INTRODUCTION

Information processing and storage in the brain relies on Ca\(^{2+}\)-dependent release of the excitatory neurotransmitter glutamate from axonal terminals. Classical studies in preparations of giant synapses that enable direct experimental access, have revealed key mechanistic relationships between neurotransmitter release, evoked Ca\(^{2+}\) entry, and resting presynaptic Ca\(^{2+}\) (Bollmann, Sakmann, Gerard, 1981).

Fluorescence lifetime imaging reveals regulation of presynaptic Ca\(^{2+}\) by glutamate uptake and mGluRs, but not somatic voltage in cortical neurons

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Funding information
Russian Global Education Program; H2020 European Research Council, Grant/ Award Number: Advanced Grant 323113; Wellcome Trust, Grant/Award Number: 323113 and 857562; European Commission; Russian Foundation for Basic Research, Grant/Award Number: 20-04-00245; Wellcome Turst, Grant/Award Number: 104033/Z/14/Z; Epilepsy Research, Grant/ Award Number: UK P1806

Abstract

Brain function relies on vesicular release of neurotransmitters at chemical synapses. The release probability depends on action potential-evoked presynaptic Ca\(^{2+}\) entry, but also on the resting Ca\(^{2+}\) level. Whether these basic aspects of presynaptic calcium homeostasis show any consistent trend along the axonal path, and how they are controlled by local network activity, remains poorly understood. Here, we take advantage of the recently advanced FLIM-based method to monitor presynaptic Ca\(^{2+}\) with nanomolar sensitivity. We find that, in cortical pyramidal neurons, action potential-evoked calcium entry (range 10–300 nM), but not the resting Ca\(^{2+}\) level (range 10–100 nM), tends to increase with higher order of axonal branches. Blocking astroglial glutamate uptake reduces evoked Ca\(^{2+}\) entry but has little effect on resting Ca\(^{2+}\) whereas both appear boosted by the constitutive activation of group 1/2 metabotropic glutamate receptors. We find no consistent effect of transient somatic depolarization or hyperpolarization on presynaptic Ca\(^{2+}\) entry or its basal level. The results unveil some key aspects of presynaptic machinery in cortical circuits, shedding light on basic principles of synaptic connectivity in the brain.

KEYWORDS

axons, calcium imaging, neurotransmitter release, presynaptic mechanisms, synapse, synaptic plasticity
& Borst, 2000; Eggermann, Bucurenciu, Goswami, & Jonas, 2012; Neher & Sakaba, 2008; Schneggenburger & Neher, 2000). In contrast, Ca²⁺ signalling at small central synapses, which are difficult to access in situ, has hitherto been explored mainly by monitoring the fluorescence intensity of Ca²⁺-sensitive indicators. The intensity-based approach has been instrumental in relating dynamic changes in presynaptic Ca²⁺ to use-dependent plasticity of neurotransmitter release (reviewed in Regehr, 2012; Zucker & Regehr, 2002). However, intensity measures are prone to uncontrolled concomitants, such as changes in local dye concentration, photobleaching, tissue light scattering, or laser power fluctuations. These limitations could be critical for Ca²⁺ concentration ([Ca²⁺]₀) measurements whereas the accuracy of ratiometric Ca²⁺ indicators in optically turbid media, such as brain tissue, is compromised by the strong dependence between the wavelength and scattering/absorption of light. Thus, monitoring [Ca²⁺] inside individual axons, in particular the nanomolar range basal Ca²⁺ levels, has been a challenge.

A breakthrough came with exploring fluorescence lifetime sensitivity of some Ca²⁺ indicators to free Ca²⁺ (Wilms & Eilers, 2007; Wilms, Schmidt, & Eilers, 2006). As a time-domain measure, fluorescence lifetime imaging (FLIM) is not influenced by light scattering, dye concentration, focus drift or photobleaching. We have recently advanced and validated an approach that optimizes FLIM-based readout of such indicators in experimental settings in situ (Jennings et al., 2017; Zheng et al., 2015; Zheng, Jensen, & Rusakov, 2018). This method has enabled dynamic monitoring of presynaptic [Ca²⁺] in individual axons in situ, with nanomolar sensitivity (Jensen et al., 2019; Jensen, Zheng, Tyurikova, Reynolds, & Rusakov, 2017). Here, equipped with this approach, we asked, first, whether the excitatory synapses supplied by individual axons of cortical neurons show evenly distributed functional features of presynaptic Ca²⁺ signalling, or whether these features change along the axon. This quest has been an important line of enquiry into fundamental traits of circuit formation and function (Bakkum et al., 2013; Debanne, Guerineau, Gahwiler, & Thompson, 1997; Guerrero et al., 2005; Kukley, Capetillo-Zarate, & Dietrich, 2007).

Second, we sought to understand whether and how the local excitatory activity affects presynaptic Ca²⁺. Glutamate released from axons is rapidly buffered and taken up, mainly by astroglial transporters (Danbolt, 2001); this keeps its extracellular concentration at the nanomolar level (Herman & Jahr, 2007) while limiting activation of intra- and extrasynaptic metabotropic glutamate receptors (mGlRs) (Huang & Bordey, 2004; Min, Rusakov, & Kullmann, 1998). Axons of cortical neurons often express group 2 mGlR2 and mGlR3, but also group 1 mGlR1 and mGlR5 subtypes of mGlRs (Cartmell & Schoepp, 2000; Gereau & Conn, 1995), with recent evidence implicating group 2 mGlRs in presynaptic inhibition in human cortex pyramidal cells (Boccio et al., 2018). These two receptor subgroups enable cellular cascades that trigger, respectively, either inhibition or mobilization of presynaptic Ca²⁺ signalling (Cartmell & Schoepp, 2000; Pinheiro & Mulle, 2008; Reiner & Levitz, 2018). The net effect of such signalling, in terms of presynaptic [Ca²⁺] changes, remains poorly understood.

Finally, our aim was to establish whether somatic depolarization (or hyperpolarization) of the host neuron affects its axonal Ca²⁺ signalling. This issue has long been a subject of debate. It has been shown that depolarizing central neurons can boost glutamate release from distant axonal boutons (Alle & Geiger, 2006; Christie, Chiu, & Jahr, 2011; Scott, Ruiz, Henneberger, Kullmann, & Rusakov, 2008; Shu, Hasenstaub, Duque, Yu, & McCormick, 2006). However, axonal Ca²⁺ imaging (using fluorescence-intensity measures) has suggested that, in hippocampal granule cells, somatic depolarization reduces spike-evoked presynaptic Ca²⁺ entry in proximal axonal segments (Ruiz et al., 2003; Scott et al., 2014) while having no detectable effect distally (Scott et al., 2008). In contrast, in cortical pyramidal cells, somatic depolarization was proposed to boost spike-evoked presynaptic Ca²⁺ entry (Christie et al., 2011; Shu et al., 2006) whereas it was presynaptic hyperpolarization that enhanced transmission between cortical or hippocampal pyramidal cells (Rama et al., 2015). The role of the underlying Ca²⁺ mechanisms has therefore remained debateable, mainly because of the limitations imposed by the traditional fluorescence intensity-based Ca²⁺ measures. We therefore thought that it was important to explore the FLIM-based approach, in the context.

## MATERIALS AND METHODS

### 2.1 Animal experimentation

All experiments involving animals were carried out in accordance with the European Commission Directive (86/609/EEC) and the United Kingdom Home Office (Scientific Procedures) Act (1986) under the Home Office Project Licence PPL P2E0141 E1. C57BL/6 mice (Charles River Laboratories; IMSR Cat#JAX_000664, RRID: IMSR_JAX-000664) of both sexes (60% male and 40% female) were group housed in a controlled environment as mandated by the locally approved guidelines, on a 12 hr light cycle and with food and water provided ab libitum. This study was not pre-registered.

### 2.2 Brain slice preparation

Acute 300 μm thick coronal brain slices were obtained from 47 3–4 week old C57BL/6 mice (15–20 g), in full compliance with national guidelines on animal experimentation, in accord with Schedule I humane procedures. Animals were anaesthetized by 5% isoflurane inhalation, deep anaesthesia was ensured by a lack of pedal reflexes; after cessation of breathing animals were decapitated for brain isolation and removal. The locally approved isoflurane anaesthesia is sufficiently potent to provide muscle relaxation adequate for ascribed procedure and produces less cerebral vasodilation than analogues (e.g. by halothane); absorption and elimination of isoflurane inhalation occur through the lungs and allow rapid and reliable aesthetic induction. Slices were prepared in an ice-cold slicing solution containing (in mM): NMDG, 92 (Sigma-Aldrich; Cat#M2004); KCl, 2.5 (Sigma-Aldrich; Cat#60130); NaH₂PO₄, 1.25 (Sigma-Aldrich; Cat#58282); HEPES, 20 (Sigma-Aldrich; Cat#54457); thiourea, 2 (Sigma-Aldrich; Cat#PHR1758);
sodium ascorbate, 5 (Sigma-Aldrich; Cat#PHR1279); sodium pyruvate, 3 (Sigma-Aldrich; Cat#P8574); MgCl₂, 10 (Sigma-Aldrich; Cat#63069); D-glucose, 25 (Sigma-Aldrich; Cat#G8270); NaHCO₃, 30 (Sigma-Aldrich; Cat#56297); CaCl₂, 0.5 (Sigma-Aldrich; Cat#21115); and sucrose, 1 (Sigma-Aldrich; Cat#S0389). For recovery slices were left for 15–20 min in slicing solution and for 40 min at 34°C ACSF solution, before being transferred to a submersion chamber for storage in an extracellular solution containing (in mM): NaCl, 125 (Sigma-Aldrich; Cat#57653); KCl, 2.5; NaH₂PO₄, 1.25; NaHCO₃, 26 (Sigma-Aldrich; Cat#56297); D-glucose, 18; CaCl₂, 2; MgSO₄, 1.3 (Sigma-Aldrich; Cat# 63138) (osmolarity adjusted to 295–310 mOsm with D-glucose). All solutions were continuously bubbled with 95% O₂/5% CO₂. Slices were allowed to rest for at least 60 min before recordings started.

2.3 | Electrophysiology, axon tracing and Tornado scanning in pre-synaptic boutons

We used a Femto2D-FLIM two-photon excitation (2PE) imaging system (Femtonics, Budapest), integrated with patch-clamp electrophysiology (Scientifica, UK) and optically linked to two femtosecond pulse lasers MaiTai (SpectraPhysics-Newport), with electrophysiology (Scientifica, UK) and optically linked to two femtosecond pulse lasers MaiTai (SpectraPhysics-Newport), with 4–5 mOsm resistance. Internal solution contained (in mM): KCl, 70; NaCl, 20; CaCl₂, 2; NaHCO₃, 10 (Sigma-Aldrich; Cat#21115); MgCl₂, 1; sucrose, 10 (Sigma-Aldrich; Cat#S0389). For recovery slices were left for 15–20 min in slicing solution and for 40 min at 34°C ACSF solution, before being transferred to a submersion chamber for storage in an extracellular solution containing (in mM): NaCl, 125 (Sigma-Aldrich; Cat#57653); KCl, 2.5; NaH₂PO₄, 1.25; NaHCO₃, 26 (Sigma-Aldrich; Cat#56297); D-glucose, 18; CaCl₂, 2; MgSO₄, 1.3 (Sigma-Aldrich; Cat# 63138) (osmolarity adjusted to 295–310 mOsm with D-glucose). All solutions were continuously bubbled with 95% O₂/5% CO₂. Slices were allowed to rest for at least 60 min before recordings started.

2.4 | 2PE Tornado-FLIM readout of Ca²⁺ concentration in small axonal boutons

In slice preparations, we thus identified and patched pyramidal neurons located in layer 2/3 of the visual cortex. Cell axons were followed, as described above, to focus on individual boutons; during individual trials (typically lasting 2 s), continuous tornado line scans were collected. The scan data were recorded by the standard analogue integration in Femtonics MES (RRID: SCR_018309), and by TCSPC in Becker and Hickl SPCM (RRID: SCR_018310) using dual HPM-100 hybrid detectors. Next, we used the fast-FLIM analysis procedure described previously (Zheng et al., 2015, 2018) to handle individual Tornado scans. We routinely collected and stored FLIM line scan data in a t × x × y × T data cube representing an x-y image with the distribution of nanosecond decay timestamps (t) of individual photons, pixel-by-pixel over the frame duration (T). However, for the purposes of this study, we collapsed all spatial information thus boosting photon counts per scan cycle. The FLIM data represented therefore the average signal over the bouton area (approximately the entire profile) covered by the scan. Post-hoc FLIM analyses were performed in a custom-made data analysis toolbox, which is available online (https://github.com/shengkaiyu/FIMAS; RRID: SCR_018311). The fluorescence decay curve (lifetime photon counts) was integrated over the 9 ns period post-pulse, and normalized to the maximum value, as detailed earlier (Zheng et al., 2018). Data from up to 5–10 neighbouring pixels were averaged to ensure that the FLIM decay traces had sufficient counts towards the tail of the decay (8–12 ns post-pulse). Data from a single trial were normally sufficient for boutons located closer to the surface of the tissue; for deeper-located boutons, several trials were required to estimate accurately the Ca²⁺ dynamics evoked by an AP.

2.5 | Estimating action potential evoked presynaptic Ca²⁺ entry

The (steady-state) basal presynaptic [Ca²⁺]₀ was directly estimated from FLIM readout over the averaging interval of ~500 ms before an action potential. However, the rapid rise of presynaptic [Ca²⁺] (1–2 ms) was faster than the averaging time of FLIM recording (5–10 ms). Therefore, to improve the signal-to-noise ratio in measuring presynaptic Ca²⁺ entry Δ[Ca²⁺], the spike-evoked peak presynaptic [Ca²⁺]ₚ was estimated using both FLIM and intensity recordings as follows. First, the saturated OGB-1 fluorescence value Fmax was estimated as

\[
F_{\text{max}} = \frac{F_{\text{max}}}{F_{0}^{\text{sat}}}
\]

where [Ca²⁺]₀ is measured directly with FLIM, and Kᵢ = 0.24 μM and γ = 6 are Ca²⁺ affinity and dynamic range of OGB-1, respectively (Scott & Rusakov, 2006). Second, [Ca²⁺]ₚ (equilibrated over 1–2 ms) was calculated as [Ca²⁺]ₚ = Kᵢ [Ca²⁺]₀ (Maravall, Mainen, Sabatini, & Svoboda, 2000; Tsien, 1989), so that Δ[Ca²⁺] = [Ca²⁺]ₚ - [Ca²⁺]₀. As an extra validation step, the fluorescence intensity decay was checked for a match with the FLIM readout decay, in the linear range of OGB-1 sensitivity to [Ca²⁺].
2.6 Statistical analysis

During axonal tracing with 2PE imaging, axonal boutons were sampled in an arbitrary manner, as they appeared in the focal plane showing distinct varicose morphology and clear action potential induced Ca\(^{2+}\) responses. No exclusion criteria were applied to animals or slices; unhealthy patched cells were excluded according to the criteria described above. Blinding was not applicable to experimental manipulations during live recording. Thus no strict randomization procedures were applicable during 3D axonal tracing. In experiments comparing independent samples in control condition (branch order comparisons), both two-way ANOVA and conservative non-parametric Kruskal-Wallis ANOVA tests were applied as described. In the real-time experiments involving application of a ligand or a voltage change, the statistical unit was individual boutons, with the effect of experimental manipulation being the only factor of interest; normally, 1–4 boutons were recorded from individual cells, 1–2 cells were recorded per slice/animal. The paired ‘baseline-effect’ comparison was therefore employed in all such experiments, in accord with the electrophysiological convention. The sample size was not predetermined because the variability of measured parameters was not known a priori. Shapiro–Wilks tests for normality produced varied results across raw data samples. Accordingly, we used either the paired-sample t-test, or the paired-sample non-parametric Wilcoxon Signed Ranks test, as indicated. The statistical software in use was Origin 2019 (Origin Lab; RRID: SCR_014212).

3 RESULTS

3.1 Monitoring presynaptic [Ca\(^{2+}\)] using FLIM-based readout

To calibrate FLIM readout for absolute [Ca\(^{2+}\)] measurements on a designated two-photon excitation (2PE) microscopy imaging system, we employed the protocol established for OGB-1 previously (Zheng et al., 2015, 2018). The procedure uses the ratiometric Normalized Total Count (NTC) method in which photon counts are integrated under the lifetime decay curve (over its Ca\(^{2+}\)-sensitive span), and the result is related to the peak value (Materials and Methods; Figure 1a). The outcome confirmed high sensitivity of the readout in the 0–300 nM [Ca\(^{2+}\)] range, providing a quantitative reference to the microscopy measurements (Figure 1b). This calibration outcome was similar to the data set obtained previously for a different 2PE system (Zheng et al., 2015, 2018), arguing for the robustness of the present approach.

We next held individual layer 2/3 pyramidal cells in whole-cell mode dialysing them with 300 μM OGB-1, and traced their axons up to a distance of 250–300 μm from the soma, in two-photon excitation (2PE) mode (Figure 1c). Once focussed on individual axonal boutons, we used spiral (tornado) line scan (at 500–1000 Hz) covering the bouton profile (Figure 1d), to record Ca\(^{2+}\)-sensitive photon count data, before and after triggering a somatic spike (Jensen et al., 2017, 2019). With the averaging of the spatial scan data (Methods), this type of recording provides stable photon count acquisition from a small region of interest during repeated trials over ~20 min (Figure 1e). This was consistent with the previously documented FLIM recording stability, in similar settings, for up to 60 min (Jensen et al., 2019; Zheng et al., 2018). Thus, decoding the recorded FLIM data provided robust traces of resting basal [Ca\(^{2+}\)]\(_{0}\) and spike-evoked presynaptic [Ca\(^{2+}\)] dynamics, in the 10–300 nM range, for boutons located at axonal branch orders 1–3, at different distances from the soma (Figure 1f).

3.2 Resting Ca\(^{2+}\) and evoked Ca\(^{2+}\) entry change with axonal branch order

We thus collected data on resting presynaptic [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{0}\)) and spike-evoked Ca\(^{2+}\) entry (concentration increment Δ[Ca\(^{2+}\)]) from 61 axonal boutons in 25 pyramidal cells. First, the results indicated no overall dependence of either [Ca\(^{2+}\)]\(_{0}\) or Δ[Ca\(^{2+}\)] on the distance from the soma (Figure 2a). This data set uncovered significant heterogeneity of both [Ca\(^{2+}\)]\(_{0}\) (detected range ~10–100 nM) and, especially, Δ[Ca\(^{2+}\)] (detected range ~10–300 nM) across the axonal population.
3.3 | Glutamate uptake and metabotropic glutamate receptors differentially affect [Ca\textsuperscript{2+}]\textsubscript{0} and Δ[Ca\textsuperscript{2+}]

To understand whether and how glutamate uptake affects presynaptic Ca\textsuperscript{2+} signalling, we documented changes in [Ca\textsuperscript{2+}]\textsubscript{0} and Δ[Ca\textsuperscript{2+}] in response to the pharmacological blockade of astroglial glutamate transporters. Application of the transporter inhibitor TBOA (Tsukada, Lino, Takayasu, Shimamoto, & Ozawa, 2005) had no detectable effect on [Ca\textsuperscript{2+}]\textsubscript{0} while depressing spike-evoked Δ[Ca\textsuperscript{2+}] by ~70% (Figure 3a and b). This suggests that the extracellular glutamate level elevated by TBOA application can inhibit Ca\textsuperscript{2+} entry through presynaptic Ca\textsuperscript{2+} channels, either through an ionotropic (electrogenic) mechanism, such as membrane depolarization, or through the action of presynaptic metabotropic glutamate receptors, or both. To distinguish between these two mechanisms, we recorded [Ca\textsuperscript{2+}]\textsubscript{0} and Δ[Ca\textsuperscript{2+}] in individual axonal boutons in baseline conditions, 15 min after washing in the selective group 1/2 mGluR blocker S-MCPG, and 15 min after the subsequent application of TBOA.

S-MCPG application reduced [Ca\textsuperscript{2+}]\textsubscript{0} by ~25% (Figure 3c and d, left), suggesting that group 1 or group 2 mGluRs, by being...
persistently (constitutively) activated, contribute an additional Ca\textsuperscript{2+} source to the equilibrated presynaptic basal Ca\textsuperscript{2+}. To distinguish between the two receptor subtypes, we repeated these tests with the specific group 2 mGluR blocker LY341495 and found no effect on [Ca\textsuperscript{2+}]\textsubscript{0}, thus indicating the prevalent role of group 1 mGluR in the constitutive control of [Ca\textsuperscript{2+}]\textsubscript{0}. The blockade of glutamate transporters in the presence of S-MCPG had little further effect on [Ca\textsuperscript{2+}]\textsubscript{0}, consistent with no effect of TBOA in control conditions (Figure 3b, left). The fact that boosting the extracellular glutamate level has no effect on [Ca\textsuperscript{2+}]\textsubscript{0} (Figure 3b, left) whereas blocking mGluRs reduces it (Figure 3d, left) suggests that, firstly, constitutive activation of group 1 mGluRs does not depend on glutamate and, secondly, once glutamate-activated, the receptor suppresses evoked Ca\textsuperscript{2+} entry (Figure 3b, right). However, S-MCPG application did reduce Δ[Ca\textsuperscript{2+}] by ~50%, which was further depressed by TBOA (Figure 3d, right). This result suggests, firstly, that the TBOA-induced decrease in Δ[Ca\textsuperscript{2+}] (Figure 3b, d; right) does involve group 1/2 mGluRs. Secondly, it relates constitutive activation of these receptors to increased evoked presynaptic Ca\textsuperscript{2+}. In our tests, the effect of the specific group 2 mGluR blocker LY341495 on Δ[Ca\textsuperscript{2+}] was inconclusive as the cells became unstable during spike initiation (see Discussion). Overall, these findings may reflect a complex nature of presynaptic Ca\textsuperscript{2+} control by different mGluR subtypes (see Discussion).

### 3.4 Subthreshold somatic depolarization (or hyperpolarization) has no consistent effect on [Ca\textsuperscript{2+}]\textsubscript{0} or Δ[Ca\textsuperscript{2+}]

To understand the effect of somatic depolarization on presynaptic Ca\textsuperscript{2+} dynamics, we documented [Ca\textsuperscript{2+}]\textsubscript{0} and Δ[Ca\textsuperscript{2+}] in individual axonal boutons when the presynaptic cell was either depolarized, or hyperpolarized, by ~15 mV either way for 500 ms prior to evoking an action potential (Figure 4a). In each selected axonal bouton, all three conditions were tested in an arbitrary sequence, to avoid any longer term effects. Overall, we found no consistent effect of somatic voltage manipulation on either [Ca\textsuperscript{2+}]\textsubscript{0} or Δ[Ca\textsuperscript{2+}] in n = 19 boutons recorded in eight pyramidal cells (Figure 4b and c).

### 4 DISCUSSION

In the present study, we employed an imaging method that could detect changes in presynaptic [Ca\textsuperscript{2+}] with virtually nanomolar sensitivity in the concentration range between 10-300 nM (Zheng et al., 2015, 2018). We have documented average [Ca\textsuperscript{2+}]\textsubscript{0} values in baseline conditions between 30 and 60 nM, which is consistent with earlier high-sensitivity Ca\textsuperscript{2+} measurements in neuronal processes (Canepari, Vogt, & Zecevic, 2008; Helmchen, Imoto, & Sakmann, 1996), including axons (Ernolyuk et al., 2013), that employed alternative Ca\textsuperscript{2+} imaging methods. Similarly, the range of Δ[Ca\textsuperscript{2+}] between 50 and 300 nM reported here corresponds to the equilibrated presynaptic [Ca\textsuperscript{2+}] after a very brief (~1 ms) local ‘hotspot’ entry, and is fully in line with previous estimates based on fluorescence-intensity measures (Ernolyuk et al., 2013; Helmchen et al., 1996; Rusakov, Saitow, Lehre, & Konishi, 2005; Scott & Rusakov, 2006). However, the FLIM-based method has several advantages over previous approaches, which enables us to explore presynaptic [Ca\textsuperscript{2+}] dynamics in greater detail, as discussed earlier (Wilms et al., 2006; Zheng et al., 2018; Zheng & Rusakov, 2015).

The quest to identify a systematic pattern of functional synaptic features along the axon has been an important line of enquiry into fundamental traits of circuit formation and function (Bakkum et al., 2013; Debanne et al., 1997; Guerrero et al., 2005; Kukley et al., 2007). One of the most common questions asked in this context has been whether the increasing sparsity of longer cell-cell connections in the cortex is compensated by their increased synaptic efficacy. We have recently employed multiplexed imaging of glutamate release and presynaptic Ca\textsuperscript{2+} in organotypic brain slices to find that [Ca\textsuperscript{2+}]\textsubscript{0} and Δ[Ca\textsuperscript{2+}] are positively correlated with release probability (Jensen et al., 2019). Thus, the present data appear to argue against increased release efficacy with greater distances from the soma, but they do support the idea that in cortical pyramidal cells, axonal branches of higher orders host more efficient release sites (Figure 2). Clearly, imaging glutamate release at individual axonal boutons should provide further clarity on the subject. However, no known time-resolved (FLIM-based) optical sensors of glutamate are available at present. Therefore, to gauge accurately glutamate release efficacy in the turbid medium of acute cortical slices or in vivo, a special effort would be required to avoid multiple concomitants of the fluorescence intensity signal, for its unbiased interpretation.

We have found that the blockade of the group 1 mGluRs, which occur in cortical axons (Cartmell & Schoep, 2000; Gereau & Conn, 1995), reduces presynaptic basal [Ca\textsuperscript{2+}], suggesting that these receptors are constitutively active, in a glutamate-independent manner. These receptors are known to trigger a powerful molecular cascade initiating local IP\textsubscript{3}-receptor dependent release from Ca\textsuperscript{2+} stores, both in neurons (Pinheiro & Mulle, 2008; Reiner & Levitz, 2018) and in astroglia (Bazargani & Attwell, 2016; Verkhratsky & Kettenmann, 1996), and their ligand-independent persistent activity has long been known documented (Ango et al., 2001). The inhibiting action of the group 1 mGluR blockade on basal Ca\textsuperscript{2+} indicates that, by acting either directly or indirectly on the axons under study, these receptors maintain an additional constant source of internal presynaptic Ca\textsuperscript{2+}, be it a Ca\textsuperscript{2+} channel or internal Ca\textsuperscript{2+} store leaking Ca\textsuperscript{2+}, a reduced capacity or affinity of the Ca\textsuperscript{2+} pump, or else.

Interestingly, group 1/2 mGluR blockade also reduced the spike-evoked Ca\textsuperscript{2+} entry. Because the contributing role of presynaptic Ca\textsuperscript{2+} stores to presynaptic Ca\textsuperscript{2+} entry has long been demonstrated (Emptage, Reid, & Fine, 2001; Galante & Marty, 2003; Shimizu et al., 2008; Sylantyev, Jensen, Ross, & Rusakov, 2013), the possible mechanism of receptor action could be related to their well-documented Ca\textsuperscript{2+} store control. At the same time, blocking glutamate uptake, which dramatically increases extrasynaptic...
actions of glutamate (Asztely, Erdemli, & Kullmann, 1997; Shih et al., 2013; Zheng, Scimemi, & Rusakov, 2008) boosting its average extracellular level, also decreased evoked Ca\(^{2+}\) entry, with or without group 1/2 mGluR blocked. One plausible mGluR-independent mechanism explaining the TBOA-dependent decrease in presynaptic Ca\(^{2+}\) is an increase in extracellular K\(^+\) under prolonged TBOA application (Larsen, Holm, Vilsen, & MacAulay, 2016; Lebedeva, Plata, Nosova, Tyurikova, & Semyanov, 2018; Shih et al., 2013), which would depolarize axonal terminals thus altering the contribution of axonal Na\(^+\) and K\(^+\) channels to Ca\(^{2+}\) entry (Scott et al., 2014). Intriguingly, unlike mGluR blockade, TBOA application had no effect on the basal presynaptic Ca\(^{2+}\) level. This observation lends support to the hypothesis that the mGluR-dependent sustained source of presynaptic Ca\(^{2+}\) is not sensitive to glutamate-receptor binding. The underpinning molecular mechanism of this functional dichotomy remains an open question.

Finally, we have found that transient (500 ms long) somatic depolarization of cortical pyramidal cells, which should mimic subthreshold

**Figure 4** Somatic membrane potential has little influence on presynaptic Ca\(^{2+}\) dynamics in cortical neurons. (a) Example of whole-cell (current clamp) recording trace illustrating three conditions: baselines (black), 500 ms depolarization pulse (red, approximately +15 mV), and 500 ms hyperpolarization pulse (blue, approximately −15 mV) applied prior to the evoked action potential, in current clamp configuration. (b) Characteristic time course (two-bouton example) of presynaptic [Ca\(^{2+}\)] (FLIM readout) in the three conditions, as indicated, for two individual axonal boutons; colour bars indicate period of somatic depolarisation (red) and hyperpolarisation (blue). (c) Summary of experiments shown in [a and b]: dots, individual boutons; bars, mean ± SEM. Left: Average values of basal Ca\(^{2+}\) level [Ca\(^{2+}\)]\(_0\) (mean ± SEM: 46 ± 2, 49 ± 3, and 47 ± 3 nM, in control, depo-, and hyperpolarization conditions, respectively; \(n = 19\) boutons, 8 cells, recorded from three animals). Right: Average spike-evoked Ca\(^{2+}\) entry \(\Delta\) [Ca\(^{2+}\)] (log scale: 169 ± 26, 176 ± 31, and 173 ± 32 nM in control, depo-, and hyperpolarization conditions, respectively; \(n = 19\) boutons, 8 cells, from three animals). Lines connect data points from the same bouton.
excitation of the cell, does not consistently affect $[\text{Ca}^{2+}]_0$ or $\Delta[\text{Ca}^{2+}]$ in their axons (Figure 4). Previous studies in cortical pyramidal cells and hippocampal granule cells have shown that somatic depolarization enhances release probability in their axons (Alle & Geiger, 2006; Christie et al., 2011; Scott et al., 2008; Shu et al., 2006). However, in the hippocampus, subthreshold somatic excitation had no effect on $\Delta[\text{Ca}^{2+}]$ in remote (giant) boutons (Scott et al., 2008) although it did inhibit $\Delta[\text{Ca}^{2+}]$ in proximal axonal segments (Ruiz et al., 2003; Scott et al., 2014). In contrast, in cortical pyramidal cells, the fluorescent intensity readout of intracellular Fluo-5F ($K_d \approx 2.3 \mu M$) (Christie et al., 2011), or indirect tests with Ca\textsuperscript{2+} chelators in the presynaptic cells (Shu et al., 2006), led to a conclusion that somatic depolarization should boost $\Delta[\text{Ca}^{2+}]$. It might be important to establish reasons for the disparity between the present data and the previous observations.

ACKNOWLEDGEMENTS

This study was supported by the Wellcome Trust Principal Fellowship (212251_Z_18_Z), ERC Advanced Grant (323113), and European Commission NEUROTWIN grant (857562) to DAR; Russian Foundation for Basic Research (RFBR 20-04-00245) and Russian Global Education Program (GEP) to OT; Wellcome Trust Grant 104033/Z/14/Z, Epilepsy Research Grant UK P1806, to KEV.

All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST

The authors declare no known conflict of interest.

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