Maternal High-fat Diet Leads to Persistent Synaptic Instability in Mouse Offspring via Oxidative Stress during Lactation

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Abbreviations: HFD, high-fat diet; ND, normal diet

1 **Abstract**

2 Maternal obesity has negative effects on the neurodevelopment of the offspring. Pups
3 from high-fat diet (HFD)-fed mice exhibit peroxidized lipid accumulations in the brain
4 and behavioral impairments. However, the synaptic basis of maternal HFD-induced
5 brain dysfunction in offspring remains unclear. In the present study, we focused on the
6 dynamics and morphology of postsynaptic dendritic spines and filopodia in the
7 offspring of HFD-fed mouse dams, using *in vivo* two-photon imaging, chosen because
8 of the involvement of peripheral organs and non-neuronal cells in the abnormal
9 metabolic state. We observed instability of dendritic spines and filopodia in the cerebral
10 cortex of offspring from HFD-fed dams. Interestingly, the synaptic instability persisted
11 into adulthood with a lower spine density even when the offspring were fed with a
12 normal diet after weaning. HFD-fed offspring from HFD-fed dams showed a severe
13 disruption of dendritic spines. Synaptic instability and loss of spines were caused even
14 by HFD exposure exclusively during lactation. The treatment of ascorbic acid, an
15 antioxidant, during lactation ameliorated the synaptic impairments. These results
16 suggest that maternal obesity leads to persistent synaptic impairments in the offspring,
17 which may be associated with behavioral deficits in adulthood, and that these synaptic
18 deficits may be due to oxidative stress from peroxidized lipid accumulations during the
19 lactation period.

20

21 **Keywords**

22 Dendritic spine; *in vivo* two-photon imaging; high-fat diet; maternal environment;
23 lactation; antioxidant

24 **1. Introduction**

25 Obesity is a worldwide health problem and a major contributor to the increased
26 incidence of coronary artery disease, hypertension and type 2 diabetes (Kopelman,
27 2000). Epidemiological studies have shown that maternal obesity has negative effects
28 on the neurodevelopment of children (Van Lieshout et al., 2011). Animal studies have
29 also demonstrated the effects of maternal obesity on the brain, as well as in the
30 peripheral organs of offspring (Williams et al., 2013). However, little is known about
31 the effects of maternal obesity on brain development in offspring. We previously
32 reported that mouse pups from dams fed with a high-fat diet (HFD) show peroxidized
33 lipid accumulations in many brain regions, impaired adult neurogenesis in the
34 hippocampus and deficits in spatial learning performance (Tozuka et al., 2010; Tozuka
35 et al., 2009). Other animal model studies have also demonstrated cognitive impairment
36 of offspring from HFD-fed dams (Rodriguez et al., 2012; Wu et al., 2013). However, the
37 synaptic basis for maternal HFD-induced brain dysfunction remains unclear. It is
38 possible that postsynaptic dendritic protrusions may be impaired in offspring born to
39 HFD-fed dams, because many behavioral deficits are associated with protrusion
40 impairment (Penzes et al., 2011). In our preceding study, peroxidized lipid
41 accumulations in the brain of the offspring from HFD-fed dams disappear when the
42 offspring are raised on a normal diet (ND) after weaning, whereas impaired progenitor
43 cell proliferation in the hippocampus and elevated locomotor activity persist into
44 adulthood (Tozuka et al., 2010; Tozuka et al., 2009). Thus, there may be reversible and
45 irreversible components of maternal HFD-induced brain impairment in offspring.
46 Accordingly, these lines of evidence raise the following questions about the impact of
47 maternal HFD on the synapses of the offspring: Are the synaptic impairments induced

48 by maternal HFD reversible or persistent? When is the critical period for the synaptic
49 impairment in the offspring from HFD-fed dams? Is oxidative stress from peroxidized
50 lipids during the critical period a key factor in developing synaptic impairment?

51 In the present study, we analyzed the dynamics and morphology of dendritic
52 protrusions, classified into mature spines or immature filopodia, using *in vivo*
53 two-photon laser-scanning microscopy. We evaluated the synaptic deficits induced by
54 HFD *in vivo* because animals exposed to HFD show various pathologies in many
55 peripheral organs and non-neuronal cells in the brain (Bilbo and Tsang, 2010; Williams
56 et al., 2013). We used not only ND-fed offspring from HFD-fed dams but also HFD-fed
57 offspring from HFD-fed dams, to model western eating habits. Furthermore, in order to
58 elucidate the critical period when maternal HFD disrupts the synapses of the offspring,
59 we used ND-fed offspring from HFD-fed dams exclusively during lactation. We
60 examined the involvement of oxidative stress from peroxidized lipids during the critical
61 period by antioxidant-treatment. Our findings demonstrate that maternal HFD lead to
62 synaptic instability in the offspring mediated by oxidative stress during lactation, and
63 that the synaptic instability persisted into adulthood with developing loss of dendritic
64 spines. The present study is the first to examine the synaptic basis of brain dysfunction
65 in offspring born to HFD-fed dams.

2. Materials and Methods

2.1 Experimental animals

Mice expressing yellow fluorescent protein (YFP) predominantly in layer (L) 5 pyramidal neurons (*Thy1-YFP* H-line) were purchased from the Jackson Laboratory and backcrossed with C57BL/6J mice. Mice were housed four per cage under controlled temperature ($25 \pm 1^\circ\text{C}$) and lighting (12 h light/dark cycle), and were provided with food and water ad libitum. The procedure of maternal HFD feeding and the macronutrient composition and caloric distribution in the ND and HFD have been described previously (Tozuka et al., 2009). Briefly, heterozygous *Thy1-YFP* mice were fed ND (CE-2, CLEA Japan) or HFD (HFD-32, CLEA Japan) (Oike et al., 2005) for 6 weeks until mating. The same diet was maintained during pregnancy and for 15 days of lactation. From lactational day 16, when pups first start to eat a solid food, pups were assigned as follows: those born to HFD-fed dams but consuming ND (HFD/ND), those from ND-fed dams but consuming HFD (ND/HFD), those from HFD-fed dams and consuming HFD (HFD/HFD), and those from ND-fed dams and consuming ND (ND/ND). Pups born to HFD-fed dams exclusively during lactation and consuming ND were assigned to HFD_{lac}/ND group. Pups from HFD-fed dams treated with 1 g/L *L*-ascorbic acid (Vitamin C; Nacalai Tesque, Japan) in drinking water during lactation and consuming ND were assigned to HFD_{AA}/ND group. The fluid intake of mice received normal drinking water or drinking water supplemented with ascorbic acid is approximately 10 mL/day (Abdel-Wahab et al., 2002). On the day of parturition (day 0), litters were culled to 6–8 offspring per mother. Pups were kept with their dams until weaning on day 22. Only male offspring heterozygous for YFP were used for all analyses. To avoid autoxidation of the HFD, diets were stored at

90 –80°C until use, and food was renewed every 2 d. Animal procedures were in strict
91 accordance with the guidelines of the National Institute of Neuroscience, National
92 Center of Neurology and Psychiatry (Japan).

93

94 2.2 *Surgical procedure for in vivo imaging*

95 We used the thinned-skull cranial window technique (Yang et al., 2010) because it is
96 less invasive than the open-skull method (Xu et al., 2007). Male pups expressing YFP
97 were deeply anesthetized with urethane (2 mg/g body weight, i.p.). Body temperature
98 was maintained at 37°C with a heating pad during the surgery and following imaging.
99 After the scalp incision, both eyes were lubricated with eye ointment, and the primary
100 somatosensory area (1.1 mm posterior to bregma and 3.4 mm lateral from midline) was
101 identified with stereotactic coordinates. A small metal plate with a round hole was glued
102 onto the skull with cyanoacrylate glue and acrylic resin dental cement (UNIFAST; GC,
103 Japan), and mice were fixed to a custom-made skull immobilization stage via the metal
104 plate. The skull above the imaging area, located in the center of the hole in the metal
105 plate, was thinned to a thickness of approximately 20 µm with a high-speed microdrill
106 (UG23A/UC210C; Urawa, Japan) and microsurgical blade (USM-6400; Sable
107 Industries, Vista, CA, USA). The hole in the metal plate was filled with artificial
108 cerebrospinal fluid during surgery and imaging.

109

110 2.3 *In vivo transcranial two-photon imaging*

111 *Thy1-YFP* pups were imaged under anesthesia using two-photon laser scanning
112 microscopy (FV1000-MPE; Olympus, Japan) with a water-immersion objective lens
113 (25×, NA 1.05) at 8× digital zoom yielding high-magnification images suitable for

quantification of dendritic spines. A Ti-sapphire laser (MaiTai HP DeepSee-OL; Spectra-Physics, Mountain View, CA, USA) was tuned to 950 nm. Laser intensity was kept in the range of 10–30 mW at the focus to minimize phototoxicity. Image-stacks (512×512 pixels; 0.124 μm /pixels; 0.75 μm z-step) were taken at approximately 70 μm below the pial surface, where L1 dendrites of L5 pyramidal neurons are located. Dendritic segments were imaged for each experiment at time 0, and then the same dendritic segments were imaged again after an interval of 1 h. All images were acquired carefully to approximate similar fluorescence levels across imaged regions within each experiment and across imaging sessions in different animals. The animals were sacrificed immediately after imaging sessions.

124

2.4 *Image analysis*

The turnover rate, density, head width, and neck length of dendritic protrusions were analyzed with Neurolucida (MicroBrightField, Williston, VT, USA) from three-dimensional two-photon z-stacks. Morphometric analysis of dendritic protrusions was in accordance with a previous report (Grutzendler et al., 2002). Dendritic protrusions were classified into two groups, spines and filopodia, and formation/elimination rate were analyzed in each group. Filopodia were identified as long, thin structures (ratio of head width to neck width $< 1.2:1$; ratio of length to neck width $> 3:1$). The remaining protrusions were classified as spines. These protrusions were considered the same between two views on the basis of their spatial relationship to adjacent landmarks and their relative position to immediately adjacent protrusions. Protrusions were considered different if they were $> 0.7 \mu\text{m}$ from their expected positions based on the first view. The formation and elimination rates for the protrusions

138 were defined as the fraction of protrusions that appeared and disappeared, respectively,
139 between two successive frames, relative to the total protrusion number. Protrusion
140 turnover rate was defined as the sum of the protrusions formed and eliminated divided
141 by twice the total number of protrusions. Data were collected from 10–21 dendrites and
142 approximately 1,000 protrusions in four to seven mice.

143

144 2.5 *Statistical analysis*

145 To determine statistical significance, we used Student's *t* test, a one-way ANOVA
146 followed by Tukey's multiple-comparison test, a two-way ANOVA followed by
147 Bonferroni's multiple-comparison test, or a Kolmogorov–Smirnov test. All statistical
148 analyses were performed with GraphPad Prism (GraphPad Software Inc, La Jolla, CA,
149 USA). All data are presented as mean $\pm$ SEM, where *n* = number of dendrites.

150 3. Results

151 3.1 *Maternal HFD leads to instability of dendritic spines and filopodia in ND-fed* 152 *adult offspring*

153 In order to evaluate the effects of maternal HFD on the synapses in adult offspring
154 consuming ND, we performed *in vivo* two-photon imaging in L1 dendrites in the
155 primary somatosensory cortex of 10-week-old ND-fed offspring of ND- or HFD-fed
156 dams (ND/ND or HFD/ND) (Figure 1a). We analyzed the formation and elimination of
157 dendritic spines and filopodia within 1 h (Figure 1b). Compared with ND/ND mice, the
158 elimination rate of spines in 10-week-old HFD/ND mice was significantly greater, while
159 the formation rate was not significantly different (Figure 1c; formation rate: ND/ND:
160 $2.9 \pm 0.9\%$, $n = 11$; HFD/ND: $5.3 \pm 0.7\%$, $n = 21$; $p = 0.0516$; elimination rate: ND/ND:
161 $1.1 \pm 0.4\%$, $n = 11$; HFD/ND: $7 \pm 1\%$, $n = 21$; $p = 0.0025$; unpaired t test). Both
162 formation and elimination rate of filopodia were greater in HFD/ND mice (Figure 1d;
163 formation rate: ND/ND: $6 \pm 3\%$, $n = 11$; HFD/ND: $28 \pm 4\%$, $n = 21$; $p = 0.0020$;
164 elimination rate: ND/ND: $2 \pm 2\%$, $n = 11$; HFD/ND: $32 \pm 6\%$, $n = 21$; $p = 0.0035$;
165 unpaired t test). These data show that maternal HFD causes synaptic instability in adult
166 offspring even though the offspring are fed ND after weaning.

167

168 3.2 *Offspring exposed to maternal HFD develop synaptic instability and loss of* 169 *dendritic spines during synaptic maturation*

170 To reveal how the synapses are impaired after weaning, we investigated the effects of
171 maternal HFD on synaptic development during juvenile and adulthood in ND/ND and
172 HFD/ND mice at 4, 6, 8 and 10 weeks of age. Because normal dendritic spines are

173 gradually stabilized across neuronal circuitry development (Grutzendler et al., 2002),
 174 we analyzed the turnover rate of spines and filopodia at each age during synaptic
 175 development. In HFD/ND mice, spine turnover rate was significantly greater than in
 176 ND/ND mice throughout synaptic development (Figure 2a; 4 wks: $p < 0.0001$; 6 wks: p
 177 < 0.0001 ; 8 wks: $p < 0.0001$; 10 wks: $p = 0.0148$; two-way ANOVA followed by
 178 Bonferroni's test), and filopodium turnover rate was greater at 6, 8, and 10 weeks of age
 179 in HFD/ND mice (Figure 2b; 4 wks: $p = 0.3612$; 6 wks: $p < 0.0001$; 8 wks: $p < 0.0001$;
 180 10 wks: $p < 0.0001$; two-way ANOVA followed by Bonferroni's test). No significant
 181 difference was observed in filopodium turnover rate at 4 weeks of age. This may be due
 182 to the high turnover rate of filopodia at this immature stage of synaptic development
 183 even in the control group. Note that, in ND/ND mice, turnover rates of spines and
 184 filopodia were gradually decreased with normal maturation. Next, we analyzed the
 185 density of dendritic spines and filopodia during synaptic development. In 4-week-old
 186 HFD/ND mice, only filopodium density was significantly greater than that in ND/ND
 187 mice, while the densities of total protrusions and spines were not different between
 188 groups (Figure 2c; protrusions: ND/ND: 0.38 ± 0.02 protrusions/ μm , $n = 13$; HFD/ND:
 189 0.40 ± 0.03 protrusions/ μm , $n = 18$; $p = 0.6432$; spines: ND/ND: 0.31 ± 0.02 spines/ μm ,
 190 $n = 13$; HFD/ND: 0.29 ± 0.02 spines/ μm , $n = 18$; $p = 0.3919$; filopodia: ND/ND: 0.070
 191 ± 0.006 filopodia/ μm , $n = 13$; HFD/ND: 0.11 ± 0.01 filopodia/ μm , $n = 18$; $p = 0.0243$;
 192 unpaired t test). At 6 weeks of age, there were no significant differences in protrusion,
 193 spine, or filopodium density between ND/ND and HFD/ND mice (Figure 2c;
 194 protrusions: ND/ND: 0.39 ± 0.02 protrusions/ μm , $n = 12$; HFD/ND: 0.38 ± 0.02
 195 protrusions/ μm , $n = 18$; $p = 0.8050$; spines: ND/ND: 0.33 ± 0.02 spines/ μm , $n = 12$;
 196 HFD/ND: 0.31 ± 0.02 spines/ μm , $n = 18$; $p = 0.5866$; filopodia: ND/ND: 0.06 ± 0.01

197 filopodia/ μm , $n = 12$; HFD/ND: 0.065 ± 0.007 filopodia/ μm , $n = 18$; $p = 0.5995$;
 198 unpaired t test). In 8-week-old mice, spine density was significantly lower whereas
 199 filopodium density was greater in HFD/ND mice than in ND/ND mice; however, total
 200 protrusion density was not significantly different (Figure 2c; protrusions: ND/ND: 0.39
 201 ± 0.01 protrusions/ μm , $n = 15$; HFD/ND: 0.36 ± 0.02 protrusions/ μm , $n = 20$; $p =$
 202 0.2621 ; spines: ND/ND: 0.35 ± 0.01 spines/ μm , $n = 15$; HFD/ND: 0.30 ± 0.01
 203 spines/ μm , $n = 20$; $p = 0.0194$; filopodia: ND/ND: 0.039 ± 0.004 filopodia/ μm , $n = 15$;
 204 HFD/ND: 0.061 ± 0.008 filopodia/ μm , $n = 20$; $p = 0.0340$; unpaired t test). In
 205 10-week-old HFD/ND mice, spine density was considerably lower than in ND/ND mice,
 206 while filopodium density was not different; therefore, total protrusion density was
 207 significantly lower (Figure 2c; protrusions: ND/ND: 0.39 ± 0.02 protrusions/ μm , $n = 11$;
 208 HFD/ND: 0.32 ± 0.01 protrusions/ μm , $n = 21$; $p = 0.0208$; spines: ND/ND: 0.35 ± 0.01
 209 spines/ μm , $n = 11$; HFD/ND: 0.28 ± 0.01 spines/ μm , $n = 21$; $p = 0.0005$; filopodia:
 210 ND/ND: 0.03 ± 0.02 filopodia/ μm , $n = 11$; HFD/ND: 0.047 ± 0.004 filopodia/ μm , $n =$
 211 21 ; $p = 0.3034$; unpaired t test). Note that, in normal synaptic development, the ratio of
 212 filopodia to spines is gradually decreased, but HFD/ND mice showed persistent higher
 213 ratio of filopodia to spines. These results indicate that the normal development of
 214 dendritic spines and filopodia in HFD/ND mice is impaired by maternal HFD, and the
 215 impairment of synaptic dynamics and morphology persists into adulthood.

216

217 3.3 *HFD-fed offspring from HFD-fed dams show a severe loss of dendritic spines*

218 Several reports indicate that HFD exposure in adulthood without maternal dietary effect
 219 causes serious damage to cognitive function, synaptic plasticity and spine density
 220 (Stranahan et al., 2008; Valladolid-Acebes et al., 2013; Wu et al., 2004). To evaluate the

221 damage of a western-style eating habit, in which both mothers and children are exposed
 222 to HFD throughout life, we investigated dynamics and morphology of dendritic spines
 223 and filopodia in maternal HFD-exposed offspring given HFD after weaning. We
 224 performed *in vivo* imaging in L1 dendrites of HFD-fed 8-week-old pups from HFD-fed
 225 dams (HFD/HFD), and compared the results with age-matched ND/ND, HFD/ND and
 226 ND/HFD groups (Figure 3a). ND/HFD group is a model of HFD exposure exclusively
 227 after weaning and is assigned to a control group. We analyzed the formation and
 228 elimination of dendritic spines and filopodia within 1 h (Figure 3b). Both formation and
 229 elimination rates of spines in HFD/HFD mice were significantly greater than those in
 230 ND/ND mice (Figure 3c; formation rate: ND/ND: $1.7 \pm 0.4\%$, $n = 15$; HFD/HFD: $6 \pm$
 231 1% , $n = 12$; $p = 0.0021$; elimination rate: ND/ND: $1.4 \pm 0.5\%$, $n = 15$; HFD/HFD: $11 \pm$
 232 2% , $n = 12$; $p = 0.0001$; one-way ANOVA followed by Tukey's test). There were no
 233 significant differences in spine formation and elimination rate among HFD/ND,
 234 ND/HFD and HFD/HFD mice (Figure 3c; formation rate: HFD/ND: $5.6 \pm 0.8\%$, $n = 20$;
 235 ND/HFD: $7.2 \pm 0.9\%$, $n = 16$; HFD/HFD: $6 \pm 1\%$, $n = 12$; elimination rate: HFD/ND:
 236 $10 \pm 1\%$, $n = 20$; ND/HFD: $12 \pm 1\%$, $n = 16$; HFD/HFD: $11 \pm 2\%$, $n = 12$; one-way
 237 ANOVA followed by Tukey's test). Formation and elimination rates of filopodia were
 238 significantly greater in HFD/HFD mice than those in ND/ND mice (Figure 3d;
 239 formation rate: ND/ND: $5 \pm 2\%$, $n = 15$; HFD/HFD: $25 \pm 6\%$, $n = 12$; $p = 0.0003$;
 240 elimination rate: ND/ND: $9 \pm 3\%$, $n = 15$; HFD/HFD: $28 \pm 5\%$, $n = 12$; $p = 0.0009$;
 241 one-way ANOVA followed by Tukey's test). There were no significant differences in
 242 rate of filopodium formation or elimination among HFD/ND, ND/HFD and HFD/HFD
 243 mice (Figure 3d; formation rate: HFD/ND: $40 \pm 4\%$, $n = 20$; ND/HFD: $46 \pm 5\%$, $n = 16$;
 244 HFD/HFD: $33 \pm 5\%$, $n = 12$; elimination rate: HFD/ND: $36 \pm 4\%$, $n = 20$; ND/HFD: 38

245 $\pm 6\%$, $n = 16$; HFD/HFD: $32 \pm 5\%$, $n = 12$; one-way ANOVA followed by Tukey's test).
 246 Note that HFD feeding after weaning caused synaptic instability even without the effect
 247 of maternal HFD. These data indicate that pre- and post-weaning HFD exposure leads
 248 to synaptic instability in later life, respectively, and that they have no additive effects on
 249 the dynamics of dendritic spines and filopodia. The density of dendritic spines in
 250 HFD/HFD group was notably lower than any other group (Figure 3e; spines: ND/ND:
 251 0.35 ± 0.01 spines/ μm , $n = 15$; HFD/ND: 0.30 ± 0.01 spines/ μm , $n = 20$; ND/HFD: 0.33
 252 ± 0.02 spines/ μm , $n = 16$; HFD/HFD: 0.23 ± 0.01 spines/ μm , $n = 12$; $p < 0.0001$
 253 (compared to ND/ND), $p = 0.0105$ (compared to HFD/ND), $p = 0.0004$ (compared to
 254 ND/HFD); one-way ANOVA followed by Tukey's test). There were no significant
 255 differences among all groups in filopodium density (Figure 3e; filopodia: ND/ND:
 256 0.039 ± 0.004 filopodia/ μm , $n = 15$; HFD/ND: 0.060 ± 0.008 filopodia/ μm , $n = 20$;
 257 ND/HFD: 0.064 ± 0.007 filopodia/ μm , $n = 16$; HFD/HFD: 0.060 ± 0.008 filopodia/ μm ,
 258 $n = 12$; one-way ANOVA followed by Tukey's test). As a result, total protrusion density
 259 in HFD/HFD group was lower than the others (Figure 3e; protrusions: ND/ND: $0.39 \pm$
 260 0.01 protrusions/ μm , $n = 15$; HFD/ND: 0.36 ± 0.02 protrusions/ μm , $n = 20$; ND/HFD:
 261 0.40 ± 0.03 protrusions/ μm , $n = 16$; HFD/HFD: 0.29 ± 0.01 protrusions/ μm , $n = 12$; $p =$
 262 0.0055 (compared to ND/ND), $p = 0.0429$ (compared to HFD/ND), $p = 0.0030$
 263 (compared to ND/HFD); one-way ANOVA followed by Tukey's test). Note that
 264 significant loss of dendritic spines is displayed only in HFD/HFD group. Interestingly,
 265 synaptic instability of HFD/HFD mice is equal to that of HFD/ND and ND/HFD mice
 266 although spine density was significantly lower only in HFD/HFD mice.

267

268 3.4 *Synaptic instability and loss of dendritic spines are caused even by limited*

269 *HFD exposure during lactation*

270 In order to elucidate the critical period when maternal HFD impairs the synaptic
271 dynamics and morphology in the mature offspring, we evaluated dynamics and
272 morphology of dendritic spines and filopodia in L1 dendrites of the offspring exposed to
273 maternal HFD exclusively during lactation (HFD_{lac}/ND) (Figure 4a). We analyzed the
274 formation and elimination of dendritic spines and filopodia over 1 h in 8-week-old
275 HFD_{lac}/ND mice *in vivo* (Figure 4b). Both formation and elimination rates of spines in
276 HFD_{lac}/ND mice were greater than those in ND/ND mice (Figure 4c; formation rate:
277 ND/ND: $1.7 \pm 0.4\%$, $n = 15$; HFD_{lac}/ND: $8 \pm 1\%$, $n = 15$; $p < 0.0001$; elimination rate:
278 ND/ND: $1.4 \pm 0.5\%$, $n = 15$; HFD_{lac}/ND: $11 \pm 2\%$, $n = 15$; $p < 0.0001$; one-way
279 ANOVA followed by Tukey's test). Furthermore, HFD_{lac}/ND mice showed greater
280 formation rate of spines than HFD/ND mice (Figure 4c; $p = 0.0401$; one-way ANOVA
281 followed by Tukey's test). Formation and elimination rates of filopodia were
282 significantly greater in HFD_{lac}/ND mice than those in ND/ND mice (Figure 4d;
283 formation rate: ND/ND: $5 \pm 2\%$, $n = 15$; HFD_{lac}/ND: $47 \pm 5\%$, $n = 15$; $p < 0.0001$;
284 elimination rate: ND/ND: $9 \pm 3\%$, $n = 15$; HFD_{lac}/ND: $44 \pm 8\%$, $n = 15$; $p = 0.0002$;
285 one-way ANOVA followed by Tukey's test). There were no significant differences in
286 rate of filopodium formation or elimination between HFD/ND and HFD_{lac}/ND mice
287 (Figure 4d). These data indicate that synaptic instability of the mature offspring is
288 triggered even by maternal HFD exposure exclusively during lactation. There were a
289 significant difference in spine density only between ND/ND and HFD_{lac}/ND group, and
290 spine density in HFD_{lac}/ND mice was lower than ND/ND mice (Figure 4e; spines:
291 ND/ND: 0.35 ± 0.01 spines/ μm , $n = 15$; HFD_{lac}/ND: 0.29 ± 0.02 spines/ μm , $n = 15$; $p =$
292 0.0273 ; one-way ANOVA followed by Tukey's test). There were no significant

293 differences among ND/ND, HFD/ND and HFD_{lac}/ND in filopodium density (Figure 4e;
294 filopodia: ND/ND: 0.039 ± 0.004 filopodia/ μm , $n = 15$; HFD/ND: 0.060 ± 0.008
295 filopodia/ μm , $n = 20$; HFD_{lac}/ND: 0.06 ± 0.01 filopodia/ μm , $n = 15$; one-way ANOVA
296 followed by Tukey's test) and total protrusion density (Figure 4e; protrusions: ND/ND:
297 0.39 ± 0.01 protrusions/ μm , $n = 15$; HFD/ND: 0.36 ± 0.02 protrusions/ μm , $n = 20$;
298 HFD_{lac}/ND: 0.35 ± 0.02 protrusions/ μm , $n = 15$; one-way ANOVA followed by Tukey's
299 test). It is interesting that the synaptic dynamics and morphology are more disrupted in
300 HFD_{lac}/ND mice than in HFD/ND mice.

301

302 3.5 *Antioxidant-treatment during lactation ameliorates the synaptic impairment* 303 *in the offspring from HFD-fed dams*

304 Several lines of evidence show that HFD-induced cognitive impairment is due to
305 oxidative stress in the brain (Souza et al., 2007; Zhang et al., 2005). Furthermore, we
306 have previously demonstrated that maternal HFD leads to peroxidized lipid
307 accumulations in the brain of the offspring during early postnatal development (Tozuka
308 et al., 2009). An index of lipid peroxidation, 2-thiobarbituric acid reactive substances, is
309 increased approximately 1.5-fold by maternal HFD in our experimental conditions
310 (Tozuka et al., 2009). Peroxidized lipid accumulations in the brain are significantly
311 decreased by the treatment of ascorbic acid, an antioxidant, in drinking water (Harrison
312 et al., 2010). According to those reports and the present results, we hypothesized that
313 maternal HFD-induced oxidative stress during lactation, when synapses are immature,
314 causes persistent impairments in synaptic development of the offspring, even if they are
315 fed with ND after weaning. In order to elucidate the involvement of lipid-mediated
316 oxidative stress during lactation in the synaptic impairments of the offspring, we treated

317 the HFD-fed dams with ascorbic acid, exclusively during lactation, and performed *in*
 318 *vivo* imaging in L1 dendrites of the 10-week-old offspring (HFD_{AA}/ND) (Figure 5a).
 319 ND_{AA}/ND mice are offspring from ND-fed and ascorbic acid-treated dams, and used as
 320 a control group. We analyzed the formation and elimination of dendritic spines and
 321 filopodia within 1 h (Figure 5b). Both formation and elimination rates of spines in
 322 HFD_{AA}/ND mice were significantly lower than those in HFD/ND mice (Figure 5c;
 323 formation rate: HFD/ND: $5.3 \pm 0.7\%$, $n = 21$; HFD_{AA}/ND: $1.7 \pm 0.5\%$, $n = 10$; $p =$
 324 0.0063 ; elimination rate: HFD/ND: $7 \pm 1\%$, $n = 21$; HFD_{AA}/ND: $1.7 \pm 0.4\%$, $n = 10$; $p =$
 325 0.0004 ; one-way ANOVA followed by Tukey's test). There were no significant
 326 differences in spine formation and elimination rate among ND/ND, ND_{AA}/ND and
 327 HFD_{AA}/ND mice (Figure 5c; formation rate: ND/ND: $2.9 \pm 0.9\%$, $n = 11$; ND_{AA}/ND:
 328 $2.0 \pm 0.7\%$, $n = 10$; HFD_{AA}/ND: $1.7 \pm 0.5\%$, $n = 10$; elimination rate: ND/ND: $1.1 \pm$
 329 0.4% , $n = 11$; ND_{AA}/ND: $0.9 \pm 0.3\%$, $n = 10$; HFD_{AA}/ND: $1.7 \pm 0.4\%$, $n = 10$; one-way
 330 ANOVA followed by Tukey's test). Although only elimination rate of filopodia was
 331 significantly decreased between HFD/ND and HFD_{AA}/ND mice (Figure 5d; formation
 332 rate: HFD/ND: $28 \pm 4\%$, $n = 21$; HFD_{AA}/ND: $17 \pm 2\%$, $n = 10$; $p = 0.2140$; elimination
 333 rate: HFD/ND: $32 \pm 6\%$, $n = 21$; HFD_{AA}/ND: $10 \pm 3\%$, $n = 10$; $p = 0.0315$; one-way
 334 ANOVA followed by Tukey's test), there were no significant differences in filopodium
 335 formation and elimination rate among ND/ND, ND_{AA}/ND and HFD_{AA}/ND mice (Figure
 336 5d; formation rate: ND/ND: $6 \pm 3\%$, $n = 11$; ND_{AA}/ND: $16 \pm 3\%$, $n = 10$; HFD_{AA}/ND:
 337 $17 \pm 2\%$, $n = 10$; elimination rate: ND/ND: $2 \pm 2\%$, $n = 11$; ND_{AA}/ND: $12 \pm 4\%$, $n = 10$;
 338 HFD_{AA}/ND: $10 \pm 3\%$, $n = 10$; one-way ANOVA followed by Tukey's test). In spine
 339 density, although there were no significant differences between HFD_{AA}/ND and
 340 HFD/ND mice, there were also no significant differences among ND/ND, ND_{AA}/ND

341 and HFD_{AA}/ND mice (Figure 5e; spines: ND/ND: 0.35 ± 0.01 spines/ μm , $n = 11$;
 342 HFD/ND: 0.28 ± 0.01 spines/ μm , $n = 21$; ND_{AA}/ND: 0.37 ± 0.02 spines/ μm , $n = 10$;
 343 HFD_{AA}/ND: 0.32 ± 0.02 spines/ μm , $n = 10$; one-way ANOVA followed by Tukey's test).
 344 There were no significant differences among all groups in filopodium density (Figure
 345 5e; filopodia: ND/ND: 0.03 ± 0.02 filopodia/ μm , $n = 11$; HFD/ND: 0.047 ± 0.004
 346 filopodia/ μm , $n = 21$; ND_{AA}/ND: 0.044 ± 0.007 filopodia/ μm , $n = 10$; HFD_{AA}/ND:
 347 0.045 ± 0.006 filopodia/ μm , $n = 10$; one-way ANOVA followed by Tukey's test). In
 348 protrusion density, there were no significant differences between HFD_{AA}/ND and
 349 HFD/ND mice, and there were also no significant differences among ND/ND,
 350 ND_{AA}/ND and HFD_{AA}/ND mice (Figure 5e; protrusions: ND/ND: 0.39 ± 0.02
 351 protrusions/ μm , $n = 11$; HFD/ND: 0.32 ± 0.01 protrusions/ μm , $n = 21$; ND_{AA}/ND: 0.42
 352 ± 0.02 protrusions/ μm , $n = 10$; HFD_{AA}/ND: 0.37 ± 0.03 protrusions/ μm , $n = 10$;
 353 one-way ANOVA followed by Tukey's test). These data indicate that ascorbic acid
 354 treatment during lactation completely rescued the instability of dendritic spines, and
 355 partially ameliorated the filopodium instability and spine density.
 356

357 4. Discussion

358 A typical diet in the developed world is an energy-rich diet containing high levels of fats
359 and sugars. Chronic consumption of this type of diet is associated with adverse effects
360 on health, and perinatal HFD exposure also causes similar effects on the offspring.
361 Many women are overweight or obese by the time they reach childbearing age (Huda et
362 al., 2010; Kanagalingam et al., 2005). Therefore, there is a great need to elucidate the
363 mechanisms by which maternal HFD can affect synaptic development and brain
364 function. In the present study, we found that maternal HFD led to instability of dendritic
365 spines and filopodia in the cerebral cortex of juvenile offspring even, when they were
366 fed with a ND after weaning. These negative effects persisted into adulthood with a
367 decline in dendritic spines. HFD-fed offspring from HFD-fed dams showed a severe
368 decline in dendritic spines, whereas synaptic instability in HFD/HFD mice was equal to
369 that in HFD/ND and ND/HFD mice. Synaptic instability and loss of spines in the
370 offspring exposed to maternal HFD exclusively during lactation were greater than or
371 equal to those in the offspring exposed to pre- and postnatal maternal HFD.
372 Antioxidant-treatment during lactation ameliorated the synaptic impairment in the
373 offspring born to HFD-fed dams. These results suggest that maternal obesity causes
374 sustained synaptic impairments in the offspring, which may be associated with brain
375 dysfunction in adulthood, and that these impairments may be due to oxidative stress
376 from peroxidized lipid accumulations during lactation. The perinatal metabolic milieu is
377 one of the most important factors that have an impact on neuronal circuitry development
378 and brain function in adulthood. This study provides the first evidence of the synaptic
379 basis of the brain dysfunction in the offspring from obese dams, using *in vivo* imaging
380 while maintaining the internal environment of the body.

381 4.1 *Animal models of synaptic development in children of obese mothers*

382 In the present study, we used a well-established animal model of maternal obesity.
383 Dams were fed HFD 6 weeks prior to mating until weaning. The protocol is known to
384 replicate human pathology in the offspring, such as abnormal insulin and glucose
385 metabolism, energy balance, cardiovascular function and adiposity (Elahi et al., 2009;
386 Ferezou-Viala et al., 2007; Samuelsson et al., 2008; Tozuka et al., 2010; Tozuka et al.,
387 2009). We have previously demonstrated that the offspring from HFD-fed dams shows
388 increased body weight and blood glucose without any changes in food intake (Tozuka et
389 al., 2009). Other models of maternal obesity also demonstrate converging effects on the
390 brain and peripheral organs (Williams et al., 2013). We can compare the results of these
391 different model studies in the context of maternal obesity.

392 In order to evaluate the synapse pathologies of maternal obesity, we
393 performed *in vivo* two-photon imaging because there are large contributions from
394 peripheral organs and non-neuronal cells in the offspring from HFD-fed dams showing
395 abnormal metabolic homeostasis (Bilbo and Tsang, 2010; Williams et al., 2013).
396 Furthermore, analyzing the dynamics of dendritic spines and filopodia in living animals
397 enabled us to detect minute synaptic impairments; there are some cases in which only
398 protrusion dynamics change, without any changes in protrusion number when synaptic
399 plasticity and pathological changes are elicited (Cruz-Martin et al., 2010; Trachtenberg
400 et al., 2002). Perinatal and chronic HFD exposure leads to inflammation in the brain and
401 activation of microglia (Bilbo and Tsang, 2010; Tapia-Gonzalez et al., 2011) that
402 remove synapses (Trapp et al., 2007). Even in a resting state, microglia regulate
403 synaptic pruning during development (Paolicelli et al., 2011) and plasticity (Tremblay et
404 al., 2010). Therefore, we used the thinned-skull method allowing excitation and

405 emission lights to penetrate the skull without inflammatory responses (Xu et al., 2007).

406

407 4.2 *Synaptic basis of maternal HFD-induced brain dysfunction in the adult*
408 *offspring*

409 Previous studies have demonstrated the effects of perinatal HFD exposure on the brain
410 by evaluating molecular and behavioral alterations (Franco et al., 2012; Mischke et al.,
411 2013; Peleg-Raibstein et al., 2012; Rodriguez et al., 2012). We previously reported
412 deficits in hippocampal neurogenesis and dendritic differentiation of newborn neurons
413 in the brain of offspring exposed to HFD perinatally (Tozuka et al., 2010; Tozuka et al.,
414 2009). However, the synaptic basis of brain dysfunction in the offspring from HFD-fed
415 dams remained unclear. In the present study, we investigated the synaptic
416 endophenotype of the offspring exposed to maternal HFD to fill the gap between the
417 molecular and behavioral phenotype, and found that the offspring showed synaptic
418 instability and loss of dendritic spines. The loss of dendritic spines may be due to the
419 slightly higher elimination rate of dendritic spines than formation rate; however,
420 long-term *in vivo* imaging lasting for more than a week would be required to elucidate
421 the relationship between the dynamics and density of dendritic spines. The lower
422 density of spines and the higher density of filopodia suggest the deficits in synaptic
423 maturation because the ratio of filopodia to spines decreases with development
424 (Grutzendler et al., 2002).

425 Maternal HFD feeding exclusively during lactation disrupts metabolic
426 homeostasis in the offspring via impairing neuronal circuitry formation in the
427 hypothalamus (Vogt et al., 2014). HFD exposure during adolescence leads to cognitive
428 dysfunction, whereas exposure during young adulthood has no effect

429 (Valladolid-Acebes et al., 2013). These reports and our present study suggest that the
430 synaptic developmental stage is one of the most susceptible periods to HFD toxicity
431 impairing brain function in the adult offspring. Maternal HFD exposure during
432 pregnancy confers adaptive protection against mesenteric artery dysfunction caused by
433 maternal HFD during lactation (Khan et al., 2005). This report is in consistent with our
434 result that HFD exposure exclusively during lactation induces more severe deficits than
435 HFD exposure during gestation and lactation.

436 Several lines of evidence suggest that perinatal HFD exposure causes
437 developmental delay in the brain including decreased neurogenesis, apoptosis, neuronal
438 differentiation, and behavioral deficits (Giriko et al., 2013; Niculescu and Lupu, 2009;
439 Tozuka et al., 2010; Tozuka et al., 2009). The turnover rate of dendritic spines and the
440 ratio of filopodia to total protrusions decrease with neuronal circuitry development
441 (Grutzendler et al., 2002). In a mouse model of fragile X syndrome, a genetic
442 neurodevelopmental disorder, increased turnover rate and immature morphology of
443 dendritic protrusions in the mice are considered manifestations of developmental delay
444 (Cruz-Martin et al., 2010). In this context, our present findings that the offspring from
445 HFD-fed dams exhibit higher turnover rate of dendritic protrusions throughout synaptic
446 development and more filopodium density at the juvenile stage suggest that a delay in
447 neuronal circuit development may be one of the synaptic mechanisms of maternal
448 HFD-induced brain dysfunction. Increased risk of developmental disorders, such as
449 ADHD (Ray et al., 2009; Rodriguez et al., 2008) and autism spectrum disorder (Dodds
450 et al., 2011; Krakowiak et al., 2012) in children from obese mothers also supports the
451 above hypothesis.

452 Maternal obesity is associated with inflammation and excess levels of insulin

453 and leptin, causing perturbations in the development of neural circuitry (Sullivan et al.,
454 2014), but the mechanisms for HFD programming of synaptic development and
455 behavior remain largely unknown. Our preceding study showed that maternal HFD
456 leads to peroxidized lipid accumulations in the cerebral cortex, and the product of the
457 lipid peroxidation reduces neural progenitor cell proliferation (Tozuka et al., 2009).
458 Peroxidized lipid accumulations in the cerebral cortex are reduced by the treatment of
459 ascorbic acid in drinking water (Harrison et al., 2010). Furthermore, oxidative stress
460 precedes the onset of HFD-induced obesity and insulin resistance (Matsuzawa-Nagata
461 et al., 2008). Together, the results from our previous and present studies suggest that
462 peroxidized lipid accumulation is one of the earliest steps in HFD-induced pathogenesis,
463 and exposure to oxidative stress during neuronal circuitry development may result in
464 subsequent synaptic deficits and behavioral impairments.

465

466 *4.3 Convergence of synapse pathology in maternal obesity and other*
467 *neuropsychiatric diseases: implications for comorbidity*

468 Many animal models of neuropsychiatric disorders including Alzheimer's disease (Tsai
469 et al., 2004), Parkinson's disease (Villalba and Smith, 2010), vascular dementia (Sigler
470 and Murphy, 2010), autism spectrum disorder (Hatanaka et al., 2015; Hung et al., 2008;
471 Isshiki et al., 2014), schizophrenia (Fenelon et al., 2013), depression (Gourley et al.,
472 2013; Liston and Gan, 2011) and anxiety disorder (Buras et al., 2013) share pathologies
473 of the synapses: protrusion instability and/or decreased mature dendritic protrusions.
474 These synaptic pathologies are also shown in our model of maternal obesity, and
475 maternal obesity is linked to an elevated risk of these neuropsychiatric disorders
476 (Sullivan et al., 2014; Van Lieshout et al., 2011). Such findings suggest that synaptic

477 instability and immaturity may underlie the comorbidity between maternal obesity and
478 neuropsychiatric disorders. According to this commonality in the synapses, peripheral
479 pathologies used as biomarkers of perinatal HFD exposure could be applicable in the
480 diagnosis of neuropsychiatric diseases. A combined model study of maternal obesity
481 and neuropsychiatric disorders is necessary to clarify the underlying mechanisms of
482 their comorbidity.

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497

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679

680 **Legends**

681 **Figure 1.** Maternal HFD leads to instability of dendritic spines and filopodia in the
682 adult offspring. **a**, Schematic diagram of maternal and offspring diet protocols. In the
683 ND/ND group, dams were fed ND throughout gestation and lactation. In the HFD/ND
684 group, dams were fed HFD throughout gestation and lactation. In both groups, dams
685 and pups were fed ND from lactational day 16. **b**, *In vivo* two-photon time-lapse
686 imaging of L1 dendrites from L5 pyramidal neurons in 10-week-old ND/ND ($n = 11$
687 dendrites from four mice) and HFD/ND ($n = 21$ dendrites from seven mice) mice.
688 Repeated images of the identical dendrites over 1 h in each group show increased
689 formation (filled arrowheads) and elimination (open arrowheads) of dendritic spines
690 (filled arrow) and filopodia (open arrow). Images are best projections (3–7 optical
691 sections, 0.75 μm apart). **c**, Percentages of spines formed or eliminated as a proportion
692 of total spines. HFD/ND mice showed a greater elimination rate of spines. **d**,
693 Percentages of filopodia formed or eliminated as a proportion of total filopodia. Both
694 formation and elimination rate were greater in HFD/ND mice. Note that both the
695 formation and elimination rates of filopodia are much higher than those of spines in the
696 control (ND/ND) group. Data are presented as mean $\pm$ SEM. ** $p < 0.01$, Student's
697 t -test. Scale bar, 5 μm .

698

699 **Figure 2.** Maternal HFD-exposed offspring develop synaptic instability and loss of
700 dendritic spines during synaptic maturation. **a**, Turnover rates of spines (ratio of spines
701 formed and eliminated to twice the total number of spines) at 4 weeks (ND/ND: $n = 13$
702 dendrites from five mice; HFD/ND: $n = 18$ dendrites from five mice), 6 weeks
703 (ND/ND: $n = 12$ dendrites from four mice; HFD/ND: $n = 18$ dendrites from five mice),

8 weeks (ND/ND: $n = 15$ dendrites from five mice; HFD/ND: $n = 18$ dendrites from seven mice) and 10 weeks of age. HFD/ND mice showed higher spine turnover rates than ND/ND mice throughout maturation. **b**, Turnover rates of filopodia at 4, 6, 8 and 10 weeks of ages. HFD/ND mice showed higher filopodium turnover rates than ND/ND mice from 6 weeks of age. **c**, The density of dendritic protrusions including both spines and filopodia, spines and filopodia in ND/ND and HFD/ND mice at each age. In HFD/ND mice, the loss of dendritic spines was developed with aging whereas filopodium density was slightly higher at each age. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA followed by Bonferroni's test (**a**, **b**) or Student's *t*-test (**c**).

Figure 3. HFD exposure both before and after weaning causes significant loss of dendritic spines. **a**, Experimental paradigm for maternal and offspring diets. In the ND/ND group, both dams and pups were fed ND until imaging. In the HFD/ND group, dams were fed HFD during gestation and lactation, and pups were fed ND after weaning. In the ND/HFD group, dams were fed ND, and pups were fed HFD after weaning. In the HFD/HFD group, both dams and pups were fed HFD. **b**, *In vivo* two-photon imaging over 1 h of L1 dendrites from L5 pyramidal neurons in 8-week-old ND/ND, HFD/ND, HFD/ND ($n = 16$ dendrites from four mice) and HFD/HFD ($n = 12$ dendrites from four mice) mice. Repeated images of the identical dendrites over 1 h in each group show a higher rate of formation (filled arrowheads) and elimination (open arrowheads) of dendritic spines and filopodia in HFD/ND, ND/HFD and HFD/HFD groups. Images are best projections (3–7 optical sections, 0.75 μm apart). **c**, Percentages of spines formed or eliminated as a proportion of total spines. HFD/HFD mice exhibited greater

728 spine formation and elimination rates than ND/ND mice. **d**, Percentages of filopodia
729 formed or eliminated as a proportion of total filopodia. HFD/HFD mice showed greater
730 rates of filopodium formation and elimination than ND/ND mice, but there were no
731 significant differences in spine and filopodium dynamics among HFD/ND, ND/HFD
732 and HFD/HFD groups. **e**, Dendritic protrusion density in ND/ND, HFD/ND, ND/HFD
733 and HFD/HFD mice. HFD/HFD mice showed a lower total protrusion density as a
734 result of having significantly fewer spines. HFD/HFD mice had fewer spines than both
735 HFD/ND and ND/HFD mice. Data are presented as mean $\pm$ SEM. ** $p < 0.01$, *** $p <$
736 0.001 (compared with ND/ND), $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ (compared with HFD/ND), $\ddagger p$
737 < 0.05 , $\ddagger\ddagger\ddagger p < 0.001$ (compared with ND/HFD), one-way ANOVA followed by
738 Tukey's test. Scale bar, 5 μm .

739

740 **Figure 4.** Maternal HFD exclusively during lactational stage leads to synaptic
741 instability and loss of dendritic spines. **a**, Schematic diagram of maternal and offspring
742 diets. In the HFD_{lac}/ND group, dams were fed HFD exclusively during lactation, and
743 pups were fed ND after weaning. **b**, *In vivo* two-photon imaging over 1 h of an identical
744 L1 dendrite from L5 pyramidal neurons in 8-week-old HFD_{lac}/ND group ($n = 15$
745 dendrites from seven mice). Formation (filled arrowheads) and elimination (open
746 arrowhead) of dendritic spines and filopodia are indicated. Images are best projections
747 (3–7 optical sections, 0.75 μm apart). **c**, Percentages of spines formed or eliminated as a
748 proportion of total spines in ND/ND, HFD/ND and HFD_{lac}/HFD mice. HFD_{lac}/ND mice
749 exhibited greater spine formation and elimination rates than ND/ND mice. **d**,
750 Percentages of filopodia formed or eliminated as a proportion of total filopodia.
751 HFD_{lac}/ND mice showed higher rates of filopodium formation and elimination than

752 ND/ND mice. **e**, Dendritic protrusion density. HFD_{lac}/ND mice showed a lower spine
753 density than ND/ND mice. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$,
754 *** $p < 0.001$ (compared with ND/ND), † $p < 0.05$ (compared with HFD/ND), one-way
755 ANOVA followed by Tukey's test. Scale bar, 5 μ m.

756

757 **Figure 5.** Antioxidant intake during lactation ameliorates the synaptic instability
758 induced by maternal HFD in the adult offspring. **a**, Schematic diagram of maternal and
759 offspring diets. In the ND_{AA}/ND group, dams were fed ND with administration of
760 ascorbic acid in drinking water during lactation, and pups were fed ND after weaning.
761 In the HFD_{AA}/ND group, dams were fed HFD throughout gestation and lactation with
762 administration of ascorbic acid during lactation, and pups were fed ND after weaning. **b**,
763 *In vivo* two-photon imaging over 1 h of identical L1 dendrites from L5 pyramidal
764 neurons in 10-week-old ND_{AA}/ND ($n = 10$ dendrites from six mice) and HFD_{AA}/ND (n
765 $= 10$ dendrites from four mice) mice. Formation (filled arrowheads) and elimination
766 (open arrowhead) of dendritic spines and filopodia are indicated. Images are best
767 projections (3–7 optical sections, 0.75 μ m apart). **c**, Percentages of spines formed or
768 eliminated as a proportion of total spines in ND/ND, HFD/ND, ND_{AA}/ND and
769 HFD_{AA}/ND mice. HFD_{AA}/ND mice showed lower spine formation and elimination rates
770 than HFD/ND mice. **d**, Percentages of filopodia formed or eliminated as a proportion of
771 total filopodia. HFD_{AA}/ND mice showed slightly lower rates of filopodium formation
772 and elimination than HFD/ND mice. **e**, Dendritic protrusion density. HFD_{AA}/ND mice
773 exhibited slightly higher density of dendritic spines than HFD/ND mice, but there was
774 no significant difference between the two groups. Data are presented as mean $\pm$ SEM.
775 ** $p < 0.01$, *** $p < 0.001$ (compared with ND/ND), † $p < 0.05$, †† $p < 0.01$, ††† $p <$

776 0.001 (compared with HFD/ND), one-way ANOVA followed by Tukey's test. Scale bar,

777 5 μm .

Figure 1.

a

Spine Turnover Ratio (%)

Age in Weeks

ND/ND (dashed line, open squares)

HFD/ND (solid line, filled squares)

*** p < 0.001, ** p < 0.01, * p < 0.05

Age in Weeks	ND/ND (%)	HFD/ND (%)
4	~4.0	~12.5
6	~2.0	~8.0
8	~2.0	~8.0
10	~2.0	~6.0

b

Filopodium Turnover Ratio (%)

Age in Weeks

ND/ND (dashed line, open squares)

HFD/ND (solid line, filled squares)

*** p < 0.001

Age in Weeks	ND/ND (%)	HFD/ND (%)
4	~28.0	~35.0
6	~12.0	~35.0
8	~8.0	~38.0
10	~5.0	~30.0

c

4-week-old

Protrusion Density (protrusions/ μ m)

ND/ND (white bars)

HFD/ND (black bars)

* p < 0.05

Protrusion Type	ND/ND (protrusions/ μ m)	HFD/ND (protrusions/ μ m)
Protrusions	~0.38	~0.40
Spines	~0.31	~0.30
Filopodia	~0.07	~0.11

d

6-week-old

Protrusion Density (protrusions/ μ m)

ND/ND (white bars)

HFD/ND (black bars)

Protrusion Type	ND/ND (protrusions/ μ m)	HFD/ND (protrusions/ μ m)
Protrusions	~0.39	~0.38
Spines	~0.33	~0.32
Filopodia	~0.06	~0.07

e

8-week-old

Protrusion Density (protrusions/ μ m)

ND/ND (white bars)

HFD/ND (black bars)

* p < 0.05

Protrusion Type	ND/ND (protrusions/ μ m)	HFD/ND (protrusions/ μ m)
Protrusions	~0.39	~0.37
Spines	~0.35	~0.30
Filopodia	~0.04	~0.07

f

10-week-old

Protrusion Density (protrusions/ μ m)

ND/ND (white bars)

HFD/ND (black bars)

* p < 0.05, *** p < 0.001

Protrusion Type	ND/ND (protrusions/ μ m)	HFD/ND (protrusions/ μ m)
Protrusions	~0.39	~0.33
Spines	~0.35	~0.28
Filopodia	~0.03	~0.05

Figure 3.

Figure 4.

Figure 5.