

1 **Presynaptically silent synapses are modulated by the density of surrounding
2 astrocytes**

4 Kohei Oyabu¹, Kotomi Takeda¹, Hiroyuki Kawano², Kaori Kubota^{1,3}, Takuya
5 Watanabe^{1,3}, N. Charles Harata², Shutaro Katsurabayashi^{1,*}, Katsunori Iwasaki^{1,3}

7 1. Department of Neuropharmacology, Faculty of Pharmaceutical Sciences, Fukuoka
8 University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

9 2. Department of Molecular Physiology and Biophysics, University of Iowa Carver
10 College of Medicine, Iowa City, Iowa 52242, United States of America

11 3. A.I.G. Collaborative Research Institute for Aging and Brain Sciences, Fukuoka
12 University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

14 *Correspondence should be addressed to:

15 Shutaro Katsurabayashi, Ph.D.

16 Department of Neuropharmacology, Faculty of Pharmaceutical Sciences,
17 Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan.

18 E-mail: shutarok@fukuoka-u.ac.jp

19 Tel: (+81) 92-871-6631 (ext. 6634)

20 Fax: (+81) 92-863-0389

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27 **ABSTRACT**

28 The astrocyte, a major glial cell type, is involved in formation and maturation of
29 synapses, and thus contributes to sustainable synaptic transmission between neurons.
30 Given that the animals in the higher phylogenetic tree have brains with higher density of
31 glial cells with respect to neurons, there is a possibility that the relative astrocytic
32 density directly influences synaptic transmission. However, the notion has not been
33 tested thoroughly. Here we addressed it, by using a primary culture preparation where
34 single hippocampal neurons are surrounded by a variable but countable number of
35 cortical astrocytes in dot-patterned microislands, and recording synaptic transmission by
36 patch-clamp electrophysiology. Neurons with a higher astrocytic density showed a
37 higher amplitude of evoked excitatory postsynaptic current (EPSC) than that of neurons
38 with a lower astrocytic density. The size of readily releasable pool of synaptic vesicles
39 per neuron was significantly higher. The frequency of spontaneous synaptic
40 transmission (miniature EPSC) was higher, but the amplitude was unchanged. The
41 number of morphologically identified glutamatergic synapses was unchanged, but the
42 number of functional ones was increased, indicating a lower ratio of presynaptically
43 silent synapses. Taken together, the higher astrocytic density enhanced excitatory
44 synaptic transmission by increasing the number of functional synapses through
45 presynaptic un-silencing.

46

47 **Keywords**

48 astrocyte, excitatory synaptic transmission, neurotransmitter release, silent synapse

49

50 **Abbreviations**

51 ANLS: astrocyte-neuron lactate shuttle
52 APV: (2R)-amino-5-phosphonovaleric acid
53 CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione
54 DAPI: 4',6-diamidino-2-phenylindole
55 EPSC: excitatory postsynaptic current
56 FM1-43: N-(3-triethylammoniumpropyl)-4-(4- (dibutyl amino) styryl) pyridinium
57 dibromide
58 HDG: high-density group
59 LDG: low-density group
60 LED: light-emitting diode
61 MAP2: microtubule-associated protein 2
62 mEPSC: miniature excitatory postsynaptic current
63 PBS: phosphate-buffered saline
64 Pvr: vesicular release probability
65 RRP: readily releasable pool
66 TTX: tetrodotoxin
67 VGLUT1: vesicular glutamate transporter 1
68 Vh: holding potential
69

70 **INTRODUCTION**

71 While neurons are indispensable for information processing through neural circuits, it
72 has been reported that glial cells play essential roles in brain physiology and
73 development (Barres, 2008). Dynamic information processing in the brain is not only
74 due to the interaction between neurons, but also to the interaction between neurons and
75 astrocytes, a major type of glial cells. In particular, the structure and function of
76 synapses, the basic information-processing units of neurons, are closely regulated by
77 astrocytes. For example, astrocytes are involved in the regulation of synaptic strength
78 (Haydon, 2001) and synapse formation (Nedergaard et al., 2003). Neurons co-cultured
79 with astrocytes have higher synaptic efficacy compared with neurons in the absence of
80 astrocytes, based on direct contact with and humoral factors secreted by the astrocytes
81 (Pfrieger et al., 1997; Ullian et al. 2001; Hama et al., 2004; Crawford et al., 2012;
82 Sobieski, et al, 2015). Furthermore, astrocytes are essential in long-term memory
83 acquisition (Suzuki et al., 2011). These findings suggest that astrocytes perform an
84 essential role in higher brain functions such as memory and learning.

85 Glial cells and neurons possess one notable evolutionary feature. The ratio of the
86 number of glia to that of neurons in the brain (glia/neuron ratio) is higher for animal
87 species in the higher phylogenetic tree (Nedergaard et al., 2003). For example, the ratio
88 is 0.03-0.05 in the leech ganglia, but it increases to approximately 0.3 in rodent cerebral
89 cortex, and reaches approximately 1.4 in human cerebral cortex. Thus, this evolutionary
90 principle seems to be associated with the complexity of brain functions. The glia/neuron
91 ratio is loosely correlated with the brain size (higher ratios with larger brains), but is
92 tightly and negatively correlated with neuronal density (higher ratios with lower
93 neuronal densities) (Herculano-Houzel, 2009, 2014; von Bartheld et al., 2016). The low
94 neuronal density in human brains is considered to reflect the large size of the neurons
95 (Herculano-Houzel, 2014). In support of these anatomical findings, human cerebral
96 cortical neurons are larger than the rat counterparts, and the longer human dendrites
97 were shown to increase electrical compartmentalization, change the neuronal input-
98 output properties, and therefore influence the synaptic integration and computation
99 (Beaulieu-Laroche, et al, 2018). These findings illustrate that the glia/neuron ratios are
100 linked with key functional differences of neurons, and are important in comparing
101 different animals' species. However, the glia/neuron ratios also vary in different brain
102 regions of a given species, including humans (Azevedo et al., 2009). The direct impact
103 of such intra-species variations in glia/neuron ratios has been unexplored.

104 This study thus evaluated the influence of different astrocyte densities on synaptic
105 transmission in a given animal species. This was achieved by co-culturing single mouse
106 neurons on microislands of mouse astrocytes, and recording synaptic transmission in
107 each microisland by patch-clamp electrophysiology. By counting the number of
108 astrocytes, we were able to define the astrocyte/neuron ratio for each microisland. The

109 electrophysiological results were separated into two groups, according to the low or
110 high densities of astrocytes. We show that the excitatory synaptic transmission is
111 enhanced with the increased astrocyte density, through a presynaptic mechanism.
112
113

114 **EXPERIMENTAL PROCEDURES**

115 **Animals**

116 All procedures regarding animal care were performed in strict accordance with the rules
117 of the Experimental Animal Care and Welfare Committee of Fukuoka University,
118 following approval of the experimental protocol (Permit Numbers: 1602907 and
119 1712128). Timed-pregnant Jcl:ICR mice (Catalogue ID: Jcl:ICR, CLEA Japan, Inc.,
120 Tokyo, Japan) were purchased at gestational day 15 from the Kyudo Company (Tosu,
121 Japan). Fifteen to seventeen-week-old pregnant Jcl:ICR mice were used. The
122 bodyweights of the pregnant mice were not recorded. A pregnant mouse was housed
123 individually in a plastic mouse-cage in temperature-controlled rooms ($23 \pm 2^\circ\text{C}$) at our
124 animal facility with a 12-hour light-dark cycle. Food (CLEA Rodent Diet, CE-2, CLEA
125 Japan, Inc., Tokyo, Japan) and water were provided *ad libitum*.
126

127 **Autaptic neuron culture**

128 Astrocytes and neurons from newborn timed-pregnant Jcl:ICR mice were cultured as
129 described previously (Bekkers and Stevens, 1991; Oyabu et al. 2019). In brief, cerebral
130 cortices were obtained from newborn mice of either sex at postnatal days 0–1. The
131 cerebral cortices were trypsinized and dissociated. Cells were cultured in 75 cm² culture
132 flasks (Corning Inc., Corning, NY, USA). After 2 weeks, non-astrocytic cells were
133 removed by tapping the culture flask several times. After that, astrocytes were isolated
134 and plated at a density of 6,000 cells/ cm² per well onto 22-mm coverslips (thickness
135 no. 1; Matsunami, Osaka, Japan) in 6-well plates (TPP, Switzerland). The coverslips
136 were first coated with 0.5% agarose, and then were stamped with a 1:1 mixture of rat-
137 tail collagen (final concentration 1.0 mg/mL, BD Biosciences, San Jose, CA, USA) and
138 poly-D-lysine (final concentration 0.25 mg/mL, Sigma-Aldrich, St Louis, MO, USA) in
139 300-μm square islands. After 1 week, neurons were obtained from the hippocampus of
140 another newborn mouse of either sex at postnatal days 0–1. The dissociated neurons
141 were plated at a density of 1,500 cells/cm² per well onto the micro-island astrocytes.
142 The cells were cultured in a humidified incubator at 37°C with 5% CO₂ for 13–18 days
143 before they were used for electrophysiological, immunocytochemical and functional
144 imaging experiments.
145

146 **Electrophysiology**

147 Synaptic responses were recorded from autaptic neurons, using a patch-clamp amplifier

148 (Multi-Clamp 700B, Molecular Devices, Sunnyvale, CA, USA), in the whole-cell
149 configuration under the voltage-clamp mode, at a holding potential (V_h) of -70 mV, and
150 room temperature (23 ± 2°C) in all cases. Patch-pipette resistance was 4–5 MΩ, and
151 70%–90% of access resistance was compensated. Autaptic neurons showed the evoked
152 synaptic transmission in response to an action potential elicited by a brief (2 ms)
153 somatic depolarization pulse (to 0 mV) from the patch pipette. The synaptic responses
154 were recorded at a sampling rate of 20 kHz and were filtered at 10 kHz. Data were
155 excluded from analysis if a leak current of >300 pA was observed. The data were
156 analyzed offline using AxoGraph X 1.2 software (AxoGraph Scientific, Berkeley, CA,
157 USA). Miniature excitatory postsynaptic currents (mEPSCs) were recorded for 100 sec.
158 They were detected with an amplitude threshold of 5 pA, using AxoGraph X 1.2
159 software. The evoked EPSC and mEPSC were confirmed to be mediated by the
160 excitatory neurotransmitter glutamate, based on an effective block of the EPSCs by
161 CNQX (data not shown)

162

163 **Nuclear staining in live cells**

164 Immediately before patch-clamp recording, the nuclei in live cells were stained using
165 NucBlue™ Live ReadyProbes™ reagent (Thermo Fisher Scientific, Waltham, MA,
166 USA). Briefly, two drops of NucBlue Live ReadyProbes Reagent were added to the
167 culture dish per milliliter of medium. The cells were then incubated in a humidified
168 incubator at 37°C with 5% CO₂ for 20 min, under protection from ambient light. The
169 stained cells were observed on an inverted microscope (Eclipse-Ti2-U, Nikon, Tokyo,
170 Japan), equipped with a 365-nm LED light source (KSL70, Rapp OptoElectronic,
171 Hamburg, Germany) and a filter cube (375/28-nm excitation, 415-nm dichroic long-
172 pass, 460/60-nm emission).

173

174 **Immunocytochemistry**

175 Autaptic neurons were immuno-stained, as described previously (Kawano et al., 2012;
176 Oyabu et al. 2019). Primary antibodies were used at the following dilutions: anti-
177 microtubule-associated protein 2 (MAP2), 1:1,000 (guinea pig polyclonal, antiserum,
178 Synaptic Systems, Göttingen, Germany), anti-vesicular glutamate transporter 1 (anti-
179 VGLUT1), 1:2,000 (rabbit polyclonal, affinity-purified, Synaptic Systems). Appropriate
180 secondary antibodies conjugated to Alexa Fluor 488 (anti-guinea pig) or 594 (anti-
181 rabbit) (Thermo Fisher Scientific, Waltham, MA, USA) were used at a dilution of 1:400.
182 Cell nuclei were visualized by counterstaining with DAPI contained in the mounting
183 medium (ProLongH Gold antifade mounting reagent, Thermo Fisher Scientific,
184 Waltham, MA, USA). Autaptic neurons were observed using a confocal microscope
185 (LSM710, Carl Zeiss, Oberkochen, Germany) with a 40× objective lens (C-
186 Apochromat, numerical aperture 1.2) to count the number of excitatory glutamatergic

187 synapses (for Figure 4).

188

189 **Identification of presynaptically active synapses using FM1-43**

190 Presynaptic terminals that actively release neurotransmitters, namely, active synapses,
191 were quantified in autaptic neuronal cultures using N-(3-triethylammoniumpropyl)-4-
192 (4-(dibutyl amino) styryl) pyridinium dibromide (FM1-43, Thermo Fisher Scientific,
193 Waltham, MA, USA), similarly as in our previous report (Kawano et al., 2012). Briefly,
194 the synaptic recycling vesicles were loaded with 10 μ M FM1-43 in a high-potassium
195 (45 mM) extracellular solution containing the NMDA receptor antagonist (2R)-amino-
196 5-phosphonovaleric acid (APV, 25 μ M, Sigma-Aldrich, St Louis, MO, USA) and the
197 AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μ M,
198 Sigma Aldrich), for 2 min at room temperature. The coverslip was washed three times
199 for 2 minutes each with a standard extracellular solution containing 1 μ M tetrodotoxin
200 (TTX), a Na⁺ channel blocker, to remove excess FM1-43. Autaptic neurons were then
201 fixed in a 4% paraformaldehyde solution of phosphate-buffered saline (PBS) for 10 min.
202 To minimize the loss FM1-43 signals (e.g. by photobleaching by ambient light), the
203 images were captured soon after the neurons were fixed. Sixteen-bit images were
204 acquired with a scientific CMOS camera (pco.edge 4.2, pco, Kelheim, Germany) on an
205 inverted microscope (Eclipse-TiE, Nikon, Tokyo, Japan) with a 40 \times objective lens (Plan
206 Apo λ , numerical aperture 0.95). FM1-43 was excited using a white LED (Lambda HPX,
207 Sutter Instruments, Novato, CA, USA) at 100% of maximum intensity, and imaged
208 using a filter cube (470/40-nm excitation, 595-nm dichroic long-pass, 535/50-nm
209 emission). In each sample, ten images were captured with the exposure time of 300 ms
210 per image, averaged, and used for analysis based on the average-intensity of the pixels.

211

212 **Identification of presynaptically silent synapses**

213 After taking images of FM1-43 puncta, the fixed autaptic neuron was blocked and
214 permeabilized with PBS containing 5% normal goat serum and 0.1% Triton X-100 for
215 30 min in the microscope chamber. After blocking and permeabilizing, it was confirmed
216 that the FM1-43 puncta were completely de-stained (data not shown). The samples were
217 then immuno-stained for VGLUT1 and MAP2 as described above. This step was also
218 performed in the microscope chamber (Kawano et al., in preparation). Although the
219 emission spectrum of Alexa Fluor 488 considerably overlapped that of FM1-43, the
220 acquisition of one signal did not interfere with the acquisition of the other. This was
221 because the FM1-43 signal was lost completely by treatment with 0.1% Triton X-100,
222 which was necessary for immuno-staining procedure before Alexa Fluor 488 signal was
223 acquired. Alexa Fluor 488 (for MAP2) was therefore imaged using the same optical
224 system as for the FM1-43. Alexa Fluor 594 (for VGLUT1) was excited using a white
225 LED at 100% of maximum intensity, and imaged using a filter cube (560/40-nm

226 excitation, 595-nm dichroic long-pass, 630/60-nm emission). Ten images per sample
227 were captured as for the FM1-43 puncta, with the exposure time of 300 ms per image,
228 and then averaged.

229 To identify presynaptically silent synapses, the image of FM1-43 was merged with
230 that of VGLUT1 and MAP2 using ImageJ (1.48v, Wayne Rasband, NIH, available at
231 <http://imagej.nih.gov/ij/>). For this purpose, the grayscale images of MAP2, FM1-43 and
232 VGLUT1 were converted to pseudo-color images in blue, green and red, respectively
233 (for Figure 5). The VGLUT1 puncta that were not stained with FM1-43 were defined as
234 presynaptically silent synapses.

235

236 **Solutions**

237 The standard extracellular solution was: 140 mM NaCl, 2.4 mM KCl, 2 mM CaCl₂, 1
238 mM MgCl₂, 10 mM glucose, 10 mM HEPES (pH 7.4, 320 mOsm). The extracellular
239 solution for application of FM1-43 was: 97.4 mM NaCl, 45 mM KCl, 2 mM CaCl₂, 1
240 mM MgCl₂, 10 mM glucose, 10 mM HEPES (pH 7.3, 320 mOsm). Patch pipettes were
241 filled with an intracellular solution (146.3 mM K-gluconate, 0.6 mM MgCl₂, 2.4 mM
242 ATP-Na₂, 0.3 mM GTP-Na₂, 50 U/ml creatine phosphokinase, 12 mM phosphocreatine,
243 1 mM EGTA, 17.8 mM HEPES, pH 7.4). Miniature excitatory postsynaptic currents
244 (mEPSCs) were recorded using the standard extracellular solution containing 1 μM
245 TTX. Hypertonic solutions for determining the size of the readily releasable pool from
246 synaptic vesicles (RRP) were prepared by adding 0.5 M sucrose to the standard
247 extracellular solution. The extracellular solutions were applied using a fast-flow
248 application system (SF-77B, Warner Instruments, Hamden, CT, USA). Each flow pipe
249 has a large diameter (430 μm), ensuring that the solution is applied to all parts of an
250 autaptic neuron on an astrocytic micro island (300 × 300 μm). This configuration was
251 suitable for the application of sucrose to induce synaptic responses from all nerve
252 terminals of the recorded neuron. All chemicals were purchased from Sigma Aldrich (St
253 Louis, MO, USA), except where otherwise specified.

254

255 **Statistical analysis**

256 Data were expressed as the mean ± SEM. Statistical analysis was performed using
257 Student's unpaired *t*-test for the comparison of two groups. Significance was considered
258 when *p* < 0.05.

259

260

261 **RESULTS**

262 **Excitatory synaptic transmission is enhanced with high density of astrocytes**

263 We used autaptic cultures (Bekkers et al. 1991, Oyabu et al. 2019) to assess whether a
264 difference in astrocyte densities around neurons affects synaptic transmission. This

265 preparation has two major advantages. First, it allows single neurons to be plated on
266 variable numbers of astrocytes per microisland. By combining this advantage with live-
267 cell nuclear staining, we can count the number of astrocytes in each microisland and
268 classify the synaptic transmission phenotypes based on the astrocytic density. Second,
269 the neurotransmitter release from any nerve terminal of a single identified neuron can be
270 detected by patch-clamp electrophysiological recording, albeit with different
271 sensitivities due to different distances of postsynaptic receptors from the recording
272 electrode. Figure 1 shows representative images of phase-contrast optics and nuclear
273 staining of microislands with low (Fig. 1A-C) and high numbers of astrocytes (Fig. 1D-
274 F). Because we used the microislands that contained only single neurons, the ratio of the
275 number of astrocytes to that of neurons (astrocyte/neuron) is simply the number of
276 astrocytes in each dot-patterned microisland. In this study, we classified the
277 microislands into two broad experimental groups: those with astrocyte/neuron = 1 to 10
278 were defined as a low-density group (LDG, Fig. 1A-C), and those with astrocyte/neuron
279 = 20 to 30 were defined as a high-density group (HDG, Fig. 1D-F). All the subsequent
280 analyses were based on the comparison between these two groups which were cultured
281 in the same culture dishes.

282 We recorded and compared the evoked excitatory postsynaptic currents (EPSCs) in
283 LDG and HDG (Fig. 2A). The evoked EPSC amplitude was significantly larger in the
284 HDG than that of the LDG (LDG, 4.62 ± 0.53 nA; HDG, 7.12 ± 0.71 nA; Fig. 2B).
285 Next, mEPSCs were recorded in the presence of TTX (Fig. 2C). Since mEPSCs
286 correspond to the activation of postsynaptic receptors by neurotransmitters
287 spontaneously released from single synaptic vesicles (Katz, 1979. Bekkers, 1995), it is
288 commonly understood that the changes in mEPSC frequencies mostly reflect the
289 changes in the number of functional nerve terminals or the probability of release from
290 nerve terminals, whereas the changes in mEPSC amplitudes mostly reflect those in the
291 number or properties of postsynaptic receptors. We found that the mEPSC frequency
292 was significantly higher in the HDG than that of the LDG (LDG, 7.07 ± 0.84 Hz; HDG,
293 9.88 ± 1.01 Hz; Fig. 2D), whereas the mEPSC amplitude was identical in both groups
294 (LDG, 29.1 ± 1.01 pA; HDG, 29.0 ± 1.43 pA; Fig. 2E). These results show increases in
295 the evoked EPSC amplitude and mEPSC frequency by high-density astrocytes, and
296 imply that these increases in excitatory synaptic transmission originated from changes
297 in presynaptic properties.

298

299 **Astrocyte density differences do not change synaptic release machineries and 300 synapse formation**

301 Because our data showed that the high astrocyte density affected presynaptic properties,
302 we measured the size of the readily releasable pool (RRP) of synaptic vesicles (Fig.
303 3A). The RRP size was determined by the application of hypertonic 0.5 M sucrose

304 solution to a single microisland (Rosenmund and Stevens, 1996; Kawano et al, 2012).
305 RRP size was significantly increased in HDG (LDG, 0.96 ± 0.15 nC; HDG, 1.38 ± 0.15
306 nC; Fig. 3B). Since all the functional nerve terminals in a microisland contribute to the
307 RRP measurement by our method, the increase in RRP may be due to an increase in the
308 functions of individual nerve terminals or the number of nerve terminals in each
309 autaptic neuron.

310 In order to further examine presynaptic functions, we designed two experiments.
311 First, we analyzed the vesicular release probability (Pvr). Pvr was defined as the
312 fraction of vesicles releasable by an action potential among the RRP vesicles. It was
313 calculated by dividing the electric charge of an action potential-induced EPSC (i.e. area
314 of the evoked EPSC trace) by the electric charge of sucrose-induced transient EPSC (i.e.
315 area of the transient component of the trace). Pvr was not statistically different between
316 the two groups (LDG, $7.98 \pm 1.39\%$; HDG, $9.91 \pm 1.83\%$; Fig. 3C). Second, we
317 measured the paired-pulse ratio of evoked EPSCs, by giving two depolarizing pulses
318 (action potentials) with an inter-pulse interval of 50 ms. This parameter was calculated
319 as the amplitude ratio of the second to the first response ($EPSC_2 / EPSC_1$). This
320 parameter is inversely correlated with the probability of release, and is generally
321 thought to reflect multiple factors, such as the amount of Ca^{2+} influx following an action
322 potential, kinetics of the increase in cytosolic Ca^{2+} concentration, and the availability or
323 depletion of synaptic vesicles for release (Xu-Friedman and Regehr, 2004). The paired-
324 pulse ratio was not different between the two groups (LDG, 1.00 ± 0.02 ; HDG, $1.02 \pm$
325 0.03 ; Fig. 3D). A lack of changes in the Pvr or the paired-pulse ratio indicates that the
326 astrocyte density did not affect the presynaptic functions, specifically the properties
327 associated with neurotransmitter release from functional or active nerve terminals.

328 Because functions of individual nerve terminals did not change, the increased
329 excitatory synaptic transmission may have resulted from an increased number of
330 synapses. Nerve terminals of excitatory neurons in the hippocampus predominantly
331 express the vesicular glutamate transporter 1 (VGLUT1) (Wojcik et al., 2004).
332 Therefore, we identified the excitatory nerve terminals as the punctate structures stained
333 positively with VGLUT1, and counted their number. Surprisingly, the numbers were not
334 different between the two groups (LDG, 358.29 ± 32.32 ; HDG, 412.21 ± 43.29 ; Fig.
335 4B). Thus, in the HDG group, there was enhancement of functions of the collective
336 nerve terminals (Figs. 2D, 3B), whereas there was no change in the functions of the
337 individual nerve terminals that released neurotransmitters (Figs. 3C, D) or in the
338 numbers of morphologically identified terminals (Fig. 4).

339

340 **The ratio of presynaptically silent synapses was reduced by increasing the**
341 **astrocyte density.**

342 The above results are consistent with the concept that the astrocytic density modified

343 presynaptically silent synapses. Presynaptically silent synapses are morphologically
344 mature, but their synaptic vesicles do not exocytose (i.e. they do not release
345 neurotransmitters) at all even when challenged with a robust depolarizing stimulus or
346 Ca^{2+} influx (Crawford and Mennerick, 2012). In other words, the presynaptically silent
347 synapses are not detectable electrophysiologically, and therefore they do not contribute
348 to the release parameters, such as EPSCs, RRP and Pvr. We therefore posited that the
349 synaptic transmission was enhanced by a decreased ratio of presynaptically silent
350 synapses in the HDG.

351 To test this hypothesis directly, we identified the presynaptically silent synapses, by
352 using double staining with FM1-43 and VGLUT1. In this experiment, FM1-43 was used
353 to locate functional nerve terminals by loading into recycling synaptic vesicles that
354 undergo endocytosis after stimulus-induced exocytosis, while the VGLUT1
355 immunostaining was used to locate mature nerve terminals morphologically regardless
356 of the capability of endocytosis. In previous studies, synapses labeled with VGLUT1
357 but lacking FM1-43 loading were identified as presynaptically silent synapses (Moulder
358 et al., 2004; Kawano et al., 2012). The ratio of presynaptically silent synapses
359 (VGLUT1 +, FM1-43 -) among all excitatory synapses (VGLUT1 +) was significantly
360 smaller in the HDG (LDG, $26.81 \pm 3.68\%$; HDG, $20.35 \pm 1.86\%$; Fig. 5B). This result
361 supports the hypothesis that the astrocytic density influences presynaptically silent
362 synapses, such that the number of functional nerve terminals is increased by high
363 astrocytic density.

364

365

366 **DISCUSSION**

367 In this study, we evaluated the impact of astrocytic density on excitatory synaptic
368 transmission, using single autaptic neurons that were co-cultured with astrocytes at
369 different densities. The HDG exhibited significant increases in the factors that reflect the
370 collective properties of functional nerve terminals in our experimental system: the
371 evoked EPSC amplitude (Fig. 2B), mEPSC frequency (Fig. 2D), and RRP size (Fig.
372 3B). In contrast, there was no change in the functions of individual nerve terminals that
373 released neurotransmitters (i.e. functions of presynaptically active synapses): the vesicle
374 release probability (Fig. 3C) and the paired-pulse ratio (Fig. 3D). There was no change
375 in a postsynaptic factor: the mEPSC amplitude (Fig. 2E). Considering an unchanged
376 number of morphologically identified, excitatory glutamatergic nerve terminals (Fig.
377 4B), all these features introduced by different astrocyte densities can be explained by a
378 change in a single factor: the ratio of presynaptically silent synapses (Fig. 5B). In the
379 HDG, this factor was significantly decreased, i.e. the number of functional nerve
380 terminals was increased due to un-silencing or awakening of silent ones (Crawford and
381 Mennerick, 2012). This change led to an enhancement of the evoked and miniature

382 excitatory synaptic transmission (Fig. 2B, C), without a change in the functions of
383 individual nerve terminals. Increase in the collective properties can be interpreted to be
384 due to the increased number of functional nerve terminals, each of which did not change
385 any release property. Naturally, there can be more complex scenarios, e.g. the functional
386 properties of unsilenced and the already active nerve terminals changed in opposite
387 directions and canceled each other, leading to the apparent lack of functional changes.
388 However, the original scenario shown above is the simplest one and is compatible with
389 every finding in our system.

390 Overall, this study underscores the important roles of astrocyte/neuron ratio in
391 regulating presynaptic features of synaptic transmission. When synaptic transmission
392 had been recorded from neurons co-cultured with astrocytes, little attention had been
393 paid to the density of astrocytes surrounding neurons. This study prompts us to exercise
394 caution in interpreting the efficiency of synaptic transmission under multiple
395 experimental conditions. In combination with the experiments where astrocytes were
396 deficient (Pfrieger et al., 1997; Ullian et al. 2001; Hama et al., 2004; Crawford et al.,
397 2012; Sobieski, et al, 2015), our study indicates that astrocytic densities have a wide
398 spectrum of effects on synaptic transmission.

399 Changes in the neuronal activity have been reported to affect the ratio of
400 presynaptically silent synapses, as a part of homeostatic synaptic plasticity to counteract
401 the initial changes. For example, prolonged depolarization of cultured hippocampal
402 neurons increases presynaptically silent synapses (Moulder et al., 2004). This change
403 was accompanied by a reduction in the evoked EPSC amplitude, mEPSC frequency, and
404 RRP size (in a manner opposite in polarity to our findings), and by no change in the
405 mEPSC amplitude or the number of morphologically identified glutamatergic nerve
406 terminals (similar to our findings). Conversely, a blockade of action potentials with
407 TTX reduces the presynaptically silent synapses in the same preparation (Moulder et al.,
408 2006). The cAMP signaling cascade is involved in both presynaptic terminal silencing
409 and un-silencing. For example, increasing cAMP signaling by forskolin reduces the
410 ratio of presynaptically silent synapses at rest, whereas decreasing it by Rp-cAMPS
411 increases the ratio (Moulder et al., 2008).

412 It is unclear how the presynaptically silent synapses are modified by different
413 astrocytic densities. One pioneering work in this aspect evaluated the effect of astrocyte-
414 derived soluble factors in inducing the presynaptically silent synapses (Crawford et al.,
415 2012). The authors compared the autaptic synaptic transmission when the neurons were
416 plated on "astrocyte-rich" standard microislands or "astrocyte-poor or -deficient"
417 microislands prepared by chemical fixation of only astrocytes. They identified
418 thrombospondins as the potential secreted agent that induces presynaptic silencing
419 through the cAMP signaling cascade. However, secreted agents are not expected to be
420 responsible for the results in our system, because the two density groups were cultured

421 together in the same culture dish, and thus they must have shared the astrocyte-derived
422 secreted agents. At least one possibility can be speculated, still based on the cAMP-
423 dependent mechanisms described above. The astrocyte-neuron lactate shuttle (ANLS)
424 proposed by Pellerin and Magistretti (1994) may be involved in the reduction of
425 presynaptically silent synapses in our study. Lactic acid produced by astrocyte
426 glycolysis is supplied to neurons where it is converted to pyruvate, which is then
427 metabolized in the mitochondria via oxidative phosphorylation to produce ATP. In the
428 HDG of our study, activation of ANLS may increase the energy supply to neurons,
429 activate the cAMP signaling pathway, and may reduce the ratio of presynaptically silent
430 synapses. Clearly, mechanistic insights into the effect of different astrocytic densities
431 await further studies.

432

433 The roles that the presynaptically silent synapses play in regulating the function of
434 the physiological and pathological neuronal network still remain under intense
435 investigations (Crawford and Mennerick, 2012). We propose that the astrocyte density
436 also contributes to the regulation of the ratio of presynaptically silent synapses. In this
437 context, it is of interest to note that the glia/neuron ratio is approximately 1.5 in the gray
438 matter of human prefrontal cerebral cortex (von Bartheld et al., 2016), but the ratio
439 varies drastically across brain regions. For example, the glia/neuron ratio is
440 approximately 0.2 in the cerebellum, 3.8 in the cerebral cortex, and 11.4 in the rest of
441 the human brain (Azevedo et al., 2009). Thus, the astrocyte/neuron ratio can be involved
442 in brain-regional difference of information processing through synaptic transmission.
443 Clarifying how the astrocytes are involved in regulating presynaptically silent synapses
444 could be essential for understanding the functions of the neuronal network and the brain.

445

446

447 DECLARATIONS OF INTEREST

448 None

449

450

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456

457

458 FIGURE LEGENDS

459 **Figure 1.** Single autaptic hippocampal neurons co-cultured with different numbers of

460 astrocytes
461 (A) A representative phase-contrast image of an autaptic neuron cultured with a low
462 density of astrocytes. (B) Nuclear staining of live astrocytes and a single neuron shown
463 in (A). (C) A merged image of phase-contrast and nuclear staining images of the same
464 observation field. (D) A representative phase-contrast image of an autaptic neuron
465 cultured with a high density of astrocytes. (E) Nuclear staining of live astrocytes and a
466 single neuron shown in (D). (F) A merged image of phase-contrast and nuclear staining
467 images of the same observation field. All scale bars indicate 100 μ m.
468

469 **Figure 2.** Excitatory synaptic transmission was enhanced with the increased astrocyte
470 density.

471 (A) Representative traces of evoked excitatory postsynaptic currents (EPSCs) recorded
472 electrophysiologically from autaptic neurons (LDG and HDG). Averaged trace of eight
473 stimuli at 0.2 Hz is shown. Depolarization-induced action currents have been removed
474 for clarity. (B) Average amplitudes of evoked EPSCs in autaptic neurons co-cultured
475 with LDG (n = 66 neurons /10 cultures) or HDG (n = 60 neurons /10 cultures). (C)
476 Representative traces of miniature EPSCs (mEPSCs) in the LDG or HDG. (D) The
477 frequency of mEPSCs in the LDG (n = 66 neurons /10 cultures) or HDG (n = 60
478 neurons /10 cultures). (E) The amplitude of mEPSCs in the LDG (n = 66 neurons /10
479 cultures) or HDG (n = 60 neurons /10 cultures). *p < 0.05.
480

481 **Figure 3.** Synaptic release machinery was unaltered by differences in astrocyte density.

482 (A) Representative traces of the responses to 0.5 M sucrose solution (8 sec) in the
483 LDG or HDG. (B) The averaged size of the readily releasable pool (RRP), measured by
484 the response to sucrose, in the LDG (n = 66 neurons /10 cultures) or HDG (n = 60
485 neurons /10 cultures). (C) The vesicular release probability in the LDG (n = 66 neurons
486 /10 cultures) or HDG (n = 60 neurons /10 cultures). (D) The paired-pulse ratio (EPSC₂ /
487 EPSC₁) in the LDG (n = 46 neurons /7 cultures) or HDG (n = 42 neurons /7 cultures).
488 *p < 0.05.
489

490 **Figure 4.** Differences in astrocyte density did not affect the number of excitatory
491 synapses.

492 (A) Representative images of autaptic neurons immuno-stained for the dendritic marker,
493 microtubule-associated protein 2 (MAP2) (in green) and the excitatory synapse marker,
494 vesicular glutamate transporter 1 (VGLUT1) (in red). The scale bars indicate 10 μ m. (B)
495 The number of VGLUT1 puncta in the LDG (n = 31 neurons /4 cultures) or HDG (n =
496 24 neurons /4 cultures).
497

498 **Figure 5.** Ratio of presynaptically silent synapses is reduced by increasing astrocyte

499 density.
500 (A) Images of MAP2 immunostaining (in blue), VGLUT1 immunostaining (in red), and
501 FM1-43 labeling of presynaptic terminals (in green), in LDG (top) and HDG (bottom).
502 In the merged images, the arrows indicate presynaptically silent synapses, stained
503 positively for VGLUT1 but negatively for FM1-43. All scale bars indicate 5 μ m. (B)
504 The ratio of presynaptically silent synapses in the LDG (n = 13 neurons / 11cultures) or
505 HDG (n = 13 neurons /11 cultures), *p < 0.05.

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507

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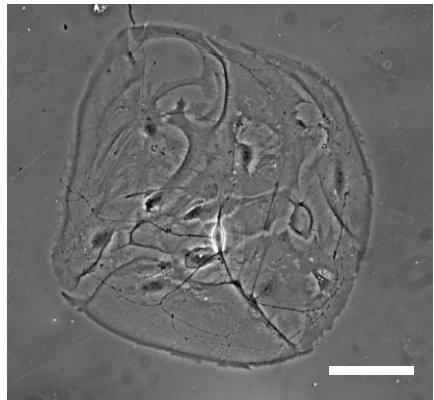
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Figure.1

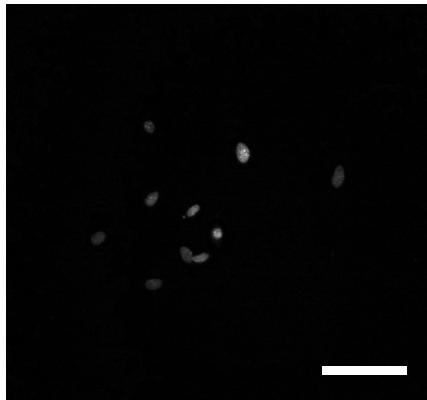
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Low-density groups (LDG)

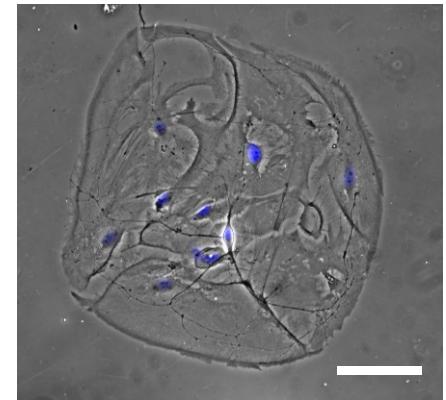
A



B

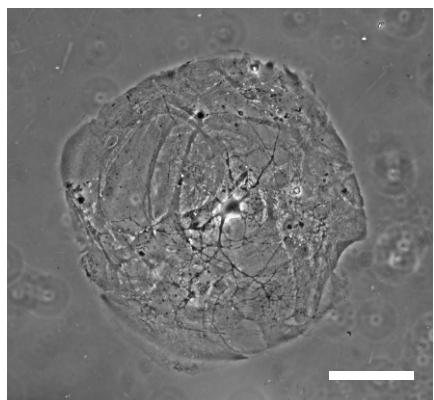


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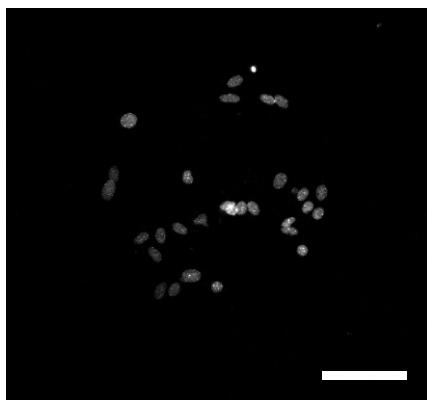


High-density groups (HDG)

D



E



F

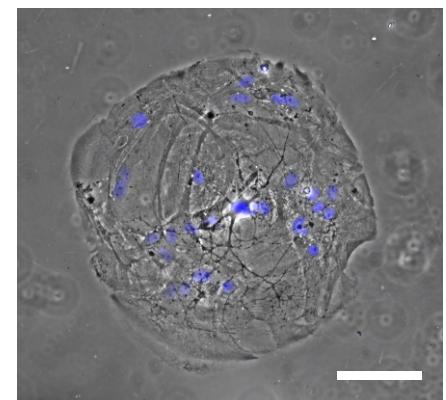


Figure.2

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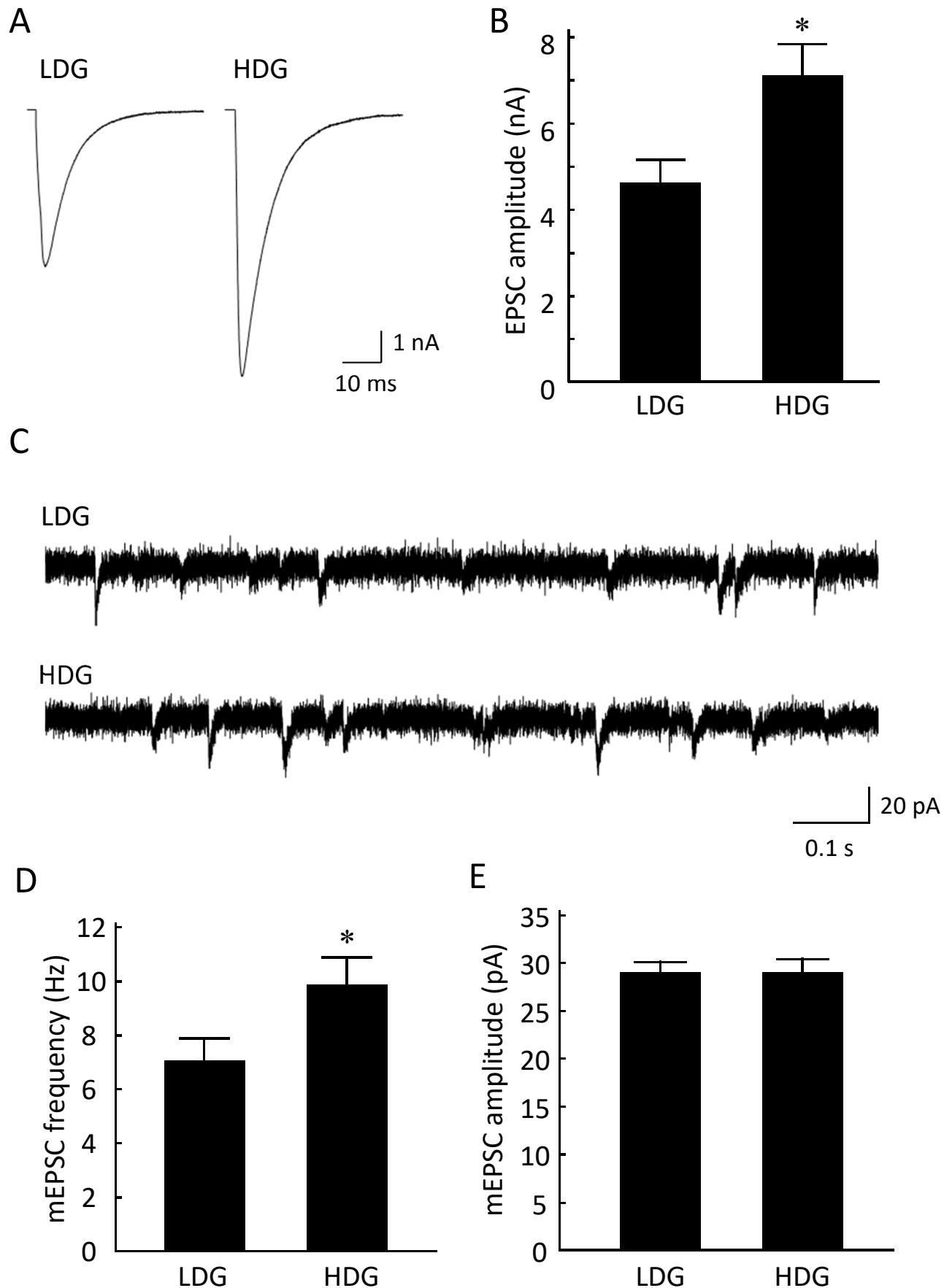


Figure.3

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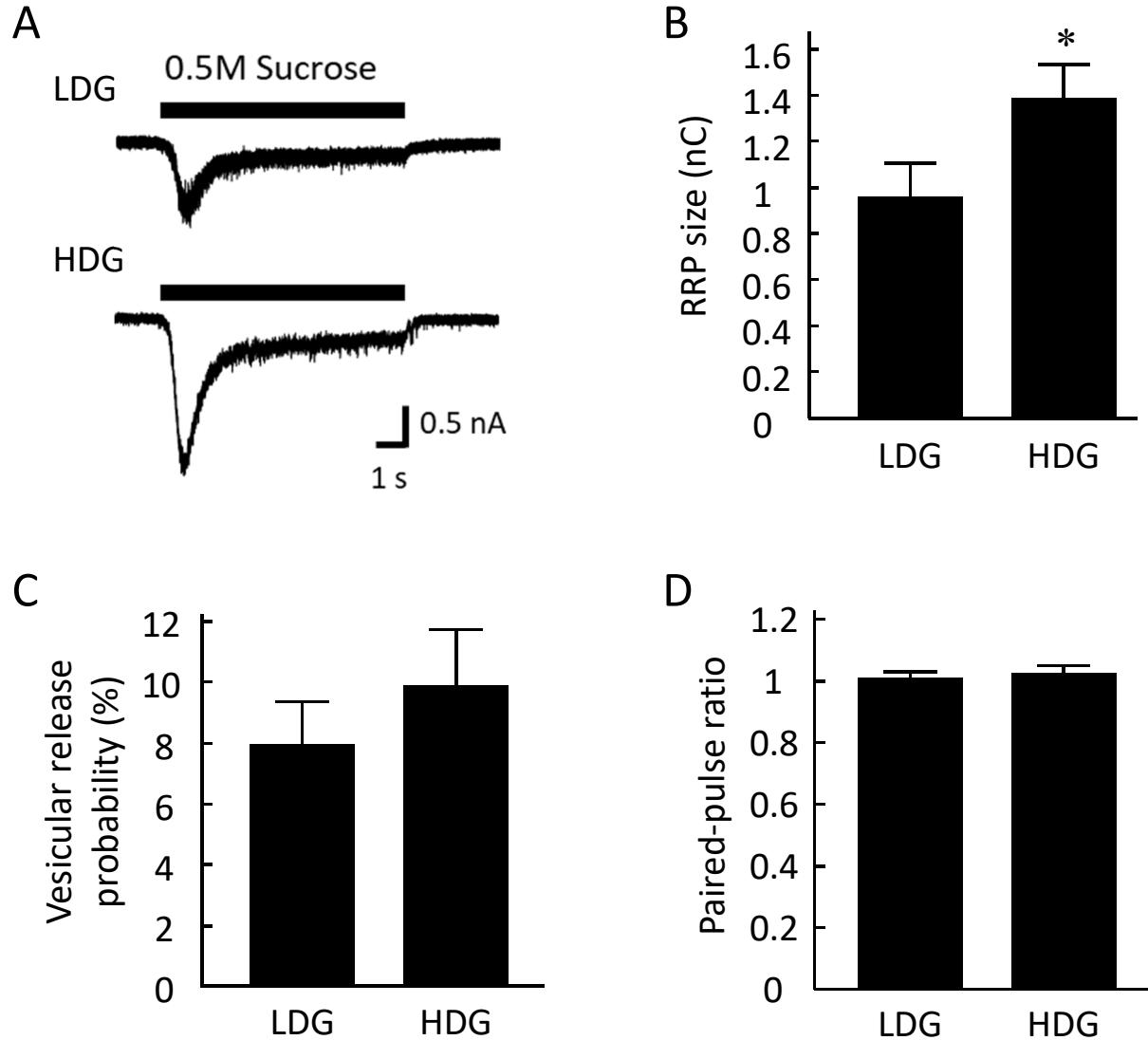


Figure.4

Oyabu et al

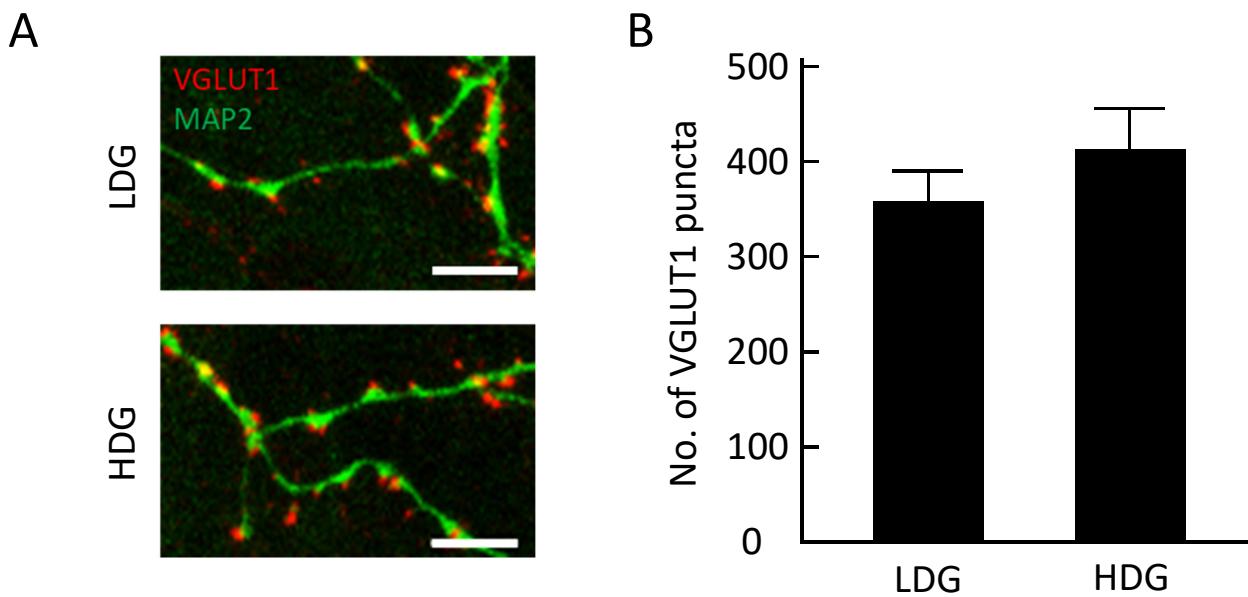
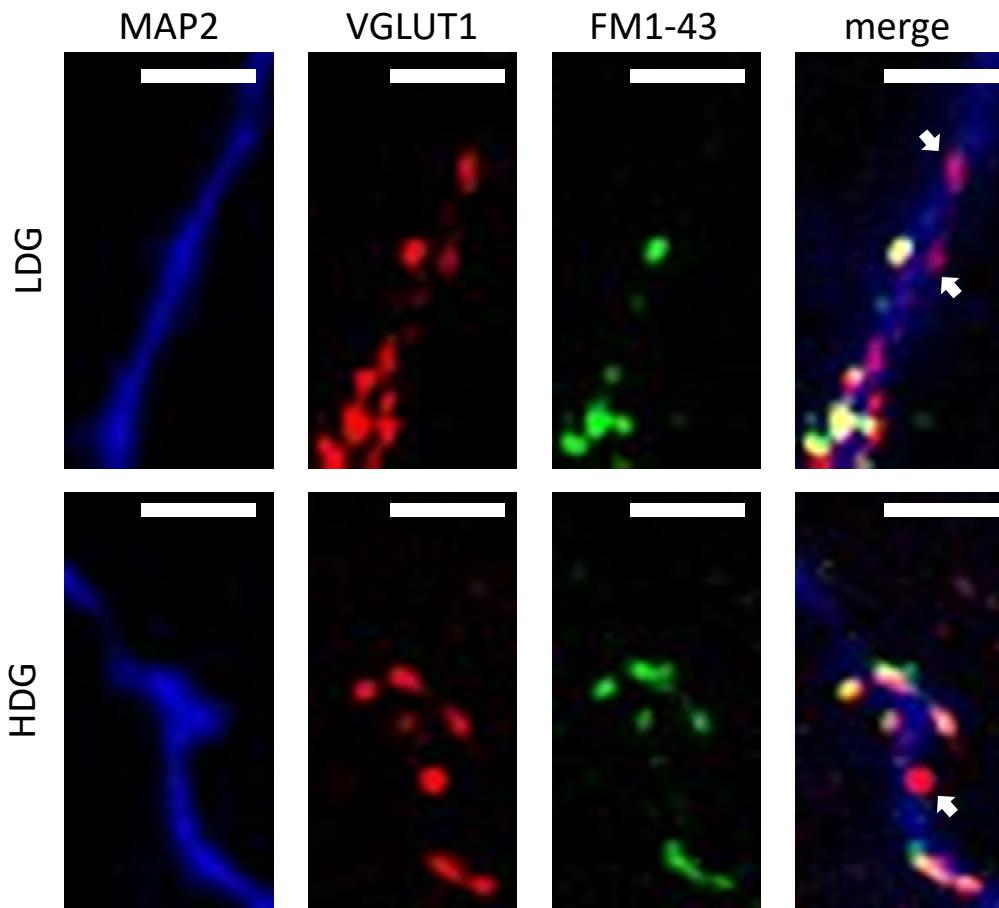


Figure.5

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A



B

