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Single-Cell Electroporation for Gene Transfer In Vivo

Neurotechnique

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Summary

We report an electroporation technique for targeting gene transfer to individual cells in intact tissue. Electrical stimulation through a micropipette filled with DNA or other macromolecules electroporates a single cell at the tip of the micropipette. Electroporation of a plasmid encoding enhanced green fluorescent protein (GFP) into the brain of intact *Xenopus* tadpoles or rat hippocampal slices resulted in GFP expression in single neurons and glia. In vivo imaging showed morphologies, dendritic arbor dynamics, and growth rates characteristic of healthy cells. Coelectroporation of two plasmids resulted in expression of both proteins, while electroporation of fluorescent dextrans allowed direct visualization of transfer of molecules into cells. This technique will allow unprecedented spatial and temporal control over gene delivery and protein expression.

Introduction

Recent large-scale genetic screens have uncovered a multitude of genes implicated in brain development, learning and memory, regeneration, and neurological diseases. Determining the function of these genes in vivo necessitates advanced techniques for controlling the timing and location of gene expression, combined with specific assays of neuronal function or morphology. In some cases it will be necessary to introduce genes into postmitotic cells, which are refractory to most available gene transfer methods.

The potential for elucidating gene function by observing the consequences of altered gene expression on neuronal structure is exemplified by in vivo imaging studies of neuronal development in *Xenopus* following viral gene transfer (Zou and Cline, 1996; Nedivi et al., 1998; Wu and Cline, 1998; Li et al., 2000) and in transgenic *Drosophila* (Zito et al., 1999). Likewise, electrophysiological studies have used side-by-side comparison of the synaptic properties of individual infected and uninfected neurons to determine the effects of gene expression on neuronal function (Hayashi et al., 2000). Although single-cell resolution of gene expression and its effects are potentially powerful for elucidating gene function, current techniques to spatially and temporally control gene delivery to single cells within the CNS are limited. One versatile technique applicable to this problem is electroporation.

Electroporation is now a widespread means of introducing macromolecules, including DNA, RNA, dyes, and proteins, into cells (Neumann et al., 1999). Electroporation refers to the permeabilization of cell membranes by application of short-duration electric field pulses, traditionally between two relatively large plate electrodes (Ho and Mittal, 1996; Neumann et al., 1999). Large external electric fields induce high transmembrane potentials, leading to the formation of minute pores (20–120 nm diameter) restricted to small regions of the cell membrane (<0.1%) adjacent to the electrodes (Ho and Mittal, 1996; Neumann et al., 1999). During the electric pulse, charged macromolecules, including DNA, are actively transported by electrophoresis across the cell membrane through these pores (Neumann et al., 1996). Noncharged molecules can also enter through the pores by passive diffusion (Neumann et al., 1998). Upon pulse termination, pores reseal over hundreds of milliseconds, as measured by recovery of normal membrane conductance values (Ho and Mittal, 1996).

Although electroporation is an established method for transferring macromolecules into both prokaryotic and eukaryotic cells, its application to neurons in intact tissues has been relatively limited. Electroporation-induced gene transfer into large numbers of neurons has been demonstrated in chick (Sakamoto et al., 1998; Atkins et al., 2000; Koshiba-Takeuchi et al., 2000) and mouse embryos (Akamatsu et al., 1999; Miyasaka et al., 1999). Electroporation is attractive for neuronal gene transfer, since it has many advantages over the more common transfection methods, including viral gene transfer, gene gun biolistics, lipofection, and microinjection. Electroporation lacks the potentially toxic effects of viruses and lipofection and the physical damage produced by biolistic gene gun and microinjection. In contrast to viruses, which are often limited by cell- or species-specific infection, there appears to be no restrictions on cell types susceptible to electroporation. In addition, few viruses are available that can infect postmitotic neurons. Electroporation is also significantly more efficient than either lipofection, microinjection, or gene gun biolistics in terms of numbers of transfected cells and the intensity of foreign gene expression (Muramatsu et al., 1997).

Current transfection techniques are insufficient for controlled spatial and temporal expression of multiple genes in single cells within intact tissues. Coexpression of multiple genes is often required to study the interaction of multiple proteins. Coexpression of a fluorescent protein from an independent plasmid along with a gene of interest is particularly valuable to identify the transfected cells in living tissue when expression of a tagged fusion protein may interfere with its function. Assays of the effects of gene expression on neuronal structure are facilitated by the coexpression of fluorescent proteins, which distribute throughout neuronal processes. Low-titer virus can be used to sparsely infect cells, but viral infection is subject to the limitations mentioned above. Similarly, gene gun biolistics can deliver multiple genes; however, gene gun biolistics is limited in its application, because it cannot be used in vivo. Gene transfer by

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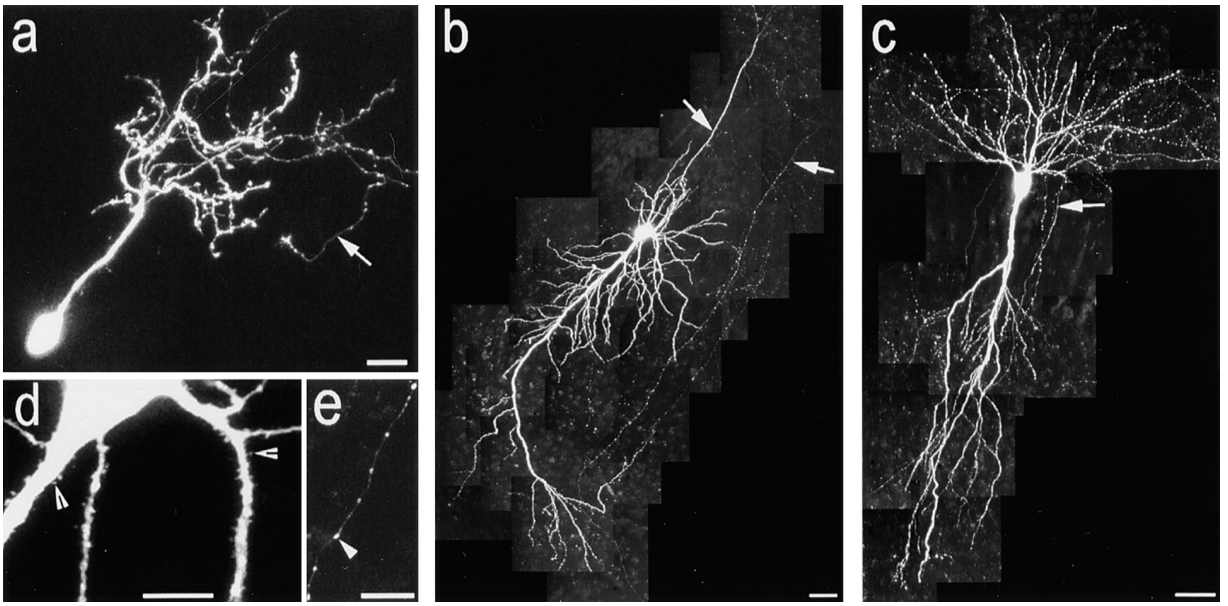

Figure 1. Single-Cell Electroporation with pEGFP Targets Gene Delivery to Individual Neurons

Confocal images of a single GFP-labeled neuron in the tadpole optic tectum (A) and CA1 (B) and CA3 (C) regions of rat hippocampal slice cultures. Arrows point to axons in each cell. Higher magnification of the CA1 pyramidal cell from (B) demonstrates that GFP expressed following electroporation completely fills dendritic spines ([D], open arrowheads) and axonal varicosities ([E], closed arrowhead). Scale bars = 10 μm in (A) and (D), 50 μm in (B) and (C), and 20 μm in (D) and (E).

microinjection requires that DNA be delivered into the cell nucleus and, therefore, is only applicable to cultured cells in which a micropipette can be visualized as it penetrates the nucleus.

While electroporation is a promising transfection technique, precise targeting is not feasible using traditional, large electrodes. One of the powerful attributes of electroporation, however, is the ability to localize transfection by controlling exposure to either DNA or the electric field. We have utilized this feature to develop a novel micropipette electroporation technique to target transfection to a single cell within the brain of intact anesthetized animals or in brain slices. Due to its versatility, this single method can be used to deliver genes, antisense oligonucleotides, fluorescent dyes, drugs, and other macromolecules to a variety of cell types, including neurons and glia in the intact animal. Application of this technique allows unprecedented spatial and temporal control of gene delivery and permits experiments that cannot be performed with current gene transfer technology.

Results

Single-Cell Electroporation

We have targeted gene delivery to single cells within intact tissue by electroporating individual cells using electrical pulses from a DNA-filled micropipette. Micropipette electroporation with the plasmid carrying the gene for enhanced green fluorescent protein (pEGFP) resulted in single GFP-expressing cells in the *Xenopus* tadpole brain and the cultured rat hippocampal slice (Figure 1). We found no apparent restrictions on the species or types of cells susceptible to electroporation. In the tadpole, both neurons and glia were readily transfected, including both newly differentiated and mature cells. In the rat hippocampal slice, both pyramidal cells and interneurons were transfected in CA1 and CA3 regions. Following transfection of neurons with pEGFP, bright GFP fluorescence could be detected within 12 hr. In hippocampal pyramidal cells, GFP completely filled all processes, including dendritic spines and fine axons (Figures 1D and 1E). GFP fluorescence increased over 2–3 days, even with repeated imaging (see Figure 3A), and remained high for more than 2 weeks.

Optimal micropipettes for electroporation of individual cells in the *Xenopus* tadpole brain and rat hippocampal slice had a tip diameter of 0.6–1 μm and resistances of 10–15 MΩ. We found that these small diameter micropipette tips resulted in a high frequency of single-cell transfections (and a low incidence of transfecting neighboring cells), while clusters of cells were transfected with larger pipettes. Electrode resistance and the current delivered through the micropipette were monitored with an oscilloscope in parallel with the electroporation circuit (Figure 2A). For single-cell electroporation (SCE) in the *Xenopus* tadpole, micropipettes were inserted into the dense cell body layer of the optic tectum (Figure 2B). Cells throughout the tadpole brain were transfected independent of depth (up to 200 μm), suggesting that this technique is applicable to cells deep within other tissues, as long as they are accessible to the micropipette.

Single-cell electroporation efficiency depended on stimulation parameters including pulse shape, the number of pulses delivered, and voltage amplitude (Figure 2C). Three types of stimuli were tested, based on success in other electroporation paradigms: individual

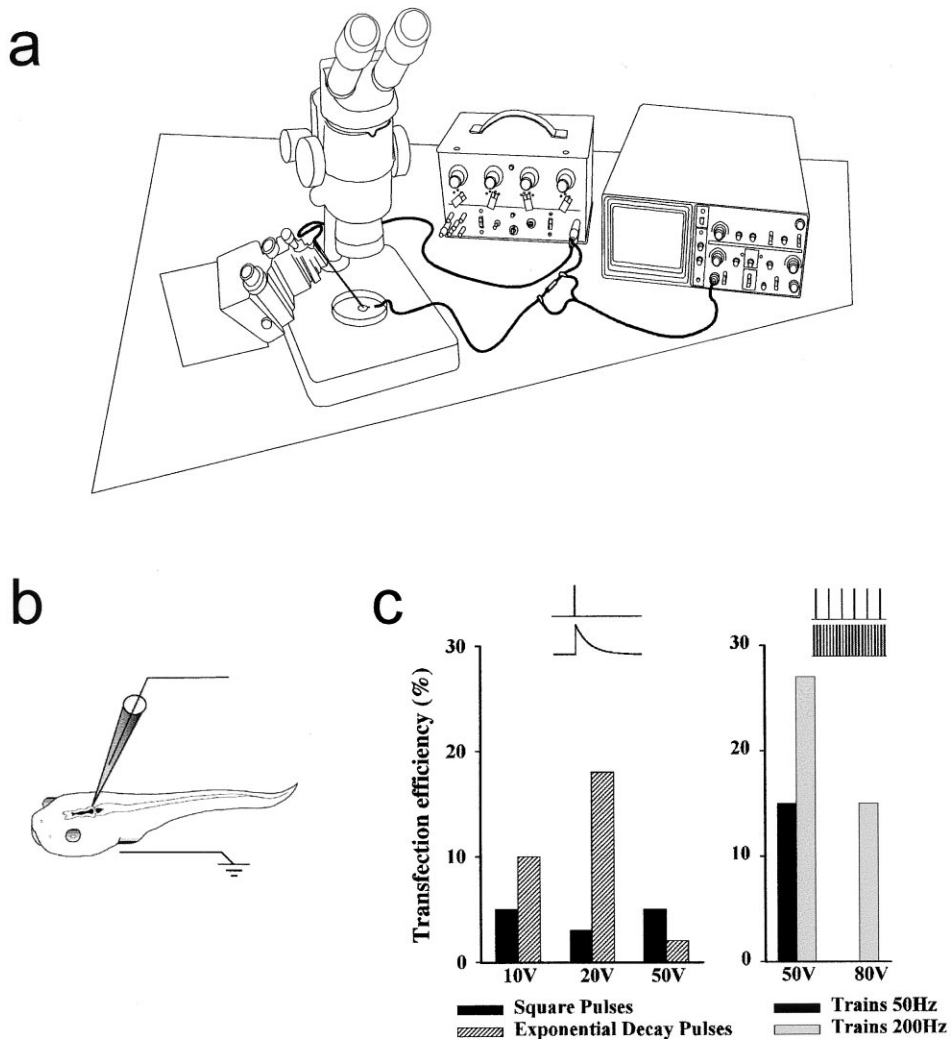

Figure 2. Single-Cell Electroporation Setup and Stimulation Parameters

(A) The single-cell electroporation setup includes a micropipette filled with a solution of DNA or other molecules to be delivered, a pipette holder, a micromanipulator, a microscope, a voltage stimulator, and wire connections. The shape and amplitude of the current passing through this circuit can be monitored by measuring the voltage drop across a known resistor with an oscilloscope.

(B) Single-cell electroporation of neurons in vivo was carried out by inserting a glass micropipette filled with DNA solution into the brain of an anesthetized tadpole. Stimulation delivered between the micropipette and an external ground electroporated a single cell at the micropipette tip.

(C) The efficiency of transfection using different stimulation parameters is presented as the percentage of electroporation attempts yielding individual cells expressing GFP in the in vivo tadpole brain 24 hr after stimulation. Exponential decay pulses (10V and 20V) with $\tau = 70$ ms were significantly more effective than the corresponding 1 ms square pulses ($p < 0.01$), and 20V exponential decay pulses were significantly more effective than 10V exponential decay pulses ($p < 0.01$). Highest transfection efficiencies were produced with 1 s long trains of 1 ms square pulses delivered at 200 Hz. Trains at 200 Hz produced significantly more transfected cells than 50 Hz trains ($p < 0.001$). All stimuli, whether individual pulses or trains, were delivered five times, with a 1 s interstimulus interval.

square pulses (Sakamoto et al., 1998; Teruel et al., 1999; Koshiba-Takeuchi et al., 2000), individual exponential decay pulses produced by capacitance discharge (Muramatsu et al., 1997), and high-frequency trains of square pulses (Atkins et al., 2000). Initial experiments indicated that a sequence of three to five individual pulses or trains with an interstimulus interval of ~1 s was much more effective than a single pulse or train (data not shown). Therefore, for comparisons of stimuli shapes and voltage amplitudes and for all subsequent transfections, five repeated pulses or trains were delivered.

Few transfected cells were produced from 1 ms square pulses at voltages ranging from 10V to 50V (Figure 2B). Transfection efficiency was significantly enhanced by delivering exponential decay pulses ($\tau = 70$ ms). Transfection rates were further increased by delivering trains of 1 ms square pulses. The efficiency of transfection with trains was frequency and voltage dependent, with the highest transfection rates (about 30%) obtained with 200 Hz trains of 1 ms square pulses at 50V. This voltage corresponded to currents of 1–5 μA. Since we were not able to see the micropipette tip in the brain of the intact animal, transfection failures were probably due to misalignment of the micropipette tip and cell body. We increased our transfection rate to 80% of animals by electroporating four sites in each

tadpole. Since pipette tips tended not to clog, the same micropipette could be used for electroporation at multiple sites and in multiple animals. The process of electroporating each site and moving to another site required only a few seconds.

In the rat hippocampal slice cultures, trains of 1 ms pulses of 20V (1.3–2 μA) at 200 Hz were the most effective stimuli for transfection of individual neurons, including pyramidal cells and interneurons in CA1 and CA3 (Figure 1). Neurons expressing GFP appeared healthy up to 4 days after electroporation—the longest time examined. The transfection rate in hippocampal slices was about 20% of attempts. The transfection rate would be significantly improved with direct visualization of the pipette and neuronal somata, using a visualized patch setup rather than a dissecting microscope. As in the tadpole brain, the easy ability to electroporate multiple sites per slice increased the overall transfection rate.

Repeated confocal imaging of neurons in anesthetized tadpoles allowed us to monitor neuronal morphology, dendritic arbor branch dynamics at 2 hr intervals, and dendritic arbor growth rates over several days (Figure 3). We compared these parameters from GFP-expressing cells with those from cells labeled with the lipophillic dye 0.02% 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI), a dye commonly used for imaging neuronal morphology (Wu and Cline, 1998). In both DiI-labeled cells and neurons electroporated with GFP, dendritic arbors grew continuously without signs of dendritic or axonal swelling or degeneration. Dendritic arbor branch additions and retractions measured at 2 hr intervals were comparable between the two groups (additions: GFP = 55.1 ± 7.6 branches/2 hr, DiI = 52.2 ± 6.5 branches/2 hr; retractions: GFP = 48.6 ± 7.2 branches/2 hr, DiI = 39.8 ± 4.5 branches/2 hr, expressed as mean ± SEM). Dendritic arbor growth rate over 24 hr was also similar between these two groups (GFP = 193.7 ± 21.7 μm/24 hr; DiI = 229.5 ± 38.1 μm/24 hr). These data indicate that single-cell electroporation and expression of GFP do not interfere with structural dynamics or neuronal dendritic arbor elaboration. We did not observe GFP-positive degenerating cells or cell debris, suggesting that electroporated cells expressing GFP remained healthy.

Coelectroporation of pEGFP and pDsRed

Single-cell electroporation with two independent plasmids encoding GFP and DsRed resulted in coexpression of both fluorescent proteins in 92% of cells (Figure 4). While GFP fluorescence could be detected within 12 hr and steadily increased in intensity over 3 days, detection of DsRed expression was relatively delayed (Baird et al., 2000). We initially detected DsRed 24–36 hr after electroporation, and fluorescence increased over the next 4–5 days. Bright GFP and DsRed fluorescence persisted for more than 2 weeks, the longest time examined. At 4 days following coelectroporation, 46 of 50 cells expressed detectable levels of both GFP and DsRed. The remaining four cells exhibited dim GFP with no detectable DsRed. In all cases, GFP was brighter than DsRed, and DsRed was never detected without GFP. The relative brightness of DsRed is four times less than GFP (Matz et al., 1999). Therefore, it is possible that DsRed is expressed at undetectable levels in these cells.

Electroporation of Fluorescent Dextrans

In addition to its utility for gene delivery, electroporation can be used to transfer molecules other than DNA into cells. Here, we demonstrate transfer of fluorescent dextrans into tadpole tectal neurons and glia (Figures 4D and 4E). Electroporation of fluorescent dextrans required lower stimulation amplitude and duration than those required for transfection with plasmid DNA. These smaller molecules can easily pass through smaller pores and do not require the multiple membrane binding and translocation steps associated with DNA delivery into cells (Neumann et al., 1996, 1998). A range of stimulus conditions (see Experimental Procedures) was tested by directly observing transfer of fluorescent dextrans into cells during electroporation. Pulses with a duration of 40 ms and amplitude of 10V–15V consistently labeled single cells. Electroporation caused rapid filling of cells with fluorescent dextran and labeling in distal processes within 30 min to 1 hr. By directly visualizing the transfer of fluorescent dextrans into cells with a 40× objective, it was clear that the micropipette was close to but not entering the cell membrane.

Discussion

We demonstrate the ability to target gene expression to individual neurons within the intact central nervous system using single-cell electroporation. Transfection is focused to individual cells by confining the extent of exposure to DNA and the applied electric field to the tip of a micropipette. Single-cell electroporation has broad application to different cell types in different species. We have been able to transfect individual optic tectal neurons and glia in the *Xenopus* tadpole brain and pyramidal cells and interneurons in the rat hippocampal slice. In addition, we demonstrate the versatility of this technique for the transfer of molecules other than DNA into cells. Single-cell electroporation of fluorescent dextrans allows rapid imaging of neuronal morphology minutes after dye filling, in contrast to the longer times required for expression of fluorescent protein following transfection.

We assessed the health of electroporated neurons using in vivo time-lapse imaging of dendritic arbor development and structural plasticity. Our previous studies have shown that these parameters are sensitive indicators of neuronal health. Furthermore, dendritic arbor growth rates are subject to changes in glutamatergic synaptic transmission (Rajan and Cline, 1998), intracellular signaling pathways (Wu and Cline, 1998; Zou and Cline, 1999; Li et al., 2000), and gene expression (Nedivi et al., 1998). Therefore, the normal dendritic arbor growth rates and branch dynamics that we observed in electroporated neurons indicate that these cellular processes have not been affected by electroporation.

Electroporated neurons expressing GFP had typical morphologies, and repeated in vivo time-lapse imaging demonstrated continuous growth for the entire imaging period, up to 6 days following electroporation. In addition, the short-term (2 hr) dendritic arbor branch dynamics and 24 hr growth rates of neurons following single-cell electroporation were similar to those from DiI-labeled cells. These similar growth rates and structural plasticity in

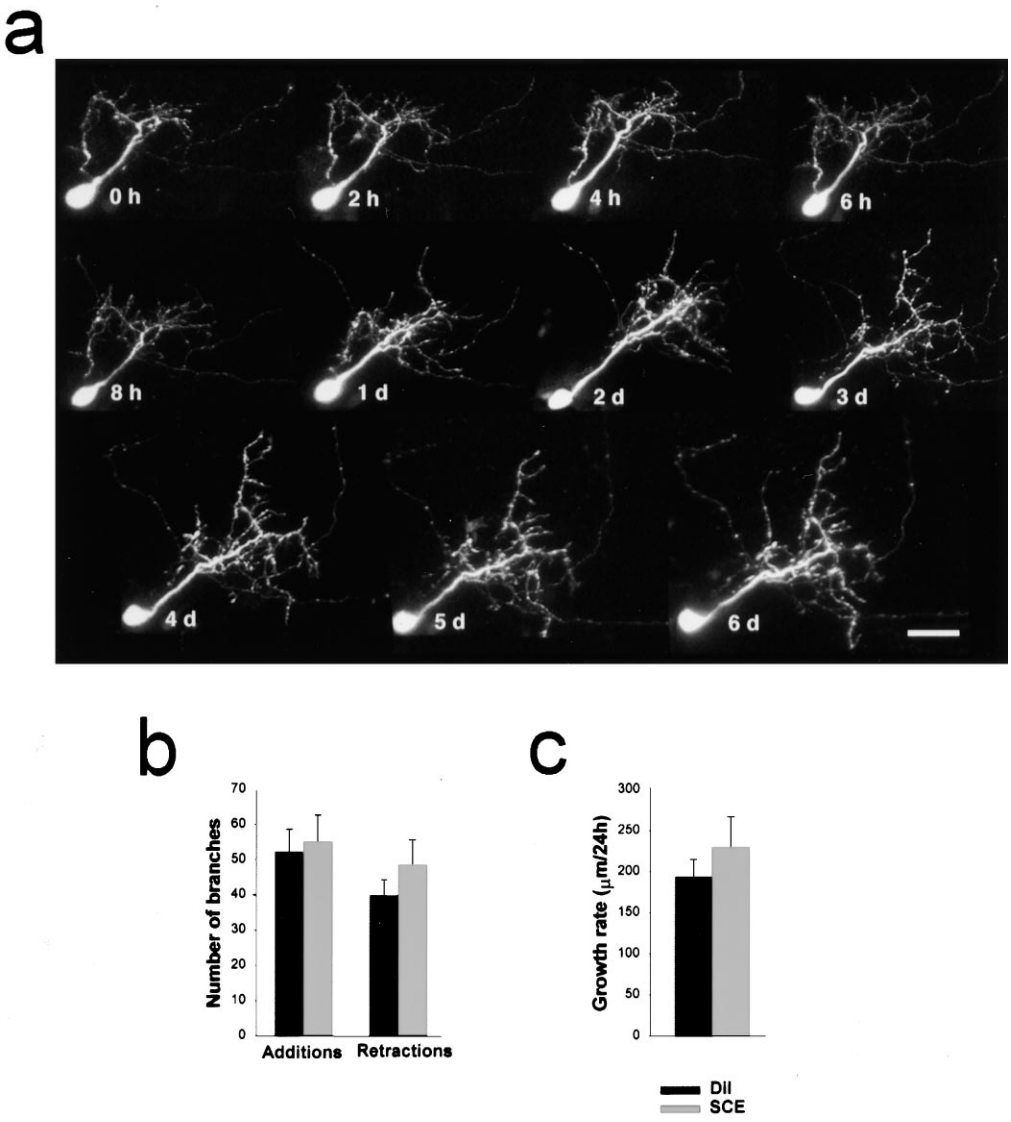

Figure 3. Neurons Labeled by Single-Cell Electroporation of pEGFP Have Typical Morphologies and Dendritic Arbor Dynamics

(A) In vivo time-lapse confocal images of an individual GFP-labeled optic tectal neuron in the tadpole collected at 2 hr intervals starting 24 hr after electroporation. The cell exhibits typical morphology and rapid axonal and dendritic extensions and retractions. Daily imaging up to 6 days following single-cell electroporation demonstrates that neurons grow continuously and appear healthy. Scale bar = 20 μm.

(B) Rates of dendritic branch additions and retractions in GFP-expressing neurons labeled by SCE are comparable to those seen in DiI-labeled neurons.

(C) The total dendritic arbor growth rates over 24 hr of neurons labeled with GFP by SCE were also similar to neurons labeled with DiI, indicating that electroporation does not adversely affect tectal cell development.

neurons visualized with DiI or by pEGFP electroporation demonstrate that electroporation, plasmid DNA delivery, and GFP expression driven by a strong CMV promoter do not interfere with neuronal function. The ability to restrict electrical stimulation to the cell body at the tip of the micropipette prevents the activation of surrounding axons and their terminals, which might lead to altered synaptic transmission. A previous study of the electroporation of hippocampal neurons in culture using trains of 1 ms square pulses demonstrated that electroporated neurons maintain normal synaptic transmission and electrophysiological properties (Teruel et al., 1999).

It is likely that optimal stimulation parameters for single-cell electroporation for gene delivery is specific to each cell type and tissue preparation. Effective stimulation parameters must balance the requirements for temporary pore formation and DNA electrophoresis against the damaging effects of strong electric fields (Ho and Mittal, 1996; Neumann et al., 1998). Cell damage may be caused by excessive electric field strengths, which result in the formation of too many pores or pores too large to reseal. While relatively brief, high-voltage pulses are required to disrupt electrostatic forces maintaining the lipid bilayer structure, longer duration, lower amplitude voltage pulses are required for efficient translocation of DNA across the cell membrane (Neumann et al.,

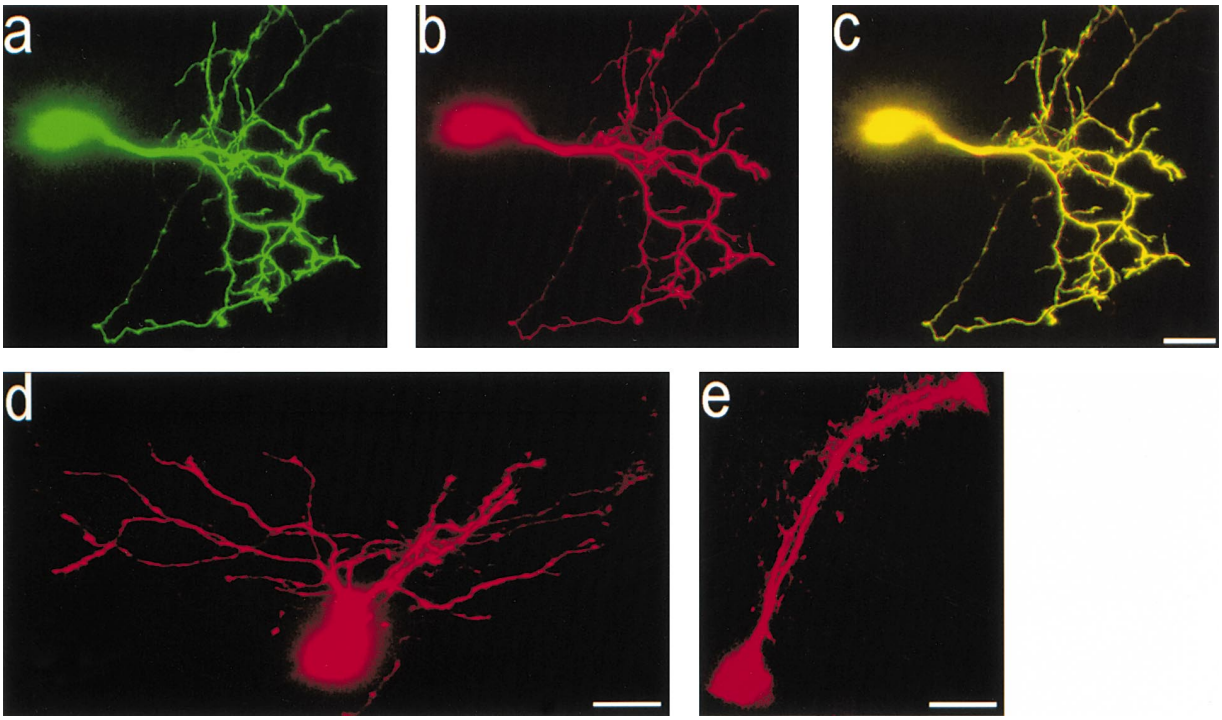

Figure 4. Single-Cell Electroporation Efficiently Transfers Multiple Plasmids into Individual Cells In Vivo

Images in (A)–(D) were collected on a confocal microscope. Image in (E) was collected on a two-photon microscope. Images in (D) and (E) were taken 30 min after electroporation. Scale bars = 20 μm.

(A–C) Cotransfection of pEGFP and pDsRed in a tadpole optic tectal neuron using single-cell electroporation results in coexpression of GFP (green, [A]) and DsRed (red, [B]; yellow = overlay of both, [C]).

(D and E) Single-cell electroporation of tetramethylrhodamine dextran into a neuron (D) and a glia radial cell (E) in the tadpole brain in vivo.

1996). For this reason, we have tested the efficiency of pulses generated by capacitance discharge, which have a high initial peak voltage followed by an exponential decay. Indeed, exponential decay pulses yielded high transfection rates. The highest transfection efficiency, however, was elicited by high-frequency trains of short-duration square pulses. This higher efficiency was most likely due to the more effective electroporation by the train of stimuli rather than the use of square pulses per se. We could not stimulate with a train of exponential decay pulses because the slow time course of the discharge was incompatible with the frequency of the pulses in the train.

We also demonstrate the high efficiency of coelectroporating individual neurons with two plasmids. In the majority of neurons, we detected expression of both GFP and DsRed upon coelectroporation of the two plasmids. These experiments indicate that electroporation of a reporter plasmid can be used to identify cells cotransfected with a second plasmid expressing a gene of interest. This represents a significant improvement over the use of dicistronic IRES constructs, where expression levels of the gene downstream of the IRES are quite variable.

Transfection following stimulation from DNA-containing micropipettes is not likely to be due to simple iontophoresis of macromolecules inside or outside of the cell. Intracellular iontophoresis is improbable, since tadpole tectal cells (10–20 μm diameter) do not survive penetration by the relatively large micropipettes (0.6–1 μm tip diameter). Furthermore, direct visualization of fluorescent dextrans during electroporation demonstrates movement from the micropipette into single cells. Successful single-cell labeling occurs when the micropipette is close to but not inside the cell membrane. Electrical stimulation results in instantaneous filling of the cell body with dye, which is too rapid and restricted to be explained by uptake of extracellularly deposited dye and endocytosis. In contrast to electroporation, we have observed that extracellular pressure injection of fluorescent dextrans at up to ten times the concentration used for electroporation results in diffuse labeling.

The single-cell electroporation technique should be applicable to a wide assortment of neurobiology preparations. Virtually any cell accessible by a glass micropipette is amenable to this method. This technique is relatively inexpensive to set up and easy to perform. Equipment required is common to many neuroscience laboratories, including a micropipette puller, a microscope, a micromanipulator, a voltage stimulator, and an oscilloscope. Voltage stimulators that are components of electrophysiological setups typically have the capacity to generate trains of square pulses of 50V and are therefore sufficient for single-cell electroporation. This circumvents the need to purchase expensive electroporators.

Electroporation lacks residual vector agents such as

virus, lipofection reagents, and gene gun particles, which might interfere with neuronal function. In addition, the ease of coexpression of different genes is an advantage over virus-mediated gene transfer, which is limited by the amount of foreign DNA that can be introduced into recombinant virus. Furthermore, the construction of expression plasmids for electroporation is relatively routine and inexpensive, in contrast to the time and cost required for constructing recombinant viral vectors.

The ability to target transfection to single cells provides a powerful tool for the study of gene function in intact nervous systems. Micropipette electroporation can transfect cells deep within tissue inaccessible to gene gun biolistics or lipofection. The use of GFP expression in individual neurons allows imaging of neuronal morphology without interference of fluorescence from neighboring cells. In addition, single-cell transfection ensures that any induced changes are likely due to cell-autonomous effects of the exogenous genes and not secondary influences from transfected neighbors. The ease of multiple gene transfer using electroporation allows for the study of the concerted actions of different proteins. While electroporation of plasmid DNA can increase protein expression, the potential to electroporate a wide assortment of macromolecules, including antisense oligonucleotides, allows the introduction of agents that decrease specific protein concentration or function. Single-cell electroporation is an important and timely advance in neuronal transfection, since the role of many genes in dendritic and axonal development awaits elucidation.

Experimental Procedures

Preparation of Organotypic Hippocampal Slices

Organotypic slice cultures of hippocampus were prepared as described (Stoppini et al., 1991). In brief, hippocampal slices (400 μm) were cut from postnatal 6- to 8-day-old rats with a tissue chopper and incubated on Millicell filters (Millipore) at 37°C and exposed to a combination of 95% O_2 and 5% CO_2 (Musleh et al., 1997). Slices were used for single-cell electroporation after 5–7 days in culture. After transfection (1–4 days), hippocampal slice cultures were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 15 min, rinsed with 0.1 M PB, and mounted on glass slides with VectaShield (Molecular Probes, Eugene, OR).

Single-Cell Electroporation

Transfection of single cells was accomplished using electroporation from DNA-filled micropipettes. Micropipettes with a tip diameter of 0.6–1 μm were pulled on a P-87 Micropipette Puller (Sutter Instrument Company, CA). The same micropipettes were used for electroporating cells in the tadpole brain and in the rat hippocampal slice. Micropipettes were filled with solution containing plasmid DNA, purified using Promega Wizard Plus MidiPreps DNA purification system (Promega, Madison, WI), and resuspended at concentrations of 0.5–1.0 μg/μl in dH_2O. Transfection efficiency was not noticeably affected by DNA concentration in the range of 0.25–2.0 μg/μl, the inclusion of $CaCl_2$ in the DNA solution, or resuspension of DNA in 0.1 M phosphate buffer (pH 7.4). A silver wire (0.25 mm diameter) was placed inside the micropipette in contact with the DNA solution. The micropipette holders were mounted on a three-axis, manual micromanipulator (Figure 2). Voltage pulses generated by a Grass SD9 Stimulator (Grass-Telefactor, West Warwick, RI) were delivered between the micropipette electrode and a silver wire ground electrode placed under the tadpole or brain slice. For delivery of negatively charged DNA, the micropipette was connected to the negative terminal (anode), and the ground electrode was connected to the positive terminal (cathode) of the voltage stimulator.

The current passing through the micropipette was determined by placing an oscilloscope in parallel with the electroporation circuit (Figure 2), with leads on either side of a known resistor (we used a 1 KΩ resistor). The current passing through the pipette was determined by measuring the voltage drop across this resistor and by applying Ohm's Law ($I = V/R$). The resistance of the micropipettes was 10–15 MΩ when filled with *Xenopus* whole-cell patch clamp internal solution containing 80 mM cesium methanesulfate, 10 mM EGTA, 20 mM tetraethylammonium, 5 mM $MgCl_2$, 2 mM adenosine triphosphate, 0.3 mM guanosine triphosphate, and 20 mM HEPES (pH 7.2) with CsOH. When filled with DNA solution (1 μg/μl in dH_2O), these micropipettes had resistances of 10–30 MΩ when inserted into the tadpole brain and 10–15 MΩ when inserted into the rat hippocampal slice.

For single-cell electroporation in the intact tadpole brain, stage 46–48 tadpoles were anesthetized with 0.01% 3-aminobenzoic acid ethyl ester (MS222, Sigma) in Steinberg's solution (pH 7.4). An anesthetized tadpole was placed on top of a moistened Kimwipe on the stage of an Olympus BX50 microscope equipped with fluorescence. The DNA-containing micropipette was inserted under visual guidance into the cell body layer of the optic tectum, using a 20× long working distance lens. For transfection of single cells in hippocampal slice cultures, a Millicell culture well containing slices was placed in a 35 mm petri dish containing culture medium on the stage of a dissecting microscope. The micropipette containing DNA was inserted into the stratum pyramidale of areas CA1 or CA3 within the hippocampal slice. Transfection efficiency was measured by expression of fluorescence following electroporation with the plasmid pEGFP (Clontech Laboratories, Palo Alto, CA), a plasmid carrying the gene for GFP driven by a CMV promoter. Tadpoles and brain slices were examined for GFP expression at least 12 hr after electroporation, using a Nikon Optiphot microscope with epifluorescence.

A range of stimulation parameters for single-cell electroporation, including voltage pulse amplitude, duration, and shape, were tested for transfection efficiency in the tadpole brain. For each condition, 10–15 animals were tested. We tested voltage pulse amplitudes from 10V–80V, single and multiple pulses with durations of 1–150 ms, and single and multiple trains of stimuli from 50–200 Hz. Two voltage pulse shapes were compared: square pulses and capacitance discharge pulses with a high initial peak followed by an exponential decay. Square pulses were generated with the Grass SD9 stimulator and were delivered as individual or multiple pulses or as trains of 1 ms square pulses up to 1 s long. To form exponential decay pulses, voltage pulses from the Grass stimulator were used to charge a capacitor (0.4–3 μF). Charging the capacitor was independent of the voltage pulse frequency or duration. Once charged to the applied voltage, the capacitor was connected to the electroporation circuit using a manual switch. The time constant (τ) of decay of the capacitance discharge was controlled by a variable resistor in parallel to the electroporation–capacitor circuit. For details, see http://www.cshl.org/labs/cline/sce.html.

Electroporation of Multiple Plasmids

In order to determine the efficiency of cotransfecting multiple independent genes, 50 neurons in the tadpole optic tectum were transfected using micropipettes containing a mixture of two plasmids (pEGFP and pDsRed; Clontech Laboratories; 0.5 μg/μl of each). Anesthetized tadpoles were examined for GFP and DsRed fluorescence by confocal microscopy 12–96 hr after electroporation.

Electroporation of Fluorescent Dextrans

Single tectal cells were electroporated with either fluorescein or tetramethylrhodamine dextrans (3000 MW, 2 mM in PB; Molecular Probes, Eugene, OR), using positive voltage pulses (the opposite polarity to that used for transfer of DNA). The following range of stimulation parameters were tested by observing dye transfer into cells: single or multiple square pulses with durations of 1–200 ms and voltages of 10V–70V; single or multiple pulses with exponential decays; and trains (20–200 Hz) of square pulses with voltages of 10V–70V, as used to electroporate DNA. Electroporation of dextrans requires shorter duration and lower amplitude stimuli than those required for DNA. Stimulation with one to three square pulses of

20–40 ms and amplitude of 10V–15V consistently labeled single cells.

The ability to directly visualize electroporation of fluorescent dextrans is of great use when initially setting up single-cell electroporation in a new system. The development of correct micropipette tips can require many trials. Since the same micropipettes can be used for electroporating fluorescent dextrans and DNA, it is easier and faster to optimize micropipette shapes and electrical pulses by testing with electroporation of fluorescent dextrans than DNA. Once these parameters are optimized for dextrans, they can then be easily modified for electroporation of DNA plasmids.

Confocal and Two-Photon Imaging

Cells labeled with fluorescent compounds were imaged with a Noran XL laser-scanning confocal attachment mounted on an upright Nikon Optiphot microscope, using a 40× Nikon oil immersion lens (1.30 NA). Tadpoles were anesthetized with 0.01% MS222 for imaging and recovered from anesthetic between imaging sessions. Hippocampal slice cultures were fixed and mounted on slides before imaging. Images were collected through the entire extent of the labeled neuron, at steps of 1 or 2 μm in the z dimension. For each optical section, 8–16 frames were averaged. GFP was excited at 488 nm, and emitted light between 500 and 550 nm was collected. DsRed was excited at 568 nm, and emitted light between 578 and 632 nm was collected. DiI was excited with 568 nm, and emitted light greater that 590 nm was collected. In some cases, cells were imaged with a custom-built two-photon laser-scanning microscope modified from an Olympus Fluoview confocal scan box mounted on an Olympus BX50WI microscope. A Tsunami femtosecond-pulsed Ti:sapphire laser (Spectra Physics, Mountain View, CA) pumped by a 10-W solid-state laser (Millenia X, Spectra Physics) was used as a light source. Images on the two-photon microscope were taken with a 40×, 0.8 NA water immersion objective, using the image acquisition software Fluoview (Olympus).

Neuronal Growth and Dendritic Branch Dynamics

Time-lapse confocal imaging was used to determine whether single-cell electroporation and subsequent protein expression interferes with the development of normal neuronal morphology and growth. Both short-term (2 hr) dendritic branch dynamics and total dendritic arbor growth over 24 hr were compared between neurons transfected with pEGFP by single-cell electroporation and single neurons labeled with DiI (Molecular Probes). Eleven optic tectal neurons were transfected with pEGFP (0.5 μg/μl), using the optimal stimulation parameters (200 Hz trains, 50V). Fifteen neurons in the optic tectum were labeled by iontophoresis of DiI dissolved in ethanol, using three to ten pulses of 200 ms positive-current pulses (1–10 nA). After electroporation (24 hr), cells were imaged five times at 2 hr intervals and then imaged the following day. In addition, some cells were subsequently imaged once a day for up to 6 days. Dendritic arbors were reconstructed by tracing the portion of the neuron in each optical section onto an acetate sheet until the entire neuron was drawn. Total dendritic branch length was measured from scanned drawings of cells, using the program NIH Image 1.61. Dendritic arbor growth rate was determined by subtracting the total dendritic branch length on the first day of imaging from the total dendritic branch length on the second day of imaging. To analyze the arbor dynamics, drawings of cells from sequential time points were superimposed to identify added and retracted branches.

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