Differentiation (2002) 70:148–154

Kurt Haas · Kendall Jensen · Wun Chey Sin · Lisa Foa
Hollis T. Cline

Targeted electroporation in *Xenopus* tadpoles *in vivo* – from single cells to the entire brain

Accepted in revised form: 31 January 2002

Abstract Electroporation is becoming more popular as a technique for transfecting neurons within intact tissues. One of the advantages of electroporation over other transfection techniques is the ability to precisely target an area for transfection. Here we highlight this advantage by describing methods to restrict transfection to either a single cell, clusters of cells, or to include large portions of the brain of the intact *Xenopus* tadpole. Electroporation is also an effective means of gene delivery in the retina. We have developed these techniques to examine the effects of regulated gene expression on various neuronal properties, including structural plasticity and synaptic transmission. Restriction of transfection to individual cells aids in imaging of neuronal morphology, while bulk cell transfection allows examination of the affects of gene expression on populations of cells by biochemical assays, imaging, and electrophysiological recording.

Key words electroporation · synaptic plasticity · transfection · functional genomics · neuronal imaging

Introduction

The rapid identification of novel genes from large-scale sequencing projects and expression assays has far outpaced research to decipher each gene's function. Elucidation of the role of genes in cellular processes requires techniques for spatial and temporal control of gene expression combined with assays of gene function. While transgenic approaches have been fruitful, creating transgenic organisms is time-consuming, expensive, and is limited to a few species. Acute transfection-mediated gene expression provides an alternative method for rapid gene delivery. In the field of neuroscience, gene delivery within intact tissues has most often been performed by infection with recombinant virus, lipofection, or gene gun biolistics. More recently, electroporation has proven to be a versatile method with superior properties and higher transfection efficiencies (Muramatsu et al., 1997; 1998; Itasaki et al., 1999; Inoue and Krumlauf, 2001). Electroporation is particularly favorable since it can be used to deliver a variety of macromolecules in addition to DNA and is able to co-transport multiple genes carried by independent plasmids into the same cells (Itasaki et al., 1999; Haas et al., 2001). Electroporation delivers DNA without leaving residual delivery agents which may confound experiments or cause cell damage.

The relatively limited application of electroporation to neurons *in vivo* has been due, in part, to challenges associated with targeting specific cell populations within intact animals. This problem was first addressed in the chick embryo by controlling the extent of tissue exposed to exogenous DNA and the applied electric field (Muramatsu et al., 1997). The DNA was compartmentalized by injecting a solution of DNA into the lumen of the neural tube. The area of tissue exposed to the electric field was controlled by the size of parallel electrodes placed on either side of the tube. Stimulation parameters, including the size and duration of the applied field, are important variables in controlling the number of cells transfected, while the polarity of pulses can be used to restrict transfection to one side of the adjacent neural tube (Briscoe et al., 2000; Manzanares et al., 2000). During stimulation, the negatively charged DNA is attracted to the positive electrode and is electrophoretically driven through the lumen wall into the neural tube tissue. Delivering pulses of alternating polarities transfects cells on both sides of the neural tube.

A further advantage of electroporation is its lack of

K. Haas (✉) · K. Jensen · W. C. Sin · L. Foa · H. T. Cline
1 Bungtown Rd, Beckman Building, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA
e-mail: haas@cshl.org
Tel: +1 516 367 6948, Fax: +1 516 367 6805

selectivity for species, cell type, or maturational stage. Bulk electroporation techniques have been used for widespread delivery in *Drosophila* (Kamdar et al., 1995), zebrafish (Buono et al., 1992), *Xenopus* (Eide et al., 2000; Foa et al., 2001), chick (Yasuda et al., 2000; Yasugi and Nakamura, 2000; Swartz et al., 2001), and mouse embryos (Akamatsu et al., 1999; Miyasaka et al., 1999). For example, bulk neuronal electroporation *in vivo* has been very effective in the study of pattern formation in the development of neural tissues by the misexpression of, or interference with, regulatory cues (Sharma et al., 1998; Araki et al., 1999; Briscoe et al., 2000; Koshiba-Takeuchi et al., 2000; Watanabe and Nakamura 2000; Inoue et al., 2001).

In our laboratory, we use electroporation to examine the function of genes in the morphological and electrophysiological development of neurons in the *Xenopus laevis* tadpole brain. By adapting techniques worked out in the chick embryo and by devising new delivery methods, we have been able to control electroporative transfection in the *Xenopus* brain to small or large brain regions, or to individual cells. In general, we employ two distinct transfection strategies: one to maximize the numbers of neurons transfected in a specific brain region or throughout the brain, and an opposing strategy designed to restrict transfection to a single neuron.

Whole-brain electroporation in the albino *Xenopus laevis* tadpole

Bulk transfection is often preferred for use in gene expression assays that require large numbers of cells, such as biochemical assays of cell homogenates, immunostaining, and gene chip assays. In addition, effects of gene expression may only be observable in a small fraction of transfected cells. For example, widespread expression in a heterogeneous cell population may be required to detect phenotypic changes in a small fraction of transfected neurons. Bulk transfection is also effective when examining the electrophysiological affects of altered gene expression since it provides a large population of potential cells to sample.

DNA purification is a major factor in successful electroporation and expression levels. We have found that the removal of endotoxins using EndoFree Plasmid Maxi Kit (Qiagen) increases transfection efficiency. We have found little effect of varying the composition of the solution in which the DNA is dissolved. DNA diluted in dH_2O, 20–200 mM NaCl or 2 mM $CaCl_2$ had no observable effect on transfection rate. Therefore, we typically dilute DNA in dH_2O. The concentration of DNA in the pipette solution in the range of 0.5 to 2 μg/μl yields high transfection rates. Transfection efficiency is tested by electroporating a vector containing the gene for green fluorescent protein (GFP) driven by a strong CMV promoter (pEGFP, Clontech Laboratories, Palo Alto, CA). Stage 44–48 albino *Xenopus laevis* tadpoles are anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222, Sigma) in Steinberg's solution (pH 7.4) and placed on a moistened Kimwipe on the stage of a dissecting microscope. A micropipette containing a solution of plasmid DNA is inserted into the brain ventricle, and approximately 75–125 nl of DNA solution is pressure injected using a picospritzer (Picospritzer II, General Valve Corporation). Fast Green (0.01%) is added to the DNA solution to monitor the DNA injection into the ventricles. The micropipette is then removed, and a custom-made electrode composed of two parallel flat platinum plates, is placed vertically on the skin with the two platinum electrodes on either side of the brain (Fig. 1). The electrodes used for bulk cell transfection were constructed by cutting two 1 mm squares of platinum foil and soldering each square to a separate wire. The soldered junction was insulated with nail polish, and the platinum plates are fixed to a metal post such that the platinum plates are parallel to each other and separated by 1 mm. We have tested a range of stimulation parameters including square pulses of durations ranging from 1 to 50 ms and exponential decay pulses with time constants ranging from 20 to 70 ms, at various voltages. Voltage pulses are generated by a Grass SD9 stimulator coupled to a capacitor. A simple circuit was constructed in which the voltage stimulator charges the capacitor which is then allowed to discharge across the platinum electrodes (also see www.cshl.org/labs/cline/sce.html). In the tadpole, we find that delivery of 5 exponential pulses with a time constant of 70 ms, a field strength between 200–400 V/cm, and a 1 sec interpulse interval produces the highest transfection efficiencies with the least cell damage or tadpole death. In the chick embryo, square voltage pulses have been found to be most effective for high transfection efficiency and cell survival. The difference in sensitivity to pulse shape in the tadpole may be due to the tissue or differences in the types of electrodes employed. After electroporation, the tadpole is quickly returned to rearing solution, where it usually recovers from anesthesia within 10 minutes. Transfection success is tested by detecting GFP expression with epifluorescent microscopy 24 h after electroporation. Whole brain electroporation in the *Xenopus laevis* tadpole consistently produces high transfection rates as indicated by GFP expression (Fig. 1). GFP expression can be detected within 4 hours after electroporation, reaches peak levels between 24 and 48 h, and persists for more than 2 weeks.

The area of the brain transfected by bulk electroporation can be controlled by adjusting the size of the electrodes, the polarity of stimuli, and the site of DNA injection. Relatively large electrodes (1 mm long) which span the length of the tadpole brain can be used to transfect cells throughout the extent of the brain (Fig. 1). Smaller electrodes (0.5 mm long) restrict transfection to specific brain regions, such as the optic tectum. Pulses of a single polarity transfect cells only on the side of the

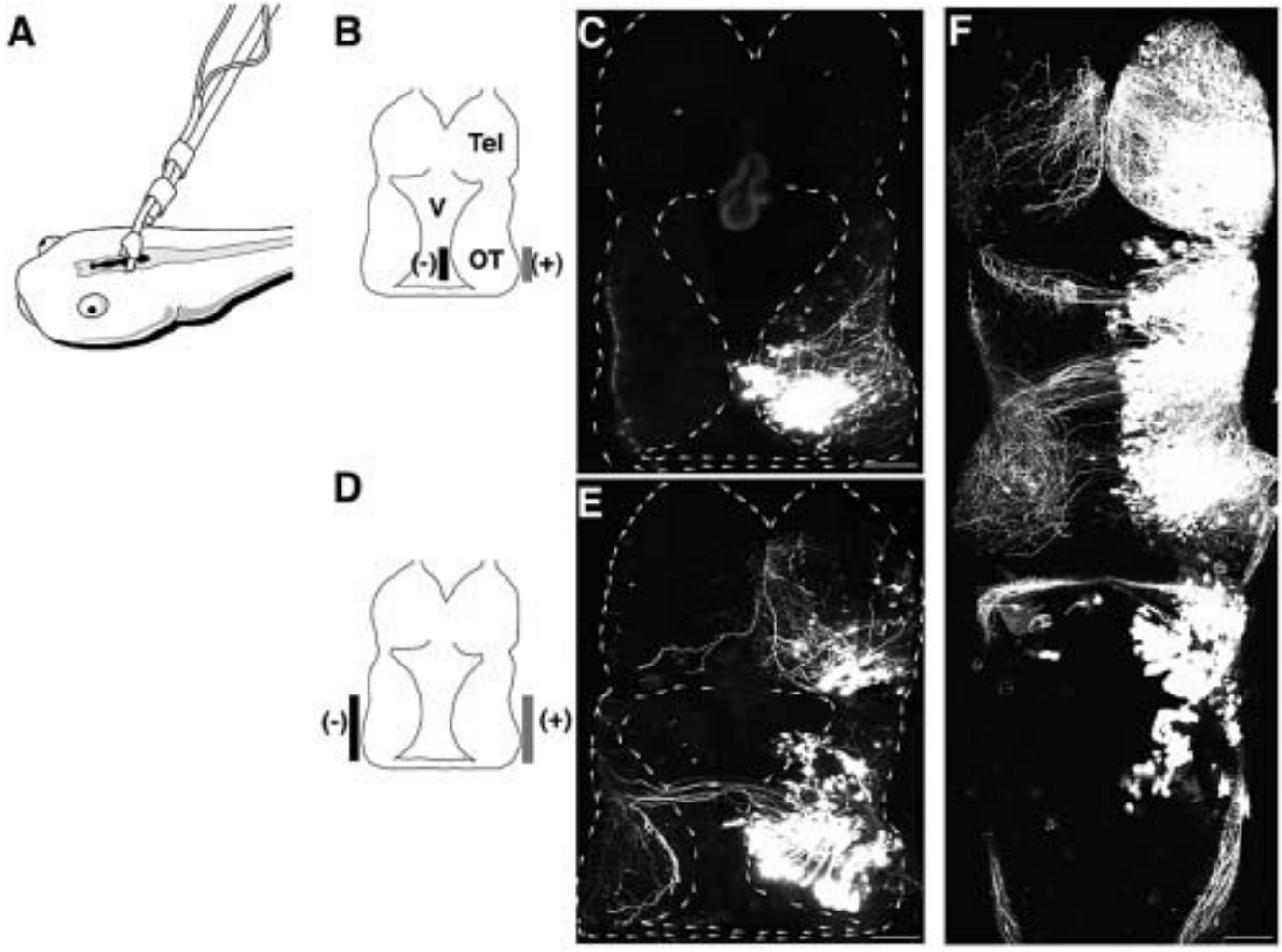

Fig. 1 Whole brain electroporation in the *Xenopus laevis* tadpole. **A**. Schematic of the custom-made electrode used for bulk electroporation of the tadpole brain. Platinum foil plate electrodes are gently placed on the tadpole skin on either side of the brain. **B**. Diagram of a horizontal view of the stage 47 tadpole brain (*rostral at top*) showing placement of small electrodes (*vertical bars*, 0.5 mm long and 0.5 mm apart) for targeting transfection to the optic tectum (*OT*). Plasmid DNA is injected into the brain ventricle (*V*). **C**. GFP expression in the right optic tectum following targeted electroporation. **D**. Larger electrodes (1 mm long and 1 mm apart) on either side of the brain are used to electroporate larger regions of the brain. **E**. Voltage pulses deliver DNA into cells between the ventricle and the positive electrode. More cells are transfected with the larger electrodes. **F**. Increased electrical field strength induces transfection in larger brain regions. Monopolar stimuli almost completely restrict transfection to half the brain. *Bar*=100 μm.

brain adjacent to the positive electrode. This indicates that the epithelial lining of the ventricle effectively prevents diffusion of DNA from the ventricle into the brain tissue, but electrophoresis can drive DNA through this barrier and deep within the tissue. Stimulation with pulses of both polarities transfects cells on both sides of the ventricle. An alternative method to restrict the extent of transfection is to inject a small bolus of DNA directly into the neuronal tissue. The strength of the applied electric field can also be used to control the number of transfected cells. However, there is a relatively small range of effective voltages which must be determined empirically for each electrode. Low voltages will induce transfection of few, or no cells, and expression levels per cell are usually low, as indicated by dim GFP fluorescence. Slightly higher voltages yield extensive transfection and higher expression levels with little or no cell death or tissue pathology. Further increases in the applied voltage, however, produce damage and cell death.

To test for the efficiency of co-electroporation of two independent plasmids, we mix pGFP with pDsRed (Clontech) in a 1:1 ratio. pDsRed is a vector carrying the gene for the red fluorescent protein DsRed driven by the same CMV promoter in the pEGFP vector. Co-electroporation of pEGFP and pDsRed produced a co-transfection rate of approximately 70%±10%, determined by screening for simultaneous expression of GFP and DsRed within 2 days after electroporation. In most cases of single plasmid expression, GFP was expressed. The slower expression rate and lower intensity of DsRed fluorescence may contribute to an apparent expression of GFP in the absence of DsRed.

Transfection techniques for retinal ganglion cells (RGCs) in our laboratory previously consisted of either lipofection or viral infection using vaccinia virus. Lipofection must be done at very early developmental stages, limiting temporal expression of the protein. In addition, lipofected animals require several days to reach an experimental stage. Viral infection rates of RGCs are frequently variable and difficult to assess *in vivo*, due to low expression levels, requiring conformation of infection rate post-experimentally. Electroporated RGCs reach peak expression of GFP along the full length of the axon and axonal arbor at 48 hours. RGCs are electroporated using the same electrodes that are used for whole brain electroporation and the same stimulation parameters.

Little damage is produced by whole-brain electroporation

The voltage amplitudes needed to induce high transfection efficiency produce little tissue damage. Slightly higher voltage amplitudes can produce local disruption of skin tissue directly in contact with the electrodes, as well as disruption of internal blood vessels. We tested for cell death following whole brain electroporation with pEGFP by staining the brain with propidium iodide, a nuclear dye that only crosses compromised cell membranes. Propidium iodide (0.5 μg/ml) was injected into the brain ventricle of anesthetized tadpoles 30 min. after whole brain electroporation using the same stimulation protocols which yield high transfection efficiency. Tadpoles were sacrificed 30 min later; brains were removed and fixed with 4% paraformaldehyde. Control tadpoles received ventricular injections of pEGFP but were not stimulated. Whole-mounted brains were examined with epifluorescent and confocal microscopy for propidium iodide positive cells. The stimulation parameters producing the highest transfection efficiencies did not alter the number of propidium iodide-positive cells compared to controls. Brains from both groups had few propidium iodide positive cells. Therefore, whole brain electroporation, under conditions that yield optimal transfection, does not induce cell death in brains within 30 min after stimulation.

Single-cell electroporation in *Xenopus laevis*

We developed single-cell electroporation (SCE) as a method to introduce plasmids and macromolecules into individual neurons, glia, and muscle fibers. SCE is particularly useful in experimental systems that allow direct imaging of cell morphology in living tissue. One of the strengths of SCE, when imaging neuronal structure, is that it allows an individual neuron's axonal and dendritic processes to be distinguished without interference from neighboring processes. SCE also allows one to determine whether the effects of gene expression on the transfected cell are cell autonomous or due to secondary effects from interaction with transfected neighbors.

Restriction of electroporation to single neurons within the intact tadpole brain is achieved by restricting both the DNA and the electric field to the tip of a glass micropipette (Fig. 2). Borosilicate glass capillary tubing (outer diameter = 1.5 mm, inside diameter = 0.86 mm, filament, Warner Instrument Corp.) is pulled to a fine tip (tip diameter = 0.6 μm) with a pipette puller (P-87 Micropipette Puller, Sutter Instrument Company, CA) and filled with a solution of plasmid DNA (typically 1 μg/μl in dH_2O, range 0.5–2 μg/μl). A silver wire (0.25 mm diameter) is inserted into the pipette in contact with the DNA solution. An anesthetized tadpole is placed on a Kimwipe moistened with Steinberg's solution on the stage of an upright Olympus BX50 microscope equipped with a 20× long working-distance objective. A silver wire ground electrode is placed under the Kimwipe. The distance between this ground electrode and the tadpole is not significant as long as it is in contact with the wet Kimwipe. The silver wire in the pipette is connected to the negative terminal of a Grass SD9 Stimulator (Grass-Telefactor, West Warwick, RI), and the ground electrode is connected to the positive terminal (also see www.cshl.org/labs/cline/sce.html). Under visual guidance, the tip of the DNA-filled micropipette is inserted into the brain of the anesthetized tadpole into a region containing dense cell bodies. Since tadpole tectal cells are difficult to discern using the 20× objective, we do not directly monitor the position of the pipette tip relative to a target cell. Although this may lower transfection efficiency at each site due to tip placements which are not directly adjacent to cell somata, the long working distances allows rapid pipette placement at multiple sites in the tissue. Therefore, the relative ease of quickly electroporating multiple sites yields high transfection rates per brain or slice. The use of higher strength objectives allows for more precise positioning of the pipette near a target cell body for increased transfection efficiency, but the associated shorter working distances reduces maneuverability between sites. Once the pipette is in position, trains of square pulses (1 ms long, 200 Hz) are delivered for 1 s. Other stimulation parameters, including repeated square and exponential decay pulses also induce single-cell electroporation, but are not as efficient as high frequency trains of short pulses. While transfection efficiency can vary greatly between pipette tips and is dependent on multiple parameters, including DNA preparation and target cell density, optimized parameters yield transfection of single cells in 20% of stimulation attempts.

An important variable for successful transfection is the applied current, which can be monitored by sampling the voltage drop across a known resistor in series with the stimulation circuit. For example, a resistor can be placed between the ground electrode and the positive

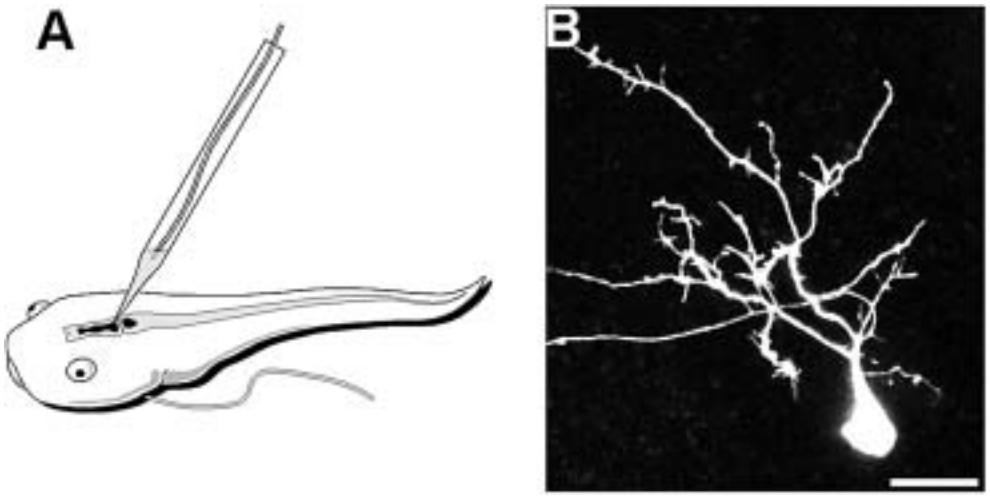

Fig. 2 Single-cell electroporation in the tadpole. **A**. Single-cell targeting is achieved by inserting a DNA-filled micropipette into the brain of an anesthetized tadpole. Electrical stimuli are delivered between the pipette and an external ground (under tadpole) which electroporates the cell at the tip of the pipette and delivers DNA into the cell. **B**. A neuron in the optic tectum of the tadpole brain 48 h after single-cell electroporation of pEGFP, imaged *in vivo* with 2-Photon microscopy. *Bar*=20 μm.

pole of the stimulator. Connecting an oscilloscope on either side of this resistor provides a measure of the voltage drop across the resistor, from which the current can be determined using Ohm's Law (V = IR). The most effective current for single-cell transfection is 1 to 4 μA. It is important to monitor the current while stimulating since cellular debris or particles in the pipette solution can clog the pipette tip causing an increase in the pipette resistance and a decrease in the applied current. By watching the circuit current, changes in pipette resistance can be monitored and compensated for by adjusting the applied voltage. In some cases, clogging can be alleviated by applying brief current pulses of alternating polarity. In the tadpole, the same pipette can typically be reused for multiple sites (up to 40 times). Other tissues, including organotypic hippocampal slices may produce more rapid clogging.

Rates of co-transfection of pGFP and pDsRed is higher with single-cell electroporation (96%) than whole brain electroporation. Efficiency of single-cell electroporation was not noticeably affected by the ionic composition of the resuspension solution (2 mM $CaCl_2$, 20–200 mM NaCl, or dH_2O) or DNA concentrations ranging from 0.1 to 5 μg/μl.

We have tested the health of neurons in the tadpole brain following single-cell electroporation by examining dendritic growth over six days (Haas et al., 2001). These cells grew by the dynamic extension and retraction of small branches resulting in large complex arbors (Wu et al., 1999). Dendritic arbor growth of electroporated cells was indistinguishable from cells labeled with DiI. Indicators of ill health, such as blebbing or a net retraction of dendritic branches is not seen. These data suggest that single-cell electroporation has no significant effects on cell health.

Single-cell electroporation for *in vivo* neuronal imaging

The combination of targeted gene transfection with time-lapse *in vivo* microscopy provides a powerful means to examine the role of genes in the development of neuronal morphology and connectivity. Co-electroporating plasmids carrying genes of interest along with plasmids encoding GFP facilitates identification of transfected cells and allows imaging of the effects of gene expression on neuronal morphology. Highly efficient co-electroporation of independent plasmids to the same cell decreases the need to make GFP-fusion proteins which may alter protein function or restrict GFP localization to specific cell compartments. In cases where the cellular distribution of an exogenously expressed protein is being examined, a plasmid carrying the GFP-fusion gene can be co-electroporated with a plasmid expressing another fluorescent protein, such as DsRed. In this case, expression of the GFP-fusion protein will demarcate sites of protein localization, while DsRed completely fills the cell for imaging morphology. Since the electroporation technique is relatively simple and transfected genes can be expressed within hours, this technique offers a rapid method for screening gene function. Two-Photon imaging of GFP expressed in individual neurons produces high-resolution images of neuronal morphology without inducing significant phototoxic damage (Haas, et al., 2001). This allows repeated imaging of neurons at short intervals for the study of axonal and dendritic arbor growth and dynamics. With the ability to deliver multiple genes into the same cell, it is possible to express one or more genes of interest along with pEGFP to examine gene function in neuronal growth and structural plasticity.

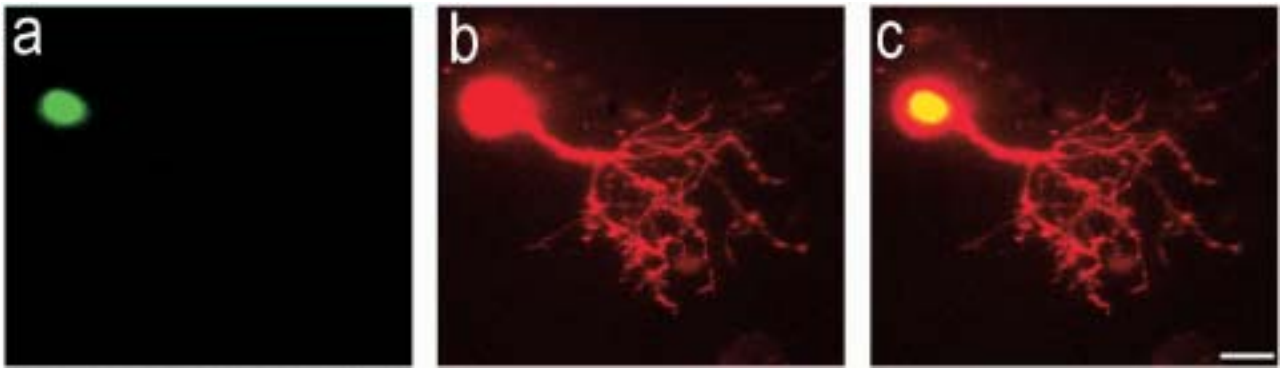

Fig. 3 Single-cell electroporation mediated delivery of tetramethylrhodamine-tagged dextran (*red*, **b**) and carboxyfluorescein-conjugated morpholino oligonucleotides (*green*, **a**) into a neuron within the tadpole brain *in vivo*. Overlay of images in *yellow* (**c**). *Bar*=10 μm.

Single-cell electroporation of fluorescent dextrans

Single-cell electroporation can also be used to label cells with fluorescently-tagged dextrans (Molecular Probes, Eugene, OR). Delivery of dyes, like tetramethylrhodamine dextran, FITC dextran, or calcium indicators such as calcium green dextran, can be directly visualized with epifluorescent microscopy. Pipettes are filled with dextrans at concentrations ranging from 100 μM to 2 mM dissolved in dH_2O. Since dextrans are relatively small compared to plasmid DNA, the electrical stimuli required for electroporation of dextrans is much less than for DNA. We typically use single square pulses, 10–40 ms in duration, at 1 μA to fill single cells. Direct visualization demonstrates immediate dye-filling of cell somata during stimulation, followed by complete diffusion through dendritic processes within 30 min.

Single-cell electroporation of morpholino oligonucleotides

We have also used the single-cell electroporation technique to deliver morpholino oligonucleotides tagged with the fluorescent marker carboxyfluorescein (200 μM, Gene Tools, Corvallis, OR) into single cells within the intact *Xenopus* tadpole brain (Fig. 3). Morpholino oligonucleotides are synthetic antisense sequences, which are insensitive to nuclease digestion. They have been used to knock down gene expression (Summerton and Weller 1997; Nasevicus and Ekker, 2000). To demonstrate the feasibility of delivering morpholino oligonucleotides by electroporation, we used a random sequence composed of 25 nucleotides (5′-CCTCTTACCTCAGTTACAATTTATA) which is not complimentary to any known mRNA. We have co-electroporated the carboxyfluorescein-conjugated morpholino oligonucleotides along with tetramethylrhodamine dextran (3000 MW, 2 mM) for cell imaging. Following co-electroporation, the fluorescent dextran completely fills cell processes while the morpholino oligonucleotides can be detected in the cell soma and proximal dendritic shaft. While we demonstrate the utility of single-cell electroporation as a tool for oligonucleotide delivery, assays for reduction of protein levels need to be performed in each case to demonstrate that these anti-sense oligonucleotides interfere with mRNA translation.

We have successfully applied single-cell electroporation to other preparations including rat organotypic hippocampal slice, chick embryo, zebrafish, and *Drosophila* embryos. While the same stimulation parameters are effective in each case, the pipette tip dimensions often require adjustments specific to each preparation. The dimensions of the pipette shank must balance requirements for preventing the pipette from breaking when entering tissue like the tadpole skin (wider shank) and reducing tissue damage from the pipette insertion (thinner shank). When initially testing single-cell electroporation in a new preparation, it is useful to first electroporate fluorescently-tagged dextrans. Direct visualization of dye transfer into cells aids in finding correct tip dimensions and tip position relative to cell bodies. Pipettes which are successful for electroporation of dextrans can then be used for transfection of DNA.

Conclusion

The main challenges faced by the study of neuronal gene function are the ability to regulate the spatial and temporal expression of specific genes using techniques which are rapid and easy to perform. Electroporation has proven to be a highly efficient method for neuronal transfection which can be used for rapid assays of gene function. Recent advances in methods to control DNA and electric field exposure provide a powerful means to

control the pattern of foreign gene expression (Atkins et al., 2000; Haas et al., 2001). Further control over spatial expression may be achieved by electroporating genes driven by cell type-specific promoters (Itasaki et al., 1999). Temporal control of exogenous gene expression can be achieved by the type of genetic material electroporated. DNA will be expressed within a few hours, while RNA can be translated within minutes. Electroporated proteins (Rols, 1998) can act immediately. Alternatively, inducible promoter systems can enhance temporal control. The ability to block or decrease the activity of gene products is also essential in order to understand gene function. Reducing gene function is possible through a number of electroporation techniques. Dominant negative forms of proteins can be expressed (Itasaki, 1999), or electroporation can be used to deliver antisense agents like morpholino oligonucleotides, or peptide and drug inhibitors. Electroporation provides researchers with a very versatile set of tools for the study of gene function.

Acknowledgements The work from our laboratory described in this review was supported by grants from the National Eye Institute (KH and HTC), and the Hoffritz Fund (HTC). We also thank Jim Duffy for graphic arts assistance with figure production.

References

Akamatsu, W., Okano, H.J., Osumi, N., Inoue, T., Nakamura, S., Sakakibara, S., Miura, M., Matsuo, N., Darnell, R.B. and Okano, H. (1999) Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems. Proc Natl Acad Sci USA 96:9885–9890.

Araki, I. and Nakamura, H. (1999) Engrailed defines the position of dorsal di-mesencephalic boundary by repressing diencephalic fate. Development 126:5127–5135.

Atkins, R.L., Wang, D. and Burke, R.D. (2000) Localized electroporation: a method for targeting expression of genes in avian embryos. Biotechniques 28:94–6, 98, 100.

Briscoe, J., Pierani, A., Jessell, T.M. and Ericson, J. (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101:435–445.

Buono, R.J. and Linser, P.J. (1992) Transient expression of RSVCAT in transgenic zebrafish made by electroporation. Mol Mar Biol Biotechnol 1:271–275.

Eide, F.F., Eisenberg, S.R. and Sanders, T.A. (2000) Electroporation-mediated gene transfer in free-swimming embryonic *Xenopus laevis*. FEBS Lett 486:29–32.

Foa, L., Rajan, I., Haas, K., Wu, G.Y., Brakeman, P., Worley, P. and Cline, H. (2001) The scaffold protein, Homer1b/c, regulates axon pathfinding in the central nervous system in vivo. Nat Neurosci 4:499–506.

Haas, K., Sin, W.C., Javaherian, A., Li, Z. and Cline, H.T. (2001) Single-cell electroporation for gene transfer in vivo. Neuron 29:583–591.

Inoue, T. and Krumlauf, R. (2001) An impulse to the brain-using in vivo electroporation. Nat Neurosci 4(Supp 1):1156–1158.

Inoue, T., Tanaka, T., Takeichi, M., Chisaka, O., Nakamura, S. and Noriko, O. (2001) Role of cadherins in maintaining the compartment boundary between the cortex and striatum during development. Development 128:561–569.

Itasaki, N., Bel-Vialar, S. and Krumlauf, R. (1999) 'Shocking' developments in chick embryology: electroporation and in ovo gene expression. Nat Cell Biol 1:E203–207.

Kamdar, K.P., Wagner, T.N. and Finnerty, V. (1995) Electroporation of *Drosophila* embryos. Methods Mol Biol 48:239–243.

Koshiba-Takeuchi, K., Takeuchi, J.K., Matsumoto, K., Momose, T., Uno, K., Hoepker, V., Ogura, K., Takahashi, N., Nakamura, H., Yasuda, K. and Ogura, T. (2000) Tbx5 and the retinotectum projection. Science 287:134–137.

Manzanares, M., Wada, H., Itasaki, N., Trainot, P.A., Krumlauf, R. and Holland, P.W. (2000) Conservation and elaboration of Hox gene regulation during evolution of the vertebrate head. Nature 408:854–857.

Miyasaka, N., Arimatsu, Y. and Takiguchihayashi, K. (1999) Foreigngene expression in an organotypic culture of cortical anlage after in vivo electroporation. Neuroreport 10:2319–2323.

Muramatsu, T., Mizutani, Y., Ohmori, Y. and Okumura, J. (1997) Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. Biochem Biophys Res Commun 230:376–380.

Muramatsu, T., Nakamura, A. and Park, H.M. (1998) In vivo electroporation: a powerful and convenient means of nonviral gene transfer to tissues of living animals (Review). Int J Mol Med 1:55–62.

Nasevicius, A. and Ekker, S.C. (2000) Effective targeted gene 'knockdown' in zebrafish. In Process Citation. Nat Genet 26:216–220.

Rols, M.P., Delteil, C., Golzio, M., Dumond, P., Cros, S. and Teissie, J. (1998) In vivo electrically mediated protein and gene transfer in murine melanoma. Nat Biotechnol 16:168–171.

Sharma, K., Sheng, H.Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H. and Pfaff, S.L. (1998) LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. Cell 95:817–828.

Summerton, J. and Weller, D. (1997) Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev 7:187–195.

Swartz, M., Eberhart, J., Mastick, G.S. and Krull, C.E. (2001) Sparking new frontiers: using in vivo electroporation for genetic manipulations. Dev Biol 233:13–21.

Watanabe, Y. and Nakamura, H. (2000) Control of chick tectum territory along dorsoventral axis by Sonic hedgehog. Development 127:1131–1140.

Wu, G.Y., Zou, D.J., Rajan, I. and Cline, H. (1999) Dendritic dynamics in vivo change during neuronal maturation. J Neurosci 19:4472–4483.

Yasuda, K., Momose, T. and Takahashi, Y. (2000) Applications of microelectroporation for studies of chick embryogenesis. Dev Growth Differ 42:203–206.

Yasugi, S. and Nakamura, H. (2000) Gene transfer into chicken embryos as an effective system of analysis in developmental biology. Dev Growth Differ 42:195–197.