Nontoxic, double-deletion-mutant rabies viral vectors for retrograde targeting of projection neurons

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Recombinant rabies viral vectors have proven useful for applications including retrograde targeting of projection neurons and monosynaptic tracing, but their cytotoxicity has limited their use to short-term experiments. Here we introduce a new class of double-deletion-mutant rabies viral vectors that left transduced cells alive and healthy indefinitely. Deletion of the viral polymerase gene abolished cytotoxicity and reduced transgene expression to trace levels but left vectors still able to retrogradely infect projection neurons and express recombinases, allowing downstream expression of other transgene products such as fluorophores and calcium indicators. The morphology of retrogradely targeted cells appeared unperturbed at 1 year postinjection. Whole-cell patch-clamp recordings showed no physiological abnormalities at 8 weeks. Longitudinal two-photon structural and functional imaging in vivo, tracking thousands of individual neurons for up to 4 months, showed that transduced neurons did not die but retained stable visual response properties even at the longest time points imaged.

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ince their introduction to neuroscience in 20071, recombinant rabies viral vectors have come to occupy a unique role in the field. Their most important and widespread use is in monosynaptic tracing, which allows identification and study of neurons directly presynaptic to single neurons or targeted cell types2–12.

Another important application is retrograde infection of projection neurons, to express transgene products such as fluorophores13,14,18 or activity indicators19–22 selectively within groups of neurons targeted on the basis of their axonal projections. Both of these applications have yielded insights into the organization of neural circuits in a wide variety of regions, ranging from cerebral cortex to spinal cord.

These vectors, however, come with the major downside of causing nontrivial cytotoxicity. All rabies viral (RV) vectors used to date for retrograde targeting and monosynaptic tracing (which we refer to below as ‘first-generation’ or ‘ΔG’ rabies viral vectors) have a single gene, G (NCBI symbol: RABVgp4), encoding the viral envelope glycoprotein, deleted from their genomes, with the other four viral genes left intact1 (Fig. 1a). Because the glycoprotein is only required for the final steps of the viral life cycle, this deletion prevents the virus from spreading beyond initially infected cells, but it does not prevent the virus from completing the entirety of its replication cycle up to that point. This is a double-edged sword. On the one hand, it results in rapid and high-level expression of transgenes, which can be extremely useful for such applications as imaging fine cellular processes or optogenetically manipulating neuronal activity18,23,24. On the other hand, it also results in fairly rapid cytotoxicity, with death of many or most infected cells occurring on a timescale of ~2 weeks1. This, of course, prevents the vectors from being used for long-term imaging or optogenetic experiments.

Efforts to reduce the toxicity of RV vectors have been made previously. It has recently been reported, for example, that first-generation RV vectors from a different parent strain (CVS N2c, rather than SAD B19) allow infected neurons to survive longer23. However, to our knowledge no claim has been made, and no evidence suggests, that the use of CVS N2c or any other strain completely eliminates the toxicity of first-generation vectors. It has also recently been reported that addition of a proteasome-targeting destabilization domain to the viral nucleoprotein removes viral toxicity25. However, because individual infected neurons were not tracked over time in these studies, it is difficult to assess whether cytotoxicity was completely abolished.

Here we take a quite different approach toward eliminating toxicity (Fig. 1b). We introduce a new class of RV vector, from the genome of which we have deleted a second and arguably much more fundamental gene. Known as L (for ‘large protein’ gene (NCBI symbol: RABVgp5), P being already taken as the name of the viral phosphoprotein gene), this second deleted gene encodes the viral polymerase, which is required both for transcription of viral genes and for replication of the viral genome26. We hypothesized that deletion of the polymerase gene would reduce expression of viral genes and transgenes to miniscule levels, resulting in a vector that would cause no detectable cytotoxicity whatsoever. We further hypothesized that, if such vectors encoded recombinases, the tiny remnant transcription levels (resulting from the activity of the few starter copies of the polymerase that are packaged in each viral particle27)
**Fig. 1** Deletion of the RV polymerase gene reduces gene expression to trace levels but leaves recombinase-encoding vectors still able to cause reporter expression in cell culture. (a, b) Strategy for eliminating the toxicity of rabies viral vectors. (a) In first-generation vectors, only the G gene, encoding the envelope glycoprotein, is deleted. This prevents the virus from spreading beyond directly infected cells but still allows the virus to replicate within them, resulting in rapid, high transgene expression as well as severe toxicity on a timescale of 1–2 weeks. (b) In the second-generation RV vectors introduced here, the L gene, encoding the viral polymerase, has also been deleted to reduce expression from the viral genome to trace levels. Because transgene expression should therefore also be reduced, the gene for a recombinase, such as Cre or FLP, is used to allow the expected very low expression levels to cause subsequent expression of a reporter at experimentally useful levels. (c–i) Results in cell culture: images and flow-cytometric histograms show EGFP fluorescence in cells infected by first- and second-generation RV vectors. Left images and histograms in c–f show native EGFP fluorescence; middle images and right histograms in c–f show anti-GFP immunolabeling with Alexa Fluor-594 (AF 594) secondary antibody; right images show overlay of left and middle images. X axes of histograms are log scale with arbitrary units. (c) Negative control: HEK 293T cells not infected with any virus do not express EGFP, as indicated by the single peak at a very low fluorescence level. (d) A first-generation RV vector encoding EGFP causes infected HEK 293T cells to fluoresce brightly, as shown by the peak far to the right of the histogram (the leftmost peak represents uninfected cells in the same well). (e,f) Second-generation RV vectors encoding EGFP cause almost undetectable EGFP expression in infected cells, as shown by histograms that resemble those of the uninfected cells shown in c; immunostaining for EGFP shows that virus is nonetheless present and producing EGFP at low levels. This is true whether the EGFP gene is placed in the most highly expressing locus at the start of the viral genome (e) or in the ‘usual’ transgene locus of the deleted G gene (f). (g–i) We then constructed second-generation RV vectors encoding Cre recombinase and tested them in reporter cells that express EGFP when Cre is expressed. (g) Negative control: uninfected reporter cells express very little EGFP. (h,i) Reporter cells infected with second-generation RV vectors encoding Cre express abundant EGFP. This is true whether the Cre gene is inserted at the start of the RV genome (h) or into the G locus (i). Experiments in this figure were performed twice with similar results each time. Scale bars, 33 μm.
would nevertheless be sufficient to achieve site-specific recombination in genomic reporter alleles in reporter mouse lines.

We show that these new (‘second-generation’ or ‘ΔGL’) viral vectors can be used to express both Cre and Flp recombinases at levels sufficient to effect reporter gene expression in widely available reporter mice and in wild-type animals when used in combination with Cre-dependent adeno-associated viral (AAV) vectors, that the new vectors retain the first generation’s characteristic ability to retrogradely infect projection neurons, and that targeted neurons appear morphologically normal up to a year postinjection. Using whole-cell recording in slices containing retrogradely targeted basolateral amygdala neurons, we show that transduced neurons exhibit normal membrane properties at 2 months postinjection. We use longitudinal two-photon structural and functional imaging in vivo in mouse primary visual cortex to show that targeted neurons exhibit no signs of toxicity and that responses to visual stimuli remain stable for at least 4 months, the longest time point in our dataset. Finally, we find that the new RV vector class has broader tropism for corticocortical projections than two other viral vector species commonly used for retrogradely targeting projection neurons.

This new class of vectors is immediately useful as a means of retrogradely targeting projection neurons to express Cre or other recombinases with no apparent toxic effects, allowing systematic selection of different classes of neurons for nonperturbative long-term anatomical or physiological study. This work also lays the foundation for the construction of a future second-generation monosynaptic tracing system that will leave transsynaptically transduced neurons alive and healthy indefinitely.

RESULTS

Testing in cell culture. We began by testing our hypothesis that L deletion would reduce vector expression to trace levels. Because genes in the rabies viral genome are expressed at levels that decrease with their positional order in the genome, we made and tested two variants of ΔGL rabies virus encoding enhanced green fluorescent protein (EGFP)37. In the first, RVΔGL-1EGFP, the EGFP gene was inserted in the highest-expressing locus, at the beginning of the genome before the remaining viral genes (note that the numbers in the vector names refer to the position of the transgene with respect to those of the other genes in the viral genome). In the second, RVΔGL-4EGFP, the EGFP gene was inserted in the lowest-expressing locus, at the end of the viral genome. Histograms of EGFP fluorescence in HEK 293T cells show that, whereas first-generation vectors encoding EGFP cause very bright fluorescence in infected cells (Fig. 1d; cf. uninfected negative control in Fig. 1c), second-generation vectors encoding EGFP (Fig. 1e,f) caused cells to express so little EGFP as to be nearly indistinguishable from uninfected controls, although anti-GFP immunostaining (Fig. 1c–e) confirmed that EGFP was indeed present at low levels. This was true whether the EGFP gene was inserted in the highest-expressing (Fig. 1e) or lowest-expressing (Fig. 1f) locus in the vector genome, indicating that L deletion indeed reduced gene expression to levels far below those of first-generation vectors, confirming our expectations.

However, these results raised the possibility that L deletion could reduce transgene expression so much that it would render the new vectors useless for neurobiological applications. Our next step was therefore to test whether second-generation RV vectors encoding a recombinase, as opposed to a fluorophore, are capable of expression at levels sufficient to activate reporter gene expression when used in the presence of a suitable recombinase-dependent reporter construct. We constructed two ΔGL vectors encoding Cre recombinase36, the first (RVΔGL-1Cre) with the Cre gene inserted into the highest-expressing locus and the second (RVΔGL-4Cre) with it inserted into the lowest-expressing locus. We tested the viruses on a reporter cell line that expresses EGFP following Cre recombination, and the results were encouraging. Whereas uninfected control cells were not fluorescent (Fig. 1g), cells infected with either of the ΔGL vectors fluoresced brightly (Fig. 1h,i), indicating successful recombination of the reporter cells’ EGFP expression cassette by viral expression of Cre. Because even RVΔGL-4Cre, the virus with the Cre gene in the lowest-expressing locus, was able to recombine the reporter cassettes, we used this version for the subsequent in vivo assays and refer to it simply as ‘RVΔGL-Cre’ below.

Anatomical testing in vivo. For our tests of the new class of vectors in vivo, we had three primary questions. (i) Are ΔGL vectors encoding recombinases capable of causing reporter expression in brains? (ii) Are the new vectors retrogradely infectious like the first-generation ones, so that projection neurons can be selectively targeted? (iii) Do the new vectors leave infected neurons alive and apparently healthy, and if so for how long?

Our in vivo experiments gave positive results on all three counts. To test the versatility of the new ΔGL vector class, we constructed two versions: the Cre-encoding vector RVΔGL-Cre, described above, and RVΔGL-Flpo, encoding Flp recombinase31,32. We injected these vectors into the somatosensory thalamus in mice of two reporter strains expressing the red fluorophore tdTomato33 after reporter cassette recombination by the corresponding recombinase (see Methods). Note that all vectors described in this paper are

Fig. 2 | Second-generation RV vectors encoding Cre and Flp retrogradely infect projection neurons, activate reporter expression and cause no apparent morphological abnormalities even up to a year after injection. a,b, Cortical neurons retrogradely labeled by second-generation RV vectors encoding either (a) Cre (7-d survival) or (b) Flp (14-d survival) recombinase injected into somatosensory thalamus of tdTomato reporter mice. c–d, Cortical neurons retrogradely labeled by second-generation RV vectors encoding Cre (c) or Flp recombinase (d) injected into the thalamus of reporter mice 1 year before perfusion. e,f, High-magnification images of dendrites and other fine processes of cortical neurons from the same sections imaged for c and d, showing apparently normal morphology with a complete absence of decomposition, blebbing or other abnormalities. These results indicate (i) that second-generation RV vectors are capable of expressing recombinases at levels sufficient to activate downstream transgene expression in readily available reporter mice; (ii) that second-generation RV vectors are capable of efficient retrograde infection of projection neurons; (iii) that the new vectors can be used to express different recombinases, allowing a variety of potential intersectional applications; and (iv) that the new vectors do not appear to cause cytotoxicity even on time scales comparable to the lifetime of the animal. Experiments in this figure were performed in 2 or 3 animals per condition, with similar results in all cases per condition. Scale bar in a–d, 250 μm; scale bars in e,f, 5 μm.
coated with the native rabies virus envelope protein, which confers on first-generation rabies viral vectors the ability to retrogradely infect large numbers of neurons that project to the injection site. The result of these thalamic injections was bright fluorescence in hundreds of corticothalamic neurons in layer 6 of overlying somatosensory cortex for both RVΔGL-Cre (Fig. 2a,c,e) and RVΔGL-FLpo (Fig. 2b,d,f). Even with short survival times (Fig. 2a,b), abundant cortical neurons were labeled. This indicated two notable facts: first, that RVΔGL-Cre and RVΔGL-FLpo are quite capable of activating reporter gene expression in common reporter mice and, second, that second-generation RV vectors efficiently and retrogradely infect projection neurons, as do their first-generation counterparts. Also, the fact that the efficacy of ΔGL vectors is not restricted to use with a single recombinase indicates that they will likely be useful for a variety of intersectional and simultaneous targeting approaches.

We also investigated whether the second-generation rabies viral vectors are compatible with recombinase-dependent AAV vectors, so that their use would not be restricted to applications in reporter mice. We found that this approach worked well in both mice and rats, as shown in Supplementary Figs. 1 and 2. This indicates that the vectors can be used in wild-type animals of multiple species for targeting expression of fluorophores and opsins to projection neurons selected on the basis of the locations of both their somata and axonal arborizations.

While these results demonstrate that the new second-generation rabies viral vectors retain the ability of first-generation ones to retrogradely infect projection neurons and that they are capable of causing recombinase-dependent expression of reporter genes, it remained to be seen to what degree L deletion achieved the main desired effect, namely, the elimination of the cytotoxicity that characterizes previous RV vectors. For a preliminary, qualitative answer to the question of preservation of the health of infected neurons, we simply conducted a series of thalamic injections of RVΔGL-Cre in Ai14 mice and RVΔGL-FLpo in Ai65F mice, killing them for analysis after increasing lengths of time. Even after leaving mice for an entire year after injection, brightly fluorescent cortical neurons were found in large numbers throughout layer 6 of somatosensory cortex (Fig. 2c,d). At higher magnification (Fig. 2e,f), dendritic spines and other fine processes were clearly intact and showed no blebbing or otherwise altered structures indicative of toxicity.

Longitudinal two-photon structural imaging. Although it appeared from these results that the second-generation rabies viral vectors do not kill neurons, there remained the possibility that only some of the labeled neurons survived over long periods, with others having died off. For a more rigorous examination, we conducted longitudinal two-photon imaging in vivo of labeled neurons in visual cortex to monitor individual neurons over the course...
of months. This stringent test allowed us to see how many of the labeled neurons survived for long periods.

For these longitudinal imaging experiments, we compared the effects of the second-generation virus RV\textsuperscript{ΔG}Cre to those of two first-generation (ΔG) control viruses. The first control virus, RVΔG-tdTomato, contains the gene for tdTomato in its genome, while the second control virus, RVΔG-Cre, encodes Cre in its genome. We injected each virus into primary visual cortex of mice (Ai14-ΔG vector, which encoded Cre instead of tdTomato. This control vector RV vectors, the second-generation vector RV\textsuperscript{ΔGL-Cre} to those of two

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Ex vivo electrophysiology. While it was clear at this point that the second-generation vector was not killing neurons, it remained to be seen whether neurons transduced with the second-generation vector, and those remnant neurons that survived infection with its first-generation (RVΔG-Cre) counterpart, were physiologically normal. As expected, most neurons infected by the first-generation control virus, which encoded Cre instead of tdTomato. This virus killed a large fraction of infected neurons but by no means all of them (Fig. 3a–c and Supplementary Video 2). Approximately 50% of cells infected with RVΔG-Cre survived up to 4 months post-infection (Fig. 3b,c). This result, that simply encoding Cre instead of tdTomato made for a much less toxic first-generation rabies viral vector, is consistent with a previous report that some neurons infected with a replication-competent RV encoding Cre can survive infection, albeit with altered gene expression\textsuperscript{36}. Nevertheless, even this less-toxic ΔG vector killed neurons over the first few weeks of imaging, and, as will be seen from the physiological results below (Figs. 4 and 5), we found signs that at least some of the surviving neurons had abnormal membrane and visual response properties.

In contrast to the complete or partial lethality of the first-generation control vectors, the second-generation vector RVΔG-Cre did not kill neurons at all (Fig. 3a–c and Supplementary Video 3). The numbers of detected neurons actually increased over the first several imaging sessions and remained constant thereafter, presumably reflecting the gradual buildup of tdTomato fluorescence above detection threshold (note the relative dimness of the cells in the week 1 image compared to that in subsequent images). After the population of labeled cells stabilized, every cell in large imaged volumes (Fig. 3d and Supplementary Video 4) could be found at later time points, and we saw no cell death in any imaged FOV across the longitudinal study.
normal. We therefore injected first-generation (RVΔG-Cre) and second-generation (RVΔG-Cre) vectors into the nucleus accumbens of Ai14 (tdTomato) reporter mice, along with fluorescent retrobeads in the contralateral nucleus accumbens of each mouse, then conducted whole-cell patch-clamp electrophysiology in slices from retrogradely labeled basolateral amygdala neurons at 8 weeks postinjection (Fig. 4). We found no significant difference in any measured membrane property between the neurons labeled with the second-generation vector versus the retrobead-labeled controls after 8 weeks of expression (Fig. 4d–f). In contrast, neurons that survived labeling with the first-generation vector had significantly more negative action potential thresholds than retrobead-labeled neurons (Fig. 4f). These data indicated that the second-generation vector RVΔG-Cre did not produce detectable changes in the membrane properties of neurons, whereas the first-generation Cre-encoding control vector RVΔ...
Fig. 6 | Second-generation RV vectors retrogradely infect more types of corticocortical cells than either CAV or rAAV2-retro vectors. a–e, CAV2-Cre (a,b), RVΔGL-Cre (c,d), or rAAV2-retro-EF1α-Cre (e) was injected into anterior cingulate area (ACA) of Ai75 mice, so that cells across the brain that provide input to ACA were labeled via Cre-induced nuclear tdTomato expression. The approximate center of the injection site for each experiment is shown in the top row (left side of images). Most brain regions containing retrogradely labeled cells from ACA were consistently identified across all three types of virus experiments, with at least one notable exception (CA1 contained labeled cells in RVΔGL images). Most brain regions containing retrogradely labeled cells from ACA were consistently identified across all three types of virus experiments, with at least one notable exception (CA1 contained labeled cells in RVΔGL, shown in c,d, row v). However, within the same areas, specifically in the cortex, we observed differences suggesting tropism or other biases in the specific types of neurons labeled. For example, labeled input cells in the contralateral ACA (boxes in row i) were predominantly in layer 5 (L5) for CAV2-Cre (a, row i), but were located more uniformly across layers with RVΔGL (c, row i) and AAV-retro-EF1α (e, row i). Similar differences between Cre+ input cells across layers were observed in most cortical areas, including ventrolateral orbital cortex (ORBvI, row iii), retrosplenial cortex (RSP, rows iv and v), and anterolateral visual cortex (VISal, rows v and vi). Quantification of the number of labeled cells per layer indicated that, in all cases, CAV2-Cre labeling was biased toward L5 neurons, whereas RVΔGL resulted in input cells labeled across all layers providing long-range interareal inputs (L2/3, L5, L6, row vii). In contrast to RVΔGL and CAV2-Cre in VISal and RSP, AAV-retro-EF1α appeared to be biased toward L2/3, with only sparse labeling in L5 (c,d, row vii). Cre+ input cells were also observed in subcortical structures, including several thalamic nuclei (for example, mediodorsal nucleus (MD), posterior complex (PO) and the central lateral nucleus (CL), seen in row iv, consistent with known connectivity of ACA. The more prominent labeling in RVΔGL and AAV-retro-EF1α cases in the thalamus (c–e, row iv) may be due to true differences in efficiency between viruses, or in the amount of viral uptake for each experiment; further experiments are necessary to explore this observation. Images in a and b were acquired by serial two-photon tomography. Images in c–e were acquired by epifluorescence microscopy and sections counterstained with DAPI (blue). Graphs in a–d, row vii, show the mean and full range of values (min to max, n=2 independent experiments for CAV2-Cre and RVΔGL-Cre; n=1 experiment for AAV-retro-EF1α-Cre). Boxplots in e, row vii, indicate the median and full range (min to max) in all four regions from a–d, row vii (n=5 independent experiments). Scale bars: rows i and iii–v, 1mm; rows ii and vi, 500μm.
Longitudinal two-photon functional imaging. To determine whether neurons transduced with the second-generation vector retained normal circuit response properties long-term, and to determine the degree to which those cells that survive transduction with the first-generation vector encoding Cre do as well, we conducted longitudinal in vivo two-photon calcium imaging of visual responses using GCaMP6f, again with repeated imaging of the same FOVs. We attempted to conduct long-term imaging of neurons infected with first-generation virus encoding GCaMP6f directly (RVΔG-4GCaMP6f) but found no responsive neurons beyond the 2-week time point when using this virus (see Supplementary Fig. 3g–i). We therefore injected either first-generation (RVΔG-Cre) or second-generation (RVΔGL-Cre) virus into the visual cortex of GCaMP6f reporter mice and imaged the visual response properties of transduced neurons to drifting gratings presented to the awake animal at time points up to 4 months.

At 16 weeks after injection, we found that neurons transduced with the second-generation vector had completely normal response properties. Those that responded to drifting gratings were well tuned to specific orientations (Fig. 5a), most showed spontaneous activity and there were no bright—but-inactive (bright—inactive) neurons in any FOV or time point (Fig. 5c–e and Supplementary Video 5). Tuning preferences of highly responsive individual neurons were stable over sessions spanning the entire 4 months of imaging (Supplementary Fig. 4). By contrast, neurons surviving infection with the first-generation vector RVΔG-Cre often had abnormal response properties. Many RVΔG-Cre-infected neurons displayed the bright—inactive phenotype (Fig. 5b–d) of very high fluorescence levels with no calcium transients or indication of spiking activity during visual stimulation. Some showed blebbing. Further (Fig. 5c), some cells showed occasional calcium transients followed by long, sustained increases in brightness that were distinct from the bright—inactive phenotype (see Supplementary Video 6 for examples of these pathologies). None of these aberrant physiological profiles were observed in any neurons labeled with the second-generation vector. As in the structural imaging experiments with tdTomato, neurons transduced with second-generation vector showed a gradual buildup of GCaMP6f, with the dimmer baseline fluorescence (F) reflected in higher signal amplitude ΔF/F at the earliest time points (Fig. 5f). Excluding cells with gross pathologies like bright—inactivity and sustained activity from the analysis, the population of neurons infected with the first-generation vector also showed a tendency toward more ‘sluggish’ calcium dynamics, with a large mean increase (29.1%) in the decay-time constant (τ) of calcium transients between the 2-week and 8-week imaging sessions (Fig. 5g). By contrast, neurons labeled with the second-generation vector showed stable time constants with no significant change in τ. All of these results indicate that the neurons labeled with the second-generation vector retained stable response properties at 8 and 16 weeks postinjection, whereas neurons infected with the first-generation vector, even when they survived for long durations, showed substantial changes and dysfunction over time.

Comparison to other vector species. As a final characterization, we conducted a direct comparison in vivo of the retrograde transduction ability of our second-generation rabies virus to two other viral species used for this application: canine adenovirus type 2 (CAV2) and the newly developed rAAV2-retro. We injected each of the three viruses in the anterior cingulate cortex of Ai75 reporter mice, in which nuclear-localized tdTomato is expressed following Cre recombination (Fig. 6). While all three viruses retrogradely labeled many cells in primary visual cortex, CAV2-Cre exhibited a pronounced preference for layer 5 cells, whereas rAAV2-retro labeled many cells in upper and lower layers but very few in layer 5. In contrast, RVΔGL-Cre labeled many cells in all cortical areas. Injections of the three vectors in other cortical areas showed similar results (Supplementary Fig. 5). These data are consistent with previous reports of both CAV2-Cre and rAAV2-retro having strong tropisms for particular cell types over others (in projections other than the ones examined here) and suggest that tropism may be less of an issue with second-generation RV than with these other two vector species.

DISCUSSION

Here we have introduced a class of RV vectors that do not cause any detectable structural or functional abnormalities in transduced neurons, in stark contrast to their first-generation counterparts. We have shown that these second-generation RV vectors encoding either Cre or Flp recombine also are effective at causing reporter-gene expression both in cell culture and in vivo in readily available reporter mouse lines and in wild-type animals with reporter AAVs, that they retain first-generation vectors’ ability to efficiently retrogradely transduce projection neurons far from the injection site, with wide tropism, and that these retrogradely transduced neurons appear morphologically and physiologically normal even at the longest time points examined. We expect that this new class of vectors will be a useful new tool for neuroscientists investigating neurons selected on the basis of their extrinsic projections.

Requirements for second-generation monosynaptic tracing. Given that these second-generation RV vectors do not structurally or functionally perturb neurons over any time scale examined and can be used to express recombinases in retrogradely infected projection neurons, an exciting potential application for this new class of vectors would be monosynaptic tracing. Monosynaptic tracing with first-generation RV vectors already allows neurons directly presynaptic to a targeted neuronal population or single neuron to be labeled for short-term anatomical identification, optogenetic manipulation, or activity imaging, but the toxicity of first-generation RV vectors confines these experiments to short-term experiments. A monosynaptic tracing system based on the second-generation RV vectors should allow labeled presynaptic networks to be imaged or optogenetically manipulated for months or even years. First-generation monosynaptic tracing requires coexpression of the single deleted gene (G), while a second-generation monosynaptic tracing system will also require expression of the deleted polymerase gene (L). In some contexts, this is theoretically very simple. Single-cell monosynaptic tracing, for example, currently entails introduction into single neurons, via a patch pipette, a mixture of plasmids encoding G, the receptor TVA (for allowing entry of the EnvA-enveloped RV into the targeted neuron) and a fluorophore to mark the targeted cell. Second-generation RV would only require inclusion of a fourth plasmid, encoding L. While such direct transfection of single cells is the conceptually simplest implementation of monosynaptic tracing, the much more accessible and widely adopted approach is to label inputs to groups of neurons, using Cre or Flp mouse lines in combination with adenovirus–associated ‘helper viruses’ that express G, TVA, and a fluorescent protein in the recombine-expressing cells. This approach has allowed, for example, whole-brain mapping of inputs to dopaminergic neurons in the midbrain and noradrenergic neurons in the locus coeruleus. Adaptation of the helper virus approach to a second-generation version is, in principle, also straightforward. However, simply including an L-expressing AAV vector in the mix is problematic, because, at 6 kb in length, L exceeds the packaging capacity of AAV. Alternative approaches currently being pursued include generating L-expressing mouse lines and splitting the polymerase into two complementary halves, each within the packaging capacity
of AAV. An aspect of RV biology that is likely to be very helpful to these efforts is the fact that the polymerase gene is expressed at very low levels by wild-type rabies virus, much lower than those of the other four viral genes, including G[46,47]. Achieving sufficient expression levels of L from the complementing systems—whether through direct transfection, genomic alleles or helper viruses—therefore appears unlikely to be a problem.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-018-0091-7.

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Author contributions

S.C. helped design experiments, performed two-photon imaging and quantified and analyzed the results, assisted by S.Y. and A.C. They also produced RV vectors in vivo for anatomical experiments in reporter mice and, in combination with FLEX AAV in rats; they also processed the tissue for these experiments, assisted by J.E.M. T.K.L. also conducted confocal imaging. K.R.B. bred mice for the anatomical experiments, and S.C. helped design experiments, performed two-photon imaging and quantified and analyzed the results. H.A.S. produced all RV vectors, S.C. helped design experiments, performed two-photon imaging and quantified and analyzed the results. B.J.M. and R.C.R. oversaw these comparative and combinatorial experiments, and S.Y. and A.C. produced retrobeads for these experiments and assisted with cloning viral vectors. G.A.M., A.B., and G.G.C. performed experiments (injections, histology, imaging and analysis) comparing RV with rAAV2-retro stocks. I.R.W. conceived and designed the study; I.R.W. and S.C. wrote the manuscript with input from other authors. Research reported in this publication was supported primarily by the National Institute of Mental Health under award number U01MH106018 (BRAIN Initiative) to I.R.W. and in part by the National Institute on Aging under award number R01AG047589 to J.A.H. and the National Eye Institute under award numbers R01EY010115 and R01EY018472 to R.C.R. K.M.T. was supported in part by a New York Stem Cell Foundation Robertson Investigator award and McKnight Scholar Award, and G.G.C. was supported by a JFDP fellowship and a NARSAD Young Investigator Award. The content of this paper is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Competing interests

The authors declare no competing interests.

Additional information

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**Methods**

**Animals.** All experimental procedures related to the use of mice were conducted according to NIH guidelines. Experiments performed at the Allen Institute were approved by the Institutional Animal Care and Use Committee (IACUC) of the Allen Institute for Brain Science. Experiments performed at MIT were approved by the MIT Committee for Animal Care (CAC). Animals were housed singly postinjection, and were kept on a normal light/dark cycle for all experiments except awake two-photon imaging.

**Cloning.** Expression vectors for rabies viral genes and the T7 polymerase gene as well as the polymerase gene as well as the polymerase gene were made by cloning into pSP-C-SP-PW-GFP (Addgene #13337) the CAG promoter and a Fp-dependent ‘FLEX’ construct consisting of pairs of compatible FRT sites flanking a back-to-back fusion of the reverse-complemented gene for TagRFP-T immediately followed by the forward gene for EGFP.

First-generation vector genome plasmids were made by insertion of the tdTomato or mammalian codon-optimized Cre gene into the T7 promoter cassette of the pEGFP-N3 vector plasmid (Clontech Laboratories). The Flp reporter construct pLV-CAG-F14F15S-Switch was made by cloning into pSP-C-SP-PW-GFP (Addgene #13337) the CAG promoter and a Fp-dependent ‘FLEX’ construct consisting of pairs of compatible FRT sites flanking a back-to-back fusion of the reverse-complemented gene for TagRFP-T immediately followed by the forward gene for EGFP.

Production of second-generation rabies viral vectors (RVΔG-EGFP, RVΔG-tdTomato and RVΔG-Cre) were made as described. Briefly, HEK 293T cells (ATCC, CRL 11288) were transfected with expression vectors for the rhabdovirus-flankned viral gene (pRVΔG-EGFP, pRVΔG-tdTomato or pRVΔG-Cre), rabies viral genes (pCAG-B19N, pCAG-B19P, pCAG-B19G and pCAG-B19L) and the T7 polymerase (pCAG-T7pol).

Survival imaging and flow cytometric analysis of HEK 293T cells. HEK 293T cells infected with serial dilutions of RVΔG-EGFP and RVΔG-tTomato, as described, were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton-X (Sigma) in 3% normal donkey serum (Jackson Immuno, #017-000-121), and incubated with 1:10,000 chicken anti-GFP polyclonal primary antibody (Aves Labs, GFP-1020) for 1 h followed by washing and incubation with 1:500 Alexa Fluor 568-conjugated donkey anti-chicken secondary antibody (Jackson Immuno, #037-585-155). Images were collected on a Zeiss 710 confocal microscope, and analyzed using the Zeiss Zen software.

**Rabies viral vector production and titration.** First-generation rabies viral vectors (RVΔG-EGFP, RVΔG-tdTomato and RVΔG-Cre) were made as described. Briefly, HEK 293T cells (ATCC, CRL 11288) were transfected with expression vectors for the rhabdovirus-flankned viral gene (pRVΔG-EGFP, pRVΔG-tdTomato or pRVΔG-Cre), rabies viral genes (pCAG-B19N, pCAG-B19P, pCAG-B19G and pCAG-B19L) and the T7 polymerase (pCAG-T7pol).

Supernatants were collected 4–7 days after transfection, filtered and pooled, then passed 3–4 times on BHK-B19G cells and at a multiplicity of infection of 2–5. Purification, concentration and titration were done as described, with the Cre reporter cell line 293T-Switch2 used for titrating RVΔG-Cre.

Production of second-generation rabies viral vectors (RVΔG-EGFP, RVΔG-tdTomato and RVΔG-Cre) proceeded similarly but with the supernatants from the transfection plates passed not on BHK-B19G cells but on HEK 293T cells transfected with pCAG-B19G and pCAG-B19L. Purification, concentration and titration were done as described, with RVΔG-Cre titred on 293T-Switch2 cells and RVΔG-Fp0 titered on HEK 293T cells transfected with the Flp reporter construct pLV-CAG-F14F15S-Switch.

**Immunostaining, imaging and flow cytometric analysis of HEK 293T cells.** HEK 293T cells infected with serial dilutions of RVΔG-EGFP and RVΔG-tTomato, as described, were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton-X (Sigma) in 3% normal donkey serum (Jackson Immuno, #017-000-121), and incubated with 1:10,000 chicken anti-GFP polyclonal primary antibody (Aves Labs, GFP-1020) for 1 h followed by washing and incubation with 1:500 Alexa Fluor 568-conjugated donkey anti-chicken secondary antibody (Jackson Immuno, #037-585-155). Images were collected on a Zeiss 710 confocal microscope, and analyzed using the Zeiss Zen software.

**Mouse thalamic injections, histology, & confocal imaging.** Mouse lines used for thalamic injections were the Cre-dependent tdTomato reporter line Ai14 for RVΔG-Cre and RVΔG-Cre structural imaging studies; the Cre-/-tdTomato-dependent GCaMP6F reporter line A193 (Jackson Laboratory #024124) crossed with the Ira driver line CamK2a-Ira (Jackson Laboratory #008190) for RVΔG-Cre and RVΔG-Cre functional imaging studies; and C57BL/6 mice (Jackson Laboratory #000664) for all RVΔG-tdTomato and RVΔG-GCaMP6F experiments.

Animals of both sexes (5–15 months old; male and female) were anesthetized under isoflurane (3% induction, 1.25% maintenance, 100% O2) and ketamine/xylazine (70 mg/kg, i.p.). An incision was made in the scalp and a headplate was implanted with Metabond (Parkell) using aseptic technique. A craniotomy (5–7 mm diameter) and durotomy were performed over left hemisphere V1 and higher visual areas (MI, 3.1 mm, AP, 1.3 mm from lambda), and a glass window was cemented over exposed cortex.

Following the mice a recovery period of 7 days to recover from surgery and allow viral injection into marked territories, and to allow dendritic arbor remodeling, two-photon imaging was performed over left hemisphere V1 and higher visual areas with widespread intrinsic signal imaging of autofluorescence under 470-nm excitation as described. The emitted fluorescence was collected by a 4× objective (Olympus, NA 0.28, long-pass filtered 500–550 nm cutoff) and recorded on a PCO.edge CMOS camera (60-Hz frame rate; 2,160×1,920 pixels). The awake, head-fixed animal was shown 7 retinotopic mapping conditions on an LCD screen (60-Hz refresh rate) positioned 25 cm from the contralateral eye. Each condition consisted of a circular patch (30° diameter) of square-wave drifting grating on a black background (100% grating contrast, 8 directions shown in quick succession, 0.05 cycles/degree, drifting at 4 Hz) for 5 s, followed by 15 s of black background. The conditions varied only in screen position (two rows of three evenly spaced patches placed, spanning the screen, followed by a seventh patch in the center). The stimulus was set repeated 5 times. After downsampling the imaging data to 2 Hz, ΔF/F for each condition was calculated as the difference between fluorescence during the stimulation period (taken as the average signal of the first 4 s) and the blank period (9–20 s), divided by fluorescence during the blank, averaged over all repeats. This procedure clearly delineated the boundaries of visual areas V1, V2, V3 and V4.

Using these intrinsic signal images, we targeted all injections to the part of V1 mapped to the center of the LCD screen. The animal was anesthetized under 1.25% isoflurane in 100% O2 and the glass window removed. A replacement window with nonresonant galvanometric scanning mirrors (512×512 pixels, 700 Hz per mirror) was cemented over cortex before recovering the animal.

**Structural two-photon imaging and data analysis.** Imaging was performed using a 2× objective (Olympus) with a 1.25× optical zoom factor. Two-photon imaging was performed on a custom-built microscope with nonresonant galvanometric scanning mirrors (512×512 pixels, 700 Hz per mirror) coupled to a mode-locked Ti:sapphire laser (Chameleon Ultra II) emitting 1.020 μm. Image acquisition was controlled with a modified version of ScanImage™. Laser power exiting the 16X water-immersion objective (Nikon, NA 0.8) varied between 30 and 70 mW, depending on depth. Two volumes were measured.

**Data analysis.** Two-photon imaging was selected with a 2× objective (Olympus, NA 0.28) with a 50× oil immersion objective (Olympus, NA 1.25). The images were analyzed using the custom-written ImageJ macros and the FLEX plugin. Images were analyzed with MATLAB. The data analysis was performed using custom-written ImageJ macros and the FLEX plugin. Images were analyzed with MATLAB.
imaged in each area at each time point: one at low magnification (600×600 µm per frame, from pial surface down to 300–600 µm, 1-µm spacing between frames), used to record labeling efficiency, for alignment checks and for the volume renders (for example, Fig. 3a); and a second, smaller volume (100×100 µm), taken at higher magnification (300×300 µm per frame, 1-µm spacing). The latter was used for cell counts. The main criterion for deciding how many frames to include in the high-magnification stacks was reducing somatic overlap when a maximum-intensity z-projection was taken of the stack, so that individual cells could be unambiguously identified in the projection. The maximum projections for different time points of an individual imaged area were aligned using TurboReg® in ImageJ. An affine transformation was generally appropriate, since slight cortical deformations did occur over 16 weeks. Each alignment was checked, with attention paid to dendritic processes and other unique identifiers. After alignment across time points, cell bodies were manually counted in Vaa3D®. Vaa3D was also used for full-volume renders of the low-magnification stacks. Images were pseudocolored for presentation in ImageJ.

Fifteen cell-counting FOVs from eight animals were followed over time: five RVΔG-Cre FOVs from three animals; four RVΔG-Cre FOVs from two animals; and six RVΔG-tTomato FOVs from three animals, giving us a total starting population of 1,025 cells. All FOVs and time points are shown in Fig. 3c. No injected animals that survived beyond 4 weeks were excluded, so statistical analyses of cell counts (paired t tests; Fig. 3b) were performed for all FOVs at weeks 1 and 4.

Imaging was only terminated before 16 weeks in case of animal health reasons or death, or in the case of RVΔG-tTomato because very few cells remained.

Functional two-photon imaging and data analysis. A week after virus injection, the center of the brightest GCaMP6f label in left-hemisphere V1 (assessed under conventional epifluorescence) was chosen as the imaging area. Two-photon functional imaging was performed using a custom-built, resonant-scanning microscope (30-Hz frame rate, 512 lines per frame) with the same objective and laser settings as at 940 nm in the structural imaging experiments. Imaging was only terminated before 16 weeks in case of animal health reasons or death, or in the case of RVΔG-tTomato because very few cells remained.

Decay time constants were estimated for cells segmented with the CNMF package using data from the first 4 min of each imaging session. Only cells that had a peak inferred δF/F > 0.25 with no plateaus of elevated fluorescence (for example, because of some activity on a bright–inactive cell), and that were clearly imaged at their cell bodies and not apical dendrites (which generally exhibited different time constants than somatic signals), were included in the population analysis.

Seven animals were used in these functional imaging experiments (21 total FOVs); three injected out to 16 weeks, two out to 8 weeks, and two RVΔG-Cre cases, each imaged out to at least 8 weeks postinjection (with one injection; and two RVΔG-GCaMP6f cases, imaged out to only 4 weeks because of very few surviving cells (see Supplementary Supplementary Fig. 3). Since week 2 had brighter GCaMP6f labeling than week 1 in the two Cre conditions, population analyses over time (Fig. 3d, 4) were done between weeks 2 and 16. Imaging termination criteria were the same as in the structural imaging experiments.

Whole-cell patch-clamp recordings. For brain slice electrophysiology recordings, 6 adult male Ai14 transgenic mice (aged 8 or 14 weeks) were used. Retrobeads (Lumafour Inc.) or the first- or second-generation rabies viral vector encoding GFP-Cre recombinase was injected into the nucleus accumbens (NAc) at the following stereotaxic coordinates from bregma: +1.30 mm AP, ±0.80 mm ML, and −4.70 mm DV.

After 8 weeks, mice were anesthetized with 90 mg/kg sodium pentobarbital and perfused transcardially with 200–300 ml artificial cerebral spinal fluid (ACSF: 125 mM NaCl, 2.5 mM KCl, 1.3 mM NaHPO4, 7 mM MgCl2, 0.5 mM CaCl2, 25 mM HCO3, and 5 mM ascorbic acid). The brain was extracted and glued (Roti cell; Carh Roth GmbH, Karlsruhe, Germany) to the platform of a semiautomatic vibrating blade microtome (VT1200; Leica, Buffalo Grove, IL), which was placed in a slicing chamber containing modified ACSF at 4 °C. Coronal sections of 300-µm conal thickness were cut in the NAc and BAL were collected and placed in a holding chamber filled with ACSF saturated with 95% O2 and 5% CO2, containing (in mM): 126 NaCl, 2.5 KCl, 1.24 NaNH4PO4, 1.0 MgCl2, 2.4 CaCl2, 26.0 NaHCO3, and 10 glucose. Recordings commenced at least 1 h after slicing, and the temperature was maintained at approximately 31 °C both in the holding chamber and recording chamber. All NAc injection sites were verified and imaged with a camera (Hamatsu Photonics K.K., Japan) attached to a microscope (BX51; Olympus, Center Valley, PA).

Recordings were made from visually identified neurons expressing tTomato or containing retrobeads. Voltage- and current-clamp recordings of BLA neurons projecting to the NAc were conducted using sharp, glass microelectrodes shaped with a horizontal puller (P-1000, Sutter, CA) and filled with a solution containing (in mM): 125 potassium glutonate, 20 HEPES, 10 NaCl, 3 MgATP, 8 biocytin and 2 Alexa Fluor-350 (pH 7.33; 287 mOsm). Recorded signals were amplified using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Analog signals were digitized at 10 kHz using a Digidata 1440 and recorded using pClamp10 software (Molecular Devices, Sunnyvale, CA). Oxygenated ACSF was perfused onto the slice via a peristaltic pump (Minipuls3; Gilson, Middleton, WI) at −3 mL/min. The membrane properties of each cell were evaluated in voltage-clamp and current-clamp modes. The access resistance (Rs), membrane capacitance (Cm), membrane resistance (Rm) and time constant (τ) were measured using a two-test conductance method, in which 100-µs, 5-mV voltage steps were delivered at a frequency of 5 Hz. We also measured the holding current at a holding potential of −70 mV and calculated the current/voltage relationship in voltage-clamp using a series of 200-mV voltage steps, deviating from the −70 mV holding potential by −30, −25, −20, −15, −10, −5, 0, 5, 10, 15, 20, 25, 30, 35, and 40 mV. In order to evaluate the action potential threshold and rheobase, a 1-s, 300- to 750-pA current ramp was delivered in current-clamp mode. Finally, to evaluate the relationship between injected current and firing frequency, we delivered a series of 2-s current pulses in current-clamp, in which the first pulse was 50 pA above the holding current required to keep the cell at −70 mV and each subsequent pulse increased by 25 pA to a maximum of 525 pA. Any significant changes in Rs or holding current were interpreted as signs of poor cell health and recordings were terminated.

Offline analysis was performed using Clampfit 10.6.1.1 (Molecular Devices, Sunnyvale, CA) or Matlab R2013b (seal test analysis). Statistical significance was assessed with GraphPad Prism 6 (GraphPad Software, Inc.) using a one-way analysis of variance (ANOVA), with Neuman–Keuls post hoc tests where appropriate.

Comparative viral vector injections, imaging and quantification. CAV2-Cre with a titer of 1.0 × 1012 genome copies/mL was obtained from the Viral Vector Production Unit at the Universitat Autonoma de Barcelona and was produced from a vector enabling expression of Cre under control of the CMV promoter. rAAV2-retro-Cre was generated at the Allen Institute by the plaque isolation of HEK 293T cells with pAAV-EF1α-Cre-WPRE-hGpHoloA, pHelper and pAAV-retro®13, followed by iodixanol gradient purification. The Cre coding sequence, with the Kozak consensus sequence of GCCGCGGCC, was inserted into the Xbal and EcoRI restriction sites to generate the pAAV-EF1α-Cre-WPRE-hGpHoloA construct. The titer of rAAV-retro-EF1α-Cre was 5.0 1012 genome copies/mL.
as measured by qPCR. The titer of the undiluted RV-AGL-Cre was $4.26 \times 10^{10}$ infectious units/mL. rAAV2/1-CAG-LEX-EGFP-WPRE-bGH stock number V5749 with a titer of $1.34 \times 10^{10}$ genome copies/mL (AAV-FLEX-EGFP) was obtained from the Penn Vector Core.

Viruses were injected into the left hemisphere of adult (P55–134) male and female Ai75 (RCL-nT; Jackson Laboratory #025106) mice using stereotactic coordinates or intrinsic signal imaging to locate the target region. A total of 7 mice were injected in ACA and 8 in AM, with representative data shown from each animal between Fig. 5 and Supplementary Fig. 5. Stereotactic coordinates were used for all three traces in ACA (mm from bregma: AP +0.5, ML –0.25, and DV +0.9 from brain surface), and for RV-AGL-Cre and AAV-retro-EE1a-Cre injections into area AM (AP –2.06, ML –0.17, and DV +0.8 from brain surface) using pressure injection. The two injections were performed during a single surgical procedure. All retrograde viruses were injected via pressure using a Nanoject II system (CAV2-Cre: 100–200 nL; RV-AGL-Cre: 250 nL; rAAV2-retro-EE1a-Cre, 50 nL). Twenty-one days after virus injection, mice were perfused with 4% paraformaldehyde (PFA), then brains were dissected and postfixed in 4% PFA.

For paired intersectional injections in mice, AAV-FLEX-EGFP was injected into ACA at (mm from bregma: AP +1.1, ML –0.25, and DV 0.9 from brain surface) using iontophoresis (5 µA, 5 min at each depth) and RV-AGL-Cre was injected into RSP at (mm from bregma: AP –2.06, ML –0.17, and DV 0.8 from brain surface) using pressure injection. The two injections were performed during a single surgical procedure. All retrograde viruses were injected via pressure using a Nanoject II system (CAV2-Cre: 100–200 nL; RV-AGL-Cre: 250 nL; rAAV2-retro-EE1a-Cre, 50 nL). Twenty-one days after virus injection, mice were perfused with 4% paraformaldehyde (PFA), then brains were dissected and postfixed in 4% PFA at room temperature (22–25 °C) for 3–6 h, followed by overnight at 4 °C. CAV2-Cre injected brains were stored in 10% glutaraldehyde (injection depths 0.3 and 0.6 mm from brain surface with a 20° pipette angle; see also methods in ref. 8). For injection into AM (AP –2.18, ML –1.6, DV +0.9 from brain surface), and for RV-AGL-Cre into AM were also methods in ref. 75.

Twenty-one days after virus injection, mice were perfused with 4% paraformaldehyde (PFA), then brains were dissected and postfixed in 4% PFA at room temperature (22–25 °C) for 3–6 h, followed by overnight at 4 °C. CAV2-Cre injected brains were stored in 10% glutaraldehyde (injection depths 0.3 and 0.6 mm from brain surface with a 20° pipette angle; see also methods in ref. 8).

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The number of retrogradely labeled cells per layer was determined by counting the number of tdTomato+ nuclei in each figure panel, except in rows iii and iv (ORBvl and RSP) of Fig. 6, where a 1 x 1 mm ROI was drawn on the image and counting was performed inside the ROI. Layer boundaries were determined by expert visualization of DAPI labeled nuclei, where available, and pia–white matter boundary. To estimate the uptake volume of viral injections, we manually drew polygons around the borders of the densely labeled cells on the images for all tissue sections covering the rostrocaudal extent of the injection site. The area of the polygons was measured using ImageJ, and these areas were converted from pixels to microns and then multiplied by the section thickness (100µm) to estimate the total volume of the injection site. The volume of injection spread did not differ between viruses (mean ± s.d. = CAV2-Cre: 1.4 ± 0.5 mm³, RV-AGL-Cre: 1.1 ± 0.6 mm³, AAV-retro-EE1a-Cre: 0.6 ± 0.4 mm³; P = 0.22) but was dependent on injection site (1.5 ± 0.4 mm³ for ACA, 0.7 ± 0.4 mm³ for VISam; P = 0.02, two-way ANOVA). All injections spanned the entire thickness of the cortex.

Statistics and reproducibility. Analysis of cell counts (Fig. 3) used two-sided paired t tests. Analysis of membrane property differences (Fig. 4) used one-way ANOVA, with Neuman–Keuls post hoc tests where appropriate. Analysis of in vivo response properties (Fig. 5) used a two-sample z-score proportion test. Analysis of differences between injection uptake volumes (Fig. 6) used two-way ANOVA. All tests for significance used a cutoff of P = 0.05. No statistical methods were used to determine sample size, but sample sizes for each type of experiment in this study are consistent with previously published work.
**Life Sciences Reporting Summary**

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Please do not complete any field with "not applicable" or n/a. Refer to the help text for what text to use if an item is not relevant to your study. For final submission: please carefully check your responses for accuracy; you will not be able to make changes later.

### Experimental design

1. **Sample size**
   
   Describe how sample size was determined.

   No formal methods were used, but sample sizes for each type of experiment in this study are consistent with previously published work.

2. **Data exclusions**
   
   Describe any data exclusions.

   Animal exclusions only affected the longitudinal experiments in this work, and the criteria were animal health/death. Described in the relevant Methods sections, the exclusion criteria were pre-established in our IACUC protocols.

   Data exclusion criteria are described in the relevant Methods sections, as well as figure legends. Briefly, the whole-cell patch-clamp recording criteria are standard: any significant changes in access resistance or holding current were interpreted as signs of poor cell health, and recordings were terminated. The criteria for in vivo functional recordings were established after observing novel pathologies associated with different viruses (including unexpected calcium time courses, as described extensively in the text). Representatives of excluded data are shown (e.g., Fig 5e). No data points from the in vivo structural imaging experiments were excluded.

3. **Replication**
   
   Describe the measures taken to verify the reproducibility of the experimental findings.

   The number of times each experiment was repeated is indicated in the text, and all experimental findings were reliably reproduced.

4. **Randomization**
   
   Describe how samples/organisms/participants were allocated into experimental groups.

   No randomization was used.

5. **Blinding**
   
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   No blinding was used.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.
6. Statistical parameters
For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☒   | The exact sample size ($n$) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☒   | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☒   | A statement indicating how many times each experiment was replicated |
| ☒   | The statistical test(s) used and whether they are one- or two-sided |
| ☒   | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ☒   | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☒   | Test values indicating whether an effect is present |
| ☒   | Provide confidence intervals or give results of significance tests (e.g. $P$ values) as exact values whenever appropriate and with effect sizes noted. |
| ☒   | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☒   | Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation) |

See the web collection on statistics for biologists for further resources and guidance.

7. Software
Policy information about availability of computer code

Describe the software used to analyze the data in this study. Clampfit 10.6.1.1, GraphPad Prism 6, pClamp10, MATLAB R2013b and R2017a, CalmAn (CNMF) in Python 2.7 (https://github.com/flatironinstitute/CalmAn), Vaa3D 3.2, ImageJ 1.5

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability
Policy information about availability of materials

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

All plasmids will be made available through Addgene (nonprofit) for distribution to the scientific community at cost.

9. Antibodies
Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Chicken anti-GFP polyclonal primary antibody (Aves Labs, GFP-1020) and donkey anti-chicken secondary antibody (Jackson Immuno, #703-585-155); validation with positive and negative controls is shown in Figure 1 and described in the legend and methods.

10. Eukaryotic cell lines
a. State the source of each eukaryotic cell line used.

HEK 293T cells were obtained from ATCC (#CRL-11268); BHK-B19G2 cells were described in Wickersham et al., Nature Protocols 2010 Mar;5(3):595-606 and were originally derived from BHK-21 cells obtained from ATCC (#CCL-10).

b. Describe the method of cell line authentication used.

Cell lines were not specifically authenticated.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cell lines were tested for mycoplasma contamination by ATCC but not subsequently.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

All experiments were conducted using the following strains of Mus musculus:
- Ai14 (Jackson Laboratory #007908)
- Ai65D (Jackson Laboratory #021875)
- Meox2-Cre (Jackson Laboratory #003755)
- Ai93 (Jackson Laboratory #024103)
- Camk2a-tTA (Jackson Laboratory #007004)
- Ai75 (Jackson Laboratory #025106)
- C57BL/6 mice (Jackson Laboratory #000664)

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

No human subjects were used.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

▶ Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

▶ Methodological details

5. Describe the sample preparation. HEK 293T cells fixed in 1% PFA and resuspended
6. Identify the instrument used for data collection. BD Biosciences LSR II
7. Describe the software used to collect and analyze the flow cytometry data. BD FACSDiva
8. Describe the abundance of the relevant cell populations within post-sort fractions. No sorting was used.
9. Describe the gating strategy used. No gating was used for sorting because no sorting was used.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. □