Transgene Expression in Target-Defined Neuron Populations Mediated by Retrograde Infection with Adeno-Associated Viral Vectors

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Tools enabling the manipulation of well defined neuronal subpopulations are critical for probing complex neuronal networks. Cre recombinase (Cre) mouse driver lines in combination with the Cre-dependent expression of proteins using viral vectors—in particular, recombinant adeno-associated viral vectors (rAAVs)—have emerged as a widely used platform for achieving transgene expression in specified neural populations. However, the ability of rAAVs to further specify neuronal subsets on the basis of their anatomical connectivity has been reported as limited or inconsistent. Here, we systematically tested a variety of widely used neurotropic rAAVs for their ability to mediate retrograde gene transduction in the mouse brain. We tested pseudotyped rAAVs of several common serotypes (rAAV 2/1, 2/5, and 2/9) as well as constructs both with and without Cre-dependent expression switches. Many of the rAAVs tested—in particular, though not exclusively, Cre-dependent vectors—showed a robust capacity for retrograde infection and transgene expression. Retrograde expression was successful over distances as large as 6 mm and in multiple neuron types, including olfactory projection neurons, neocortical pyramidal cells projecting to distinct targets, and corticofugal and modulatory projection neurons. Retrograde infection using transgenes such as ChR2 allowed for optical control or optically assisted electrophysiological identification of neurons defined genetically as well as by their projection target. These results establish a widely accessible tool for achieving combinatorial specificity and stable, long-term transgene expression to isolate precisely defined neuron populations in the intact animal.

Introduction

The ability to experimentally target defined neuronal subpopulations is critical for understanding brain function. Such tools as targeted gene knock-ins and Cre recombinase (Cre) mouse driver lines in combination with the Cre-dependent expression of proteins allow for the genetic specification and manipulation of neural circuits and systems in the intact brain (Luo et al., 2008; Madisen et al., 2010, 2012; Yizhar et al., 2011). Nonetheless, the ability to target increasingly precise neuronal subsets for robust and stable changes in gene expression is limited by the selectivity of known promoters and by limitations in gene transduction methods. For example, functionally discrete neuronal populations are rarely defined by expression of a single gene and many promoters fail to drive transgene expression sufficiently to alter neuronal function (Luo et al., 2008; McGarry et al., 2010; Zhao et al., 2011).

Viral vectors have the potential to overcome these limitations as they support both genetic and anatomical specification of neuronal subpopulations by incorporating cell type-specific promoters or Cre-dependent expression switches and can drive strong transgene expression (Luo et al., 2008; Betley and Sternson, 2011). Viral vectors that retrogradely transduce neurons via their axon terminals allow for even more precise targeting of neuronal subsets defined by axonal projections or synaptic connectivity (Larsen et al., 2007; Wickersham et al., 2007; Callaway, 2008). Combining retrograde viral infection with Cre-dependent expression enables a combinatorial approach to targeting neuronal subpopulations (for example, isolating a subset of genetically specified cortical pyramidal neurons projecting to a given target), although achieving this has been difficult. To date, most retrograde vectors are based on rabies or herpes virus for retrograde infection, but only a few such vectors mediate Cre-dependent gene expression (Wall et al., 2010; Lo and Anderson, 2011) and their eventual lethality to the host cell limits their use in long-term functional studies or gene-therapy strategies (Wickersham et al., 2007; Osakada et al., 2011). In contrast, recombinant adeno-associated virus (rAAV) vectors are a widely used platform for the delivery of transgenes to CNS neurons due to their ability to mediate long-lasting, relatively stable gene expression and their relative ease of production and handling (Betley and Sternson, 2011). With a few exceptions, however (Kaspar et al., 2002; Passini et al., 2005; Masamizu et al., 2011; Zhang et al., 2013), rAAVs are reported to be weak or ineffective at retrogradely infecting CNS neurons (Chamberlin et al., 1998; Burger et al., 2004;

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Salegio et al., 2013), and retrograde infection by rAAVs using Cre-dependent switches has not been systematically assayed.

Here, we report robust retrograde infection by a variety of widely used rAAV vectors, especially those incorporating Cre-dependent expression switches. Transgene expression via retrograde infection was successful in multiple neuron classes and using multiple rAAV serotypes and enabled the *in vivo* isolation of genetically defined neuronal subsets with anatomically distinct axonal projections. These results highlight the potential for further development of rAAVs as tools to target specific populations of CNS neurons for experimental or therapeutic applications.

Materials and Methods

Animals. The following mouse strains of either sex were used, as specified in the text: GAD2-IRES-Cre (Taniguchi et al., 2011), Jax stock #010802; PCdh21-Cre (Nagai et al., 2005), Gene Expression Nervous System Atlas (GENSAT) Project, Mutant Mouse Regional Resource Centers (MMRRC) stock #030952-UCD; Slc6a4 [serotonin reuptake transporter (SERT)]-Cre, GENSAT Project, MMRRC stock #031028-UCD, CCK-IRES-Cre (Taniguchi et al., 2011), Jax Stock #012706; Ai9 (Madisen et al., 2010), Jax stock #007905; TH-Cre, Jax stock #008601; Emx1-IRES-Cre (Gorski et al., 2002) Jax stock #005628. All procedures were performed following National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Utah Institutional Animal Care and Use Committee.

Viral vectors. Viral vectors used were obtained from the viral vector cores of the University of Pennsylvania or the University of North Carolina, as specified. All vectors were from stock batches available for general distribution. Injection of Cre-dependent vectors was performed in mice that were homozygous for the allele driving Cre expression; injections in heterozygous animals were also successful but are not described here. Virus injection was performed by stereotaxic targeting of specified brain areas using previously described procedures (Wachowiak et al., 2013). The specified volumes of virus were delivered through a 33 or 30 gauge metal needle at a rate of 0.1 μ l/min. Mice were between 4 and 12 weeks of age at the time of virus injection and individually housed after injection for 14–28 d before evaluating for transgene expression. The viral vectors used, with their abbreviated names as used in the text, were as follows: AAV2/1.hSynap.FLEX.GCaMP3.3.WPRE.SV40 (2/1.FLEX.GCaMP3); AAV2/1.CAGGS.FLEX.ChR2-tdTomato.SV40 (2/1.FLEX.ChR2-tdTomato); AAV2/1.hSynap.FLEX.GCaMP5G(GCaMP3-T302L.R303P.D380Y).WPRE.SV40 (2/1.FLEX.GCaMP5G); AAV2/1.hSynap.EGFP.WPRE.bGH (2/1.EGFP); AAV2/1.CB7.CI.mCherry.WPRE.rBG (2/1.mCherry); AAV2/5.EF1a.DIO.hChR2(H134R)-EYFP.WPRE.hGH (2/5.DIO.hChR2-EYFP); AAV2/9.EF1a.DIO.hChR2(H134R)-EYFP.WPRE.HGHpA (2/9.DIO.hChR2-EYFP); AAV2/1.hSynap.FLEX.GCaMP6f.WPRE.SV40 (2/1.FLEX.GCaMP6f); AAV2/1.hSynap.GCaMP5G(GCaMP3-T302L.R303P.D380Y).WPRE.SV40 (2/1.GCaMP5G); AAV2/5.EF1a.DIO.mCherry (2/5.DIO.mCherry); AAV2/5.EF1a.DIO.hChR2(H134R)-mCherry(2/5.DIO.hChR2-mCherry); AAV2/1.hSyn.FLEX.iGluSnFr.WPRE.SV40 (2/1.FLEX.iGluSnFr); AAV2/1.CAG.FLEX.tdTomato.WPRE.bGH (2/1.FLEX.tdTomato).

Histology. Mice were deeply anesthetized and perfused with 4% paraformaldehyde. To evaluate transgene expression, brains were processed and vibratome-sectioned as described previously (Wachowiak et al., 2013) and expression evaluated from native fluorescence without immunohistochemical amplification. To assay for coexpression with the mitral/tufted cell (MTC)-specific marker Tbx21, brains were cryosectioned and processed for immunohistochemistry as described in Wachowiak et al. (2013). Briefly, cryoprotected brains were embedded in optimum cutting temperature compound (Tissue-Tek, Sakura-Fintek) and cut coronally (15–30 μ m) with a cryostat. Sections were incubated with primary antibody (rabbit anti-Tbx21, 1:1000; kindly provided by Y. Yoshihara, RIKEN) overnight at 4°C, followed by incubation with Alexa543-conjugated goat anti-rabbit secondary antibody (1:1000; A-11010, Invitrogen) at room temperature (RT) for 1 h. Viral-driven GCaMP expression was enhanced by staining with FITC-conjugated GFP antibody (1:150; #ab6662, Abcam) for 4 h at RT to counter deteriorated native fluorescence due to cryotreatment. For display and for

cell counts, image stacks were obtained with an Olympus FV10i confocal laser scanning microscope.

Recordings and optical stimulation. For *in vivo* recordings, mice were anesthetized with pentobarbital (50 mg/kg) and secured in a stereotaxic device (Kopf Instrument) or custom head bar. Body temperature was maintained at 37°C. A small ($\sim 1 \times 1$ mm) craniotomy was performed over one olfactory bulb (OB) and the dura removed. Extracellular recordings were obtained from units in the dorsal OB using a 16-channel electrode (A1x16-5mm50-413-A16, NeuroNexus) and an RZ5 digital acquisition system [Tucker Davis Technologies (TDT)]. Electrode depth was monitored with a digital micromanipulator (MP-225, Sutter Instruments). Custom scripts in TDT were used to control data acquisition and optical stimulation. Criteria for selecting units for analysis were as described in Carey and Wachowiak (2011). *In vivo* whole-cell recordings were performed using 4–6 M Ω glass electrodes drawn on a horizontal puller (P97, Sutter Instruments) and filled with an intracellular solution consisting of the following (in mM): 135 K-gluconate, 4 KCl, 10 HEPES, 10 phosphocreatine, 4 MgATP, 0.3 GTP, and buffered to pH 7.3 with KOH (Sigma-Aldrich). In some experiments, internal solution contained 20 μ M sulforhodamine 101 (Invitrogen) and the following (in mM): 120 K-gluconate, 20 KCl, 10 HEPES, 7 diTrisPhCr, 4 Na2ATP, 2 MgCl2, 0.3 Tris-GTP, 0.2 EGTA, and buffered to pH 7.3 with KOH (Sigma-Aldrich). For *in vitro* recordings, OB slices (300 μ m thickness) were prepared from adult mice using previously published protocols (Wachowiak et al., 2013). Slices were maintained at 33°C and recordings made using the same pipette resistance and internal solution as for the *in vivo* recordings. All whole-cell recordings were performed in current-clamp mode using a Multiclamp 700A amplifier (Molecular Devices) and data acquisition and optical stimulation were controlled with pClamp10 (Molecular Devices). For optical stimulation, light was presented using a 470 nm LED and controller (LEDD1B, Thorlabs) and a 1 mm optical fiber or a 473 nm diode-pumped solid-state laser (LaserWave) and 200 μ m fiber, each positioned within 1 mm of the surface of the dorsal OB or the OB slice. Total light power emitted was typically 7–12 mW for the 1 mm fiber and ~ 1 mW for the 200 μ m fiber.

Results

Retrograde infection of OB projection neurons by rAAVs

We first tested the ability of rAAVs to drive transgene expression via retrograde axonal infection in the mouse olfactory pathway. The olfactory bulb (OB) was used as a test bed for characterizing retrograde infection because its principal neurons, mitral/tufted cells (MTCs), constitute an anatomically and functionally heterogeneous group with subpopulations projecting to multiple forebrain targets and having distinct dendritic and laminar organization (Macrides and Schneider, 1982; Nagayama et al., 2010; Igarashi et al., 2012). To selectively target MTCs, we used a transgenic mouse line (PCdh21-Cre) that, within the forebrain, expresses Cre primarily in MTCs (Nagai et al., 2005; Mitsui et al., 2011; Wachowiak et al., 2013). Injection of rAAV containing Cre-dependent expression constructs (e.g., 2/1.FLEX.GCaMP3; 2/1.FLEX.ChR2-tdTomato) into the OB leads to expression that appears restricted to MTCs, with the majority of somata in the mitral cell layer and deep glomerular layer (Fig. 1A) and fewer somata in the external plexiform layer. This expression pattern matches that described for mRNA expression of PCdh21 in the OB (Nakajima et al., 2001), indicating that the rAAV2/1 serotype has sufficient tropism to enable efficient transduction of the Cre-dependent transgene in OB MTCs. The PCdh21+ MTC population likely includes superficial and external tufted cells with somata in the glomerular layer, whose axonal projections either remain intrinsic to the OB or project only to the most anterior portions of olfactory cortex (Schoenfeld et al., 1985; Shipley et al., 2004; Ghosh et al., 2011; Igarashi et al., 2012).

To test for retrograde infection of OB MTCs with Cre-dependent rAAV2/1 constructs, we injected virus into anterior

Figure 1. Cre-dependent and target-specific transgene expression in OB output neurons via retrograde rAAV infection. **A**, Expression of GCaMP3 in MTCs after injection of Cre-dependent virus (2/1.FLEX.GCaMP3) into the OB of a PCdh21-Cre mouse. Left image shows low-magnification view of the OB. Right image shows higher-magnification view of the dorsal OB. Expression is strongest in the dorsal half of the OB and apparent in somata in the mitral cell layer (MC) and the superficial external plexiform layer (EPL)/deep glomerular layer (GL). Dendritic processes are also apparent throughout the EPL. **B**, GCaMP3 expression after injection of 2/1.FLEX.GCaMP3 into aPC of a PCdh21-Cre mouse. Left, Low-magnification, epifluorescence image showing heavy expression in MTCs throughout the OB. Middle, Confocal stack showing dense expression in cells predominately in the MC layer (compare with **A**). Right, Axon terminals of GCaMP3-expressing neurons in piriform cortex. Note the lack of cell bodies expressing GCaMP3 in piriform. **C, D**, GCaMP3 expression after injection of 2/1.FLEX.GCaMP3 into (**C**) posterior piriform cortex (pPC) and (**D**) posterolateral cortical amygdala (PLCo). Injection in both areas leads to preferential expression in mitral and deep tufted cells, with dendritic branching restricted to the deep half of the EPL. **E**, Expression of GCaMP3 after 2/1.FLEX.GCaMP3 injection into medial amygdala (MeA). Expression is strongest in mitral cells of the accessory OB (AOB).

piriform cortex (aPC), a major target of MTC axons, in PCdh21-Cre mice. We injected a relatively large volume (400–1000 nL) of virus to cover a large extent of aPC. Injection of AAV2/1.FLEX.GCaMP3 into aPC led to widespread expression of GCaMP3 in MTCs throughout the OB (Fig. 1*B*, left; $n = 25$ animals). Somata at the injection site in aPC, as well as centrifugal neurons projecting from aPC to the OB (Haberly and Price, 1978; Shipley and Adamek, 1984; Boyd et al., 2012) do not express the transgene as they do not contain Cre (Fig. 1*B*, right). Transgene expression after retrograde rAAV infection occurred in MTCs throughout the entire extent of the OB, in contrast to localized expression seen with direct OB injection (Fig. 1*A*, left). Retrograde infection also led to preferential expression in neurons in the mitral cell layer compared with direct OB infection. In retrogradely infected mice, 85% of labeled somata (412 cells, 10 mice) were located in the mitral cell layer, with the remainder in the external plexiform layer. In mice injected directly into the OB, only 61% of counted somata (885 cells, six mice) were located in the mitral cell layer, with the remaining in the external plexiform layer or glomerular layer (Fig. 1*A*). This difference likely reflects the exclusion of PCdh21+ cell types that do not project to aPC. Retrograde infection was robust, typically leading to expression in a high fraction of all PCdh21+ mitral cells: in PCdh21-Cre mice expressing GCaMP after retrograde infection into aPC, 81% of neurons in the mitral cell layer that expressed the MTC-specific marker Tbx21 (Nagai et al., 2005; Mitsui et al., 2011) were also positive for GCaMP (351 of 433 cells, three mice). Retrograde infection was also reproducible: in >50 injections into aPC in PCdh21-Cre mice, we have achieved widespread expression in MTCs in ~80% of cases, with most failures attributable to errors in stereotaxic targeting. Retrograde infection was also effective using injection volumes as small as 100 nL (two mice, four injections), though fewer MTCs were labeled.

To establish the usefulness of retrograde infection for isolating subsets of genetically defined neurons based on their axonal projections, we injected virus into different olfactory cortical areas known to be targeted by distinct populations of OB MTCs. These experiments also helped to confirm that transgene expression was in fact due to retrograde infection rather than bulk spread of injected virus from forebrain areas to the OB. One subpopulation of MTCs includes neurons with somata in the mitral cell layer and lateral dendrites that remain restricted to the deep half of the external plexiform layer and which project to posterior piriform cortex and cortical amygdala (Haberly and Price, 1977; Scott et al., 1980; Schneider and Scott, 1983). Another subpopulation of PCdh21+ MTCs includes mitral cells of the accessory OB, which project to medial amygdala but not to piriform cortex (Scalia and Winans, 1975). Consistent with these anatomically defined divisions, we found that Cre-dependent rAAV injection (2/1.FLEX.GCaMP3) into posterior piriform (seven mice, 12 injections) or cortical amygdala (two mice, four injections) led to expression almost exclusively in mitral cells, with 85 and 90% of GCaMP3-expressing neurons ($n = 124$ and 142 cells) having somata in the mitral cell layer for posterior piriform and cortical amygdala injections, respectively. Furthermore the lateral dendrites of GCaMP3-expressing neurons were restricted to the deep external plexiform layer of the main OB (Fig. 1*C, D*). Injection into medial amygdala, in contrast, led to expression solely in mitral cells of the accessory OB (Fig. 1*E*; two mice, four injections). These results argue that the observed expression is not due to bulk spread of virus from the injection site, and that retrograde viral infection can be used to isolate neuronal subpopulations defined on the basis of their axonal projection patterns. We also note that the distance from cortical or medial amygdala to the OB is ~5.3–6.1 mm, indicating that retrograde infection is effective over a considerable distance.

Table 1. rAAV constructs tested for retrograde infection in OB projection neurons^a

Construct	rAAV serotype	Source	Titer	n (mice)	Retrograde infection
Cre-dependent					
FLEX.GCaMP3	2/1	University of Pennsylvania	5.6×10^{12} – 2.1×10^{13}	25	Yes
FLEX.GCaMP5G	2/1	University of Pennsylvania	1.1×10^{13} – 1.5×10^{13}	30	Yes
FLEX.GCaMP6f	2/1	University of Pennsylvania	1.7×10^{12} – 2.9×10^{13}	14	Yes
FLEX.ChR2-tdTomato	2/1	University of Pennsylvania	5.3×10^{11} – 1.4×10^{12}	2	Yes
FLEX.iGluSnFr	2/1	University of Pennsylvania	1.1×10^{13}	1	Yes
DIO.hChR2-EYFP	2/5	University of Pennsylvania	6.7×10^{11} – 2.0×10^{13}	4	Yes
DIO.hChR2-mCherry	2/5	University of North Carolina	6.0×10^{12}	2	Yes
DIO.mCherry	2/5	University of North Carolina	4.0×10^{12}	2	Yes
Cre-independent					
GCaMP5G	2/1	University of Pennsylvania	2.3×10^{12}	11	No
mCherry	2/1	University of Pennsylvania	5.3×10^{12}	2	Yes
eGFP	2/1	University of Pennsylvania	2.5×10^{13}	2	No

^aTable summarizes rAAV constructs that were injected into anterior piriform cortex and evaluated for expression in OB projection neurons. Reported *n* is for number of mice but we typically performed two injections per animal. Vectors are classified as mediating successful retrograde expression if we observed expression in >100 OB neurons in all injections in which successful stereotaxic targeting was confirmed. Typically, however, unsuccessful vectors displayed <8 labeled neurons per injection. Additional constructs and serotypes tested in different brain regions are described in the text and Table 2.

A aPC injected (PCdh21-Cre). 2/1.GCaMP5G **B** aPC injected. 2/1.EGFP **C** 2/1.FLEX.GCaMP5G

2/1.mCherry

overlay

Figure 2. Differential ability of constitutive rAAV vectors to drive retrograde expression in OB projection neurons. **A**, GCaMP5G expression in centrifugal axons in the OB after injection of the constitutive rAAV 2/1.GCaMP5G into aPC of a PCdh21-Cre mouse. Left, Middle, Low-magnification and high-magnification images showing strong labeling of axonal processes in the granule cell layer (GCL) throughout the OB but not cell bodies expressing GCaMP5G. Right, Image of aPC, near injection site, showing numerous GCaMP5G-expressing cell bodies. **B**, Similar labeling of centrifugal axons in the OB after injection of rAAV2/1.EGFP into aPC. Here, axon terminals are apparent in the GCL as well as around the periphery of glomeruli in the glomerular layer (GL), but no cell bodies are apparent in the OB. **C**, Robust retrograde expression mediated by the constitutive rAAV 2/1.mCherry. 2/1.mCherry was coinjected with 2/1.FLEX.GCaMP5G into aPC of a PCdh21-Cre mouse. Left image shows GCaMP5G expression in the OB (green). Middle image shows mCherry expression (red). Right image shows overlay. GCaMP5G expression is apparent primarily in mitral cells, while mCherry expression is apparent in mitral cells and in axons in the granule cell layer, consistent with both retrograde infection of mitral cells and direct infection of neurons projecting from aPC to the OB.

Robust retrograde infection by rAAVs

Because retrograde infection by rAAVs has been reported as weak or ineffective in some cases and effective in others (Chamberlin et al., 1998; Burger et al., 2004; Wachowiak et al., 2013; Zhang et al., 2013), we evaluated the robustness of this phenomenon by testing for expression of different Cre-dependent transgene constructs and serotypes. We observed similar success of retrograde infection for identical Cre-dependent constructs encoding multiple transgenes and using multiple promoters (Table 1), and across multiple production lots obtained from the same source over 24 months. Both 2/1 and 2/5 serotypes resulted in similarly strong retrograde expression levels (Table 1).

We next tested the ability of constitutive (Cre-independent) constructs to drive transgene expression after retrograde infection using the same injection protocol in PCdh21-Cre mice. Injection of the constitutive GCaMP5G construct (2/1.GCaMP5G) led to expression in neurons throughout aPC and in the terminals of corticofugal axons in the granule cell and glomerular layers of the OB (Fig. 2A), indicating effective viral transduction around the injection site followed by anterograde labeling of axonal projections from piriform. However, this virus failed to drive expression in MTCs of the OB (11 mice, 22 injections; eight cells total could be identified in the MCL in 14 sections in five of the mice). Likewise, injection of 2/1.EGFP, the construct used in a large-

Figure 3. Retrograde transgene expression in cortical pyramidal neurons projecting to different targets. **A–F**, Low-magnification epifluorescence images (top) and higher-magnification confocal stacks (bottom) of mCherry fluorescence in S1 after injection of rAAV2/1.mCherry into superior colliculus (**A**, **D**, SC), thalamus (**B**, **E**), and contralateral S1 (**C**, **F**, cS1). For all images, the pial surface is toward the top. mCherry-expressing layer 5 pyramidal neurons are apparent in each preparation. In the contralateral-injected animal (**C**, **F**), the brightest fluorescence is from anterograde labeling of contralateral-projecting neurons infected at the injection site, but mCherry-expressing cell bodies are apparent in layer 5 (**F**). **G**, Injection of two different Cre-dependent viruses into SC and cS1 in the same Emx1-Cre mouse labels distinct populations of pyramidal neurons. 2/1.FLEX.tdTomato (red) was injected into cS1; 2/1.FLEX.GCaMP3 into SC. Low-magnification image (top) shows GCaMP3-expressing somata and anterograde mCherry labeling in contralaterally projecting axon terminals. Retrogradely expressing somata from cS1 are less clear at this magnification and in this section, but are apparent in higher-magnification confocal stacks (bottom). Retrogradely labeled pyramidal neuron populations from SC and cS1 are nonoverlapping.

scale effort to map anterograde projection patterns across the mouse brain (Allen Brain Institute, <http://help.brain-map.org/display/mouseconnectivity/Documentation>), also showed robust expression in aPC neurons and their corticofugal projections to the OB but no expression in MTCs (two mice, four injections; no cells could be identified in the MCL in eight sections; Fig. 2*B*). These data are consistent with previous results showing an absence of retrograde labeling when using rAAVs with constitutive constructs (Chamberlin et al., 1998; Skorupa et al., 1999; Heuer et al., 2002; Passini et al., 2002). These results also suggest that retrograde infection is not facilitated by the presence of Cre recombinase in infected neurons.

Surprisingly, however, a third Cre-independent construct, 2/1.mCherry, was highly effective at driving transgene expression via retrograde infection. To characterize this, we coinjected 2/1.mCherry virus into aPC of PCdh21-Cre mice along with the Cre-dependent 2/1.FLEX.GCaMP3 vector described above (two mice, four injections). In these experiments, coexpression of mCherry and GCaMP3 was prevalent in OB MTCs (Fig. 2*C*), with clear coexpression in 92% (292 of 317 GCaMP3-expressing cells) of visible cells in the mitral cell layer. While mCherry was visible in the axon terminals of corticofugal projections from aPC to the OB, we observed no mCherry expression in neurons of the granule cell layer, as might be expected if the virus spread by bulk movement to the OB. These experiments demonstrate that at least some Cre-independent rAAV vectors are capable of driving robust transgene expression through retrograde infection via axonal projections, consistent with a recent report for 2/1.hChR2 (Zhang et al., 2013). Notably, these results also demonstrate that simultaneous infection by two different rAAVs and retrograde expression of distinct transgenes is possible in the same neuron.

Retrograde transgene expression in target-defined cortical pyramidal neuron subsets

We next assessed the ability of rAAVs to retrogradely infect other projection neuron types. Layer 5 pyramidal neurons are a major source of output from neocortex and project to diverse targets. There is evidence that layer 5 subpopulations projecting to different targets have distinct functional and morphological properties (Hattox and Nelson, 2007; Larsen et al., 2007). To assess the ability of retrograde rAAV infection to define target-specific cortical neuron populations, we investigated projections from mouse primary somatosensory cortex (S1), in which different subpopulations of layer 5 pyramidal neurons have been previously identified using retrograde tracing with a recombinant rabies virus (Larsen et al., 2007). We used the constitutive rAAV 2/1.mCherry construct, injected into three different targets of S1 layer 5 pyramidal neurons: superior colliculus, thalamus, and contralateral S1.

2/1.mCherry injection into these three targets (six mice; one injection in each) led to mCherry expression patterns that largely recapitulated those achieved with recombinant rabies virus (Larsen et al., 2007). Injection into superior colliculus led to strong expression in layer 5 pyramidal neurons within a spatially restricted region of S1 (Fig. 3*A*, *D*). Injection into thalamus also drove strong mCherry expression in layer 5 pyramidal neurons, although across a broader area of S1 (Fig. 3*B*, *E*). Injection into S1 led to mCherry expression in layer 5 pyramidal neurons in contralateral S1 (Fig. 3*C*, *F*). S1 injection also drove strong expression in layer 5 neurons around the injection site, whose axons could be seen coursing through the corpus callosum and terminating throughout contralateral S1 (Fig. 3*C*, *F*). This last experiment demonstrates the ability of rAAVs to infect neurons directly

A OB injected (CCK-Cre)

B DB injected (GAD2-Cre)

2/1.FLEX.GCaMP3

OB injected

C BLA injected (SERT-Cre)

2/9.DIO.hChR2-EYFP

Figure 4. Retrograde transgene expression in diverse populations of centrifugal projection neurons. **A**, Retrograde expression of GCaMP3 in CCK-positive neurons projecting from AON to the OB. Left image shows confocal stack of GCaMP3 fluorescence in the ipsilateral AON after injection of 2/1.FLEX.GCaMP3 into the OB of a CCK-Cre mouse, with dense expression in neurons throughout AON. Right images show confocal stack of GCaMP3 fluorescence in the contralateral AON of the same mouse, showing weaker but still clear expression in numerous AON neurons. Right, Zoom of outlined region showing details of GCaMP3-expressing neurons. **B**, Retrograde expression of GCaMP3 in GABAergic neurons projecting from the basal forebrain to the OB. Left, Image of GCaMP3 fluorescence in the nucleus of the diagonal band of Broca (DB) after injecting 2/1.FLEX.GCaMP3 into DB of a GAD2-Cre mouse. Right, Image of GCaMP3 fluorescence in the HDB after injection of rAAV2/1.Flex.GCaMP3 into the OB of a GAD2-Cre mouse, showing appreciable numbers of GCaMP3-expressing neurons and their processes. Section from atlas (Paxinos and Franklin, 2001) illustrates targeted injection sites and source of labeled projections. **C**, Retrograde expression of ChR2-EYFP in serotonergic neurons projecting to basolateral amygdala. Image shows EYFP fluorescence in the dorsal raphe after injection of 2/9.DIO.hChR2(H134R)-EYFP into the basolateral amygdala (BLA) of a SERT-Cre mouse. Numerous ChR2-EYFP-expressing somata are apparent clustered along the midline.

through their somata, in contrast to recombinant rabies variants, which selectively infect neurons via their axon terminals (Kelly and Strick, 2000). The number of retrogradely infected pyramidal neurons apparent after superior colliculus or thalamus injection appeared similar to or greater than those reported using recombinant rabies virus (Larsen et al., 2007), indicating a robust ability

of this construct to drive transgene expression after axonal infection. Finally, we demonstrated that the retrograde infection capabilities of these rAAVs enables the differential labeling of pyramidal neuron subpopulations projecting to distinct targets in the same animal. We used Emx1-Cre mice, which express Cre in glutamatergic neurons of neocortex (Gorski et al., 2002) and

A PAG injected (2/1.mCherry) **B CPu injected (TH-Cre)** **C PrL/IL and NAc injected (TH-Cre)**

Figure 5. Retrograde transgene expression of projections to and from midbrain nuclei. **A**, mCherry fluorescence after injection of 2/1.mCherry into PAG. Left, Middle, Low-magnification epifluorescence (top) and higher-magnification confocal stacks (bottom) from frontal association (FrA) and cingulate cortices (Cg). Right, Confocal stacks from amygdala (top) and hypothalamus (bottom). All images are taken from the same mouse, indicating retrograde infection of somata in widespread regions that project to PAG. **B**, Retrograde expression of GCaMP3 in dopaminergic neurons projecting to the striatum. Image shows GCaMP3 fluorescence in the substantia nigra pars compacta (SNC) after injection of 2/1.FLEX.GCaMP3 into the striatum (CPu) of a TH-Cre mouse. Numerous GCaMP3-expressing somata are apparent in SNC. **C**, Retrograde expression of GCaMP3 and tdTomato in separate neuron subsets of the VTA after injection of 2/1.FLEX.GCaMP3 into the nucleus AcbSh and 2/1.FLEX.tdTomato into PrL/IL cortex of the same TH-Cre mouse.

injected AAV2/1 Cre-dependent vectors into contralateral S1 and superior colliculus in the same mouse ($n = 2$ mice). As predicted from the single-injection experiments and from prior retrograde tracing studies (Larsen et al., 2007), these injections resulted in labeling of nonoverlapping populations of pyramidal neurons (Fig. 3G).

Retrograde expression in target-defined subsets of centrifugal projection neurons

Retrograde viral infection is also useful for isolating subsets of neurons that mediate centrifugal feedback or neuromodulation in a particular brain region. We thus tested the ability of rAAVs to isolate target-defined populations of centrifugal neurons. First we tested two systems that modulate olfactory processing in the OB: descending projections from the anterior olfactory nucleus (AON; Shipley and Adamek, 1984; Markopoulos et al., 2012) and GABAergic projections from the basal forebrain (Ma and Luo, 2012; Zaborszky et al., 2012). A large population of descending AON projection neurons target the OB, with neurons throughout AON projecting to the ipsilateral and, to a lesser extent, contralateral OB (Shipley and Adamek, 1984). AON projections also target other forebrain areas, such as piriform cortex (Brunjes et al., 2005; Hagiwara et al., 2012). Because of evidence that AON projection neurons may contain the peptide transmitter cholecystokinin (CCK; Burgunder and Young, 1990; Schiffmann and Vanderhaeghen, 1991), we used CCK-Cre mice to attempt to retrogradely label neurons projecting from AON to the OB by injecting Cre-dependent rAAV (2/1.FLEX.GCaMP3) into the OB on one side (two mice, two injections). In addition to expression in CCK-positive tufted cells of the OB due to direct infection (Seroogy et al., 1985), virus injection led to strong expression in large numbers of neurons with somata throughout the ipsilateral AON (Fig. 4A), as well as sparser expression in neurons of the contralateral AON (Fig. 4A, inset). Likewise, we isolated GABAergic projections from the horizontal limb of the diagonal band of Broca (HDB), the source of GABAergic basal forebrain projections to the OB (Shipley and Adamek, 1984; Zaborszky et al., 2012) by injecting 2/1.FLEX.GCaMP3 into the OBs of mice expressing Cre in GAD65-positive neurons (GAD2-Cre mice; three mice, six injections). In addition to the expected expression in GABAergic interneurons of the OB (Wachowiak et al., 2013), GCaMP3 expression also occurred in neurons throughout HDB (Fig. 4B). Retrograde expression was more sparse than was evident after direct virus injection into HDB (Fig. 4B; 32% of neurons labeled by direct HDB injection;

mean of 75 vs 235 neurons per counted section; four injections, two mice) consistent with only a subpopulation of these neurons projecting to the OB.

We also tested for retrograde infection of serotonergic projection neurons, which project to multiple targets throughout the brain from the midbrain raphe nuclei. Cre-dependent rAAV

Table 2. Projection neurons subpopulations showing retrograde transgene expression with rAAV constructs^a

Projection target	Source	Distance (mm)	Expression locus (–Cre mouse)	Vector
CPu	SNC	3.5	TH	2/1.FLEX.GCaMP3
AcbSh	VTA	4.4	TH	2/1.FLEX.GCaMP3
PrL/IL	VTA	5.0	TH	2/1.FLEX.GCaMP3; 2/1.FLEX.tdTomato
BLA	DR	5.4	SERT	2/9.DIO.hChR2-EYFP
aPC	OB	2.7	PCdh21	See Table 1
pPC	OB	5.3	PCdh21	See Table 1
PLCo	OB	6.8	PCdh21	See Table 1
MeA	AOB	4.6	PCdh21	See Table 1
OB	AON	2.2	CCK	2/1.FLEX.GCaMP3
OB	HDB	4.3	GAD65	2/1.FLEX.GCaMP5G
OB	HDB	4.3	ChAT	2/5.DIO.hChR2-EYFP
cS1BF (contra)	S1BF	6.0 (lateral)	WT, Emx1 (cortical pyramidal neurons)	2/1.FLEX.GCaMP3; 2/1.FLEX.tdTomato; 2/1.mCherry
SC	S1BF	1.9	WT, Emx1 (cortical pyramidal neurons)	2/1.FLEX.GCaMP3; 2/1.mCherry
Thalamus	S1BF	2.5 (diagonal)	WT (cortical pyramidal neurons)	2/1.mCherry

^aTable summarizes virus injection sites and Cre expression loci resulting in retrograde transgene expression after infection by rAAV constructs. Viral constructs were injected into the projection targets of cells located in the source area. Reported distance between the injection site and source area is the straight-line distance in the anterior–posterior direction unless otherwise noted. Expression locus represents mouse line expressing Cre from the designated locus. For WT mice, the targeted neuron types are listed. AOB, Accessory olfactory bulb; BLA, basolateral amygdaloid nucleus, anterior part; CPu, caudate putamen (striatum); DR, dorsal raphe nucleus; MeA, medial amygdaloid nucleus, anterior part; PLCo, posterolateral cortical amygdaloid nucleus; pPC, posterior piriform cortex; S1BF, primary somatosensory cortex, barrel field; SC, superior colliculus; SNC, substantia nigra, pars compacta.

(2/9.DIO.ChR2-EYFP) was injected into the basolateral amygdala (three mice, six injections) of Slc6a4 (SERT)-Cre mice, which express Cre selectively in serotonergic neurons (Zhuang et al., 2005). Basolateral amygdala receives relatively strong innervation from serotonergic raphe neurons (Vertes, 1991). Consistent with this, virus injection led to ChR2-eYFP expression in substantial numbers of neurons throughout the raphe nuclei ($n = 671$ cells; 17 sections, two mice), with the majority of expressing somata in the dorsal raphe (Fig. 4C). Together these results indicate that retrograde infection using rAAVs can also be used to isolate otherwise indistinguishable subpopulations of centrifugal or neuromodulatory projection neurons based on the target of their axonal projections.

Retrograde transgene expression in neurons projecting to and from midbrain nuclei

We next targeted projections to and from specific nuclei of the midbrain, a phylogenetically more primitive brain structure that receives input from and sends projections to diverse brain regions. Isolating subsets of midbrain projection neurons based on projection target or source is thus a useful application of retrograde viral tracing. First we used the constitutive AAV 2/1.mCherry to test for retrograde infection of neurons projecting to the periaqueductal gray (PAG). The PAG represents an interface between the forebrain and the lower brainstem and receives input from diverse brain areas (Benarroch, 2012). AAV 2/1.mCherry injection into the PAG resulted in, besides expression in PAG neurons themselves, mCherry expression in substantial numbers of neurons with somata in frontal association cortex, cingulate cortex, hypothalamus, and the amygdala (Fig. 5A). Neurons with somata along the ventricles between the midbrain and frontal cortex showed no infection, indicating specific retrograde tracing and not an unspecific diffusion along the ventricle.

We next tested for retrograde infection of dopaminergic midbrain projection neurons, which originate in several midbrain nuclei and project to multiple forebrain targets. First, injection of 2/1.FLEX.GCaMP3 into the striatum (caudate putamen) of TH-Cre mice (four mice, four injections) labeled neurons in substantia nigra pars compacta (Fig. 5B) that were pyramidal in shape with rostrocaudally oriented dendrites, matching earlier descriptions of substantia nigra neurons projecting to striatum (Fallon et al., 1978; Faull and Mehler, 1978). Notably, these results differ from recent studies finding that constitutive vectors using the

native AAV2 serotype failed to drive retrograde expression in nigrostriatal projection neurons (Ciesielska et al., 2011; Salegio et al., 2013) but appear similar to the levels of retrograde expression mediated by other retrogradely infecting AAV serotypes (Ciesielska et al., 2011; Masamizu et al., 2011; Salegio et al., 2013). Second, we labeled neurons projecting from the ventral tegmental area (VTA) to distinct forebrain targets (Margolis et al., 2006) by injecting 2/1.FLEX.tdTomato or 2/1.FLEX.GCaMP3 into either prelimbic/infralimbic cortex (PrL/IL; three mice, three injections) or nucleus accumbens shell (AcbSh; four mice, four injections) of TH-Cre mice. In each case, injection led to expression in sparsely distributed neurons in VTA. Injection of virus into PrL/IL and AcbSh in the same TH-Cre mouse resulted in labeling of distinct (though still sparse) subsets of VTA neurons (Fig. 5C); these results are consistent with previous retrograde labeling studies using histochemical markers (Margolis et al., 2006).

Table 2 summarizes all virus injection sites used to evaluate retrograde infection capabilities of rAAV constructs. Together these results indicate that retrograde infection by a variety of rAAVs can be used to isolate diverse subpopulations of neurons based on the target of their axonal projections.

Optogenetic identification of target-defined projection neurons *in vivo* using retrograde expression of ChR2

We have recently demonstrated that expression of GCaMPs via retrograde infection yields sufficient expression levels to support the selective imaging of spontaneous and odorant-evoked activity from aPC-projecting MTCs *in vivo* (Wachowiak et al., 2013). Here, we tested whether this approach yields sufficient expression levels to support the identification and physiological characterization of cell types based on projection target. This process is equivalent to the ChR2-assisted circuit mapping approach described recently (Petreanu et al., 2007) but in the retrograde direction. ChR2(H134R) was expressed in MTCs projecting to aPC via retrograde rAAV infection and “blind” whole-cell or extracellular recordings were obtained from neurons in the OB. For *in vivo* extracellular or whole-cell recordings, neurons in the dorsal OB were targeted and brief (5–10 ms) light pulses were delivered to the dorsal OB to test for ChR2 expression in the recorded cells. For *in vitro* whole-cell recordings, mitral cells were visually targeted in OB slices using differential interference contrast optics and light pulses (3–5 ms) delivered to the entire slice. In all three cases, ChR2-expressing neurons were distinguished by reliable

(95–100%) light-evoked spikes (for extracellular recordings) or depolarizing potentials and spike bursts (for whole-cell recordings) occurring with latencies of <10 ms and low jitter (SD of spike time, 0.2–1.9 ms; Fig. 6), consistent with other characterizations of responses of ChR2-expressing neurons to light flashes (Arenkiel et al., 2007; Zhang et al., 2013). In the OB slice recordings, five of seven recorded mitral cells (in two mice) showed light-evoked responses meeting these criteria. In the *in vivo* whole-cell recordings, five of eight cells (in five mice) responded reliably to light stimulation; one of these five was recovered and confirmed to be a mitral cell. Two of the three cells showing no or longer-latency responses to light stimulation were recovered and identified as a superficial tufted cell and a granule cell (data not shown). These results demonstrate that retrograde viral infection with optogenetic transgenes can enable the isolation of genetically specified cell types projecting to specific target locations for *in vivo* or *in vitro* analysis.

Discussion

AAV vectors based on the AAV2 serotype have emerged as a widely used platform for the delivery of transgenes to neurons of the CNS for experimental as well as therapeutic applications (Wu et al., 2006; Betley and Sternson, 2011; Salegio et al., 2012). Several earlier studies have reported efficient retrograde transduction mediated by other AAV serotypes (for example, AAVs 5, 6, and 9, but ineffective retrograde transduction by AAV2; Paterna et al., 2004; Chen et al., 2013; Salegio et al., 2013). Recombinant, pseudotyped AAVs (rAAVs) engineered to improve transduction efficiency and tissue tropism using capsid genes from other AAV serotypes (Gao et al., 2002; Rabinowitz et al., 2004) have been reported to mediate retrograde transduction in certain projection neuron populations in the rodent and primate CNS (Kaspar et al., 2002; Passini et al., 2005; Taymans et al., 2007; Yasuda et al., 2007; Towne et al., 2009; Masamizu et al., 2011; Zhang et al., 2013). However, other studies have found pseudotyped rAAVs to be poor or ineffective at retrograde transduction (Chamberlin et al., 1998; Burger et al., 2004); indeed, rAAVs based on the AAV2 serotype (e.g., AAV2/1) have been the vector of choice for the large-scale mapping of anterograde axonal projections in the mouse brain (Allen Brain Institute, <http://help.brain-map.org/display/mouseconnectivity/Documentation>; Harris et al., 2012). Recent iterations of rAAVs feature DNA constructs with optimized promoters, conditional expression switches, and novel fluorophores, as well as optimized methods for purification of high-titer virus (Ayuso et al., 2010; Betley and Sternson, 2011); these vectors have been widely adopted for applications aimed at the functional dissection and manipulation of genetically defined neuronal subpopulations in the intact brain (Luo et al., 2008; Yizhar et al., 2011). Here, we present an extensive study of the retrograde infection abilities of many of the most widely used rAAV vectors for such applications. We tested 13 different rAAVs from two different vector core production facilities (the University of Pennsylvania and the University of North Carolina) and targeted neurons projecting from 14 different brain structures.

We found that many of rAAV vectors—in particular, though not exclusively, those using Cre-dependent expression switches—show a robust capacity for retrograde infection and subsequent transgene expression in the mouse. The substantial distances between injection site and expressing somata (up to almost 7 mm; Table 2) and the lack of expression in neurons in between these two areas rule out the possibility that these results could be mediated by bulk movement of virus from the injection site. We obtained robust and efficient retrograde expression in Cre-

Figure 6. Optogenetic identification of target-defined OB projection neurons after retrograde expression of ChR2. **A**, *In vivo* extracellular recording from an MTC in the mouse OB after injection of 2.1.DIO.hChR2(H134R)-EYFP into aPC of a PCdh21-Cre mouse. Top, Three trials of 50 light pulses (10 ms duration, 10 Hz, 200 μ m optical fiber, $\sim$ 1 mW total power at the fiber tip) elicits a single spike following each pulse with near 100% reliability. Bottom, Pulse-triggered spike histogram showing spike latencies of 8–10 ms with little jitter. **B**, *In vitro* whole-cell recording from a mitral cell in an OB slice after retrograde ChR2 expression in MTCs (PCdh21-Cre mouse, as in **A**). Top trace shows responses to a train of light pulses (5 ms duration, 5 Hz, 1 mm optical fiber, $\sim$ 24 mW total power), with each pulse eliciting a spike. Lower trace shows overlay of the response to each pulse in the train, showing short latency to depolarization onset and low jitter in spike time. **C**, *In vivo* whole-cell recording from an MTC after retrograde ChR2 expression, as in **A** and **B**. Left trace shows continuous recording of response to train of 10 pulses (11 mW total power) delivered as in **B**. In this cell, the light pulse does not always evoke a spike; right traces show overlay of responses to pulses that fail to evoke a spike. Failed responses consist of a depolarization with short latency (2–3 ms) and no detectable jitter in onset time. Higher intensities ($\leq$ 25 mW total power) or longer durations elicited spikes to each pulse (data not shown).

expressing projection neurons using multiple rAAV serotypes and transgenes, multiple Cre mouse lines, and in multiple neuron types including olfactory projection neurons, cortical pyramidal cells, corticofugal projection neurons, and neurons projecting from neuromodulatory centers to different forebrain targets. Retrograde transduction efficiency appeared comparable to or greater than that for the same projection neuron populations targeted in earlier studies using recombinant rabies virus as a “benchmark” retrograde viral tracer (Larsen et al., 2007); likewise we observed retrograde expression patterns in corticothalamic and nigrostriatal projection neurons that appeared qualitatively similar and occurred over a similar or shorter incubation time to those mediated by other native AAV serotypes identified as efficient retrograde transduction vectors (Ciesielska et al., 2011; Masamizu et al., 2011; Salegio et al., 2013). Retrogradely mediated transgene expression levels were strong enough to support optical imaging or

optogenetic activation of target-defined projection neuron subpopulations *in vivo*.

Surprisingly, we found dramatic differences in the ability of rAAVs of identical serotype to mediate retrograde transduction depending on the vector construct. For example, 2/1.FLEX.GCaMP5G drove strong retrograde expression in Cre-expressing MTCs of the OB, but the identical construct without the FLEX switch (2/1.GCaMP5G) yielded no retrograde expression in the same mice. Indeed, every Cre-dependent vector tested was capable of driving strong retrograde transgene expression in OB projection neurons and Cre-dependent vectors of the appropriate serotype were effective at driving expression in diverse projection neuron populations. Likewise, one of the three constitutive vectors tested (2/1.mCherry) was highly effective at retrograde infection while others (e.g., 2/1.eGFP) were ineffective. These results speak against the possibility that the presence of Cre aids in axonal transport or genome processing, as some constitutive constructs failed to retrogradely express in Cre-expressing neurons while others (e.g., 2/1.mCherry) led to strong retrograde expression in wild-type mice. Thus, differences in retrograde expression success appear to be determined by the rAAV itself. While viral titer and purification method can affect transduction efficiency (Wu et al., 2006; Towne et al., 2010), reported titers were similar for all constructs tested here and purification method was identical (http://www.med.upenn.edu/gtp/vectorcore/quality_control.shtml).

The apparent importance of vector DNA composition in the efficiency of retrograde infection by rAAVs is surprising and, to our knowledge, has not been previously reported. Instead, residues in the genome encoding the rAAV capsid are thought to be the chief determinants affecting the neurotropism and intracellular processing of the viral genome and genome unpackaging is thought to occur only after retrograde transport and nuclear entry of the virus particle (Wu et al., 2006; Naumer et al., 2012; Nonnenmacher and Weber, 2012). At present, however, the factors affecting viral transduction efficiency remain unclear and the relationship between vector genome and retrograde transduction requires further exploration (Wu et al., 2006; Naumer et al., 2012; Nonnenmacher and Weber, 2012).

Despite this uncertainty, the robust capacity of the latest generation of rAAVs for retrograde transduction constitutes an important expansion of the toolbox for cell-type-specific isolation and manipulation of neurons in the CNS. For example, Cre-dependent rAAV vectors allow for a combinatorial strategy for defining neuronal populations based on genetic and anatomic criteria and offer advantages over rabies-based and herpes-based vectors in their relative ease of production and handling and their relatively stable, long-term expression of transgenes (Howarth et al., 2010; Betley and Sternson, 2011). The retrograde expression abilities of these vectors also enables the simultaneous expression of different fluorophores, optical reporters, or optogenetic probes in distinct projection streams from the same brain area. Finally, the ability to retrogradely (or directly) drive expression of more than one transgene in the same neuron allows for additional combinatorial possibilities for circuit mapping as well as genetically manipulating neuronal function based on axonal projection patterns. Given the recent surge in efforts to map connectivity and probe circuit function in the intact brain (Luo et al., 2008; Arenkiel and Ehlers, 2009; Yizhar et al., 2011; Alivisatos et al., 2012; Osten and Margrie, 2013), further optimization of retrograde gene transfer using rAAVs is a promising avenue for improved experimental approaches to understanding brain circuits as well as for the continued development of gene therapy

applications to treating CNS disorders (Kaspar et al., 2002; Han and Friedman, 2012; Weinberg et al., 2013).

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