# NMDA receptor-independent control of transcription factors and gene expression

J. Paige Adams, Rachel A. Robinson, Eric D. Hudgins, Erin M. Wissink and Serena M. Dudek

**Consolidation of synaptic plasticity seems to require transcription, but how the nucleus is informed in this context remains unknown. As NMDA receptor antagonists have been shown to interfere with action potential generation, the issue of whether or not a synaptically generated signal is required for nuclear signaling is currently unresolved. Here, we show that pharmacological maintenance of action potentials during NMDA receptor blockade allows for NMDA receptor-independent transcription factor binding and *arc* gene expression, both of which were previously thought to be NMDA receptor dependent. These data suggest that types of signaling in the nucleus previously attributed to NMDA-receptor-dependent synapse-to-nucleus signals can be initiated in the absence of NMDA receptor-dependent synaptic plasticity.** *NeuroReport* **20:1429–1433 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Laboratory of Neurobiology, National Institute of Environmental Health Sciences, National Institutes of Health, North Carolina, USA

Correspondence to Dr Serena M. Dudek, PhD, Laboratory of Neurobiology, National Institute of Environmental Health Sciences, National Institutes of Health, PO Box 12233, MD F2-04, Research Triangle Park, NC 27709, USA
Tel: +1 919 541 3275; fax: +1 919 541 4611; e-mail: dudek@niehs.nih.gov

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## Introduction

Transcription seems to be critical for the late phase of long-term potentiation (LTP) [1,2], but how do we determine the mechanisms used by synapses to signal to the nucleus that potentiation has occurred? A widely held view is that a signal must be generated upon NMDA receptor activation and physically transported from the synapse to the nucleus after LTP is induced. In this scenario, a signal is created at the synapse after LTP induction and is transported to the nucleus, where it enters and either directly or indirectly regulates transcription (i.e. NF-kB, [3]). Evidence supporting this view is that blockade of NMDA-type ionotropic glutamate receptors, which are important for most types of LTP induction at synapses, inhibits transcription of many genes [4–6]. Recently, however, we have shown that NMDA receptor blockade has other effects in the cell, namely the inhibition of action potential generation from synaptic stimulation resulting in a blockade of signaling cascades, such as the ERK/MAP kinase pathway [7]. These data indicate that an alternative model should be considered seriously, one in which action potentials signal the nucleus directly through the resulting calcium influx from voltage-gated calcium channel signaling [8]. Thus, one cannot infer that NMDA receptor blockade of transcription is because of signals transported from the synapse unless action potentials are carefully controlled [7].

However, the specific genes critical for LTP consolidation have yet to be identified, and therefore in the interim, we consider the regulation of certain transcription factors (TFs) and the activity-regulated gene *arc/arg3.1*. *Arc* is rapidly upregulated after a learning episode [9] and has been closely associated with the later phases of learning and LTP [10,11], or long-term depression and homeostatic plasticity [12,13]. Induction of this gene previously has been shown to be NMDA receptor dependent [5]. Here, we show that when action potentials are pharmacologically maintained during an LTP-inducing stimulation, TF binding and *arc* induction do not require NMDA receptors. These data show that synaptic stimulation induces many biochemical events related to transcription and that NMDA receptors need not be directly involved.

## Methods

### Slice preparation and electrical stimulation

Hippocampal slices (350 μm) were prepared from 5 to 7-week-old Sprague–Dawley rats. Slices were cut on a vibratome at 4°C in artificial cerebrospinal fluid (ACSF) containing (in mM): KCl, 4; sucrose, 240; $NaH_2PO_4$, 1.25; $NaHCO_2$, 26; $CaCl_2$, 1; $MgCl_2$, 3; glucose, 10; bubbled with 95/5% $O_2/CO_2$. Minislices of CA1 were microdissected in ice-cold cutting ACSF, after which they were placed in an interface recording chamber and perfused with standard ACSF (in mM: NaCl, 124; KCl, 4; $NaH_2PO_4$, 1.25; $NaHCO_2$, 26; $CaCl_2$, 2.5; $MgCl_2$, 1.5; and glucose, 10) at 34°C for 2–3 h before stimulation. After application of 10 μM bicuculline or bicuculline plus 50 μM APV (2-amino-5-phosphovalerate) for 20–40 min, a

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concentric bipolar stimulating electrode (FHC Inc., Bowdoin, Maine, USA) placed in the stratum radiatum was used to stimulate the minislices with a θ-burst pattern (130 μs duration, 100 μA). This pattern of stimulation is known to induce LTP in CA1 pyramidal neurons [14] and induces action potentials resistant to the inhibitory effects of APV [7]. LTP was effectively blocked by this concentration of APV, even in the presence of bicuculline ($n = 3$, data not shown). This stimulus intensity and duration was found to evoke population spikes to the edge of the minislices, resulting in an estimated 60–80% of the cells being activated based on phospho-ERK staining [15]. Five (electrophoretic mobility shift assay, EMSA) or 15 (quantitative PCR) min after electrical stimulation, slices were removed from the chamber, snap-frozen on dry ice, and stored at −80°C. One control (nonstimulated) slice was removed and frozen for each stimulated slice to match for time after cutting and drug exposure. Electrical stimulation without bicuculline did not reliably induce *arc*, which was likely to be because of the fact that more action potentials are evoked across a greater area of the slice in the bicuculline case. Therefore, because the two conditions were not thought to represent similar degrees of stimulation, comparisons between cases stimulated in bicuculline and those stimulated without were not made.

### Preparation of nuclear proteins

Nuclear proteins were isolated using a modification of the protocol as directed in the CelLytic NuCLEAR Extraction kit (Sigma Aldrich, St. Louis, Missouri, USA). Twenty-one to 24 minislices from three to four rats were pooled for homogenization and nuclear preparation. Slices were homogenized in 600 μl lysis buffer [0.6% Igepal, 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, protease inhibitor cocktail III (Calbiochem, Gibbstown, New Jersey, USA), and phosphatase inhibitor cocktail II (Calbiochem)] and spun at 9000*g* for 20 min at 4°C to pellet nuclei. Nuclear proteins were extracted by resuspending the resulting pellet into 7 μl extraction buffer (extraction buffer from kit, 166 mM NaCl, 1 mM DTT, protease and phosphatase cocktails) and shaking the tubes on ice for 30 min. Nuclei were again spun at 18 000*g* for 15 min at 4°C, and the supernatant was divided into 1 μl aliquots and frozen for future EMSAs. A second extraction with the same buffer was performed on the pellet and the phosphorylation of ERK was determined by Western blot to confirm electrical stimulation [7].

### Electrophoretic mobility shift assays

Consensus oligonucleotides [AP-1, CBF, NF-κB, SRE, CREB, and Ets (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA)] were end-labeled using a T4 polynucleotide kinase and γ-32P ATP according to the manufacturer's instructions (New England Biolabs, Ipswich, Massachusetts, USA). Nuclear extracts from control and stimulated slices were incubated at room temperature with radiolabeled oligonucleotides in Gel Shift Binding Buffer (Promega, Madison, Wisconsin, USA) and subsequently resolved by nondenaturing electrophoresis on a 6% TBE gel at 4°C. Gels were dried overnight and visualized using a phosphorimager. Samples containing the bound DNA/protein complex were retarded within the gel. Images were analyzed using ImageQuant software (Amersham Biosciences, Pittsburgh, Pennsylvania, USA), and bands representing specific binding were identified using mutant oligonucleotides that do not bind to the specified TFs (Santa Cruz Biotechnology Inc.). These bands are indicated in Fig. 1 with an arrow.

### Quantitative real-time PCR

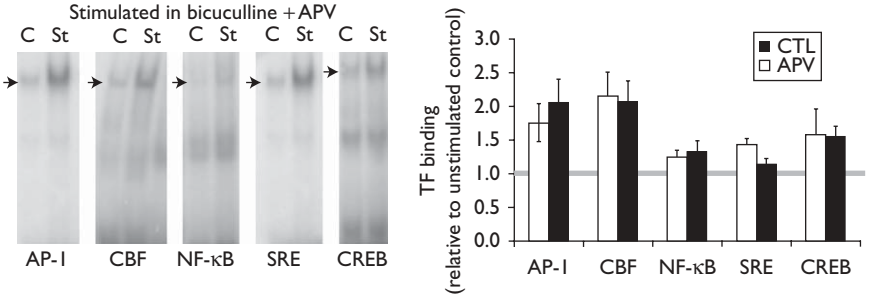
Total RNA was isolated from 21 to 26 frozen slices using RNAeasy Lipid Tissue Mini kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions, converted to cDNA, and stored for further analysis. Quantitative real-time PCR experiments were performed using a Perkin Elmer ABI Prism 770 Sequence Detector using 1–2.5 μl cDNA with Power SYBR Green (Applied Biosystems, Foster City, California, USA) and forward and reverse primers (forward: 5′-TGA CTC ACA ACT GCC ACA CA-3′; reverse: 5′-TGA GGA AGC CAA AGG TGT TC-3′). GAPDH was used for normalization as an internal control. Data are presented as a mean fold change over the unstimulated control slices.

## Results

Activity-dependent genes such as *arc* are quickly transcribed after LTP-inducing stimulation, and therefore the TFs regulating those genes can be studied using the EMSAs. In an earlier work from our laboratory, we used TF arrays to identify TFs of interest from rat hippocampal slices that had been electrically stimulated to induce LTP (Hudgins and Dudek, unpublished). Using oligonucleotides with the consensus sequences to TF-binding sites identified in the arrays and others known to be in the *arc* promoter region [16], we performed EMSAs on similar nuclear extracts to test for the role of the NMDA receptors. To test whether or not LTP-inducing stimulation could continue to activate TFs when action potentials were maintained, extracts were made from slices that had been electrically stimulated either in the presence or in the absence of APV, an NMDA receptor antagonist. This treatment consisted of eliminating fast synaptic inhibition with bicuculline, a GABA-A receptor antagonist that preserved action potentials during the continued NMDA receptor blockade.

Five minutes poststimulation we found that NMDA receptors were not required for the increase in TF binding to AP-1, CBF, CREB, or NF-κB consensus sequence oligonucleotides when action potentials were

**Fig. 1**

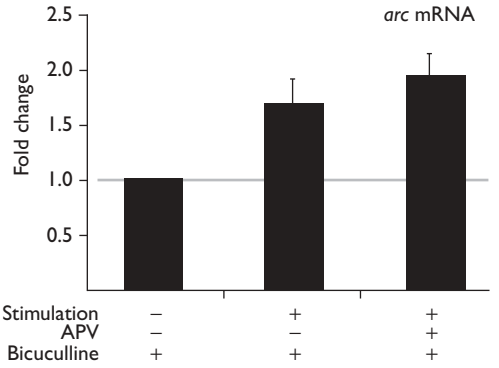



Stimulation-induced transcription factor (TF) binding does not require NMDA receptors (nor long-term potentiation), if action potentials are maintained. To insure that action potentials were maintained during NMDA receptor antagonist exposure (50 μM D-APV), synaptic stimulation was delivered in a θ-burst pattern of stimulation with bicuculline [7]. Hippocampal CA1 minislices were sampled 5 min after stimulation. Nuclear protein extracts from unstimulated (C) and stimulated (St) slices were assessed for TF binding by electrophoretic mobility shift assays (EMSAs). Plotted on the right are data from eight to 11 (control; CTL) and seven to eight (APV) separate EMSAs. TF binding in APV-treated slices are not significantly different from the slices without the drug. Binding to SRE is not affected by APV at this time point ($P = 0.04$, $\alpha = 0.01$).

maintained (Fig. 1). The effect of NMDA receptor blockade on SRE binding trended toward, but did not reach, significance ($P = 0.04$, $\alpha = 0.01$). Thus, synaptic stimulation induces rapid TF binding to, at least several of, the consensus sequences related to plasticity-regulated genes, and the binding is independent of NMDA receptor activation.

On the basis of these data, we sought to determine whether a similar response could be observed with a gene whose induction had been previously shown to be NMDA receptor dependent [5,17]. Using real-time quantitative PCR analysis, we tested whether *arc*, an immediate early gene, could be induced in the absence of NMDA receptor activation while maintaining action potentials with bicuculline. Like TF binding, we found that LTP-inducing stimulation in the presence of bicuculline led to an increase in *arc* transcription, and it was unaffected by the NMDA receptor blockade (Fig. 2). L-type voltage sensitive calcium channels were likely contributing, at least in part, to the calcium levels in the postsynaptic neurons: nifedipine, a blocker of voltage-gated calcium channels, significantly reduced the stimulation-induced increase in *arc* in APV and bicuculline (APV/bicuculline: $1.53 \pm 0.12$; APV/bicuculline/10 μM nifedipine: $1.25 \pm 0.11$; $P < 0.018$). We conclude that NMDA receptors are not necessary for stimulation-induced changes in *arc* transcription when action potentials are preserved.

**Fig. 2**




Stimulation-induced *arc* expression does not require NMDA receptors, if action potentials are maintained. To insure that action potentials were maintained during NMDA receptor antagonist exposure (50 μM D-APV), synaptic stimulation was delivered in a θ-burst pattern of stimulation with bicuculline [7]. Hippocampal CA1 minislices were sampled 15 min after stimulation. mRNA was purified from pooled slices, cDNA prepared and real-time quantitative PCR was performed. Plotted are data from four separate determinations. Stimulated *arc* levels in the APV-treated slices are not significantly different from the stimulated levels in slices without the drug.

## Discussion

LTP, particularly its later transcription-dependent phases, has been a major focus of research in the search for the cellular mechanisms of long-term memory. Owing to the evidence showing that new RNA is transcribed in the nucleus in response to LTP and learning [9,18], we have examined how synapses signal a need for new RNA synthesis in response to synaptic plasticity. On the basis of the experiments showing that NMDA receptor antagonists inhibit transcription of many genes [4,5,19], a reasonable conclusion has been that a signal originating at synapses translocates to the nucleus to induce transcription. In addition, certain proteins have recently been shown to be capable of such translocation from synapses [3,20]. The inhibitory effects of NMDA receptor antagonists on action potential generation, however, had not been considered yet [7]. This inhibitory effect on action potentials only becomes an issue if one proposes that action potentials, through calcium, are instead the critical signals regulating rapid transcription of plasticity-related genes. As action potentials alone are sufficient to induce a myriad of signaling pathways and rescue of

late-phase LTP [21], though, the idea of action potential-dependent transcription must now be considered. Here, we have provided evidence consistent with the idea that an NMDA receptor-independent signal is sufficient to signal the nucleus and commence transcription of an important plasticity-related gene. These data support our hypothesis that NMDA receptor-independent mechanisms, such as calcium increases generated by action potentials, can provide a large, fast signal in the nucleus that would favorably permit rapid induction of transcription. Signals transported from synapses would have little or no targeting advantage over a cell-wide signal, as synaptic 'tagging' after LTP would be critical no matter how a signal reached the nucleus [*22*,*23*].

These data support our hypothesis that gene expression can occur irrespective of NMDA receptor-dependent plasticity for at least one immediate early gene. As bicuculline may have some effects on the integrative properties of dendrites that we are not measuring, our data do not rule out a role for aberrant activation of another signaling pathway, such as activation of ERK through the brain derived neurotrophic factor. The pathway would, however, be synaptic plasticity independent – recall that LTP (and long-term depression) are blocked in our experiments with APV. We do know, though, that bicuculline by itself, in the absence of electrical stimulation, does not lead to the activation of ERK [7]. One way relevant transcription could be initiated is with the activation of kinases by calcium: CAMKIV is concentrated in the nucleus and has been implicated in late-LTP and learning [24], whereas CAMKI reportedly is able to translocate to the nucleus from the cytosol very rapidly [25]. Similarly, calcium-dependent cyclic AMP synthesis and subsequent activation of PKA could also mediate TF binding, as PKA activity is necessary for late-LTP and memory [26]. However, PKA's speed of activation in the nucleus in response to neuronal activity is unknown. Further study will be required to work out the details of nuclear engagement once specific necessary genes and TFs are identified.

## Conclusion

These data indicate that when action potentials are carefully controlled, NMDA receptor-dependent signals from synapses are unnecessary for fast stimulation of TF binding and transcription of *arc*. We propose that an action potential-mediated calcium increase, or other similar non-NMDA receptor-dependent processes, initiate the rapid regulation of genes necessary for consolidation of synaptic plasticity.

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