1 **3,5,6,7,8,3',4'-heptamethoxyflavone, a citrus flavonoid, on**
2 **protection against memory impairment and neuronal cell death**
3 **in a global cerebral ischemia mouse model**

4
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25 **Abstract**

26 The present study evaluated the effects of treatment with the citrus flavonoid,
27 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF) on protection against memory impairment
28 and neuronal death in a global cerebral ischemia mouse model. The results showed that
29 HMF, administrated for three days immediately after ischemic surgery, protected
30 against ischemia-induced memory dysfunction, rescued neuronal cell death in the CA1
31 cell layer, increased the production of BDNF, stimulated the auto phosphorylation of
32 CaMK II and suppressed microglial activation in the hippocampus. These results
33 suggest that HMF has a neuro protective effect after brain ischemia by inducing BDNF
34 production and anti-inflammatory effects.

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36

37 **Key words:**

38 Heptamethoxyflavone; global cerebral ischemia; hippocampus; BDNF; neuronal cell
39 protection; anti-inflammation

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41

42 **1. Introduction**

43 Ischemic stroke is a serious risk to human health because it is accompanied by
44 cognitive impairments (Horn and Schlote, 1992; Zola-Morgan et al., 1986).
45 Ischemia/reperfusion leads to neuronal cell death due to reductions in ATP levels,
46 glutamate excitotoxicity, membrane depolarization, increases in cellular calcium levels,
47 and the formation of reactive oxygen and nitrogen species that culminate in oxidative
48 stress (Dirnagl et al., 1999; Juurlink and Sweeney, 1997). After ischemic onset,
49 inflammatory cells such as blood-derived leukocytes and microglia are activated and
50 accumulate within brain tissue, subsequently leading to inflammatory injury. Delayed
51 neuronal cell death commences several hours after ischemic stroke and lasts for several
52 days (Broughton et al., 2009), with the main vulnerable regions of delayed neuronal cell
53 death being the cerebral cortex, striatum, and hippocampus (Yonekura et al., 2004). As
54 the hippocampus is an important region for learning and memory (Neves et al., 2008),
55 the majority of stroke/ischemia survivors suffer from various cognitive dysfunctions.
56 Thus, the prevention and treatment of neuronal cell death following ischemia have
57 become increasingly important. Evidence has recently been accumulated to show that
58 brain-derived neurotrophic factor (BDNF), one of the representative neurotrophic
59 factors in the brain, potentially plays a neuroprotective role in ischemic brains (Larsson

60 et al., 1999).

61 We previously demonstrated that 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF; Fig.1),
62 a citrus flavonoid, significantly induced the activation (phosphorylation) of cAMP
63 response element-binding protein (CREB), which is known as a transcription factor that
64 regulates BDNF gene expression (Furukawa et al., 2012). In our subsequent study, we
65 administered HMF for five days before bilateral common carotid artery occlusion
66 (2-vessel occlusion: 2VO), and showed, using brain tissues dissected three days after
67 surgery (Okuyama et al., 2012), that 1) HMF induced CREB phosphorylation in the
68 hippocampus in ischemic brains; 2) HMF increased BDNF expression in the
69 hippocampus, and that most BDNF-positive cells were merged with glial fibrillary
70 acidic protein (GFAP)-positive astrocytes; and 3) HMF increased neurogenesis in the
71 dentate gyrus subgranular zone. These results indicated that HMF could be a good
72 candidate for a preventive drug against cerebral ischemia. In the present study, we
73 investigated the therapeutic effect of treatment with HMF on the ischemic brain
74 starting immediately after 2VO surgery though the previous experimental design was
75 scheduled to start HMF treatment five days before the ischemic surgery (Okuyama et
76 al., 2012).

77 Here, we successfully demonstrated that HMF increased the production of BDNF,
78 followed by the suppression of neuronal death and protection against ischemia-induced
79 memory dysfunction.

80

81

82 **2. Material and methods**

83 **2.1 Animals**

84 Nine-week-old male C57BL/6 strain mice were purchased from Japan SLC
85 (Hamamatsu, Japan). Mice in all groups were kept at 23 ± 1 °C on a 12-h light/dark cycle
86 (light on 8:00-20:00). Tap water and feed were freely available during the experimental
87 period. All animal experiments were carried out in accordance with the Guidelines for
88 Animal Experimentation and approved by the Animal Care and Use Committee of
89 Matsuyama University.

90

91 **2.2 Global cerebral ischemia procedure**

92 Mice were anesthetized with 1.0-2.0 % isoflurane and subjected to bilateral
93 common carotid artery occlusion (2-vessel occlusion: 2VO) as previously reported
94 (Okuyama et al., 2012, 2013a). To induce transient global ischemia, micro-aneurysm
95 clips (#14120, 30 g pressure; World Precision Instruments, Sarasota, FL) were applied
96 for 12 min to occlude arteries and stop blood flow. Sham control animals received the
97 same surgical treatment without arterial occlusion. Core body temperature was
98 maintained during surgery with a rectal probe and a heated blanket (37.0 ± 0.5 °C), and
99 brain temperature was monitored with a tympanic membrane probe into the ear and

100 maintained at 36.5 ± 0.2 °C. Mice were excluded if their brain temperatures were out of
101 range of the criteria during ischemic surgery because hypo or hyperthermia have been
102 shown to attenuate ischemic damage in the brain (Harukuni and Bhardwaj, 2006;
103 Lampe and Becker, 2011). After surgery, all mice were placed in a recovery cage under a
104 heat lamp and had free access to drinking water.

105

106 **2.3 Drug treatment**

107 HMF was prepared from commercial orange oil (Wako, Osaka, Japan) as described
108 previously (Furukawa et al., 2012), and dissolved in DMSO/PEG300 (1:1) solution.
109 There were five experimental groups (n=9 in each groups); sham-control group (Sham),
110 2VO-control group (2VO), 2VO surgery and HMF (25 mg/kg/day)-treatment group
111 (HMF25), 2VO surgery and HMF (50 mg/kg/day)-treatment group (HMF50), and 2VO
112 surgery and edaravone (3-methy-1-phenyl-2-pyrazolin-5-one; Toronto Research
113 Chemicals Inc., Toronto, Canada; 3 mg/kg/day)-treatment group (EV3). Drugs or vehicle
114 (DMSO/PEG300) were subcutaneously administrated using an Alzet osmotic pump
115 (1003D, 1.0µL/h; DURECT Corporation, Cupertino, CA). Osmotic pump implantation
116 started just following ischemic surgery and was continued for three days.

117

118 **2.4 Y-maze test**

119 The Y-maze device consisted of three arms (L × W × H: 35 × 8.5 × 15 cm) at 120
120 degrees to each other. Each mouse was placed at the end of one arm and was allowed to
121 move freely through the maze during an 8-min session. The total distance travelled
122 (locomotor activity) and alternation behavior was analyzed with the ANY-maze Video
123 Tracking System (Stoelting, Wood Dale, IL) connected to a USB digital camera. An
124 alternation was defined as entries into all three arms on consecutive occasions. The
125 percentage of alternations was calculated as (number of alternations/total arm
126 entries-2) × 100.

127

128 **2.5 Immunofluorescence for confocal microscopy**

129 Mice were transcardially perfused with ice-cold PBS three days after the surgery.
130 Brains were removed and postfixed as described in our previous study (Sato et al., 2008;
131 Xu et al., 2010). Sagittal sections at 30 µm were incubated with the following primary
132 antibodies; rabbit anti-BDNF (dilution 1:150; Epitomics, Burlingame, CA), mouse
133 anti-GFAP (1:200; Sigma-Aldrich, St. Louis, MO), goat anti-doublecortin (DCX, 1:50;
134 Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho
135 calcium-calmodulin-dependent protein kinase II (p-Thr286 CaMK II, 1:500;

136 Sigma-Aldrich), mouse anti-neuronal nuclei (NeuN, 1:300; Millipore, Billerica, MA).
137 Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1:300; Invitrogen, Carlsbad, CA), Alexa
138 Fluor 488 donkey anti-goat IgG (H+L) (1:300), Alexa Fluor 568 goat anti-rabbit IgG
139 (H+L) (1:300), and Alexa Fluor 568 goat anti-mouse IgG (H+L) (1:300) were used as
140 secondary antibodies. A mounting medium with DAPI was used (Vectashield; Vector
141 Laboratories, Burlingame, CA), and images were captured with a confocal fluorescence
142 microscopy system (LSM510; Zeiss, Oberkochen, Germany). Fluorescence signals in the
143 images were quantified using Image J software (NIH, Bethesda, MD) as described
144 previously (Okuyama et al., 2012, 2013a). The location of the captured images and
145 quantification shows with square in the figure.

146

147 **2.6 Immunohistochemistry for optical microscopy**

148 Sagittal sections at 30 μ m were incubated in 3 % H₂O₂, followed by 5 % normal goat
149 serum blockade, and were immune stained at 4 °C overnight using a rabbit polyclonal
150 antibody against ionized calcium binding adaptor molecule 1 (IBA1, 1:1000; Wako,
151 Osaka, Japan) to stain microglia. EnVision-plus system-HRP labeled polymer (anti
152 rabbit; Dako, Glostrup, Denmark) was used as the secondary antibody.
153 Immunoreactivity was developed and visualized using DAB substrate (SK-4100; Vector

154 Laboratories), and quantified using Image J software (NIH). The location of the
155 captured images and quantification shows with square in the figure.

156 A mouse monoclonal antibody against microtubule-associated protein-2 (MAP-2,
157 1:500; Sigma-Aldrich) was used for neuronal cell dendrites staining and the secondary
158 antibody was EnVision-plus HRP labeled polymer (anti mouse; Dako). Sections on slides
159 were immersed in 0.1 % Cresyl violet solution for Nissl staining. After coverslipping
160 slides, the number of viable-looking neurons with defined cell membranes and nuclei in
161 the hippocampal pyramidal cell layer of CA1 region were counted under a microscope
162 field (CX21; Olympus, Tokyo, Japan) at 1000× magnification.

163

164 **2.7 Statistical analysis**

165 Data for individual groups are expressed as means $\pm$ SEM. Data were analyzed by a
166 one-factor ANOVA followed by Bonferroni's Multiple Comparison Test (Prism 5;
167 GraphPad Software, La Jolla, CA). A value of $P < 0.05$ was considered to be significant.

168

169

170 3. Results

171 3.1 Suppressive effect of HMF on ischemia-induced behavioral abnormalities

172 The effect of HMF on ischemic-induced memory dysfunction was examined as
173 spontaneous alternation behavior in the Y-maze, which is considered to reflect
174 short-term memory (Li et al., 2010). Figure 2A shows that the percentage of
175 spontaneous alternations in the 2VO-control group (54.3 ± 6.7 %) was significantly
176 lower ($*p<0.05$) than that in the Sham group (75.1 ± 2.3 %). The percentage of
177 spontaneous alternations in the HMF25 group (76 ± 5.5 %) and HMF50 group ($74.3 \pm$
178 5.6 %) was significantly higher ($\#p<0.05$) than that in the 2VO-control group. As a
179 positive control group in this experiment, edaravone, a radical scavenger, was also
180 administered to mice after 2VO surgery (EV3 group). The percentage of spontaneous
181 alternations in the EV3 group was 79.4 ± 8.8 % ($\#p<0.01$ vs. 2VO group).

182 As locomotor hyperactivity has been known to frequently occur after cerebral
183 ischemia (Bobyne et al., 2005), we next analyzed the effect of HMF on ischemic-induced
184 locomotor hyperactivity as the total distance travelled in the Y-maze. Figure 2B shows
185 that the total distance travelled by the 2VO group (30.2 ± 2.9 m) was significantly
186 longer ($*p<0.05$) than that by the Sham group (21.3 ± 1.3 m). Total distances travelled
187 by the HMF25 group and HMF50 group were 27.8 ± 2.3 m and 20.3 ± 2.9 m, respectively,

188 which indicated that ischemia-induced hyperactive behavior was significantly
189 suppressed ($p<0.05$) in the HMF50 group. The administration of edaravone slightly
190 suppressed locomotor hyperactivity ($p=0.094$, vs 2VO). These results demonstrate that
191 HMF has the ability to ameliorate ischemia-induced memory dysfunction and
192 behavioral abnormalities.

193

194 **3.2 Neuroprotective effect of HMF in the hippocampal CA1 region of ischemic brains**

195 It has been established that pyramidal cells in the CA1 region of the hippocampus
196 are vulnerable to transient global cerebral ischemia (Bobyn et al., 2005; Lu et al., 2012;
197 Olsson et al., 2003); therefore, we investigated whether HMF contributes a
198 neuroprotective effect in ischemic brains using immunohistochemical methods
199 (MAP-2/Nissl staining). Figure 3A shows that the pyramidal cells with dendrites, which
200 stained with both Cresyl violet and the anti-MAP-2 antibody, was less in the 2VO group
201 (Fig. 3A-b) than in the Sham group (Fig. 3A-a), and the number of intact cells,
202 viable-looking neurons with defined cell membranes and nuclei, in the hippocampal
203 pyramidal cell layer of CA1 region was significantly reduced (Fig. 3B, $***p<0.001$). On
204 the other hand, the number of intact neuronal cells was maintained in HMF
205 treated-groups (Fig. 3A-c, d). In this experiment, we used edaravone, a novel radical

206 scavenger drug that is widely used in Japan for acute ischemic stroke, as a positive
207 control because it was shown to prevent cerebral ischemia-induced neuronal cell death
208 (Lu et al., 2012). Figure 3A-e shows that the number of intact neuronal cells was
209 maintained in the EV3 group. As shown in Figure 3B, HMF at a dose of 25 mg/kg/day or
210 50 mg/kg/day had a neuroprotective effect ($\#p<0.05$ and $###p<0.001$, respectively)
211 equivalent to that of the EV3 group ($###p<0.001$). Other than the hippocampus, the
212 striatum is known to be a fragile area in the global ischemia 2VO model (Olsson et al.,
213 2003). We then investigated the neuroprotective effect of HMF in the striatum. Figure
214 3A-f-j showed that neuronal cell protection by HMF was also observed in the striatum.
215 These results indicated that HMF had a neuroprotective effect against cerebral
216 ischemia, and its effect was equivalent to that of edaravone.

217

218 **3.3 Stimulatory effect of HMF on BDNF production in ischemic brains**

219 We previously showed that pretreatment with HMF for five days before 2VO
220 increased the expression of BDNF in the hippocampus three days after surgery, and
221 that most BDNF signals were also stained with GFAP (Okuyama et al., 2012). In the
222 present experiment, BDNF immunoreactivity (green) was minimal in the dentate gyrus
223 of the Sham group (Fig. 4A-a), but was higher in the 2VO group (Fig. 4A-d) as

224 previously reported (Okuyama et al., 2012; Sharp et al., 2000). Similarly, activated
225 astrocytes (red) were rarely observed in the Sham group (Fig. 4A-b), but were markedly
226 increased in the 2VO group (Fig. 4A-e). Most BDNF positive signals merged with
227 anti-GFAP antibody staining (Fig. 4A-c, f). As well as in the case of pretreatment with
228 HMF (Okuyama et al., 2012), BDNF immunoreactivity in the hippocampus was further
229 increased in ischemic brains with the HMF treatment (Fig. 4A-g, j), and they were also
230 merged with GFAP signals (Fig. 4A-i, l). As shown in Figure 4B, signal counts showed
231 that the ability of HMF to induce BDNF expression was dose-dependent, and that the
232 values observed in HMF-treated groups were significantly higher than those in the 2VO
233 group (HMF25 group, $^{##}p<0.01$; HMF50 group, $^{###}p<0.001$). These results showed that
234 the HMF treatment induced BDNF synthesis in astrocytes activated by ischemia.

235

236 **3.4 Enhancement of neurogenesis by the HMF treatment in ischemic brains**

237 BDNF has been shown to stimulate neurogenesis in the dentate gyrus subgranular
238 zone (SGZ) following global ischemia (Kee et al., 2001; Liu et al., 1998). We previously
239 demonstrated that pretreatment with HMF accelerated neurogenesis in the dentate
240 gyrus SGZ and subventricular zone (SVZ) after ischemia (Okuyama et al., 2012). We
241 then examined whether treatment with HMF affected neurogenesis in ischemic brains

242 in a similar manner to pretreatment with HMF. Figure 5 shows the immunoreactivities
243 of DCX, a marker of neuronal precursor cells, in the SGZ. DCX-positive cells (green)
244 were rarely observed in the 2VO group (Fig. 5-b), but markedly increased in number
245 with the HMF treatment (Fig. 5-c, d). These results suggest that the HMF treatment
246 accelerated neurogenesis in the SGZ following ischemia similar to that by pretreatment
247 with HMF (Okuyama et al., 2012).

248

249 **3.5 Restoratory effect of HMF on ischemia-induced inhibition of CaMK II** 250 **autophosphorylation**

251 CaMK II, a multifunctional serine/threonine protein kinase in neuronal tissues, is
252 highly abundant in the hippocampus (Erondy and Kennedy, 1985) and is known to be
253 essential for hippocampus-dependent memory formation. Total CaMK II activity in the
254 hippocampus was shown to decrease following transient brain ischemia (Yamamoto et
255 al., 2009), which was correlated with the extent of neuronal damage after ischemia
256 (Hanson et al 1994). These findings prompted us to evaluate the effect of HMF on the
257 expression of phosphorylated CaMK II (p-CaMK II) in the hippocampal
258 CA1-CA3-dentate gyrus region of ischemic brains. Figure 6 shows that the
259 immunoreactivity of p-CaMK II (green) was observed in the pyramidal cell layer of the

260 CA1 region, along mossy fibers projecting to the CA3 region, and in granular cells of the
261 dentate gyrus (Fig. 6-a); however, signals in the 2VO group were very weak in all
262 regions (Fig. 6-d). In contrast, the immunoreactivity of p-CaMK II in HMF-treated
263 groups increased in a dose-dependent manner (Fig. 6-j). Phosphorylated CaMK
264 II-positive cells (Fig. 6-a,d,g,j) and NeuN-positive neurons (Fig. 6-b,e,h,k) were merged
265 (Fig. 6-c,f,i,l). These results suggested that the HMF treatment significantly protected
266 the ischemia-induced decrease in phosphorylated CaMK II in neurons in the
267 hippocampus.

268

269 **3.6 Suppressive effect of HMF on ischemia-induced inflammation**

270 Inflammation is a pathophysiological phenomenon that is involved in ischemia
271 (Wang et al., 2007). On the other hand, HMF was recently shown to have an
272 anti-inflammatory effect on human monocytes stimulated by lipopolysaccharide (LPS)
273 (Manthey et al., 1999) and LPS-injected mice (Manthey and Bendele, 2008). Therefore,
274 we evaluated the effect of HMF on microglial activation in ischemic brains.

275 Microglia are known to be responsible for inflammatory reactions in the central
276 nervous system, and activated microglial has been shown to increase in ischemic brains
277 (Kim et al., 2009; Uno et al., 1997; Okuyama et al., 2013a). Microglial cells

278 immunostained with the IBA1 antibody were observed in a ramified form (an
279 inactivated form) in the hippocampus of the Sham group (Fig. 7A-a, b). However,
280 microglial cells were condensed and in a hypertrophied form (an activated form called
281 ameboid microglia) in the 2VO group (Fig. 7A-c, d), and the expression of IBA1 was
282 significantly increased (Fig. 7B, *** $p < 0.001$). The ramified form of microglia was also
283 observed in the HMF25 group (Fig. 7A-e, f), and the signals of the hypertrophied form of
284 microglia (Fig. 7B) was significantly ($p < 0.05$) less than that of the 2VO group. In the
285 HMF50 group, the hypertrophied form of microglia further decreased (Fig. 7A-g, h), and
286 the IBA1 positive signals (Fig. 7B) of these cells was significantly (#### $p < 0.001$) less than
287 that of the 2VO group.

288

289

290 4. Discussion

291 It has been established that 1) pyramidal cells in the CA1 region of the hippocampus
292 are vulnerable to transient global cerebral ischemia (Bobyn et al., 2005; Lu et al., 2012;
293 Olsson et al., 2003); 2) the expression of neurotrophins including BDNF is up-regulated
294 in the brain after an ischemic insult (Sharp et al., 2000); and 3) BDNF is critically
295 involved in protection against ischemic cell damage (Schabitz et al., 1997). The results
296 of the present study showed that treatment with HMF immediately following 2VO
297 surgery effectively increased the expression of BDNF by astrocytes (Fig. 4) and
298 enhanced neurogenesis (Fig. 5) in the mouse hippocampus, similar to that by
299 pretreatment with HMF (Okuyama et al, 2012). Non neuronal-BDNF producing cells
300 have been shown to differ according to the delay after stroke induction; namely, neuron
301 and ependymal cells at 4 h and 24 h, microglial cells and endothelial cells at 24 h, and
302 then astrocytes (Béjot et al., 2011), suggesting a possible involvement of these cells in
303 the mechanism of neuronal survival after ischemia. We also demonstrated here that the
304 HMF treatment significantly protected ischemia-induced neuronal cell death in the
305 CA1 cell layer (Fig. 3), which then contributed to rescue against ischemia-induced
306 memory dysfunction and behavioral abnormalities (Fig. 2). This is the first report
307 showing that HMF had a neuroprotective effect in mice following brain ischemia.

308 Regarding the mechanism for the neuroprotective effect of HMF, we suggest that it
309 was mediated through CREB phosphorylation, followed by the up-regulation of BDNF
310 synthesis, and subsequent autophosphorylation of CaMK II. CREB is a transcription
311 factor that regulates BDNF gene expression, and we previously showed that HMF had
312 the ability to induce a sustained increase in the expression of phosphorylated CREB and
313 its upstream kinase, extracellular signal-regulated kinases 1/2 (ERK1/2), in ischemic
314 brains (Okuyama et al., 2012) and cultured neurons (Furukawa et al., 2012).
315 Phosphodiesterase (PDE)-3 inhibitor increases CREB phosphorylation (Tanaka et al.,
316 2010), and HMF shows inhibitory effect of PDE3 and PDE4 *in vitro* (Takeda et al., 2009;
317 Manthey et al., 1999). As for CaMK II, we showed that the HMF treatment induced a
318 sustained increase in the expression of phosphorylated CaMK II in ischemic brains (Fig.
319 6). As BDNF has previously been shown to increase CaMK II activity (Blanquet and
320 Lamour, 1997), we suggest that the elevation in BDNF expression of HMF was followed
321 by the induction of CaMK II phosphorylation. On the other hand, CaMK II is a
322 downstream kinase of the N-methyl-D-aspartate (NMDA) glutamate receptor, and the
323 stimulation of NMDA receptors has been shown to elevate intracellular Ca²⁺ levels in
324 cultured neurons and subsequently increase the levels of CaMK II autophosphorylation
325 (Fukunaga et al., 1989). Whether HMF directly affects glutamate receptors such as the

326 NMDA receptor has not yet been investigated. A previous study showed that nobiletin
327 (5,6,7,8,3',4'-hexamethoxy flavone; NBT), another *citrus* polymethoxyflavone, had a
328 neuroprotective effect against delayed neuronal death in the hippocampal CA1 region
329 10 days after ischemia through CaMK II and CREB phosphorylation (Yamamoto et al.,
330 2009), and that NBT also had the ability to phosphorylate the GluR1 receptor
331 (Matsuzaki et al., 2008).

332 This is the first report to show that HMF may act as an anti-inflammatory agent in
333 the brain (Fig. 7), and we presumed the suppression of inflammation is the additional
334 important mechanism for the neuroprotective effect of HMF. There are several reports
335 that polymethoxylated flavones demonstrate anti-inflammatory effects. For example, 1)
336 HMF has anti-inflammatory ability (i.e., reduced LPS-induced TNF- α production in
337 human monocytes; Manthey et al., 1999); 2) nobiletin, another polymethoxylated
338 flavone, shows anti-inflammatory response in cultured microglia (i.e., suppressed
339 LPS-induced TNF- α and IL-1 β production, and inhibited NF κ B signaling; Cui et al.,
340 2010). In the experiments using human monocytes (Manthey et al., 1999), HMF has
341 been shown to suppress the inflammation by PDE-dependent and -independent
342 mechanism. In addition, PDE inhibitors are known to suppress tumor necrosis factor
343 (TNF)- α production (Semmler et al., 1993), and our preliminary experiment showed that

344 HMF has the ability to suppress LPS-induced expression of TNF- α in cultured
345 astrocytes (unpublished data). On the whole, we suggested that HMF has a possibility
346 to suppress inflammation in the brain in part by suppression of PDE activity. In
347 addition, it is well known that proinflammatory cytokines are regulated at the
348 transcriptional levels by several transcription factors, such as nuclear factor-kappa B
349 (NFkB). Effect on this issue of HMF remains to be resolved.

350 In order to use HMF as a therapeutic agent, HMF has to pass through the
351 blood-brain barrier (BBB). We previously showed that the administration of HMF to
352 mice treated with an NMDA receptor antagonist MK-801, and HMF protected the
353 MK-801-induced deterioration in spatial learning performance in the Morris
354 water-maze task (Furukawa et al., 2012) and in the open field test (Okuyama et al.,
355 2013b). This result suggested that HMF may pass through the BBB because it is not
356 impaired in these model mice (Imer et al., 2009; Kuhlmann et al., 2009). To support this
357 finding, recent studies showed that 1) NBT, which has 6 methoxy groups instead of 7 in
358 HMF, was detected in the brains of mice following its *i.p.* or *p.o.* administration (Saigusa
359 et al., 2011); and 2) 4'-demethylnobiletin, a bioactive metabolite of NBT, was detected in
360 the brains of mice following its *i.p.* administration (Al Rahim et al., 2009).

361

362 **5. Conclusions**

363 When HMF was administrated immediately after ischemic surgery, it 1) rescued
364 against ischemia-induced memory dysfunction; 2) protected neuronal cell death in the
365 CA1 cell layer; 3) increased the production of BDNF in the hippocampus; 4) stimulated
366 neurogenesis in the dentate gyrus subgranular zone; 5) activated the
367 autophosphorylation of CaMK II; and 6) suppressed microglial activation in the
368 hippocampus. These results suggest that HMF has a neuroprotective effect after brain
369 ischemia by inducing BDNF/CaMK II and anti-inflammatory effects. HMF may be a
370 candidate as a novel neuroprotective drug candidate against cerebral ischemia.

371

372

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377

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566

567 **Figure legends**

568 **Figure 1**

569 Structure of 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF).

570

571 **Figure 2**

572 The effect of HMF on ischemia-induced impairments in short-term memory (A) and
573 ischemia-induced locomotor hyperactivity (B) in the Y-maze test. Values are means $\pm$
574 SEM (n=9 for each group). Symbols are significantly different for the following
575 conditions; vs Sham (*p<0.05) and vs 2VO (#p<0.05, ##p<0.01).

576

577 **Figure 3**

578 The effect of HMF on the expression of MAP-2 immunoreactivity counterstained
579 with Cresyl violet solution in the ischemic mouse hippocampus CA1 region and
580 striatum. (A) Sagittal sections three days after bilateral common carotid artery
581 occlusive ischemic surgery were stained with the anti-MAP-2 antibody. Pictures show
582 the hippocampal CA1 region (a~e) and striatum (f~j). Scale bar shows 10 μ m. (B)
583 Quantitative analysis of the number of intact cells in the hippocampal CA1 region. The
584 location of the captured images and quantification shows with square in the figure.

585 Values are means $\pm$ SEM (12 sections in each group). Symbols are significantly different
586 for the following conditions; vs Sham (** $p < 0.001$) and vs 2VO ($\#p < 0.05$, $###p < 0.001$).

587

588 **Figure 4**

589 The effect of HMF on the expression of BDNF and GFAP immunoreactivity in the
590 ischemic mouse hippocampus. (A) Sagittal sections three days after bilateral common
591 carotid artery occlusive ischemic surgery were stained with specific antibodies, either
592 anti-BDNF (green; a, d, g, j) or anti-GFAP (red; b, e, h, k), and nuclei with DAPI (blue).
593 Scale bar shows 100 μ m. (B) Quantitative analysis of BDNF positive signal counts using
594 Image J software. The location of the captured images and quantification shows with
595 square in the figure. Values are means $\pm$ SEM (18 sections in each group). Symbols are
596 significantly different for the following conditions; vs Sham ($*p < 0.05$) and vs 2VO
597 ($\#p < 0.01$, $###p < 0.001$).

598 **Figure 5**

599 The effect of HMF on the expression of DCX immunoreactivity in the ischemic
600 mouse hippocampal dentate gyrus. Sagittal sections three days after bilateral common
601 carotid artery occlusive ischemic surgery were stained with the anti-DCX antibody
602 (green) and nuclei with DAPI (blue). Scale bar shows 100 μ m.

603 **Figure 6**

604 The effect of HMF on the expression of phosphorylated CaMK II and NeuN
605 immunoreactivity in the ischemic mouse hippocampus. Sagittal sections three days
606 after bilateral common carotid artery occlusive ischemic surgery were stained with
607 specific antibodies, either anti-p-CaMK II (green; a, d, g, j) or anti-NeuN (red; b, e, h, k).
608 Scale bar shows 200 μ m.

609

610 **Figure 7**

611 The effect of HMF on the expression of IBA1 immunoreactivity in the ischemic
612 mouse hippocampus. (A) Sagittal sections three days after bilateral common carotid
613 artery occlusive ischemic surgery were stained with the anti-IBA1 antibody. Lower
614 magnification pictures were taken at 100 $\times$ magnification (a, c, e, g) and higher were at
615 400 $\times$ magnification (b, d, f, h). Scale bar shows 100 μ m (Low) and 10 μ m (High). (B)
616 Quantitative analysis of IBA1-positive signals using Image J software. The location of
617 the captured images and quantification shows with square in the figure. Values are
618 means $\pm$ SEM (12 sections in each group). Symbols are significantly different for the
619 following conditions; vs Sham (**p<0.001) and vs 2VO (#p<0.05, ###p<0.001).

620

Figure 1

Figure 2

(A)

(B)

Figure 3

(A)

(B)

Figure 4

(A)

(B)

Figure 5

Figure 6

Figure 7

(A)

(B)