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Supplemental information

Third-generation rabies viral vectors allow nontoxic retrograde targeting of projection neurons with greatly increased efficiency

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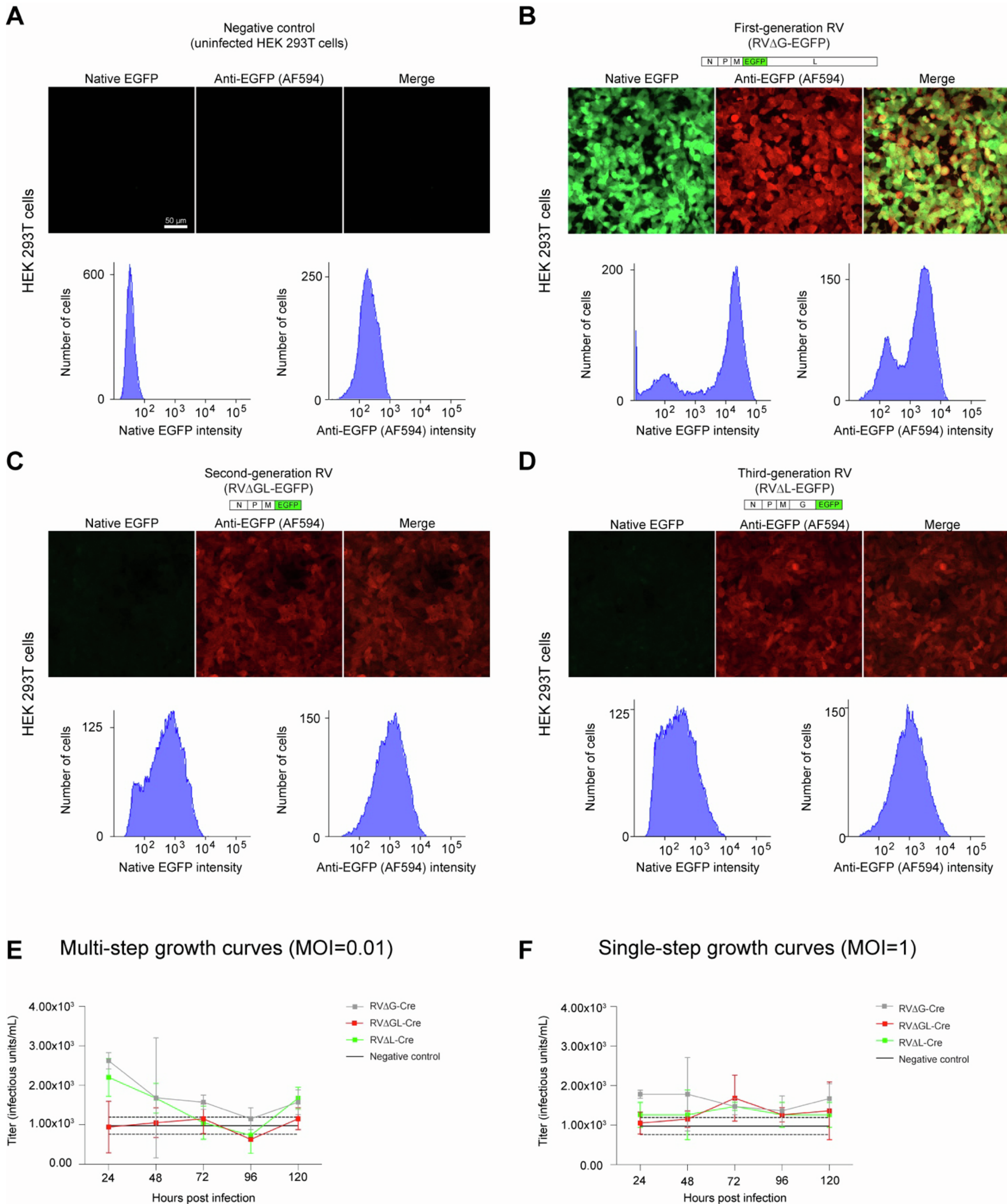
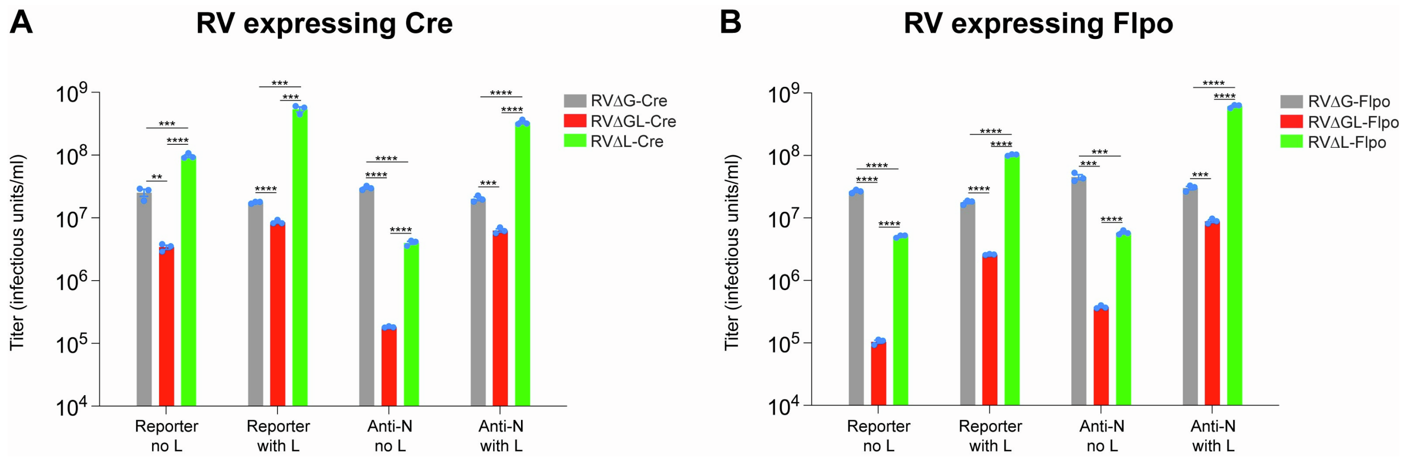
SUPPLEMENTAL INFORMATION



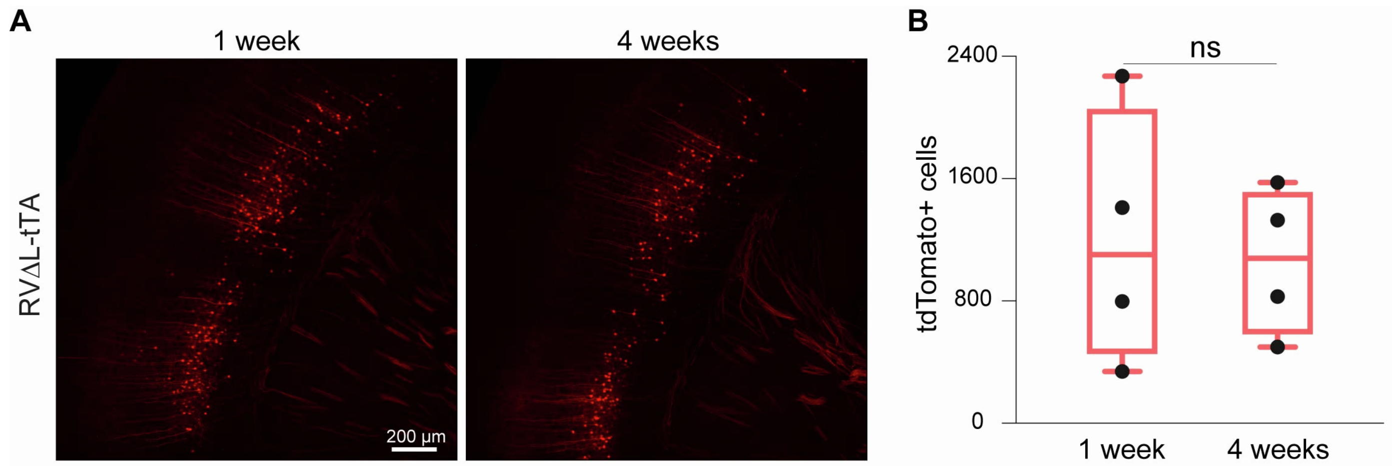
Supplementary Figure S1. ΔL and ΔGL viruses express EGFP at similarly low levels, and ΔG, ΔGL, and ΔL viruses do not propagate in non-complementing cells, Related to Figure 1.
Confocal images and flow cytometric histograms showing native and immunostained EGFP signal in uninfected cells (A) and cells infected with first-generation (ΔG) virus (B), second-generation (ΔGL) virus (C), or third-generation virus (D) expressing EGFP. Scale bar: 50 µm, applies to all images. (E-F), Viral titers in supernatants of BHK-21 cells not expressing any rabies viral genes, infected with ΔL, ΔGL, or ΔG viruses at a multiplicity of infection (MOI) of 0.01 ("multi-step growth curves", panel E) or 1 ("single-step" growth curves, panel F), with supernatants collected every 24 hours for five days. Graphs show mean ± s.e.m. .Black lines show negative control "titers" calculated from uninfected reporter cells (mean ± s.e.m. of 10 samples). Note that the titers in these graphs are 3-4 orders of magnitude lower than those obtained on complementing cells (Figure 1).



Supplementary Figure S2. Direct comparisons of titers of ΔG, ΔGL, and ΔL viruses expressing Cre and Flpo, each titered in four different ways, Related to Figure 1.
In order to directly compare the titers of the Cre- and Flpo-expressing versions of each of the three generations of rabies viral vectors, we made a fluorescent reporter cell line (293T-FLEX-F14F15S-BC) in which either Cre or Flpo activity causes mCherry expression (see Methods). We further made a version of this reporter line that expresses the rabies viral polymerase gene (293T-FLEX-F14F15S-BC-TTBL, see Methods), in order to amplify the low recombinase expression from ΔGL and ΔL vectors and thereby unmask subthreshold expression. In the case of ΔL viruses, but not ΔGL ones, this L expression would also allow replication and spread between cells, confounding interpretation; despite this, we titered the vectors of all three generations with and without L for completeness. After making side-by-side preparations of six viruses, RVΔG-Cre, RVΔGL-Cre, RVΔL-Cre, RVΔG-Flpo, RVΔGL-Flpo, and RVΔL-Flpo (see Methods), we titered them in triplicate by serial dilution on the above two dual reporter cell lines. For an additional readout that was independent of recombinase efficacy, we also immunostained for the rabies virus nucleoprotein, giving a total of four titering methods per virus (mCherry expression versus anti-nucleoprotein signal, with and without L). Noteworthy findings include the following:

1) The titer of each of the two ΔL viruses significantly exceeds that of the corresponding ΔGL virus using any of the four titering methods: i.e., the titer of RVΔL-Cre is significantly greater than that of RVΔGL-Cre, and the titer of RVΔL-Flpo is significantly greater than that of RVΔGL-Flpo, regardless of titering method.
2) For both ΔGL and ΔL vectors (but not ΔG vectors), the titer of the Cre-expressing version was significantly higher than that of the Flpo-expressing versions, when assessed by fluorescent reporter expression: i.e., the measured titer of RVΔL-Cre is significantly greater than that of RVΔL-Flpo, and the titer of RVΔGL-Cre is significantly greater than that of RVΔGL-Flpo, when titering based on reporter expression (and regardless of whether L was expressed *in trans*). However, when nucleoprotein staining was used to determine the presence of virus, the titer of each of the Flpo-expressing ΔGL and ΔL viruses were actually higher than that of the corresponding Cre-expressing one: that is, the titer of RVΔL-Flpo is significantly greater than that of RVΔL-Cre, and the titer of RVΔGL-Flpo is significantly greater than that of RVΔGL-Flpo, when titering using nucleoprotein staining, regardless of whether L was expressed *in trans*. These findings are consistent with an interpretation that the lower efficacy of Flpo versus that of Cre, coupled with the intentionally low expression levels of ΔGL and ΔL vectors, result in lower *de facto* titers (as judged by the percentage of mCherry-labeled reporter cells) and lower numbers of retrogradely labeled neurons *in vivo* (See Figure 2).
3) Expression of L *in trans* significantly increases the measured titers of all four ΔGL and ΔL viruses, whether measured by fluorescent reporter expression or nucleoprotein staining. This is also consistent with the above hypothesis that the intentionally low expression levels characteristic of these vectors may result in artificially low measured titers because of subthreshold expression of recombinase or nucleoprotein.

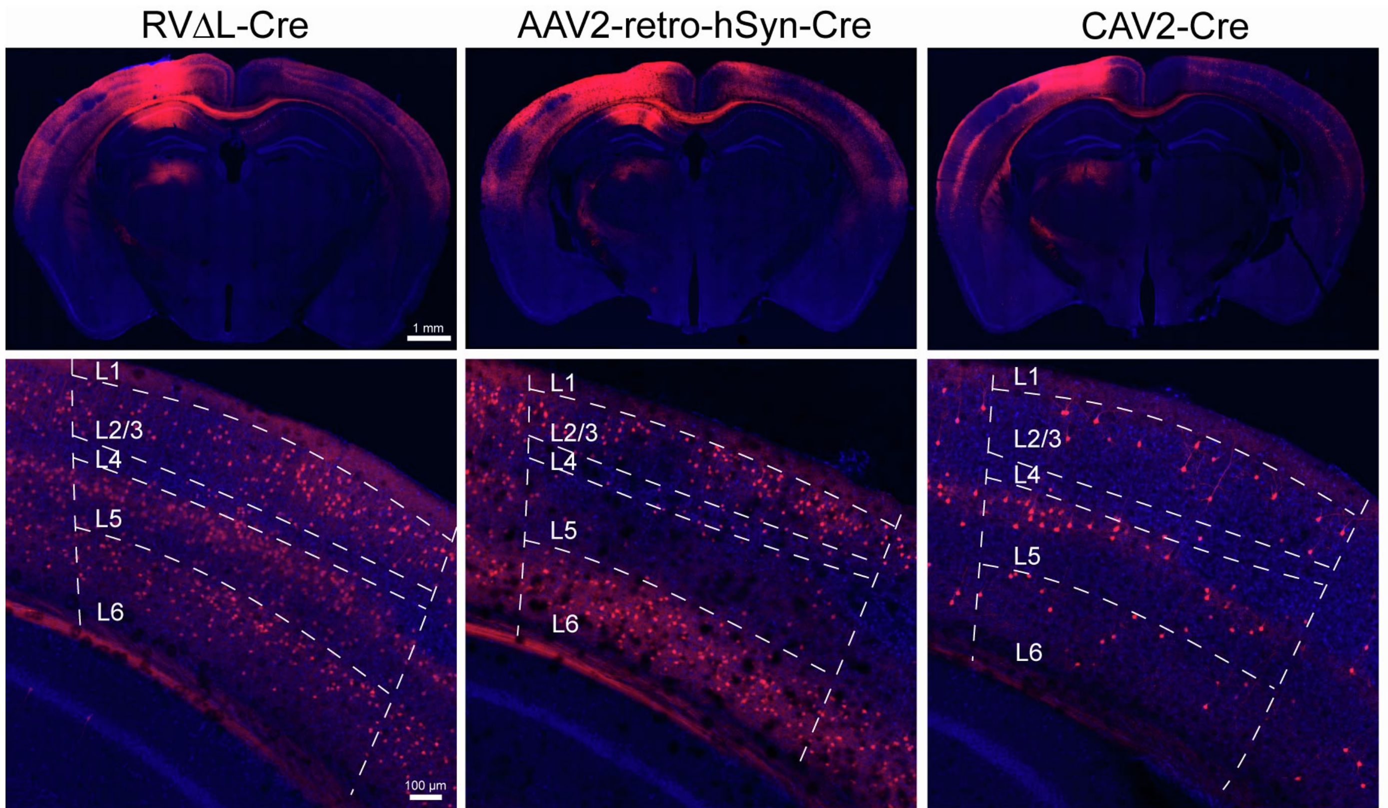
See Supplementary File S1 for titers and statistical comparisons.

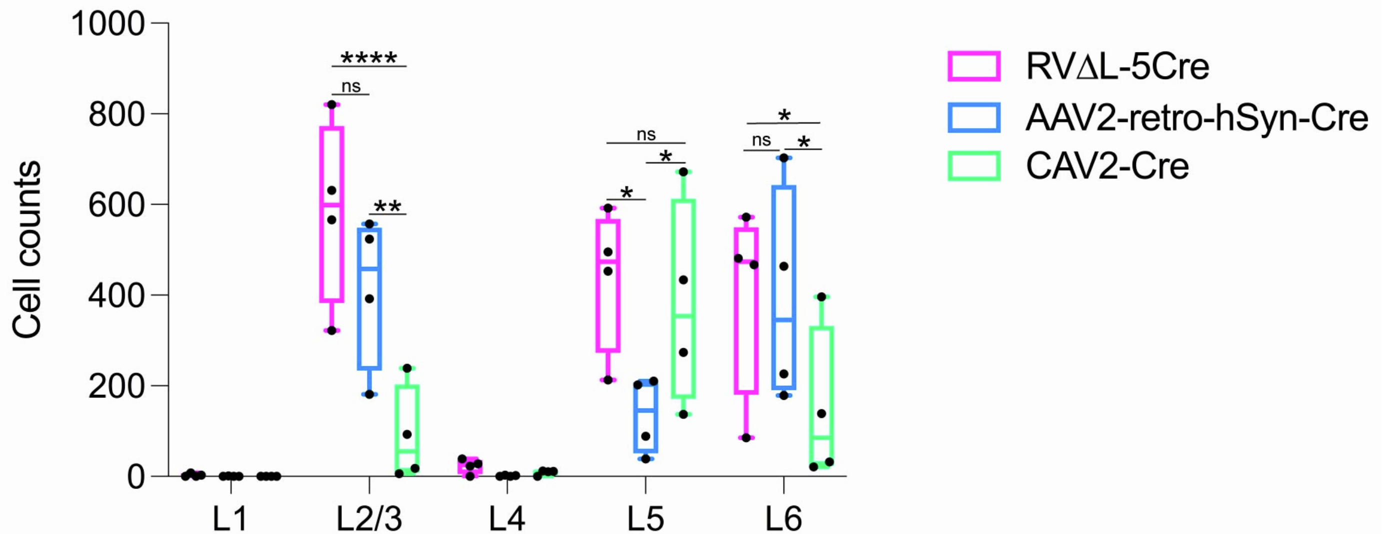


Supplementary Figure S3. Retrograde targeting with third-generation (ΔL) rabies virus expressing the tetracycline transactivator, Related to Figure 2.

(A) Corticothalamic neurons retrogradely labeled by a ΔL virus expressing tTA injected in the somatosensory thalamus of Ai63 reporter mice (tdTomato driven by TRE-tight) 1 week (left image) or 4 weeks (right image) prior to perfusion. Scale bar: 200 µm, applies to both images.

(B) Counts of labeled cortical neurons, with each data point being the total number found in one series consisting of every sixth 50 µm section from a given brain - see Methods). Numbers are not significantly different between the two time points (single factor ANOVA, $p = 0.772$, $n = 4$ mice per group). See Supplementary File S1 for counts and statistics).





Supplementary Figure S4. Retrograde labeling by ΔL rabies virus compared to rAAV2-retro and CAV-2: injections in anteromedial visual cortex (AM), Related to Figure 3.
RVΔL-Cre, rAAV2-retro-hSyn-Cre (from Addgene), or CAV-Cre (from the Plateforme de Vectorologie de Montpellier) was injected undiluted into the cortical anteromedial area (AM) of reporter mice, with injections being of equal volumes (200 µl); after a 4-week survival time, brain sections were imaged and labeled neurons in several brain regions were counted. Note that each data point is the total number in one series consisting of every sixth 50 µm section from a given brain (see Methods) so that the total number of labeled S1 neurons in each brain would be approximately six times the corresponding number shown here. In contralateral primary somatosensory cortex, RVΔL labeled more cells in layers 2/3, 5, and 6 than did either of the other two viruses, although the difference between RVΔL and the "runner up" in each case (rAAV2-retro in layers 2/3 and 6, CAV-2 in layer 5) was not statistically significant. Few neurons were labeled in layers 1 and 4, and the differences in these layers were not significant. See Supplementary File S1 for all counts and statistical comparisons**.** See also Supplementary Files S2a – S4b for sets of high-resolution confocal images of series of coronal sections from mice labeled with each of the three viruses injected in AM.