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One nuclear calcium transient induced by a single burst of action potentials represents the minimum signal strength in activity-dependent transcription in hippocampal neurons

Yan Yu, Kristin Oberlaender, C. Peter Bengtson, Hilmar Bading *

Department of Neurobiology, Interdisciplinary Center for Neurosciences, Heidelberg University, 69120 Heidelberg, Germany

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ABSTRACT

Neurons undergo dramatic changes in their gene expression profiles in response to synaptic stimulation. The coupling of neuronal excitation to gene transcription is well studied and is mediated by signaling pathways activated by cytoplasmic and nuclear calcium transients. Despite this, the minimum synaptic activity required to induce gene expression remains unknown. To address this, we used cultured hippocampal neurons and cellular compartment analysis of temporal activity by fluorescence in situ hybridization (catFISH) that allows detection of nascent transcripts in the cell nucleus. We found that a single burst of action potentials, consisting of 24.4 ± 5.1 action potentials during a 6.7 ± 1.9 s depolarization of 19.5 ± 2.0 mV causing a 9.3 ± 0.9 s somatic calcium transient, is sufficient to activate transcription of the immediate early gene *arc* (also known as *Arg3.1*). The total *arc* mRNA yield produced after a single burst-induced nuclear calcium transient was very small and, compared to unstimulated control neurons, did not lead to a significant increase in *arc* mRNA levels measured using quantitative reverse transcriptase PCR (qRT-PCR) of cell lysates. Significantly increased *arc* mRNA levels became detectable in hippocampal neurons that had undergone 5–8 consecutive burst-induced nuclear calcium transients at 0.05–0.15 Hz. These results indicate that a single burst-induced nuclear calcium transient can activate gene expression and that transcription is rapidly shut off after synaptic stimulation has ceased.

1. Introduction

In the nervous system, synaptic activity-induced changes in gene expression are critical for long-lasting adaptive processes including consolidation of memory formation and extinction, acquired neuroprotection, and the development of chronic pain [1,2]. Fast acting synapse-to-nucleus communication pathways, triggered by calcium entry into neurons, couple neuronal excitation to gene transcription-regulating events [3]. The dialogue between the synapse and the nucleus is mediated by two principal pathways [4]. They are the ERK-MAP kinase cascade that is activated by a near-plasma membrane calcium signal [5,6] and cell nucleus-invading calcium transients activating nuclear calcium/calmodulin-dependent enzymes, including CaMKIV and CaMKII [7–12]. Additional protein-based messengers contribute to activity dependent gene expression in neurons [13]. It is well documented that transcription-regulating biochemical events such as CREB phosphorylation on its activator site serine 133 [14,15], phosphorylation of MeCP2 [12,16,17], or nuclear export of class IIa histone deacetylases (HDACs) [18,19] take place within minutes after electrical stimulation. However, surprisingly little is known about the precise relationship between a synaptic input and the evoked induction of transcription, i.e., the *de novo* synthesis of mRNA. Mechanistic studies of signal-regulated neuronal gene expression are usually done in cultured neurons and the stimulation paradigms generally involve prolonged, many minutes to hours-lasting exposures to synaptic activity-inducing compounds or membrane depolarizing conditions. Here we used a very sensitive method that allows detection of nascent transcripts of the *arc* gene (activity-regulated cytoskeleton-associated protein), also known as Arg3.1 [20,21] in individual cell nuclei in order to deter-

Abbreviations: catFISH, temporal activity by fluorescence in situ hybridization; qRT-PCR, quantitative reverse transcriptase PCR; CaMKIV, Calcium/calmodulin-dependent protein kinase type IV; CaMKII, Calcium/calmodulin-dependent protein kinase type II.

* Corresponding author at: Department of Neurobiology, Interdisciplinary Center for Neurosciences, Heidelberg University, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany.

E-mail addresses: yuyan1248@gmail.com (Y. Yu), Oberlaender@nbio.uni-heidelberg.de (K. Oberlaender), Bengtson@nbio.uni-heidelberg.de (C.P. Bengtson), Bading@nbio.uni-heidelberg.de (H. Bading).

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mine the minimum synaptic activity needed to switch on gene transcription.

2. Material and methods

2.1. Hippocampal cell culture and treatments

Hippocampal neurons from newborn Sprague-Dawley rats were cultured as previously described [22] except that growth media was supplemented with B27 (Invitrogen), 1% rat serum, and 1 mM glutamine. Neurons were plated onto 12 mm glass coverslips or 35 mm dishes at a density of 400–600 cells per mm^2. All experiments were performed on day *in vitro* (DIV) 10–11 when neurons have developed a rich network of processes, are expressing functional NMDA-type and AMPA/kainate-type glutamate receptors and have formed synaptic contacts [6,23–25]. Bursts of action potential (AP) firing throughout the neuronal network was induced by treating the culture with the $GABA_A$ receptor antagonist bicuculline (Axxora) at a concentration of 50 μM. Bicuculline was dissolved in DMSO; final concentration of DMSO in the media did not exceed 0.1%. Network bursting was halted by bath application of 1 μM tetrodotoxin (TTX, Tocris).

2.2. DNA transfection

DNA transfection was done on DIV 10 by using Lipofectamine 2000 (Invitrogen) as described [26]. Cells were left for additional 48 h before analysis. The following plasmids were used in this study: mCherry.NLS and CaMBP4-Flag. Both were cloned into a recombinant adeno-associated virus (rAAV) construct with a CaMKIIα promoter. The fluorescence of mCherry was completely bleached after the catFISH protocol. The expression of mCherry.NLS and CaMBP4 was identified by immunocytochemistry with a polyclonal antibody against DsRed (Clontech) and a polyclonal antibody against FLAG (Sigma-Aldrich), respectively, followed with an Alexa Fluor® 488-conjugated secondary antibody (Invitrogen) staining.

2.3. Quantitative reverse transcriptase PCR (qRT-PCR)

After treatments, cells were harvested for total RNA extraction using an RNase kit (Qiagen) with an additional on-column DNase I digestion according to the manufacturer's instructions. cDNAs were synthesized from 1.2 μg total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems®). QRT-PCR was done on an Applied Biosystems 7300 Real-Time PCR System using TaqMan® Gene Expression Master Mix with TaqMan® Gene Expression Assays for the *arc* gene (Rn00571208_g1). Expression levels of *arc* were normalized to the expression of the housekeeping gene *GusB* (Rn00566655_m1).

2.4. CatFISH (cellular compartment analysis of temporary activity by fluorescence in situ hybridization)

CatFISH was performed as previously described [27,28] with minor changes. Briefly, sense and antisense mRNA probes were transcribed *in vitro* with a Transcription Kit (DIG RNA labeling Kit SP6/T7), and T3 polymerase (Roche) from rat Arc/Arg3.1 cDNA (a kind gift from Paul F. Worley, Johns Hopkins University, Baltimore, Maryland, USA). Coverslips with cells were fixed with ice-cold 4% PFA (pH 7.2–7.6) for 10 min. After washing steps, samples were incubated with 1× pre-hybridization solution at room temperature for 30 min. The RNA probes (1 ng/ul) were denatured by heating at 90 °C for 5 min and followed immediately with chilling in an ice bath. Hybridization was performed in a humid chamber in a 56 °C oven for at least 16 h. Coverslips were then washed, and then treated with 10 ug/ml RNase A (Roche) in 2× SSC at 37 °C and 2% H_2O_2 in 1× SSC to quench endogenous peroxidase activity. The signals were detected with a horseradish peroxidase (HRP) conjugated antibody to DIG (1:100, anti-Digoxigenin-POD, Fab fragments from sheep, Roche) and amplified using a Cyanine-3 Tyramide Signal Amplification Kit (1:100, PerkinElmer). At the end, cells were counterstained with Hoechst 33258 (2 μg/ml, Serva) and mounted with Mowiol. The catFISH signals were detected with a Leica SP2 confocal microscope (Leica) in an xyz scanning mode spanning the whole nucleus with a depth interval of 1 μm. The images were merged to get the maximal signal with ImageJ software and analyzed for positive *arc* signal or signal location. The percent of neurons possessing the *arc* catFISH signal were normalized to the total population of neurons in the imaging field identified by staining with Hoechst 33258. Since the gene induction reference time point is ten minutes after bicuculline stimulation, only one or two foci of intensive *arc* catFISH signals were detected per cell.

2.5. Electrophysiology

Whole-cell patch clamp recordings were made from primary cultures of rat hippocampal neurons plated on coverslips secured with a platinum ring in a recording chamber (OAC-1, Science Products GmbH) continuously perfused with artificial cerebrospinal fluid (aCSF; in mM: NaCl, 125; KCl, 3.5; $MgCl_2$, 1.3; NaH_2PO_4, 1.2; $CaCl_2$, 2.4; glucose, 10; $NaHCO_3$, 26; gassed with 95% O_2 and 5% CO_2) and mounted on a fixed-stage upright microscope (BX51WI, Olympus). Differential interference contrast optics, infrared illumination and a CCD camera (Photometrics Coolsnap HQ, Visitron Systems) were used to view neurons on a computer monitor using a software interface (Metamorph, Molecular Devices). Patch electrodes (3–4 MΩ) were made from borosilicate glass (1.5 mm, WPI) and filled with (in mM): KCH_3SO_4, 135; NaCl, 8; KCl, 12; HEPES, 10; K_2-phosphocreatine, 10; Mg_2-ATP, 4; Na_3-GTP, 0.3; pH 7.35 with KOH). Recordings were made with a Multiclamp 700 B amplifier, digitized through a Digidata 1322A, acquired and analyzed using pClamp software (Molecular Devices). All membrane potentials have been corrected for the junction potential of −11 mV.

2.6. Calcium imaging

Calcium transients were imaged on DIV10-11 with either a membrane permeable, small molecule indicator, Fluo3-AM (Invitrogen), or a genetically encoded calcium sensor, GCaMP3 [29] targeted to nuclei with a nuclear localization sequence (NLS) [30,31]. GCaMP3.NLS was cloned into a recombinant adeno-associated virus (rAAV) construct with a CaMKIIα promoter and packaged into virus with a serum type of AAV1/2. Cells were infected with the virus on DIV4. Fluo-3AM was dissolved in pluronic acid and DMSO and loaded either at 3 μM for 20 min at room temperature or at 1.5 μM for 30 min at 37 °C. Following washout, coverslips were left for 30 min for de-esterification and transferred to a custom made chamber at the microscope. Confocal imaging was performed using a CO_2 independent buffer (NaCl 140 mM, KCl 6.8 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, HEPES 10 mM, Glycine 1 mM, Glucose 30 mM, Na pyruvate 0.5 mM and Phenol red 0.2%) on a Leica SP2 microscope with a 40 x objective (NA 1.25 oil, Leica) with a 488 nm excitation laser light, 512 × 512 format, 8 bit resolution, xyt scan mode, 1.0 airy pinhole, 400 Hz scan speed and one frame per second. Wide-field calcium imaging was performed with the same microscope and solutions used for electrophysiology using an XLUMPLFLN20xW objective (NA 1.0, Olympus), a 470 nm LED excitation light (pE2, CooLEDs) and excitation (470/40 nm) and emission (525/50 nm) filters (Chroma Technologies). Confocal data are presented as $(F-F_0)/F_0$ where F represents the average fluorescence intensity in a somatic region of interest (ROI) and F_0 is the