Recurrent connections between CA2 pyramidal cells

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20 **Abstract**

21 Recurrent excitatory synapses are shown theoretically to play roles in memory storage
22 and associative learning, and such recurrent synapses are well described to occur in the
23 CA3 region of the hippocampus. Here, we report that the CA2 region also contains
24 recurrent excitatory monosynaptic couplings. Using dual whole-cell patch-clamp
25 recordings from CA2 pyramidal cells in mouse hippocampal slices under differential
26 interference contrast microscopic controls, we evaluated monosynaptic excitatory
27 connections. Unitary excitatory postsynaptic potentials occurred in 1.4% of 502 cell
28 pairs. These connected pairs were located preferentially in the superficial layer and
29 proximal part (CA2b) of the CA2 region. These results indicate that recurrent excitatory
30 circuits are dense in the CA2 region as well as in the CA3 region.

31

32 **Introduction**

33 Theoretical studies have suggested that reentrant positive-feedback excitation of the
 34 neuronal network is critical for nonlinear information processing, including memory
 35 storage, associative learning, and pattern separation/completion (Amari et al., 1996;
 36 Hebb, 1949; Hopfield, 1982; Kohonen, 1998). The hippocampal CA3 region is one of
 37 the candidate brain regions that anatomically contain self-associative excitatory
 38 networks and functionally exert such information processing (Guzman et al., 2016;
 39 MacVicar and Dudek, 1981; Miles and Wong, 1986; Nakazawa et al., 2004; Treves and
 40 Rolls, 1994). However, only a few studies have directly measured monosynaptic
 41 recurrent connections in the hippocampus (Deuchars and Thomson, 1996; Guzman et al.,
 42 2016; Miles and Wong, 1986), particularly in the CA2 region (Mercer et al., 2012).

43 The CA2 region of the hippocampus is unique in terms of spatial representation
 44 and social memory (Hitti and Siegelbaum, 2014; Mankin et al., 2015), but it has been
 45 less investigated because it is not included in the classical tri-synaptic pathway and
 46 because the CA2 region is anatomically small, which limits experimental access.
 47 Notably, whether CA2 pyramidal cells are mutually excited at the monosynaptic level
 48 remains controversial. Some reports suggest that the CA2 region contains recurrent
 49 excitatory circuits (Lu et al., 2015) because CA2 neurons elaborate highly arborized
 50 dendrites (Dudek et al., 2016) and because CA2 neurons can initiate sharp wave/ripple
 51 oscillations (Oliva et al., 2016).

52 In the present study, we directly measured excitatory monosynaptic
 53 connections between CA2 pyramidal cells using double whole-cell patch-clamp
 54 recordings. We observed monosynaptic unitary excitatory postsynaptic potentials

(EPSPs) between 1.4% of the CA2 pyramidal cell pairs. The CA2-to-CA2 connections existed preferentially in the CA2b subarea and exhibited a preferential spatial direction.

Results and Discussion

Recent histological studies have broadened the conventional definition (Lorente de Nó, 1934) of the CA2 region based on specific molecular expressions, such as those of G-protein signaling protein 14 (RGS14) and striatal-enriched protein tyrosine phosphatase (STEP) (Dudek et al., 2016; Kohara et al., 2014; Lein et al., 2005; Noguchi et al., 2017). We also confirmed that RGS14 immunoreactivity indicated the macroscopic location of the CA2 region in transverse slices of the hippocampus. At the single cell level, however, RGS14-positive cells were sparse near the CA1/CA2 and CA2/CA3 borders (Figure 1). This sparsity was found particularly in the ventral hippocampus. This salt-and-pepper distribution of RGS14-positive cells indicates that a significant number of non-CA2 pyramidal cells (presumably, CA1 or CA3 pyramidal cells) are present in the CA2 region.

We investigated recurrent CA2 connectivity by recording unitary EPSPs between two CA2 pyramidal cells. We visually targeted pyramidal cells using a differential interference contrast microscope. The recorded neurons were intracellularly filled with biocytin through patch-clamp pipettes and were then immunostained using an antibody against RGS14 or STEP (Figure 2A). A train of four action potentials was evoked at 20 Hz in one of the recorded cells, and the evoked membrane potentials were monitored for the other cells (Figure 2B). In the 502 pairs tested, we found 7 chemical synaptic connections and no electrical coupling; that is, the CA2-to-CA2 synaptic connection probability was 1.4%. The unitary EPSPs had a mean peak amplitude of

79 0.43 $\pm$ 0.14 mV and a mean transmission failure rate of 54 $\pm$ 24% (mean $\pm$ SD of 7
80 connections). Three of the 7 connections exhibited short-term depression in response to
81 four presynaptic spikes (Figure 3). We also patched 108 CA3 pyramidal cell pairs and
82 found 3 chemical synaptic connections (2.8%), whose unitary EPSPs had a mean peak
83 amplitude of 0.75 $\pm$ 0.54 mV and a mean failure rate of 45 $\pm$ 22% (3 connections).
84 These parameters of the CA3 pairs are similar to those reported in previous studies of
85 CA3 connections (Guzman et al., 2016; Miles and Wong, 1986), and they did not differ
86 significantly from those of the CA2 connections in the present study ($P > 0.05$,
87 Student's *t*-test).

88 During the whole-cell recordings, we measured the physical distances between
89 the somata of two recorded cells in the images taken with a differential interference
90 contrast microscope. The inter-soma distances were not correlated with the connection
91 probability (Figure 4). This result appears similar to the previously reported spatial
92 patterns of CA3 connectivity (Guzman et al., 2016) but is different from those of
93 neocortical connectivity, in which more adjacent pairs had a higher probability of
94 connections (Peng et al., 2017; Perin et al., 2011; Song et al., 2005). When considering
95 the connectivity, however, one needs to consider the anatomical size of the CA2 region;
96 the majority of CA2 cell pairs have inter-soma distances of less than 100 μ m; thus, we
97 cannot rule out the possibility that this sampling restriction masked the distance
98 dependence.

99 CA1 and CA3 pyramidal cells are heterogeneous along both transverse
100 (Ishizuka et al., 1990; Lee et al., 2015; Lu et al., 2015; Sun et al., 2017) and radial axes
101 (Kohara et al., 2014; Lee et al., 2014; Valero et al., 2015). Similar heterogeneity has also
102 been reported in the CA2 region (Oliva et al., 2016). We thus investigated whether

103 CA2-to-CA2 connections are spatially biased in the CA2 stratum pyramidale. For one
 104 of the 7 connections, we failed to accurately identify the relative loci of the recorded
 105 cells in the RGS14-positive CA2 region. Thus, we analyzed the remaining 6 pairs. The
 106 relative location of each recorded cell was determined in the CA2 stratum pyramidale,
 107 which was rectangularly standardized according to the RGS14-positive or
 108 STEP-positive areas (Figure 5A). In this cell map, we noticed two structural tendencies.
 109 First, the directions from presynaptic cells to postsynaptic cells were spatially biased
 110 (Figure 5B; $V = 2.05$, $P = 0.02$, $n = 6$ pairs, V -test). A given CA2 pyramidal cell tended
 111 to make a synaptic connection with a CA2 pyramidal cell that was more proximal to the
 112 CA3 regions. This tendency suggests that compared to distal CA2 cells, more proximal
 113 CA2 pyramidal cells receive more recurrent inputs from other CA2 pyramidal cells.

114 Second, the connected pairs were located preferentially in the superficial layer
 115 and the proximal side of the CA2 region (Figure 5C). The difference between the
 116 superficial and deep layers of the stratum pyramidale has not been well studied in the
 117 CA2 region. A recent study demonstrated that the CA2 deep layer initiates sharp
 118 wave/ripple oscillations (Oliva et al., 2016). Recurrent excitation is proposed as one of
 119 the substrates of sharp wave/ripple generation (Traub et al., 1989), but our results
 120 indicate that the recurrent connections of the CA2 pyramidal cells are nearly exclusively
 121 located in the superficial layer. The generation of sharp waves/ripples is a complex
 122 process, including dense recurrent circuits, timed interneuron activation (Stark et al.,
 123 2014) and extrahippocampal controls (Buzsaki, 2015). The CA2 region may trigger
 124 sharp waves/ripples independently of local recurrent excitation.

125 We found that the proximal CA2 (CA2b) subarea, but not the distal CA2
 126 (CA2a) subarea, has dense recurrent connections, similar to the CA3 region. According

127 to the most recent definition (Dudek et al., 2016; Lein et al., 2005), the CA2b subarea
 128 encompasses the stratum lucidum and receives monosynaptic inputs from the dentate
 129 gyrus (Kohara et al., 2014; Sun et al., 2017); however, the unitary EPSP sizes are
 130 smaller in CA2 pyramidal cells than in CA3 pyramidal cells (Sun et al., 2017). In
 131 addition, CA2 pyramidal cells receive strong excitatory inputs from layer III neurons in
 132 the entorhinal cortex (Chevalleyre and Siegelbaum, 2010). These extrahippocampal
 133 inputs contribute to the unstable dynamics of CA2 neuronal activity (Lee et al., 2015;
 134 Lu et al., 2015; Mankin et al., 2015). The CA3 network contains clustered circuit motifs
 135 (Guzman et al., 2016). The neocortex also contains rich clustered connections (Peng et
 136 al., 2017; Perin et al., 2011; Song et al., 2005). In CA2 pyramidal cells, we did not find
 137 bidirectionally connected pairs. This may be because the numbers of pair recordings are
 138 simply not enough.

139 A previous study reported a very low connection probability (0.22%) between
 140 CA2 pyramidal cells (Mercer et al., 2012); however, this study did not identify CA2
 141 pyramidal cells histologically using the CA2 cell markers (RGS-14 or STEP). Given
 142 that CA2 pyramidal cells are sparse even in the CA2 pyramidal cell layer, the authors
 143 may have underestimated the true CA2 connectivity. Another possibility is that they
 144 might have focused more on the CA2a subarea, which was conventionally defined as
 145 "the CA2 region". This sampling bias could also lead to an underestimation of the CA2
 146 connectivity.

147 The CA2 region was reported to contain a higher density of GABAergic
 148 interneurons than the CA1 or CA3 region (Leranth and Ribak, 1991; Mercer et al.,
 149 2007). Interestingly, within the CA3 region, the CA3a subarea receives more inhibitory
 150 inputs and has denser recurrent connectivity than the CA3b subarea (Sun et al., 2017).

151 These observations suggest similar circuit properties of the CA2b and CA3a subareas.
152 The high density of recurrent excitatory connections may be counter-balanced by strong
153 inhibitory inputs and maintain the excitatory-to-inhibitory balance in the CA2 local
154 circuit.

155

156 **Methods**

157 *Animal experiment ethics*

158 Experiments were performed with the approval of the Animal Experiment Ethics
159 Committee at the University of Tokyo (approval no. P29-9) and according to the
160 University of Tokyo guidelines for the care and use of laboratory animals. Institute of
161 Cancer Research (ICR) mice (SLC) were housed in cages under standard laboratory
162 conditions (12 h light/dark cycle, ad libitum access to food and water). All efforts were
163 made to minimize the animals' suffering and the number of animals used.

164

165 *Acute slice preparation*

166 Acute slices were prepared from the hippocampi of ICR mice (17-26 postnatal days).
167 The mice were anesthetized with isoflurane and then decapitated. The brains were
168 removed and placed in an ice-cold oxygenated solution consisting of (in mM) 222.1
169 sucrose, 27 NaHCO₃, 1.4 NaH₂PO₄, 2.5 KCl, 1 CaCl₂, 7 MgSO₄, and 0.5 ascorbic acid.
170 The brains were sliced horizontally at a thickness of 400 μm using a VT1200S
171 vibratome (Leica). The slices were allowed to equilibrate at room temperature for at
172 least 0.5 h while submerged in a chamber filled with oxygenated aCSF consisting of (in
173 mM) 127 NaCl, 26 NaHCO₃, 1.6 KCl, 1.24 KH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂, and 10

174 glucose. The slices were mounted in a recording chamber and perfused at a rate of 1.5–3
175 ml/min with oxygenated aCSF.

176

177 *In vitro electrophysiology*

178 All recordings were performed at 32–34°C. Whole-cell recordings were collected from
179 up to four CA2 pyramidal cells using a MultiClamp 700B amplifier and a Digidata 1550
180 digitizer controlled by pCLAMP10.6 software (Molecular Devices). Borosilicate glass
181 pipettes (3–6 MΩ) were filled with a solution containing (in mM) 135 K-gluconate, 4
182 KCl₂, 0.3 EGTA, 10 HEPES, 10 Na₂-phosphocreatine, 4 MgATP, 0.3 Na₂GTP and 2.0
183 biocytin. The signals were gained 10-fold, low-pass filtered at 1 kHz and digitized at 20
184 kHz. The existence of synaptic connectivity was assessed by averaging 50 successive
185 traces in which 4 spikes at 20 Hz were induced by current injection in presynaptic cells.

186

187 *Histology*

188 CA2 pyramidal cells were perfused intracellularly with 2 mM biocytin for whole-cell
189 recordings. After the recordings, the slices were fixed for at least 20 h at 4°C in 0.1 M
190 Na₃PO₄, pH 7.4, containing 3% (w/v) formaldehyde. The sections were incubated with
191 2 µg/mL streptavidin-Alexa Fluor 594 conjugate and 0.2% Triton X-100 for 6 h,
192 followed by incubation with 0.4% NeuroTrace 435/455 blue-fluorescent Nissl Stain
193 (Thermo Fisher Scientific; N21479) overnight. The tissue sections were incubated
194 subsequently with mouse primary antibodies for RGS-14 (NeuroMab; N133/21; 1:500)
195 or STEP (Cell Signaling Technology; 4396S; 1:500) for 16 h at 4°C, followed by
196 incubation with a secondary goat antibody to mouse IgG (Thermo Fisher Scientific;
197 A-11001; 1:500) for 6 h at 4°C.

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289

Figure 1 | Distributions of CA2 pyramidal cells along the longitudinal axis of the mouse hippocampus. A) A whole hippocampus was sectioned transversely at the dorsal, middle, and ventral areas, indicated by the broken white lines. **B-D)** The transverse sections at the dorsal (B), middle (C), and ventral (D) areas were labeled with an anti-RGS14 antibody (*green*, a CA2 marker) and NeuroTrace 435/455 Nissl Stain (*blue*). The bottom photos are magnified images of the CA2 parts, indicating that the CA2 borders next to the CA1 and CA3 regions are ambiguous because of the sparse distribution of the CA2 pyramidal cells, particularly in the ventral hippocampus.

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302

303 **Figure 2 | Monosynaptic recurrent connection of a CA2-CA2 pair.** A) *Top:*
304 Confocal images of a synaptically connected pair of CA2 pyramidal cells that were
305 filled with biocytin (*red*). *Middle:* The CA2 region was immunostained for RGS14
306 (*green*). *Bottom:* RGS14 was expressed in the recorded cells. B) Unitary EPSPs of the
307 connected pair. *Top:* Mean trace of a train of current injection-induced action potentials
308 of the presynaptic cell (blue, averaged for 50 traces). *Middle:* Representative
309 postsynaptic membrane responses recorded in the current-clamp mode for the
310 successive trials (black). *Bottom:* an average of 50 recorded traces for the postsynaptic
311 cell.

312

Figure 3 | Unitary EPSP properties of all connected pairs. *Top:* Percentages of synaptic failures in the responses to a presynaptic spike train (4 pulses at 20 Hz) for 7 connected pairs. Train stimuli were repeated 50 times. *Center:* Synaptic efficacies across the 50 trains (including failure events). *Bottom:* Synaptic potencies (without failure events).

321

322

323 **Figure 4 | Lack of correlation of CA2-CA2 connections with their inter-cell**
 324 **distances.** The top histogram indicates the distributions of the distances between the
 325 centroids of the cell bodies of all recorded pairs. The center histogram indicates the
 326 same distribution as the top, but for only the synaptically connected pairs. The bottom
 327 graph plots the connection probabilities (*i.e.*, the center histogram divided by the top
 328 histogram) as a function of the inter-soma distance (red). The black line is the mean
 329 connection probability (1.4%). The gray area is the 95% confidence interval estimated
 330 by 10,000 randomly resampled surrogates.

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334 **Figure 5 | Spatial bias of CA2-CA2 connections.** **A)** The stratum pyramidale of the
 335 CA2 region was normalized as a rectangle according to RGS14-positive or
 336 STEP-positive areas. Gray dots and lines indicate the soma location of the recorded
 337 CA2 pyramidal cells and their potential connections (unconnected), respectively. Red
 338 lines represent the synaptic connections ($n = 6$). Red filled and open circles indicate the
 339 soma location of the presynaptic and postsynaptic cells, respectively. **B)** A polar plot of
 340 the inter-soma directions of the synaptically connected pairs. Each black line indicates
 341 the direction from the soma of one presynaptic cell to the soma of its postsynaptic cell.
 342 The red arrow represents the mean vector of all 6 connected pairs. $V = 2.05$, $P = 0.02$,
 343 V -test. **C)** Cumulative distributions of the midpoints between two soma locations of
 344 connected pairs (red line) and unconnected pairs (black line) along the transverse axis
 345 (left) and the radial axis (right). The gray areas indicate the 95% confidence intervals
 346 estimated by Kaplan-Meier method.

347

A

Normalized CA2 region

● Pre
○ Post **B** **C**