INTRODUCTION

Calcium (Ca\(^{2+}\)) signalling plays a central role in synaptic function in the central nervous system (CNS). Action potential triggers entry of extracellular Ca\(^{2+}\) through voltage-gated calcium channels (VGCCs), leading to presynaptic neurotransmitter release (Südhof, 2012a, 2012b). On the other side of the synapse, depolarization of the membrane by opening of the neurotransmitter receptors opens up VGCCs as well as NMDA-type glutamate receptors (NMDARs) in the dendritic spine. Resulting postsynaptic Ca\(^{2+}\) entry then triggers a complex cascade of signalling pathways, regulating multiple aspects of neuronal cell biology such as gene expression, membrane trafficking and protein turnover (Higley & Sabatini, 2012).

NMDARs and VGCCs are the two main sources of Ca\(^{2+}\) entry into the synapse. VGCCs are complex channel proteins possessing multiple transmembrane domains, which open to allow passage of Ca\(^{2+}\) once the cell membrane has depolarized beyond a threshold level (Catterall, 2011; Südhof, 2012a). NMDA receptors sense both depolarization of the membrane and release of glutamate, which triggers them to open allowing for entry of various cations, including Ca\(^{2+}\) (Hunt & Castillo, 2012; Paoletti et al., 2013). The properties of these two channel types are therefore quite different. Furthermore, their respective localization also displays notable differences: while NMDARs are mainly located in the postsynaptic environment of the dendritic spine, VGCC can be found across the cell.

Abstract

Regulation of extracellular Ca\(^{2+}\) influx by neuronal activity is a key mechanism underlying synaptic plasticity. At the neuronal synapse, activity-dependent Ca\(^{2+}\) entry involves N-methyl-D-aspartate receptors (NMDARs) and voltage-gated calcium channels (VGCCs); the relationship between NMDARs and VGCCs, however, is poorly understood. Here, we report that neuronal activity rapidly (1h) regulates recruitment of R-type VGCCs in hippocampal neurons through synaptic NMDAR signalling. This finding reveals a link between two key neuronal signalling pathways, suggesting a feedback mode for regulation of synaptic Ca\(^{2+}\) signalling.

KEYWORDS

calcium channels, NMDA receptors, synapse
Fast high-voltage activated VGCCs containing a CaV2 subunit (CaV2-VGCCs) are of particular importance to synaptic Ca2+ signalling due to their spatial proximity to the synapse. Among the three known types of CaV2-VGCCs, P/Q-type (CaV2.1) and N-type (CaV2.2) VGCCs are found presynaptically (Glebov et al., 2017; Holderith et al., 2012; Indriati et al., 2013; Nakamura et al., 2014), while R-type (CaV2.3) channels may operate on both sides of the synapse (Bloodgood & Sabatini, 2007a; Parajuli et al., 2012; Wormuth et al., 2016). Despite the well-established roles for both NMDARs and CaV2-VGCCs, the relationship between their signalling pathways remains poorly understood. Local NMDAR and VGCC signalling can be immediately coupled through short-term biophysical mechanisms (Bloodgood & Sabatini, 2007b; Theis et al., 2018). On a timescale of days, chronic neuronal activity engages the mechanisms of homeostatic plasticity to regulate levels of all three types of CaV2-containing VGCCs (Glebov et al., 2017; Lazarevic et al., 2011). However, the link between NMDAR activity and synaptic CaV2-VGCCs recruitment remains unclear, as do the underlying cell biological mechanisms.

2 | MATERIALS AND METHODS

2.1 | Materials

Cell culture reagents were from Invitrogen (UK). Anisomycin, MK801 and memantine were from Sigma Aldrich (UK). TTX, NBQX, APV and gabazine were from Tocris (UK). Syn-PER reagent was from Thermo Fisher (China). The list of the antibodies used in this study can be found in Table 1.

2.2 | Neuronal culture

Dissociated hippocampal neuronal cultures were isolated from rat embryos at day 18 of gestation and grown according to the Brewer method. Culture medium was Neurobasal with GlutaMax and B-27. No anti-mitotic agents or antibiotics were used during culture. All experiments were carried out at 16–21 days in culture. Cells were cultured on 13 mm poly-L-lysine coated round glass coverslips with 1.5 thickness placed into 35 mm Petri dishes, 4 coverslips per dish. To minimize variability in culture conditions, each experiment was carried out using the cells from the same dissection and cultured within the same Petri dish. All experimental protocols were performed following the guidelines of the local Research Ethics Committee.

2.3 | Immunostaining and confocal microscopy

All steps were performed at room temperature (RT). After treatment, coverslips were fixed with 2% (when probing for Psd95) or otherwise 4% paraformaldehyde dissolved in phosphate buffered saline (PBS). Fixation was carried out for 15–20 min and was followed by the permeabilization/blocking step. Permeabilization/blocking was carried out in 0.2% Triton-X100 dissolved in PBS supplemented with 5% horse serum, for 10 min. All subsequent incubations were carried out in 0.2% Triton-X100 dissolved in PBS supplemented with 5% horse serum. For primary labelling, coverslips were incubated with appropriate primary antibodies for 45–90 min, then washed four times in PBS. For secondary labelling, coverslips were incubated with the appropriate secondary antibodies labelled with AlexaFluor-488 and AlexaFluor-647 at a concentration of 0.3 μg/ml each for 45–90 min. Coverslips were then mounted in Fluoromount-G mounting medium and imaged on a Zeiss LSM710 or a Nikon C2+ laser confocal microscope equipped with a standard set of lasers.

Imaging system was perfomed using the relevant proprietary software. Acquisition parameters were kept as follows: apochromatic 60/63x oil objective, regions of interest sized

| Antigen      | Conjugation | Species | Manufacturer               | Cat. No. |
|--------------|-------------|---------|---------------------------|----------|
| Cav2.3       | Rabbit      | Synaptic Systems | 152403               |
| Cav2.3       | Rabbit      | Alomone | ACC-006                  |
| PSD95        | Mouse       | Abcam   | 13552                    |
| Bassoon      | Mouse       | Abcam   | 82958                    |
| Cav2.1       | Rabbit      | Synaptic Systems | 152103               |
| Cav2.2       | Rabbit      | Alomone | ACC-002                  |
| Rabbit IgG   | Cy5         | Donkey  | Jackson Immuno           | 711-175-152 |
| Mouse IgG    | AlexaFluor488 | Goat | Invitrogen              | A-11001  |
| Actin        | Rabbit      | Bioss   | BS-0061R                 |
| Rabbit IgG   | HRP         | Goat    | Jackson Immuno           | 111-035-144 |
| Mouse IgG    | HRP         | Goat    | Jackson Immuno           | 115-035-003 |

TABLE 1 Antibodies used in this study
1024 × 1024 pixels (65.8 nm/pixel), 12-bit, speed 7, averaging setting 2. Excitation laser wavelengths were 488 and 633 nm. Bandpass filters were set at 500–550 nm and 650–750 nm for Alexa Fluor 488 and Alexa Fluor 647, respectively. Pinhole size was limited to 1–2 Airy units. Detector gain settings were optimized to ensure appropriate dynamic range, low background and sufficient signal:noise ratio. Fields of view for imaging were determined using visual examination of the Alexa Fluor 488 immunostaining through the eyepiece of the microscope. To minimize bias, the experimenter remained agnostic with regards to the Alexa Fluor 647 staining until the completion of image acquisition.

2.4 | Image analysis

Image analysis was carried out using the ImageJ software package, version 1.42. In experiments involving Psd95 immunostaining, non-synaptic regions with high background fluorescence (e.g., cell bodies) were manually excluded from analysis. For quantification of CaV2.3 in the cell bodies, the mean intensity of CaV2 across the whole cell body was measured by manually selecting the relevant region of interest, excluding regions containing any punctate staining for synaptic markers.

For identification of synapses, images were thresholded using the “Moments” setting. Individual synapses were then identified using the “Analyse Particles” command, yielding 200–1000 regions of interest (ROIs). In order to enrich the sample for synaptic particles, parameters were set to identify ROIs with areas of 0.1–2 μm². To minimize bias and variability, the resulting multiple ROIs were then automatically combined into one compound ROI, using the ROI Manager interface and the “Combine” and “Add” functions thereof. Following this, quantification of mean signal intensity in each channel was performed using the “Measure” function. Background subtraction was performed as appropriate.

2.5 | Synaptosomal preparation and western blotting

Synaptosomal preparation was carried out using the Syn-PER synaptic protein extraction reagent according to the manufacturer’s protocol. Briefly, rat brain cortex tissue was homogenized in a Dounce homogenizer, cell debris was removed by low speed centrifugation, the synaptosomal fraction was pelleted by high speed centrifugation and stored at −80°C.

Protein separation was performed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Acrylamide percentage in the separating gel was 10% for beta-actin detection and 6% for Bassoon (Bsn) and CaV2.3 detection. Proteins were transferred onto Immobilon polyvinylidene fluoride membranes and blocked with 5% nonfat dry milk in Tris-buffered saline Tween-20 buffer. Antibodies were used at following dilutions: 1/4000 (anti-actin), 1/1000 (anti-Bsn), 1/1000 (anti-CaV2.3), 1/1000 (HRP-conjugated secondary antibodies). Immobilon™ Western Chemiluminescent HRP Substrate was used for signal visualization.

2.6 | Statistical analysis

All the experiments were repeated three to five times, and five images were acquired per condition. For statistical analysis, Prism 6.0c software package (GraphPad Software) was used. Data distributions were assessed for normality using d’Agostino and Pearson omnibus tests. Student’s t test and one-way analysis of variance were used for normally distributed datasets to assess statistical significance; for not normally distributed datasets, Mann-Whitney rank test was used. Dunn’s post-test was used to assess statistical significance of the treatment effects relative to the untreated control samples. Data were presented as box & whisker plots; error bars denote the 10–90 percentile range.

3 | RESULTS AND DISCUSSION

Blockade of action potential firing by tetrodotoxin (TTX; 2 μM) for 1 hr caused a significant increase in the CaV2.3 signal co-localizing with the puncta of a canonical synaptic marker Psd95 (Figure 1a,b), although the intensity of the Psd95 signal was unchanged (Figure S1a). Blockade of inhibitory transmission by a GABA receptor blocker gabazine (50 μM) also increased the CaV2.3 signal intensity overlapping the Psd95-positive puncta, while moderate depolarization of the neuronal membrane by elevated (15 mM) concentration of KCl had no significant effect (Figure 1a,b). Colocalization between the Psd95 and CaV2.3 signals was significantly increased in gabazine-treated cells (Figure 1c). This indicated that neuronal activity regulated recruitment of R-VGCCs towards the synapse.

To investigate the mechanism coupling neuronal activity and synaptic R-VGCCs, AMPA-type glutamate receptors, which carry out most of glutamatergic synaptic transmission in hippocampus, were blocked using 2,3-dioxo-6-nitro-7-sulfamoyl-benzo[fl]quinoxaline (NBQX; 20 μM). The observed increase in CaV2.3 signal resembled that of TTX, suggesting that R-VGCCs levels were regulated by excitatory synaptic transmission (Figure 1d). To further elucidate the signalling mechanism regulating R-VGCCs downstream of synaptic transmission, NMDAR signalling was inhibited by a specific blocker (2R)-amino-5-phosphonovaleric acid (APV; 50 μM). The resulting effect was similar to that of TTX and NBQX, indicating that blockade of NMDARs was sufficient to...
FENG and GLEBOV demonstrated by western blotting, both CaV2.3 and Bsn were localized in the close vicinity of the synapse (Biesemann et al., 2014; Richter et al., 2018). To investigate whether their timescale of their recruitment matched that of R-VGCCs, immunostaining for the pore-forming subunits CaV2.1 and CaV2.2 was performed in cultures treated with APV for 1 hr. Synaptic intensities for both CaV2.1 and CaV2.2 were not significantly increased, suggesting that the accumulation of VGCCs at the synapse triggered by the 1 hr NMDAR blockade was specific to R-VGCCs (Figure 2a-d).

Other types of CaV2-containing VGCCs, namely N-VGCCs and P/Q-VGCCs, have been shown to slowly accumulate at the synapse over the course of 24–48 hr upon blockade of activity, consistent with the timescale of homeostatic plasticity (Glebov et al., 2017; Lazarevic et al., 2011). To investigate whether their timescale of their recruitment matched that of R-VGCCs, immunostaining for the pore-forming subunits CaV2.1 and CaV2.2 was performed in cultures treated with APV for 1 hr. Synaptic intensities for both CaV2.1 and CaV2.2 were not significantly increased, suggesting that the accumulation of VGCCs at the synapse triggered by the 1 hr NMDAR blockade was specific to R-VGCCs (Figure 2a-d).

This study reports that neuronal activity controls recruitment of R-VGCCs to the synapse through excitatory neurotransmission and synaptic NMDAR signalling. In contrast to previously reported fast biophysical coupling between postsynaptic R-VGCCs and NMDARs, the timescale of...
the observed effect (1 hr) is consistent with long-distance NMDAR signalling (Dieterich et al., 2008; Karpova et al., 2013). This notion is further supported by the observed increase in Cav2.3 amount in the cell body (Figure S1c), suggesting that global NMDAR signalling may exert control over Cav2.3 across the entire cell, including both pre- and postsynaptic locales (Bloodgood & Sabatini, 2007b; Dietrich et al., 2003; Ermolyuk et al., 2013; Parajuli et al., 2012). The 1 hr timescale, however, is considerably faster than the previously reported slow (24–48 hr) mechanisms of homeostatic plasticity regulating P/Q and N-type VGCCs which are primarily presynaptic (Glebov et al., 2017; Lazarevic et al., 2011; Zhao et al., 2011), indicating that activity-dependent regulation of different VGCCs may engage different processes.

The mechanistic aspects of the connection between synaptic NMDARs and R-VGCCs will warrant further investigation. Potential involvement of translational regulation (Figure 1h) is especially relevant in the light of the recent studies showing the link between postsynaptic mGluR activation and R-VGCC translation (Gray et al., 2019; Park et al., 2010), given the primarily postsynaptic localization of NMDARs (Paolelli et al., 2013). Also, recent findings have demonstrated considerable abundance of Cav2.3 mRNA in the presynaptic compartment compared to other CaV-containing VGCCs (Hafner et al., 2019), suggesting that Cav2.3 translation may also occur at the presynaptic site.

Our results show that blockade of inhibitory neurotransmission mimics the effect of NMDAR blockade, increasing Cav2.3 (Figure 1a,b). As blockade of inhibitory transmission increases neuronal firing and glutamate release (Wiegert et al., 2009), this effect may reflect Cav2.3 upregulation through an alternative NMDAR-independent glutamate signalling pathway involving group I metabotropic glutamate receptors (Gray et al., 2019). Moreover, this observation is also consistent with mGluR-dependent long-term depression (LTD) of NMDAR signalling (Snyder et al., 2001), and may even represent the earliest stages of homeostatic downscaling of synaptic NMDARs (Ehlers, 2003; Watt et al., 2000), highlighting the complexity of the pathways linking neuronal activity, neurotransmitter signalling and synaptic composition.

Feedback regulation of synaptic Ca²⁺ signalling is likely to be a key factor allowing for dynamic integration of multiple synaptic signalling pathways in response to neuronal activity (Bloodgood & Sabatini, 2007a). However, given that our findings are based on global pharmacological blockade in neuronal cultures, further research into the link between NMDAR and VGCC signalling will benefit from physiologically relevant approaches, for example, localized activity manipulation, electrophysiology and calcium imaging in slices or in vivo. This will allow to investigate potential contributions of NMDAR-regulated R-VGCC signalling to key post- and presynaptic processes, namely Ca²⁺ transients in the dendritic spine and neurotransmitter release from the synaptic terminal (Bloodgood & Sabatini, 2007b; Ermolyuk et al., 2013), as well as neuronal development (Nishiyama et al., 2011), certain forms of synaptic plasticity, and neuronal
pathology (Weiergraber et al., 2006; Wormuth et al., 2016; Yokoyama et al., 2004).

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CONFLICT OF INTEREST
None.

AUTHORS CONTRIBUTION
Z.F. contributed to experiments and data analysis. O.O.G. contributed to experiments, data analysis and manuscript preparation.

PEER REVIEW
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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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