

Region-directed phototransfection reveals the functional significance of a dendritically synthesized transcription factor

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Multiple nuclear transcription factors including E-26-like protein 1 (Elk-1) have been found in neuronal dendrites, yet the functional significance of such localization has not yet been explained. Here we use a focal transfection procedure, 'phototransfection', to introduce *Elk1* mRNA into specific regions of live, intact primary rat neurons. Introduction and translation of *Elk1* mRNA in dendrites produced cell death, whereas introduction and translation of *Elk1* mRNA in cell bodies did not produce cell death. Elk-1 translated in dendrites was transported to the nucleus, and cell death depended upon transcription, supporting the dendritic imprinting hypothesis and highlighting the importance of the dendritic environment on protein function. Our demonstration of the utility of phototransfection for spatially controlled introduction of mRNAs opens the broader opportunity to use this method to introduce selected quantities of small molecules into discrete regions of live cells to assess their biological functions.

An understanding of the complexities of dendrite physiology is critical to understanding both normal and pathological cell signaling in the brain, and the development of sensitive technologies has allowed for a more thorough characterization of the contributions dendrites make to neuronal function. For example, studies have shown that dendrites contain a diverse population of mRNAs and can translate protein independent of the cell body^{1–5}, a finding with important implications for the relative autonomy of dendrites in synaptic responsiveness and plasticity.

Recently, multiple nuclear transcription factors have been found in neuronal dendrites; these transcription factors include Elk-1 (ref. 6), which is known to be involved in differentiation⁷, cell proliferation⁸, tumorigenesis⁹ and apoptosis⁸. Few studies have been carried out to determine how transcription factors that are localized in dendrites, far from their known site of action, may influence neuronal function. The 'dendritic imprinting' hypothesis¹⁰ posits that transcription factors in dendrites travel to the

nucleus to modulate gene expression in response to region-specific activation^{10–12}. It is hypothesized that dendritic imprinting could be used to transmit signals from locally restricted elevations in calcium levels¹¹, or that it could preserve different spatial or temporal information than what is captured by kinase cascades typically thought of as mediating synapse-to-nucleus signaling¹⁰. Previous studies have shown that transcription factor proteins, including CREB¹⁰ and p65 (ref. 11) can traffic from dendrite to nucleus. Given the spatial polarity of neurons, the dendritically synthesized transcription factor is hypothesized to be functionally distinct from that which exists in the cell soma.

One of the serious limitations in testing the dendritic imprinting hypothesis has been the experimental difficulty associated with delivery of transcripts to specific subcellular regions. Here we describe a phototransfection technique, which permits reliable introduction of mRNA into experimenter-controlled subcellular regions. Using this approach to introduce *Elk1* mRNA specifically into distal dendrites or cell bodies of live, intact neurons, we demonstrated the importance of the subcellular localization of *Elk1* mRNA for the control of neuronal function.

RESULTS

Elk-1 localization in neuronal dendrites

Immunocytochemistry on primary rat hippocampal neurons (Fig. 1a), and immunohistochemistry on adult rat brain sections (Fig. 1; Supplementary Fig. 1 online) revealed Elk-1 immunoreactivity in proximal and distal dendrites. Using an antibody that recognizes the somatodendritic kinase MAP2, we confirmed the dendritic localization of Elk-1. We observed the pattern of Elk-1 immunoreactivity with multiple antibodies, and our observations were consistent with previous reports of Elk-1 immunoreactivity in dendrites⁶. Phosphorylation is known to potentiate Elk-1 transcriptional activation¹³, and we detected immunoreactivity for Elk-1 phosphorylated at Ser383 (Fig. 1b) in a punctate pattern in proximal and distal dendrites, indicating

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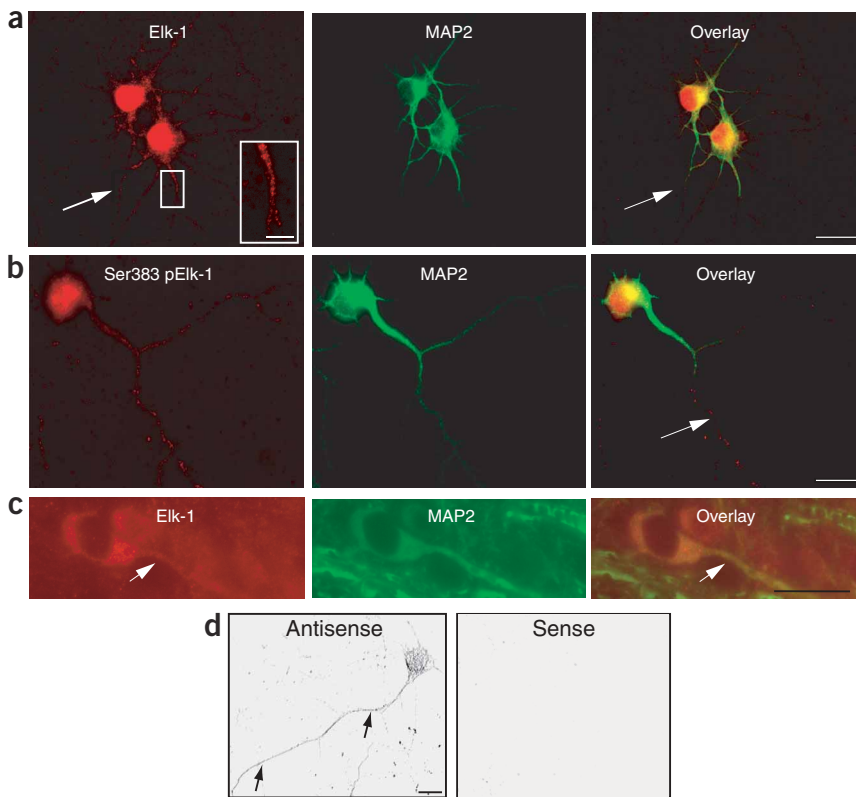


Figure 1 | Elk-1 protein and *Elk1* mRNA are localized in dendrites. **(a,b)** Immunoreactivity of Elk-1 (rabbit polyclonal; Cell Signaling; **a**) and of Elk-1 phosphorylated at Ser383 (**b**) in proximal and distal dendrites in primary rat hippocampal neurons. Inset in **a** is a magnified image of an individual dendrite; scale bar, 5 μm . Signal in the cell body is saturated to show signal in dendrites. **(c)** Elk-1 immunoreactivity in dendrites from adult rat brain sections. An antibody recognizing Elk-1 was detected using Cy3 (left) and an antibody recognizing somatodendritically localized MAP2 was detected using Alexa 488 (middle). Merged images (right) show immunological detection of Elk-1 and MAP2, or phospho-Elk-1 and MAP2. Arrows highlight regions of Elk-1 immunoreactivity in dendrites. **(d)** *In situ* hybridization on primary rat hippocampal neurons using 2.5 ng/ μl of 400-bp digoxigenin-labeled antisense and sense *Elk1* RNA probes followed by alkaline phosphatase treatment for probe visualization. Arrows indicate alkaline phosphatase signal in both proximal and distal dendrites. Scale bars, 20 μm .

phototransfection technique uses a titanium-sapphire laser to induce small temporary holes in lipid membranes of primary neurons (**Fig. 2**).

We estimated the number of mRNA molecules that diffuse into a phototransfected dendrite to be between 10 and 30 based on the following conditions: the concentration of mRNA in the phototransfection chamber is 10–15 $\mu\text{g}/\text{ml}$, the volume of the solution is 1 ml, the diffusion coefficient for an RNA of $\sim 1,000$ bases is 3 $\mu\text{m}^2/\text{s}$, the phototransfected area is 0.16 μm^2 for each 5-ms pulse period, and we used 8–16 laser pulses for each experiment. This may be an overestimate for a variety of reasons including the increase in diffusion coefficient ($\sim 20\times$) when the RNA moves from an aqueous solution to the cellular cytoplasm. Given that translation is an endogenous biological amplification, such small amounts of RNA may give rise to physiologically relevant levels of protein.

We initially bathed primary hippocampal neurons in extracellular solution containing Lucifer yellow. Using a titanium-sapphire laser at a power of 70 mW (at the back aperture of the lens), we

that dendrites also contain post-translationally modified versions of Elk-1. Preincubation with control peptides blocked Elk-1 and Ser383 phospho-Elk-1 immunoreactivity (**Supplementary Fig. 1**) and omission of the primary antibody resulted in an absence of staining (data not shown). We next used *in situ* hybridization to assess the presence of *Elk1* mRNA in dendrites. We hybridized 400-bp sense and antisense digoxigenin-labeled *Elk1* RNA probes to primary rat hippocampal neurons and visualized them using fluorogenic alkaline phosphatase substrates. We detected the signal for *Elk1* mRNA when we probed with the antisense probe ($n = 3$) but did not detect it when probing with the sense control ($n = 3$), indicating that *Elk1* mRNA is present in dendrites (**Fig. 1d**).

Phototransfection of discrete regions of live, intact neurons

To assess the role of dendritically localized Elk-1, we focally introduced *Elk1* mRNA into distal dendrites versus cell bodies of live neurons. We accomplished this using a technique similar to, but distinct from, previous techniques using a violet diode laser¹⁴ or titanium-sapphire laser¹⁵ to transfect cell lines with plasmid DNA, or a titanium-sapphire laser as a tool for non-invasive nanodissection¹⁶. We customized the laser-induced cellular poration of neurons for the introduction of mRNA (phototransfection) into cell-attached dendrites, facilitating a functional analysis of dendritic protein synthesis in the intact neuron. This

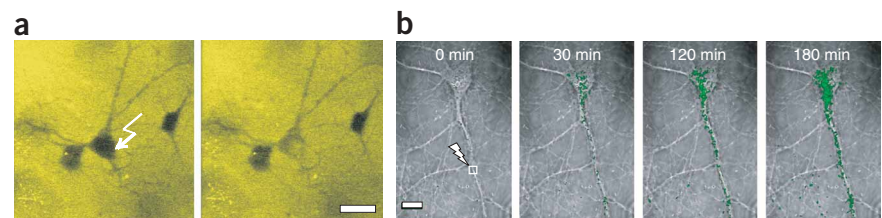


Figure 2 | Phototransfection successfully introduces exogenous constructs into neurons. **(a)** Live hippocampal neurons before poration (arrow); note they are not labeled with Lucifer yellow (left). After poration of the center neuron the cell becomes fluorescent around the site of poration showing that the cell has taken up the Lucifer yellow (right). Also note that the other neurons remain nonfluorescent because photons were not delivered to their processes, demonstrating the spatial control provided by poration. **(b)** Neurons were phototransfected in the presence of 10 $\mu\text{g}/\text{ml}$ capped GFP mRNA. The box roughly outlines the area of phototransfection. DIC images showing neuron before phototransfection and at indicated times after phototransfection are overlaid with the GFP fluorescent signal. Scale bars, 20 μm .

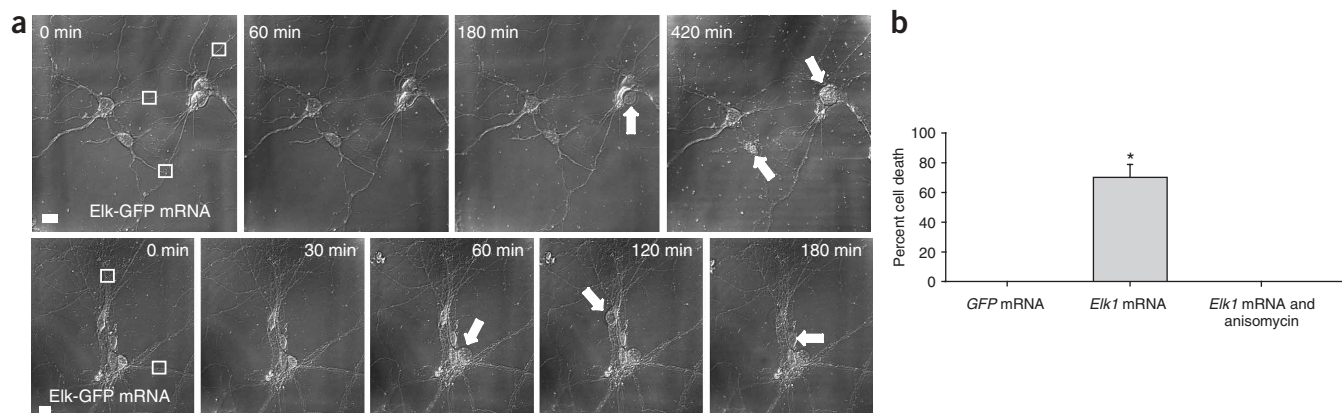


Figure 3 | Phototransfection of *Elk1* mRNA into neuronal dendrites initiates cell death. **(a)** Phototransfection of *Elk1-GFP* mRNA into distal dendrites in culture. DIC images (top) of neurons before and at indicated time after phototransfection (boxes indicate regions of phototransfection). Scale bars, 20 μ m. **(b)** Percent cell death based on morphological observations after phototransfection of *GFP* mRNA ($n = 13$), *Elk1-GFP* mRNA ($n = 17$) or *Elk1-GFP* mRNA after preincubation with anisomycin ($n = 6$). * $P = 0.0000334$ when comparing phototransfections with *GFP* mRNA versus *Elk1* mRNA.

induced the formation of small pores (light-induced poration) and allowed the intracellular accumulation of the Lucifer yellow dye (Fig. 2a). Although the sites of poration were too small to visualize directly, we observed intracellular dye accumulation in milliseconds after poration as measured through line scans of fluorescence intensity (data not shown), indicating successful membrane permeabilization and intracellular diffusion of the extracellular dye.

Using mRNA sequences encoding fluorescent proteins^{2,5,17}, we were able to determine time course and location of translation in live neurons after phototransfection of mRNA. We bathed primary hippocampal neurons in extracellular solution containing *GFP* mRNA (Fig. 2b). After poration of a single dendrite at a power of 30 mW to ensure preservation of cell viability, we detected GFP fluorescence, indicating that exogenous mRNA was introduced into neurons and translated. We called this combination of poration with extracellular delivery of mRNA phototransfection. We observed GFP fluorescence 30 min after phototransfection, and fluorescence continued to increase in a time-dependent fashion, which is consistent with previous studies using the isolated dendrite transfection assay^{2,17}. As translation is a biological amplifier, signal could be detected from *GFP* mRNA using phototransfection with lower power settings than what were used with Lucifer yellow. Neurons phototransfected with *GFP* mRNA remained viable for at least 24 h, indicating that this experimental manipulation did not compromise cell viability. In the absence of phototransfection, no detectable GFP signal was produced, as bath incubation of mRNA does not promote mRNA entry into neurons (data not shown).

Elk-1-induced neuronal death

We phototransfected dendrites with an mRNA construct encoding Elk-1 with an in-frame C-terminal GFP tag (*Elk1-GFP*) at regions at least 40 μ m distal to the cell body and observed cell death occurring as a function of time after phototransfection (Fig. 3a). We could detect morphological signs of cell death, including cell-body swelling and dystrophic dendrites by 60 min after phototransfection of *Elk1-GFP* mRNA, and over the course of several hours, we detected variable rates of death in individual neurons (Fig. 3a). Based on morphological observations, 0/13 neurons

phototransfected with control *GFP* mRNA compared to 12/17 neurons phototransfected with *Elk1-GFP* mRNA displayed characteristics associated with cell death (Fig. 3b). By preincubating neurons with the translational inhibitor anisomycin, we were able to block the *Elk1*-induced cell death ($n = 6$; Fig. 3b), indicating that translation of the mRNA, not just mRNA introduction, is required to produce changes in cell viability.

Using a cell viability and cytotoxicity assay we confirmed our morphological observations of cell death. Neurons phototransfected with *Elk1-GFP* mRNA produced nuclear dye accumulation of the dead-cell stain, confirming our previous finding of cell death after phototransfection with *Elk1-GFP* mRNA (Fig. 4a). In contrast, neurons phototransfected in the absence of *Elk-GFP* mRNA did not produce nuclear dye accumulation (Supplementary Fig. 1). Using the cell viability and cytotoxicity assay, only 1/7 neurons phototransfected in the absence of *Elk1-GFP* mRNA displayed nuclear dye accumulation, compared to 9/11 neurons phototransfected in the presence of *Elk1-GFP* mRNA (Fig. 4a). Neurons phototransfected with *Elk1* mRNA displayed the same morphological changes and nuclear dye accumulation as neurons phototransfected with *Elk1-GFP* mRNA, indicating that the addition of a GFP tag was not impacting neuron viability (data not shown). Phototransfection of mRNA encoding the transcription factor c-fos also did not produce cell death in the phototransfected neurons (Fig. 4a). Phototransfection of other mRNAs including those encoding DS-RED, Venus fluorescent protein¹⁸ and an unrelated *Xenopus* elongation factor 1 α (pTRI-Xef), did not produce cell death in the phototransfected neurons (data not shown) indicating that phototransfection of *Elk1* mRNA in dendrites is unique in its ability to initiate cell death. We added 25 μ M dihydroxyphenylglycine (DHPG) after both control and *Elk1* mRNA phototransfections to stimulate protein synthesis, and in control studies this did not influence neuron viability.

Several pieces of evidence indicate that dendritically translated *Elk1-GFP* mRNA was the cause of cell death after phototransfection. Studies designed to assess anterograde transport of mRNA in hippocampal neurons estimate anterograde mRNA transport to be 10–20 μ m/h^{19–21}. If one assumes retrograde transport of mRNA to occur at the same rate, *Elk1-GFP* mRNA phototransfected into

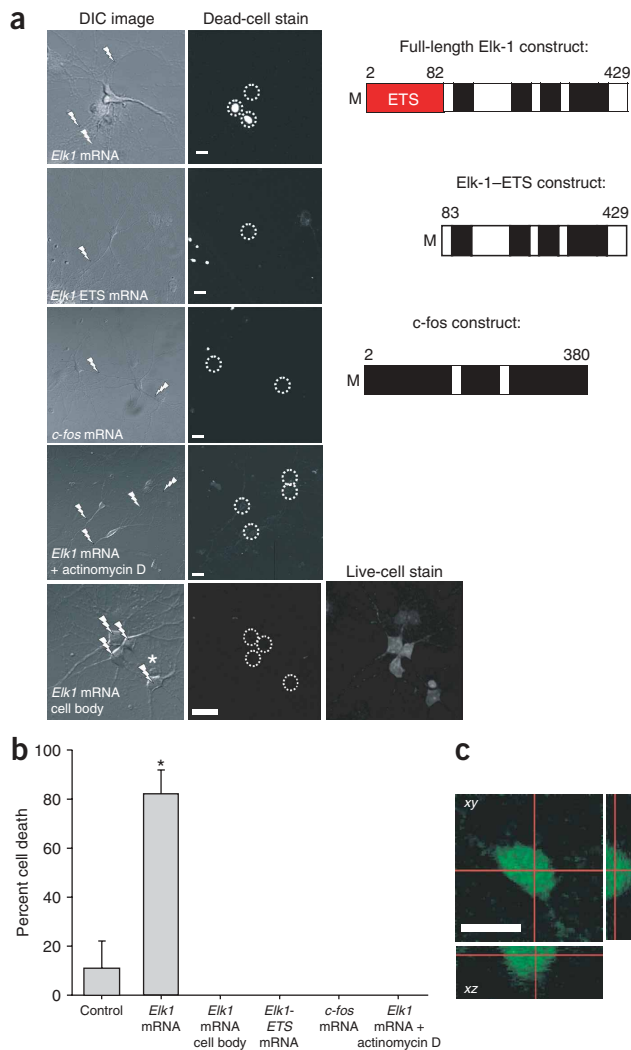


Figure 4 | Cell death initiated by *Elk1* mRNA depends on transcription.

(a) Lightning bolts in images represent regions of phototransfection. A cell viability and cytotoxicity assay was used to confirm cell death or cell viability after phototransfection; dotted circles show the area where one would expect to see nuclear dye accumulation indicative of cell death in the phototransfected neurons. Images were taken approximately 4 h after phototransfection. Scale bars, 20 μm . Illustrated on the right are the constructs used for phototransfection experiments. The red box in the full-length *Elk-1* schematic shows the position of the ETS domain of the *Elk-1* protein. Black boxes represent other known functional domains. (b) Percent cell death associated with phototransfection of the mRNAs indicated in a under the following conditions: control ($n = 7$; phototransfection in dendrites without mRNA), *Elk1* mRNA ($n = 11$; phototransfection in dendrites with *Elk1-GFP* mRNA), *Elk1* mRNA cell body ($n = 6$; phototransfection of *Elk1-GFP* mRNA into cell bodies), *Elk1-ETS* mRNA ($n = 12$; phototransfection in dendrites with an *Elk-1* construct lacking its DNA binding domain), *c-fos* mRNA ($n = 12$; phototransfection in dendrites with *c-fos* mRNA), *Elk1* mRNA + actinomycin D ($n = 7$; phototransfection in dendrites with *Elk1-GFP* mRNA after preincubation with actinomycin D). * $P = 0.0085$ when comparing control phototransfections versus those with *Elk1* mRNA. (c) GFP fluorescence through the *xy*, *yz* and *xz* planes in a neuron after phototransfection of *Elk1-GFP* mRNA into a distal dendrite. Scale bar, 20 μm .

To further assess *Elk1* mRNA translation in dendrites, we used an isolated dendrite assay as previously described^{1,17,24}. After mechanical isolation of individual cultured dendrites, lipid-coated *c-myc*-tagged *Elk1* mRNA was blown onto isolated dendrites. After a 30-min application of DHPG (50 μM), which increases translation through stimulation of Group I mGluRs²⁵, we assessed *c-myc* immunoreactivity ($n = 3$). We detected *c-myc* immunoreactivity in isolated dendrites, indicating that exogenous *Elk1* mRNA can be locally translated (Supplementary Fig. 1).

Given that *Elk1* mRNA introduced into dendrites initiated cell death, we next assessed whether *Elk1* mRNA introduced into cell bodies would have the same effect. Phototransfection of *Elk1-GFP* mRNA directly into cell bodies alone did not produce cell death (Fig. 4a). By assessing the time-dependent appearance of GFP fluorescence, we were able to confirm that the construct was being translated after phototransfection in cell bodies (Supplementary Fig. 1). This indicates that *Elk1* mRNA is capable of producing dramatically different cellular effects when introduced into distinct subcellular compartments. This result is also consistent with *Elk-1* being translated in the dendrite, because if *Elk1* mRNA introduced into dendrites was simply transported into the cell body and then translated, one would expect that the same result would be obtained with *Elk1* mRNA introduction directly into the cell body.

To assess whether cell death associated with dendritically synthesized *Elk-1* was transcriptionally dependent, we phototransfected full-length *Elk1-GFP* mRNA into dendrites in the presence of the transcriptional inhibitor actinomycin D. Preincubation with actinomycin D inhibited *Elk-1*-induced cell death (Fig. 4a,b). To assess whether *Elk-1* transcriptional activity was required to produce cell death, we generated a construct encoding *Elk-1* lacking its DNA binding domain (*Elk1-ETS*). The deletion of the *Elk-1* DNA binding domain has previously been used to eliminate *Elk-1* DNA binding and subsequent *Elk-1* transcriptional activity²⁶. Phototransfection of the *Elk1-ETS* mRNA construct into dendrites did not produce any change in cell viability in contrast to the full-length *Elk1* mRNA construct, consistent with *Elk1* transcriptional activity being required to produce changes in cell viability (Fig. 4a,b). By following the fluorescent signal produced from

dendrites would not reach the cell body before the initiation of cell death, as we phototransfected at least 40 μm away from the cell body and could see evidence of cell death between 30 min and 1 h after phototransfection. This suggests that translation of *Elk1-GFP* mRNA was occurring in the dendrites during the time course in which cell death was initiated. In contrast to mRNA transport, anterograde protein transport is estimated to occur on the order of micrometers per second rather than micrometers per hour^{22–23}. If one assumes retrograde transport to occur at a similar rate, protein translated in the dendrite could be trafficked back to the cell body during the time course in which cell death was initiated. If the protein were being translated in the cell body, one would expect all processes to show equivalent GFP fluorescence. Instead, the primary GFP signal was seen in the phototransfected dendrites, not the surrounding dendrites (Fig. 2b), again suggesting that local translation was occurring in the phototransfected dendrites. Lastly, by looking at the change in fluorescence intensity in cell bodies versus dendrites over time after phototransfection, we observed fluorescent signal on average first appearing in dendrites, which again suggests that the *Elk1-GFP* mRNA was being translated in the dendrite before reaching the cell body (Supplementary Fig. 1).

the GFP tag, we found GFP fluorescence visible in the cell body and nucleus over time after *Elk1-GFP* mRNA phototransfection in dendrites. We localized Elk-1–GFP in the cell soma from a neuron, which had been phototransfected in the dendrite with Elk-1–GFP, by confocal microscopy, and we were able to view the cell from various visual planes (Fig. 4c). GFP fluorescence was observed in all three dimensions throughout the field, indicating that Elk-1–GFP synthesized from the dendritically phototransfected mRNA was present in the nucleus of the cell. This is consistent with the movement of Elk-1–GFP protein to the nucleus where it would be available for subsequent nuclear transcriptional changes.

DISCUSSION

The phototransfection technique, used here for the first time to focally transfect mRNA, highlights a unique aspect of Elk-1 function in neuronal dendrites. There are several possible explanations for why *Elk1* mRNA phototransfected into dendrites led to cell death, whereas *Elk1* mRNA phototransfected into cell bodies did not. One possibility is that different post-translational modifications can occur in the dendrite versus the cell body. Based on our immunocytochemical data, post-translationally modified versions of Elk-1 do exist in dendrites (Fig. 1b). Indeed, Elk-1 can undergo SUMOylation²⁷ and there are nine Ser/Thr phosphorylation sites on Elk-1 (ref. 13), any combination of which may activate Elk-1 to induce cell death. These modifications could regulate Elk-1 protein-protein interactions, protein-DNA interactions and/or direct movement of Elk-1 to different subcellular regions. We are currently investigating the types of stimulation that may increase translation of endogenous *Elk1* mRNA or modify Elk-1 phosphorylation state, to pinpoint what physiological conditions would activate Elk-1 specifically in neuronal dendrites. It is possible that with normal synaptic activity, Elk-1 has a nonpathological role in neurons, but that excessive or toxic stimuli would modify or increase translation of *Elk1* mRNA to a level that would allow Elk-1 to initiate cell death. Whatever the specific mechanism, this data represents an exciting area for future research into the effects of the dendritic environment on protein function.

The dependence of cell death on Elk-1 transcriptional activity is consistent with the previously proposed dendritic imprinting hypothesis^{10–12}. Elk-1 can be activated by multiple MAP kinase cascades in response to diverse cellular inputs including growth factors stimulation and glutamate receptor activation. It may be that Elk-1 is localized in the dendrite where it would respond to distinct synaptic stimuli and achieve protein modifications that would then differentially affect gene transcription to produce unique cellular consequences, such as cell death. The identified gene targets of Elk-1 are mostly immediate early genes, and it is unclear what transcripts resulting from Elk-1 transcriptional activity are associated with its regulation of cell death. Elk-1 can act as both a transcriptional activator and as a transcriptional repressor⁸. Because of this, teasing apart the specific downstream transcriptional consequences of Elk-1 dendritic localization will require a more detailed analysis of the cadre of genes regulated by Elk-1.

These data highlight the importance of subcellular localization on protein function. The ability to phototransfect mRNA into discrete regions of neurons promises to facilitate a more detailed analysis of the specific biological consequences of a local environment. Notably, this technique allows for the introduction of small amounts of mRNA by manipulation of the concentration of mRNA

in the bath solution and through regulation of the laser intensity to modulate the pore size and duration of opening. With the introduction of small amounts of mRNA, more physiologically relevant manipulations of translated protein abundance can be achieved, and the issue of overexpression artifacts can be avoided. This technique will also permit a functional genomics analysis of multiple mRNAs simultaneously in live cells. For the first time, we will be able to introduce various types of RNA including siRNA and miRNA into discrete subcellular regions in known controllable quantities. Further, it should be possible to phototransfect the same cell multiple times so time courses of RNA function may be examined. Finally, phototransfection of RNA into cells in live brain slices should permit detailed analysis of RNA influences on systems biology.

METHODS

Cell culture. We plated neuron-enriched primary rat hippocampal cultures from E18.5 embryos at 100,000 per ml in Neurobasal medium (Invitrogen) with B-27 supplement (Sigma) on 12-mm round German Spiegelglas coverslips (Bellco) or grided coverslips (Eppendorf) as previously described²⁸. For a subset of phototransfection experiments, we also used primary rat hippocampal neurons from Cambrex Corporation.

Phototransfection. To confirm membrane permeabilization after phototransfection, we looked for diffusion of Lucifer yellow (Molecular Probes) from the extracellular space into the live cell and then synthesized the following capped mRNAs for use in the phototransfection experiments (Ambion mMessage mMachine): GFP-tagged *Elk-1*, *GFP* (Clontech), *DS-RED* (Clontech), *Venus* fluorescent protein¹⁸, *pTRI-Xef* (Ambion), *c-fos* (IMAGE clone 2582234) and *Elk-1-ETS*. *Elk-1-ETS* contains *Elk-1* with the initiator methionine but without its DNA binding domain (generated by PCR; verified by sequencing). We bathed primary rat hippocampal neurons in 10–15 μ g capped mRNA in 1 ml extracellular saline (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgSO₄, 10 mM HEPES and 10 mM D-glucose) at 37 °C and imaged and phototransfected neurons using a Prairie Technologies, Inc. Ultima multiphoton scanhead attached to an Olympus BX61 fixed-stage upright microscope equipped with a 40 \times water immersion lens (LUMPlanFI/IR, numerical aperture (NA) 0.8). We used two sets of galvanometers independently for imaging and phototransfection simultaneously. Based on transmitted light gradient contrast image or weak autofluorescence excited by 840 nm excitation, we selected 8 to 16 points over an area for phototransfection. We then used a titanium-sapphire laser (Mai-Tai, Spectral-physics) to make transient poration sites by delivering 100-fs pulses (80 MHz repetition rate) for 1–5 ms at a power of 30 mW (at the back aperture of the lens). Approximately 80% of the power was transmitted to the focused spot, and we moved the laser pulses over 8–16 points randomly with 10-ms intervals between each pulse. The pulse duration and interval was controlled by Pockel's cell. The focused beam size was diffraction limited. After phototransfection, we treated samples with 25 μ M DHPG (Tocris) to increase translation and transferred the samples to an Olympus IX81 inverted microscope and FluoView 1000 confocal microscope for simultaneous GFP fluorescence and differential interference contrast (DIC) imaging. All images were processed with Metamorph (Universal Imaging Corporation)

image processing software. For cell death assays, we used a cell viability and cytotoxicity kit (Molecular Probes) according to the manufacturer's instructions. Drug treatments: 5 μ M anisomycin and 1 μ g/ml actinomycin D (Sigma). Rats were used as a cell source for these studies with approval of the University of Pennsylvania Institutional Animal Care and Use Committee.

Additional methods. Descriptions of immunocytochemistry and immunohistochemistry, *in situ* hybridization and *Elk-1* translation experiments are available in **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

We thank C. Garner for providing the polyclonal MAP2 antibody, M. Maronski for preparation of primary neuronal cultures, and R. Pittman and C. Lai as well as members of the Eberwine laboratory for helpful discussions. This work was funded by grants MH74169 (to L.E.B.), DA015395 and DA09082 (to E.V.B.), P30NS047321, P20MH071705, R01NS043142 and R37NS037585 (to P.G.H.), and AG9900 and MH58561 (to J.H.E.).

AUTHOR CONTRIBUTIONS

L.E.B. and J.-Y.S. planned and performed experiments, H.T. performed experiments, E.J.B. planned and interpreted the electron microscopy, and P.G.H. and J.H.E. planned and interpreted experiments.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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