

Electroporation-mediated gene transfer system applied to cultured CNS neurons

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Electroporation is effective in transferring foreign genes into immature neurons in intact brain tissue. We utilized this approach to transfect genes into developing rodent hippocampi. Transfected hippocampi were subsequently dissociated and allowed to differentiate in culture. By optimizing developmental stage of the hippocampus, promoters to drive the marker cDNA, and culture conditions, neurons kept strong expression of multiple marker genes for more than two weeks after electroporation. We could

also induce transient expression in mature neurons by combining electroporation of plasmids containing loxP-flanked stopper sequences and infection of Cre-producing recombinant adenoviruses. The system described here is useful in analyzing biological roles of multiple genes in specific stages of neuronal development. *NeuroReport* 15:971–975 © 2004 Lippincott Williams & Wilkins.

Key words: Adenovirus; Cre recombinase; Electroporation; Green fluorescent protein; Hippocampus; Transfection

INTRODUCTION

Efficient and convenient methods for introducing foreign genes into primary neurons in culture are required to efficiently screen a large number of candidate proteins for their effects on cellular processes, such as signal transduction, organelle transport, and synapse maturation. Several techniques have already been developed for introducing foreign genes into neurons. Microinjection [1] and particle-mediated gene transfer [2] are preferential methods to obtain a small number of transfected cells in culture. It is possible to obtain large numbers of transfected cells using lipid-mediated and calcium-phosphate-mediated transfection methods [3,4]. However, exposure of cultured neurons to solutions containing lipids or calcium-phosphate precipitates often induces neurotoxicity, especially when neurons were maintained without astroglial feeder cells. Recombinant adenoviruses can be utilized to express foreign genes in up to 90% of primary hippocampal neurons in culture [5]. However, the construction of adenoviruses is labor-intensive and long-term toxic effects should be taken into account.

Electroporation has been shown to be an effective method for transferring DNA into neurons in culture and in slices [6,7]. Recently, electroporation-induced gene transfer has been demonstrated in intact neural tissue of both chick and mouse embryos [8,9]. Here we describe a novel strategy to introduce foreign genes into hippocampal neuronal culture. Immature neurons within intact embryonic hippocampal tissue were transfected by electroporation and subsequently

dissociated and plated for long-term culture. Because foreign genes were already introduced into cells before plating, no perturbation of differentiating cells during culture was necessary. Induction of foreign genes at a given time point in culture can be achieved by electroporation of loxP-flanked stopper sequence-containing expression plasmids and subsequent expression of Cre-recombinase by infection of recombinant adenoviruses. Our studies suggest that the electroporation-based DNA transfection combined with Cre-loxP system is a powerful approach to study stage-specific functions of macromolecules in CNS neurons.

MATERIALS AND METHODS

Preparation of expression constructs: We generated expression vectors of enhanced green fluorescent protein (eGFP) and monomeric red fluorescent protein 1 (mRFP1) [10] under the control of cytomegalovirus immediate early gene (CMV) promoter (CMV-eGFP, CMV-mRFP1), chimeric promoter of CMV and β -actin (CAG promoter; CAG-eGFP, CAG-mRFP1) [11] and chicken β -actin promoter (β -actin-eGFP, β -actin-mRFP1) [12]. To enhance expression of tubulin $T\alpha 1$ promoter [13] we generated four tandem repeats of 1 kb enhancer sequences of $T\alpha 1$ promoter upstream of either eGFP or mRFP1 coding sequences ($T\alpha 1X4$ -eGFP and $T\alpha 1X4$ -mRFP1). A stopper fragment from plasmid AxCALNLZ [14] contains transcriptional/translational terminating signals with two loxP sites at the 5' and 3' ends. The stopper fragment was placed 5' to the eGFP coding sequences of

β -actin-eGFP plasmid to generate β -actin-loxPSTOP-eGFP. Coding region of eGFP-tagged PSD-Zip45 was replaced with eGFP sequences of β -actin-eGFP to generate β -actin-eGFP-PSD-Zip45. Coding region of eGFP was from pEGFP-N1 vector (Clontech, Palo Alto, CA, USA) and CMV promoter sequences were from pCMV β -Clontech). A plasmid encoding mRFP1 was provided by Dr. Tsien and Tubulin α 1 promoter was from Dr. Miller. Cre-producing recombinant adenoviruses (AxCANCre) [14,15] were provided by Dr. Okado.

Dissection of hippocampus and electroporation: Hippocampi were dissected from E15.5 mouse embryos and kept in cold Ca^{2+} - and Mg^{2+} -free Hank's balanced salt solution (HBSS: 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4 , 0.34 mM Na_2HPO_4 , 4.15 mM NaHCO_3 , 5 mM glucose, 1 mM sodium pyruvate, 15 mM Hepes, pH 7.4). Figure 1 shows a schematic view of the electroporation device. An electroporation chamber has a 2 mm² electrode surrounded by a silicone rubber sheet. A Millipore filter membrane was placed on the electrode and the gap between the electrode and the membrane was filled with HBSS. The dissected hippocampus was placed on the filter with a drop of HBSS and excess buffer was carefully removed. Expression plasmid constructs were dissolved in 5 μ l EP buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgCl_2 , 10 mM glucose, 20 mM Hepes, pH 7.4) at a concentration of 1 mg/ml and applied to the tissue fragment. A tungsten needle was placed just above the specimen. An electroporator (CUY21EDIT, Nepa gene, Japan) was used to apply five rectangular pulses (15 V, 5 ms duration with an interval of 995 ms) for 5 s. After the first train of electrical pulses, the direction of electrical field was reversed and the second train of five pulses was applied with identical settings.

Cell culture: After electroporation, hippocampi were incubated in cold HBSS for 20 min and mechanically dissociated. Dissociated cells were plated onto poly-L-lysine-coated coverslips or glass-bottom 35 mm dishes. Culture conditions were identical to our previous experiments with the E16.5 embryonic hippocampus [16]. In some experiments, intact hippocampi were placed on Millipore

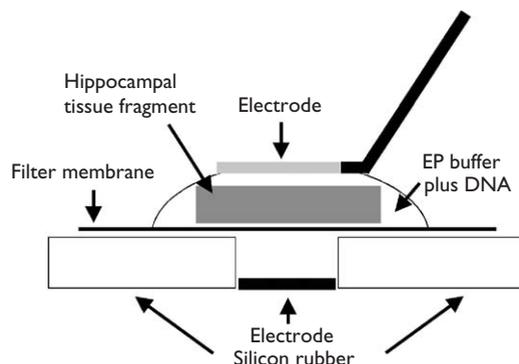


Fig. 1. Schematic representation of an electroporation set-up. A fragment of the mouse embryonic hippocampus was placed on a Millipore membrane filter and 5 μ l EP buffer containing 1 mg/ml of plasmid DNA was applied onto the tissue. A tungsten needle was attached to the surface of a droplet. After application of square pulses the tissue fragment was returned to a petri dish containing ice-cold HBSS solution.

filter membranes with DMEM supplemented with 10% FCS at 37°C in a humidified incubator with 5% CO_2 /95% air.

Immunocytochemistry and imaging: Cells were fixed in 2% paraformaldehyde in PBS for 30 min, extracted with 0.1% Triton X-100 for 5 min. Cells were blocked with 5% NGS and incubated with anti-GFP antibody (Molecular Probes, Eugene, OR, USA) and anti-MAP2 antibody (Sigma, St. Louis, MO, USA). The first antibody was visualized by secondary antibodies conjugated with Cy3 or Alexa488. Images were obtained using an Olympus Fluoview confocal scanning microscope with a $\times 60$ oil-immersion objective lens. Hippocampi maintained on Millipore filter membranes were fixed in 2% paraformaldehyde in PBS for 45 min, washed three times in PBS and placed at the bottom of glass-bottom 35 mm dishes. Images were obtained using the confocal microscope with a $\times 20$ objective. Time-lapse imaging of living neurons and retrospective immunocytochemistry was performed as described previously [17].

RESULTS

We initially evaluated electroporation efficiency of eGFP expression plasmids by culturing tissue fragments of embryonic hippocampi without dissociation on Millipore filter membranes for 2–3 days and subsequent visualization of eGFP-positive cells under a fluorescence microscope. We found that the level of eGFP expression was strongly dependent on promoters. Hippocampal tissue fragments transfected with CMV-eGFP showed weak expression of eGFP in non-neuronal cells. In contrast, transfection of CAG-eGFP, $\text{T}\alpha$ 1X4-eGFP, and β -actin-eGFP resulted in strong expression of eGFP in a large number of immature neurons (Fig. 2a–c). We measured the fluorescence intensity per unit area of hippocampal tissue fragments and there were significant differences in fluorescence intensity between CMV promoter and β -actin promoter and also between CMV promoter and CAG promoter (Fig. 2h). We next evaluated whether transfection efficiency is dependent on developmental stages. E15.5 mouse hippocampi showed higher transfection efficiency than E16.5 mouse hippocampi (Fig. 2i). Another important parameter of transfection efficiency was number and duration of electrical pulses applied to the tissue. To achieve higher transfection efficiency, multiple rectangular pulses of 5 V and 5 ms duration were necessary. However, >10 rectangular pulses induced acute swelling of the tissue close to the electrode and resulted in the decrease of viable cells after tissue dissociation. Therefore we utilized the protocol of 5 pulses of 5 V and 5 ms duration and subsequent application of another 5 pulses with reversed direction of electrical field.

We next evaluated survival and differentiation of transfected E15.5 hippocampal cells after dissociation and plating onto glass substrate coated with poly-L-lysine. In a culture condition of serum-containing medium without glial feeder cells, differentiation of hippocampal pyramidal neurons was comparable to that of the preparation without electroporation [16]. We determined transfection efficiency by anti-GFP staining of cells electroporated and maintained for 4 days after plating (Table 1). Among three promoters evaluated, the β -actin-eGFP construct showed the highest transfection efficiency. Two weeks after plating, pyramidal neurons transfected with β -actin-eGFP plasmid showed bright eGFP

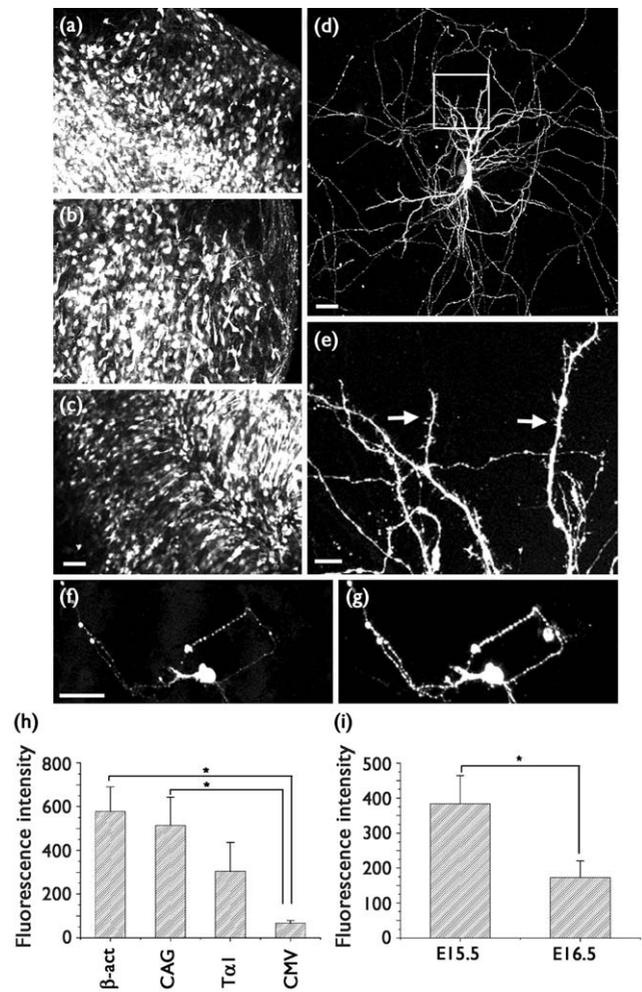


Fig. 2. Electroporation-mediated expression of fluorescent proteins in hippocampal neurons. (a–c) Organ culture of hippocampal tissue fragments three days after electroporation with CAG-eGFP (a), Tα1X4-eGFP (b), and β-actin-eGFP (c) expression constructs. (d,e) A mature hippocampal neuron maintained 14 days in dissociated culture after electroporation of a β-actin-eGFP expression construct. Higher magnification view of the region marked by a rectangle in (d) reveals dendritic spines on the surface of dendritic shafts in (e). (f,g) A hippocampal neuron 7 days after electroporation of 1:1 mixture of Tα1X4-eGFP and Tα1X4-mRFP1. Both eGFP fluorescence (f) and mRFP1 fluorescence (g) can be observed in a single cell. (h) Relative fluorescence intensity of hippocampal tissue fragments after electroporation of eGFP-expression plasmids with four different promoter sequences. The tissue fragments were maintained in culture for 4 days, fixed and observed under a confocal microscope. Fluorescence intensities per unit area of the tissue fragments were determined. (i) Relative fluorescence intensity of hippocampal tissue fragments isolated at two different developmental stages and electroporated with β-actin-eGFP. Tissue fragments were maintained for 4 days in culture and subsequently fixed. Fluorescence intensities were measured using a confocal microscope. Bars=50 μm (a–d,f,g); 10 μm (e).

fluorescence that filled all dendritic and axonal processes (Fig. 2d,e). Higher magnification view of the dendritic segments revealed mature morphology of dendrites including numerous spines protruding from dendritic shafts. Pyramidal neurons transfected with CAG-eGFP or Tα1X4-eGFP showed comparable levels of eGFP expression. In contrast, we could identify few eGFP-positive cells after

Table 1. Percentage of GFP-positive cells with different expression constructs.

Expression vector	Percentage of GFP(+) cells/total cells	Percentage of MAP2(+) cells/GFP(+) cells	Percentage of MAP2(–) flat cells/GFP(+) cells
β-actin	3.8%	84%	5.3%
CAG	1.1%	81%	6.3%
Tα1X4	0.7%	100%	0.0%

Cells were transfected with each expression construct and the number of transfected cells was measured 4 days after transfection. Neurons were identified by MAP2 immunoreactivity. Multiple samples ($n=4-8$) were examined to calculate the ratio of cells positive with GFP or MAP2.

electroporation of CMV-eGFP and a majority of these cells were non-neuronal. Electroporation with two independent plasmids encoding eGFP and mRFP1 under the control of Tα1X4 promoter sequences resulted in coexpression of both fluorescent proteins in the same cells (Fig. 2f,g). About 90% of GFP-positive cells also expressed mRFP1. Bright eGFP and mRFP1 fluorescence could be detected 2 days after transfection and persisted for > 2 weeks. A small number of glial cells were present in our culture preparation. In the case of CAG-eGFP, 6.3% of transfected cells showed flat morphology without expression of neuronal marker MAP2. These cells are likely to be immature glial cells in culture (Table 1). In contrast, no eGFP-positive cells showed flat morphology of glial cells after electroporation with Tα1X4-eGFP. This observation indicates the advantage of using Tα1X4 promoter for neuron specific expression of exogenous proteins. The level of eGFP fluorescence varied among individual neurons after electroporation-mediated transfection. Presence of transfected neurons showing low to moderate levels of eGFP fluorescence was advantageous for time-lapse imaging of eGFP-tagged proteins in living neurons. To see whether transfected neurons with low levels of expression are appropriate for imaging GFP-tagged postsynaptic molecules, we transfected hippocampal neurons with an expression construct of eGFP-tagged PSD-Zip45 (GFP-PSD-Zip45). PSD-Zip45 is a member of Homer family proteins localized at the postsynaptic density (PSD) and directly interacts with type I metabotropic glutamate receptors [18–20]. We performed time-lapse recording of pyramidal neurons 1 week after transfection (Fig. 3). Neurons expressing a low level of GFP-PSD-Zip45 showed a punctate pattern of GFP fluorescence, which was indistinguishable from endogenous distribution of PSD-Zip45. Time-lapse image sequences of GFP-PSD-Zip45 positive neurons revealed rapid remodeling of fluorescent clusters within dendrites. This observation was consistent with our previous data on the dynamics of GFP-PSD-Zip45 in hippocampal pyramidal neurons [17]. By infecting hippocampal neurons with Cre-producing recombinant adenoviruses, induction of gene expression from transfected DNA constructs containing loxP-flanked stopper sequences was possible. In this experimental paradigm, neurons were transfected with 1:1 mixture of β-actin-mRFP1 and β-actin-loxPSTOP-eGFP. Eight days after transfection, the neurons were exposed to Cre-producing recombinant adenoviruses. Two days after adenovirus infection, specific induction of eGFP fluorescence was observed in neurons expressing mRFP1 (Fig. 4). This induction of eGFP fluorescence was specific to the infected neurons and no GFP positive cells were observed in control culture.

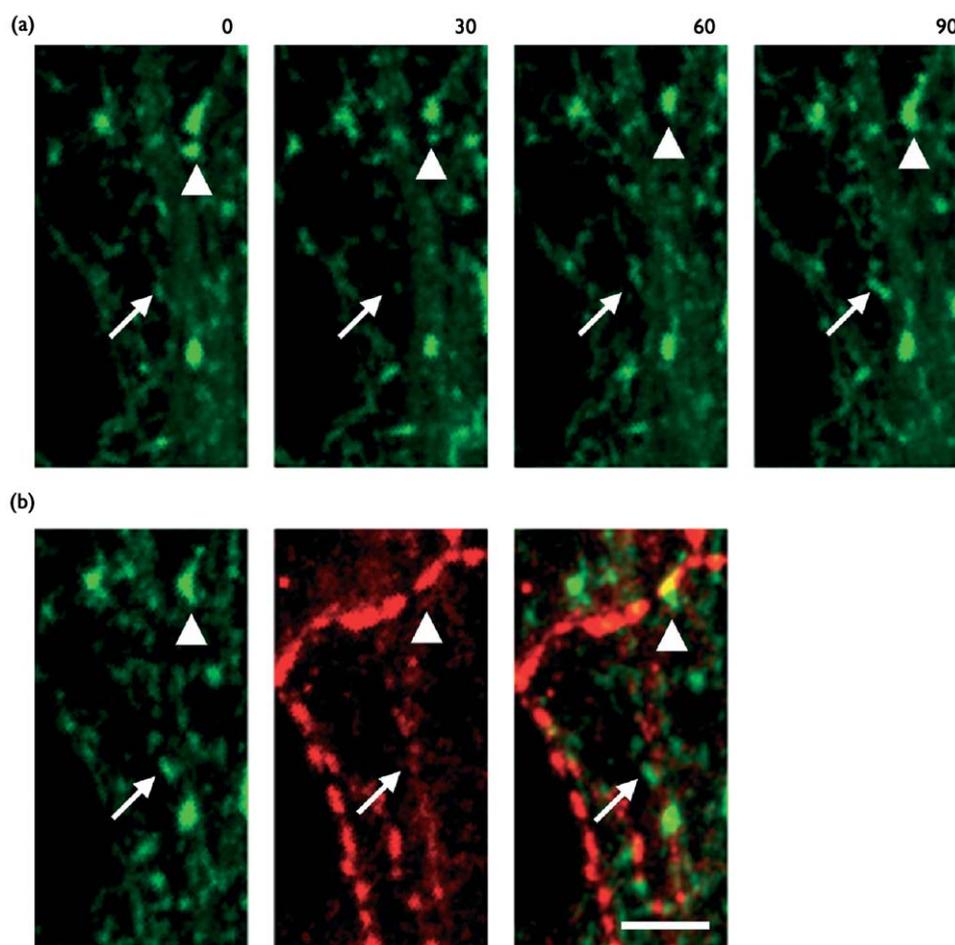


Fig. 3. Time-lapse confocal images of a neuron transfected with β -actin-GFP-PSD-Zip45 and maintained for 7 days in culture. (a) Formation of new PSD-Zip45 clusters within dendritic processes (arrows) and possible dissociation of preexisting clusters (arrowheads) were observed. Time stamps are shown in minutes in the top right corners. (b) Retrospective immunocytochemistry with anti-synapsin I antibody (red) revealed association of a newly formed PSD-Zip45 cluster with the synapsin I-immunoreactive axon (arrow in the merged color image). Bar=5 μ m.

DISCUSSION

In this study we applied electroporation-mediated transfection technique to deliver plasmid DNA into hippocampal neurons. By subsequent dissociation of transfected cells and culture in serum-containing medium, fully differentiated hippocampal excitatory neurons expressing variable levels of reporter molecules could be obtained. Efficient gene activation can be achieved by inserting loxP-flanked stopper sequences into the expression DNA constructs and subsequent Cre-mediated recombination using recombinant adenoviruses. This protocol offers the following advantages. (1) Toxicity to neurons is minimal. Because plasmid DNA is delivered to immature neurons before plating, no perturbation of cells by exchanging medium and exposure to toxic substances is necessary. (2) Co-expression of different proteins in the same cell is possible. After chemical transfection of two expression plasmids into primary neurons, only some of the transfected neurons express both proteins. We found that > 90% of neurons expressed two types of fluorescent proteins after electroporation of 1:1 mixture of plasmid DNA. Effects of multiple protein molecules on cellular processes can be easily analyzed in our expression system. (3) Induction of transfected genes is

possible by Cre-producing recombinant adenovirus [14,15]. The major drawback of persistent gene expression after electroporation is its pleiotropic effects on the early development of CNS neurons. In this sense, availability of gene activation system after neuronal maturation is important. By combining this approach with GFP-based imaging system, morphological changes of neurons before and after activation of transfected genes can be evaluated efficiently.

We identified three promoter sequences that can drive expression of exogenous genes in fully mature neurons maintained in culture. CMV promoter was not effective to support expression of eGFP. Other three promoter sequences, CAG, T α 1X4, and β -actin, supported comparable levels of eGFP in mature hippocampal neurons. Among these three promoter sequences, β -actin promoter showed slightly higher expression level of eGFP in hippocampal neurons maintained for > 2 weeks after plating. This observation is consistent with strong activity of β -actin promoter in CA1 pyramidal neurons in the postnatal period [12] and suggests its advantage in experiments analyzing long-term effects of transfected genes in mature hippocampal neurons.

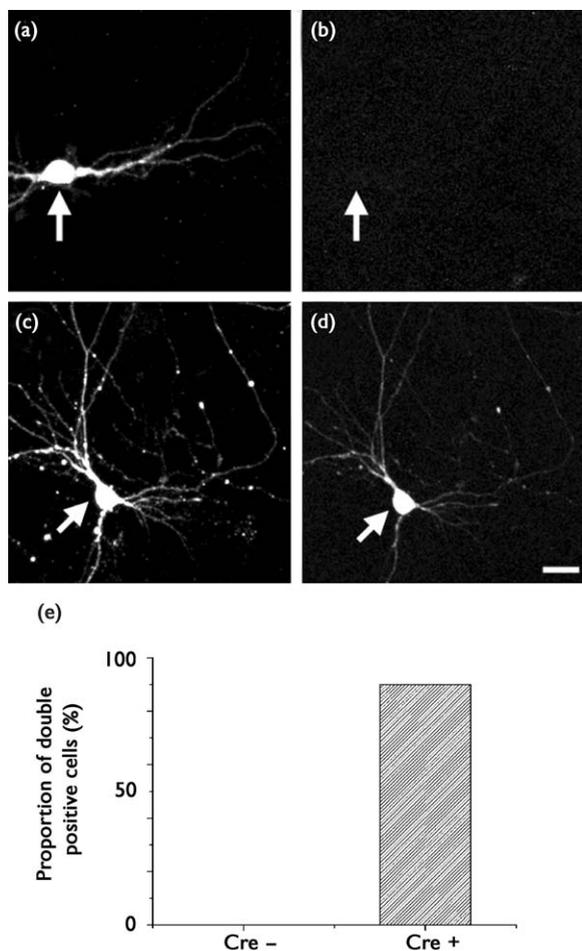


Fig. 4. Activation of a transfected gene by the expression of Cre-recombinase. (a,b) Absence of eGFP expression in an mRFPI-positive neuron (arrows) without infection of Cre-producing recombinant adenoviruses. Expression of mRFPI (a) suggests the presence of β -actin-loxPSTOP-eGFP DNA in the same cell, which did not show eGFP fluorescence (b). (c,d) Activation of β -actin-loxPSTOP-eGFP after infection of Cre-producing recombinant adenoviruses. A neuron positive with mRFPI fluorescence (arrow in c) also expressed eGFP (arrow in d). (e) Proportion of neurons double positive with eGFP and mRFPI fluorescence. Expression of Cre-recombinase induced expression of eGFP in 90% of neurons already expressing mRFPI. There were no double-positive hippocampal neurons in control culture preparations. Bar=25 μ m.

CONCLUSION

We have developed a protocol that transfer plasmid DNA molecules into intact embryonic hippocampi and allow stable expression of exogenous genes after long-term culture of dissociated neurons *in vitro*. In combination with the technique of gene activation by Cre-mediated excision of stopper sequences from introduced DNA, this protocol will enable new experimental designs especially for GFP-based real-time imaging.

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