined using the standard curve and further normalized to that of β-actin in the same sample. The primer and probe sequences for these genes are shown in **Table S5**. We defined “GluA2's RNA amount/(GluA1+GluA3+GluA4's RNA amount) ratio” as Ca²⁺-permeability index.

Sanger sequencing

Templates for the Sanger sequencing of the GluA2 editing site were prepared by amplifying cDNA with the following primer pair: forward primer, GAGGAATTTGAAGATGGAAGAGA, and reverse primer, AGGAGGAGATGATGATGAGGGT. PCR products (10%) were confirmed by agarose gel electrophoresis. After confirming the appropriate anticipated size, the PCR product was purified using the innuPREP PCRpure Lite Kit (Analytik Jena, Jena, Germany) according to the manufacturer's instructions before direct sequencing. Cycle sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and each of the primers mentioned above. Sanger sequencing analysis was performed using ABI3500 (Applied Biosystems, CA, USA) and sequence viewer software 4Peaks.

Editing assays

The Q/R editing state of GluA2 was analyzed using the QuantStudio™ 3D digital PCR system with a TaqMan® custom SNP genotyping assay (Thermo Fisher Scientific, Wilmington, DE). TaqMan probes

were synthesized by Thermo Fisher Scientific and VIC-labeled probe was used to detect non-edited (Q) sequences and FAM-labeled probe was used to detect edited (R) sequences (5' CATCCTTGCTGCATAAA 3' and 5' ATCCTTGCCGCATAAA 3', respectively). The following primer pairs for amplification were used for PCR reaction: mice forward primer 5' TTGGGATTTTAAATAGTCTCTGGTTTTCTT 3' and mice reverse primer 5' GACCAACCTTGGCGAAATATCG 3'. cDNA of 5–50 ng was mixed with digital PCR master mix (Thermo Fisher Scientific, Wilmington, DE) and the mixed TaqMan probes were loaded onto the QuantStudio™ 3D digital PCR 20K Chip (Thermo Fisher Scientific, Wilmington, DE, USA) using an automatic chip loader according to the manufacturer's instructions. Loaded chips were amplified using the Gene Amp 9700 PCR system (Thermo Fisher Scientific, Wilmington, DE, USA) under the following conditions: 96°C for 10 min, 39 cycles at 56°C for 2 min, and 98°C for 30 s, followed by a final extension step at 60°C for 2 min. After amplification, the chips were imaged using a QuantStudio 3D Instrument (Thermo Fisher Scientific, Wilmington, DE, USA). For analysis of the chip data, QuantStudio 3D Analysis Suite Cloud Software (Thermo Fisher Scientific, Wilmington, DE) was used for the relative and quantitative data analysis.

Golgi staining and acquisition of images of the whole hippocampus

The coronal sectioned mice brains from Bregma -0.94 mm to Bregma -4.04 mm (including dorsal to ventral, medial to lateral whole hippocampus, 5 mm in width) was fixed by 4% paraformaldehyde for 2 days, then stained by a Golgi Cox stained system (FD Rapid GolgiStain™ Kit, MD 21041, USA). The impregnated tissues were cut into 100 µm sections and counterstained with crystal violet, and the total number of the 15 µm spines in the apical dendrites of CA1 and DG, as well as the percentage of morphological spines (thin, stubby, and mushroom), were examined using Axio Observer Z1 (Carl Zeiss, Germany). Another set of paraformaldehyde-fixed 5 mm width coronal sections of the brain including the whole hippocampus was processed using the passive CLARITY technique method (PACT) [36] and examined through Lightsheet Z.1 (Carl Zeiss, Germany) using a 10X clearing objective, and 3D images of the whole hippocampus of Thy1-YFPH transgenic wild type mouse (The Jackson Laboratory, stock number: 003709, strain name: B6.Cg-Tg.(Thy1-YFP)16Jrs/J) [37, 38], HFD-fed mice, and HFD-fed mice with PER-treatment were obtained, respectively. We acquired the maximum intensity projection image along the z-, time-, or channel dimensions, which created an output image where the pixels contained the maximum value over all images in the stack at a particular pixel location. With the Z-stack function, a series of XY images in different focus positions can be acquired, resulting in a Z-stack. In this way, a 3D dataset 400 µm × 400 µm × 400 µm from the specimen in each dorsal to lateral and medial to lateral CA1, CA3, and DG was obtained. The gallery view displays images from the z-stack within a time series.

Quantitative analysis of Thy1-YFPH⁺-cell number

3D reconstruction was completed using the Arivis Vision four-dimensional (4D) software. Blob finder (filter) was used to the rounded 2D and 3D segments close to the sphere-like shapes in a noisy image. Using the Gaussian scale, we found the object seeds and a watershed algorithm to identify the object

boundaries. We set the average size of the structure of interest to 20 μm and the threshold to 5. High-resolution rendering is an approach for visualizing the current view in a higher image and data resolution.

Immunohistochemical analysis

Mouse brains were perfusion-fixed in 4% paraformaldehyde and embedded paraffin and cut into 4 micrometer-thick sections used for the immunohistochemical analysis of monoclonal antibodies for DCX (E-6 monoclonal, 1:50, Santa Cruz Biotechnology Inc., Dallas, USA), MAP2ab (AP-20 monoclonal, 1:100, SIGMA-ALDRICH, St. Louis, USA), GluA1 (AB1540 polyclonal, 1:100, EMD Millipore Corp., Burlington, USA), and GluA2 (AB1768-1 polyclonal, 1:5, EMD Millipore Corp., Burlington, USA), respectively.

Quantitative immunostaining area by ZEISS ZEN Intellesis software.

Acquisition of immunostained area in each MAP2, GluA1, and GluA2 expression area was performed using AxioVision (Carl Zeiss, Germany) microscopy (20X objective lens) with a ZVI format. Generating data image processing with image segmentation was performed using machine learning with ZEN Intellesis software. More than 1000 cells were examined in each group. One-way ANOVA was used to test the variance among the three groups, excluding data of 3SD or more. Then, we used the following sample numbers: HFD series: MAP2 CD n=731, HFDs n=288, HFD+PER n=225; GluA1 CD n=220, HFD n=156, HFD+PER n=94; GluA2 CD n=78, HFD n=114, HFD+PER n=182; GluA2/GluA1 CD n=78, HFD n=94, HFD+PER n=94. The Bonferroni method was used for post-hoc testing to analyze significant differences among the groups.

RNA-seq analyses

The sequenced raw RNA-seq fastq reads were aligned to the mouse GRCm38 (ensemble release 104, http://ftp.ensembl.org/pub/release-104/fasta/mus_musculus/dna/) genome using HISAT2 (v.2.2.0). The average mapping rate for all the samples was 94.21% (range 88.70–96.30%). Aligned reads were quantified using Salmon (v.0.14.2), and TPM values were calculated using StringTie (v.2.1.2). Variant calling was performed according to GATK Best Practices for RNAseq short variant discovery using the GATK package (v.3.8). Adding read group information, sorting, marking duplicates, and indexing were carried out using Picard's tools. The GATK tool Split N Cigar Reads was used for splitting reads into exon segments and hard clipping any sequences overhanging into the intronic regions. Variant calling and filtration were performed using GATK HaplotypeCaller and VariantFiltration, respectively. Functional annotation of the output variant was performed using the SnpEff (v.4.3). The RNA-editing level was calculated as the ratio of the total number of reads aligned to the GluA2 R/Q editing site (chr3: 80706912) to the number of reads with T-to-C conversion at this site.

Statistical analysis

In the animal model experiments, one-way ANOVA followed by Bonferroni multiple comparisons test and/or two-tailed t-test were used for the statistical analysis. Statistical significance was set at $p < 0.05$. In the human experiments, the relationship between BMI, GM volume of the whole brain, body weight, and percentage of correct responses to task conditions (new, lure, and same) was analyzed using partial

correlation analysis. Differences between mean brain activity and correct answer rate under three (new, lure, and same) task conditions were analyzed using one-way ANOVA.

Results

HFDs stimulate calcium entry through α -3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA) in mice hippocampus

We first examined mouse obesity models treated with HFDs for 12 weeks after weaning (the composition of the diets [39] is shown in **Table S1**) and whether consumption of HFDs during the juvenile/adolescent periods altered the dynamics of calcium signaling in the hippocampus by direct measurement of intracellular calcium concentration ($[Ca^{2+}]_i$) in acute brain slices in the hippocampus with peri-hippocampal regions (Fig. 1a, **Movie S1**). Mice fed with HFDs exhibited marked activation of calcium signaling through AMPAR by the application of 100 μ M AMPAR with 100 μ M cyclothiazide (CTZ, a channel desensitizer) in the entorhinal cortex (EC), cornu ammonis 1 (CA1), CA2, CA3, and dentate gyrus (DG) estimated by maximal value of normalized F525 (NF525) (Fig. 1b, c, d and e, and **Table S2**).

Inactivation of N-methyl-D-aspartate (NMDA) receptors induced by intracellular Ca^{2+} through AMPAR

In contrast, calcium signaling through 50 μ M NMDA with 10 μ M glycine (a channel coactivator) was inactivated in the hippocampus derived from the mice with HFDs, whereas control diet (CD)-fed mice exhibited activation through the hippocampus (Fig. 1f, g, h, i, and j; **Table S2**; and **Movie S2**). In the hippocampal slices derived from mice fed with HFDs plus perampanel (PER) (Fycompa®, Eisai, Japan) (5 mg/kg per day, orally delivered by hydrogel), a novel noncompetitive AMPAR antagonist [16], we identified normalized glutamatergic transmission through AMPAR (Fig. 1d, **Table S2**), and simultaneous recovery of NMDAR-mediated signaling (Fig. 1i, **Table S2**, and **Movie S3**). A similar change in $[Ca^{2+}]_i$ through AMPAR was observed in HFD-fed mice for at least 7 days (**Fig. S1a, b, c**). Interestingly, these mice retained their activation through NMDAR. We found that a rise of $[Ca^{2+}]_i$ through AMPAR was normalized by the treatment of PER (**Fig. S1b**). These pathological AMPAR-mediated increases in $[Ca^{2+}]_i$ were completely abolished by the addition of 100 μ M PER, and similarly, for other AMPAR antagonists such as 100 μ M GYKI47261 (Tocris, UK, Bristol) and 20 μ M NASPM, a selective antagonist of CP-AMPA receptors (Tocris, UK, Bristol) (**Fig. S1d**). As the signals through AMPAR and NMDAR of hippocampal slices derived from control mice fed with CDs plus PER did not show any significant difference compared with those fed with CDs alone (**Fig. S9 a, b**), PER treatment effects reversal of HFD-induced impairments as opposed to PER having effects on its own. We anticipated Ca^{2+} -dependent inactivation (CDI) [15,40,41] observed in obesity mice is probably regulated by Ca^{2+} -permeability of AMPARs and endogenous Ca^{2+} -buffer capacity. Indeed, AMPAR-mediated NMDAR inactivation was abolished in the presence of intracellular fast Ca^{2+} -buffer BAPTA or in Ca^{2+} -free extracellular solution (Fig. 2a, b). On the other hand, bath application of ionomycin (1 μ M) [42] with 2mM Ca^{2+} induced CDI of NMDAR (Fig. 2c, d). These results suggest that HFD-

induced impairments in NMDA receptor function in the hippocampus was mediated by increased Ca^{2+} permeability of AMPA receptors.

Upregulation of calcium-permeable AMPAR by HFDs

AMPA is composed of four subunits, GluA1–4, which mediate fast excitatory neurotransmission and determine the functional properties of the AMPAR channel. Ca^{2+} permeability is regulated by the relative extent of GluA2 expression, which is essentially Ca^{2+} impermeability in relation to the extent of other Ca^{2+} -permeable subunits of GluA1, GluA3, and GluA4, as well as the abundance of GluA2, and the status of GluA2 at the Q/R site within its re-entrant M2 membrane loop region edited by adenosine deaminase acting on RNA type 2 (ADAR2) responsible for adenosine (A) to inosine (I) editing of double-stranded RNA, and lastly the extent of GluA1 trafficking to the membrane [7,8,14]. NMDARs are heteromeric composition assembled by NR1 and NR2A-D, and highly permeable to Ca^{2+} . Properties of NMDAR are regulated by the relative extent of GluN2A-D subunits: open probability of GluN2A subunit containing NMDAR is 5 times or 50 times larger than that of the GluN2B or GluN2C/N2D subunit containing NMDAR respectively, single channel conductance of GluN2A/N2B subunit containing NMDAR is larger than that of the GluN2C/N2D subunit containing NMDAR respectively [43]. NMDARs play critical functions both in physiological and pathological processes in the CNS, and synaptic plasticity such as LTP, and LTD, which forms underlying mechanism for learning and memory associated with dendritic spine remodeling during their processes; LTP corresponds to mushroom and/or stubby spines, and LTD, shrinkage of their size. Their synaptic dysfunction induces cognitive decline in various CNS disorders including neurodegenerative, neuropsychiatric, demyelinating diseases, and so forth [44]. Thus, we sought to clarify the mRNA level of subunit composition of AMPAR, and NMDAR by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and editing level of GluA2 at the Q/R site in subregions of the hippocampus. We found significant elevation of GluA1, GluN1, and GluN2B expression in the CA3 region, and GluN2B expression in the DG region associated with HFD-fed mice ($p < 0.05$), respectively and a significant elevation of GluA2 in CA1 in HFD-fed mice with PER treatment ($p < 0.05$) (Fig. 3a). We introduced the index of calcium permeability of AMPA receptors (**See Materials & Methods**). The reduction in the Ca^{2+} -permeability index (Fig. 3b, **Table S3**) by HFD-fed mice ($n=5$) was normalized to CD-fed mice level ($n=5$) by treatment with PER ($n=5$) (Fig. 3b, **Table S3**). These data would suggest that the extent of GluA1 expression of AMPAR in the hippocampus increased under the HFD condition compared with the CD condition and recovered under the HFD with PER condition. Up-regulation of GluA1 content of AMPAR might play a crucial role in the upregulation of Ca^{2+} permeability of AMPARs in HFD mice (Fig. 1a, c). Cross-linking assays using BS³, a cross-linker that can be used to determine surface receptors and intracellular receptors, showed an elevation of three-fold the amount of GluA1 surface receptors in the HFD-fed group compared with the CD group ($n=4$), and further declined to basal levels following treatment with PER ($n=4$) in CA3 (Fig. 3c) (**Fig. S4 a-c**). In editing analysis at residue 607, we found that genomic glutamine (Q607) was converted to an arginine (R) codon of >99.9% in both CD-fed and HFD-fed mice through CA1, CA2, CA3, DG, and EC (Fig. 3d) (**Fig. S 4d**), respectively. These findings altogether indicate the Ca^{2+} -permeability change of AMPAR in HFD-fed mice was mainly

dependent by the subunit composition change not dependent by the Q/R editing of GluA2 subunit. Up regulation of NMDA receptor mRNA in CA1,CA2, and CA3 but not DG and EC was detect in HFD-fed mice, although we could not find characteristic subunit compositional change. These results indicated that as reported by [15], increased Ca^{2+} - permeability of AMPA receptors may cause inactivity of NMDA receptors via the interaction of AMPA and NMDA receptors in HFD mouse. The change in the index of calcium permeability of AMPA receptors at the mRNA level are consistent with CP-AMPA subunits composition and may cause secondary changes in NMDA receptor function.

Structural reorganization of the brain of mice fed with HFDs

We monitored the dietary intake and weight of mice after weaning (6 weeks) maintained on CD, HFDs, and HFDs with PER (Fycompa®, Eisai, Japan) (5 mg/kg per day) to 18 weeks, respectively (Fig. 4a, b). Although dietary caloric volume was not significantly different among the three groups, we found that the significant suppression of weight in the HFDs with PER group started from 16 weeks compared with the HFD alone group, although the significant body weight gain in the HFD group started from 8 weeks compared with the CD group. To clarify the effect of HFDs on cognitive behavior, we conducted an open field test (Fig. S2a-c), elevated plus maze test (Fig. S2 d, e), contextual fear conditioning test (Fig. S2f), novel object recognition test (Fig. 4c), and five-trial social interaction tests (social memory) (Fig. 4d). Among these tests, we found a significant difference (cognitive decline) between the CD and HFD groups in the latter two tests ($p < 0.005$). Hippocampal NMDARs were reported to play an important role in the retrieval of spontaneous object recognition memory [3, 4, 45]. Cognitive decline of object recognition memory in HFDs -fed mice should be due to the dysfunction of NMDA receptors in hippocampus (Fig. 1). As the hippocampal CA2 region has been reported to play an essential role in social memory [25] and social memory is dependent on NMDA receptor function [46], we found an abnormal social memory in HFD mice (Fig. 4d), which may be induced by the NMDAR dysfunction of the hippocampal CA2 region in HFD mice (Fig. 1). Indeed, PER treatment restored social memory disturbance in HFD-fed mice in accordance with the normalized NMDAR dependent $[\text{Ca}^{2+}]_i$ signal of CA2 neuron. Water Maze test is strongly correlated with hippocampal synaptic plasticity and NMDA receptor function [47] and associative memory recall need to the activation of hippocampal CA3 NMDA receptors [48]. We found a reduced pattern completion ability (a retrieval ability to create explicit memory from partial cues of stored memory) in HFD mice, which was recovered in HFD with PER treatment mice associated with the recovery of Ca^{2+} entry through NMDAR of CA3 neuron (Fig. 1 & Fig. 4e-g). Our data suggest that conversion from AMPAR to CP-AMPA plays a key role in the disruption of pattern completion ability in HFD mice.

Quantitative volumetric analysis of the brain

Volumetric analysis is one of the fundamental approach to brain function [18]. In the quantitative volumetric analysis of the brain by 11.7 T magnetic resonance imaging (MRI) (Bruker BioSpec 117/11, Bruker BioSpin GmbH, Germany), we found that all mice ($n=6$, 16 weeks old) fed with HFDs after weaning showed hydrocephalus (Fig. 5a), a notable sign of brain atrophy caused by the apparently reduction of total brain volume in every HFD-fed mice. Indeed, there was a significant reduction of GM and increase of

WM in HFD mice (n=6) compared to CD-fed mice ($p<0.01$). Increase of WM, and disappearance of hydrocephalus in HFD mice was found by the PER treatment (Fig. 5a, **and b**). Furthermore, there was a significant reduction of the left CA1, bilateral CA3 and bilateral EC in HFD mice (n=6) compared to CD-fed mice (Fig. 5b). We found that PER treatment in HFD-fed mice (n=6) was significantly restored in the left and right sides of the hippocampal CA1 and DG and CA1, CA3, and DG regions, respectively (Fig. 5b). These volumetric increased hippocampal subregions were involved in the networks for associated and spatial memory, and we could see rescue effect of these ability in mice treated with PER (Fig. 4d-e). In addition, Golgi- Thy1-eYFP transgenic mice [37] were adopted using clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue-hydrogel (CLARITY) [49], and Z1 fluorescent microscopy (Carl Zeiss, Germany) was used to quantify neural cells in the hippocampal subregions. The reduced number of Thy-1⁺-YFP neuronal cells in CA3, a center for associative memory recall, of HFD mice was significantly restored by PER treatment ($p<0.001$) (n=4) (Fig. 5c, d, **Movies S4-S 7**) (**Fig. S8**): down regulation of neuronal number of hippocampal CA3 region may have a possible effect for the down regulation of NMDAR function in hippocampal CA3 region (Fig. 1) and the reduced pattern completion ability in HFD mice (Fig. 4e, f **and g**). Our data indicated that hippocampal volume is not related to the cognitive function. In contrast, NMDARs was governed by the shape (length and radius) of the spine neck, spine neck morphology is related to the synaptic strength [50, 51]. The spines on the dendrites are classified into mushroom type (with a large spine neck), thin type (with a medium size spine neck), and stubby type (without a spine neck) according to their shape. It has been reported that the density of AMPA receptors at synaptic sites depends on spine size [52, 53].

By Golgi-Cox impregnation staining, we found loss of integrity of dendritic arbors in CA1 in HFD mice, which was restored by PER (Fig. 6a, **left upper panel**). Similarly, the configuration of dendritic processes stained by anti-MAP2 antibody in CA1 showed the recovery of integrity of dendritic arbor by PER (Fig. 6a, **left upper panel**). There was no significant number of spines among CD, HFD and HFD plus PER treatment mice group, but a significant decrease of thin type spine, and a significant increase of stubby spine type was observed in HFD mice compared with CD mice. Furthermore, PER treatment in HFD mice increased mushroom spines, suggesting CP-AMPA inhibits spine maturation. The immunoreactivity of GluA1 in CA1 was significantly enhanced in HFD mice but reduced with PER treatment, coordinating the elevation of GluA2/GluA1 ratio. In the DG, Golgi-stained cells were recovered in HFD mice treated with PER (Fig. 6a, **left middle low**), exhibiting simultaneously apical spine maturation (Fig. 6a, **lower low**). As shown in Fig. 6b, a significant increase in the immunoreactive area of GluA1 was observed in HFD mice, but not in GluA2. Since the ratio of GluA2 / GluA1 is significantly increased in HFD plus PER treatment mice compared with HFD mice, the change in the ratio of subunit composition contributes to the restoration of calcium permeability of AMPA receptor. In addition we also found that the increased doublecortin-positive cells in HFD plus PER hippocampus indicating regulation on calcium permeability by PER treatment induces neurogenesis in DG (Fig. 6c, **left corner**) and furthermore proceeds the maturation of DCX⁺-positive neural progenitors (**Fig. S3a**). These data suggest that synaptic immaturity, judged by increased stubby type, of the DG region under the HFD conditions was restored by the PER treatment (Fig. 6a).

Higher body mass index (BMI) impacts negatively on human gray matter (GM) volume

To investigate the correlation between BMI and brain GM volume (gv), we enrolled 122 healthy Japanese subjects (including 84 [BMI: <25], 27 [BMI: 25–29.9], and 11 [BMI: >30] subjects) (**Table S4**) and applied volumetric analysis obtained by brain T2 MRI. We found a significant negative correlation between BMI and gv in men compared with women. Regarding higher BMI (~ 30.2) and lower BMI (~ 21.1) values in healthy individuals (Fig. 7a, b), we could observe a tendency for a decrease in gv with the BMI value. There was a significant negative correlation between “gv and ages,” and “gv and body weights” (**Fig. S5, S7 a-c**). In contrast, we could not identify the significant correlation between “BMI and ages.” However, there was a significant positive correlation between “BMI and body weights” (Fig. 7f) (**Fig. S6**). We also found a significant negative correlation between gv and BMI (Fig. 7g) using partial correlation analysis to minimize the effects of age and body weight.

Next, we examined the impact of BMI elevation on human hippocampal synaptic transmission by monitoring the fluctuation of blood oxygenation level-dependent (BOLD) response, hippocampal memory function of pattern completion, and pattern separation capabilities (the formation of distinct representations of similar inputs) using fMRI behavioral tasks (Fig. 7c-e) (**Table S 6**) [17, 18]. Amplitude of Bold response was related to neural activity of both NMDAR and AMPAR in model animals [54], thus our data would indicate the modulation of AMPA and/or NMDAR would happen with the higher BMI condition in human. A negative correlation between BMI and pattern completion and ability for memory recall, whose basis of structural networks is CA3 and CA1 subregions that play a central role in ensemble dynamics [55], was observed in healthy human volunteers (Fig. 7h). Importantly, the overweight group did not reveal a significant cognitive decline compared with the control group (Fig. 7i); in contrast, people with BMI $>30 \text{ kg/m}^2$ also showed a marked decline of pattern completion capabilities (Fig. 7j) (**Table S7**).

Human pattern completion ability for memory recall is inversely correlated with higher BMI

The significance of resting-state functional MRI (fMRI) for biomarkers of human cognition, especially in hippocampal memory function, is increasing [17]. Glutamatergic synapse could regulate cognitive function and brain connectivity [56], thus we clarified the functional network connectivity in resting-state in human. Among the intrinsically organized large-scale networks in resting-state fMRI data, default mode network dynamically controls the salience and central executive networks in healthy individuals, and this pattern of interaction has remained largely intact in overweight people; however, disorganization of this interaction in obese individuals results in cognitive decline (Fig. 8a). Lastly, we applied a quantitative analysis of human networks based on the graph theory in normal, overweight, and obese examinees. We created a functional connectivity map of each group by seed-based analysis and set the posterior cingulate cortex and precuneus in the default mode network and the anterior cingulate cortex and the cerebellar lobules Crus/ in the hippocampal memory function as regions of interest [17]. The network connectivity varied among subjects in each classified group, as illustrated in Fig. 8b. Specific alternation

of greater networks and between centrality mapping associated with the disruption of module autonomy became evident in the overweight and obese groups compared with the normal BMI group, resulting in failure of correct memory recall. The betweenness centrality of the anterior cingulate cortex and the paracingulate gyrus increased more than in the other two groups. Connectivity between different functional networks (DMN, SN, CEN, CBN, and VN) was extended in the obese groups. The total number of nodes in the normal weight, overweight, and obese groups was estimated to be 17, 21, and 23, respectively (Fig. 8b, c).

Discussion

A central role for CP-AMPA in obesity-associated cognitive decline

Here, we demonstrate that HFDs stimulate hippocampal calcium dynamics through AMPAR and subunit rearrangement from non-CP-AMPA to CP-AMPA, which leads to altered NMDAR calcium signaling to downregulate Ca^{2+} entry. Inactivated transmission through NMDAR function may correspond to cognitive decline. CP-AMPA also triggered a reduction in hippocampal size and cell number, especially in CA3, dendritic integrity of CA1, and spine maturation in the DG region. Intake of HFDs for at least 7 days influenced the hippocampal memory circuit through calcium signaling. For this short-term feeding, calcium dynamics through NMDAR were maintained, but calcium entry through AMPAR became evident in the hippocampus. With intake for a long-term duration of more than 8 weeks after weaning with HFDs, calcium entry through NMDAR completely diminished in accordance with the alteration of calcium dynamics through AMPAR. Suppression of NMDAR-dependent calcium influx in synapses is modulated by intracellular calcium concentration irrespective of the source of calcium, such as AMPARs, voltage-gated calcium channels, internal stores, and NMDAR [15]. Above all, AMPARs are colocalized with NMDAR and commit excitatory synaptic response on a tenths millisecond time scale, and HFDs induced an increase of calcium entry by these receptors, which finally resulted in reducing magnitude of calcium current through NMDARs reported to play a key role for the regulation of appetite and food preference [57] on to the induction of synaptic plasticity (LTP/LTD) [58] that stimulates a vicious cycle for binge eating, a reaction by failure in food restriction, and increased obesity [59] finally further accelerating cognitive decline.

Alteration of glutamatergic synaptic transmission impacts on functional network connectivity in resting-state fMRI data

Higher BMI is associated with cognitive disturbance in younger adults [60] in middle-aged adults, overweight and obesity increases the future risk of cognitive decline in old age. For people in old age, higher BMIs are paradoxically associated with better cognition and decreased mortality [61]. HFDs induce obesity, which leads to hypertension, type 2 diabetes, and cardiovascular events, and patients with these diseases suffer from an increased risk of cognitive decline [62, 63] which probably hinders effective treatment and disease control due to the failure of proper lifestyle management, including self-regulation of eating behavior. In medicine, developing an effective intervention for obesity-associated cognitive

decline a key to better disease control. The correlation between the decreased resting-state activity in the precuneus and posterior cingulate and simultaneous anti-correlated inactivity of the dorsolateral prefrontal cortex and insula was disrupted in the brains of overweight and obese individuals (Fig. 8). A similar disruption was observed in subjects with metabolic syndrome [64].

AMPA is an attractive target for obesity-associated cognitive decline

Of note, a high incidence of hydrocephalus was found among the early onset obese mice group. In humans, sustained obesity starts in early childhood between 2 and 6 years of age [65], and most children who are obese at that age are obese in adolescence. Proper and effective interventions are urgently needed for human health and disease. Importantly, application of HFDs with PER treatment restored the dysregulated calcium signaling through both AMPARs and NMDARs, while simultaneously inducing the recovery of brain size and behavioral memory capability. The prevention of weight gain in obesity induces health benefits. However, food restrictions that are reported to induce CP-AMPA in the nucleus accumbens [66] usually rebound and result in weight gain and overeating. Taken together, these findings suggest that AMPARs are an attractive therapeutic target for human obesity-associated cognitive decline.

Declarations

Data availability

The datasets that support the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions

YM contributed qRT-PCR, Q/R, rodent behavioral analysis, and planning of animal experimental design. KF contributed Immunohistochemical analysis. KH contributed Rodent MRI imaging. DU contributed NGS analysis. CK contributed Western blot analysis. MN contributed Human and rodent MRI imaging and network analysis. HT contributed Ca^{2+} imaging and editing of the manuscript. SH contributed Z1 imaging, Golgi staining, project administration, funding acquisition, conceptualization and writing, and review and editing of the manuscript. All authors have no competing interests.

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Ethics Approval

All animal experiments were performed in accordance with the guidelines of the Animal Experiment Ethics Committee of the University of the Ryukyus (approval number: A2019239).

All experiments were approved by the ethical committee of the University of the Ryukyus for medical and health research involving human subjects and were performed in accordance with guidelines of human experiment regulations at University of the Ryukyus (approval number:111).

Consent to Participate

Not applicable.

Consent for Publication

All authors have seen and approved the manuscript and contributed significantly to this work.

Competing Interests

The authors declare no competing interests.

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Figures

Figure 1

Perampanel (PER) rescued the alternation of AMPAR and NMDAR function in hippocampus of mice fed with HFDs.

a, Fluo-3 fluorescent image of hippocampus before and after AMPA with CTZ application under CD, HFD, and HFD with PER treatment conditions. Green subtraction images from after to before image. Mouse: 17

weeks old; HFD or HFD with PER treatment: from 8 weeks to 17 weeks. **b-d**, AMPA dependent change of NF525 in hippocampal CA1, CA2, CA3, DG, and EC region; CD (**b**), HFD (**c**), and HFD with PER treatment (**d**) conditions, respectively. **e**, Averaged maximal value of NF525 after AMPA application from different slice preparations (n=5).*: p<0.05, two tailed t-test. **f**, Fluo-3 fluorescent image of the hippocampus before and after NMDA with glycine application under CD, HFD, and HFD with PER treatment conditions and subtraction image. **g-i**, NMDA-dependent change of NF525 in hippocampal CA1, CA2, CA3, DG, and EC regions; CD (**g**), HFD (**h**), and HFD with PER treatment (**i**) conditions. **j**, Average maximal value of NF525 after NMDA application from different slice preparations (n=5). **: p<0.01; *: p<0.05, two tailed t-test.

Figure 2

Ca²⁺-dependent inactivation of NMDAR under the HFD condition

a, Rescue effect by BAPTA-AM treatment or Ca²⁺ free conditions for HFD induced inactivation of NMDA receptor function. Hippocampal slice was pre-treated under the BAPTA-AM 3μM conditions or Ca²⁺-free conditions for 60min. before Ca²⁺ imaging experiments.

b, Statistical comparison of NMDA receptor function among HFD (n=4), BAPTA-AM treatment (n=3) and Ca^{2+} free conditions (n=3) NMDA:50 μM ; Glycine:10 μM ;APV:50 μM

Mouse: 17 weeks old, from 15 week to 17 week HFD.

c, Pre-treatment (for 10min) of ionomycin (1 μM) with 2mM Ca^{2+} induced the inactivation of NMDAR function

d, Statistical comparison of NMDA receptor function between pre-treatment of ionomycin (1 μM) with 2mM Ca^{2+} group (n=5) and no pre-treatment of ionomycin group (n=4).

NMDA:50 μM ; glycine:10 μM ; APV:50 μM Mouse: 14-15 weeks old

* $p < 0.05$

Figure 3

Transcriptional profiling in the hippocampal subregion, AMPAR and NMDAR subunit expression in mice fed with HFD.

a, Expressions of AMPAR subunits, NMDAR subunits, and ADAR2 in CA1, CA3, DG, and EC hippocampal subregions by qRT-PCR. Note the GluA1 at CA3 in mice fed with CD, HFDs, and HFDs with PER treatment.

Mouse: 17 weeks old; HFD or HFD with PER treatment: from 8 weeks to 17 weeks. **b**, Ca^{2+} -permeability index of CA1, CA3, DG, and EC hippocampal subregions fed by CD, HFDs, and HFDs with PER treatment. **c**, Surface (center column) and intracellular (right column) expression of GluA1 protein in the hippocampal CA3 region in mice fed with HFDs increased, while that in mice fed with HFDs with PER treatment was downregulated. **d**, Q/R editing analysis of CA1, CA2, CA3, DG, and EC hippocampal subregions in CD and HFD mouse. VIC: VIC-labeled probe to detect non-edited (Q) sequence, FAM: FAM-labeled probe to detect edited (R), Q & R ratio (%) (left upper and row) and copy number of each subregion (the box on the far right of the bottom row). Mouse: 17 weeks old; HFD or HFD with PER treatment: from 6 weeks to 17 weeks.

Figure 4

Behavioral changes in mice fed with HFD

a, Change in body weight of animals fed with CD, HFDs, and HFDs with PER treatment (n=6) . Mouse: 6-17 weeks old; HFD or HFD with PER treatment: from 6 weeks to 17 weeks. **b**, Food intake of animals fed with HFDs and HFDs with PER treatment (n=4).

c, Average investigation time of new object and familiar object in mice fed with CD (n=11), HFDs (n=12), and HFDs with PER treatment (n=12). The symbol overlapping the bar graph indicates the individual data. Mouse: 12 weeks old; HFD or HFD with PER treatment: from 4 weeks to 12 weeks. **d**, Interaction time of five-trial social interaction tests on each day of testing in mice fed with CD, HFDs, and HFDs with PER treatment. Mouse: 16 weeks old; HFD or HFD with PER treatment: from 8 weeks to 16 weeks. Data are shown as mean $\pm$ SD. *: $p < 0.05$; **: $p < 0.01$, two tailed t-test. **e-g**, Representative examples of the swim path to the phantom platform (blue) for the probe trials (end point of trajectory represents the start point) (**e**), probe trials with cue manipulation (**f**). Goal arrival time of the Morris water maze pattern completion test (**g**) on each day of testing in mice fed with CD, HFD, and HFD + PER, respectively. Mouse: 17weeks old.

Figure 5

Brain weight and cell number alternation of the hippocampus by HFDs.

a, Coronal section of the MRI image under BL/6 (16 weeks old), HFDs, and HFDs with PER treatment conditions. White asterisk and diamond (HFD-fed mouse): dilated third and bilateral lateral ventricles. Right bar graph shows mean volume of total intracranial volume (TIV), whole brain gray matter (GM), and

whole brain white matter (WM) per TIV of mouse. The Y-axis on the left and on the right show the volume of TIV (mL) and the ratio of GM or WM to TIV, respectively. **b** Mean volume of GM per TIV of hippocampal CA1, CA3, DG, and EC. Left and right bar graph indicate left and right hippocampus, respectively. The Y-axis on the left shows the ratio of GM to TIV of CA1, CA3, and DG and on the right shows EC. **c, d**, 3D image of Thy-1 mouse (17 weeks old) hippocampus, length of cubic box; sagittal length axis (anterior to posterior): 4000 μm , width: 3000 μm , and height: 3000 μm (**c**) and hippocampal CA1, CA3, and DG regions, each length of the cubic box is 400 μm (width, depth, and height) (**d**) under BL/6, HFDs (from 4 weeks to 17 weeks), and HFDs with PER (from 4 weeks to 17 weeks) treatment conditions. Right bar graph showing cell density of the Thy-1⁺-FYP mouse hippocampal CA1, CA3, and DG regions, respectively.

Figure 6

Micro level morphological alternation of the hippocampus by HFDs

a-Cc, Golgi staining of CA1 and DG region (**a**, left column) , schematic diagram of spine (**a**, right lower column) and percentage of thin, stubby, and mushroom-type spine (**a**, right upper column); anti-MAP2 Ab (antibody), anti-GluA1 Ab, and anti-GluA2 Ab staining (**b**, upper column); immuno-reactive area of anti-MAP2 Ab (antibody), anti-GluA1 Ab, and anti-GluA2 Ab staining in hippocampal DG region and GluA2/GluA1 ratio (**Bb**, lower column); anti DCX⁺ staining (**c**, left column); and number of DCX⁺ positive cell/HFP (**c**, right column) in hippocampal DG region under BL/6, HFDs, and HFDs with PER treatment condition. Bar: 200 μm (upper **a**), 100 μm (middle **a**), 1 μm (lower **a**), 50 μm (**b**), and 100 μm (left **c**) and 50 μm (left **c**). Mouse age: 17 weeks old, HFD or HFD with PER treatment: from 4 weeks to 17 weeks. *, **, and ***: $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Figure 7

Increased BMI influences human memory function and synaptic fluctuation. **a**, Anatomy of a subregion of the right hippocampus on a T2-weighted image, indicated by cornu ammonis 1 (CA1), green; cornu ammonis 2 (CA2), magenta; cornu ammonis 3 (CA3), yellow; cornu ammonis 4 and dentate gyrus (CA4/DG), red; and subiculum (SUB), blue. **b**, Correlation in the activation of CA3 and CA1 in the right hippocampus under recognition task (normal weight [n=84], overweight [n=27], and obese [n=11]). **c-d**,

Correlation between percentage value of BOLD signal in CA3 of the right hippocampus and the percentage of correct response of the recognition task (**c**); percentage value of the BOLD signal of the bilateral hippocampal CA3 regions (**d**) (male: 52; female: 65; total: 117; average age = 37.8 ± 19.6 years, range 18–86 years). **e**, Mean value of the correct response rate under the recognition task. **f, g**, Correlation between BMI and the correct response rate under the recognition task ($r=-0.22$, $p<0.05$) (**f**). Correlation between BMI and volume of whole brain gray matter ($r=-0.28$, $p<0.05$) (**g**). In normal weight, overweight, and obese groups. **h-j**, Activation map and BOLD signal change in the left and right CA3, and the results of the correct response rate of the hippocampal memory task (New, Lure, Same) from left to right in normal weight (**h**), overweight (**i**), and obese (**j**) groups. Color bars: T-value. *: $p<0.001$.

Figure 8

Relationships between network connectivity and BMI in humans, **a**, DMN map (upper panel) and anticorrelation map to DMN (lower panel) on normal weight, overweight, and obesity groups. Color bars indicate T-value. **b**, Functional network connectivity among normal weight (left column), overweight (center column), and obese groups (right column). The diameter of each circle indicates the value of betweenness centrality. Blue, red, yellow, green, cyan, and orange nodes indicate Default mode network (DMN), Salience network (SN), Central executive network (CEN), Emotional network (EN), Cerebellar network (CBN), and Visual network (VN), respectively. **c**, Demonstrating the mean and standard deviation of the betweenness centrality and the degree of each node. a.u.: arbitrary unit.

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