

Abundant GFP Expression and LTP in Hippocampal Acute Slices by In Vivo Injection of Sindbis Virus

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D'Apuzzo, Massimo, Georgia Mandolesi, Gerald Reis, and Erin M. Schuman. Abundant GFP expression and LTP in hippocampal acute slices by in vivo injection of sindbis virus. *J Neurophysiol* 86: 1037–1042, 2001. Virus-mediated gene transfer into neurons is a powerful tool for the analysis of neuronal structure and function. Recombinant sindbis virus has been previously used to study protein function in hippocampal neuron cultures as well as in hippocampal organotypic slice cultures. Nevertheless, some concern still exists about the physiological relevance of these cultured preparations. Acute hippocampal slices are a widely used preparation for the study of synaptic transmission, but currently recombinant gene delivery is usually achieved only through time-consuming transgenic techniques. In this study, we show that a subregion of the CA1 area in acute hippocampal slices can be specifically altered to express a gene of interest. A sindbis virus vector carrying an enhanced green fluorescent protein (EGFP) reporter was injected in vivo into the hippocampus of adult rats. After 18 h, rats were killed, and acute hippocampal slices, infected in the CA1 field, were analyzed morphologically and electrophysiologically. Infected slices showed healthy and stable electrophysiological responses as well as long-term potentiation. In addition, infected pyramidal cells were readily recognized in living slices by two-photon imaging. Specifically, the introduction of an EGFP-Actin fusion protein greatly enhanced the detection of fine processes and dendritic spines. We propose this technique as an efficient tool for studying gene function in adult hippocampal neurons.

INTRODUCTION

Several molecules have been proposed to play a role in adult synaptic plasticity (Malenka and Nicoll 1999). Long-term potentiation (LTP) at the Schaffer collateral-CA1 synapses is a well-established form of synaptic plasticity. Many of the molecules involved in LTP have been identified through pharmacological manipulations of adult hippocampal brain slices (Schuman 1996). Alternatively, the manipulation of genes through the generation of transgenic animals provides specificity. In addition, the introduction of the Cre/loxP recombination technique eliminates some of the concern related to potential developmental effects of the genetic manipulation (Tsien et al. 1996). Both approaches have their limitations: the pharmacological approach is limited by the existence of compounds with a specific activity, while the transgenic approach is expensive and time consuming. To overcome these limitations, we developed a different strategy based on virus-mediated gene transfer. Several viral vectors including adenovirus,

herpes simplex virus, vaccinia virus, adeno associated virus, lentivirus, and sindbis virus have been shown to effectively infect cultured hippocampal neurons as well as hippocampal slice cultures (Ehrengruber et al. 1999; Griesbeck et al. 1997; Kammesheidt et al. 1996; Miyaguchi et al. 2000; Moriyoshi et al. 1996). Reports of successful infection of acute slices exist (Kantor et al. 1996) but remain plagued by concerns for neuronal specificity, health, and viability (Easton et al. 1998). The sindbis virus was chosen as a vector because it possesses an efficient promoter and readily infects neurons (Ehrengruber et al. 1999). An enhanced green fluorescent protein (EGFP)-based reporter was introduced into the CA1 area of the adult hippocampus by intra-cranial stereotaxic injection of virus solution. On the day following this in vivo infection in anesthetized rats, acute hippocampal slices were prepared from the infected area and late-phase LTP was successfully induced. Furthermore, the introduction of an EGFP-actin construct allowed the resolution of dendrites and spines in living neurons using two-photon microscopy.

METHODS

Virus construction

Sindbis virus was generated using the sindbis expression kit (Invitrogen). The fragments coding for EGFP and EGFP-actin were released from pEGFP-C1 and pEGFP-Actin (Clontech), respectively, using *NheI* and *ApaI* and subcloned into the *XbaI* and *ApaI* sites of SinRep5. Capped RNA was generated and cotransfected with the DH (26S) helper RNA (invitrogen) into baby hamster kidney cells. After 36 h, the supernatant was collected and concentrated by differential centrifugation (200,000 *g* for 3 h at 4°C).

In vivo injection

Adult male Sprague-Dawley rats (48–52 days old) were anesthetized with a ketamine (90 mg/kg)/xylazine (10 mg/kg) mixture by intraperitoneal injection and placed in a stereotaxic frame (Kopf Instrument). Occasionally, additional doses of anesthetic were delivered to keep the anesthesia level stable throughout the experiment. Two holes were drilled in the skull to gain access to the pyramidal cell layer of area CA1 in the hippocampus. The injection coordinates were established using the point of intersection of the sagittal suture with the best fit along the coronal suture (Bregma) as a reference for the lateral and antero-posterior coordinates (AP: -5.5 mm; L: ± 5.2 mm). The dura mater was carefully removed to expose the brain surface,

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which was used as reference point for the vertical coordinates (V: -2.8 mm). To obtain reproducible injections, quartz micropipettes with a microfilament (1 mm OD, 0.7 OD, 10-cm-length, Sutter Instrument) were beveled (K. T. Brown Type, Sutter Instrument). Viroid solution was delivered in short pulses (20–400 ms) supplied by a picospritzer apparatus (General Valve, Picospritzer II). To obtain a reproducible volume per pressure pulse, the injection pressure was varied between 20 and 40 PSI. Multiple pulses were applied up to a total volume of 0.50 ± 0.10 μ l. The surgery was completed by suturing the scalp and allowing the rat to recover with analgesic treatment (ibuprofen PO).

Hippocampal slice preparation

Injected rats were killed 16–20 h after the *in vivo* injection. Rats were anesthetized with halothane and decapitated, and the brain was rapidly removed to ice-cold, oxygenated, artificial cerebrospinal fluid [ACSF, which contained (in mM) 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.4 CaCl₂, 1.0 NaH₂PO₄, 26.2 NaHCO₃, and 22 glucose]. Hippocampal slices were prepared using a Stoelting tissue chopper. In experiments requiring high-magnification imaging, the rats were anesthetized with a ketamine (40 mg/kg)/xylazine (5 mg/kg) mixture, and the slices were prepared with a cooled oscillating tissue slicer (OTS-300-04; FHC, Brunswick, ME). Serial sections of 500 μ m were cut. The extraneous cortical and subcortical tissue was gently dissected away with the small end of a spatula. The injected slices were identified by fluorescence microscopy and then allowed to recover in an interface chamber at room temperature for at least 2 h.

Electrophysiology

Hippocampal slices were perfused with oxygenated ACSF at room temperature in a submerged chamber. The injected area was identified with an epifluorescence microscope. Field excitatory postsynaptic potentials (fEPSPs) measured in stratum radiatum were evoked by stimulation of the Schaffer collateral-commissural afferents (1 stimulus every 15 s). The initial slope was measured. The extracellular recording electrodes were filled with 3 M NaCl. Input-output (I/O) relations were monitored by measuring the EPSP slope in response to increasing stimulation currents. Paired-pulse facilitation was measured at two different intervals: 50 and 100 ms. Tetanic stimulation was delivered at the test intensity in 1-s trains at 100 Hz with four trains 30 s apart or four trains 5 min apart, for short- and long-term potentiation, respectively. Ensemble average plots represent group means of each EPSP for all the experiments, aligned with respect to the time of the LTP induction. Error bars indicate the standard error of the mean (SE) calculated for the entire data set for a given time point. Paired or unpaired *t*-tests were used to calculate the statistical significance of within group or between group comparisons, respectively. A one-way ANOVA was used to calculate the statistical significance of potential differences between the I/O curves.

Imaging

During imaging, the slices were placed into a 35-mm glass-bottom dish (MatTek) coated with poly-lysine (1 mg/ml) and perfused with ACSF gassed with 95% O₂-5% CO₂ at room temperature. To immobilize the sample during perfusion, a Millicell-CM filter (0.4 μ m, Millipore) was laid on the slice. A custom-built two-photon laser-scanning microscope (TPLSM) was used to acquire high-resolution images of three-dimensional neuronal structures deep within brain slices (50–200 μ m). The two-photon light source provided by a Ti:Sapphire laser (Tsunami, Spectra Physics) mode-locked at a wavelength of 860 nm was delivered to the scan-head of an Olympus Fluoview confocal microscope. High-resolution images were acquired through an inverted Olympus IX70 microscope supplied with $\times 60$ water-immersion objective corrected for infrared light (UplanApo/IR

60 X/1.2 Olympus). Images were then manipulated with Image Space software (Molecular Dynamics).

RESULTS

Specific targeting of hippocampal CA1 neurons

The complex organization of the hippocampal cortex makes the intracranial targeting of the virus particularly challenging. *In vivo* injections of the virus are most easily targeted to the antero-septal portion of the hippocampus. However, the most reliable field recordings are obtained from slices situated in the middle of the hippocampal formation (Amaral and Witter 1994; Schuman 1996). The stereotaxic coordinates chosen in our studies represent a compromise between these two situations. Holes were drilled under stereotaxic guidance in the parietal bone at the median edge of the insertion of the temporal muscle. The injection spot, 5.2 mm lateral from the longitudinal fissure and 5.5 mm posterior to the Bregma point (intersection of the coronal and longitudinal fissures), was reproducibly localized in adult rats (48- to 52-day old). Great care was taken in establishing the vertical coordinates. The targeted central portion of the hippocampus, area CA1, runs at a very steep angle; thus a small change in the vertical plane will result in a misplaced injection. We found that the surface of the brain, if left undamaged, can be used as a reliable reference. The stratum pyramidale of the CA1 area was reached by gently lowering the tip of the injection pipette 2.8 mm below the pial membrane. The viral solution was delivered with an average of 10 pressure pulses applied through a picospritzer apparatus. The duration of the generated pulses and the pressure applied to the electrode was chosen to obtain a minimal injected volume per stroke, generally <50 nl. We found that beveling the tip of the electrode to an aperture of 1–2 μ m was crucial in obtaining reliable injections. Pipettes with a smaller diameter allowed a more precise control of the injected volume but tended to occlude more easily. Rats were generally killed 1–2 days postinfection. Transverse hippocampal slices (500 μ m) were cut to evaluate the extent and the localization of the viral delivery. A successful injection resulted in infection of a discrete subregion of area CA1.

EGFP was chosen as a reporter gene, allowing an effortless visualization of the expressing neurons. As shown in Fig. 1, the fluorescent signal is derived almost exclusively from pyramidal cells, extending from the hippocampal fissure to the alveus. The size of the infected area was proportional to the amount of virus injected. For example, if 500 nl of viral solution (multiplicity of infection of $10^5/\mu$ l) was injected, the resulting infection extended laterally transducing cells through two to three adjacent 500- μ m-thick slices. Such a widespread infection ensured that at least one slice was infected throughout its entire thickness. Higher injection volumes were associated with tissue damage, while lower volumes reduced the chance of cutting transverse slices that exhibited green signal on both surfaces.

The expression of the introduced gene was very rapid; proteins were detected as early as 6 h post infection and expression levels rose to steady-state levels in the subsequent 12–18 h. The rapid expression of the introduced genes led us to test whether the *ex vivo* infection of acute slices would be feasible. Acute slices 500- μ m thick were prepared from rats killed at different ages and allowed to recover for 2 h at an

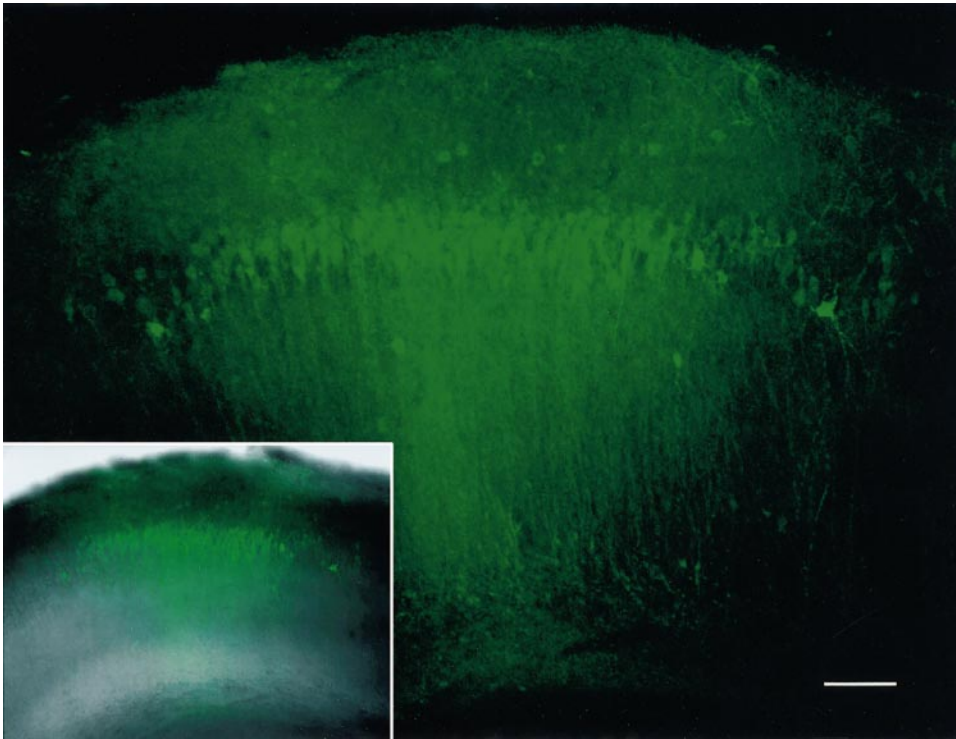


FIG. 1. Specific infection of CA1 neurons in the adult rat hippocampus. A representative transverse slice cut 18 h after infection with the enhanced green fluorescent protein (EGFP)-actin construct is shown. The image represent the maximal intensity projection of 10 sections acquired by two-photon laser-scanning microscopy (TPLSM) every 15 μm and collapsed (bar, 200 μm). An overlay of the fluorescent signal on the differential interference contrast image is shown.

interface chamber. Using a picospritzer setup similar to the one described in METHODS for intra-cranial injections, the virus was delivered *ex vivo* under microscopic guidance.

Fluorescent cells were easily identified in slices prepared from rats ≤ 12 day. Efficient infection was achieved only when the somatic area of the cell bodies of neurons was directly exposed to the viral solution (see also Ehrenguber et al. 1999). Older animals (15-, 30-, and 50-day old) were completely refractory to the virus (data not shown), underlining the necessity for *in vivo* injection in the adult animal.

Synaptic plasticity in infected slices

To establish if the infected tissue exhibited normal electrophysiological properties, fEPSPs were recorded from infected regions of s. radiatum and compared with nonfluorescent adjacent slices (Fig. 2B). Although this recording technique does not allow the resolution of responses from individual neurons, it does provide a reliable indicator of the integrity and viability of most neurons in the infected area.

The fluorescent area was visualized with an epifluorescent microscope, and the recording electrode was carefully positioned in the middle of the fluorescent s. radiatum (Fig. 2A). Infected slices exhibited healthy and stable electrophysiological responses. As showed in Fig. 2B, the waveform of fEPSP was similar in both control and virus-infected slices. On induction of LTP, a large and persistent increase in synaptic strength was observed in both infected slices and adjacent uninfected slices. In infected slices, late-phase potentiation measured 230 min after tetanus induction was $191.9 \pm 14.8\%$ of the baseline ($P < 0.0001$). The adjacent uninfected slices showed a potentiation of $174 \pm 5.7\%$ when compared with the baseline.

In addition, paired-pulse facilitation (PPF) was measured as a form of short-term plasticity (Fig. 2C). PPF, measured in the

infected slice at an interstimulus interval (ISI) of 50 or 100 ms, was not significantly different from that observed in control slices. A significant difference was observed when basal synaptic transmission of the infected slices was compared with the control adjacent slices ($P < 0.01$). Measurements of the input/output (I/O) relation indicated that the infected slices showed a "less steep" I/O curve (Fig. 2D).

High magnification two-photon imaging of infected slices

The EGFP-actin reporter was chosen to obtain high-fidelity images of dendritic spines. In healthy CA1 pyramidal cells, polymerized actin is highly concentrated at synapses where it forms a lattice underneath the postsynaptic density and is longitudinally organized in the spine neck (Matus 1999). In apoptotic neurons, the actin signal has been observed to fade from the spines to highlight some of the developing varicosities and eventually concentrate in high-intensity puncta in processes devoid of spiny protrusion (data not shown). This cytopathic phenotype was observed in most processes visualized at the slice surface (data not shown) and was probably due to the slice cutting technique. These observations are consistent with the finding that actin redistributes following neuronal distress (Halpain et al. 1998).

Two-photon microscopy was used to image viable tissue inside the slice (Denk and Svoboda 1997). As shown in images acquired 100–200 μm below the surface in the center of the infected area (Fig. 3A), 50% of the neuropil contains the reporter EGFP-actin. As expected in healthy tissue, the fluorescent signal was enriched in dendritic spines; in addition, most of the processes exhibited homogenous fluorescence. These features are more clearly observed at the periphery of the infected area, where single dendrites glow under the excitation light without substantial fluorescence from adjacent neurons (Fig. 3, B and C).

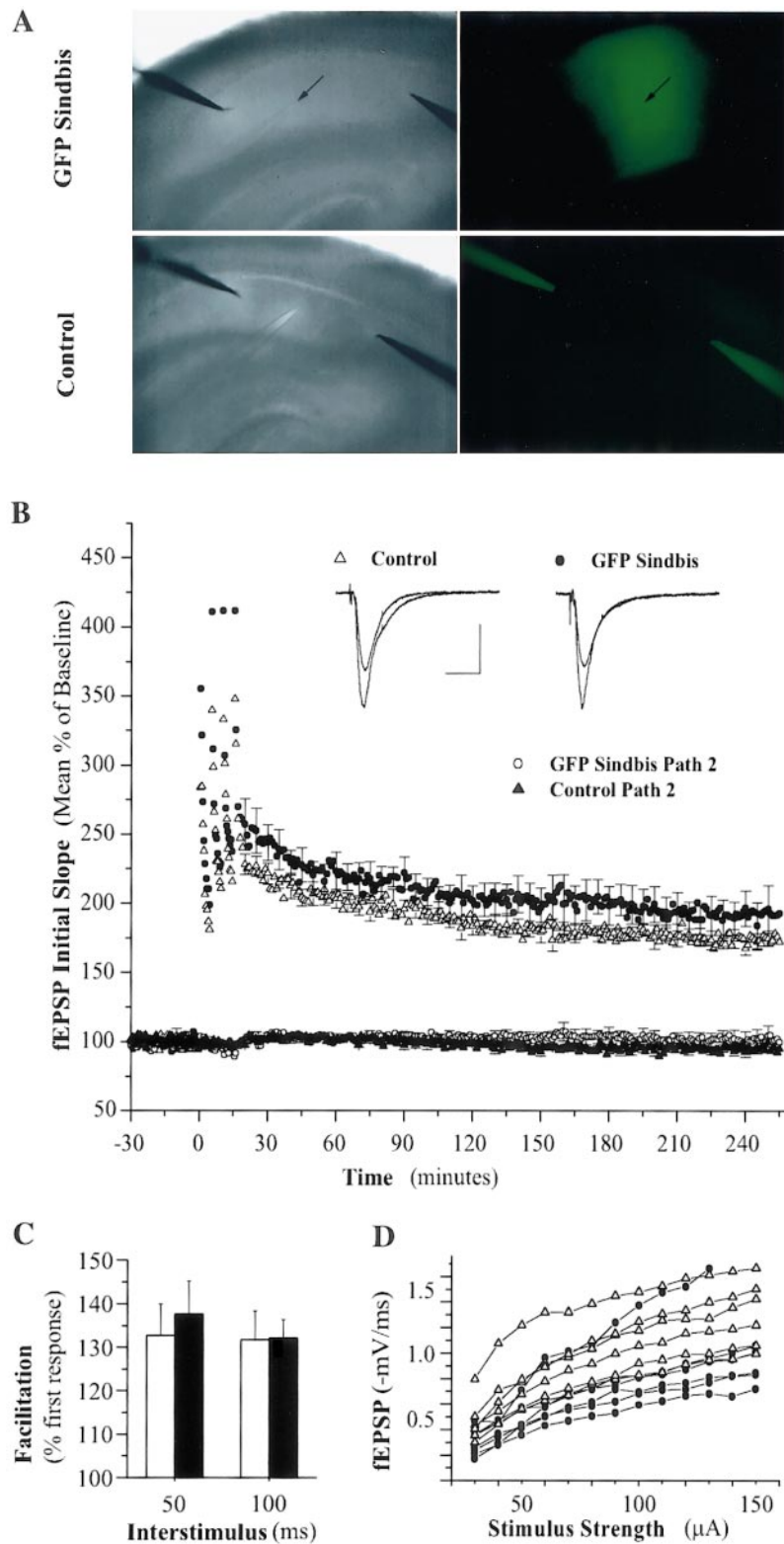


FIG. 2. Induction of long-term potentiation (LTP) in infected slices. *A*: a typical location of the recording (\downarrow) and stimulating electrodes is depicted. The picture representing the uninfected control has been acquired at a higher exposure. *B*: ensemble averages of 2 pathway experiments performed on both infected slices ($n = 5$, \circ) and adjacent uninfected slices ($n = 5$, \triangle). LTP was induced by 4 trains of tetanic stimulation at *time 0* (\triangle and \bullet). Untetanized control pathways are shown ($P < 0.05$). The superimposed representative excitatory postsynaptic potentials (EPSPs) were recorded 10 min before the tetanus and 10 min before the end of experiments (scale bars, 0.5 mV and 20 ms). *C*: paired-pulse facilitation in infected slices (\blacksquare , $n = 6$) is not significant from the uninfected adjacent slices ($n = 6$). *D*: input-output relations are significantly "less steep" in the infected (\bullet) than in the uninfected adjacent slices ($P = 0.005$).

To further confirm the morphological observations of healthy tissue, fEPSPs were recorded from the infected regions of CA1 s. radiatum and compared with nonfluorescent adjacent slices (Fig. 3D). As in the case of slices infected with EGFP virus, EGFP-actin expression is not toxic: the fEPSP waveforms were indistinguishable from controls and furthermore, LTP was induced in both control and virus-infected slices.

DISCUSSION

In this study, we describe a technique for the introduction of foreign genes into a discrete region of the brain. Several aspects of the injection technique were optimized to achieve abundant expression of the gene of interest, allowing field potential recordings in the infected area. A relatively selective

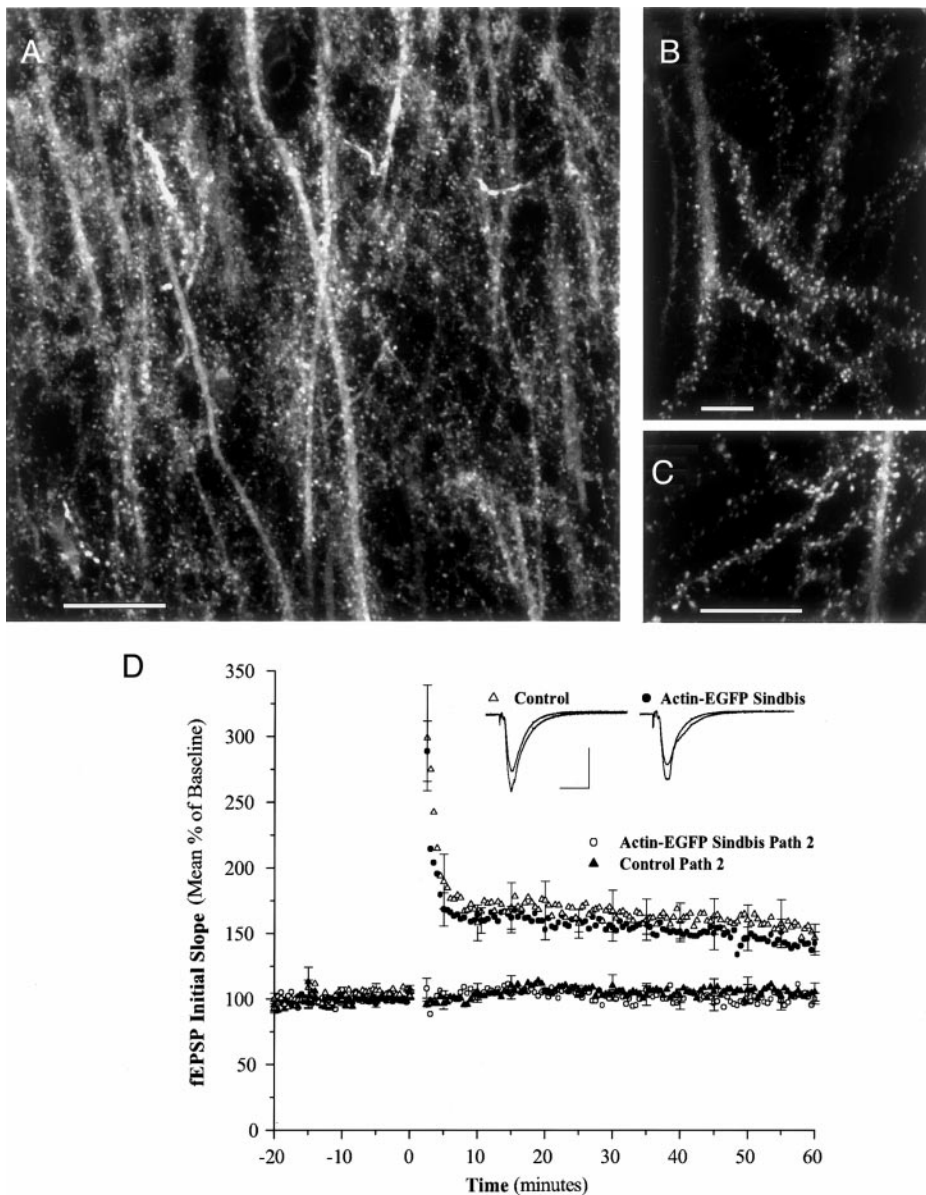


FIG. 3. EGFP-actin infected neurons exhibit a healthy morphology with clearly visible spines. *A*: high magnification of the neuropil of the stratum radiatum in the center of the infected area. A maximal intensity projection is shown after filtering 12 sections $1 \mu\text{m}$ apart with a Gaussian procedure (bar, $20 \mu\text{m}$). *B* and *C*: single sections from isolated dendrites at the periphery of the injected area (bar, $20 \mu\text{m}$). *D*: the ensemble average of 2 pathway experiments performed on both infected slices (\circ) and adjacent uninfected slices ($n = 8$ and 6 , respectively). LTP was induced by 4 trains of tetanic stimulation at *time 0* (Δ and \bullet). Untetanized control pathways are shown. The superimposed representative EPSPs were recorded 10 min before and 50 min after LTP induction (scale bars, 0.5 mV and 20 ms).

infection of neurons was achieved by using the sindbis virus and delivering it to the cell body area.

In our experiments, expression of the reporter protein was detected 6 h post infection. The fluorescent signal increased to reach a plateau ~ 24 h post infection. Although viable cells have been identified ≤ 4 days following infection, signs of cytotoxicity, assessed by TPSLM microscopy, advises against such a prolonged incubation.

As indicated in RESULTS, the coordinates chosen for the injection represent a compromise between the stereotaxic accessibility and a strong induction of field potentials. Although electrophysiological measurements were made on horizontal hippocampal slices, we found that coronal slices also show clear field potentials when the stimulating electrode is placed in close proximity to the recording electrode in the s. radiatum. Since the injection area can be easily modified to target adjacent regions (such as the hippocampal CA3 area, the amygdala or the dentate gyrus), coronal slices may be advantageous while establishing the stereotaxic

coordinates because the injected area is more easily identified.

The electrophysiological response of the infected slices was similar to control slices in the induction of short-term synaptic plasticity, assessed as PPF, and in the stimulation of a robust late phase potentiation at the CA3–CA1 synapses. Basal synaptic excitability, monitored measuring the slope of the fEPSP in response to electrical stimulation of varying current amplitudes, was significantly altered in the infected slices. Increasing stimulus strength was less efficient in increasing synaptic transmission in the infected slices when compared with the control animal. This difference is probably a consequence of the injection itself. Indeed a small percentage of dead cells was observed in the infected area even when the tissue was fixed *in vivo* by intracardiac perfusion of paraformaldehyde solution. The relative “shallowness” of the I/O curve in infected slices does not have any obvious effect on plasticity since both PPF and LTP were normal in infected slices. Nevertheless this difference in basal synaptic excitability invites caution in the

design of future experiments. Results obtained with the introduction of a foreign gene should always be compared with a control (e.g., EGFP).

The strong expression of the reporter gene under the sindbis sub-genomic promoter was used to introduce a fluorescent reporter gene. When combined with two-photon microscopy, the presented method allows the simultaneous electrophysiological manipulation and visualization of adult hippocampal tissue.

The ability to induce foreign genes in a discrete subregion of the brain can be utilized to precisely trigger recombination events. In the *Cre/loxP* system, the recombination event is generally obtained by crossing a Cre transgenic mouse line with a line expressing a floxed gene. In this case, specificity is determined by the nature of the transcriptional promoter that drives the expression of *Cre*. Viral delivery of Cre recombinase will greatly increase the flexibility of the *Cre/loxP* system as well as enhance its temporal and spatial resolution.

The high activity of the sub-genomic promoter will also ensure that dominant negative mutants will be expressed at a sufficient high level; furthermore the high number of RNA transcripts generated will be beneficial in antisense techniques.

We propose this technique as a new effective tool for studying gene function in adult hippocampal neurons.

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