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## Ultrasound-mediated gene transfer into neuronal cells

Andy J. Fischer<sup>a,\*</sup>, Jennifer J. Stanke<sup>b</sup>, Ghezal Omar<sup>a</sup>,  
Candice C. Askwith<sup>a</sup>, Richard W. Burry<sup>a</sup>

<sup>a</sup> Department of Neuroscience, The Ohio State University, College of Medicine and Public Health, 3020 Graves Hall,  
333 W. 10th Avenue, Columbus, OH 43210-1239, USA

<sup>b</sup> Integrated Biomedical Science Graduate Program, The Ohio State University, Columbus, OH 43210-1239, USA

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### Abstract

A new field of gene transfer is emerging as a simple, effective means to drive the expression foreign genes in cells: ultrasound-mediated gene transfer or sonoporation. We report here that sonoporation is an effective means of gene transfer for cultured neurons, a cell type that has been difficult to transfect. Neuronal cell types that are effectively sonoporated include chick retinal neurons, chick dorsal forebrain, chick optic tectum, PC12 cells, rat cerebellar neurons and mouse hippocampal neurons. Depending on the type of cell and conditions of sonoporation the transfection efficacy was as high as 20%. Sonoporation of plasmid DNA was effective for cells adherent to a substrate and for free-floating cells that were freshly dissociated. In the free-floating preparations, between 60 and 95% of the cells that were transfected were neuronal, as much as 90% higher than that observed for other methods of gene transfer including adenovirus and lipid-based transfection methods. We conclude that sonoporation is a simple, effective and inexpensive means by which to preferentially transfet DNA into neuronal cells.

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**Keywords:** Gene transfer; Ultrasound; Sonoporation; Retina; Cerebellum; Hippocampus; Optic tectum

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### 1. Introduction

Gene transfer has proven to be a powerful tool in biomedical research. In addition, the transfer of genes holds great potential as a therapy to treat a wide vari-

ety of diseases. Accordingly, the development of novel, effective means of gene transfer is important to basic research and clinical science. One major challenge in the field of gene transfer has been to effectively transfect postmitotic neurons. With few exceptions, this has proven to be difficult; neuronal transfections have been unreliable, cytotoxic, labor-intensive and inefficient (reviewed by Washbourne and McAllister, 2002).

\* Corresponding author. Tel.: +1 614 292 3524;  
fax: +1 614 688 8742.

E-mail address: [fischer.412@osu.edu](mailto:fischer.412@osu.edu) (A.J. Fischer).

Widely used methods of gene transfer involve recombinant viruses, calcium phosphate co-precipitation, liposome-mediated gene transfer, non-liposome-forming lipids, high molecular weight cationic polymers, particle bombardment (biollistics) or electroporation. A new method of gene transfer is slowly emerging with a broad range of potential applications. This method of gene transfer involves the use of ultrasound and sonoporation to deliver DNA into cells. Ultrasound is commonly used as a diagnostic tool in clinical medicine to non-invasively probe for tissue features, in bioreactors to facilitate biocatalysis or to apply heat to expedite tissue repair. However, ultrasound can also be used to induce sonoporation to mechanically deliver DNA into cells. Sonoporation utilizes sonic energy to induce microbubble cavitation, which can transiently open cell membranes (Marmottan and Hilgenfeldt, 2003). This transient reparable disruption of the cell membrane allows for the entry of foreign DNA or other macromolecules into the cytoplasm. To date, there are published examples of sonoporation-mediated gene transfer into cell lines (Miller et al., 2002), yeast (Wyber et al., 1997), skeletal muscle cells (Pislaru et al., 2003), cardiac smooth muscle (Bekeredjian et al., 2003, 2004; Chen et al., 2003) and the developing limbs of embryonic chickens (Ohta et al., 2003). In addition, several recent studies have assayed whether ultrasound can be used to transfect plasmid DNA in neural cells (Shimamura et al., 2004; Manome et al., 2005). Manome et al. (2005) reported sonoporation of cells in slice preparations of mouse brain. However, most of the transfected cells were glial; an unspecified low number of neurons was transfected. Similarly, Shimamura et al. (2004) used ultrasound and microbubble-mediated cavitation to transfect cells in the rat central nervous system, but failed to transfect neurons.

Here, we provide evidence for effective sonoporation-mediated gene transfer into primary neurons. We describe a method of gene transfer that is simple, fast and inexpensive by making use of a standard laboratory sonicator, commonly used to disrupt cells or homogenize liquids. We report that sonoporation can be optimized to result in little cell death, high percentages of transfected neurons, can be easily applied to adherent cells in culture and works well for primary cultures of neural precursors and postmitotic neurons.

## 2. Methods and materials

### 2.1. Animals

The use of animals in these experiments was in accordance with the guidelines established by the National Institutes of Health and The Ohio State University. Eggs were obtained from the Department of Animal Sciences at the Ohio State University. Chick embryos were staged according to guidelines established by Hamburger and Hamilton (1951). Newly hatched leghorn chickens (*Gallus gallus domesticus*) were obtained from the Department of Animal Sciences at The Ohio State University and kept on a cycle of 12-h light:12-h dark (lights on at 7:00 a.m.). Chicks were housed in a stainless steel brooder at about 30 °C and received water and Purina<sup>TM</sup> chick starter ad libitum.

### 2.2. Tissue culture

Retinas were dissected in sterile Hanks' buffered saline solution (HBSS) added with 3% d-glucose and 0.01 M HEPES buffer (HBSS+). Retinal cells were dissociated by mild trituration after incubation for 10 min at 37 °C in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS plus 0.05% trypsin. Cell density was determined by using a hemacytometer. Between 100,000 and 200,000 cells were plated onto 12 mm glass coverslips that were coated sequentially with poly-D-lysine and Matrigel (Collaborative Research) diluted to 1:100 in HBSS. Cell cultures were maintained at 37 °C and 5% CO<sub>2</sub> under culture medium (DMEM:F12 without glutamate or aspartate; Invitrogen), added with 100 units/ml penicillin and 100 mg/ml streptomycin, 0.05 M HEPES and 2% fetal bovine serum; Gibco BRL). Cultures of chick-derived cells were maintained under 1 ml of DMEM-F12 per well (using 24-well plates) for up to 14 days with 50% of the medium replaced every 48 h.

PC12 cells (PC12-N21 clone) were prepared and grown as previously described (Burry and Perrone-Bizzozero, 1993).

Cultures of cerebellar granule cortex were prepared from 3-day-old rats. After removing the cerebellum, the meninges were discarded and the cerebellar cells were dissociated in culture medium by mild trituration using a Pasteur pipette. After settling for 3 min, the supernatant with the dispersed cells was

aspirated and the dissociation process was repeated three times. Cells were collected from the supernatant by centrifugation at 175 RCF for 4 min. Cells were plated at  $4 \times 10^5$  cells/cm<sup>2</sup>. The cultures were maintained at 37 °C in 5% CO<sub>2</sub> with the media replaced twice weekly. The culture medium was a variation of Hams F12 added with 10% fetal bovine serum (Burry, 1983).

Hippocampal neurons were prepared as previously described (Askwith et al., 2004; Wemmie et al., 2002). Briefly, postnatal day 1 mouse pups were euthanized and the brain removed. The hippocampus was dissected and placed in a sterile solution of L15/BSA (0.2 mg/ml) and torn into small pieces. The hippocampal tissue was transferred into a solution of L15/BSA/papain (0.375 mg/ml) and incubated for 15 min at 37 °C with 95% O<sub>2</sub>/5% CO<sub>2</sub> blowing gently over the surface of the solution. After incubation, the tissue was washed three times with pre-warmed culture media (M5-5) (Mennerick et al., 1995), added with insulin-transferring-sodium selenite and then triturated with a series of pasture pipettes of varying diameters. Hippocampal neurons were plated onto collagen-coated coverslips in M5-5 media at a density of three hippocampi/4 ml media.

### 2.3. Virus-mediated gene transfer

Purified adeno-associated virus type 1 (AAV1), AAV2 or adenovirus serotype 5 (AdV5) were kindly provided by Dr. Reed Clark (Department of Pediatrics, The Ohio State University). Viruses were prepared and purified as described elsewhere (Schnepp and Clark, 2002; Liu et al., 2000). These viruses utilize the CMV promoter to drive expression of eGFP. The viruses were added to adherent cultures of chick retinal cells at 10<sup>6</sup> PFU/ml of media or approximately 10<sup>6</sup> PFU/200,000 chick cells.

### 2.4. Liposome-mediated gene transfer

Lipofectamine 2000™ (Invitrogen) or FuGene6™ (Roche) were mixed with plasmid DNA at 3 µl/2 µg of pCAX-eGFP in 50 µl of HBSS+. The pCAX vector is the same as described elsewhere (Chen et al., 2004). The relative quantities were optimized as per the manufacturer's instructions. These reagents were incubated at room temperature for 15 min and added to adherent

cell culture. Media was replaced 12 h after treatment with the Lipofectamine 2000™ or FuGene6™.

### 2.5. Sonoporation

We used the Sonics and Materials VC130 sonicator which produces continuous wave ultrasound at 20 kHz, has an adjustable output range of 0.5–130 W and replaceable probe tips that are 2, 6 and 12 mm in diameter across the tip. The sonicator had inherent limitations to output levels for each probe tip; the minimum output for the 2 mm probe was 0.5 W and the minimum output for the 12 mm probe was 2.5 W. The peak amplitude of the ultrasound that was delivered by the two probes (207 µm) was nearly three-fold that of the 12 mm probe (75 µm). Specifications for the probes can be found at the manufacturer's website: [http://www.sonicsandmaterials.com/Accessories/Accessories\\_7/accessories\\_7.html](http://www.sonicsandmaterials.com/Accessories/Accessories_7/accessories_7.html).

Plasmid DNA (pCAX-eGFP) was diluted in HBSS+, added to each well between 0.5 and 20 µg/ml and incubated for 5–10 min at 37 °C. Prior to placing the sonicator probe into the media, it was sterilized by sonication in 70% ethanol followed by sonication in sterile water. With 1000 µl medium per well, the sonicator probe was placed into the media and activated for between 0.5 and 30 s delivering between 0.5 and 10 W of energy. Cells were cultured for 1–14 days after the sonoporation and thereafter, fixed and processed for immunocytochemical labeling by using standard methods (Fischer et al., 1998).

### 2.6. Assay for cell survival

To assay for cell survival following sonoporation, we used the Live/Dead™ assay from Molecular Probes as per the manufacturer's specifications. In short, adherent retinal cells were sonicated using the 2 or 12 mm probes for 8 or 2 s, respectively, between 0.5 and 10 W. Immediately following sonication, the media was aspirated and cells were rinsed two times with HBSS+, added with 200 µl per well of Live/Dead™ reagents (1 µM ethidium homodimer and 1 µM Calcein AM diluted in HBSS+) and incubated for 30 min at 37 °C. Following the incubation, cells were rinsed twice in HBSS+, coverslips mounted on HBSS+ and photographs were made as described below.

### 2.7. Fixation and immunocytochemistry

Cells were fixed and immunolabeled as described elsewhere (Fischer et al., 2004; Fischer and Reh, 2001). Working dilutions and sources of antibodies used in this study included, mouse anti-visinin at 1:50 (Developmental Studies Hybridoma Bank), mouse anti-vimentin at 1:100 (H5; Developmental Studies Hybridoma Bank); rabbit anti-eGFP at 1:3000 (Dr. L. Berthiaume, University of Alberta), mouse anti-neurofilament at 1:2000 (Dr. V. Lee, University of Pennsylvania) and mouse anti-Hu at 1:200 (Monoclonal Antibody Facility, University of Oregon). Secondary antibodies included goat-anti-rabbit-Alexa568, goat-anti-mouse-Alexa568 and goat-anti-rat-Alexa488 (Molecular Probes Inc., Eugene, OR) diluted to 1:1000 in PBS plus 0.2% Triton X-100. Cells were labeled with DAPI (Sigma) at 1 µg/ml in PBS prior to mounting the cells and coverglass on Fluoromount-G.

### 2.8. Photography, measurements, cell counts and statistical analyses

Photomicrographs were taken by using a Leica DM5000B microscope equipped with epifluorescence, FITC and rhodamine filter combinations and Leica DC500 digital camera. Images were optimized for color, brightness and contrast and double-labeled images overlaid by using Adobe Photoshop<sup>TM</sup> 6.0. Cell counts were made on at least five fields of view from at least three coverslips per experimental treatment and means and standard errors calculated. Data between experimental conditions were compared statistically with ANOVA and post hoc Student's *t*-test.

## 3. Results

### 3.1. Efficacy of sonoporation with different probe tips and energy output

To determine if sonoporation efficacy was affected by the diameter of the probe tip we ran a series of experiments on cultures of embryonic chick retinal cells. Chick retinal cells were plated at 200,000 cells/cm<sup>2</sup> and grown in culture for 2 days before sonoporation. With the 2 mm probe using 8 s of sonication at 0.5 W, we found a few cells that were sonoporated, as indicated

by the expression of eGFP (Fig. 1a). In comparison to sonication at 0.5 W, a 2-fold increase in output energy resulted in a 10-fold increase in the number of eGFP-positive cells (Fig. 1b and g). However, when the output energy was further increased the number of eGFP-positive cells was decreased (Fig. 1c and g). This likely resulted from decreased survival. In addition, when the tip of the probe randomly moved over coverslip during sonication, the eGFP-positive cells were scattered randomly across the coverslip. However, when the tip of probe remained stationary near the center of the coverslip eGFP-positive cells were found concentrated in a narrow region near the center of the coverslip (data not shown). This finding indicates that sonoporation can be directed to discrete locations.

Sonication using the 12 mm probe for 2 s at 2.5 W resulted in numerous cells that were eGFP-positive (Fig. 1d). By comparison, increased output energy to 5 or 10 W resulted in decreased numbers of eGFP-positive cells (Fig. 1e–g), which likely resulted from decreased survival. There was substantial variability across the coverslips, as indicated by the width of the error bars (Fig. 1g). Sonoporated cells maintained their expression of eGFP for at least 2 weeks after treatment (data not shown).

### 3.2. Cell death and sonoporation

To assay for cell viability following sonoporation we used the Live/Dead<sup>TM</sup> assay kit from Molecular Probes. Compared to control preparations that were not sonicated (Fig. 2a), culture preparations treated with the 2 mm probe (0.5 W for 8 s) contained few dead cells that accumulated ethidium homodimer, while the vast majority of cells were alive and accumulated Calcein AM (Fig. 2b). However, sonication for 20 s at 0.5 W resulted in an increase in the number of dead cells and a decrease in the density of viable cells as indicated by areas devoid of cells (Fig. 2c). Using the 2 mm probe, 8 s of sonication at 1 W in the center of the coverglass resulted in abundant cell death near the center of the coverglass (Fig. 2d), while there was no apparent loss of cells in peripheral regions of the coverglass (Fig. 2e). Sonication for 8 s at 2 W using the 2 mm probe resulted in an obvious decrease in cell density of viable cells and an increase in the number of dead cells (Fig. 2f).

Sonication using the lowest possible energy setting (2.5 W) for the 12 mm probe resulted in increased

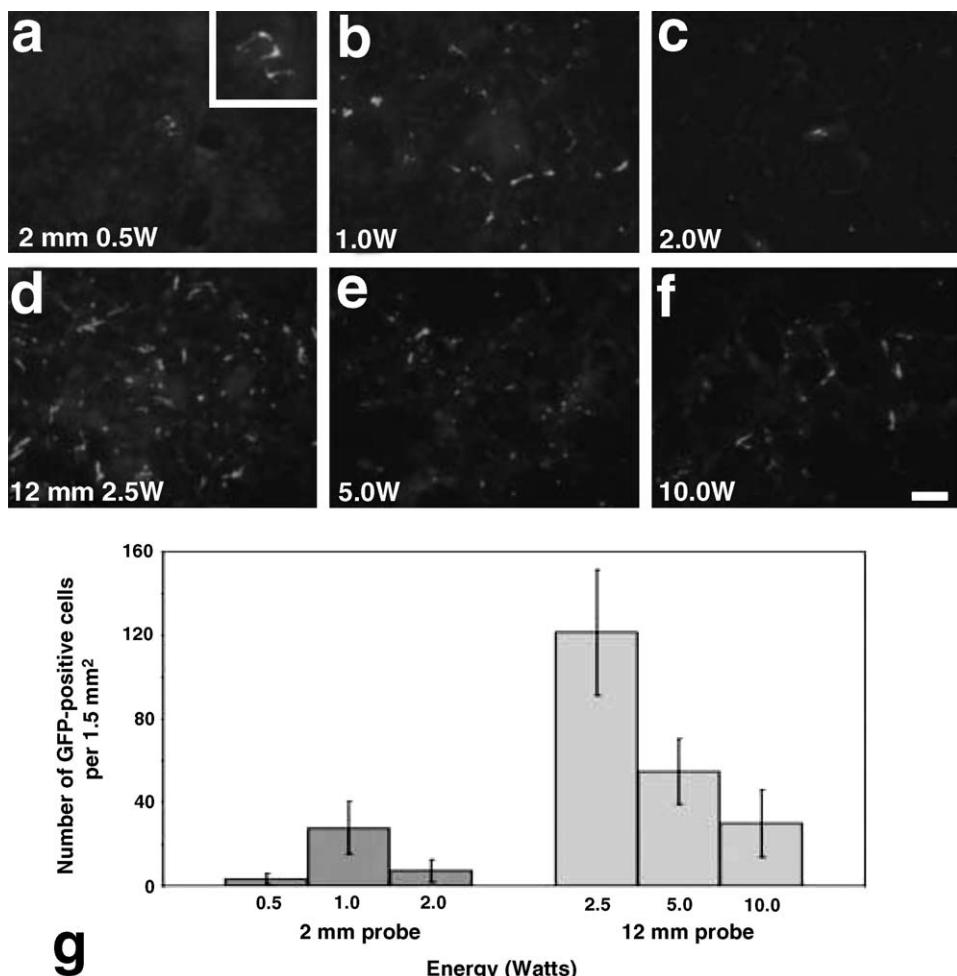
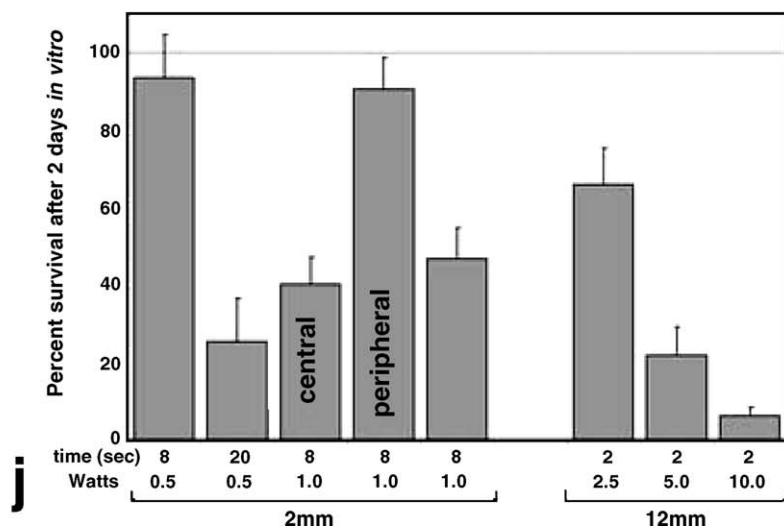
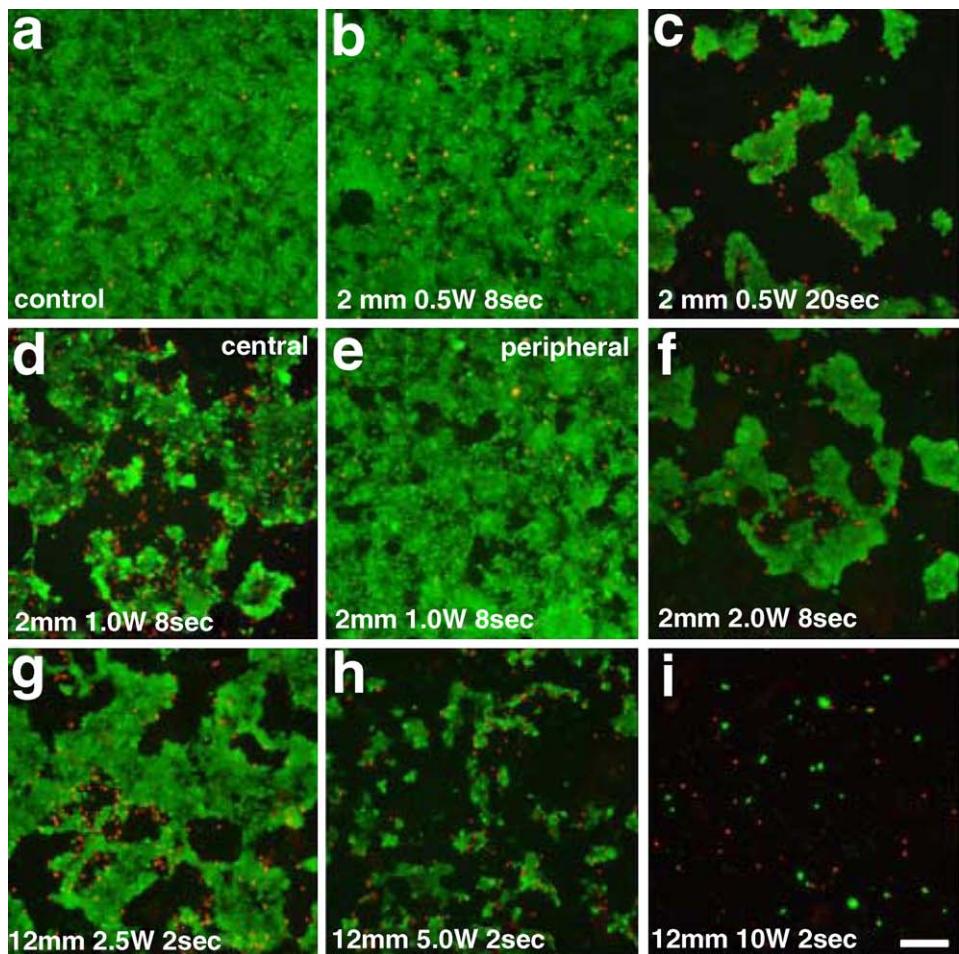


Fig. 1. Sonication of cells from the embryonic chicken retina results in transfection of expression plasmid. Dissociated cells from E7 chick retina were plated at 200,000 cells/cm<sup>2</sup> and grown in culture for 2 days. The cells were added with 4 µg/ml pCAX-eGFP and sonicated using probe tips that were 2 or 12 mm in diameter for 8 or 2 s, respectively, at 0.5–10 W. Following sonoporation, cells were maintained for 48 h in vitro. The calibration bar in panel f represents 50 µm and applies to panels a–f. Panel g is a histogram illustrating the number of eGFP-expressing cells/1.5 mm<sup>2</sup> that resulted from sonication with the different probe tips and energy levels.

levels of cell death compared with sonication using the 2 mm probe. Two seconds of sonication at 2.5 W resulted in an obvious increase in the abundance of dead cells and reduced the density of viable cells (Fig. 2g). Increasing the energy delivered to 5 or 10 W resulted in wide-spread cell death and substantial decreases in the density of viable cells (Fig. 2h and i). In summary, sonoporation at lower energy levels (<1 W) did not increase levels of cell death, unless the duration was extended to 8 s, while higher energy levels (>2 W) resulted in the wide-spread destruction of

cells, even with short periods (2 s) of sonication. Two days after sonication the percentages of surviving cells with the different sonoporation conditions correlated well with observations made using the Live/Dead™ assay immediately following sonication (Fig. 2j).

Ultrasound-mediated heating of the media was unlikely to contribute to cell death or in some way to gene transfer. Sonication of 1 ml of media with the 2 mm probe at 1.0 W for 20 s raised the temperature  $1.13 \pm 0.09$  or  $0.028^\circ\text{C}/(\text{W s ml})$ . Sonication of 1 ml of media with the 12 mm probe at 5 W for 8 s raised the



temperature  $1.73 \pm 0.22$  or  $0.043^\circ\text{C}/(\text{W s ml})$ . These changes in temperature are relatively minor compared to fluctuations in media temperature that occur with manipulating the cells during dissociation, applying fresh media and making observations on an inverted microscope. However, we cannot exclude the possibility that sonication caused large, transient and spatially restricted increments in temperature, which we failed to detect. These sonication-mediated changes in temperature may have influenced transfection or compromised cell survival.

### 3.3. Increasing plasmid concentration yields increased transfection efficacy

To assay whether plasmid concentrations influence the number of sonoporated cells, we added between 0.5 and 20  $\mu\text{g}$  of pCAX-eGFP/ml of media and sonicated cells for 2 s at 2.5 W using the 12 mm probe. In control preparations added with 1  $\mu\text{g}/\text{ml}$  of pCAX-eGFP that were not sonicated, but were adjacent to wells that received sonication, we found a few cells ( $1.8 \pm 1.6$  cells/ $1.5 \text{ mm}^2$ ) that were eGFP-positive (Fig. 3a and g). By comparison, the combination of increasing concentrations of pCAX-eGFP and sonication resulted in a significant ( $p < 0.0001$ ; ANOVA) increase in the number of eGFP-positive cells (Fig. 3b–g). There was more than a 10-fold increase in the number of eGFP-positive cells between preparations that received 0.5 and 20  $\mu\text{g}$  of expression plasmid (Fig. 3g). In preparations that received 20  $\mu\text{g}/\text{ml}$  pCAX-eGFP,  $19.7 \pm 4.1\%$  of the cells were eGFP-positive.

### 3.4. Different types of retinal neurons are transfected via sonoporation

Many of the eGFP-transfected cells had the morphology of mature retinal neurons. Based on mor-

phological criteria, these cells included cone (Fig. 4a) and rod (Fig. 4b) photoreceptors, ganglion cells (Fig. 4c) and bipolar cells or Müller glia (Figs. 4d and e). In addition, we observed numerous eGFP-positive, non-neuronal cells scattered across the coverglass or arranged in a radial pattern in rosettes (Fig. 4a and f). These cells likely were retinal progenitors, which are known to form rosettes of cells when grown as adherent cells (Reh and Levine, 1998; Anchan et al., 1991). To confirm that some of the transfected cells were neurons, we labeled eGFP-expressing cells with antibodies directed to visinin, a marker for differentiated rod and cone photoreceptors and Hu, a marker for differentiated amacrine and ganglion cells. We found many of the eGFP-transfected cells with neuronal morphology that were immunoreactive for visinin (Fig. 4g–i) or Hu (Fig. 4j–l). All of the Hu-positive cells ( $n = 374$ ) shared a common morphology: small somata (less than 8  $\mu\text{m}$  in diameter) and elaborate peripheral processes (Fig. 4j–l).

### 3.5. Sonoporation of free-floating retinal cells compared to other methods of gene transfer

Freshly dissociated retinal cells were prepared from E7 chick embryos. Expression plasmid (pCAX-eGFP) at 4  $\mu\text{g}/\text{ml}$  was added to cells suspended at 200,000 cells/ml in culture media. For a direct comparison between lipid-based transfection methods and sonoporation, retinal cells from the same dissection were used for free-floating sonoporation or plated at 200,000 cells per well on to PDL-coated glass coverslips for sonoporation as adherent cells or transfection with Lipfectamine 2000<sup>TM</sup> or FuGene6<sup>TM</sup>. In a total volume of 2 ml, the 2 mm sonicator tip was placed into the media and sonication activated. In parallel, using identical sonoporation settings and plasmid concentration, we sonoporated the adherent cells

**Fig. 2.** Increasing the energy delivered via sonoporation results in increasing levels of cell death. The viability and death of cells was assayed by using the Live/Dead<sup>TM</sup> assay from Molecular Probes. In this assay, dead cells accumulate ethidium homodimer in the nucleus and fluoresce red and cells that remain alive accumulate and metabolize Calcein AM to fluoresce green. Dissociated retinal cells were obtained from E7 chick embryos, plated at 200,000 cells/cm<sup>2</sup> and sonicated for 2–20 s at 0.5–5.0 W using the 2 and 12 mm probes. Panel d is a central region of the cover glass and panel e is a peripheral region of the same coverglass. Fluorescent micrographs were made immediately after the staining protocol was completed. The calibration bar in panel i represents 50  $\mu\text{m}$  and applies to panels a–i. Panel j is a histogram demonstrating the relative percentage of cells that survived in culture 2 days after sonoporation. Numbers of surviving cells were assayed by surveying DAPI-labeled nuclei that were not pyknotic. Percent survival was calculated from the average number of DAPI-positive nuclei/10,000  $\mu\text{m}^2$  in sonicated preparations relative to the average number of DAPI-positive nuclei/10,000  $\mu\text{m}^2$  in unsonicated samples. “For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article”.

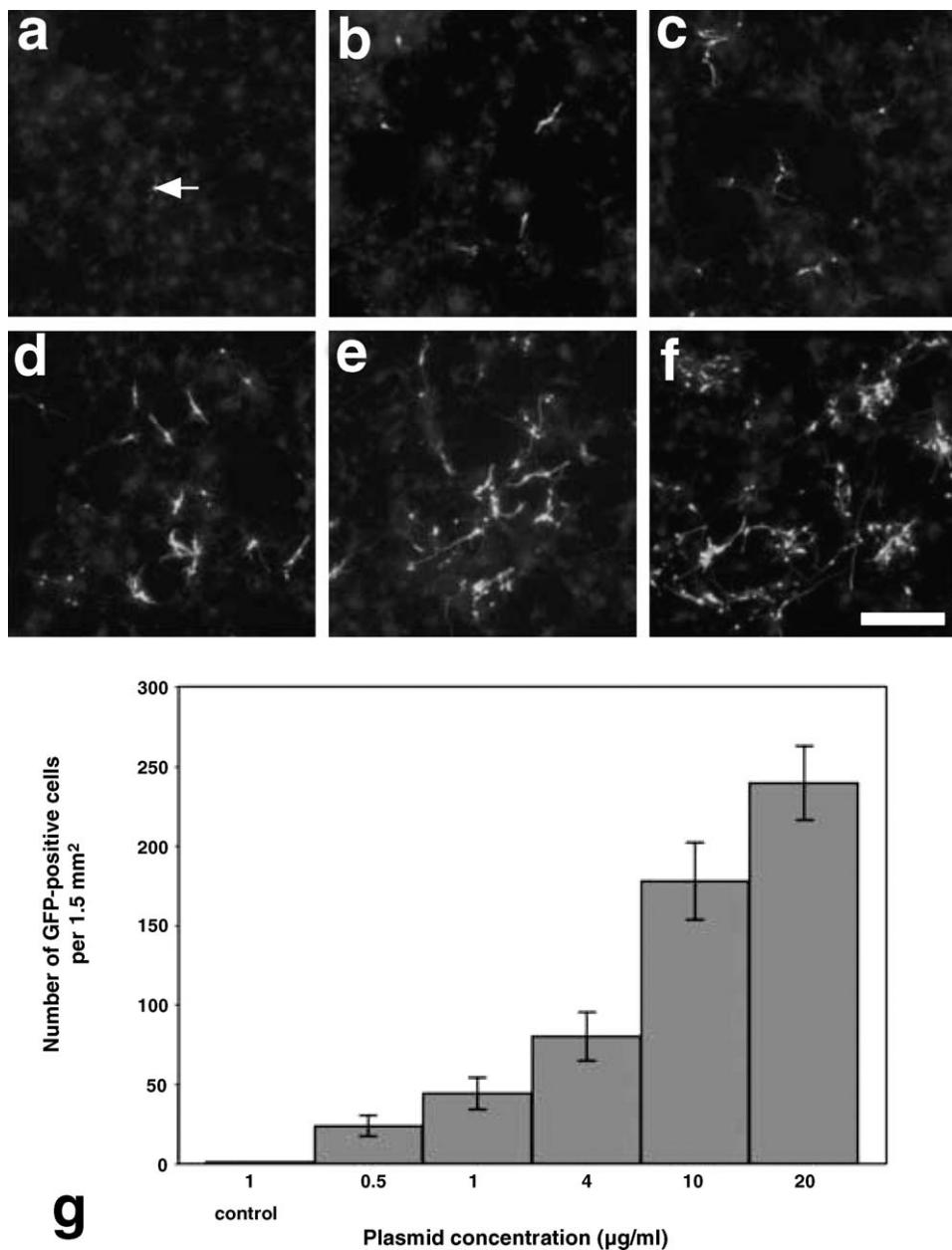
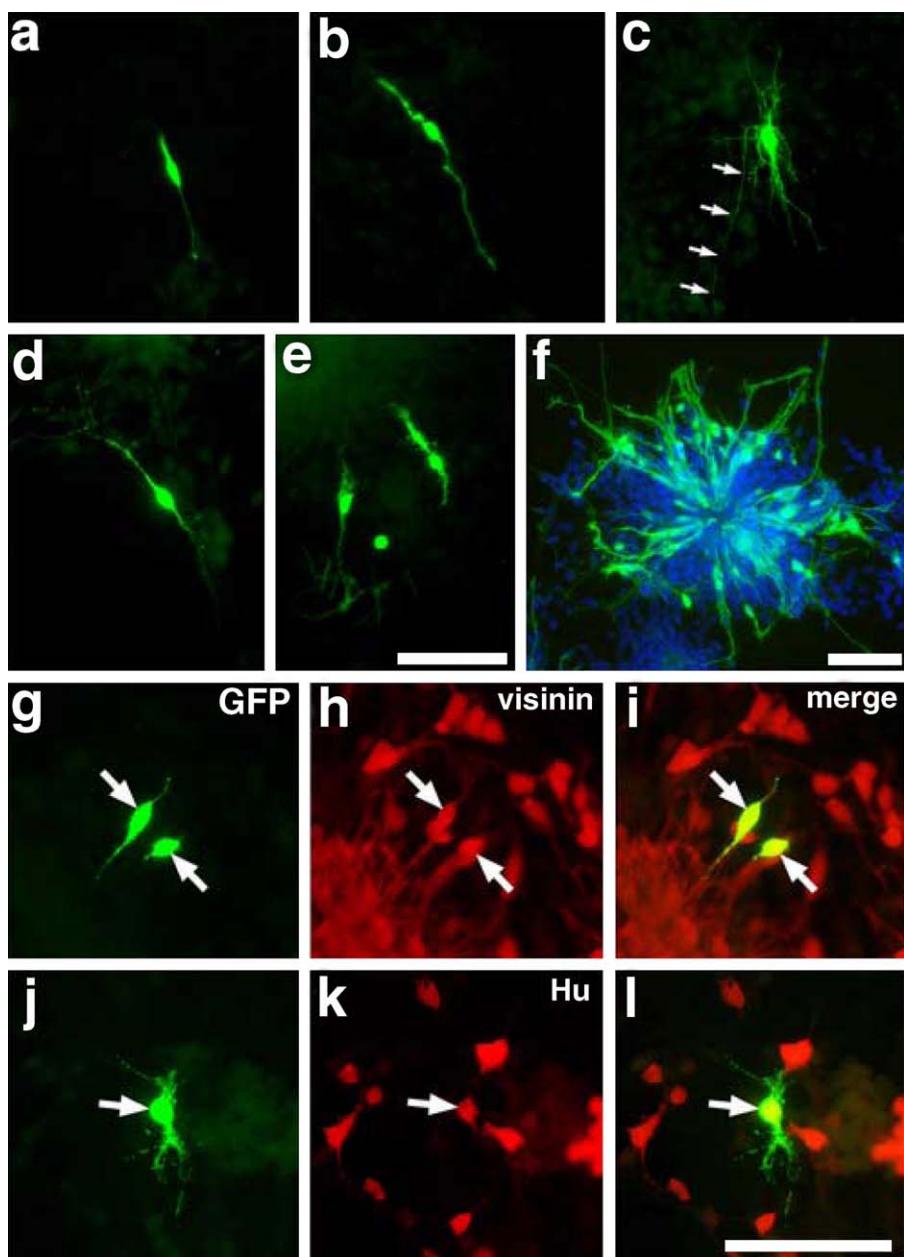


Fig. 3. Increasing plasmid concentration results in increased numbers of cells that are transfected via sonoporation. Dissociated retinal cells obtained from E7 chick embryos were plated at 200,000 cells/cm<sup>2</sup>, added with pCAX-eGFP and sonicated for 2 s at 2.5 W using the 12 mm probe. Plasmid concentrations were between 0.5 and 20  $\mu\text{g}/\text{ml}$ . For a control, cells were added with pCAX-eGFP at 1  $\mu\text{g}/\text{ml}$  and were not sonicated (panel a and the first column in panel g). The arrow in panel a indicates an eGFP-positive cell. The calibration bar in panel f represents 50  $\mu\text{m}$  and applies to panels a–f. Panel h is a histogram demonstrating the number of eGFP-positive cells/1.5 mm<sup>2</sup> that were sonoporated with increasing concentrations of pCAX-eGFP.

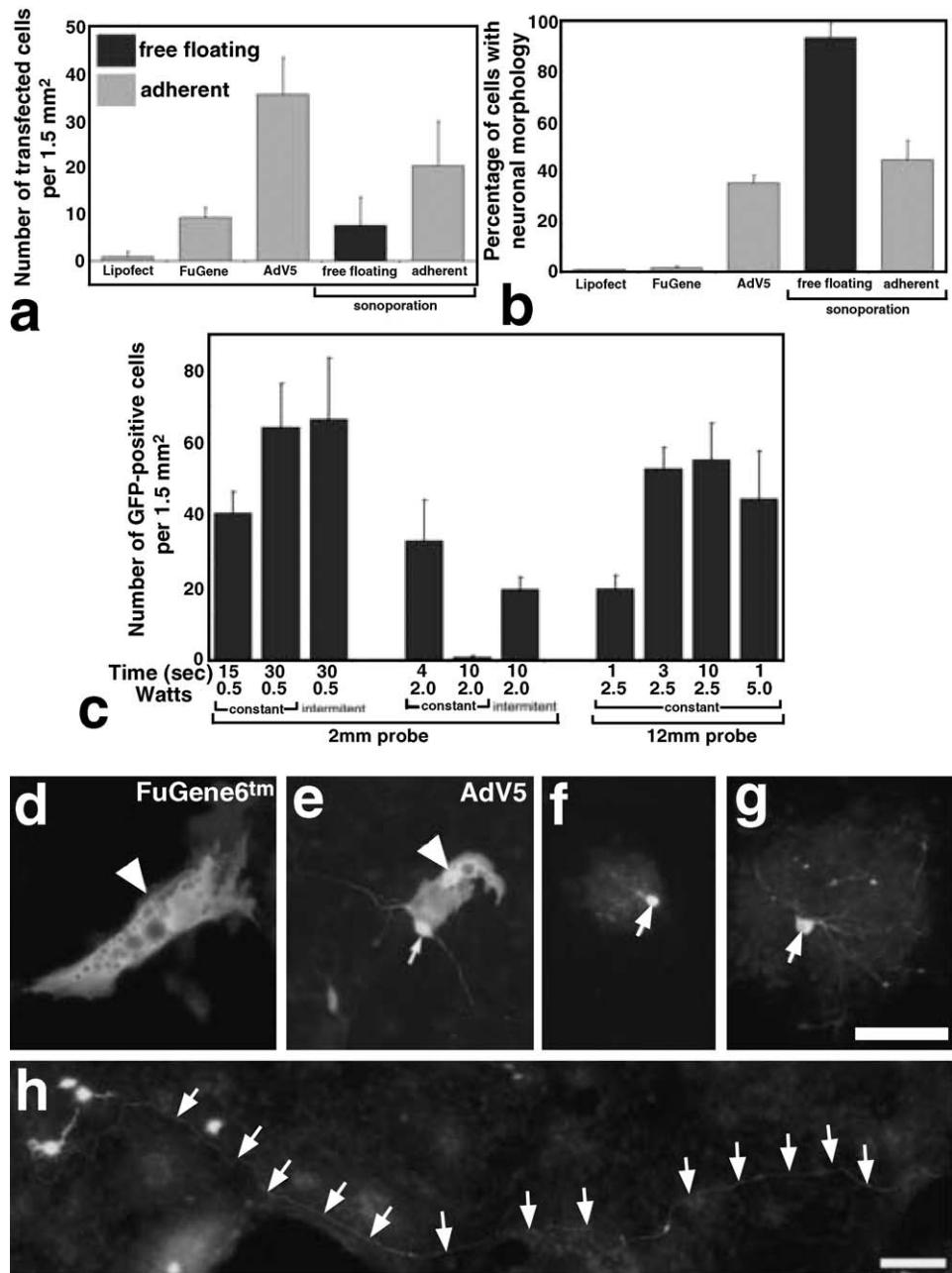


**Fig. 4.** Different types of neurons and neural progenitors from the chick retina are transfected via sonoporation. Cells were added with pCAX-eGFP at 2 µg/ml and sonicated for 2 s at 2.5 W using the 12 mm probe; 48 h after sonoporation, cells were processed for immunocytochemistry and labeled with antibodies to eGFP (green; a–g, i, j and l), visinin (a markers for rod and cone photoreceptors in red; h and i) or Hu (a marker for amacrine and ganglion cells). Cell types that were sonoporated include those with the morphology of cones (a), rods (b), ganglion cells (c), bipolar cells (d) and progenitors (e and f). The arrows in panel c indicate the axon of a ganglion cell. In panel f, DAPI (blue) was used to label the nuclei of cells; the sonoporated progenitor cells (green) are organized into a rosette. In panels g–l, arrows indicate cells that are double-labeled for eGFP and neuronal markers. The calibration bar (50 µm) in panel e applies to a–e, the bar in f applies to f alone and the bar in l applies to g–l. “For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article”.

after 1 day in culture or transfected the cells with Lipofectamine 2000<sup>TM</sup> or FuGene6<sup>TM</sup>.

We found that sonoporation of free-floating cells resulted in numbers of transfected cells greater than those observed for Lipfectamine 2000<sup>TM</sup>, but comparable to FuGene6<sup>TM</sup>-mediated transfection (Fig. 5a).

However, numbers of adherent versus free-floating cells transfected with identical sonication parameters differed significantly ( $p < 0.01$ ), with nearly a three-fold increase in the number of eGFP-positive cells observed in sonoporated adherent-cell preparations (Fig. 5a).



We compared the types of cells that were sonoporated in free-floating preparations to the types of cells that were transfected in adherent-cell preparation with sonoporation, liposomes, AAV1, AAV2 or AdV5. The cells used in these experiments were derived from the same preparation of E7 retina that was dissociated and pooled prior to allocation to the free-floating and adherent-cell paradigms. For this and subsequent experiments, we identified neuronal cells based on their morphology and their lack of vimentin expression, assuming that all progenitors and glial cells were vimentin-positive and all neuronal cells were vimentin-negative. The AAV1 and AAV2 added at  $10^6$  PFU per well failed to transfect any chick retinal cells. The vast majority of the cells transfected with Lipofectamine<sup>TM</sup>, FuGene6<sup>TM</sup> or AdV5 were large, more than 20  $\mu\text{m}$  in diameter across the somata and flat, having the morphology of progenitor or fibroblastic cells (Fig. 5b, d and e). None of cells transfect via Lipofectamine 2000<sup>TM</sup> had neuronal morphology, less than 5% of cells transfected with FuGene6<sup>TM</sup> and less than 40% of the cells transfected with AdV5 had neuronal morphology (Fig. 5b). By contrast, nearly, 95% of the cells that were sonoporated as free-floating cells had a neuronal morphology (Fig. 5b and f–g). Most of these cells had small somata (<10  $\mu\text{m}$  in diameter), formed elaborate peripheral processes and were unipolar (Fig. 5f) or multipolar (Fig. 5g). Some sonoporated neurons included cells with the morphology of ganglion cells, with numerous local processes and a lengthy (>400  $\mu\text{m}$ ) primary axon (Fig. 5h). By comparison, in adherent-cell cultures of retinal cells that were sonoporated with the

12 mm probe, about 45% of the transfected cells had neuronal morphology (Fig. 5b).

We tested whether transfection efficacy of the free-floating cells could be increased by increasing the sonication and plasmid concentration. Accordingly, we dissociated E7 retinal cells, added pCAX-eGFP at 10  $\mu\text{g}/\text{ml}$  to 2 ml of cells (at 200,000 cells/ml) suspended in culture media and sonoporated the cells with the 2 mm probe at 0.5 or 2 W for different lengths of time. one ml or about 200,000 cells, of the sonicated or control (not sonicated) cells were plated per well. The abundance of eGFP-positive cells increased nearly 60% and this increase was not influenced by reducing the duty cycle of the sonication to 50% (0.5 Hz; Fig. 5c). By comparison, sonication at 2 W was less effective, with less than 5 cells/1.5  $\text{mm}^2$  that were eGFP-positive after 10 s of sonication (Fig. 5c). This resulted from a large reduction in the number of cells that survived exposure to 10 s of sonication at 2 W.

In free-floating preparations retinal cells survived higher levels of sonication compared to retinal cells grown adherent to a substrate. There was no difference in the density of cells that were untreated and those that were sonoporated with the 2 mm probe for 30 s at 0.5 W. This finding suggests that low-energy sonoporation of free-floating retinal cells results in little or no cell death. By contrast, sonoporation at 2 W for 10 s resulted in a 60% reduction in the density of cells that survived in culture. Not surprisingly and in line with the underlying design of the sonicator, higher energy levels (>2 W) resulted in wide-spread destruction of cells. Sonoporation of free-floating cells with the 12 mm probe at 2.5 W

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**Fig. 5.** Sonoporation of free-floating retinal cells preferentially transfects neuronal cells. Freshly dissociated E7 chick retinal cells were suspended in culture media added with pCAX-eGFP and sonicated between 4 and 30 s at 0.5 or 2.0 W. Cells were plated at 200,000 cells/cm<sup>2</sup> to compare sonoporation of free-floating cells with adherent-cell cultures that were transfected via sonoporation or with Lipofectamine 2000<sup>TM</sup>, FuGene6<sup>TM</sup> or adenovirus serotype 5 (AdV5). The histogram in panel a illustrates the relative number of cells per 1.5  $\text{mm}^2$  that were added with 2  $\mu\text{g}$  of pCAX-eGFP and transfected with Lipofectamine 2000<sup>TM</sup>, Fugene6<sup>TM</sup>,  $10^6$  PFU AdV5 or sonoporation (for free-floating and adherent cells) with the 2 mm probe at 0.5 W for 5 s. Retinal cells were derived from the same pool of cells. Panel b illustrates the percentage of cells with neuronal morphology that were transfected with pCAX-eGFP via Lipofectamine 2000<sup>TM</sup>, Fugene6<sup>TM</sup>,  $10^6$  PFU AdV5, sonoporation of free-floating cells with the 2 mm probe at 0.5 W for 30 s or sonoporation of adherent cells with the 12 mm probe at 2.5 W for 2 s. For this experiment, retinal cells were derived from the same pool of cells. Panel c illustrates the number of cells per 1.5  $\text{mm}^2$  that are eGFP-positive with different sonoporation conditions. Cells were suspended at 200,000 cells/ml of media, added with 10  $\mu\text{g}/\text{ml}$  of pCAX-eGFP and sonicated using the 2 mm probe at 0.5 or 2 W for 4–30 s or the 12 mm probe at 2.5 W. Intermittent sonoporation was done by applying sonication with a duty cycle of 0.5 Hz. The non-neuronal cells that were transfected with FuGene6<sup>TM</sup> (d) or AdV5 (e) were easily distinguished from the neuronal cells. The vast majority of free-floating cells that were transfected via sonoporation had neuronal morphology, including amacrine-like cells (f and g) and ganglion cells that produced an axon (indicated by the arrows) that often coursed hundreds of micrometers across the coverglass (h). The arrows indicate neuronal cells and the arrow-heads indicate non-neuronal cells. The calibration bar (50  $\mu\text{m}$ ) in panel g applies to panels d–g and the bar in h applies to h alone.

for 3–10 s produced similar results as the 2 mm probe (data not shown).

Some cultures of sonoporated retinal cells were maintained for 2 weeks after treatment. There was no obvious decrease in the number of eGFP-expressing neurons and these cells formed elaborate and extensive dendritic and axonal processes (data not shown). These findings suggest that sonoporation-mediated gene transfer does not impact negatively on neuronal survival and the cellular functions required for neurite extension.

### 3.6. Sonoporation of chick tectal and forebrain cells

Since sonoporation of free-floating preparations of chick retinal cells preferentially transfected neurons (Fig. 5b), we assayed whether preferential transfection of neurons occurred with the sonoporation of cells from other regions of the chick brain. Forebrain and tectal cells were obtained from E7 chick embryos and sonoporated as free-floating and adherent cells. In preparations of tectal cells, about 30% of the total number of cells were non-neuronal vimentin-positive cells and about 60% of the cells in forebrain preparations were non-neuronal.

We found that the abundance and survival of sonoporated cells differed with the sonoporation conditions and between the cell types (Fig. 6). For comparison with sonoporation of free-floating preparations, we assayed whether adherent preparations of tectal and forebrain cells were amenable to transfection via sonoporation. Two days after plating, when cells were between 70 and 80% confluent, tectal and forebrain cells were added with 10 µg/ml pCAX-eGFP and sonicated for 2 s at 2.5 W using the 12 mm probe, conditions that were optimal for transfection of adherent retinal cells. In cultures of tectal cells, we found relatively few eGFP-positive cells ( $5.9 \pm 1.5$  cells/ $1.5 \text{ mm}^2$ ; Fig. 6c). About 70% of the eGFP-positive tectal cells were non-neuronal (Fig. 6d). In addition, the survival of the tectal cells was greatly reduced; about 85% of the sonoporated tectal cells did not survive (Fig. 6e). In cultures of forebrain cells, we found eGFP-positive cells ( $10.0 \pm 2.6$  cells/ $1.5 \text{ mm}^2$ ) and about 85% of these cells were non-neuronal, while the remaining 15% were neuronal (Fig. 6d). Furthermore, sonoporation dramatically reduced the survival of the forebrain cells by

more than 80% (Fig. 6e). By comparison, sonoporation of free-floating tectal and forebrain cells resulted in greater numbers of transfected cells accompanied by increased survival (Fig. 6). The abundance of transfected cells was highest for free-floating preparations of tectal and forebrain cells that were sonoporated with the 12 mm probe for 5 s at 2.5 W (Fig. 6a). Transfection efficacy was maximal using the 12 mm probe at 2.5 W for 5 s for both tectal ( $3.3 \pm 0.7\%$ ) and forebrain cells ( $5.6 \pm 0.8\%$ ).

For free-floating preparations of both tectal and forebrain cells, the majority of the sonoporated cells were neuronal, having small somata ( $<10 \mu\text{m}$ ) and forming elaborate peripheral processes (Fig. 6a and b). The non-neuronal cells had large somata ( $>20 \mu\text{m}$  in diameter), large nuclei, flattened morphology and formed few peripheral processes (arrow-heads in Fig. 6b). Regardless of probe tip, duration of sonication and energy delivered, the percentage of sonoporated tectal cells that were neuronal was about 80% for tectal cells and 60% for forebrain cells (Fig. 6b). The relative percentage of neuronal to non-neuronal cells that were sonoporated in adherent-cell preparations was significantly lower compared to the percentage of neuronal cells that were sonoporated in free-floating preparations (Fig. 6b).

Similar to the sonoporation of retinal cells, increases in the duration and energy delivered reduced the survival of tectal and forebrain cells (Fig. 6e). The survival of tectal and forebrain cells that were sonoporated as adherent cells by using the 12 mm probe at 2.5 W for 2 s was greatly reduced (>80%) compared to cells that were not sonicated (Fig. 6e). By contrast, the majority (nearly, 90%) of the tectal and forebrain cells survived sonication with the 2 mm probe at 0.5 W for 30 s (Fig. 6e). Increasing the energy delivered from 0.5 to 1 W decreased the survival of tectal cells by nearly, 70% (Fig. 6e), while the survival of forebrain cells was decreased by only 15% (Fig. 6e). Sonoporation of cells using the 12 mm probe at 2.5 W for 5 s resulted in approximately a 50% decrease in survival (Fig. 6e). Survival was further decreased by increasing the duration of sonication from 5 to 12 s (Fig. 6e).

### 3.7. Sonoporation of PC12 cells

We assayed the efficacy of sonoporation for PC12 cells, a widely used neuronal cell line, in suspension

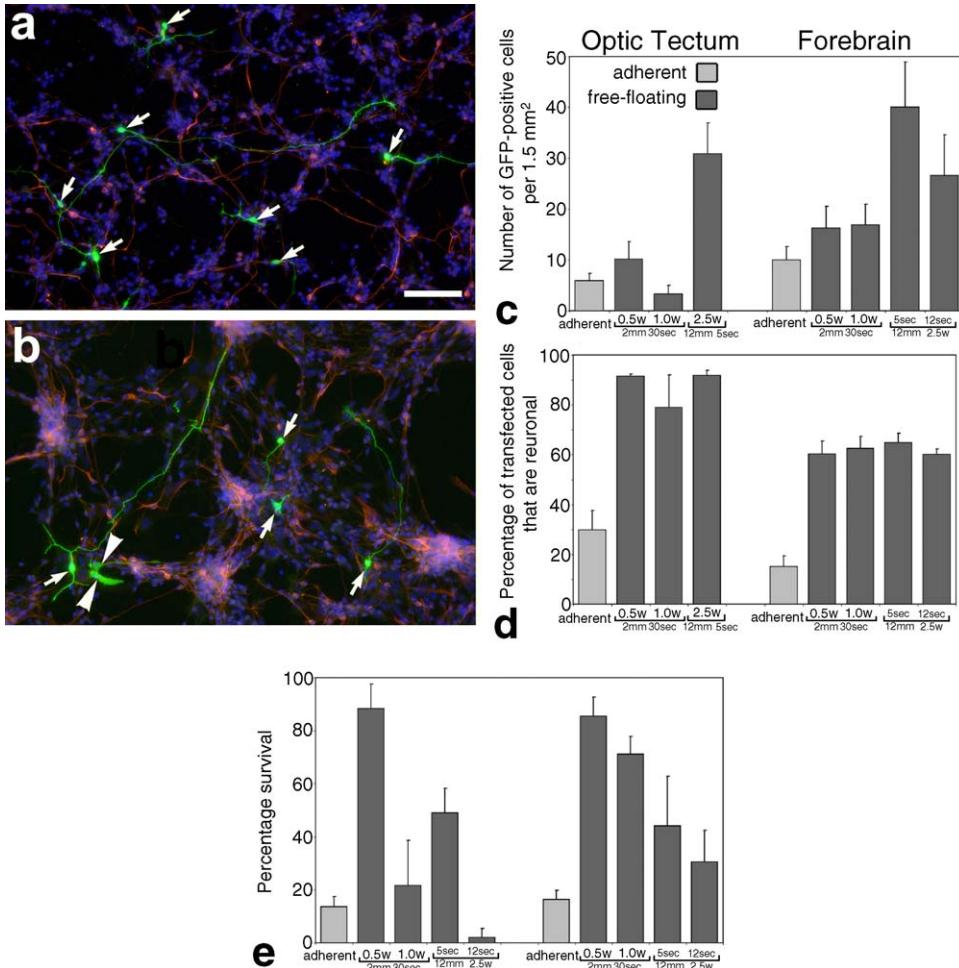


Fig. 6. Comparison of sonoporation of tectal (a) and forebrain cells (b) as adherent cultures or as free-floating preparations. Sonoporation of adherent cells was done by using the 12 mm probe at 2.5 W for 2 s and sonoporation of free-floating cells was done by using the 2 or 12 mm probes at 0.5–2.5 W for 5–30 s. All cells were maintained in culture for 2 days after sonoporation prior to fixation and processing for immunolabeling. Cells were fixed and labeled with DAPI (blue) and antibodies to neurofilament (red) or eGFP (green) (a and b). The arrows in panels a and b indicate the somata of sonoporated cells and the arrow-heads in b indicates glial or progenitor cells. The calibration bar (50  $\mu\text{m}$ ) in panel a applies to a and b. Panel c is a histogram illustrating the number of eGFP-positive cells per  $1.5 \text{ mm}^2$  that were sonoporated as adherent cells or as free-floating cells. Panel d is a histogram illustrating the percentage of sonoporated cells that are neuronal. The neuronal cells were distinguished from the non-neuronal cells based on their morphology, small cell body (<10  $\mu\text{m}$  in diameter) and elaborated peripheral processes (see Fig. 7b). Panel e is a histogram illustrating the percentage of tectal or forebrain cells that survive sonoporation. The percentage of surviving cells was determined as the number of DAPI-labeled cells (excluding those that were pyknotic)/10,000  $\mu\text{m}^2$  after 2 days of culture in after sonication as a percentage of the number of DAPI-labeled cells/10,000  $\mu\text{m}^2$  that were not sonicated. Values were calculated as the mean and standard deviation for each data set. “For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article”.

and in adherent-cell preparations. On adherent-cell preparations using the 12 mm probe at 2.5 W for 3 s,  $5.2 \pm 3.8\%$  of the PC12 cells were transfected. However, the survival of the sonoporated adherent PC12 cells was low; nearly 70% of the cells did not sur-

vive (Fig. 7d). We found that the transfection efficacy and the survival of PC12 cells was increased with sonoporation of free-floating preparations compared to sonoporation of adherent-cell preparations. Although the abundance of PC12 cells transfected in suspension

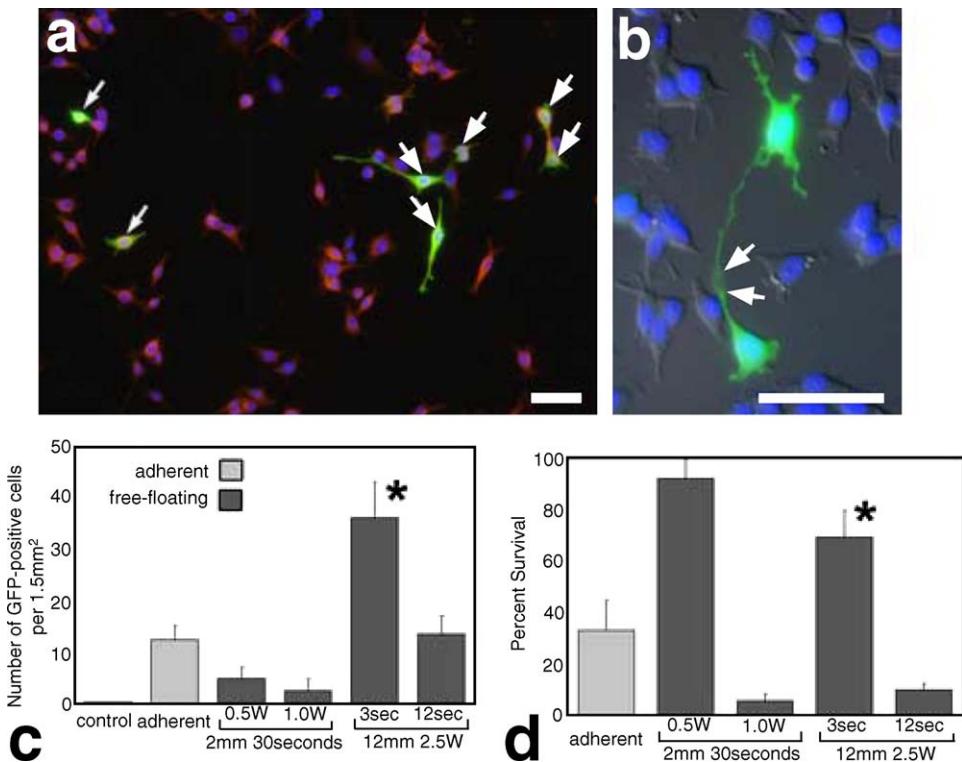


Fig. 7. Sonoporation of PC12 cells. For adherent preparations, PC12 cells were added with 10  $\mu\text{g}/\text{ml}$  pCAX-eGFP and sonoporated using the 12 mm probe for 3 s at 2.5 W. For free-floating preparations, PC12 cells were added with 10  $\mu\text{g}/\text{ml}$  pCAX-eGFP and sonoporated using the 2 or 12 mm probe for 3–30 s at 0.5–10 W; 48 h after sonoporation, cells were processed for labeling with DAPI (blue; a and b) and antibodies to neurofilament (red; a) and eGFP (green; b). The image in panel b combines fluorescence microscopy with DIC bright-field optics. The arrows in a indicate sonoporated cells that are labeled with DAPI and antibodies to neurofilament and eGFP. The arrows in b indicate growth cones. The calibration bars represent 50  $\mu\text{m}$ . Panel c is a histogram illustrating the number of eGFP-positive cells per  $1.5 \text{ mm}^2$  that resulted from different sonoporation conditions. Panel d is a histogram illustrating the percent survival following different sonoporation paradigms. Percent survival was determined as the number of DAPI-positive cells/ $10,000 \mu\text{m}^2$  that were observed 2 days after sonoporation compared to the number of DAPI-positive cells that were not sonoporated. Significance ( $^*p < 0.0001$ ) of difference was assessed by using ANOVA and a post hoc Student's *t*-test to compare the mean values between adherent and free-floating cells that were sonoporated using the 12 mm probe for 3 s at 2.5 W. "For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article".

was low ( $5.1 \pm 2.2$  cells/ $1.5 \text{ mm}^2$ ) for the 2 mm probe, survival was more than 90% at 0.5 W (Fig. 7c and d). The survival of free-floating PC12 cells was greatly reduced by increasing the duration and the energy of the sonication (Fig. 7). For example, the survival of the PC12 cells was reduced more than 80% with a two-fold increase in the energy delivered with the 2 mm probe over 30 s (Fig. 7d). Similarly, the survival of the free-floating PC12 cells was reduced nearly 60% with a four-fold increase in the duration of sonication with the 12 mm probe at 2.5 W (Fig. 7d). The abundance ( $36.4 \pm 7.6$  cells/ $1.5 \text{ mm}^2$ ), survival (nearly 70%) and

the transfection efficacy ( $7.8 \pm 1.9\%$ ) of sonoporated free-floating PC12 cells was optimal with use of the 12 mm probe at 2.5 W for 3 s in free-floating preparations. Although the transfection efficacy ( $10.9 \pm 2.0\%$ ) was higher for sonoporation using the 12 mm probe at 2.5 W for 12 s instead of 3 s, the survival of the cells was significantly reduced with the longer period of sonication (Fig. 7c and d). PC12 cells that were sonoporated as free-floating preparations extended processes and formed growth cones (Fig. 7a and b), indicating that sonoporation did not disrupt the mechanisms involved in these cellular activities.

We found that the transfection efficacy of adherent PC12 cells was much higher using Lipofectamine 2000<sup>TM</sup> compared to sonoporation. The efficacy of Lipofectamine was  $46.2 \pm 5.1\%$  compared to nearly, 11% with sonoporation of free-floating PC12 cells using the 12 mm probe for 8 s at 2.5 W. These findings suggest that PC12 cells, unlike primary neuronal cultures, are more amenable to transfection via Lipofectamine compared to sonoporation.

### 3.8. Sonoporation of primary cultures of mammalian neurons

To assay whether primary cultures of mammalian neurons could be transfected via sonoporation, we prepared cultures of postnatal rat cerebellar neurons and mouse hippocampal neurons. In adherent preparations of rat cerebellar cells (plated at 150,000 cells/cm<sup>2</sup>) that were sonicated with the 12 mm probe at 2.5 W for 3 s, we found relatively few ( $14.2 \pm 5.4$  cells/1.5 mm<sup>2</sup>) eGFP-positive cerebellar cells. Nearly, 60% ( $58.2 \pm 8.3$ ) of the eGFP-positive neurons were small (about 6  $\mu\text{m}$  in diameter) and formed many short (<50  $\mu\text{m}$ ) processes. Since sonoporation of free-floating retinal, tectal and forebrain cells proved to be effective and neuron-preferring, we tested whether sonoporation of free-floating rat cerebellar cells was effective. Dissociated cerebellar cells were sonicated at a density of 150,000 cells/ml of media added with 10  $\mu\text{g}/\text{ml}$  pCAX-eGFP. We found that the transfection efficacy of cerebellar cells was greatly increased when these cells were sonoporated in free-floating preparations. Although few cells (about two per coverslip) expressed eGFP in unsonicated, control samples (Fig. 8a and g), we found numerous eGFP-expressing cells in sonicated samples (Fig. 8b–e and g). We found between 50 and 120 eGFP-positive cells/1.5 mm<sup>2</sup> (Fig. 8). The abundance of eGFP-expressing cells and transfection efficacy ( $11.2 \pm 3.1\%$ ) was highest with sonication using the 12 mm probe at 2.5 W for 6 s (Fig. 8e and g). Sonoporation of free-floating cerebellar cells preferentially transfected neuronal cells. Despite a relatively high abundance (about 40%) of glia in preparations of cerebellar cells, more than 95% of the cells that were transfected were neuronal with all four sonoporation conditions (Fig. 8f). Sonoporation conditions that resulted in higher numbers of transfected cells also

resulted in decreased survival. Although the survival of cerebellar cells was modestly reduced (about 20%) by sonication with the 2 mm probe at 0.5 W for 30 s, increased energy levels reduced survival by more than 50% (Fig. 8h).

We next assayed whether adherent cultures of mouse hippocampal cells could be transfected via sonoporation. Cells were maintained as adherent cells in culture for 18 days prior to sonoporation. We found few neurons that were transfected; about 7–10 neurons per coverslip. By comparison, we found between 20 and 40 glial cells per coverslip that were sonoporated. All of the neurons that were transfected had elaborate dendritic arbors that were covered with spines (Fig. 9a and b). Sonoporation of acutely dissociated free-floating hippocampal cells resulted in increased numbers of sonoporated neurons (40–60 per coverslip). Similar to free-floating preparations of chick neurons and rat cerebellar cells, sonoporation of free-floating mouse hippocampal cells resulted in preferential transfection of neuronal cells. Nearly two-thirds ( $65.6 \pm 5.8\%$ ) of the transfected hippocampal cells were neuronal, while one-third were glial (Fig. 9c and d).

## 4. Discussion

Here, we report for the first time that a standard laboratory sonicator, designed to disrupt cells and homogenize solutions, can be applied to effectively transfer genes into neuronal cells. We found that sonoporation can be used to transfer plasmid DNA in different types of primary neuronal cells, including those from chick retina, forebrain, optic tectum, rat cerebellum and mouse hippocampus.

Sonoporation may be a preferred method of gene transfer for physiological studies. Since liposome-mediated gene transfer involves the fusion of synthetic lipids into the plasmid membrane, this mode of gene transfer may interfere with physiological recordings and the behavior of proteins that insert into the cell membrane. Therefore, sonoporation of neurons may be a preferable alternative to liposome-mediated gene transfer for studying the forced-expression of transmembrane proteins. In addition, sonoporation of free-floating cells preferentially transfets neuronal cells, thereby facilitating experimental manipulations of neuronal cells. Transfection of neuronal cells

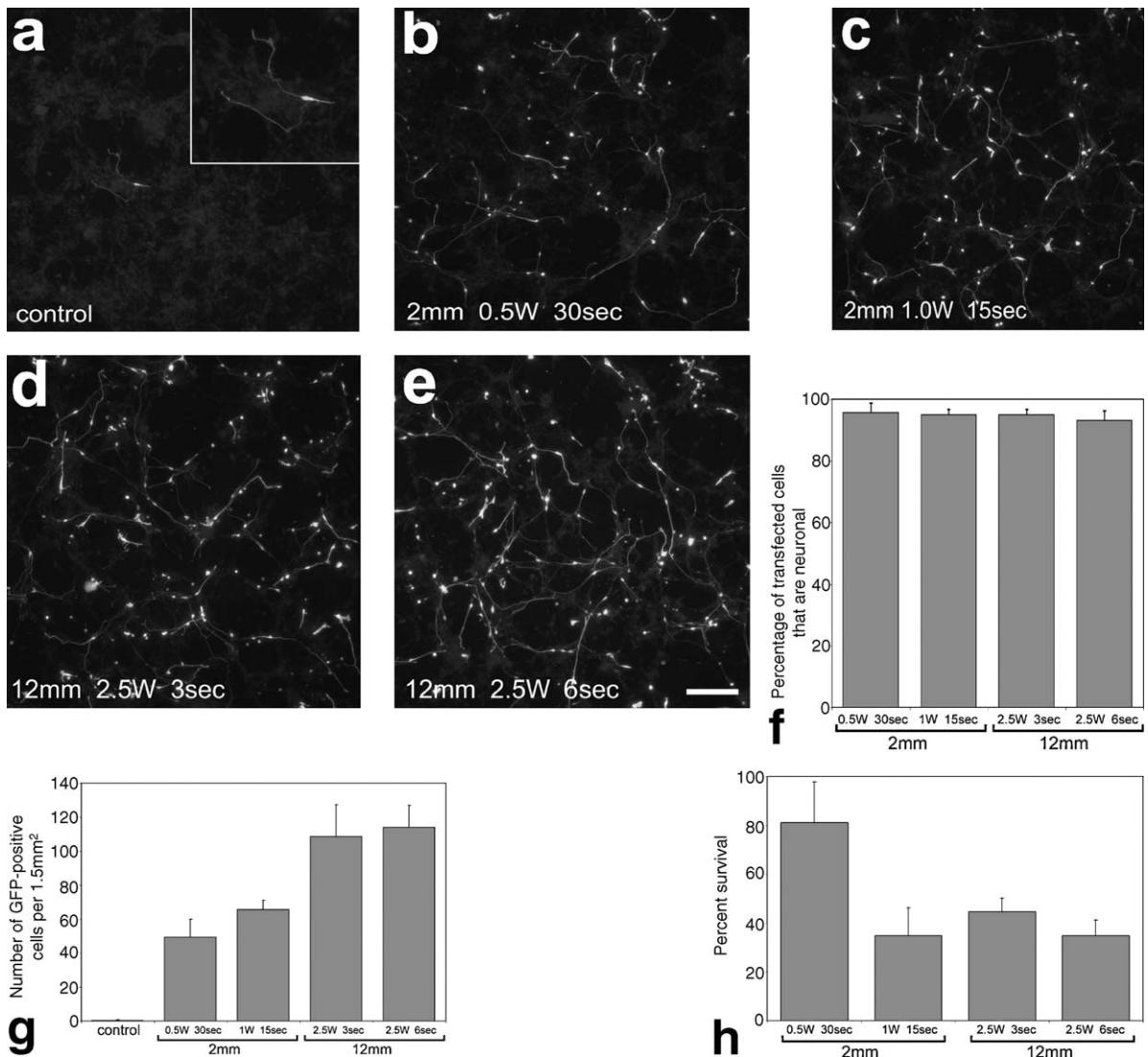
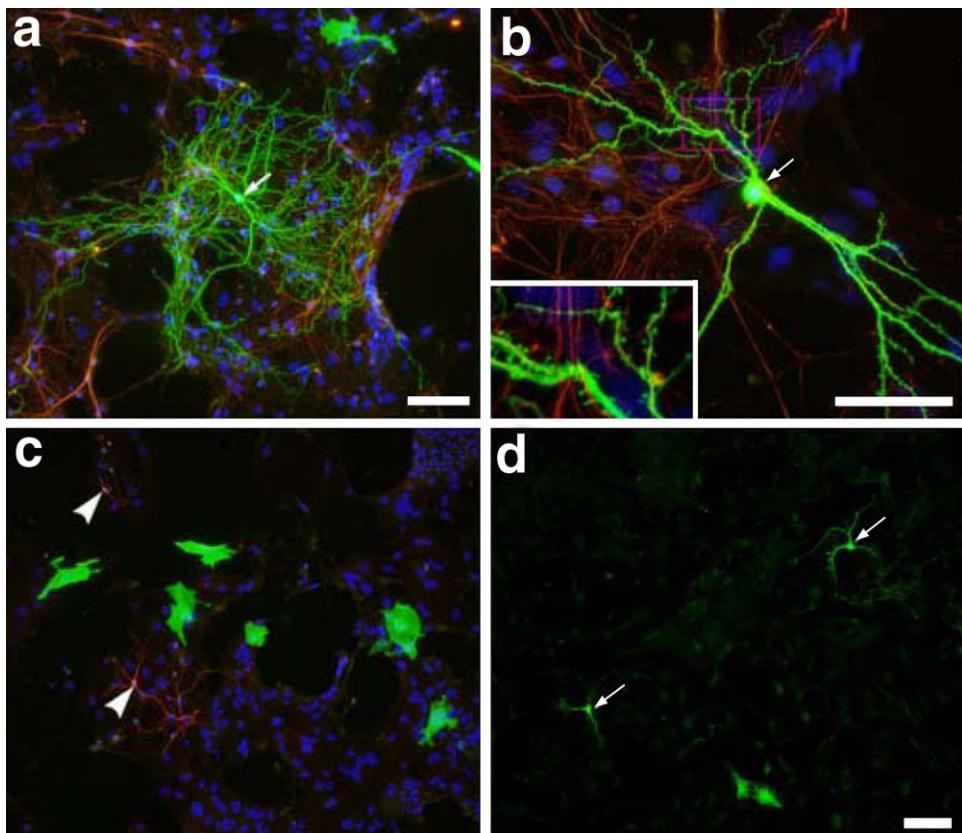


Fig. 8. Sonoporation effectively transfects free-floating rat cerebellar neurons. Dissociated cells from P2 rat cerebellum were suspended at 150,000 cells/ml of media, added 10 µg/ml pCAX-eGFP and sonoporated for 3–30 s at 0.5–2.5 W using the 2 or 12 mm probes. Two days after sonoporation cells were fixed and processed for immunolabeling. The calibration bar (50 µm) in panel e applies to panels a–e. Panel f is a histogram that demonstrates that the relative number of GFP-positive cells is greater than 95% regardless of the sonoporation conditions. Panel g is a histogram that illustrating the abundance of GFP-positive cells resulting from different sonoporation conditions. Panel h is a histogram illustrating the percent survival following sonoporation. Survival was calculated as the number of DAPI-labeled cells (excluding pyknotic nuclei)/10,000 µm<sup>2</sup> that remained in culture 2 days after sonoporation as a percentage of the number of DAPI-labeled cells/10,000 µm<sup>2</sup>.

has been notoriously difficult (Washbourne and McAllister, 2002). Nevertheless, virus-based methods of gene transfer have been the most successful for the transfection of high percentages (>70%) of neuronal cells (Berry et al., 2001; Washbourne and McAllister,

2002). However, preparation of recombinant viruses is expensive, labor-intensive and can elicit immune responses. Sonoporation provides an alternative method of gene transfer for naked plasmid DNA that prefers neuronal cells over glial cells and may avoid



**Fig. 9.** Sonoporation effectively transfects mouse hippocampal neurons. In panels a and b, cells were maintained in culture for 18 days, added with 10 µg/ml pCAX-eGFP and sonoporated as adherent cells using the 2 and 12 mm probes. Three days after sonoporation, cells were fixed and processed for labeling with DAPI (blue) and antibodies to neurofilament (red) and eGFP (green). The inset in panel b is an enlarged field of view (from the area indicated by the pink box) to indicate the presence of dendritic spines on the sonoporated neuron. In panels c and d, acutely dissociated hippocampal cells were added with 10 µg/ml pCAX-eGFP and sonicated as free-floating cells using the 12 mm probe for 5 s at 2.5 W. Five days after sonoporation, cells were fixed and processed for labeling with DAPI (blue) and antibodies to neurofilament (red) and eGFP (green). Arrows indicate the somata of sonoporated neurons and arrow-heads indicate the somata of neurofilament-immunoreactive neurons that were not sonoporated. The calibration bar (50 µm) in panel a applies to a alone, the bar in b applies to b alone and the bar in d applies to c and d. “For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article”.

unwanted side-effects that can result from alternative methods of transfection.

Our findings suggest that the efficacy of sonoporation is variable for different cell types and optimal conditions of sonoporation between the different cell types are variable. We found, for example, that transfection efficacy was optimal for adherent retinal cells, while the transfection efficacy for tectal and forebrain cells was better for free-floating preparations. Similarly, we found that sonoporation of PC12 was relatively ineffective compared to Lipofectamine, while Lipofectamine was ineffective in primary cultures of

retinal cells compared to transfection via sonoporation. We did not exhaustively vary the sonoporation conditions, and there are likely to be conditions that yield levels of transfection efficacy that are higher than those that we observed. In line with our observations, optimal transfection parameters are variable for the electroporation of different cell types (Mertz et al., 2002; Swartz et al., 2001; Washbourne and McAllister, 2002).

Regardless of the type of neuron, sonoporation of free-floating cells was highly selective for neuronal versus glial cells. For example, the percentage of the total number of transfected chicken retinal cells that

was neuronal was much higher for sonoporation compared to the percentages of neuronal cells that were transfected via adenovirus, Lipofectamine 2000<sup>TM</sup> and FuGene6<sup>TM</sup>. It should be noted that viral methods of gene transfer effectively transfect significant numbers of neurons in different regions of the central nervous system (Berry et al., 2001; Washbourne and McAllister, 2002). It remains uncertain how viral gene transfer into rodent hippocampal cells, for example, compares to sonoporation for selective transfection of neurons versus glia. The relative percentage of sonoporated cells that were neuronal varied between the tissue of origin, but was independent of the energy delivered. For example, we found that the transfection efficacy for retinal cells was highest for adherent-cell preparations, whereas the transfection efficacy for tectal and forebrain cells was highest for free-floating preparations. In addition, the relative percentage of neuronal cells that were sonoporated varied between cell types. For example, regardless of sonoporation conditions, about 95% of retinal cells, 80% of tectal cells and 60% of forebrain cells that were sonoporated were neuronal. Assuming that the relative abundance of non-neuronal cells is similar in the developing retina, tectum and forebrain, these findings suggest that the preferential sonoporation of neuronal cells from different tissue is variable and may depend on the physical properties of the neuronal types that are found in those tissues. The morphological types of neurons that are found in the retina, tecta and dorsal forebrain are distinctly different in each of these tissues.

The mechanisms underlying the preferred transfection of neuronal cells via sonoporation remain unknown. Sonoporation is believed to facilitate the entry of macromolecules into cells via microbubble-mediated cavitation and transient disruption of the plasma membrane (Marmottant and Hilgenfeldt, 2003; Miller et al., 2002). It is possible that the frequency and intensity of the ultrasound may select for particular cell types based on the diameter of the cell body. For example, our findings indicate that PC12 cells, which have a large diameter (about 20  $\mu\text{m}$ ), have attenuated survival with relatively low levels of sonication and are transfected with lower efficacy than primary neuronal cells with small diameters. In addition, our data indicate that the physical state of the cell may influence sonoporation. For example, sonoporation of free-floating cells versus adherent cells derived from the same pool of

cells resulted in preferential transfection of different types, smaller neuronal cells for free-floating preparation versus larger glial/progenitors cells for adherent-cell preparations. Similarly, the physical state of the cells influences survival following exposure to sonication. For example, there was no loss in cell density when free-floating retinal cells were sonicated with the 2 mm probe for 30 s at 1 W, whereas there was decreased survival when these sonication parameters, where applied to adherent retinal cells. The mechanisms underlying the elevated tolerance of free-floating cells to sonication requires further investigation.

Sonoporation-mediated gene transfer does not appear to disrupt basic neuronal functions such as neurite outgrowth and long-term survival. We found that sonoporated cells maintained transgene expression for at least 2 weeks after sonication. In addition, freshly dissociated free-floating cells had all peripheral processes removed, yet after sonication, plating and maintenance in culture the transfected neurons formed extensive neurites, indicating that sonoporation does not impact negatively upon normal cellular functions such as the outgrowth of dendrites and axons. In addition, we found sonoporated neurons that had well developed dendritic and axonal arbors, such as those observed with the sonoporation of adherent hippocampal pyramidal cells. This finding suggests that neurons with elaborate neurites can be transfected via sonoporation without physical disruption of the neurites. Taken together, these data suggest that sonoporation does not result in genomic instability or other forms of permanent cellular damage that would limit the utility of sonoporation to short-term applications. In addition, we propose that sonoporation would be particularly useful for biochemistry experiments of primary neuronal cultures, where glial transfection can cause considerable background.

Most reports investigating the potential of sonoporation to transfect cells have used high frequency ( $>1$  MHz) ultrasound (reviewed by Miller et al., 2002). In addition, most studies have found that ultrasound-mediated transfection efficacy is facilitated by the addition of microbubble-generating agents, compounds normally used in diagnostic ultrasound procedures to enhance contrast within fluid-filled cavities (reviewed by Miller et al., 2002). Recent studies have applied high frequency (210 kHz or 1 MHz) ultrasound and microbubble-generating agents to transfect

nervous tissue (Shimamura et al., 2004; Manome et al., 2005). However, these studies reported that very few or no neurons were transfected (Shimamura et al., 2004; Manome et al., 2005). Preliminary data indicate that the addition of the microbubble-generating agent Optison<sup>TM</sup> and plasmid DNA to retinal cell cultures did not significantly enhance the efficacy of sonoporation of neurons (data not shown). Further studies are required to assay the ability of high frequency ultrasound and microbubble agents to transfect primary neuronal cells.

We conclude that sonoporation is an effective means of gene transfer for primary cultures of neuronal cells. This method of gene transfer is simple, fast, inexpensive and utilizes a standard piece of laboratory equipment. Ultrasound-mediated gene transfer works well for adherent and free-floating preparations. We found that sonoporation of free-floating cells preferentially transfects neuronal cells, whereas conventional lipid-based and viral transfection methods preferentially transfect retinal progenitors, glia or fibroblastic cells. Neuronal cells are notoriously difficult to transfect effectively. Thus, sonoporation provides an alternative, effective means to transfer genes into neuronal cells in culture. We propose that sonoporation could be applied to the intact nervous system to transfer foreign DNA or other macromolecules for basic scientific research and possibly for therapeutic purposes.

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