A versatile microporation technique for the transfection of cultured CNS neurons

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Abstract

The application of molecular techniques to cultured central nervous system (CNS) neurons has been limited by a lack of simple and efficient methods to introduce macromolecules into their cytosol. We have developed an electroporation technique that efficiently transfers RNA, DNA and other large membrane-impermeant molecules into adherent hippocampal neurons. Microporation allowed the use of either in vitro transcribed RNA or cDNA to transfect neurons. While RNA transfection yielded a higher percentage of transfected neurons and produced quantitative co-expression of two proteins, DNA transfection yielded higher levels of protein expression. Dextran-based calcium indicators also were efficiently delivered into the cytosol. Microporated neurons appear to survive poration quite well, as indicated by their morphological integrity, electrical excitability, ability to produce action potential-evoked calcium signals, and intact synaptic transmission. Furthermore, green fluorescent protein (GFP)-tagged marker proteins were expressed and correctly localized to the cytosol, plasma membrane, or endoplasmic reticulum. The microporation method is efficient, convenient, and inexpensive: macromolecules can be introduced into most adherent neurons in a 3 mm² surface area while requiring as little as 1 μl of the material to be introduced. We conclude that the microporation of macromolecules is a versatile approach to investigate signaling, secretion, and other processes in CNS neurons.

Keywords: Electroporation; Hippocampal neurons; Quantitative co-transfection; RNA; YFP; CFP; GFP

1. Introduction

The study of signaling processes in intact cells requires the combination of molecular biological and physiological methods. Such processes have been difficult to study in neurons, in part because neurons are not readily amenable to methods such as transfection. Although several techniques have been developed for transfecting neurons, these techniques are time consuming, more difficult, and less effective in comparison to the many means available for transfection of cell lines. For instance, the construction of viruses such as vaccinia virus (Pettit et al., 1995) or adenovirus (Moriyoshi et al., 1996) is laborious and expensive. Further, these viruses often have long-term toxic actions. Microinjection (Kaang et al., 1993) or particle-mediated methods (Lo et al., 1994) produce very low yields when used in cultured central nervous system (CNS) neurons. Lipofection (Holt et al., 1990) or calcium-phosphate precipitation (Xia et al., 1996), though in many laboratories adequate for DNA transfection, cannot be used to supply RNA or other reagents.

We have attempted to circumvent these problems by developing a method based on electroporation, the transient permeabilization of the plasma membrane during application of an electric field. Electroporation is a well established technique for transfecting suspended cells such as bacteria, yeast, and cell lines. Recent application of this method to adherent, non-neuronal cells (Teruel and Meyer, 1997) suggests the possibility that electroporation might be used also on cultured neurons. Further, electroporation creates tran-
sient pores that establish direct paths from the extracellular space to the cytosol, which provides a means to deliver reagents other than DNA. In this respect, electroporation offers a broader range of applications than transfection techniques such as lipofection or calcium-phosphate precipitation, which are restricted to DNA. For example, electroporation has proven capable of introducing RNA, fluorescent dyes, peptides and proteins into cell lines (Subramanian and Meyer, 1997; Oancea et al., 1998; Shen and Meyer, 1998).

Here we describe a microvolume electroporation device (termed a ‘microporator’) derived from the apparatus described by Teruel and Meyer (1997) and show that this apparatus is effective in introducing RNA, DNA and dextran molecules into hippocampal neurons in primary culture. Furthermore, we show that by using the microporation technique with RNA, proteins can be quantitatively co-expressed in neurons. Our studies suggest that the microporation of reagents into cultured neurons is a powerful approach to study signal transduction, secretion, and other cellular processes in neurons.

2. Materials and methods

2.1. Preparation of GFP-fusion constructs

To evaluate the efficacy of transfection, we examined expression of the green fluorescent protein (GFP) or its derivatives, cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). The cDNAs for these GFP variants was kindly provided by Roger Tsien (University of California, San Diego). The cloning and in vitro transcription of elastase–GFP (ER–GFP) is described in Subramanian and Meyer (1997). The GFP used in these studies was an enhanced GFP (EGFP) with mammalian codon usage (Haas et al., 1996), green-shifted fluorescence (Ser65Thr; Heim and Tsien, 1996), and improved folding (Phe64Leu; Cormack et al., 1996). The EGFP coding sequence was amplified in a polymerase chain reaction (PCR) and then cloned into a custom-modified in vitro translation vector, pSHiro3, which was optimized for in vitro transcription in mammalian cells (Yokoe and Meyer, 1996). This vector contained 5’ and 3’ β-globin untranslated regions and a Kozak sequence. PM–GFP were made by inserting a ten amino acid sequence at the N-terminal end with a Kozak sequence. PM–GFP were made by inserting a ten amino acid sequence from the same family member, Fyn, was shown to be sufficient for plasma membrane localization through myristoylation and palmitoylation (Resh, 1994). DNA sequencing was performed for all constructs to exclude PCR error.

In vitro transcription and RNA processing of GFP and PM–GFP were performed according to the procedure described by Yokoe and Meyer (1996). Briefly, the GFP fusion constructs cloned in pSHiro3 were linearized with EcoRI, whose restriction site is downstream of the 3’ untranslated region, and in vitro transcription was performed with SP6 RNA polymerase using a mMESSAGE mMACHINE commercial kit (Ambion, Austin, TX) according to the manufacturer’s protocol. The reaction was terminated by addition of 10 mM EDTA, and the RNA was purified by an RNeasy column (Qiagen, Chatsworth, CA). Polyadenylation (addition of a poly A tail) was carried out at 37°C for 30 min in a 50-μl reaction mixture containing 40 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 2.5 mM MnCl₂, 250 mM NaCl, 0.25 mg/ml RNA, 250 μM ATP, and 5 units of poly (A) polymerase (Life Technologies, Gaithersburg, MD). EDTA 20 mM was used to terminate this reaction, and the polyadenylated mRNA was purified using an RNeasy column. The eluent (purified mRNA) was dried and dissolved at 1 μg/μl in electroporation buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 10 mM glucose, and 20 mM HEPES, pH 7.4).

For DNA transfection experiments, the cDNA was dissolved at 1–2 μg/μl in the same electroporation buffer used for the RNA electroporation described above.

2.2. Cell culture and preparation

Hippocampal neurons were cultured using modifications of the procedures described in Bekkers and Stevens (1991) and Ryan and Smith (1995). Region CA1 was dissected from the hippocampus of 2–4-day-old Sprague-Dawley rats and dissociated with papain or trypsin. Cells were plated onto coverslips coated with either Matrigel (Collaborative Biomedical Products, Bedford, MA) or 500 kDa MW poly-D-lysine (Sigma). To minimize the number of cells and amount of medium required, cells were plated in microwells formed by using a silicone sealant (Dow-Corning) to seal cloning cylinders (8 mm i.d., Belco Glass, Vineland, NJ) to the coverslip. Cells were cultured for 6–15 days before use. Culture medium consisted of minimal essential media (Gibco), 5% fetal bovine serum (FBS; Gibco), B-27 (Gibco) and Mito + serum extender (Collaborative Biomedical) at their suggested concentrations, 3.6 g/l glucose, Pen/Strep (Gibco, 5000/5000 U), and 500 μM MEM sodium pyruvate (Gibco). The cells were typically plated at densities of 20000–40000 per cloning cylinder. After 24 h in culture, the plating media was replaced with medium which also contained the antimitotic combination of 80 μM 5-fluoro-2-deoxyuridine (Sigma) and 200 μM uridine (Sigma). The cells were incubated at 37°C in a
5% CO₂ humidified chamber and were fed by exchanging 50% of the medium twice per week.

To make autapses (Bekkers and Stevens, 1991; Tong and Jahr, 1994), coverslips were coated with 0.15% agarose (Sigma), allowed to dry for 1 h, then sprayed with a fine mist of poly-D-lysine (0.5 mg/ml) and collagen (0.5 mg/ml). Cells were prepared as above and plated at 2000–5000 cm⁻². The culture medium was exchanged 24 h after plating, but antimitic agents were not added and the cells were not fed thereafter. Only neurons that were completely isolated from other neurons (typically on glial islands 200–400 μm in diameter) were used for electrical recordings.

To perform the Trypan Blue viable/non-viable cell assay, a 1:1 mixture of extracellular buffer and Trypan Blue (Sigma) was applied for 5 min to neurons that had been microporated 8 h earlier. Only the neurons that looked morphologically intact and that excluded the Trypan Blue were counted as viable.

2.3. Optical measurements and microscopy

Confocal fluorescence and differential interference contrast imaging were performed with a self-built microscope that used software and hardware modified from a design developed by Stephen Smith (Stanford University). Four hundred and forty two nanometers (HeCd laser) and 514 nm (argon-ion laser) excitation and appropriate emission filters were used for the imaging of CFP and YFP, respectively. Ca²⁺ responses evoked by electrical field stimulation were measured on an inverted Nikon Diaphot microscope coupled to an Odyssey confocal imaging system (Noran Instruments, Middleton, WI). During imaging, the culturing media was replaced with an extracellular buffer consisting of 135 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1.5 mM MgCl₂, 20 mM glucose, and 20 mM HEPES (buffered to pH 7.4). All experiments were performed at room temperature (~ 25°C).

[Ca²⁺] was monitored using the calcium indicator Calcium Green-1 dextran (70 000 MW, Molecular Probes, Eugene, OR). The following equation was used to convert the relative peak amplitude of fluorescence to an apparent intracellular Ca²⁺ concentration: [Ca²⁺] = K_d(F(t) - F_min)/(F_max - F(t)), where F(t) is the measured background-corrected intensity of Calcium Green-1 dextran fluorescence, K_d = 240 nM and F_max was measured as the fluorescence intensity after addition of ionomycin (~1 μM) in the presence of extracellular [Ca²⁺] of 1.5 mM. F_min was measured on a spectrofluorometer (Fluoromax, SPEX Industries, Edison, NJ) to be 35% of F_max.

2.4. Whole-cell patch-clamp recording

For patch clamp recording, cells were bathed in a solution containing 150 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 20 mM glucose, and 10 mM HEPES. The patch pipette solution was filled with an intracellular solution containing 100 mM gluconic acid, 5 mM MgCl₂, 5 mM NaATP, 0.3 mM NaGTP, 5 mM EGTA, and 40 mM HEPES, with the pH adjusted to 7.3 with CsOH. Currents were recorded using an EPC-9 amplifier. Series resistance was monitored and compensated ≥80%, and ranged from 5 to 20 MΩ. A second patch pipette filled with the extracellular solution (~2 MΩ) was used for extracellular stimulation of nearby neurons.

2.5. Field stimulation

Calcium transients were generated using the field stimulation electrodes described in Jacobs and Meyer (1997). This device consisted of two parallel platinum wires (3 mm apart) that were lowered until they nearly contacted the coverslip covered with neurons. Computer-controlled current pulses yielding fields of ~10 V/cm were applied in bursts, 1 s in duration and consisting of 30 1-ms pulses (i.e. 30 Hz).

3. Results

3.1. The microporator

Fig. 1A shows a schematic view of the small-volume electroporation device (microporator) that was used in our experiments. This device was modified from the design of Teruel and Meyer (1997). The foot of the microporator is placed onto a coverslip of neurons, creating a restricted space above the neurons. Reagents to be introduced into porated neurons are injected into the small space through a hole in the center of the foot structure (Fig. 1B). The volume of this space is kept small to restrict flow of electrical current and also to reduce the amount of reagent that is needed for transfection. In this version of the microporator, the distance under the foot structure was increased from 100 μm to 300 μm to reduce shear forces on the neurons during the application of the reagent and to increase the space available for the cells.

The electroporation protocols described in Teruel and Meyer (1997) were also modified for application to neurons. Before electroporation, the medium in the cloning cylinder was carefully removed and stored, and the cells were then placed in electroporation buffer containing a glutamate receptor blocker, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM). This drug was added to prevent potential excitotoxicity caused by glutamatergic synaptic transmission activated during poration. After electroporation, this buffer was removed, and the cells were once again placed in culture medium from the cloning cylinder in which they had
been cultured. The cells were then returned to the incubator for 1 h or more to recover. Good transfection results were also obtained when the neurons were left in the same medium they were cultured in during poration, recovery, and imaging and thus were not subjected to possible differences in medium/buffer osmolarity.

3.2. Transfection of hippocampal neurons by microporation

We first determined whether the microporator could be used to transfect cultured neurons with mRNA. We achieved transfection of cultured hippocampal neurons with RNA via the four-step process shown in Fig. 2A. Capped and polyadenylated (A) RNA can be used to express proteins in mammalian cell lines (Yokoe and Meyer, 1996), so we first synthesized modified RNA by in vitro transcription in the presence of a methyl cap-analog. Second, poly-(A) polymerase was added to obtain a 3’ poly(A) tail. While this step is not necessary for protein expression in *Xenopus laevis* oocytes, in mammalian cells it increases the amount of expressed proteins at least 3-fold (H. Yokoe and E. Oancea, personal communication). Third, RNA was introduced into neurons by injecting 1 µl of RNA (∼1 µg/µl) into the microporation region. For neurons 8 days old or younger, three voltage pulses—each 220 V/cm, lasting 30 ms and of opposite polarity—were given at 20 s intervals. For neurons older than 8 days, only two voltage pulses were used. Fourth, an incubation period of 2–8 h was allowed for synthesis of protein from the mRNA.

To assess the efficacy of transfection, we looked at the expression of green fluorescent protein (GFP) in neurons grown 9–15 days in culture. Following intro-
Fig. 2. RNA transfection of cultured hippocampal neurons. (A) Schematic representation of the RNA transfection technique. First, capped RNA is synthesized by in vitro transcription. Second, a 3' poly(A) tail is added to the RNA by in vitro addition of poly(A) polymerase. Third, the modified RNA is concentrated and then porated into adherent neurons using the microporator device. Fourth, the cells are left to synthesize the transfected protein for a period of at least 2 h. (B) Hippocampal neuron transfected with RNA encoding EGFP. A differential interference contrast image of a microporated neuron is shown on the left, and a confocal fluorescence section through the same neuron is shown on the right. (C) Transfection of neurons and glial cells in co-cultures. The arrows are pointing to examples of transfected glial cells. (D, E) Lower magnification images showing the high fraction of neurons transfected by this method. In many regions, greater than 50% of the neurons are transfected. The scale bar is 20 μm in all images.

Fig. 3. DNA transfection of cultured hippocampal neurons. (A) A confocal fluorescence section through hippocampal neuron transfected with DNA encoding EGFP. (B) Epifluorescence image of neurons microporated with DNA after 3 days in culture, imaged 2 weeks later.
duction of RNA by poration, large numbers of neurons and glial cells expressed GFP. Differential interference contrast and fluorescent images of a transfected hippocampal neuron are shown in Fig. 2B. This cell had a typical neuronal morphology while expressing large amounts of GFP (Fig. 2B, right). Likewise, transfected glial cells seemed unperturbed by the poration process (Fig. 2C). By comparing the intracellular fluorescence intensity of GFP to that of fluorescein-conjugated dextran (Yokoe and Meyer, 1996; Teruel et al., 1997), we estimated the maximal intracellular concentration of GFP expressed following RNA transfection to be >3 μM. The transfected area appeared to cover approximately 3 mm² following poration with a 1 μl sample of RNA, but larger volumes of reagents could be applied to electroporate larger numbers of neurons. In the areas on the coverslip exposed to RNA, more than 90% of the intact neurons were transfected (Fig. 2D, E). Under most conditions, we found that the fraction of cells transfected was greatest prior to 10 days in culture.

Because preparation of DNA is simpler and less expensive than preparing RNA, we also tested the ability of the microporator to transfect hippocampal neurons with DNA. We achieved DNA transfection using the same procedure as for RNA, except that 1 μl of 2 μg/μl plasmid DNA encoding GFP was applied. Fig. 3A, B show neurons transfected with DNA. These neurons were typical in their morphology and brightly fluorescent as a result of GFP expression. While the fraction of transfected neurons was always lower when DNA was used instead of RNA (~1–30%), the transfected neurons expressed GFP at high concentrations. When quantified using the method described above, we estimated the concentration of expressed GFP to be >10 μM in many cells following DNA transfection.

The time course of protein expression differed for the two transfection techniques. GFP was observed as little as 2 h following RNA transfection, typically reached a maximum within 8–12 h, and declined thereafter. This transient expression presumably is due to the rapid degradation of mRNA within the cell. Following DNA transfection, GFP was rarely observed within 4 h, but the concentration continued to increase over the course of at least 24 h and was maintained for many days or weeks in some neurons (Fig. 3B). The slower onset of expression following DNA transfection presumably arises from delays in transcription of the DNA into RNA. The longer duration of expression suggests that the DNA persists within the cell for at least several days.

For most experiments, we used 1 μg/μl RNA or 2 μg/μl DNA and found that these concentrations provided high rates of transfection and high levels of expression. In general, changing the concentration of RNA changed the average expression level in transfected cells, but did not affect the fraction of cells that were transfected. On the other hand, decreasing the DNA concentration to 0.1 μg/μl or below reduced the number of cells transfected; however, even at 0.1 μg/μl DNA, a few neurons were often transfected and some of these appeared to express large amounts of GFP.

The number of adherent cells could be reduced by either injection of the sample or application of the electric field. However, damage was minimized by use of the protocols and device described here, and with care, few neurons were immediately killed or detached from the coverslip. To measure the proportion of neurons that survived the procedure, we microporated neurons grown 6 days in culture and counted the viable neurons 8 h later by Trypan Blue exclusion. When the neurons were very healthy before electroporation more than 50% of the neurons survived. Together, these results demonstrate that neurons in primary culture can be efficiently transfected via electroporation. Furthermore, cells successfully transfected using this method remain healthy, displaying normal morphology and the capability for normal transcription and translation.

3.3. Quantitative co-expression of YFP and CFP by RNA transfection

We next asked whether electroporation could transfec cultured neurons with more than one construct at a time. To accomplish this, we synthesized RNAs that encoded two variants of GFP: cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). These two proteins differ in their spectral properties, permitting simultaneous and independent detection of the expression of each (Heim and Tsien, 1996). The RNAs encoding each of these proteins included a ten amino acid long targeting sequence that targeted the proteins to the plasma membrane (see below). The expression of these proteins was assessed by imaging with a confocal microscope able to excite and collect emission from each protein separately. With this setup, when CFP alone was expressed in neurons (Fig. 4A), fluorescence was easily detected in the CFP channel, but fluorescence was less than 5% as bright in the YFP channel. Conversely, expressed YFP was detected in the YFP channel but not in the CFP channel (Fig. 4B). Thus, the setup achieved parallel confocal imaging of CFP and YFP and was therefore able to assess coexpression of the two fluorescent proteins.

When neurons were microporated with a 1:1 mixture of RNA for CFP and YFP, all transfected neurons expressed both proteins (Fig. 4C). We evaluated the efficiency of cotransfection by quantifying the fluorescence intensity of each protein in many neurons. As shown in Fig. 4D, the absolute intensity of expression varied considerably from neuron to neuron; however, within a given neuron, the two proteins were always
expressed at nearly identical levels. This observation contrasted with the pattern of expression following transfection of the DNAs for the same two proteins. Following DNA cotransfection, only some of the transfected neurons expressed both proteins and the relative expression levels of the two proteins were widely scat-
tered (Fig. 4E). Thus, although either DNA or RNA can be used for co-transfection by microporation, the quantitative coexpression of multiple proteins can only be achieved by using RNA.

3.4. Microporated neurons produce action potentials and calcium signals

Although microporated neurons are morphologically intact, we were interested in determining whether poration affected their physiological properties. We first tested their membrane excitability. Whole-cell patch-clamp recordings from GFP-transfected neurons revealed that these cells have normal action potentials and ionic currents. For example, depolarization elicited voltage-dependent Na⁺ and K⁺ currents that appeared qualitatively very similar to those recorded from non-transfected neurons (Fig. 5A, left). The Na⁺ current was reversibly blocked by tetrodotoxin (500 nM), a potent blocker of Na⁺ channels (Fig. 5A, center and right). Thus, poration does not appear to affect the excitability of neurons.

We also used a fluorescent calcium indicator to test whether electrical stimulation could elevate cytosolic calcium concentration (Jacobs and Meyer, 1997). The membrane-impermeant calcium indicator, Calcium Green-1 dextran (70 kDa molecular weight), could be loaded into neurons via microporation (Fig. 5B). This shows that poration can deliver macromolecules other than RNA and DNA into neurons. A burst of depolarizing stimuli induced calcium transients (Fig. 5C; N = 50 responses out of 50 cells tested) that were 250–300 nM in amplitude (spatially averaged value) and were indistinguishable from those measured in non-porated neurons (Jacobs and Meyer, 1997). Thus, microporated neurons have intact voltage-gated calcium channels, can generate action potentials, and in response to action potentials can produce calcium signals similar to those measured in neurons that are not microporated.

3.5. Synaptic transmission is intact in transfected neurons

We next evaluated synaptic transmission in transfected neurons. For this purpose, we used whole-cell patch-clamp methods to record excitatory postsynaptic currents (EPSCs) in neurons microporated with RNA encoding GFP. EPSCs could be recorded in one GFP-transfected neuron when another transfected neuron was stimulated with an extracellular electrode (Fig. 6A; N = 10 responses out of ten cell pairs tested). These responses could be blocked reversibly by CNQX (10 µM; Fig. 6A), confirming that they arose from functional glutamate receptors. When transfected neurons were directly stimulated by applying a brief depolarizing voltage step through the patch pipette, the cell produced an action potential current (off scale in Fig. 6B). In some cases, this current was followed by prolonged responses with multiple peaks (Fig. 6B). These multiple peaks were eliminated by CNQX, indicating that they likely arose from feedback excitation of the cell through a network of nearby neurons. Because both the neuron from which the recording was made and most of its neighbors had been microporated, the presence of a synaptic response indicates that transfection does not interfere with the release of neurotransmitters.

To more closely examine presynaptic function in electroporated neurons, we cultured neurons in isolation on microislands of substrate and glia. In these conditions, the neurons form synapses upon themselves.
Fig. 6. Intact synaptic transmission in microporated hippocampal neurons. (A) EPSCs in a GFP-transfected neuron recorded in response to stimulation of a nearby GFP-transfected neuron. Traces before during and after application of CNQX (10 μM) are shown. (B) A complex, recurrent EPSC recorded in a GFP-transfected neuron in response to a brief depolarizing step applied through the patch pipette (0.8 ms step to 0 mV from 70 mV). (C) EPSCs during and 1 s after evoking 50 action potentials at 20 Hz in an isolated, autaptic neuron grown on a glial island and microporated with 70-kDa fluorescein dextran. (D–F) Amplitudes of EPSCs recorded during and after application of a train of 50 action potentials (bars). The dashed line is the average response amplitude to low frequency stimuli (0.06 Hz) given before the train. The last stimulus was given 1 s after termination of the train. Graphs illustrate responses from an isolated, autaptic neuron that had not been porated (D), responses from the porated autaptic neuron shown in C (E), and responses from the porated neuron shown in A (F).

(autapses) and provide a particularly favorable arrangement for synaptic stimulation and recording (Bekkers and Stevens, 1991). Recurrent synaptic responses were measured in every autaptic cell (N= 7 synaptic responses out of seven cells examined) that was loaded with fluorescein–dextran via microporation. An example of such a response is shown in Fig. 6C (left). Following the initial inward current, which is caused by an action potential, was an EPSC that arose from the release of glutamate from the presynaptic terminals of the neuron onto itself. This directly demonstrates that porated neurons are capable of releasing neurotransmitter.

We also examined synaptic depression, a form of plasticity that is thought to arise from the depletion of an available pool of releasable synaptic vesicles (Thies, 1965; Liley and North, 1968; Stevens and Wang, 1995). In control neurons, a high-frequency train of action potentials caused a depression in the amplitude of EPSCs, and EPSCs recovered within seconds after the train stopped (Fig. 6D). Synaptic depression appeared to be very similar in autapses porated with fluorescein dextran (Fig. 6E; N= 3 of three) as well as in a multi-cell culture transfected with GFP (Fig. 6F). In all three situations, the half-maximal decrease in EPSC amplitude occurred after approximately five action potentials and nearly complete recovery was observed within 1 s. These results indicate that the kinetics of synaptic depression are unaffected by microporation. This suggests that synaptic vesicle release and recycling (Rosenmund and Stevens, 1996), as well as presynaptic terminal calcium homeostasis (Swandulla et al., 1991), are not affected by the microporation process. We conclude that transfection affects neither the presynaptic nor the postsynaptic functions of neurons.

3.6. Targeting of proteins in transfected neurons

Because neurons are highly compartmentalized cells, we were interested in determining whether proteins expressed by our transfection procedure were localized to their correct targets. For this purpose, we asked whether proteins that are destined for the plasma membrane or endoplasmic reticulum were correctly expressed and targeted in the neurons. GFP was fused to a ten amino acid N-terminal myristoylation and palmitoylation sequence which has been shown to localize proteins to the plasma membrane (Resh, 1994). When this PM–GFP construct was expressed in neurons, it was clearly located on the neuronal plasma membrane (Fig. 7A, B). The branching pattern could be resolved very clearly in neurons expressing this protein, making it a useful technique for visualizing the fine structure of neurons. We also expressed an elastase–GFP fusion construct which localizes to the lumen of the endoplasmic reticulum in other cell types (Subramanian and Meyer, 1997). This protein showed a tubular network
staining, reminiscent of the endoplasmic reticulum, when expressed in neurons (Fig. 7C, D). The endoplasmic reticulum appeared to be continuous along the processes of some, but not all, of these transfected neurons (Terasaki et al., 1994). This construct was also expressed in glial cells (Fig. 7E). The flatter morphology of the glial cells made the continuous, as well as isolated, structures of the endoplasmic reticulum more readily apparent. Together, these examples show that neurons, as well as glial cells, can be readily transfected with different GFP-based probes and that these probes target to the correct location. This enables studies of dynamic changes in the localization of cellular proteins and organelles in intact neurons.

Fig. 7. Correct targeting of expressed GFP-tagged proteins. (A, B) Plasma membrane localization of a GFP construct with a consensus sequence for palmitoylation/myristoylation. Neurons were transfected with RNA encoding a ten amino acid myristoylation and palmitoylation sequence (from Lyn) fused to the N-terminal end of GFP. Two transfected neurons with different morphologies are shown. (C, D) Localization of a GFP construct targeted to the endoplasmic reticulum (ER–GFP). Neurons were transfected with RNA encoding a fusion protein of elastase and GFP which has been previously shown to be a marker of the lumen of the endoplasmic reticulum (Subramanian and Meyer, 1997). Reticular structures that are highly reminiscent of the endoplasmic reticulum can be seen in the cell body and processes of hippocampal neurons. (E) Confocal section below the neuronal layer from a culture transfected with RNA for ER–GFP. A fragmented and dynamically rearranging structure of the endoplasmic reticulum in glial cells is clearly visible.
4. Discussion

Our study demonstrates that microporation is an efficient method to introduce RNA, DNA and other macromolecules into neurons. This offers a new and versatile approach to neuronal transfection and makes possible other experimental approaches for the study of neuronal function. By a series of tests, we have shown that microporated neurons are functionally intact. First, using differential interference contrast and fluorescence imaging, we found that microporated neurons are morphologically indistinguishable from non-transfected neurons. Second, the transcription and translation machinery of these neurons was intact, as demonstrated by the expression of GFP via both RNA and DNA transfection. Third, we found that neurons could still fire action potentials and had intact calcium signaling. Fourth, porated cells retained intact synaptic transmission. Finally, neurons appeared to localize expressed proteins to their appropriate intracellular targets. Taken together, these measurements show that microporation of macromolecules can be used to study synaptic transmission and many other molecular and cellular processes in intact, functional neurons.

The ability to transfet with either RNA or DNA offers tremendous flexibility. The principle difference between RNA and DNA transfection is that microporated RNA has only to reach the cytosol instead of the nucleus for protein expression to occur. Thus, RNA transfection offers the following important advantages. (1) Protein expression is rapid; several micromolar of intracellular GFP can be synthesized in as little as 2 h (Yokoe and Meyer, 1996). (2) The method is efficient. Using RNA transfection, more than 50% of the neurons can often be transfected. This will enable simultaneous imaging of several neurons or even entire neuronal networks. It also should be a benefit for small-scale biochemical experiments, which usually require a large fraction of transfected cells. (3) RNA transfection is quantitative. Different cells transfected with a given amount of RNA express similar amounts of protein. Since the concentration of expressed protein falls in a more narrow range with RNA transfection compared to DNA transfection (Fig. 4), expression levels can be more easily titrated by diluting the RNA before loading in the microporator. (4) Co-expression of different proteins in the same cell is readily possible. Imaging studies of the distribution of different proteins tagged with different GFP color variants can be achieved by mixing their respective RNA. Furthermore, multiple proteins that reconstitute a signaling pathway or dominant negative inhibitors of cell function could be co-expressed in individual cells at defined relative concentrations. On the other hand, DNA is simpler and cheaper to prepare than RNA, and DNA transfection results in considerably higher protein concentrations and longer-lasting protein expression.

We showed that microporation can be used not only for transfection but also to introduce dextran-based fluorescent indicators into cultured neurons. Indeed, previous studies of cell lines suggest that the microporator can be used to introduce many different biologically active compounds or optical indicators, such as peptides (Stauffer et al., 1997) and caged compounds (Horne and Meyer, 1997). Thus, we expect that microporation will enable many new experimental strategies to unravel the cellular and molecular mechanisms underlying neuronal function.

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