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Retrograde neuronal tracing with a deletion-mutant rabies virus

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Abstract

We have constructed a deletion-mutant rabies virus encoding EGFP and find it to be an excellent tool for studying detailed morphology and physiology of neurons projecting to injection sites within the mammalian brain. The virus cannot spread beyond initially infected cells yet, unlike other viral vectors, replicates its core within them. The cells therefore fluoresce intensely, revealing fine dendritic and axonal structure with no background from partially or faintly labeled cells.

A fundamental question regarding brain organization is how the various structures are connected to each other. A standard means of addressing it has been to inject into a region of interest some 'retrograde tracer': a substance taken up by the axon terminals of neurons that project to the injection site and which allows either visualization of their anatomy or, in the case of fluorescent tracers, their targeting for physiological study[1,2]. The rise of molecular biology has allowed the use of retrogradely infectious viruses expressing genetically encoded fluorophores[3,4]. In no published cases, however, has the resulting fluorescence been bright enough to consistently provide detailed anatomical information without immunohistochemical amplification and therefore to permit high-resolution identification of live labeled neurons for subsequent physiological study. Here we present a newly created virus that, because of several unique characteristics, does achieve these goals.

Rabies virus, which has been used as a trans-synaptic tracer[5], infects neurons through axon terminals and spreads between synaptically coupled neurons in an exclusively retrograde direction. In earlier work, K.K.C. and colleagues produced a virus that had the envelope glycoprotein gene deleted from its genome but that was grown in complementing cells so that the glycoprotein itself was incorporated into the viral particles' membranes despite the lack of its coding sequence in the viral genome[6]. Such a virus can infect contacted cells normally and, because the glycoprotein plays no role in transcription and replication, can still express its remaining genes and proliferate the viral core within initially infected cells. However, with no means of synthesizing glycoprotein in these cells, the newly created progeny are unable to infect other cells[6,7], transforming the virus into a first-order retrograde tracer instead of a trans-synaptic one. To reveal detailed morphology, we substituted the gene for enhanced green

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COMPETING INTERESTS STATEMENT

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fluorescent protein (EGFP) for the gene for the glycoprotein (Fig. 1a). The result should be a virus that can infect initially contacted cells, replicate its core to high copy number and express high levels of EGFP, but be unable to spread beyond these initially infected cells. We termed this virus, which was derived from the SAD B19 vaccine strain of rabies virus, SADΔG-EGFP.

Cells infected with SADΔG-EGFP invariably display intense fluorescence filling of all of their processes; label is visible in mouse cortex at 2 d after injection into the thalamus but steadily increases in brightness thereafter (Fig. 1b). EGFP levels are high enough to easily resolve the fine processes of both dendrites and axons (Supplementary Fig. 1 online).

Other viruses besides rabies virus are also known to infect retrogradely, among them adenovirus, α-herpesviruses and lentiviral vectors pseudotyped with envelope glycoproteins of various strains of rabies virus[4,8,9]. For direct comparison, we constructed an HIV-1 lentiviral vector encoding EGFP under the control of the human cytomegalovirus promoter. This virus was pseudotyped with the envelope glycoprotein from the same strain of rabies virus (SAD B19) used to derive our recombinant version, so that both the lentivirus and the rabies virus contained the same envelope glycoprotein in their membranes. We equalized the two viruses' titers and injected equal volumes into the thalami of four mice each, then examined the brains 6 d later.

Cells retrogradely infected with the lentivirus were markedly less impressive, by eye, than those infected with the rabies virus (Fig. 2). The lentivirus-infected neurons also exhibited considerable heterogeneity in fluorescence intensity, with some cells quite bright but most extremely faint even after signal amplification by immunostaining for EGFP (data not shown). Without antibody staining, in fact, very few cells were visibly infected with the lentivirus at all—those shown in Figure 2a–c were the brightest available—whereas native fluorescence from the rabies virus (Fig. 2d) was invariably brilliant and unmistakable.

We counted labeled cells in cortex ipsilateral to the injection site, after amplification by immunostaining, in every sixth section (see Supplementary Methods online). For both viruses, labeled cells in the overlying cortex were, without exception, layer 5 and layer 6 pyramidal cells, which are established as providing the sole cortical projections to thalamus[10]. In the brains injected with rabies virus ($n = 4$), there were 210.5 ± 35.4 detectable cells (mean ± s.d.); in the lentiviral cases ($n = 4$), there were 107.3 ± 33.4. The lentiviral number is doubtless an underestimate, because the labeling in many of the cells was so faint as to be barely discernable from background. The numbers of retrogradely infected cells are therefore similar for the two viruses, indicating, intriguingly, that the glycoprotein may be the sole determinant of rabies virus's ability to retrogradely infect cells once in contact with their axons.

The difference in fluorescence intensity, however, was dramatic. To quantify this, individual somata of the fluorescent cells within the fields shown in Figure 2 were manually identified and the maximum native fluorescence intensity (without immunostain) was taken within each soma.

This comparison was heavily handicapped in favor of the lentivirus, for two reasons. First, no set of parameters was sufficient to effectively image the cells infected with lentivirus without causing saturation of the photomultiplier tube from most of the much brighter cells infected with rabies virus. This artificially lowered the measured intensity of the rabies virus–mediated fluorescence compared to that mediated by the lentivirus. Second, as far fewer lentivirus-infected cells were visible by intrinsic fluorescence than became apparent in antibody-amplified alternate sections from the same animals, only the brightest of these cells were able to be used for the quantification, artificially raising the mean intensity of the lentivirus-mediated fluorescence.

Despite this bias, the mean fluorescence intensity for the lentivirus-infected cells ($n = 18$) was 499.2 ± 556.4 (arbitrary units; see Supplementary Methods). The corresponding value for the rabies virus-infected cells ($n = 23$) was 3,938.6 ± 528.1 ($P < 5 \times 10^{-21}$; two-tailed Student's *t*-test). In other words, despite the facts that the lentiviral mean was artificially raised by selection of the few visibly bright cells and that the rabies viral mean was artificially lowered by instrument saturation, the fluorescence from the rabies virus was calculated to be eight times brighter.

In whole-cell recordings from fluorescent cortical cells in brain slices prepared from rats injected with SADΔG-EGFP into the thalamus 5–12 d previously, we found no significant difference in gross electrophysiological properties of infected cells versus those of nearby nonfluorescent cells. The resting membrane potential of infected cells was −49.6 ± 8.0 mV (mean ± s.d.; $n = 14$) while that of uninfected cells ($n = 9$) was −50.7 ± 6.0 mV ($P = 0.73$, two-tailed Student's *t*-test); action potential thresholds were −36.4 ± 9.2 mV and −39.3 ± 10.1 for the infected and control cells respectively ($P = 0.48$).

To estimate the time course of survival for neurons infected with SADΔG-EGFP, we injected mouse thalami with equal volumes each of viral solution and sacrificed them at 2-d intervals after injection to count fluorescent cells in overlying cortex. The number of extant fluorescent cells remained roughly constant out to 16 d, then dropped substantially to a much lower but persistent number thereafter (Supplementary Fig. 2 online). This dropoff was accompanied by morphological changes in many surviving neurons, such as blebbing of processes and the appearance of 'bifurcating' somata, indicative of cytotoxicity.

We propose that the recombinant rabies virus presented here is the best retrograde tracer for revealing detailed neuronal morphology that has been available to neuroscientists so far. Because the genomic deletion of this mutant is of a gene essential for cell-to-cell spread but irrelevant to transcription and genome replication, infection occurs in an all-or-nothing fashion, resulting in cells labeled brilliantly and in great detail if they are labeled at all. Because of the relatively low cytopathic effect of rabies virus, this mutant nevertheless affords potentially several weeks of anatomical or physiological study of infected cells. Although lentiviral vectors would be a better choice for long-term study of neurons with minimal impact on their health, our results indicate that, for shorter-term studies where number of visibly labeled cells and intensity of label are at all important, there is little comparison to the recombinant rabies virus, which causes every infected cell to be illuminated in great detail. Also, of course, the system could be used for retrograde expression of any other gene beside the coding sequence for EGFP, suggesting a variety of physiological studies using fluorescent reporters of activity or photosensitive proteins for optical perturbation of membrane potential of cells targeted on the basis of their axonal projection targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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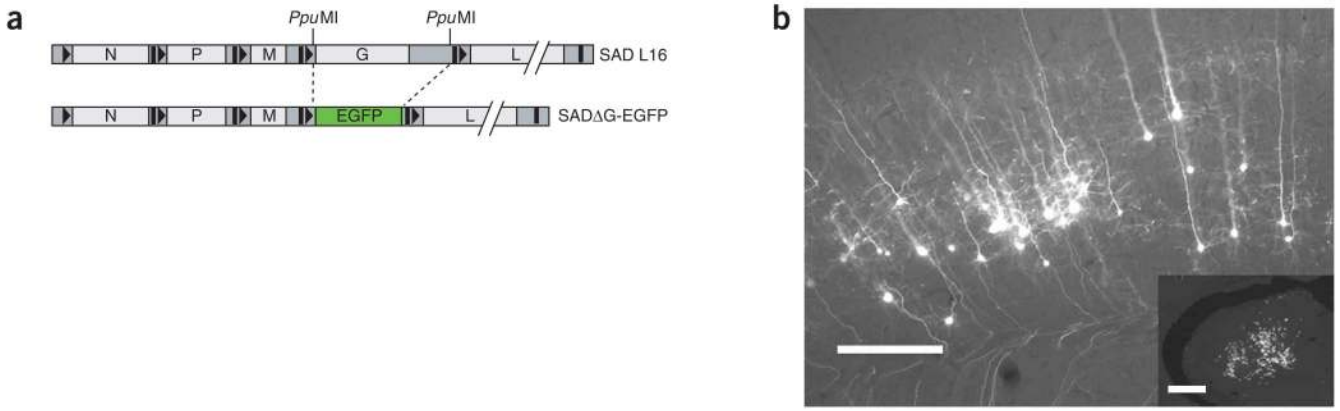

Figure 1.

The recombinant rabies virus SADΔG-EGFP. (**a**) In SADΔG-EGFP the glycoprotein (G) open reading frame is replaced with that of EGFP. EGFP mRNA is transcribed using the authentic G transcription start (black arrowhead) and stop-polyadenylation signals (black bar). Viral open reading frames, light gray boxes; noncoding sequences, gray; transcription signals, black. N, P, M and L denote the remaining viral genes. (**b**) An example of retrograde labeling with SADΔG-EGFP in mouse brain. These are widefield microscopic images of intrinsic EGFP fluorescence in unstained tissue, 6 d after injection. Top, deep-layer cortical pyramidal cells infected after injection in thalamus. Scale bar, 150 μm. Inset, injection site. Scale bar, 750 μm.

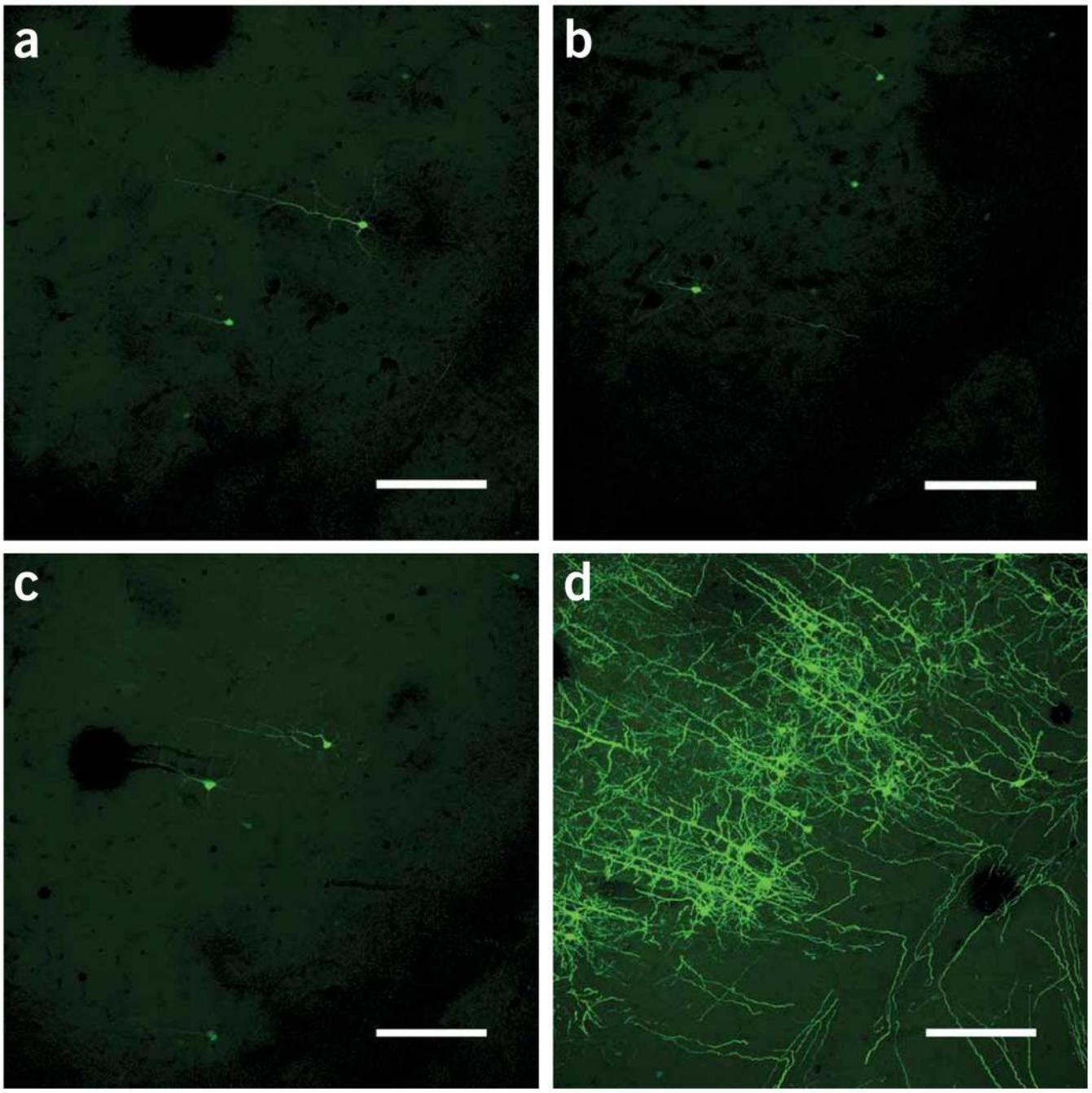

Figure 2.
Comparison of SADΔG-EGFP and lentivirus. These confocal images of intrinsic fluorescence, taken back to back using identical instrument settings, show cortical pyramidal cells retrogradely infected either with a lentivirus pseudotyped with the rabies virus glycoprotein and expressing EGFP driven by the cytomegalovirus promoter, or with the recombinant rabies virus SADΔG-EGFP. (**a**–**c**) Lentivirus. (**d**) The rabies virus SADΔG-EGFP. Scale bars, 150 μm.