

# A Modified Sindbis Vector for Prolonged Gene Expression in Neurons

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Submitted 14 May 2003; accepted in final form 25 June 2003

**Jeromin, Andreas, Li-Lian Yuan, Andreas Frick, Paul Pfaffinger, and Daniel Johnston.** A modified Sindbis vector for prolonged gene expression in neurons. *J Neurophysiol* 90: 2741–2745, 2003. First published July 9, 2003; 10.1152/jn.00464.2003. Sindbis viruses have been widely used in neurobiology to express a variety of genes in cultured neurons, in cultured slices, and in vivo. They provide fast onset and high levels of expression of foreign genes, but the expression is limited to a short time window due to a shut-off of host protein synthesis. We have used a mutation in an essential gene (nsP2) of the life cycle of Sindbis, which allows the functional analysis of changes in protein expression for  $\geq 6$  days after infection. This Sindbis mutant (nsP2) was used to express enhanced green fluorescent protein (EGFP) in hippocampal neurons in culture and in vivo without any sign of toxicity, based on two-photon imaging and electrophysiology. In addition, the EGFP mutant virus can be injected in vivo to visualize spines and other details of neuronal structure. The Sindbis mutant described here provides an improved tool in neurobiology with reduced cytotoxicity and a prolonged time window of expression for novel applications in imaging and behavior. In addition, the use of this vector for the functional expression of mammalian voltage-gated ion channels in organotypic slices is demonstrated.

## INTRODUCTION

Sindbis and the related Semliki Forest virus have gained increased popularity in neurobiology in recent years (reviewed in Ehrengruber 2002). Sindbis selectively infects neurons and confers a rapid onset and high level of expression of foreign genes in contrast to other viral vectors (for review see Washbourne and McAllister 2002). In recent years, Sindbis has been used to express various receptors, anchoring molecules, and kinases in cultured neurons and cultured slices, and also in vivo (Ehrengruber 2002; Shi et al. 2001). The high and rapid onset of expression is accomplished at the cost of shutting off protein synthesis of the infected host cell (reviewed in Huang and Schlesinger 2003). In recent years, a number of virus mutants have been isolated that are less cytopathic. Such mutants of Sindbis virus were first obtained from infected Baby Hamster kidney (BHK)-21 cells (Dryga 1997). A single change in the viral nsP2 protein from proline to serine was responsible for this phenotype. The nsP2 protease activity is required for the processing of the viral polyprotein and plays a fundamental role in its life cycle (Frolov et al. 1999). Interestingly, this nsP2 mutant has a less suppressive effect on host RNA synthesis, and incorporation of this mutant into a Sindbis virus expression vector led to higher levels of synthesis of the reporter,  $\beta$ -ga-

lactosidase, in BHK-cells than obtained with the original Sindbis virus replicon (Dryga et al. 1997).

To determine the usefulness of this Sindbis mutant for prolonged and high level expression in neurons, we constructed Sindbis virus vectors expressing enhanced green fluorescent protein (EGFP) alone and EGFP in a double subgenomic vector (2 $\times$ SgP). The expression of transgenes over extended periods of time was monitored by 2-photon-microscopy and electrophysiology to determine the utility of this virus mutant as a vector for use in primary organotypic cultures and in vivo stereotactic injection.

## METHODS

### *Viral vectors and preparation*

The pSinrep5 vector containing the nsP2 ser mutant and the helper plasmids were kindly provided by Sondra Schlesinger (Washington University, St. Louis, MO). EGFP (Clontech) was subcloned into the mutant pSinRep5 vector, and a double subgenomic vector was constructed by expressing EGFP under control of second promoter. The rat Kv4.2 cDNA was subcloned into this double subgenomic vector. Further details regarding the construction of these vectors and the vectors are available on request. Recombinant Sindbis virus was generated according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Briefly, RNA was transcribed from these plasmids and the helper plasmid using the Message Machine kit (Ambion, Austin, TX). These two RNAs were electroporated into BHK-21 cells (ATCC, Rockville, MD), and recombinant virus particles were harvested 48–72 h after electroporation. The titer of the EGFP mutant virus is indistinguishable from EGFP in the original wildtype virus.

### *Organotypic slice culture*

Organotypic slices were prepared and cultured according to the interface technique as originally described by Stoppini et al. (1991). Slices were prepared from P1 to P7 rats (Sprague-Dawley) at 325  $\mu$ m thickness using a McIlwain tissue chopper (Mickle Laboratory Engineering) and cultured on Millicell inserts (Millipore) in a MEM-based medium (Invitrogen) containing 20% horse serum (Invitrogen). Medium was changed every second day, and slices could be kept alive until  $\geq 4$  wk in culture.

### *Infection of organotypic slices with recombinant Sindbis virus and stereotactic in vivo injection*

Slices were infected with recombinant Sindbis viruses in defined areas of CA1 using a Picospritzer (General Valve, Fairfield, NJ) injected through glass pipettes pulled to an outer diameter of 5–10

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$\mu\text{m}$ . By varying the pulse duration and pressure, the multiplicity of infection (MOI) was adjusted from very few to 10–50 infected cells. (data not shown).

For *in vivo* injection, adult Sprague-Dawley rats (160–190 g) were anesthetized with a ketamine (90 mg/ml)/xylazine (10 mg/ml) mixture by intraperitoneal injection and placed into a stereotaxic frame (Stoelting, Wood Dale, IL). Two holes were drilled using a dental drill (Stoelting) to gain access to the pyramidal cell layer of CA1 of the hippocampus. The coordinates for injection were established using the intersection of the sagittal and coronal suture (Bregma) as a reference point for the anterior-posterior (AP;  $-5.5$  mm) and lateral ( $\pm 3.5$  mm) coordinates. A 26-gauge needle attached to a microsyringe (WPI) was slowly advanced to the desired depth (4 mm below the pia), and a bolus of the Sindbis virus was slowly injected into the brain tissue (3–5  $\mu\text{l}$ ). In some experiments, multiple injections of Sindbis virus were performed at different depths along the needle track. The needle was gradually withdrawn over a period of 3–5 min after completion of the injection.

The incision was closed with a surgical silk suture (Ethicon), and the rats were allowed to recover. Acute slices (500  $\mu\text{m}$ ) of the hippocampus were prepared from these injected animals as described previously (Yuan et al. 2002).

### Two-photon imaging

The basic setup for two-photon imaging has been described previously (Frick et al. 2003). We used short pulses (3–10 mW) from a compact Titan:Sapphire System (Mai Tai, Spectra Physics, Mountain View, CA) at 920 nm and a fast galvanometric scanner (Leica MP RS, Leica Microsystems, Mannheim, Germany) mounted on an upright microscope (Leica DM LFS A) equipped with a 40 $\times$  objective (HCX APO L40x/0.8W) to visualize EGFP fluorescence. Individual planes were acquired and reconstructed to yield stacks using Leica's 3D view software.

### Electrophysiology

A Zeiss Axioskop, fitted with a 40 $\times$  water-immersion objective and differential interference contrast (DIC), was used to view cultured slices. Light in the near infrared (IR) range (740 nm) was used in conjunction with a contrast-enhancing camera to visualize individual neurons. Infected neurons expressing EGFP were identified under a fluorescent microscope. The bath solution containing (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO<sub>3</sub>, 2.0 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, and 10 dextrose was constantly saturated by 5% CO<sub>2</sub>-95% O<sub>2</sub>. Recording pipettes were pulled from borosilicate glass and filled with (in mM) 120 K-gluconate, 20 KCl, 10 HEPES, 2 MgCl<sub>2</sub>, 4 Na 2-adenosine 5'-triphosphate, 0.3 Mg guanosine 5'-triphosphate, and 14 phosphocreatine (pH 7.25 with KOH). Whole cell current-clamp and voltage-clamp recordings were made with an Axoclamp 2A amplifier in "bridge" mode and an Axopatch 200, respectively. To isolate  $I_A$  in pyramidal neurons, 1  $\mu\text{M}$  TTX and 2 mM MnCl<sub>2</sub> were added to the bath to block Na<sup>+</sup> currents, Ca<sup>2+</sup> currents, and Ca<sup>2+</sup>-activated K<sup>+</sup> currents. Pipettes had resistance between 2 and 5 M $\Omega$ . Whole cell capacitance and series resistances were compensated to more than 80%, and in addition, series resistances were less than two times that of tip resistance. All experiments were done at room temperature. Pulse generation and data acquisition were controlled with custom software written in IGOR (by Dr. Richard Gray). Leakage and capacitive currents were digitally subtracted on-line.

## RESULTS

We first determined if the mutant Sindbis virus could be used for prolonged gene expression in organotypic slices from the hippocampus. CA1 neurons infected by local injection with a wildtype virus expressing EGFP showed signs of toxicity

72 h after infection as noted by the swelling of dendrites (Fig. 1A) and by changes in their electrophysiological characteristics (the resting potential of these cells was depolarized to  $-40$  to  $-45$  mV,  $n = 3$ ). In contrast, CA1 neurons in organotypic slices infected with the mutant virus expressing EGFP appeared healthy and indistinguishable from neighboring uninfected cells even 7 days after infection (Fig. 1B). These infected EGFP expressing neurons did not differ from neighboring uninfected neurons in terms of their intrinsic electrophysiological parameters, such as membrane potential, input resistance, and firing threshold for action potentials (Fig. 2). In addition, the ultrastructure of synapses based on electron microscopy did not appear to be different in EGFP-infected neurons from neighboring uninfected control neurons.

By varying the conditions of the injection, for example, the diameter of the glass capillary (typically 5–10  $\mu\text{m}$ ) and the duration and magnitude of the injection pulse, the number of infected neurons could be varied from very few to  $\leq 50$ –100 neurons per injection site. We found infected neurons as deep as 100–150  $\mu\text{m}$  below the surface of the 325- $\mu\text{m}$ -thick slice, but this can be adjusted according to experimental needs. Depending on the position of the neuron(s) relative to the injection capillary and the titer of the virus, the number of virus

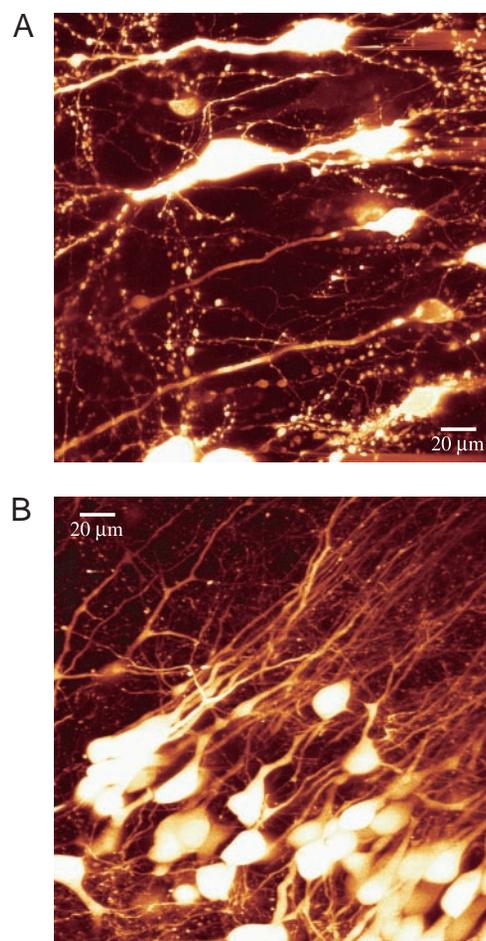


FIG. 1. Organotypic slices from P7 pups (Sprague-Dawley rats) were focally infected with either wildtype (A) or mutant (nsP2ser) (B) Sindbis viruses over the CA1 after 5–7 days in culture. A: CA1 neurons infected with enhanced green fluorescent protein (EGFP) in the wildtype Sindbis virus 72 h after infection. B: CA1 neurons infected with EGFP in the mutant (nsP2ser) Sindbis virus 6 days after infection.

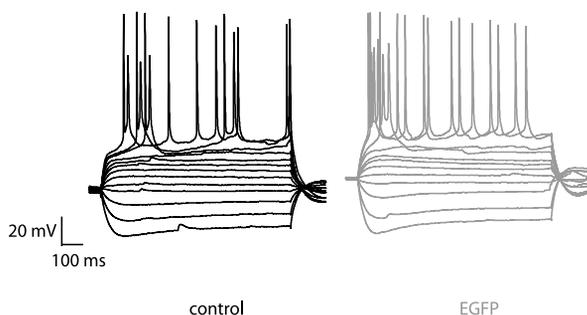


FIG. 2. Intrinsic and firing properties of EGFP-expressing neurons. A family of step currents was injected at the soma to determine the input resistance and action potential firing threshold and firing patterns. EGFP-expressing neurons and their neighboring control neurons did not show significant differences in resting potential, input resistance, and firing threshold/pattern. Input resistance was calculated from a current injection of  $-50$  pA. Firing threshold was defined as the current amplitude at which an action potential was 1st elicited. Resting membrane potential  $V_m$  was  $-61.1 \pm 1.3$  mV for control and  $-60.5 \pm 1.0$  mV for EGFP-expressing neurons, the input resistance  $R_{input}$  was  $398.1 \pm 14.7$  M $\Omega$  for control and  $406.9 \pm 26.0$  M $\Omega$  for EGFP-expressing neurons, and the firing threshold was  $67.5 \pm 3.2$  pA for control and  $56.9 \pm 4.5$  pA for EGFP-expressing neurons ( $n = 5$  for control and  $n = 4$  for EGFP-expressing neurons). These values of the control and EGFP-expressing neuron group are not significantly different ( $P < 0.05$ ).

particles infecting a neuron will vary. In particular, under in vivo conditions, the number of infected neurons decreased as the distance from the injection site increased.

We further characterized the ability of a mutant Sindbis virus vector to express EGFP off a second subgenomic promoter (SGP) incorporated into the same vector. Such a double subgenomic promoter will allow us to express any mRNA of interest off the first SGP, while simultaneously identifying the transgene expressing neuron by the expression of EGFP (Fig. 1B). In side by side comparisons between this  $2 \times$ SGP vector and our single SGP vector expressing EGFP, we observed no significant differences in the ability to identify infected cells or in the health of infected neurons  $\leq 6$  days postinfection. As further evidence for the efficacy of this vector, the EGFP fluorescence intensity appeared to increase over time, and at 5–7 days, it was at least as bright if not brighter than EGFP expressed in the wildtype after 48 h (data not shown). The level of expression of EGFP in the mutant virus was high enough to allow the visualization of individual spines (Fig. 3, A and B).

Besides the expression of EGFP, we wanted to determine if the mutant virus is suitable for the functional expression of vertebrate ion channels. We chose to express Kv4.2 type  $K^+$  channels, because they are likely to underlie the A-type  $K^+$  current ( $I_A$ ) in CA1 neurons (Yuan et al. 2002). CA1 neurons in organotypic slices were infected with a double subgenomic virus ( $2 \times$ SGP) expressing rat Kv4.2 and EGFP from a separate (subgenomic) promoter. A prepulse protocol (Fig. 4A) in the presence of TTX and  $Mn^{2+}$  was used to isolate the total A-type current from the sustained current. In EGFP-expressing neurons, the density of  $I_A$  was significantly increased compared with neighboring uninfected cells (Fig. 4).

We next determined if the mutant virus is suitable for in vivo injection experiments. Using EGFP as a marker, adult rats were stereotactically injected with a small volume of virus into the hippocampus, and acute slices were cut from these infected brains 5 days after the in vivo infection. Figure 5 shows an example of a CA1 neuron in an acute slice previously infected

in vivo and imaged under the two-photon microscope. The morphology of the EGFP-expressing pyramidal cells appears normal (Fig. 5A), without any sign of pathology. Expressed EGFP appeared to fill the entire neuron, revealing apical and basal dendrites and the proximal part of the axon. The expression of EGFP in the axon was high enough to follow it for some distance in the tissue.

## DISCUSSION

Sindbis viruses have found widespread use in expressing different foreign proteins in cultured neurons, neurons in cultured slices, and in vivo (Ehrengruber 2002 and Washbourne and McAllister 2002). In contrast to other viral vectors, Sindbis (and the related Semliki Forest virus) offers the advantage of selectively infecting neurons with a rapid onset and high levels of expression. Their cytotoxicity, however, poses limits on the time window of infection. We have tested whether a Sindbis (nsP2 ser) mutant can be used to overcome these limitations. The mutant used in this study has previously been described as being less cytotoxic in infected BHK-21 cells (Dryga et al. 1997) and having a less suppressive effect on shutting off protein synthesis of the infected host.

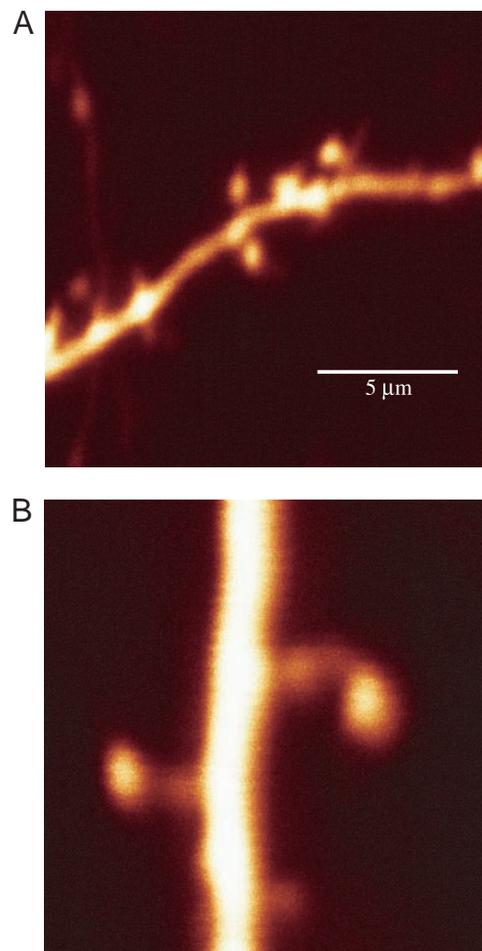


FIG. 3. High magnification images of dendritic segments of 2 CA1 pyramidal neurons infected with EGFP in the mutant (nsP2ser) virus. Organotypic slices from P7 pups (Sprague-Dawley), which were infected focally over CA1, after 7 days in culture and imaged 5 days after infection. A: dendritic segment with spines. B: different segment with 2 visible spines from a different neuron (higher magnification; scale bar =  $2 \mu\text{m}$  for B).

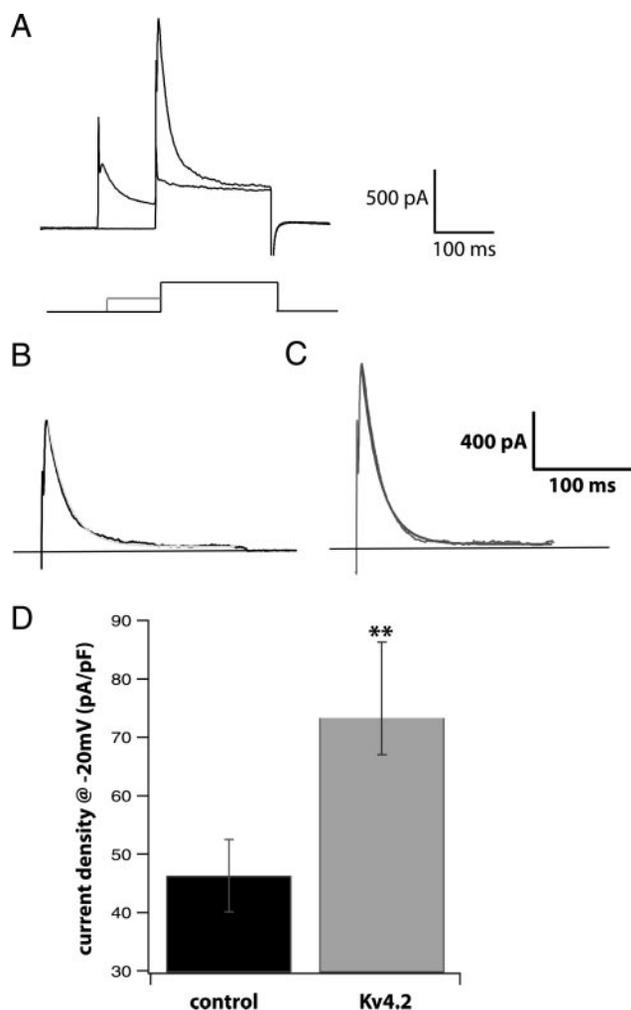


FIG. 4. Overexpression of Kv4.2 subunits in organotypic culture using the Sindbis virus mutant. *A*: to separate sustained  $K^+$  currents from the transient A-type current ( $I_A$ ), a prepulse (a voltage step to  $-40$  mV for 50–100 ms before the test pulse) was used, which revealed a prominent portion of transient  $K^+$  current in the total outward current. *B*: prepulse protocol isolated  $I_A$  at  $-20$  mV from a control CA1 pyramidal neuron with  $C_m = 13$  pF. Channel inactivation was fitted with a single exponential with a time constant  $\tau = 18$  ms (yellow). *C*:  $I_A$  from a transfected CA1 pyramidal neuron with  $C_m = 10.5$  pF. Channel inactivation was fitted with a single exponential time constant  $\tau = 19.3$  ms (blue). *D*: data summarized from control ( $n = 5$ ) and Kv4.2-expressing neurons ( $n = 4$ ). Current density of Kv4.2-expressing neurons is increased by more than 50% ( $P < 0.05$ ).

In contrast to the original wildtype virus, neurons infected with the mutant virus expressing EGFP remained healthy and indistinguishable from their uninfected neighbors (Fig. 2). In organotypic slice cultures infected with the mutant virus, expression levels of EGFP after 5–7 days were equivalent or greater than those obtained with the original wildtype virus after 48 h. In addition, the mutant virus was used to functionally express the vertebrate  $K^+$  channel Kv4.2 in CA1 neurons in organotypic slice cultures in a double subgenomic vector (2 $\times$ SGP; Fig. 4). In summary, the mutant virus combines the advantages of Sindbis virus, i.e., its neuronal specificity, with a prolonged, high level of expression and less cytopathic effects. These findings are in agreement with a preliminary report by Kim et al. (2002), demonstrating reduced cytotoxicity and an extended period of

expression in cultured neurons using a similar mutant EGFP-expressing virus. We believe that these features of reduced toxicity, prolonged high level of expression, and specificity of infection in neurons makes the mutant Sindbis virus a more generally applicable tool for expression studies. As an example, we showed the expression of the vertebrate Kv4.2-type  $K^+$  channel, which is likely responsible for the A-type current in CA1 neurons. The mutant virus might be particularly advantageous in cases where the functional expression of the transgene relies on the heteromultimerization with endogenous subunits, as in the case of some dominant-negative mutant or accessory factors. In these cases, the ability of the infected neuron to sustain cellular protein synthesis may be critical for the success of the experiments. Likewise, the extended health of the infected neurons may be critical to observe significant changes in situations where the trafficking and turnover of native proteins is slow, such as channels anchored to the postsynaptic density.

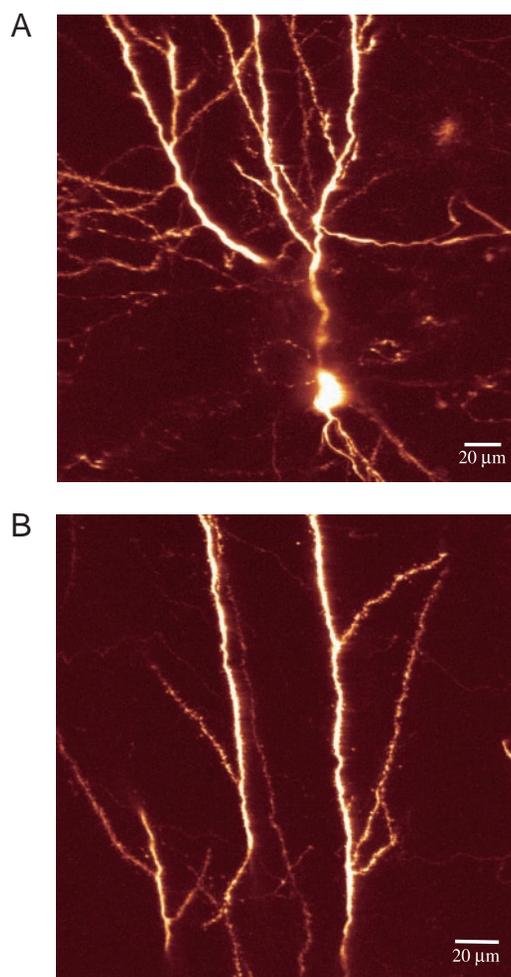


FIG. 5. In vivo injection of EGFP in the mutant (nsP2ser) virus into the hippocampus of young adult rats (Sprague-Dawley, 4–5 wk). The mutant virus was stereotactically injected into the hippocampus, and acute slices were prepared 5 days after infection and used for 2-photon-imaging. *A*: example of an EGFP-labeled CA1 pyramidal neuron. Expression of EGFP was mainly found in the dendrites; the proximal part of the axon was also labeled in this neuron. *B*: segment of the dendritic tree of the same neuron.

We thank S. Schlesinger for providing some of the original Sindbis vectors and H. Koester for help with the two-photon microscopy.

#### DISCLOSURES

This study was supported by National Institutes of Health Grants NS-37444, MH-48432, and MH-44754.

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