Heterogeneity and independency of unitary synaptic outputs from hippocampal CA3 pyramidal cells

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Abstract

The variation of individual synaptic transmission impacts the dynamics of complex neural circuits. We performed whole-cell recordings from monosynaptically connected hippocampal neurons in rat organotypic slice cultures using a synapse mapping method. The amplitude of unitary excitatory postsynaptic current (uEPSC) varied from trial to trial and was independent of the physical distance between cell pairs. To investigate the source of the transmission variability, we obtained patch-clamp recordings from intact axons. Axonal action potentials (APs) were reliably transmitted throughout the axonal arbour and showed modest changes in width. In contrast, calcium imaging from presynaptic boutons revealed that the amplitude of AP-evoked calcium transients exhibited large variations both among different boutons at a given trial and among trials in a given bouton. These results suggest that a factor contributing to the uEPSC fluctuations is the variability in calcium dynamics at presynaptic terminals. Finally, we acquired triple whole-cell recordings from divergent circuit motifs with one presynaptic neuron projecting to two postsynaptic neurons. Consistent with the independency of calcium dynamics among axonal boutons, a series of uEPSC fluctuations was not correlated between the two postsynaptic cells, indicating that different synapses even from the same neuron act independently. We conclude that the intra-bouton and inter-bouton variability in AP-induced calcium dynamics determine the heterogeneity and independency of uEPSCs.

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Abbreviations

AP, action potential; cEPSC, compound excitatory postsynaptic current; uEPSC, unitary excitatory postsynaptic current.
Introduction

The axon of a pyramidal cell in the cerebral cortex or the hippocampus elaborates thousands of regularly spaced synapses, called en passant synapses. The transmission efficacy at excitatory synapses between pyramidal neurons are heterogeneous (Debanne et al. 1999; Song et al. 2005; Bouddkazi et al. 2007; Lefort et al. 2009; Ikegaya et al. 2012). In addition, the occurrence of neurotransmitter release from these synapses is stochastic and varies from trial to trial. Therefore, the diversity and reliability of synaptic transmissions must be taken into account to accurately capture the behaviour and function of neural networks; however, several basic questions remain to be resolved: (i) whether the ‘across-synapse’ and ‘within-synapse’ variability of neurotransmission depend on the locations of the synapses; (ii) whether the neurotransmission properties interact between synaptic boutons; and (iii) what determines the neurotransmission variability. It is experimentally laborious to address these questions, because one needs to correct data from a large number of synapses and axons at distances of several hundred micrometres from the soma.

In this study, we utilised an optical probing technique referred to as reverse optical trawling (ROTing), a high-throughput, large-scale synapse mapping that can efficiently identify long-range synaptic connections (Sasaki et al. 2009; Takahashi et al. 2010). Using multiple patch-clamp recordings from synaptically coupled neurons (Sasaki et al. 2012b), we found that hippocampal synapses, even of the same axon, show a high degree of trial-to-trial fluctuations in output patterns and operate independently of one another. To examine the detailed mechanisms, we employed two recently developed methods: (i) axonal patch-clamp recording and (ii) calcium imaging from multiple axonal boutons (Sasaki et al. 2011, 2012a). These recordings revealed that the heterogeneity and independency of hippocampal synaptic transmission are rooted in the variability of calcium dynamics among and within boutons, rather than by axonal AP propagation per se.

Methods

Animal experiment ethics

All experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo (approval no. 19-43) and according to the University of Tokyo guidelines for the care and use of laboratory animals.

Hippocampal slice preparations

Postnatal day 7 Wistar/ST rats were anaesthetised with ketamine–xylazine and then chilled on ice. The brains were removed and cut horizontally into 300 μm-thick slices using a DTK-1500 vibratome (Dosaka, Kyoto, Japan). During the generation of slices, brain tissues were submerged in aerated, ice-cold Gey’s balanced salt solution supplemented with 25 mM glucose. Entorhino-hippocampal slices were cultivated for 7–14 days on Omnipore membrane filters (JHWP02500, φ25 mm; Millipore, Bedford, MA, USA) (Koyama et al. 2007). Cultures were incubated in 1 ml of 50% minimal essential medium, 25% Hanks’ balanced salt solution (Invitrogen, Gaithersburg, MD, USA), and 25% horse serum (Cell Culture Laboratory, Cleveland, OH, USA) in a humidified incubator at 37°C in 5% CO₂. The culture medium was changed every 3.5 days, and the cultured slices were analysed after 8–11 days in vitro, unless otherwise specified.

Patch-clamp recordings

A cultured slice was placed in a recording chamber and perfused at 1.5–2 ml min⁻¹ with artificial cerebrospinal fluid (aCSF) containing 127 mM NaCl, 26 mM NaHCO₃, 3.3 mM KCl, 1.24 mM KH₂PO₄, 3.0 mM MgSO₄, 3.0 mM CaCl₂, and 10 mM glucose at 30–32°C. To reduce background activity and increase the synaptic release probability, the K⁺ concentration was reduced to 3.3 mM, whereas the Ca²⁺ and Mg²⁺ concentrations were raised to 3.0 mM (Pavlidis & Madison, 1999; Awatramani et al. 2005; Lu et al. 2010; Sasaki et al. 2012a). Whole-cell recordings were performed from visually identified pyramidal cells. Patch pipettes (5–7 MΩ) were filled with 135 mM potassium gluconate, 4 mM KCl, 10 mM Hepes, 10 mM phosphocreatine, 4 mM MgATP, 0.3 mM NaGTP, and 200 μM Alexa Fluor 488 hydrazide (A-10436, Invitrogen) (pH 7.2 with KOH titration). The reversal potential of chloride ions was −91 mV. Voltages were not corrected for the theoretical liquid junction potential. In both CA1 and CA3 pyramidal neurons, the access resistance ranged from 10 to 25 MΩ. Data were discarded if access resistance changed by more than 20% during an experiment. Axonal cell-attached recordings were obtained from axonal varicosities using fluorophore-coated pipettes (Ishikawa et al. 2010; Sasaki et al. 2011, 2012a,b). Immediately before use, the glass pipettes (9–12 MΩ) were filled with aCSF, and the tips were immersed for 5–10 s in a solution containing 0.02% bovine serum albumin Alexa Fluor 488 conjugate (A-13100, Invitrogen). Action potentials were evoked by the delivery of a brief current injection (2–3 ms, 1–2 nA) into the soma. Signals were recorded at 20–100 kHz and low-pass filtered at 2 kHz using two Multiclamp 700B amplifiers (Molecular Devices, Union City, CA, USA). No further filtering was applied to these digitalized data. The signals were analysed using pCLAMP 10 software and custom
software written in Matlab (The Mathworks, Natick, MA, USA). Fast and slow pipette capacitive transients were minimised using the cell-attached configuration. All salts used were obtained from Wako Chemicals (Osaka, Japan). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), and picrotoxin (PIC) were from Sigma (St Louis, MO, USA).

**Presynaptic calcium imaging**

CA3 pyramidal cells were whole-cell patched using a pipette loaded with 250 μM Oregon Green 488 BAPTA-1 (OGB1) potassium salt (O-6806, Invitrogen) and 200 μM Alexa Fluor 594 conjugate (A-10438, Invitrogen) (Sasaki et al. 2012a). After allowing 30–60 min for intra-axonal dye diffusion, axonal branches were monitored at 50 frames per second (fps) using a Nipkow-disk confocal unit (CSU-X1, Yokogawa Electric, Tokyo, Japan), a back-illuminated CCD camera (iXon DU897, Andor, Belfast, Northern Ireland, UK), and a water-immersion objective lens (4×, 0.80 NA, Nikon, Tokyo, Japan). Fluorophores were excited at 488 nm with a laser diode (HPU50101PFS, FITEL, Tokyo, Japan) and visualised using a 507 nm long-pass emission filter. Axonal varicosities were carefully identified by eye and marked for the regions of interest (ROIs) with a diameter of 1–3 μm. Within each ROI, the fluorescence intensity was spatially averaged across pixels. No signal correction was done for the background fluorescent intensity. The AP-induced calcium increase was measured as ΔF/F = (Ft – F0)/F0, where F0 is the fluorescence intensity at time t and Ft is the baseline averaged for 10 s before and after time t. To analyse the variation in calcium transients, the variation in baseline fluorescence traces was compensated; for each ROI, the fluorescence intensity was spatially averaged across pixels. No signal correction was done for the baseline averaged for 10 s before and after time t. The coefficient of variation (CV) was calculated by dividing the standard deviation of the preceding baseline fluctuation, was calculated as the mean uEPSC amplitude divided by the standard deviation of the preceding baseline fluctuation, was calculated as the mean uEPSC amplitude divided by the standard deviation of the preceding baseline fluctuation, was calculated as the mean uEPSC amplitude divided by the standard deviation of the preceding baseline fluctuation.

\[ CV_{real}^2 = CV_{cal}^2 + CV_{base}^2, \]

where CVreal is the CV of the amplitudes of the calcium transients obtained from the raw fluorescence trace, and CVbase is the CV of the baseline fluorescence traces 2–10 s prior to AP induction. In all cases, CVbase was significantly smaller than CVreal, resulting in a positive value for CVcal.

**Reverse optical trawling (RO Ting)**

RO Ting was used to detect synaptically coupled neurons (Sasaki et al. 2009; Takahashi et al. 2010). For dye loading, a glass pipette (1–3 MΩ) was filled with aCSF containing 200 μM OGB1 AM (O-6807, Invitrogen) and was then inserted into the CA3 pyramidal cell layer. Using intra-pipette pressure, neurons were bolus-loaded with the calcium indicator. Ten micromolar glutamate was iontophoretically applied locally to the OGB1-loaded area through a glass pipette (~1 MΩ, 3–10 μA for 1–5 s each), while one or two CA3 or CA1 pyramidal cells were voltage-clamped at −70 mV. This low concentration of glutamate evoked APs in a small number of CA3 neurons. The iontophoretic pipette was slowly moved over the CA3 networks, and the evoked APs were monitored at 50 fps using a Nipkow disk confocal microscope with a water immersion objective lens (16×, 0.80 NA, Nikon, Tokyo, Japan). APs were determined based on the onset times of individual calcium transients using an automatic machine-learning algorithm (Sasaki et al. 2008). To determine which pyramidal neurons were candidate presynaptic targets for subsequent patch clamping, the AP timing was compared to the onset of the excitatory postsynaptic currents (EPSCs) recorded for the patch-clamped neurons. Analyses were performed with custom software written in Matlab.

**Data representation**

Data are reported as the mean ± standard deviation (SD).

**Results**

We used rat hippocampal slice cultures, because long-distance connections are re-established ex vivo to an extent observed in vivo (Wittner et al. 2007; Takahashi et al. 2010). To examine the properties of monosynaptic excitatory connections, we obtained simultaneous whole-cell recordings from 108 CA3-to-CA3 or 65 CA3-to-CA1 synaptic pairs of pyramidal cells (Fig. 1A). Synaptically connected pairs at the inter-neuron distance of more than 200 μm were identified using ROTing and targeted for patching. The presynaptic neurons were current-injected 50–100 times every 10 s to evoke single APs, and uEPSCs were monitored from the postsynaptic cell that was voltage-clamped at −70 mV (Fig. 1B). Monosynaptic contacts between two neurons were determined based on two following criteria: (i) the latency from presynaptic APs to the onset of postsynaptic currents was 2–5 ms for CA3-to-CA3 synapses and 4–8 ms for CA3-to-CA1 synapses; (ii) the signal-to-noise ratio of the mean current trace averaged over 50–100 trials, calculated as the mean uEPSC amplitude divided by the standard deviation of the preceding baseline fluctuation, was more than 3. Polysynaptic responses were distinguishable based on their distinctive response traces, which include several points of inflection during their rising phase with the latency of more than 8 ms after presynaptic APs. In our experimental conditions, these polysynaptic responses were rarely observed because of our extracellular concentrations of potassium, calcium and magnesium ions; these conditions were used to reduce ongoing
Figure 1. Paired patch-clamp recordings from pyramidal cells in organotypic hippocampal slice cultures
A, a confocal stack image of synaptically connected CA3 neurons. B, uEPSC traces (post) evoked by single spikes of a presynaptic neuron (pre) recorded from the synaptically connected pair shown in A. The synaptic efficacy was 24.8 pA. Twenty successive trials are superimposed. Raw current traces are shown in insets. All recordings were performed at a holding potential of −70 mV. C, trial-to-trial variation in uEPSCs at weak (top, synaptic efficacy = 6.2 pA) and strong (bottom, synaptic efficacy = 82.1 pA) synapses. D, transmission probability plotted versus the synaptic potency of CA3–CA3 (black) and CA3–CA1 (red) synapses. Data were collected from 108 CA3–CA3 and 65 CA3–CA1 pyramidal pairs. E and F, cumulative probability distribution of synaptic efficacy (E) and potency (F) of CA3–CA3 and CA3–CA1 synapses. Properties of unitary synaptic transmission do not differ between CA3–CA3 and CA3–CA1 synapses (P > 0.1, Kolmogorov–Smirnov test). G and H, variability (G) and synaptic stability (i.e., 1/CV$_{syn,eff}^2$) for various uEPSC sizes (H) are plotted versus the synaptic efficacy of CA3–CA3 and CA3–CA1 synapses. I and J, cumulative probability distribution of synaptic efficacy (I) and variability (J) of CA3–CA3 synapses recorded in higher divalent ion solution (black; 3.0 mM Ca$^{2+}$, 3.0 mM Mg$^{2+}$ and 3.3 mM K$^+$, n = 108) and lower divalent ion solution (blue; 2.0 mM Ca$^{2+}$, 2.0 mM Mg$^{2+}$ and 3.5 mM K$^+$, n = 54).
background activity in order to detect even small uEPSCs. We discarded the data for the rare cases that showed polysynaptic responses. Because the postsynaptic current is influenced by the somatic depolarisation of the presynaptic neuron due to an AP broadening effect (de Polavieja et al. 2005; Shua et al. 2006; Kole et al. 2007; Sasaki et al. 2012a), fluctuations in the subthreshold membrane potential were minimised to a range of 1 mV by using a high-calcium extracellular solution (Sasaki et al. 2009).

The amplitude of the uEPSCs varied among synapses as well as trials by over two orders of magnitude, ranging from 2 pA to 180 pA. We defined the synaptic ‘efficacy’ as the mean uEPSC amplitude of successful transmission trials; note that in each trial, transmission was considered a failure when the signal-to-noise ratio of an AP-triggered current was less than 2. Synapses with the efficacy of more than 56.7 pA (10% of the highest) and less than 9.7 pA (20% of the lowest) were defined as strong and weak synapses, respectively. Figure 1 shows that the synaptic ‘potency’ was defined as the mean uEPSC amplitude of synaptic transmission, whereas the synaptic ‘efficacy’ was defined as the mean uEPSC amplitude of successful transmission trials; note that in each trial, transmission was considered a failure when the signal-to-noise ratio of an AP-triggered current was less than 2. Synapses with the efficacy of more than 56.7 pA (10% of the highest) and less than 9.7 pA (20% of the lowest) were defined as strong and weak synapses, respectively. Figure 1 shows examples of time-varying uEPSCs at weak (Fig. 1C, top, synaptic efficacy = 6.2 pA) and strong (Fig. 1C, bottom, synaptic efficacy = 82.1 pA) synapses.

Higher synaptic potency exhibited higher probabilities of synaptic transmission; at synapses with a synaptic potency of > 30 pA, the success rate of synaptic transmission reached almost 100% (Fig. 1D). This relationship did not differ between CA3-to-CA3 (n = 108 pairs) and CA3-to-CA1 (n = 65 pairs) synapses (P > 0.1, Kolmogorov–Smirnov test). In addition, neither the synaptic efficacy (Fig. 1E) nor the synaptic potency (Fig. 1F) differed significantly between CA3–CA3 and CA3–CA1 synapses (mean synaptic efficacy: 31.3 ± 30.1 pA for 108 CA3–CA3 pairs; 28.8 ± 26.5 pA for 65 CA3–CA1 pairs; Mann–Whitney U_{108,65} = 3085.5, P = 0.18) (mean synaptic potency: 32.3 ± 29.7 pA for CA3–CA3; 30.6 ± 26.3 pA for CA3–CA1; Mann–Whitney U_{108,65} = 3171.0, P = 0.29). The average latencies of monosynaptic transmission were 3.5 ± 0.4 ms (ranging from 2.0–4.8 ms) and 4.5 ± 0.6 ms (ranging from 3.5–6.8 ms) for CA3–CA3 (n = 108 pairs) and CA3–CA1 (n = 65 pairs) synapses, respectively.

Trial-to-trial variability in synaptic transmission was measured as the CV value defined as the standard deviation divided by the mean of the uEPSCs efficacy. This CV (called here CV_{syn}) was, on average, 0.62 ± 0.31 (n = 173 synapses). Consistent with previous reports (Debanne et al. 1999; Frick et al. 2007), weaker synapses exhibited larger fluctuations in synaptic strength (Fig. 1G). The inverse square of the CV of the uEPSC size (1/CV^2) measures the stability of neurotransmission (Pavlidis & Madison, 1999; Bouddkazi et al. 2007). The 1/CV_{syn}^2 was positively correlated with synaptic efficacy, suggesting that the stability of neurotransmission increases as the mean uEPSC amplitude increases (Fig. 1H). This relationship did not differ between the CA3 and CA1 synapses (P > 0.1, comparison test for two regression parameters).

Since we used relatively higher concentrations of divalent ions in the extracellular solution, we conducted the same experiments under more physiological conditions using lower divalent ion solution (2.0 mM Ca^{2+}, 2.0 mM Mg^{2+}, and 3.5 mM K^+). In these conditions, the synaptic efficacy was lower (Fig. 1I; P < 0.05, Kolmogorov–Smirnov test), whereas CV_{syn} was higher (Fig. 1J; P < 0.05, Kolmogorov–Smirnov test), compared to those recorded in the higher divalent ion solution (3.0 mM Ca^{2+}, 3.0 mM Mg^{2+}, and 3.3 mM K^+). These differences may arise from the reduction in release probability at presynaptic terminals and enhancement of background neuronal activity.

The high transmission variability of synaptic transmission would originate from presynaptic components, because of the significant correlation between 1/CV_{syn}^2 and uEPSC size (Fig. 1H; see also Pavlidis & Madison (1999) and Bouddkazi et al. (2007)). We next focused on NMDA receptor-mediated responses. If NMDA receptor-mediated transmission exhibits a similar degree of trial-to-trial changes to AMPA receptor-mediated transmission as observed in Fig. 1, the result indicates that the transmission variability is independent of the post-synaptic receptor types and reinforces the hypothesis that the variability of transmission has the presynaptic origin.

To selectively measure NMDA receptor-mediated currents (EPSC_{NMDA}), slices were perfused with Mg^{2+}-free ACSF supplemented with 50 μM CNQX and 50 μM picrotoxin to inhibit AMPA and GABA_A receptor-mediated responses, respectively (Fig. 2). We measured compound EPSC_{NMDA} evoked by near-threshold electrical stimulation (Fig. 2A). After recording compound synaptic currents mainly mediated by AMPA receptors (cEPSC_{AMPA}) at Schaffer collateral–CA1 synapses in control drug-free condition, we pharmacologically isolated compound EPSC_{NMDA} (cEPSC_{NMDA}) at the identical synapses (Fig. 2B and C). In a representative recording shown in Fig. 2C, the trial-to-trial variability of cEPSC_{NMDA} was 0.079, which was nearly identical to the transmission variability of cEPSC_{AMPA} (0.076). Figure 2D illustrates the relationship between cEPSC_{AMPA} and cEPSC_{NMDA} variability in all recordings (n = 10 recordings). Most of the datasets were near or on the diagonal line (P < 0.01, Pearson’s correlation test), demonstrating that AMPA and NMDA receptor-mediated responses exhibited similar degrees of trial-to-trial variability within synapses. Thus, the synaptic transmission variability is unlikely to be attributable to postsynaptic receptors.

What factor determines the high variations in neurotransmitter release? In presynaptic cells, signal cascades are roughly divided into three functional components: (i) AP propagation to axonal boutons; (ii) calcium...
inflow in presynaptic boutons; and (iii) exocytosis of neurotransmitter-containing synaptic vesicles. Here, we focused on the first two potential mechanisms. Whereas APs have traditionally been considered to be digital-like ‘binary’ signals, emerging evidence has indicated that axons are capable of performing a more complex role by changing the axonal AP waveform (Debanne, 2004; Sasaki et al. 2011). To assess the waveform variation in APs propagating through axons, patch-clamp recordings were conducted from axonal varicosities 40–400 μm away from the axon hillock using fluorophore-coated pipettes (Fig. 3A). The distance from the axon hillock was measured by tracing the axonal path. APs were evoked at the soma by a current injection every 10 s, and the APs that were travelling down the axon were extracellularly captured as unit-like sharp negative currents in the cell-attached configuration (Fig. 3B). This current signal serves as a good measurement to estimate the intracellular AP width (Raastad & Shepherd, 2003; Sasaki et al. 2011, 2012a, b). All APs evoked at the soma faithfully invaded the recording sites, and the axonal APs did not vary in width (Fig. 3C, n = 11 recordings). The average AP width was 0.28 ± 0.02 ms, and the average CV of the normalised AP duration (CVAP) was 0.08 ± 0.03. These values were substantially lower than CVsyn (0.62 ± 0.31) observed in Fig. 1G, indicating that the trial-to-trial variations in synaptic transmission were unlikely to result from changes in the axonal AP waveform.

The second possible mechanism underlying the transmission variability is the calcium responses upon AP generation at presynaptic terminals. To examine this possibility, AP-evoked increases in calcium were monitored from en passant boutons of axon collaterals of hippocampal CA3 pyramidal cells. Axons were anterogradely labelled with a mixture of 200 μM OGB-1 and 200 μM Alexa 594 through whole-cell pipettes (Fig. 4A), and a Nipkow disk confocal microscope was used for high-speed, long-term and wide-field monitoring of the calcium dynamics from multiple axonal varicosities (Sasaki et al. 2012a; Takahashi et al. 2012). The arbours of the axon collaterals were distinguished from the dendrites based on their morphology, whereby axons are smaller in diameter than dendrites, project to wide areas and lack spines. Axonal varicosities at the axonal path distance of 40–250 μm from the axon hillock were selected for calcium imaging. Single APs were evoked by brief current injections into the soma every 10 s.

Figure 4B illustrates typical calcium fluorescence traces in a synaptic bouton evoked by repetitive single APs in 3.0 mM Ca2+. As shown in this example, the calcium increase in the bouton varies considerably from trial to trial; the ΔF/ΔF of the calcium increases in response to single APs from 12.2% to 48.9% with an average of 25.2%, and the CV of the amplitudes of the calcium transients (CVcal) was 0.32 (Fig. 4C). In all boutons tested, the variation in the amplitudes of the calcium transients was 0.32 (Fig. 4C). In all boutons tested, the variation in the amplitudes of the calcium transients was similar.
obtained from the raw fluorescence traces (CV_{real}) was significantly larger than that of the baseline fluorescence trace (CV_{base}), indicating that the variations in the calcium transients are not attributable to random optical noise. No apparent failures of calcium responses were detected in all boutons tested (n = 217 synaptic boutons). When the extracellular calcium ion concentration was lowered to 2.0 mM (3.5 mM K^+, 2.0 mM Mg^{2+}, and 2.0 mM Ca^{2+}), the ΔF/ΔF values were decreased, whereas the CV_{calc} values were increased, compared with those in 3.0 mM Ca^{2+} (Fig. 4D and E). These calcium-dependent changes are consistent with the results of synaptic transmission shown in Fig. 1I and J. The average ΔF/ΔF (Fig. 4D) and CV_{calc} (Fig. 4E) values were independent of the distance from the axon hillock (3.0 mM Ca^{2+}: ΔF/ΔF, r = -0.09, P > 0.1, n = 121 synaptic boutons from 9 cells; CV, r = -0.02, P > 0.1; 2.0 mM Ca^{2+}: ΔF/ΔF, r = -0.15, P > 0.1; CV, r = -0.02, P > 0.1, n = 96 synaptic boutons from 6 cells). These results might be a possible explanation for the fact that synaptic efficacy and the variability of synaptic transmission did not differ significantly between CA3–CA3 and CA3–CA1 synapses (Fig. 1G).

On average, the CV_{calc} in 3.0 mM Ca^{2+} was 0.28 ± 0.08 within a bouton (n = 121 boutons, Fig. 4E, left). Because of a power-law relationship between calcium influx and exocytosis of neurotransmitter (Augustine & Charlton, 1986; Bucurenciu et al. 2010), we expected the intra-bouton variations in the calcium increase produce larger variations in synaptic transmission. Given a power coefficient of 3.3 (Bucurenciu et al. 2010) and the data presented in Fig. 4E, the CV of synaptic transmission was estimated to be 0.66 ± 0.23 (n = 121 boutons). This estimation corresponds with those experimentally obtained from Fig. 1G (CV_{syn} = 0.62 ± 0.31). Taken together, we conclude that the intra-bouton variations in AP-induced calcium entry are a major factor responsible for the trial-to-trial differences in synaptic transmission.

Next, we sought to examine the correlation between calcium dynamics and the relative location of pre-synaptic varicosities. Within a single imaging field, we monitored AP-induced calcium transients simultaneously from 5–37 synaptic boutons (Fig. 5A). For a typical bouton pair shown in Fig. 5B, the correlation coefficient of the time-varying amplitudes was 0.01 (P > 0.05). Figure 5C shows the fluorescence increases in all 37 boutons that were numbered in an ascending order from the soma to distal axon terminals (Fig. 5C). The correlations between all possible pairs of 37 synaptic boutons are presented in a matrix form (Fig. 5D). If there is a local correlation, positive values would appear near the diagonal line in the matrix. However, the local correlations were not apparent in all neurons tested.

Figure 3. Stability of AP wavelength recorded from axons at resting potential
A, simultaneous recordings from the soma and axon of a CA3 pyramidal cell. Alexa Fluor 488 was injected into the soma of the CA3 pyramidal cell, and the axon located 220 μm from the axon hillock was targeted for cell-attached recording to record the conducting APs. B, example AP waveforms obtained from the soma and axon on trials 12, 36 and 47. All APs were evoked from resting potential (−63 mV). C, average of the normalised half-maximal width of the axonal AP from 11 different recordings. The resting membrane potential of the examined neurons was −65.2 ± 2.4 mV, on average (n = 11 cells). D, average CV_{AP} for the normalised half-maximal width of axonal APs (n = 11 recordings). For each recording, CV_{AP} was calculated from 40–60 trials.
The average correlation coefficient was $0.03 \pm 0.18$ (Fig. 5E; $n = 2452$ pairs from 129 synaptic boutons of 10 neurons). This distribution was not significantly different from that of surrogate datasets in which the fluorescence amplitudes of each cell were shuffled across trials ($P > 0.1$, Kolmogorov–Smirnov test). These results demonstrate that the trial-to-trial variations in the calcium dynamics were not correlated across synaptic boutons, even within a few micrometres of axonal length.

Because calcium rises in synaptic boutons play a key role in release of neurotransmitter, it is plausible that the inter-bouton independency of calcium signals gives rise to the independency of synaptic outputs from the same axon; however, this possibility has not been tested. Using ROTing, we searched presynaptic CA3 neurons that projected simultaneously onto two postsynaptic neurons and patched these triplets in which both postsynaptic targets were CA3 neurons (Fig. 6A and B, $1 \times$CA3-to-$2 \times$CA3 triplets) or CA1 neurons.
neurons (Fig. 6E and F, 1×CA3-to-2×CA1 triplets). The synaptic efficacies were not correlated between two postsynaptic pairs in either triplet (Fig. 6C and G; CA3–CA3 synapses, \( r = -0.10, P > 0.1 \); CA3–CA1 synapses, \( r = -0.52, P > 0.1 \)). The 20–80% rise times were not significantly correlated between the two postsynaptic pairs (Fig. 6D and H; \( r = 0.27, P = 0.09 \)). The unitary conductances recorded simultaneously in two postsynaptic neurons were plotted across trials (Fig. 6J). The trial-to-trial variation in the uEPSC sizes of one neuron was independent of that in the other neuron. The correlation coefficients of the two uEPSC fluctuations were \( 0.02 \pm 0.09 \) in 1×CA3-to-2×CA3 triplets (\( n = 9 \) recordings) and \( 0.05 \pm 0.06 \) in 1×CA3-to-2×CA1 triplets (\( n = 6 \) recordings). This mutual independence did not differ between CA1 and CA3 synapses (Fig. 6J, \( t_{19} = 0.14, P = 0.40 \), Student’s \( t \) test). Since the ROTing technique requires loading of calcium indicators into potential presynaptic cells, this process could interfere with calcium-dependent neurotransmission at presynaptic terminals. To rule out this possibility, we searched 1×CA3-to-2×CA3 triplets by random patching without the ROTing. In these synaptically connected triplets, the average correlation coefficient of uEPSC fluctuations between two postsynaptic cells was \( 0.02 \pm 0.11 \) (\( n = 6 \) recordings), which is a similar value obtained from the triplets revealed by the ROTing. We conclude that synapses act independently of other synapses on the same axons.

**Discussion**

In this study, we found that synapses are heterogeneous in efficacy and variable among trials. Moreover, we found that the output patterns exhibited by the identical axons were not correlated between synapses. AP wavelength recorded from axons was highly reliable over trials, although axonal varicosities underwent stochastic calcium fluctuations in response to every AP invasion, independently of nearby boutons. These results suggest that the diversity of synaptic efficacy between pyramidal cells is attributable to the variability of AP-induced calcium influxes both among boutons and within single boutons.

We demonstrated that synaptic weights were negatively correlated with the trial-to-trial variations in uEPSC sizes, that is weaker synapses were not merely weaker in synaptic strength but were also more stochastic in terms of their transmission events and less stable in terms of their uEPSC sizes at successful transmission. The difference
in uEPSC sizes may be accounted for by the variability of passive cable properties and/or active conductance in hippocampal pyramidal cells. In fact, we previously demonstrated that larger uEPSGs had faster waveform kinetics (Ikegaya et al. 2012). It has been hypothesised that weak synapses serve as preparative spares for future plasticity, whereas strong synapses function as channels through which neural information flows reliably (Kasai et al. 2003; Lefort et al. 2009). In accordance with this hypothesis, we recently demonstrated that single transmission at strong synapses could elicit APs in postsynaptic neurons, a phenomenon termed spike transmission, and thereby contributes to the stabilisation of sparse spiking and the network responsiveness to single neurons (Ikegaya

Figure 6. Time-varying fluctuations in synaptic transmission do not correlate between different synapses originating from identical presynaptic CA3 neurons: triple patch-clamp recordings
A and E, image showing triple whole-cell recordings from a divergent circuit in which one presynaptic CA3 pyramidal cell innervates two postsynaptic CA3 (A) and CA1 (E) pyramidal cells. B and F, traces of representative uEPSCs at CA3-to-CA3 (B) and CA3-to-CA1 (F) synapses in response to single APs in the presynaptic CA3 neuron. C and G, summary depicting the independency of uEPSC efficacy between two synaptic connections (proximal synapse no. 1 and distant synapse no. 2) (C, CA3–CA3 synapses, n = 15 triplet recordings; G, CA3–CA1 synapses, n = 6 recordings). The red dots indicate the example recordings shown in B and F. D and H, the 20–80% rise times are plotted (same as in C and G). I, representative trial-to-trial variation in the uEPSCs of two postsynaptic pyramidal cells (uEPSC$_1$ and uEPSC$_2$) evoked simultaneously by single APs in the CA3 presynaptic pyramidal cells (top, two CA3 postsynaptic neurons; bottom, two CA1 postsynaptic neurons). J, correlation coefficient of the uEPSC size between two postsynaptic neurons (n = 9 recordings for 2 CA3 pyramids with ROTing; n = 6 recordings for 2 CA3 pyramids without ROTing; n = 6 recordings for 2 CA1 pyramids).
et al. 2012). Thus, the non-uniform distribution of synaptic strengths may represent a hierarchical organisation of the network structure.

We observed no marked difference in fundamental synaptic properties between CA3-to-CA3 (close) and CA3-to-CA1 (distant) synapses. The synaptic properties were independent of the locations of the synapses along the axons. These results suggest that synaptic strength at the resting potential is uniform over axonal arbours, irrespective of the distance from the soma. Recent studies have demonstrated that subthreshold depolarisation at the soma can enhance synaptic outputs to nearby cortical pyramidal cells mainly through an AP broadening effect, implying that cortical axons carry mixed digital and analog signals (Debanne, 2004; Alle & Geiger, 2006; Shu et al. 2006; Kole et al. 2007). Thus, when the soma is depolarised to a sufficient level, synapses within hundreds of micrometres from the soma may be potentiated, allowing specific modulations of synaptic outputs depending on the location of synapses (Sasaki et al. 2012a).

The intra-bouton and inter-bouton variance in AP-induced calcium transients have been reported in various brain regions (Wu & Saggau, 1994; Frenguelli & Malinow, 1996; Mackenzie et al. 1996; Llano et al. 1997; Koester & Sakmann, 2000; Kirischuk & Grantyn, 2002; McGuinness et al. 2010). Most of these studies have speculated that the variable response is caused by the probabilistic opening of a small number of calcium channels on the presynaptic bouton, not by the fluctuations in AP waveform or propagation through the axons. However, these studies have failed to verify whether APs are, in fact, faithfully propagated along axons with a constant waveform, in spite of a report showing that APs can fail to conduct in the middle of axon tracts (Debanne et al. 1997). This occasional AP elimination could also contribute to trial-to-trial variations in synaptic outputs. We used axonal patch-clamp recordings and demonstrated directly that APs during axonal conduction did not vary in waveform among trials, except under special conditions observed in our previous study (Ishikawa et al. 2010; Sasaki et al. 2011, 2012a). These data indicate that the trial-to-trial variations in synaptic transmission cannot be accounted for by the variation in axonal AP conduction. However, it should be noted that pyramidal cells in the hippocampal CA3 region often generate burst firings, which might alter the physiology of axonal AP propagation and calcium-dependent neurotransmission at presynaptic terminals. As a result, in the bursting mode, the properties of synaptic transmission might be different from those under resting conditions. In previous studies, calcium activity was recorded from only a small number of boutons (usually far less than 10 boutons), presumably due to technical limitations. We monitored simultaneously up to 37 boutons with high spatial (5 pixels μm−1) and temporal (up to 50 Hz) resolution using Nipkow disk confocal microscopy. We then discovered the intra-bouton and inter-bouton variances for an ensemble of boutons. A drawback of our imaging method was that we could not identify the postsynaptic cells contacting the monitored boutons. It has been reported that the amplitude of calcium signals in the boutons of cortical pyramidal cells is dependent on the target cell type (Koester & Johnston, 2005). In our study, some boutons may be synapses made with inhibitory cells. Therefore, the identification of postsynaptic cell types with genetic tools and morphological data will be required for further detailed analyses of the calcium dynamics in presynaptic boutons.

In conclusion, synapses are heterogeneous in efficacy and work independently of one another. These synaptic properties are not trivial, especially when extrapolated to higher-order network operations. These findings will be helpful in modelling neural networks, because our data justify the mathematical simplification with spatiotemporal independence of synaptic parameters.

References


**Author contributions**

T.S. collected the experimental data and conducted the data analysis. T.S. and Y.I. wrote the manuscript. N.M. supervised the project and provided feedback on the manuscript.

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