

# Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems

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**Adeno-associated viruses (AAVs) are commonly used for *in vivo* gene transfer. Nevertheless, AAVs that provide efficient transduction across specific organs or cell populations are needed. Here, we describe AAV-PHP.eB and AAV-PHP.S, capsids that efficiently transduce the central and peripheral nervous systems, respectively. In the adult mouse, intravenous administration of  $1 \times 10^{11}$  vector genomes (vg) of AAV-PHP.eB transduced 69% of cortical and 55% of striatal neurons, while  $1 \times 10^{12}$  vg of AAV-PHP.S transduced 82% of dorsal root ganglion neurons, as well as cardiac and enteric neurons. The efficiency of these vectors facilitates robust cotransduction and stochastic, multicolor labeling for individual cell morphology studies. To support such efforts, we provide methods for labeling a tunable fraction of cells without compromising color diversity. Furthermore, when used with cell-type-specific promoters and enhancers, these AAVs enable efficient and targetable genetic modification of cells throughout the nervous system of transgenic and non-transgenic animals.**

~~AAVs<sup>1</sup> have been extensively used as vehicles for gene transfer to the nervous system, enabling gene expression and knockdown, gene editing<sup>2,3</sup>, circuit modulation<sup>4,5</sup>, *in vivo* imaging<sup>6</sup>, disease model development<sup>7</sup> and the evaluation of therapeutic candidates for the treatment of neurological diseases<sup>8</sup>. AAVs are well suited to these applications because they are capable of providing safe, long-term expression in the nervous system<sup>9,10</sup>. Most of these applications rely on local AAV injections into the adult brain to bypass the blood-brain barrier and to temporally and spatially restrict transgene expression.~~

Targeted AAV injections have also been used for gene delivery to peripheral neurons to test strategies for treating chronic pain<sup>11,12</sup> and for tracing, monitoring and modulating specific subpopulations of vagal neurons<sup>13,14</sup>. Many peripheral neuron populations, however, are difficult to access surgically (for example, dorsal root ganglia (DRG), nodose ganglia, sympathetic chain ganglia and cardiac ganglia) or are widely distributed (for example, the enteric nervous system), thereby limiting methods for genetic manipulation of these targets. Likewise, in the CNS, single localized injections may be insufficient to study circuits in larger species<sup>15</sup> or to test gene therapies for diseases that

involve the entire nervous system or widely distributed cell populations (for example, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, spinal muscular atrophy, Friedreich's ataxia and numerous lysosomal storage diseases)<sup>8</sup>.

Systemic AAV delivery provides a noninvasive alternative for broad gene delivery to the nervous system<sup>16</sup>; however, the high viral load required and relatively low transduction efficiency have limited wide adoption of this method. Several groups have developed AAVs that enhance gene transfer to the CNS after intravenous delivery. The recently reported AAV-AS capsid<sup>17</sup>, which utilizes a polyalanine N-terminal extension to the AAV9.47 (ref. 18) VP2 capsid protein, provides higher neuronal transduction, particularly in the striatum, which may have applications for Huntington's disease. Similarly, the AAV-BR1 capsid<sup>19</sup>, based on AAV2, may be useful for applications that require more efficient and selective transduction of brain endothelial cells. Using a cell-type-specific capsid selection method we developed called CREATE (Cre recombinase-based AAV targeted evolution), we recently identified ~~AAV-PHP.B, a capsid that transduces the majority of neurons and astrocytes across many regions of the adult mouse brain and spinal cord after intravenous injection<sup>20</sup>. While the efficiency of AAV-PHP.B opens up new possibilities for CNS-wide genetic modification<sup>21,22</sup>, it requires a substantial dose of vector (for example,  $1 \times 10^{12}$  vg per adult mouse or higher).~~

Here, we used CREATE to further evolve AAV-PHP.B for more efficient transduction of neurons throughout the adult mouse brain and spinal cord. We describe an ~~enhanced variant of AAV-PHP.B, AAV-PHP.eB, which lowers the viral load required to transduce the majority of CNS neurons. Notably, we also report the characterization of a second capsid variant, AAV-PHP.S, that displays improved tropism toward peripheral neurons, including those in the DRG, cardiac ganglia and enteric nervous system.~~

AAVs are also used for the bulk study of neuronal anatomical connectivity and morphology<sup>23</sup> and are components of multiviral strategies for tracing the relationships between bulk inputs and outputs<sup>24</sup>. At the single-cell level, ~~AAV-based multicolor labeling systems<sup>25</sup>~~ have been developed with the goal of improving tracing efforts. However, the lack of control over the labeling density and uniformity of color diversity has been a persistent challenge<sup>25</sup>. To overcome these challenges, we have developed a two-component viral vector system

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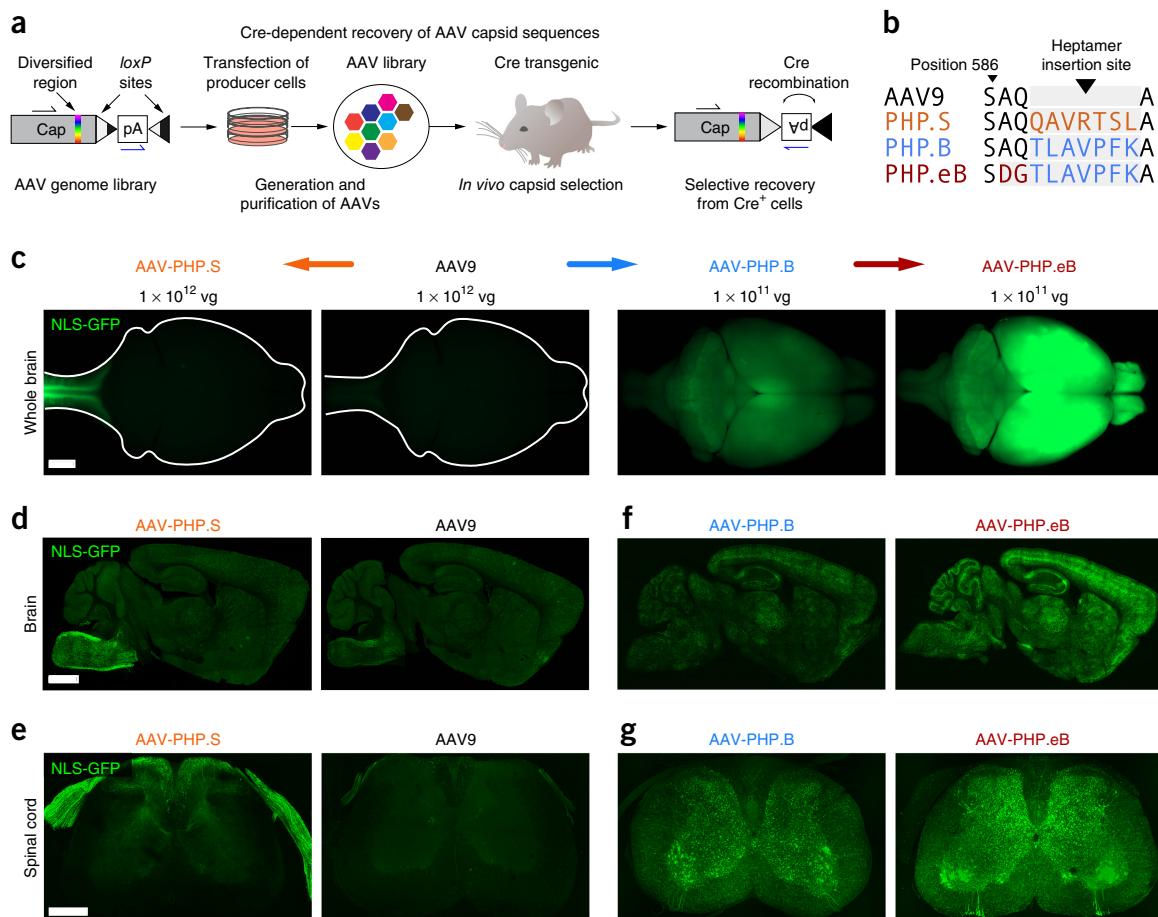
to stochastically label cells with a wide range of hues while independently controlling the fraction of cells labeled. In addition, using the new capsids reported here, we expressed a variety of fluorescent reporters under different cell-type-specific promoters, supporting the potential use of these vectors for population-wide genetic manipulations of the nervous system in Cre transgenic or wild-type mice.

## RESULTS

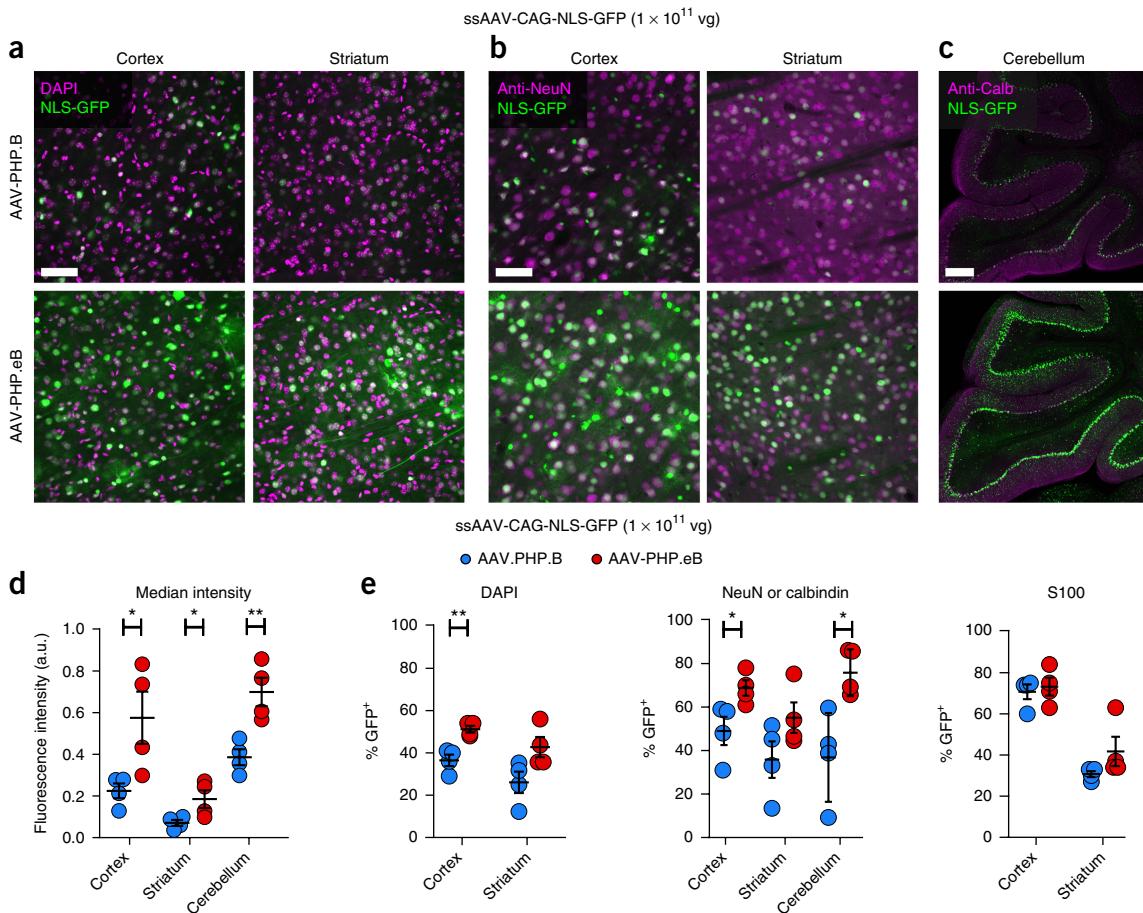
### Engineered AAV capsids for efficient transduction across the central and peripheral nervous systems

Here we report two new AAV capsids that provide increased gene transfer to the CNS and the PNS via the vasculature. We developed these vectors by applying the previously published *in vivo* capsid selection method CREATE<sup>20</sup> (Fig. 1a) to AAV-PHP.B and AAV9 as parental capsids. First, we sought to evolve the previously described AAV-PHP.B vector for more efficient tropism toward the CNS. We generated an AAV capsid library in which the AAV-PHP.B heptamer targeting sequence was modified by randomizing three consecutive amino acids in an overlapping fashion across the heptamer and flanking amino acids (Online Methods). To provide selective pressure for capsids that transduce both neurons and glia, we subjected the library to parallel *in vivo*

selections in adult Vglut2-IRES-Cre, Vgat-IRES-Cre and GFAP-Cre mice, which express Cre in glutamatergic neurons, GABAergic neurons and astrocytes, respectively. After two rounds of selection, we found that a variant comprising the sequence DGTLAVPKF in place of the AAV-PHP.B sequence AQTLAVPKF at amino acid positions 587–595 (Fig. 1b) was enriched in all three transgenic lines (11.1%, 12.1% and 15.7% of the total recovered sequences, respectively). We used this DGT substitution variant or AAV-PHP.B to package a single-stranded (ss) AAV reporter genome that expresses a nuclear GFP (NLS-GFP) from the constitutive CAG promoter (ssAAV-CAG-NLS-GFP) and delivered  $1 \times 10^{11}$  vg of the viruses by intravenous injection to adult mice. The DGT substitution variant appeared to transduce the CNS more efficiently than AAV-PHP.B as judged by wide-field fluorescence microscopy of the intact brain (Fig. 1c) and confocal microscopy on thin sections from brain (Fig. 1f) and spinal cord (Fig. 1g). We refer to this enhanced AAV-PHP.B variant as AAV-PHP.eB and present further quantitative characterization below. Second, we performed a separate selection of an AAV9-based heptamer library in GFAP-Cre mice<sup>20</sup>. After two rounds of selection, we found a variant (heptamer sequence QAVRTSL) that represented 33.3% of the total sequences recovered (Fig. 1b). As above, we evaluated the capsid



**Figure 1** Engineered AAV capsids for efficient transduction across the peripheral and central nervous systems. (a) Schematic of the CREATE selection method. (b) The amino acid sequences for the heptamer insertions and flanking AAV9 sequences for AAV-PHP.S, AAV-PHP.B and AAV-PHP.eB; the heptamer and adjacent substitutions are highlighted in colored text. (c–g) ssAAV-CAG-NLS-GFP was packaged into the indicated capsid and intravenously injected into adult mice at  $1 \times 10^{12}$  vg per mouse (AAV9 and AAV-PHP.S; c–e) or  $1 \times 10^{11}$  vg per mouse (AAV-PHP.B and AAV-PHP.eB; c,f,g). (c) Representative whole-brain fluorescence images after 3 weeks of expression. (d–g) Representative confocal images of native GFP fluorescence from sagittal brain sections (d,f) and transverse spinal cord sections (e,g) for the indicated capsids. For d–g, all imaging and display conditions are matched across panel pairs to allow comparisons. Panels d and f are 40-µm maximum intensity projections (MIPs) and panels e and g are 300-µm MIPs. Scale bars for c–e are 1 mm.



**Figure 2** AAV-PHP.eB transduces several CNS regions more efficiently than AAV-PHP.B. ssAAV-PHP.B:CAG-NLS-GFP or ssAAV-PHP.eB:CAG-NLS-GFP was intravenously injected into adult mice at  $1 \times 10^{11}$  vg per mouse. Native GFP fluorescence was assessed after 3 weeks of expression. (a,b) Representative images of native GFP fluorescence (green) and DAPI staining (a, magenta) or NeuN staining (b, magenta) in the cortex and striatum. (c) Representative images of native GFP fluorescence (green) and calbindin immunohistochemistry (magenta) in the cerebellum. (d) Mean GFP intensity in all DAPI<sup>+</sup> nuclei in the cortex ( $t_6 = 2.688$ ;  $P = 0.0361$ ) and striatum ( $t_6 = 2.536$ ;  $P = 0.0443$ ) or in calbindin<sup>+</sup> cells in the cerebellum ( $t_6 = 4.007$ ;  $P = 0.0071$ ). (e) Quantification of cell transduction. From left to right, the percentage of DAPI<sup>+</sup> cells that express GFP in the cortex ( $t_6 = 4.669$ ;  $P = 0.0034$ ) and striatum ( $t_6 = 2.390$ ;  $P = 0.0541$ ), the percentage of NeuN<sup>+</sup> cells that express GFP in the cortex ( $t_6 = 2.662$ ;  $P = 0.037$ ) and in the striatum ( $t_6 = 1.764$ ;  $P = 0.128$ ), the percentage of calbindin<sup>+</sup> cerebellar Purkinje cells that express GFP ( $t_6 = 3.328$ ;  $P = 0.039$ ), and the percentage of S100<sup>+</sup> cells that express GFP in the cortex ( $t_6 = 0.4422$ ;  $P = 0.6738$ ) and striatum ( $t_6 = 1.512$ ;  $P = 0.1814$ ). (d,e) For quantification:  $n = 4$  mice per group, mean  $\pm$  s.e.m.; unpaired, two-tailed  $t$ -test (\*\* $P < 0.01$ ; \* $P < 0.05$ ). All images are single-plane confocal sections of native GFP fluorescence. Scale bars are 50  $\mu$ m (a,b) and 100  $\mu$ m (c).

against AAV9, its parent capsid, by packaging the ssAAV-CAG-NLS-GFP reporter and delivering the vectors at  $1 \times 10^{12}$  vg per mouse. While the QAVRTSL variant and AAV9 both showed similar sparse transduction of the brain (Fig. 1c,d), the QAVRTSL variant notably appeared to strongly transduce peripheral sensory afferents entering the spinal cord and brain stem (Fig. 1c–e). We refer to this variant as AAV-PHPS. While unexpected given the selection scheme, the improved tropism for sensory neurons provided by AAV-PHPS could be useful for widespread gene transfer to cells of the PNS. We explore this possibility below.

Together these data indicate that the tropism of AAV9 can be routed toward either more efficient CNS or PNS transduction (Fig. 1c), depending on the amino acid insertion or modification. A comparison of the amino acid insertion and modification sequences of AAV-PHP.eB and AAV-PHPS, along with their parent capsids, AAV-PHP.B and AAV9, respectively, is shown in Figure 1b, and their nucleotide sequences and relative viral production efficiencies are provided in Supplementary Table 1.

#### Quantification of transduction by AAV-PHP.eB and AAV-PHPS

Next we quantified the transduction efficiency of AAV-PHP.eB and AAV-PHP.B 3 weeks after intravenous administration of  $1 \times 10^{11}$  vg of ssAAV-CAG-NLS-GFP. First, when compared with AAV-PHP.B, AAV-PHP.eB provided significantly increased (>2.5-fold) GFP fluorescence per cell nucleus throughout the cortex and striatum and (>1.8-fold) in cerebellar Purkinje cells (Fig. 2d and Supplementary Fig. 1). To quantify expression in Purkinje cells, we developed an automated cell segmentation and analysis pipeline (Supplementary Fig. 2 and Online Methods). AAV-PHP.eB also showed an increase over AAV-PHP.B in both the percentage of total cortical cells transduced (DAPI<sup>+</sup> cells that express GFP; 51  $\pm$  2% versus 37  $\pm$  3%; Fig. 2a,e) and the percentage of NeuN<sup>+</sup> neurons transduced (69  $\pm$  4% versus 49  $\pm$  7%; Fig. 2b,e). In the striatum, a similar trend toward increased transduction by AAV-PHP.eB compared to AAV-PHP.B was observed from quantifying both the percentage of DAPI<sup>+</sup> and NeuN<sup>+</sup> cells that express GFP (DAPI<sup>+</sup>: 42  $\pm$  5% versus 26  $\pm$  5%; Fig. 2a,e; NeuN<sup>+</sup>: 55  $\pm$  7% versus 36  $\pm$  8%, Fig. 2b,e). In the cerebellum,

we observed an increase in the percentage of calbindin<sup>+</sup> Purkinje cells that expressed GFP in mice injected with AAV-PHP.eB as compared with AAV-PHP.B (76 ± 5% versus 37 ± 10%, Fig. 2c,e). Both vectors transduced a similar percentage of S100<sup>+</sup> glia in the cortex as well as the striatum (Fig. 2e and Supplementary Fig. 3).

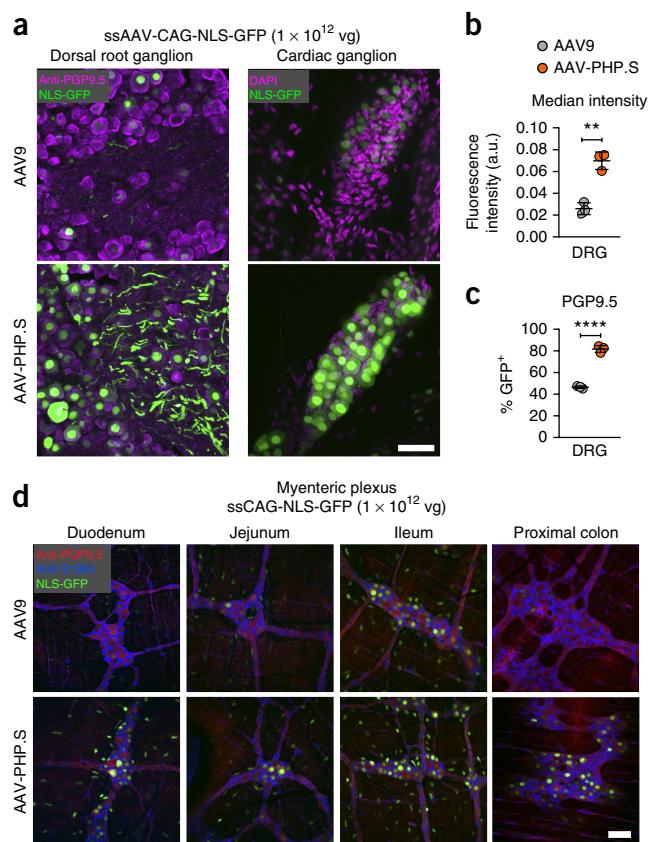
Next we examined the transduction efficiency of AAV-PHP.S as compared with AAV9, a vector known to provide broad transduction of the PNS and many peripheral organs after intravenous delivery<sup>16,26,27</sup>. We packaged ssAAV-CAG-NLS-GFP into AAV-PHP.S or AAV9 administered the viruses to adult mice by intravenous injection at  $1 \times 10^{12}$  vg per mouse and then assessed GFP expression after 3 weeks. In the DRG, AAV-PHP.S provided a significant increase in transduction over AAV9 (Fig. 3a) as quantified both by native GFP fluorescence per cell nucleus (>2.6-fold) (Fig. 3b) and by the percentage of cells positive for PGP9.5, a marker for neurons in the PNS, that coexpressed GFP (82 ± 2% versus 46 ± 0.7%) (Fig. 3c). AAV-PHP.S also transduced the cardiac ganglia (Fig. 3a) and cells within the enteric nervous system of the small intestine and colon (Fig. 3d and Supplementary Video 1) with high efficiency. To determine the identity of enteric nervous system cells transduced by AAV-PHP.S, we performed immunohistochemistry for the neuronal marker PGP9.5 and the astrocyte marker S100b. Most GFP<sup>+</sup> cells in the myenteric plexus (Fig. 3d) and submucosal plexus (Supplementary Fig. 4) of the small intestine and colon were PGP9.5<sup>+</sup>, with sparse and faint transduction seen in S100b<sup>+</sup> cells (Supplementary Video 1). Notably, we also observed robust transduction by AAV-PHP.S of cells in many organs, including the liver, lungs and heart, and of stomach cells within the muscle and myenteric plexus layers (Supplementary Fig. 4).

#### AAV-PHP.eB and AAV-PHP.S enable efficient cotransduction of neurons

The increased transduction efficiency and enhanced expression per cell observed with both AAV-PHP.eB and AAV-PHP.S, relative to their parental capsids, suggested that a large fraction of cells will be transduced by multiple AAV genomes when administered at the above doses. The transduction efficiency of these vectors may therefore enable applications that require expression from more than one AAV genome. To test this, we coadministered a cocktail of three AAV genomes with the single fluorescent proteins (XFPs) mRuby2, mNeon-Green and mTurquoise2 expressed from the human synapsin (SYN1) promoter, which limits expression to neurons. The three-vector mix (AAV-hSyn1-3XFP) was packaged into AAV-PHP.eB or AAV-PHP.S and injected into adult wild-type mice. Coadministration of these vectors resulted in dense labeling of cells with multiple colors in the brain when delivered with AAV-PHP.eB and in the enteric nervous system when delivered with AAV-PHP.S (Fig. 4a). Because cells transduced by a single vector would express only one of the three XFPs, and thereby be red, green or blue, the wide color diversity seen in both the brain and the enteric nervous system demonstrates that these capsids enable the independent and stochastic cotransduction of neurons by multiple viral vectors.

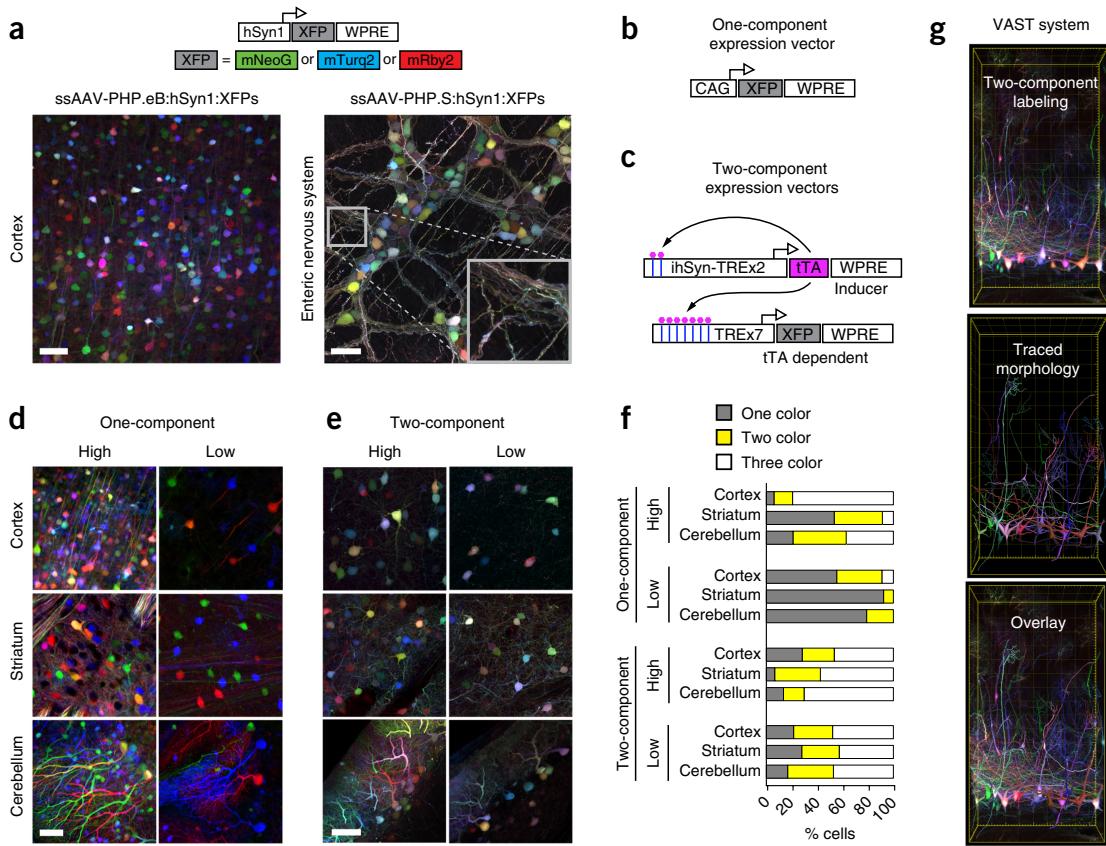
#### Tunable density multiplexed gene expression

Multicolor labeling by AAVs can benefit single-cell morphology and tracing studies<sup>28</sup>. However, direct local virus injections<sup>23,25</sup> may not provide uniform color diversity because of the non-uniform distribution of vector copies radiating outward from the injection site<sup>25</sup>. We predicted that systemically delivered AAVs may offer a solution to this challenge because the distribution of labeled cells and color diversity will be more uniform. Indeed, when we delivered three separate XFPs expressed from the CAG promoter (AAV-CAG-3XFP; Fig. 4b) we



**Figure 3** AAV-PHP.S efficiently transduces peripheral neurons. ssAAV-PHP.S:CAG-NLS-GFP or ssAAV9:CAG-NLS-GFP was intravenously injected into adult mice at  $1 \times 10^{12}$  vg per mouse. Native GFP fluorescence was assessed after 3 weeks of expression. (a) Representative images of GFP expression and neuronal PGP9.5 (left, magenta) or DAPI (right, magenta) staining in a DRG (left) and cardiac ganglion (right). (b) Quantification of the mean GFP fluorescence intensity per cell with AAV-PHP.S or AAV9 ( $t_4 = 7.814$ ; \*\* $P = 0.0014$ ). (c) Quantification of the percentage of PGP9.5<sup>+</sup> cells transduced ( $t_4 = 18.29$ ; \*\*\*\* $P < 0.0001$ ). (d) Representative images of GFP expression, with neuronal PGP9.5 (red) and astrocyte S100b (blue) staining in the myenteric plexus of the duodenum, jejunum, ileum and proximal colon. In b,c  $n = 3$  independent animals per group, mean ± s.e.m.; unpaired, two-tailed t-test. For representative images;  $n = 3$  animals (a) and 2 animals (d). All imaging and display conditions are matched in the GFP channel across panel pairs. Scale bars in a,d are 50  $\mu$ m.

observed a wide range of hues in the cortex, striatum and cerebellum (Fig. 4d, left). However, this strategy, whereby the expression of the transgene is controlled *in cis*, couples the expression level, labeling density and color diversity to the virus dose. As expected, when we lowered the virus dose we observed that the density and color diversity were reduced (that is, most cells were labeled by primary colors—red, green, or blue—likely reflecting single transduction events) (Fig. 4d, right). Therefore, to achieve stochastic, multicolor labeling while reducing the fraction of labeled cells, we sought a two-component AAV system in which the expression of XFPs in each cell is dependent on a separate inducer vector that is cotransduced at a variable dose. In this way, color diversity could be decoupled from the labeling density. We developed two inducer systems: one that uses the tetracycline (tet)-inducible system—a tet-off transactivator (tTA) and a tet-responsive element (TRE)<sup>29</sup> (Fig. 4c)—and a second that uses the FLPo-FRT system<sup>30</sup> (Supplementary Fig. 5). Both systems



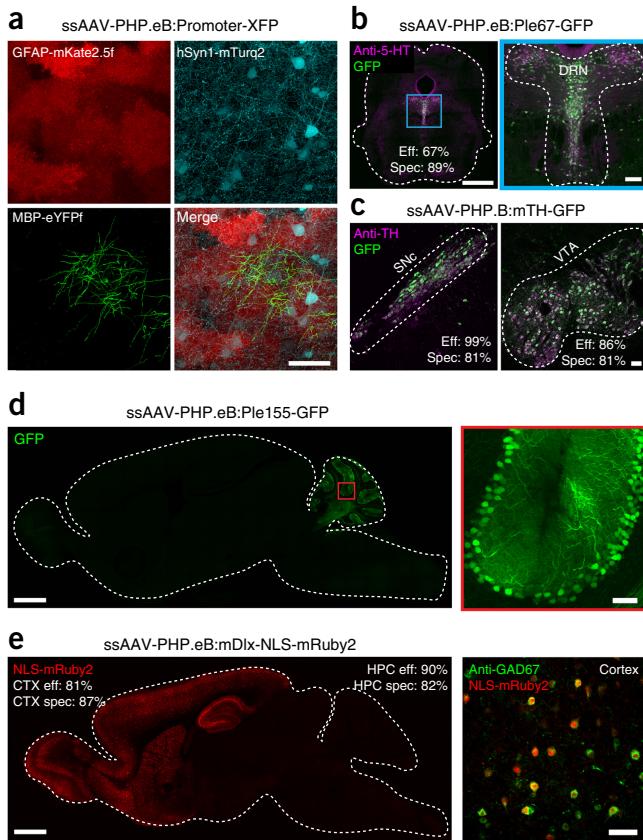
**Figure 4** AAV-PHP.eB and AAV-PHP.S transfer multiple genomes per cell and enable tunable multicolor labeling. **(a)** Coadministration of a cocktail of three ssAAV vectors that each express one of three XFPs (top, schematic) from the *SYN1* promoter labels individual cells with a range of hues. Representative images of the cortex (left, AAV-PHP.eB; total dose:  $4 \times 10^{11}$  vg) and the enteric nervous system (right, AAV-PHP.S; total dose:  $1 \times 10^{12}$  vg). The inset highlights the dense network of neurites labeled with a range of hues. **(b,c)** Schematics show one-component (**b**) and tTA-TRE-based two-component systems (**c**). Expression from the TRE-XFP vectors is dependent on cotransduction with a tTA (tet-off) inducer vector that contains a positive feedback loop to increase tTA expression (ssAAV-PHP.eB:ihSyn-tTA). **(d)** Representative images using the one-component expression system with a 3XFP cocktail expressed from the CAG promoter at an equal dose per XFP (high: total dose  $2 \times 10^{11}$  vg; low: total dose  $1 \times 10^{10}$  vg). **(e)** Representative images using the two-component system:  $1 \times 10^{12}$  total of the TRE-3XFP cocktail and a high ( $1 \times 10^{11}$  vg) or low ( $1 \times 10^{10}$  vg) dose of the tTA inducer. **(f)** Percentages of individual cells that express one, two or all three XFPs. **(g)** MIP (left) of a coronal section from the olfactory bulb of a *Tbx21-Cre* mouse transduced with a cocktail of ssAAV-PHP.eB:TRE-DIO-3XFP ( $1 \times 10^{12}$  vg total dose) and ssAAV-PHP.eB:ihSyn-tTA ( $1 \times 10^{10}$  vg). The morphologies of local neuronal dendritic arbors were traced with neuTube and imported into Imaris v8.3 for three-dimensional rendering (middle) and overlay (right). Scale bars in **a,d,e** are 50  $\mu$ m. **(d,e)** Images are MIPs of 40  $\mu$ m z-stacks. The total volume in **g** is 0.042 mm<sup>3</sup> with xy dimensions of 800  $\times$  425  $\mu$ m. All images were acquired on tissue extracted 3 weeks after intravenous AAV delivery in adult mice, except in **a** where the expression was assessed after 11 d.

allow cell-type-specific expression using FLEX/DIO vectors and the extensive collection of Cre transgenic animals available (for example, from the Jackson Laboratory, the Allen Brain Institute (ABI) and the Gene Expression Nervous System Atlas (GENSAT)).

The tTA-TRE based system relies on coadministration of three separate vectors with XFP expression driven by a TRE containing promoter, along with an adjustable dose of an AAV-tTA expression vector. To evaluate this approach, we coadministered ssAAV-TRE-mNeonGreen along with a ssAAV-CAG-tTA vector. We delivered ssAAV-TRE-mNeonGreen at the same dose across all animals while varying the dose of the ssAAV-CAG-tTA inducer. We observed a dose-dependent reduction in the density of cells expressing mNeonGreen 2 weeks after intravenous delivery of the two vectors (Supplementary Fig. 6). Similar results were achieved with the two-component FLPo-FRT system (Supplementary Fig. 5). Although both systems are viable, we focused on the tTA-TRE system because (i) the short (417 bp) TRE promoter is compatible with longer transgenes and (ii) the positive-feedback loop in the AAV-tTA vector

provides high levels of expression (Supplementary Fig. 7). However, care should be taken with long-term use of the tTA-TRE overexpression system to avoid the potential toxicity seen with overexpression of the tTA<sup>31</sup>.

We next sought to test the tTA-TRE system with multicolor labeling. Using the same 3XFP cocktail described above (Fig. 4a), we generated three ssAAV-TRE-XFP genomes and delivered the vectors at a high dose to all animals, along with either  $1 \times 10^{11}$  or  $1 \times 10^{10}$  vg per animal of a neuron-specific inducer vector (ssAAV-PHP.eB:hSyn1-tTA). Three weeks after intravenous administration we observed robust and inducer dose-dependent labeling (Fig. 4e). Even at the lower inducer dose, and in contrast with the single-component expression system (Fig. 4d), many cells expressed more than one of the three XFP transgenes. We quantified the fraction of cells that contained each XFP and found that, with the two-component system at a low inducer dose, at least 42% of the labeled cells in the cortex, striatum and the Purkinje cell population expressed all three XFPs. In comparison, in the one-component system, less than 10% of the labeled cells in the



**Figure 5** AAV-PHP.eB can be used with gene regulatory elements to achieve cell-type-restricted gene expression throughout the brain. (a) Images show cotransduction of the cortex 3 weeks after coadministration of three vectors with three different promoters driving distinct XFPs (intravenous injection of AAV-PHP.eB at  $1 \times 10^{12}$  vg per viral vector). (b) A vector providing GFP expression driven by an *FEV* (serotonergic-neuron-specific) promoter (ssAAV-PHP.eB:Ple67-GFP) was intravenously delivered at  $1 \times 10^{12}$  vg and colocalized to serotonin (5-hydroxytryptamine, 5-HT, magenta)-expressing cells in the dorsal raphe nucleus (DRN) outlined in blue and expanded for detail (right). (c) A vector providing GFP expression from a mouse tyrosine hydroxylase (*Th*) promoter (ssAAV-PHP.B:mTH-GFP) was intravenously injected at  $1 \times 10^{12}$  vg, and imaging with immunohistochemistry for *Th* (magenta) was performed after 2 weeks of expression. Images outline the substantia nigra pars compacta (SNC, left) and ventral tegmental area (VTA, right). (d) A vector with a Purkinje-cell-selective promoter (Ple155, *PCP2*) driving GFP (ssAAV-PHP.eB:Ple155-GFP) was intravenously injected at  $1 \times 10^{12}$  vg and expression was examined at 4 weeks. A whole sagittal section (left) shows native GFP fluorescence (green) in the cerebellum (left) in cells with the morphology of Purkinje cells (see higher resolution of the red boxed region, right). (e) A vector with a *Dlx5/6* forebrain GABAergic-interneuron-specific enhancer driving nuclear mRuby2 (AAV-PHP.eB:mDlx-NLS-mRuby2) was intravenously injected at  $3 \times 10^{11}$  vg and expression was examined at 8 weeks (CTX: cortex, HPC: hippocampus). Native mRuby2 expression is seen in the forebrain (red, left). Colocalization was assessed by immunohistochemistry for GABAergic cells (GAD67+, green, right). The scale bars in a, b (right), c, d (right) and e (right) are 50  $\mu$ m. The scale bars in b (left), d (left) and e (left) are 1 mm. All panels are confocal images of native XFP fluorescence. The efficiency (eff) and specificity (spec) values for transduction by *FEV* (Ple67), *Th* and *Dlx5/6* vectors are giving in the DRN, VTA, SNC, cortex (CTX) and hippocampus (HPC).

cortex and 1% of cells in the striatum and the Purkinje cell population expressed all three XFPs (Fig. 4f). Finally, we used the two-component system to trace mitral cells in the olfactory bulb of an adult Tbx21-Cre

mouse, a transgenic line that labels mitral and tufted cells in the olfactory bulb. Three weeks after intravenous administration of  $1 \times 10^{12}$  vg per animal of a cocktail of TRE-DIO-XFPs and  $1 \times 10^{10}$  vg per animal of AAV-ihSyn1-tTA, a positive-feedback inducible tTA driven by the human *SYN1* promoter, we observed multicolor labeling at a density that facilitated segmentation of several local dendritic arbors after tissue clearing (Fig. 4g). We call this approach vector-assisted spectral tracing (VAST).

#### A versatile AAV toolbox with cell-type-specific promoters

A further advantage of systemic AAV administration is that it provides a means to transduce populations of specific cell types even if the cells are distributed broadly. As a proof of concept that AAV-PHP.eB can be used to achieve cell-type-restricted expression across the brain, we coadministered three ssAAV genomes that each expressed a spectrally distinct XFP controlled by a different cell-type-specific promoter. In this way, we were able to independently express distinct transgenes in neurons (*SYN1*-driven), astrocytes (*GFAP*-driven) and oligodendrocytes (*Mbp*-driven) within the same animal (Fig. 5a). We also packaged AAV vectors containing previously published promoters that restrict reporter expression to catecholaminergic cells (mouse tyrosine hydroxylase; *Th*), serotonergic cells (*FEV* promoter; Pleiades (Ple) Promoter Project 67 (Ple67)<sup>32</sup>), Purkinje cells (*PCP2* promoter; Ple155)<sup>32</sup> or GABAergic interneurons within the forebrain (mouse distal-less homeobox enhancer; *Dlx5/6*)<sup>33</sup> and administered these intravenously (Fig. 5b–e). We provide quantification of the specificity and efficiency of reporter transduction using these gene regulatory elements in **Supplementary Table 2**.

Similarly, to achieve cell-type-restricted expression within the PNS, we used AAV-PHP.S to coadminister the 3XFP cocktail under the control of the human *SYN1* promoter (ssAAV-PHP.S:hSyn1-3XFP) or by expressing the 3XFP cocktail from the strong synthetic CAG promoter in a double-floxed inverted open reading frame (ssAAV-PHP.S:CAG-DIO-3XFP) for expression in cholinergic neurons when used with ChAT-IRES-Cre transgenic mice (Supplementary Fig. 8). Using Cre-dependent VAST, we were able to sparsely label cholinergic neurons within the myenteric plexus in the proximal colon and trace the processes of multiple neurons for several millimeters (Supplementary Fig. 8). In **Supplementary Table 3**, we summarize the available constructs presented here, along with their applications in this paper.

#### DISCUSSION

Using the previously described CREATE<sup>20</sup> selection method, we have developed and characterized two new capsids, AAV-PHP.eB and AAV-PHP.S, that enable efficient and noninvasive gene delivery throughout the CNS or PNS and visceral organs, respectively (Fig. 1 and Supplementary Fig. 4). As in our earlier study<sup>20</sup>, these variants were identified after only two rounds of *in vivo* selection, further demonstrating how CREATE can be used to identify AAV capsids with enhanced transduction properties.

Previously, we selected for AAV capsids that more efficiently crossed the blood-brain barrier and transduced GFAP-expressing astrocytes. Several of the identified variants, most notably AAV-PHP.B, also transduced neurons, although in general not with the same efficiency. Here we sought to improve neuronal transduction while maintaining strong astrocytic transduction by performing parallel selections for capsids that transduced two widely distributed glutamatergic and GABAergic neuronal populations, as well as astrocytes. This led to the development of the AAV-PHP.eB capsid, a variant that was consistently enriched through selections in each of the three Cre lines. Remarkably, AAV-PHP.eB differs from AAV-PHP.B at only two

amino acids adjacent to the original heptamer insertion, yet at the relatively low systemic dose of  $1 \times 10^{11}$  vg per mouse it transduced the majority of neurons in the cortex and striatum, as well as over 75% of cerebellar Purkinje cells. The success of this approach suggests that the continued evolution of the AAV-PHP.B-based capsid using CREATE in multiple Cre lines is a viable strategy for improving transduction of other cell types, such as oligodendrocytes, while maintaining the overall CNS tropism.

We also describe the AAV-PHP.S capsid, which we identified by selecting for capsids that transduce GFAP-expressing cells. Although the mechanism underlying the selection of this variant is not clear, its discovery is nevertheless important; there have to our knowledge been no reports of recombinant vectors capable of efficient, widespread transduction of peripheral neurons via systemic delivery in adult animals. Consequently, studies of PNS circuitry are arguably lagging behind those in the CNS. Many peripheral neuronal populations are challenging to access surgically, especially in small rodents, owing to their location or broad distribution. For example, the DRG are a key target for gene therapy or genetic manipulation in the study of pain<sup>12,34</sup>. Our finding that AAV-PHP.S transduces at least 80% of DRG neurons should enable high-coverage delivery of transgenes to many DRG. More broadly, AAV-PHP.S also transduces neurons in the enteric nervous system and in other peripheral ganglia, such as the cardiac ganglia, and could provide system-wide gene delivery to these populations, or to subsets of these populations when used with Cre transgenic lines or cell-type-specific promoters. Encouragingly, several recent studies have demonstrated that the tools developed for studying the brain can also be applied to probe the function and projections of specific cell types in the PNS<sup>11,13,14</sup>. The importance of anatomically and functionally mapping the PNS has been recognized by the recent NIH initiative for Stimulating Peripheral Activity to Relieve Conditions (SPARC), and the tropism of AAV-PHP.S may bring about new strategies to modulate PNS circuits through genetic tools such as optogenetics<sup>4</sup> or chemogenetics<sup>5</sup>. For example, systemic Flp- and Cre-dependent effectors delivered via AAV-PHP.S to Cre transgenic animals or used in combination with circuit-specific retrograde Cre viruses should allow circuit-based interrogation of PNS function and enable the types of experiments now routinely conducted in the CNS<sup>4</sup>.

The improved transduction efficiency we observed from both AAV-PHP.eB and AAV-PHP.S relative to their parental capsids was apparent from both the increased number of transduced cells and the increased expression level per cell (Figs. 2 and 3 and Supplementary Fig. 1). The higher expression in individual cells likely results from an increased mean number of vector genomes per cell, as supported by the high frequency of cells expressing multiple XFPs (Fig. 4a). This suggests that, for applications that do not require high expression levels per cell, the viral doses could be lowered from those used in this study. Lowering the viral load used for systemic gene delivery will reduce cost and production burden<sup>1</sup> and minimize the risk for adverse reactions to the viral components<sup>35</sup>.

As AAV vector engineering efforts continue to improve the efficiency of transduction of specific organs or cell populations<sup>36</sup>, widespread gene transfer will become a more viable alternative to the development of germline transgenics. To facilitate studies in nontransgenic animals, it will be necessary to develop more vehicles with improved transduction efficiency and specificity and to identify gene regulatory elements that can be used to restrict expression to defined populations. Finding short enhancers that provide the expected restriction in expression has proven challenging<sup>37</sup>, but more recent efforts<sup>32</sup> are expanding the collection of enhancer-promoters

available. Here we evaluated several gene regulatory elements and found that it was possible to achieve expression that was largely restricted to the expected cell types. If the separate vector components are translatable across species, vector-based genetic manipulations of specific cell types may become more feasible in genetically intractable organisms. Notably, intravenously administered AAV-PHP.B provided improved transduction of the rat CNS, relative to AAV9 (ref. 38). Recently, a *Dlx5/6* enhancer was found to drive expression that was largely restricted to GABAergic neurons in the telencephalon of a panel of vertebrate species<sup>33</sup>. Here, we further validate the *Dlx5/6* enhancer vector for forebrain-wide GABAergic neuron expression when delivered using intravenous AAV-PHP.eB (Fig. 5e).

Cell morphology both informs and determines cell function<sup>39,40</sup>, and many tools have been developed for morphology and connectivity applications<sup>41,42</sup>, especially in the brain<sup>43,44</sup>. To enable tracing of cellular processes, sparse labeling of cells has been a key enabling technology, whether this comes from restricted gene or dye delivery (for example, single-cell electroporation<sup>45,46</sup>) or from stochastic multicolor labeling and color-channel separation<sup>25,43,44</sup>. Both viral and transgenic approaches have been employed to address this problem. Although transgenic mice with sparse gene expression<sup>47,48</sup> are useful, AAVs<sup>25</sup> provide a versatile platform that can be quickly customized with up-to-date tools, promoters and recombinase-dependent expression control systems. Furthermore, vectors can be delivered through different routes or at a specific dose to control the location and fraction of cells labeled. For these reasons, we developed the AAV-based VAST expression system that allows for sparse multicolor labeling. VAST uses two AAV components: a cocktail of XFP encoding vectors that exist in the off state until induced by a second AAV vector that can be delivered at an empirically determined dose to provide expression in the desired fraction of cotransduced cells. We demonstrate that VAST is well suited to the use of systemically delivered AAV-PHP.eB and AAV-PHP.S to achieve relatively uniform and sparse expression in the CNS and PNS. In addition, decoupling the relationship between the labeling density and the expression level may have further applications. For example, sparse, high-level expression could be beneficial for *in vivo* monitoring of neural activity with genetically encoded activity indicators by lowering the background fluorescence during wide-field imaging<sup>21,49</sup> or large-area two-photon imaging<sup>50</sup>. Furthermore, a similar framework could be applied to the understanding of how neuronal circuitry and morphology change during normal and diseased states. Future applications of this two-component approach could also be used to achieve intersectional gene expression in defined populations through circuit-specific delivery of one or both components or through the use of unique capsid-promoter or capsid-capsid combinations that serve to restrict expression to more specific populations. Overall, the vector resources we present here expand the AAV toolbox for the genetic modification of neurons within the central and peripheral nervous systems.

## METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the [online version of the paper](#).

*Note:* Any Supplementary Information and Source Data files are available in the [online version of the paper](#).

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#### AUTHOR CONTRIBUTIONS

K.Y.C. and B.E.D. designed and performed experiments, imaged samples and analyzed data. K.Y.C. prepared figures with input from B.E.D. and V.G. M.J.J. analyzed data, prepared figures and assisted with experiments and in manuscript preparation. B.B.Y. assisted with tissue processing, imaging and virus production. A.G. helped with image analysis. N.R. assisted with molecular cloning. W.-L.W. and L.S.-G. assisted in tissue processing. C.L. and S.K.M. assisted in experimental designs. K.Y.C., B.E.D. and V.G. wrote the manuscript with support from all authors. B.E.D. and V.G. conceived the project. V.G. supervised all aspects of the work.

#### COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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## ONLINE METHODS

**Animals.** All procedures were approved by the California Institute of Technology Institutional Animal Care and Use Committee (IACUC). Mice were purchased from Jackson Laboratory (JAX): C57Bl/6J (000664), GFAP-Cre (012886)<sup>51</sup>, Vgat-IRES-Cre (016962)<sup>52</sup>, Vglut2-IRES-Cre (016963)<sup>52</sup> and Tbx21-Cre (024507)<sup>53</sup>. Male or female mice were used. Mice were randomly assigned to groups of predetermined sample size. All experiments with direct comparisons were performed in parallel to minimize variability.

**In vivo selection.** The AAV-PHP.eB capsid was identified by selecting for AAV-PHP.B variants that transduced cells in Vglut2-IRES-Cre, Vgat-IRES-Cre and GFAP-Cre mice through two rounds of selection as previously described<sup>20</sup>. Intravenous administration of AAV vectors was performed by injection into the retro-orbital sinus of adult mice (6–8 weeks of age). The AAV-PHP.B capsid was modified by partial randomization of the original AAV-PHP.B heptamer insertion and four flanking amino acids (AAV-PHP.B amino acid positions 587–597). The AAV-PHP.B-based library was generated by PCR using primer XF<sup>20</sup> and a mixture of five primers identical to the AAV-PHP.B template sequence with the exception of mixed bases at three consecutive randomized codons (NNK). In each primer mix, the position of the three randomized NNK codons was shifted by 6 bp (2 codons) such that each position in the heptamer and the two amino acids on each side of the heptamer amino acids were randomized, three amino acid at a time, in an overlapping manner. An equal amount of each of the five PCR products was assembled into the AAV-Cap-in-cis loxP acceptor genome using the NEB Hi-Fi DNA Assembly Master Mix (New England Biolabs E2621) and used to generate the AAV-PHP.B-based AAV library. AAV-PHPS was generated from an insertion of random heptamers between the 588 and 589 sites of AAV9 and was identified from two rounds of *in vivo* selection in GFAP-Cre mice<sup>20</sup>.

**Plasmids.** The backbones of all AAV plasmids that are double inverted orientation (DIO) were based on pAAV-Ef1a-DIO EYFP (Addgene, 27056), and all non-DIO plasmids were based on pAAV-Ef1a-Cre (Addgene, 55636). The backbone of pAAV-CAG-fDIO-mNeonGreen was based on pAAV-Ef1a-fDIO EYFP (Addgene, 55641). The fluorescent protein cDNAs for mTurquoise2 (ref. 54), mNeonGreen<sup>55</sup>, mRuby2 (ref. 56) or mKate2.5 were synthesized as gBlocks (Integrated DNA Technologies). The MBP-eYFP and GFAP-mKate2.5f vectors have a farnesylation sequence attached by overhang PCR. The pAAV-CAG-NLS-GFP vector was modified by PCR to add an N- and a C-terminal NLS and assembled using NEB Hi-Fi DNA Assembly Master Mix (New England Biolabs E2621). The mouse tyrosine hydroxylase (*Th*) promoter (mTH) was based on the 2.5-kb region of a published rat *Th* promoter<sup>57</sup> and myelin basic protein (*Mbp*) promoter<sup>58</sup>. Both were directly cloned from mouse genomic DNA. The human *SYN1* promoter<sup>59</sup> was cloned from pAAV-hSyn Con/Foff EYFP (Addgene, 55651). The *GFAP* (GfABC<sub>1</sub>D) promoter was previously described<sup>60</sup> and was cloned upstream of a synthetic intron. The GFAP-mKate2.5 plasmid also contained three tandem copies of sequences complementary to six miRNAs—miR-1, miR-122, miR-375, miR-196a, miR-743 and miR-10b—inserted between the WPRE and an SV40 pA sequence. The TRE was PCR amplified from a SG-TRE-containing plasmid<sup>29</sup>. The inducible human *SYN1* (ihSyn1) promoter was cloned by overlap PCR with the *SYN1* promoter, and oligonucleotides designed with a synthetic intron and three tetO binding sites. The AAV vectors expressing GFP from the Ple67 and Ple155 promoters were obtained from Addgene (49138 and 49140, respectively). The AAV-mDlx-NLS-mRuby vector was cloned by replacing the GFP reporter in pAAV-mDlx-GFP-Fishell-1 (Addgene, 83900) with a mRuby reporter fused with an N-terminal SV40 NLS.

**Virus production and purification.** AAVs were generated in HEK 293T cells (ATCC) using polyethylenimine<sup>61</sup>. The cell line identity was not validated, nor was it tested for mycoplasma. We harvested viral particles 72 h after transfection from the medium and 120 h after transfection from cells and the medium. Viral particles from the medium were precipitated with 40% polyethylene glycol (Sigma, 89510-1KG-F) in 500 mM NaCl and combined with cell pellets for processing. The cell pellets were suspended in 500 mM NaCl, 40 mM Tris, 2.5 mM MgCl<sub>2</sub>, pH 8, and 100 U/mL of salt-activated nuclease (Arcticyzmes) at 37 °C for 30 min. Afterwards, the cell lysates were clarified by centrifugation at 2,000g and then purified over iodixanol (Optiprep, Sigma; D1556) step gradients (15%, 25%, 40% and 60%)<sup>62</sup>. Viruses were concentrated using Amicon filters (EMD, UFC910024) and formulated in sterile phosphate-buffered saline (PBS). Virus titers were measured by determining the number of DNase I-resistant vg using qPCR using a linearized genome plasmid as a standard<sup>61</sup>.

**Tissue preparation, immunohistochemistry and imaging.** Intravenous administration of AAV vectors was performed by injection into the retro-orbital sinus of adult mice (6–8 weeks of age). After allowing time for expression (3 weeks unless otherwise noted in Results), mice were anesthetized with Euthasol (Virbac) and transcardially perfused first with 0.1 M phosphate buffer (pH 7.4) at room temperature and then with freshly prepared, ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The tissues were postfixed in 4% PFA overnight and afterwards transferred to PBS with 0.05% sodium azide. The brains, lungs and liver were sectioned at 100 µm and the spinal cord at 300 µm, using a Leica VT1200 vibratome. Immunohistochemistry was performed using 10% normal donkey serum (NDS), 0.1% Triton X-100 (except that no detergent was used with GAD67 staining) and 0.05% sodium azide. Primary antibodies used were rabbit calbindin (1:500; Abcam, ab11426), mouse NeuN (1:500; EMD, MAB377), rabbit S100 (1:500; Abcam ab868), rabbit tyrosine hydroxylase (1:500; Abcam, ab112), goat 5-HT (1:200; Immunostar, 20079), rabbit PGP9.5 (1:500; Abcam, ab10404) and mouse GAD67 (1:250; EMD, MAB5406). Sections were incubated with primary antibodies in blocking solution (PBS with 10% NDS and 0.05% sodium azide) for 16–24 h at room temperature and then washed in PBS overnight, followed by secondary antibody incubation (Jackson ImmunoResearch, 711-166-152, 711-606-152, 715-166-150, or 715-606-150) for 16–24 h at room temperature.

DRG were postfixed in 4% PFA at 4 °C overnight and then cryopreserved in 30% sucrose at 4 °C overnight. Tissues were then embedded in OCT (Tissue-Tek, Torrance, CA, USA), frozen in 2-methybutane on dry ice and stored at -80 °C until sectioning. For cryosectioning, the DRG were cut at a thickness of 16 µm using a Leica CM3050 and adhered to a Superfrost Plus microscope slide (Fisher Scientific, Tustin, CA, USA). Slides were stored at -80 °C until staining. For DRG immunohistochemistry, the slides were incubated in rabbit anti-PGP9.5 antibody (1:250, Abcam, ab10404) in blocking solution (10% horse serum, 0.1% Triton X-100 and 0.02% sodium azide in PBS) overnight at room temperature. The next day, sections were incubated with fluorescence-conjugated secondary antibody for 1 h at room temperature (Jackson ImmunoResearch, 711-606-152). Between each step, sections were thoroughly washed with PBS. ProLong Gold anti-fade mounting medium (P36941; Molecular Probe, Life Technologies, Carlsbad, CA, USA) was applied to the slide before coverslip mounting.

The small intestines and colons were postfixed in 4% PFA at 4 °C overnight. Then the duodenum, jejunum, ileum and proximal colon were sectioned and blocked in PBS with 0.1% Triton X-100, 3% bovine serum albumin and 0.01% sodium azide overnight at room temperature. The next day, the tissue was incubated with mouse-on-mouse blocking reagent (Vector Laboratories, MKB-2213) in PBS with 0.01% sodium azide at room temperature for 16 h. Afterwards, the tissue was stained in the initial blocking solution with guinea pig anti-PGP9.5 antibody (1:100, Abcam, ab10410) and rabbit anti-s100β antibody (1:200, Abcam, ab52642) for 48 h and then washed in PBS with 0.01% sodium azide overnight. The secondary antibodies—goat anti-guinea pig Alexa 647 (A-21450) and goat anti-rabbit Alexa 555 (A-21428) from Molecular Probes (Life Technologies, Carlsbad, CA, USA)—were incubated in the initial blocking solution for 48 h. Unbound antibodies were then washed out with PBS with 0.01% sodium azide overnight. Finally, samples were incubated in the refractive matching solution (RIMS)<sup>63</sup> for 6 h before imaging.

For **Figure 4b,g**, samples were made transparent and mounted using ScaleS4 (ref. 64). For **Figures 4a,d** and **5c** and **Supplementary Figures 5a,b** and **6b,c**, samples were made transparent and mounted using RIMS. All other samples were mounted with Prolong Diamond Antifade (ThermoFisher, P36965).

For some images, the 16-bit green channel (GFP) gamma was adjusted to enable visualization of low- and high-expressing cells while avoiding oversaturation. In all cases, changes to contrast or gamma as well as microscope laser settings remained constant across sets of images that were directly compared. Images were acquired on a Zeiss LSM 780 confocal microscope fitted with the following objectives: Fluar 5× 0.25 M27 Plan-Apochromat 10× 0.45 M27 (working distance 2.0 mm) and Plan-Apochromat 25× 0.8 Imm Corr DIC M27 multi-immersion or Zeiss Axiozoom V16 with a 0.7× objective fitted with a Hamamatsu c11440 camera.

**Image processing and data analysis.** All image processing was performed using Zen Black 2012, Adobe Photoshop and Illustrator CC, ImageJ, neuTube build 1.0z2016 and Bitplane Imaris 8.3, and custom-made scripts in Matlab. For data analysis, Microsoft Excel 2016, Matlab, Python and GraphPad Prism 7.01 were used.

To minimize bias, when direct comparisons for quantification (Figs. 2d,e and 3b,c) or direct qualitative comparisons (Fig. 3a,d and Supplementary Figs. 3a,b and 4a–c) were made, matched regions were selected for imaging by first viewing the cell-type-specific immunostaining channel, rather than the GFP expression. Manual cell counting in the striatum and cortex was performed by a blinded observer (Fig. 2e). Automated counting was performed for Purkinje cells in the cerebellum (Fig. 2e).

To mitigate any cross-talk between the three fluorescent proteins, linear unmixing was used (Fig. 4d,e). For a given imaging condition, the expected cross-talk between the three fluorophores was evaluated on the basis of the emission and excitation spectra of each fluorophore. Then, for each pixel in the RGB image, the corresponding unmixed signals were calculated using a non-negative least squares fitting.

Cell profiling was done using Imaris (Bitplane). To detect cell bodies from the image data, we manually analyzed cells in the cortex and striatum by drawing a region of interest (ROI) around each cell's nucleus and intensity values were exported into Microsoft Excel and GraphPad Prism for analysis. For analysis of the cerebellum, we developed a computational pipeline to facilitate the automation of the whole procedure, since the highly organized layout of Purkinje cells allowed high-accuracy automated counting (the true positive rate was 88.0% and the positive predictive value was 95.5% relative to manual detection).

To detect neuronal cell bodies from the image data (Supplementary Fig. 2), the custom Matlab code “GFPcount\_from\_cerebellum.m” was used and is provided in the **Supplementary Software**. In short, we applied a circular Hough transform to the maximum projection of the image stack. Prior to this process, morphological filtering with a circular structural element was performed with the images to remove thin fibrous processes. Each circular region detected from the images was considered a single cell body. To measure the ratio of GFP<sup>+</sup> cells and their intensity in the cerebellum, we applied the abovementioned cell-body detection method to the calbindin channel to detect the somata of Purkinje cells and used this to define ROIs in the GFP channel. We then subtracted the background from each ROI to exclude the expression (or leakage) of GFPs in cytosol. The background was estimated by applying a two-dimensional Gaussian smoothing kernel to each cell body image. Subsequently, the image was converted to binary and the convex hull of the connected object therein was calculated. Objects whose areas were smaller than 10% of the cell body were removed, and we considered the largest object to be the nucleus of interest if there were more than one object. Nuclei with a mean intensity higher than 0.1 (with the intensity rescaled to the range of 0–1) was counted as GFP<sup>+</sup>. The mean intensity of the nucleus was calculated by averaging the pixel values inside the object (Supplementary Fig. 1).

The pipeline for automated cell body detection was also applied to other analyses (Fig. 4f and Supplementary Fig. 7). To measure the density of labeled cells in the sparse-labeling system (Supplementary Fig. 6), the custom Matlab code “cell\_density.m” was used and is provided in the **Supplementary Software**. In short, we applied the cell body detection method to the images and measured the density of detected cells by dividing the cell number by the area of the image. We also examined the distance between the neighboring cells; for each cell, all of the distances to other cells were calculated and the minimum was selected as the distance to the closest neighbor. The values for all cells were then averaged for each region and condition. We used the same method to determine whether each cell expressed one, two or three XPFs (Fig. 4f) in the one- and two-component labeling systems by running custom Matlab code, “expressed\_channel\_count.m,” that can be found in the **Supplementary Software**. After detecting each cell body, we collected all the normalized intensity values therein from each channel. If the mean intensity of the channel was above 0.1, we considered this gene (color) to be expressed in the cell. Counting the number of expressed channels in each cell, we calculated the fraction of cells according to the number of expressed gene types.

To determine the mean GFP intensity (Figs. 2d and 3b), the automated cell body detection pipeline above was used for calbindin<sup>+</sup> cells in the cerebellum and PGP9.5<sup>+</sup> cells in the DRG, along with custom Python code “drg\_intensity.

ipynb” that can be found in the **Supplementary Software**. Manual ROIs were drawn onto DAPI<sup>+</sup> nuclei in the cortex and striatum using Bitplane Imaris 8.3, with the GFP channel turned off to minimize bias. The median fluorescence per ROI was determined and the average across all cells within each replicate were reported as the mean GFP intensity.

**Statistics.** Microsoft Excel 2016 and GraphPad Prism 7.01 were used for data analysis and graph generation. For the comparison between AAV9 and AAV-PHP.S ( $n = 5$  per group) and AAV-PHP.B and AAV-PHP.eB ( $n = 5$  per group), the animal group size was chosen on the basis of preliminary data that suggested a large effect size. One animal from each of the AAV-PHP.B and AAV-PHP.eB groups was excluded from data analysis after necropsy owing to failed retro-orbital injections. For Figure 1c–g, representative images were chosen from AAV9 ( $n = 5$ ), AAV-PHP.S ( $n = 5$ ), AAV-PHP.B ( $n = 4$ ) and AAV-PHP.eB ( $n = 4$ ). For Figures 2d,e and 3b,c, each dot is representative of one animal with three technical replicates averaged per animal. For Supplementary Figure 1a–c, each line represents one animal with three technical replicates per animal. For Supplementary Figure 6c,d, each dot represents a technical replicate for each of the three doses. For Figures 2d,e and 3b,c, unpaired, two-tailed *t*-tests were performed. Error bars show s.e.m. Data distribution was assumed to be normal, but this was not formally tested. For Supplementary Figure 1a–c, statistical significance was assessed with a Kolmogorov–Smirnov test. For Supplementary Figure 6c,d, means were compared by *t*-tests in which the *P* value is computed by fewest assumptions and did not assume consistent s.d. Statistical significance was determined by the Holm–Sidak method to correct for multiple comparisons with an  $\alpha = 0.05$ . A **Supplementary Methods Checklist** is available.

**Data and code availability.** Cell detection algorithms used in this study are available through <https://github.com/GradiaruLab/ChanNN17>. Custom Matlab code packages “GFPcount\_from\_cerebellum.m”, “cell\_density.m” and “expressed\_channel\_count.m” and custom Python code “drg\_intensity.ipynb” are provided in **Supplementary Software**. The data that support the findings of this study are available from the corresponding authors upon reasonable request. All tools and protocols are available through Addgene or the Beckman Institute for CLARITY, Optogenetics and Vector Engineering Research for technology development and broad dissemination: <http://www.beckmaninstitute.caltech.edu/clover.shtml>.

**Accession codes.** GenBank: AAV-PHP.B, KU056473; AAV-PHP.eB: MF187357, AAV-PHP.S: MF187356.

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