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Laminar specificity and coverage of viral-mediated gene expression restricted to GABAergic interneurons and their parvalbumin subclass in marmoset primary visual cortex

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Abstract

In the mammalian neocortex, inhibition is important for dynamically balancing excitation and shaping the response properties of cells and circuits. The various computational functions of inhibition are thought to be mediated by different inhibitory neuron types of which a large diversity exists in several species. Current understanding of the function and connectivity of distinct inhibitory neuron types has mainly derived from studies in transgenic mice. However, it is unknown whether knowledge gained from mouse studies applies to the non-human primate, the model system closest to humans. The lack of viral tools to selectively access inhibitory neuron types has been a major impediment to studying their function in the primate. Here, we have thoroughly validated and characterized several recently-developed viral vectors designed to restrict transgene expression to GABAergic cells or their parvalbumin (PV) subtype, and identified two types that show high specificity and efficiency in marmoset V1. We show that in marmoset V1 AAV-h56D induces transgene expression in GABAergic cells with up to 91-94% specificity and 79% efficiency, but this depends on viral serotype and cortical layer. AAV-PHP.eB-S5E2 induces transgene expression in PV cells across all cortical layers with up to 98% specificity and 86-90% efficiency, depending on layer. Thus, these viral vectors are promising tools for studying GABA and PV cell function and connectivity in the primate cortex.

eLife assessment

Unlocking the potential of molecular genetic tools (optogenetics, chemogenetics, sensors, etc.) for the study of systems neuroscience in nonhuman primates requires the development of effective regulatory elements for cell-type specific expression to facilitate circuit dissection. This study provides a **valuable** building block, by carefully characterizing the laminar expression profile of two optogenetic enhancers, one designed for general GABA+ergic neurons (h56D) and the second (S5E2) for parvalbumin+ cell-type selective expression in the marmoset primary visual cortex. This study contributes **solid** evidence to our understanding of these tools but is limited by the understandably small number of animals used.

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Introduction

The computations performed by the neocortex result from the activity of neural circuits composed of glutamatergic excitatory and GABAergic inhibitory neurons. Although representing only 15-30% of all cortical neurons, inhibitory neurons profoundly influence cortical computations and cortical dynamics. For example, they influence how excitatory neurons integrate information, shape neuronal tuning properties, modulate neuronal responses based on sensory context and behavioral state, and maintain an appropriate dynamic range of cortical excitation (Ferster and Miller, 2000; Shapley et al., 2003; Tremblay et al., 2016). These various functions of inhibition are thought to be mediated by different inhibitory neuron types, of which a large diversity has been identified in several species, each having distinct chemical, electrophysiological and morphological properties (Ascoli et al., 2008; Burkhalter, 2008; Kubota et al., 2016).

In mouse cortex, the expression of specific molecular markers identifies three major, largely non-overlapping classes of inhibitory neurons: parvalbumin-(PV), somatostatin-(SOM), and serotonin receptor (5HT3aR, a larger class which includes vasoactive intestinal peptide or VIP cells)-expressing neurons (Xu et al., 2010; Rudy et al., 2011). The creation of mouse lines selectively expressing Cre-recombinase in specific inhibitory neuron classes has led to a multitude of studies on the connectivity and function of each class (Tremblay et al., 2016; Wood et al., 2017; Shin and Adesnik, 2023). Distinct patterns of connectivity and function specific to each inhibitory neuron class are emerging from these mouse studies, but it remains unknown whether insights gained from mouse apply to inhibitory neurons in higher species such as primates. Understanding cortical inhibitory neuron function in the primate is critical for understanding cortical function and dysfunction in the model system closest to humans, where cortical inhibitory neuron dysfunction has been implicated in many neurological and psychiatric disorders, such as epilepsy, schizophrenia and Alzheimer's disease (Cheah et al., 2012; Verret et al., 2012; Mukherjee et al., 2019).

A major impediment to studying inhibitory neuron function in primates has been the lack of tools for cell-type specific expression of transgenes in this species. However, recent advances in the application to primates of cell-type specific viral technology are beginning to enable studies of inhibitory neuron types in primate cortex. In particular, two recent studies have developed specific promoters or enhancers that restrict transgene expression from recombinant adeno-associated viral vectors (AAV) to GABAergic neurons, specifically the *mDlx* enhancer

(Dimidschstein et al., 2016) and the *h56D* promoter (Mehta et al., 2019), in both rodents and primates. Viral strategies to restrict gene expression to PV neurons have also been recently developed (Mehta et al., 2019; Vormstein-Schneider et al., 2020; Mich et al., 2021).

To facilitate the application of these inhibitory-neuron specific viral vectors to studies of the primate cortex, we have performed a thorough validation and characterization of the laminar expression of reporter proteins mediated by several enhancer/promoter-specific AAVs. Here we report results from the two vector types that have shown the greatest specificity of transgene expression in marmoset primary visual cortex (V1); specifically, we have tested three serotypes of the *h56D* promoter-AAV that restricts gene expression to GABAergic neurons (Mehta et al., 2019), and one serotype of the S5E2 enhancer-AAV that restricts gene expression to PV cells (Vormstein-Schneider et al., 2020). Using injections of these viral vectors in marmoset V1, combined with immunohistochemical identification of GABA and PV neurons, we find that the laminar distribution of reporter protein expression mediated by the GABA- and PV-enhancer AAVs validated in this study resembles the laminar distribution of GABA-immunoreactive (GABA+) and PV-immunoreactive (PV+) cells, respectively, in marmoset V1. Reporter protein expression mediated by the h56D-AAV is specific and robust, but the degree of specificity and coverage depends on serotype and cortical layer. We found that about 92% of PV cells in marmoset V1 are GABA+, and reporter protein expression mediated by the S5E2-AAV shows up to 98% specificity and 86-90% coverage, depending on layer. We conclude that these viral vectors offer the possibility of studying GABAergic and PV neuron connectivity and function in primate cortex.

Results

We validated 3 serotypes (1,7,9) of a pAAV-h56D-tdTomato (Mehta et al., 2019), and the AAV-PHP.eB-S5E2.tdTomato (Vormstein-Schneider et al., 2020). We report results from 10 viral injections, of which 3 injections of AAV-h56D-tdTomato, and 7 injections of AAV-PHP.eB-S5E2.tdTomato, made in 4 marmoset monkeys (see Methods and **Supplementary Table 1**). Tissue sections through V1 were double immunoreacted for GABA and PV and imaged on a fluorescent microscope. We quantified the laminar distribution of viral-induced tdTomato (tdT) expression as well as of GABA+ and PV+ cells revealed by immunohistochemistry (IHC), and counted double and triple-labeled cells to determine the specificity and coverage of viral-induced tdT expression across marmoset V1 layers (see Methods).

V1 laminar distribution of GABA+ and PV+ neurons

We first determined the V1 laminar distribution of GABA+ and PV+ neurons identified by IHC (**Fig. 1**). To this goal, in each section used for analysis, we counted GABA+ and PV+ cells within 2×100μm-wide regions of interest (ROIs) spanning all layers in dorsal V1 anterior to the posterior pole, for a total of 6 ROIs in 3 tissue sections selected randomly. Cortical layer boundaries were determined using DAPI and/or PV staining (**Fig. 1B-C**), as we found that PV-IHC reveals laminar boundaries consistent with those defined by DAPI.

The laminar distribution of GABA+ and PV+ cells was quantified as percent of total GABA+ or PV+ cells (**Fig. 2A**), as well as cell density (number of cells per unit area; **Fig. 2B**), in each cortical layer. Both GABA+ and PV+ cell percent and density peaked in layers (L) 2/3 and 4C. There was no significant difference in the percent or density of GABA+ cells in L2/3 (33%±2, and 1,294 cells/mm^2±74.4, respectively) vs. L4C (33.4%±3.1, and 1,052 cells/mm^2±42.2, respectively), as determined by a Bonferroni-corrected Kruskall-Wallis test (p=1.00, n=6 ROIs across 3 sections, L2/3 vs L4C in **Fig. 2A**) or by a Bonferroni-corrected ANOVA test (p=1.00, n=6 ROIs across 3 sections, L2/3 vs L4C in **Fig. 2B**). Similarly, the percent and density of PV+ cells in L4C (42.3%±3.6, and 888 cells/mm^2±38.8, respectively) were not significantly different from those in L2/3 (31.3%±2.5 and 812 cells/mm^2±65, respectively; p=1.00 for both comparisons, n=6 ROIs). However, density of PV+

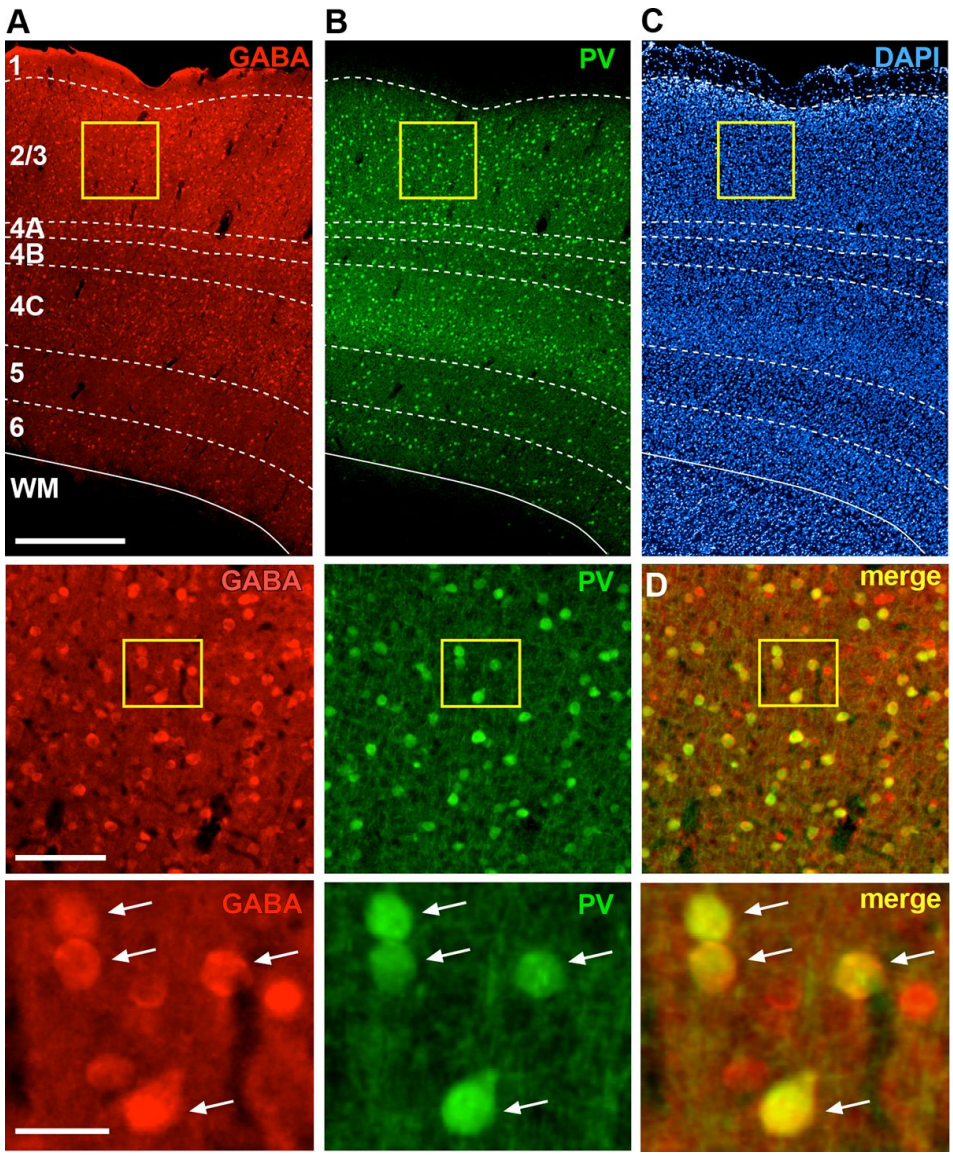

Figure 1.

Laminar expression of GABA and PV immunoreactivity in marmoset V1. Epifluorescence images of the same V1 section triple-stained for GABA-(red channel) and PV-(green channel) IHC and DAPI (blue channel), showing individual and merged channels. (A) GABA+ expression through all cortical layers (Top). *Dashed contours* mark layer boundaries; *solid contour* marks the bottom of the cortex. Cortical layers are indicated. Scale bar: 500 μm (valid for the top panels in A-C). Middle: V1region inside the *yellow box* in (A) shown at higher magnification. The cells inside the *yellow box* are shown at higher magnification in the bottom panel. Scale bar: 100μm (valid for the middle panels in A-B and the top panel in D). Bottom: scale bar: 25 μm (valid for the bottom panels in A-C). (B) Same as in (A) but for PV+ expression. (C) DAPI stain used to reveal cortical layers. (D) Merge of red (GABA) and green (PV) channels shown in the respective panels to the left. *Arrows* point to double-labeled cells.

cells in L4C was significantly higher than in all remaining layers (p=0.0280.05 for 4C vs. 4A/B, and <0.001 for 4C vs. all other layers; ANOVA test with Bonferroni correction; n= 6 ROIs), and L2/3 PV+ density was significantly higher than L1, 5 and 6 ($p=5.93E^{-11}$ for L2/3 vs. L1 and p= 0.00037 L2/3 vs. L6, p=0.002 for L2/3 vs. L5, n=6 ROIs), but not L4A/B (although percent of PV+ cells in L2/3 was significantly higher than in L4A/B; p=0.029 Bonferroni corrected Kruskall-Wallis-test, n=6 ROIs). GABA+ cell density in L2/3 was significantly higher than in all other layers except L4C (p=0.028 vs. L4A/B and p=0.013 vs L6, p=0.007 vs. L1 and p=0.005 vs. L5). GABA density in L4C did not differ from any other layers, but the percent of GABA+ cells in L4C was significantly higher than in L1 (p=0.009) and 4A/B (p=0.000022). As expected, within each layer, GABA+ cell density was significantly higher than PV+ cell density (p<0.05, one-sided t-test for equality of means, n=6 ROIs).

We compared the laminar distributions of GABA+ and PV+ cell density in marmoset V1 with previously published distributions of these two cell markers in mouse V1 (Xu et al., 2010). In marmoset V1, there is an overall higher density of PV+ cells than in mouse V1, with density peaking in L2/3 and 4C. In contrast, PV+ cell density in mouse V1 peaks in L4 and 5, and density in L2/3 is lower than in all other layers (**Fig. 2C**). GABA+ cell density in marmoset V1 peaks in L2/3 followed by 4C, whereas it peaks in L4 and 5 with a smaller third peak in L2/3 in mouse V1 (**Fig. 2D**).

Counts of cells double labeled for GABA+ and PV+ revealed that 92.3%±1.9 of PV+ cells across all layers were GABA+, and that PV+ cells represent on average 61.4%±2.7 of all GABA+ cells, ranging from 54.5%±3.7 in L6 to 78.5%±5.6 in L4C (**Fig. 2E**). This differs from mouse V1 in which PV+ cells represent about 40% of all GABA cells (Xu et al., 2010).

Laminar specificity and coverage of GABA-specific AAV-h56D

Figure 3 shows tdT expression at three injection sites, each of a different serotype (9,7,1) of the GABA-specific AAV-h56D-tdT. Identical injection volumes of each serotype, delivered at 3 different cortical depths (see Methods), resulted in viral expression regions that differed in both size as well as laminar distribution, suggesting the different serotypes have different capacity of infecting cortical neurons and layers. The AAV7 injection resulted in the smallest expression region, which additionally was biased to the superficial and deep layers, with only few cells expressing tdT in the middle layers (**Fig. 3B**). AAV9 (**Fig. 3A**) and AAV1 (**Fig. 3C**) resulted in larger expression regions which involved all cortical layers. Given identical volumes and titers used for the AAV9 and AAV7 injections (injected volume of the AAV1 was the same but titer was higher-see **Supplementary Table 1**), as well as identical post-injection survival times for all 3 serotypes, the differences in the size of the expression region are like due to different tropism and/or viral spread of the different serotypes. However, given that we only made a single injection per serotype, we cannot exclude that other factors may have contributed to the reduced spread of the AAV7.

In **Figure 4** we report quantitative counts for each serotype. Below we describe these results, but acknowledge that differences we observed between serotypes need to be interpreted with caution, given they are based on a single injection per serotype. **Figure 4A** compares quantitatively tdT expression obtained with each viral serotype, quantified as the percent of total tdT+ cells in each layer for each serotype, with the percent laminar distribution of GABA+ cells identified by IHC. Serotypes 9 and 1 overall showed similar laminar distribution as GABA+ IHC, the percent of tdT+ cells peaking in L2/3 and 4C, suggesting good specificity of viral infection (the laminar distributions of AAV9- and AAV1-induced tdT+ cells were not significantly different from the GABA+ cell distribution; p>0.05 for all comparisons, Bonferroni corrected independent-samples Median test, n=4-6 ROIs). In contrast, due to the lower capacity of AAV7 to infect the mid-layers, AAV7-induced tdT expression was relatively higher in L2/3 compared to GABA+ expression, approaching statistical significance (p=0.059; Bonferroni corrected independent-samples Median test, n=4 AAV7 ROIs and 6 GABA-IHC ROIs). The percent of AAV7-infected cells was also significantly higher than the percent of AAV9- and/or AAV1-infected cells in L2/3 (p=0.028 for both

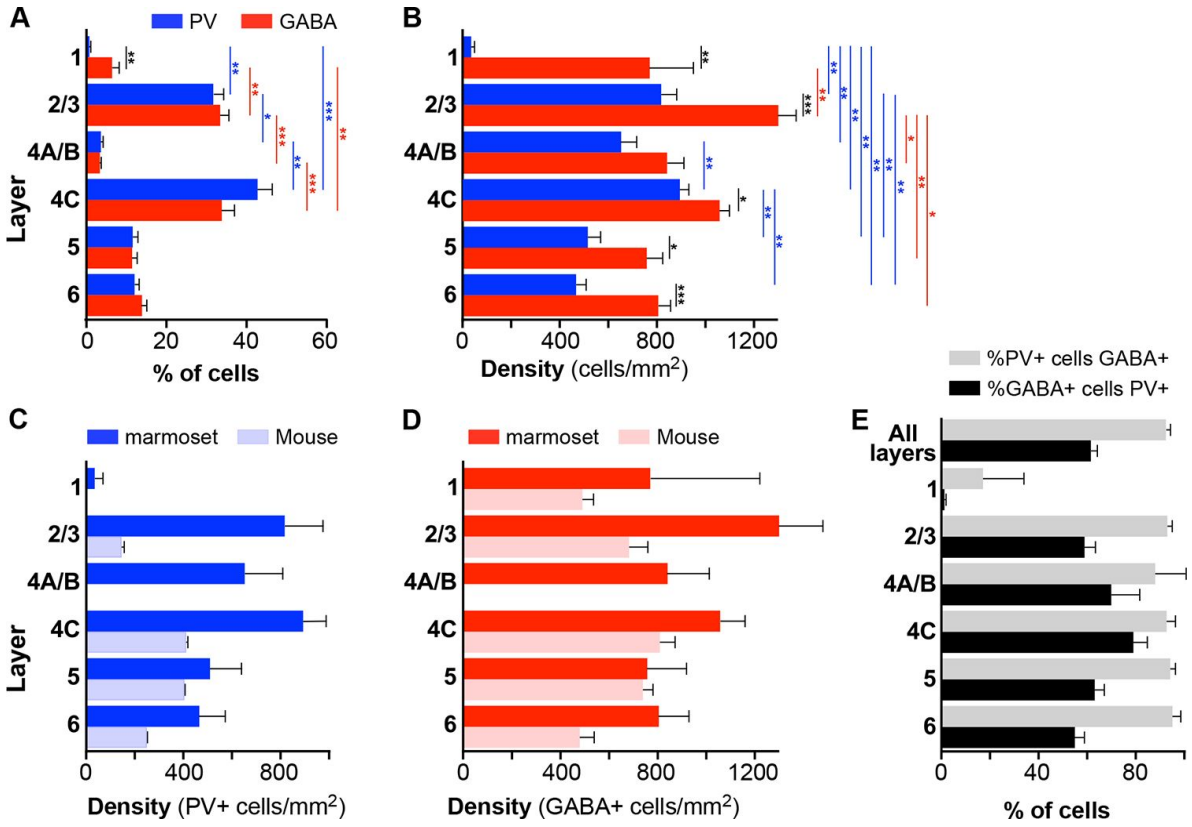

Figure 2.

Laminar distribution of GABA+ and PV+ cells in marmoset V1 **(A)** Average percent of total number of GABA+ (*red*) or PV+ (*blue*) cells in each layer. Here and in (B,E) error bars represent standard error of the mean (s.e.m.) across ROIs (n=6 ROIs in A,B,E). In all panels *asterisks* indicate statistical significance (* <0.05, **<0.01, ***<0.001). **(B)** Mean density of GABA+ and PV+ cells in each layer. **(C)** Mean density of PV+ cells in marmoset (*dark blue*) and mouse (*light blue*) V1. Here and in (D), mouse data are from Xu et al. (2010), error bars represent the standard deviation, and n=4-6 ROIs for mouse and 6 ROIs for marmoset. **(D)** Mean density of GABA+ cells in marmoset (*red*) and mouse (*pink*) V1. **(E)** Average percent of all counted PV+ cells that were double-labeled for GABA (*gray*), and average percent of all counted GABA+ cells that were double-labeled for PV+ (*black*) are shown at the top of the histogram. The percentages for each layer are shown underneath.

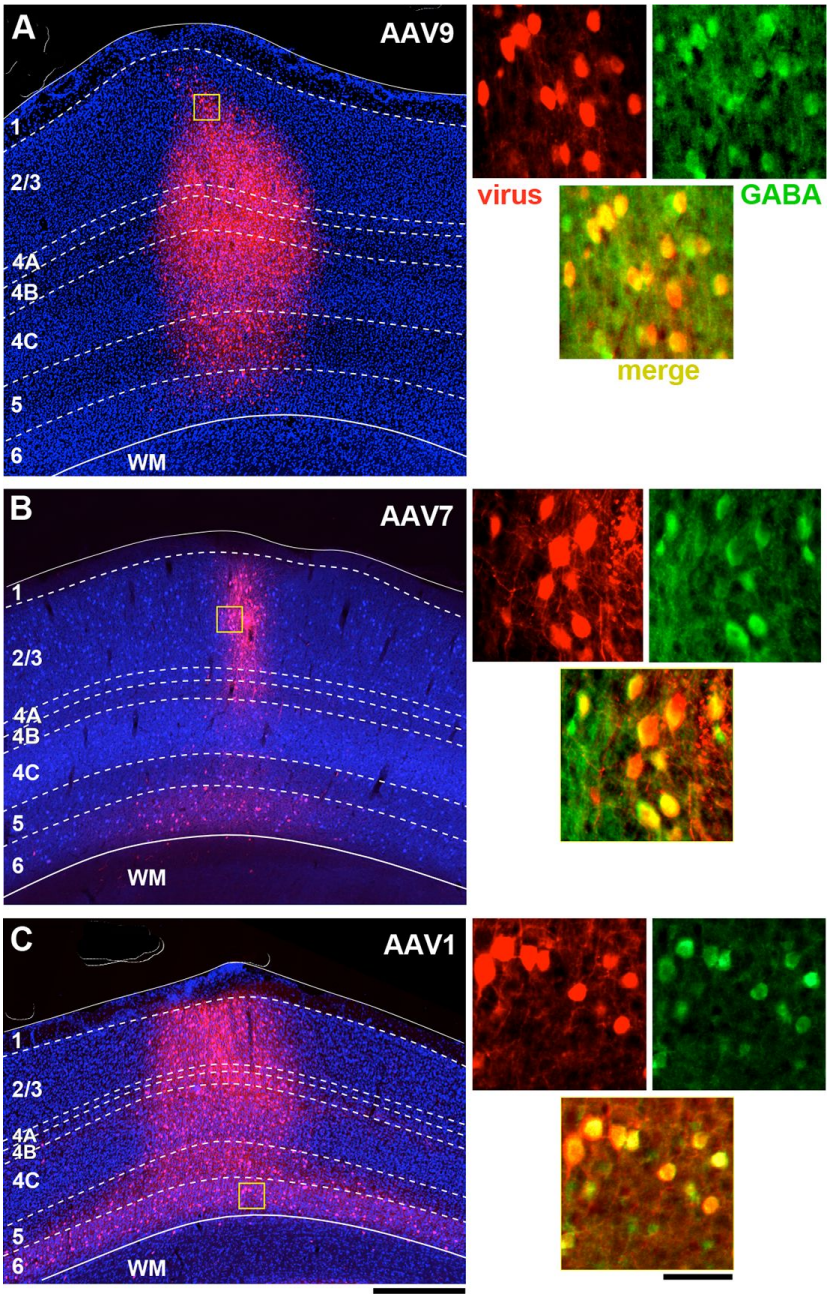

Figure 3.

Laminar profile of pAAV-h56D-mediated tdT expression in marmoset V1 **(A-C) Left**: tdT expression (*red*) across V1 layers (indicated) following injection of an identical volume of AAV-h56D-tdT serotype 9 (A), serotype 7 (B) and serotype 1 (C). The viral titers for the AAV9 and AAV7 injections were also the same, while titer was higher for AAV1 (see **Supplementary Table 1**). The tdT expression region in panel A is a merge of two adjacent sections, because the tdT expression region did not encompass all layers in individual sections. TdT expression in other panels, instead, is from a single section. *Dashed contours* mark layer boundaries; *solid contours* mark the top and bottom of the cortex. Layers were identified based on DAPI counterstain (*blue).* Note that the cortical thickness varies across cases because these sections are from different regions of V1. *Yellow box* in each panel is the region shown at higher magnification on the right. Scale bar: 500μm (valid for A-C). **Right:** Higher magnification of the V1 region inside the yellow box in each respective left panel, showing individual channels (*red:* viral-mediated tdT expression; *green:* GABA+ IHC) and the merge of these two channels (*yellow*). Scale bar: 50μm (valid for A-C).

comparisons) and in L6 (p=0.028 for AAV7 vs. AAV1), and significantly lower than the percent of AAV9- and AAV1-infected cells in L4C (p=0.028 for both comparisons; Bonferroni corrected independent-samples Median test, n=4 ROIs).

To quantify the specificity of tdT expression induced by each serotype, i.e. the accuracy in inducing tdT expression selectively in GABA cells, for each serotype separately we measured the percent of tdT-expressing cells that colocalized with GABA expression revealed by IHC (**Fig. 4B**). Overall, across all layers, AAV9 showed the highest specificity (82.3%±1.1) followed by AAV7 (79.2%±5.4), and AAV1 (75.3%±2.6), and there was a statistically significant difference in overall specificity between AAV9 and AAV1 (p=0.014; Bonferroni corrected independent-samples Median test, n=4 ROIs for each serotype). The specificity of AAV9 did not differ significantly across layers, ranging from 80.4%±21 in L4C to 93.8%±6.3 in L4A-B. In contrast, specificity for the other two serotypes varied by layer. AAV7 showed highest specificity in L1 (100% but there were only 2 tdT+ cells in this layer), L4C (90.1%±5.9) and L6 (87.1%%±8.1), and lowest in L4A/B (50%±35.4). AAV1 specificity was highest in L6 (85.4%±8.8) and L4C (81.6%±1.4) and lowest in LA-B (38.3%±21.7). There was a tendency for AAV9 to be more specific than AAV1 in L4A-B (93.8±6.3 vs 38.3±21.7), and L5 (88.8%±6.6 vs 59.4±8.3) but these differences did not reach statistical significance.

To quantify the efficiency of the virus in inducing tdT expression in GABA cells, for each serotype separately we measured the viral coverage as the the percent of GABA+ cells within the viral injection site that colocalized with tdT expression (**Fig. 4C**). Overall, across all layers, AAV9 and AAV1 showed significantly higher coverage (66.1±3.9 and 64.9%±3.7) than AAV7, which showed much lower coverage values (34%±5.6; p=0.014 for both comparisons; Bonferroni corrected independent-samples Median test, n=4 ROIs across 2 sections for each serotype). AAV9 and AAV1 coverage was similar across layers, and both showed slightly higher coverage in superficial (AAV9: 67.4%±2.5; AAV1: 69%±6.5) and middle layers (AAV9: 78.5%±9.1; AAV1: 76.9%±7.4), compared to deep layers (AAV9:50-55%, AAV1: 44-60%). Instead, AAV7 showed very low coverage values in L4A-B (8.3%±8.3) and L4C (14.4%±6.7) and highest values in L6 (47.9%±4.3) followed by L2/3 (44.6%±9). AAV7 coverage was significantly lower than AAV9 coverage in L2/3 (p=0.014), L4A/B (p=0.014) and L4C (p=0.014), and was significantly lower than AAV1 in L4C (p= 0.014; Bonferroni corrected independent-samples Median test, n=4 ROIs for each serotype). Thus, our results suggest that AAV9 is the serotype of choice for marmoset studies of GABAergic neurons requiring highest specificity and coverage across all layers, but AAV7 may be a better choice for studies intending to restrict transgene expression to L6 or L2/3 GABA cells with good specificity.

Laminar specificity and coverage of PV-specific AAV-PHP.eB-S5E2

We assessed the laminar specificity and coverage of the AAV-PHP.eB-S5E2-tdT following injections of different viral volumes ranging from 90 nl to 585 nl (see **Supplementary Table 1**). **Figure 5** shows fluorescent images of tdT expression at the site of viral injection for an example 105 nl injection (**Fig. 5A**) and an example 315 nl injection (**Fig. 5B**).

Figure 6A compares tdT expression resulting from injections of different volumes, quantified as the percent of total tdT+ cells in each layer for each volume, with the percent laminar distribution of PV+ cells identified by IHC. Cell counts from injections of 315-585nl volumes were pooled as injections ≥315nl produced similar results (see **Supplementary Table 2**). We found that the distribution of tdT expression resulting from all injection volumes did not differ significantly from the distribution of PV+ IHC, all distributions similarly peaking in L2/3 and 4C (p>0.1 for all comparisons; Bonferroni corrected Kruskall-Wallis test; n=8-12 PV-AAV ROIs, and 6 PV-IHC ROIs), suggesting good viral specificity.

The specificity of tdT expression induced by different injection volumes is quantified in **Figure 6B**, separately for 3 groups of injection volumes: group 1= 90-105nl, group 2= 180 nl, group 3= 315-585nl. Results from individual injection cases are reported in **Supplementary Table 2**. The degree of viral specificity was high at all volumes, but depended slightly on injection volume.

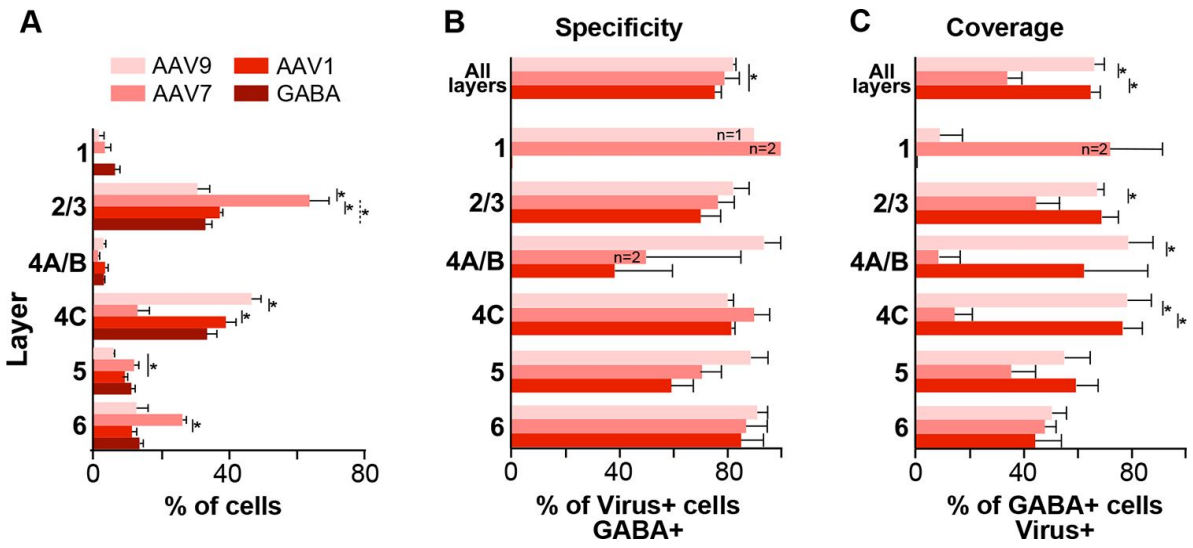

Figure 4.

Laminar distribution, specificity, and coverage of tdT expression induced by 3 different serotypes of pAAV-h56D. **(A)** Average percent of total number of GABA immunoreactive cells, and average percent of total number of tdT-expressing cells after injections of 3 different serotypes of the GABA-AAV vector, in each V1 layer. **(B)** Specificity of tdT expression induced by each serotype across all layers and in each layer. Specificity is defined as the percent of viral-mediated tdT expressing cells that colocalize with GABA immunoreactivity. **(C)** Coverage of each viral serotype across all layers and in each layer, defined as percent of GABA immunoreactive cells that co-express tdT. In all panels, error bars represent s.e.m. across ROIs (n= 4 for AAV9, 4 for AAV7, 4 for AAV1, 6 for GABA-IHC), and *asterisks* indicate statistically significant differences at the $p<0.05$ level.

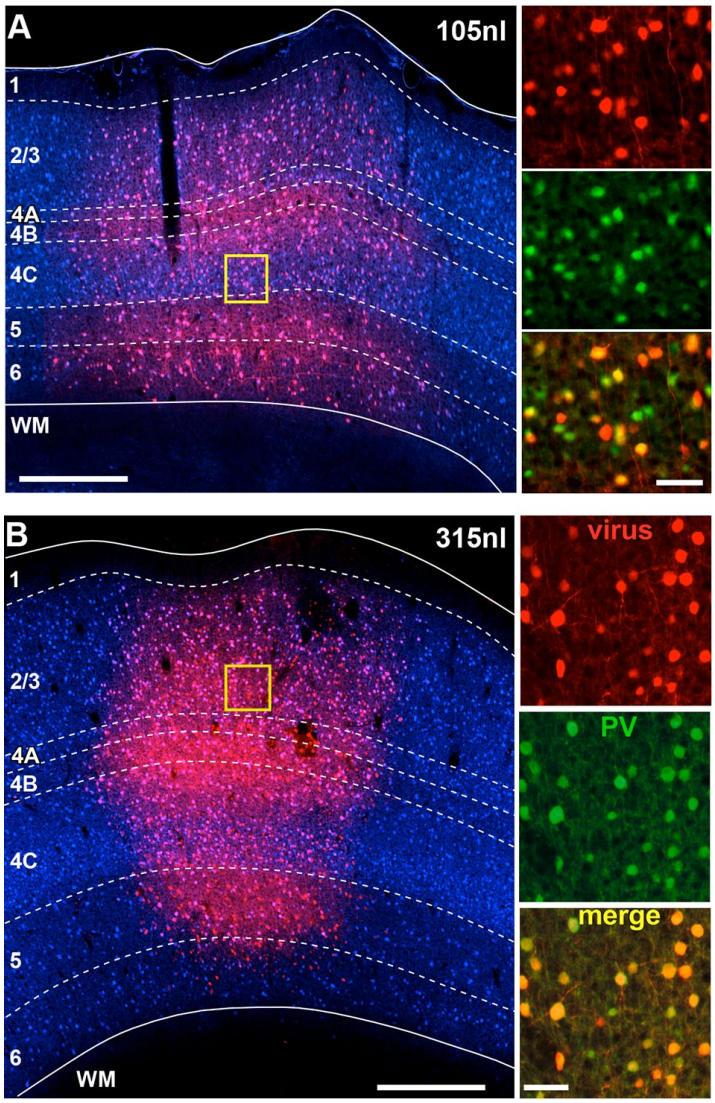

Figure 5.

Laminar profile of AAV-PHP.eB-S5E2-mediated tdT expression in marmoset V1. **(A) Left:** tdT expression (*red*) across V1 layers following an injection of 105 nl volume of AAV-PHP.eB-S5E2-tdT. *Dashed contours* mark layer boundaries; *solid contours* mark the top and bottom of the cortex. Layers were identified based on DAPI counterstain (*blue). Yellow box* is the region shown at higher magnification in the right panels. Scale bar here and in the left panel in (B): 500μm. **Right:** Higher magnification of the V1 region inside the yellow box in the left panel, showing individual channels (*red:* viral-mediated tdT expression; *green:* PV+ IHC) and the merge of these two channels (*yellow*). Scale bar here and in the right panels in (B): 50μm. **(B)** Same as in (A) but for an injection volume of 315nl.

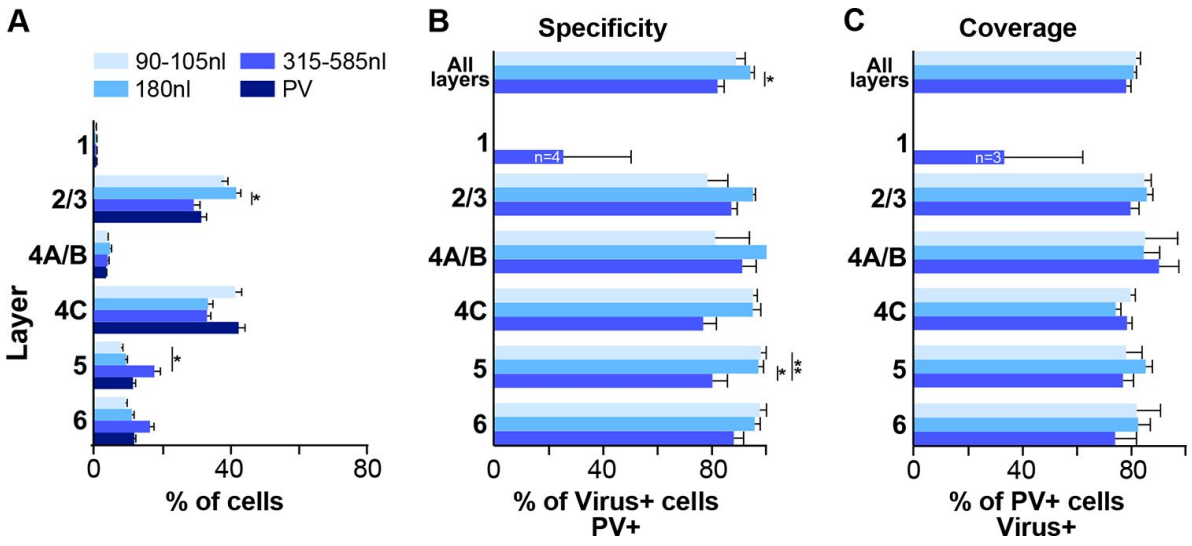

Figure 6.

Laminar distribution, specificity, and coverage of tdT expression induced by 3 different injection volumes of AAV-PHP.eB-S5E2. (A) Average percent of total number of PV immunoreactive cells, and average percent of total number of tdT-expressing cells after injections of 3 different volumes of the PV-AAV vector, in each V1 layer. (B) Specificity of tdT expression induced by each injection volume across all layers and in each layer. (C) Coverage of each viral injection volume across all layers and in each layer. In all panels, error bars represent s.e.m. across ROIs (n= 8 for 90-105nl, 8 for180 nl, 12 for 315-585nl PV-AAV injection volumes and 6 for PV-IHC), and *asterisks* indicate statistically significant differences.

Overall, across all layers, group 2 (180nl) showed the highest specificity (94.7%±1.6), which differed significantly from the specificity of group 3 volumes (≥315nl; 82%±3.2; p=0.01, Bonferroni corrected Kruskall-Wallis test, n=8-12 ROIs). Specificity was similar across layers for all volumes, but volumes ≥315nl resulted in slightly lower specificity than smaller volumes in L4C (76.6%±5.6 for >315nl volumes vs 95.2%±1.7 and 94.9%±3 for 90-105nl and 180nl volumes, respectively) and L5 (80.1±5.8 for ≥315nl vs 97.9%±2.1 and 97%±1.9 for 90-105 and 180nl, respectively), and these differences in L5 were statistically significant (p=0.013 and 0.005; Bonferroni corrected Kruskall-Wallis test; n=8-12 ROIs).

The viral coverage resulting from each injection volume is quantified in **Figure 6C** separately for the three different volume groups, and shown for each individual injection case in **Supplementary Table 2**. Coverage of the AAV-PHP.eB-S5E2-tdT was high, did not depend on injection volume, and it was similar across layers for all volumes. Overall, across all layers coverage ranged from 78%±1.9 to 81.6%±1.8.

Reduced GABA and PV immunoreactivity at the viral injection site

Qualitative observations of tissue sections seemed to indicate slightly reduced expression of both GABA and PV immunoreactivity at the viral injection sites, extending beyond the borders of the injection core (**Supplementary Fig. 1**). To quantify this observation, we counted GABA+ and PV+ cells at the site of the viral injections (n=12 ROIs across 6 sections for AAV-h56D injection sites (pooled across serotypes), and 28 ROIs across 14 sections for AAV-S5E2 injection sites) and at sites located several millimeters beyond the viral injection borders (n=6 ROIs across 3 sections). We found that both the number and density of GABA+ and PV+ cells were reduced across all layers at the site of the AAV-h56D (**Fig. 7A-D**) and AAV-S5E2 (**Fig. 7E-H**) injections compared to control tissue away from the injection sites. The magnitude of the reduction in immunoreactivity depended on the viral type, with the AAV-h56D virus inducing an overall greater and more significant reduction in GABA immunoreactivity (28.1% and 21.5% % reduction in mean GABA+ cell number and density across all layers, respectively, p=0.024 in **Fig. 7A**, and p=0.013 in **Fig. 7B**, Mann Whitney U test) than in PV immunoreactivity (20.5% and 10.2% reduction in mean PV cell number and density across all layers, respectively; p=0.041 in **Fig. 7C**, and p=0.125 in **Fig. 7D**, Mann Whitney U test) and vice versa for the AAV-S5E2 virus, which reduced PV immunoreactivity (33.3% and 25.4% reduction in mean PV+ cell number and density across all layers, respectively, p<0.001 in **Fig. 7G**, and p=0.013 in **Fig. 7H**) more than GABA immunoreactivity (27.4% and 20.2% reduction in mean GABA+ cell number and density across all layers, respectively, p=0.005 in **Fig. 7E and p**=0.042 in **Fig. 7F**). The reduced GABA and PV immunoreactivity caused by the viruses imply that the specificity of the viruses we have validated in this study is likely higher than estimated in **Figs. 4**,**6**. Moreover, reduced GABA and PV immunoreactivity could at least partly underlie the apparent reduction in specificity observed for larger PV-AAV injection volumes (see Discussion).

Discussion

Understanding the connectivity and function of inhibitory neurons and their subtypes in primate cortex requires the development of viral tools that allow for specific and robust transgene expression in these cell types. Recently, several enhancer and promoter elements have been identified that allow to selectively and efficiently restrict gene expression from AAVs to GABAergic neurons and their subtypes across several species, but a thorough validation and characterization of these enhancer-AAVs in primate cortex has lacked. In particular, previous studies have not characterized the specificity and coverage of these vectors across cortical layers. In this study, we have characterized two main enhancer-AAV vectors designed to restrict expression to GABAergic cells or their PV subtypes, which show high specificity and coverage in marmoset V1. Specifically, we have shown that the GABA-specific AAV9-h56D (Mehta et al., 2019) induces transgene

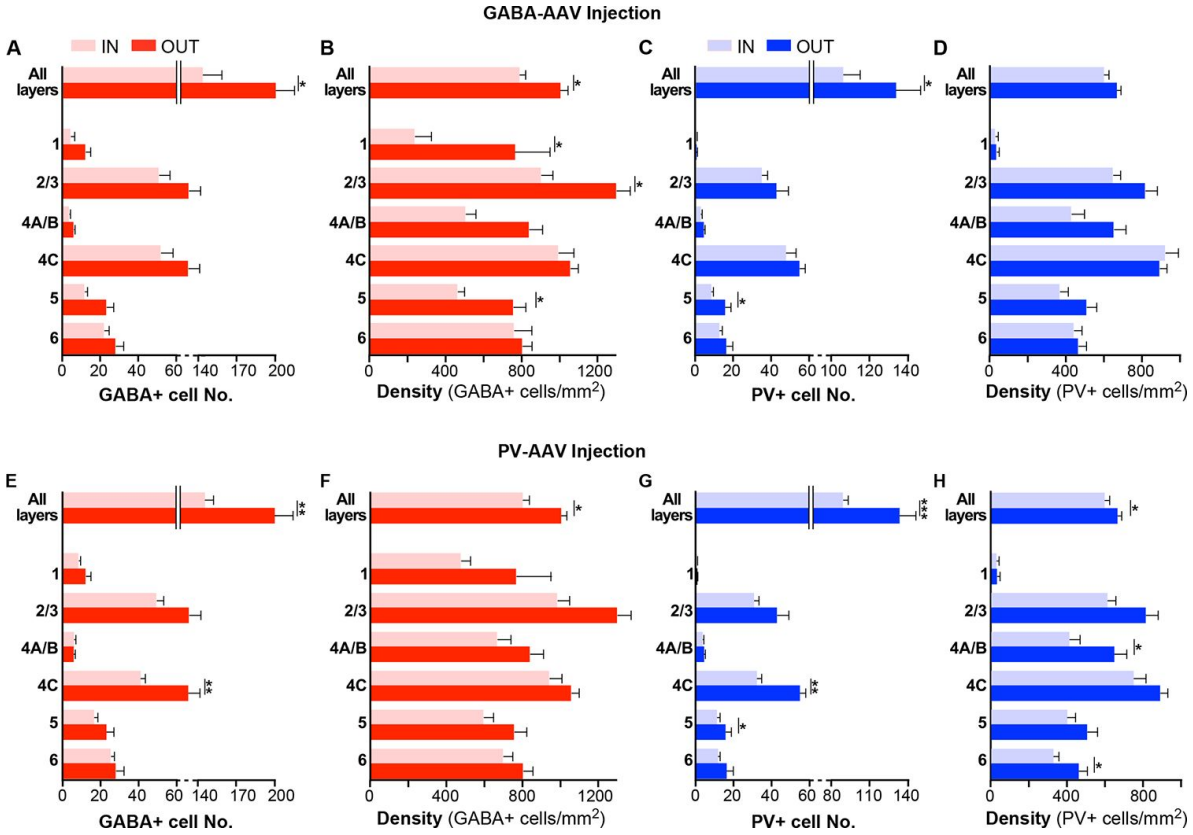

Figure 7.

Reduced GABA and PV immunoreactivity at the viral injection site **(A,B)** Number (A) and density (B) of GABA+ cells inside (*pink*; n=12 ROIs across 6 sections) and outside (*red*; n=6 ROIs across 3 sections) the GABA-AAV injection sites. **(C**,**D)** Number (C) and density (D) of PV+ cells inside (*light blue*; n= 12 ROIs across 6 sections) and outside (*dark blue*; n=6 ROIs across 3 sections) the GABA-AAV injection sites. **(E,F)** Number (E) and density (F) of GABA+ cells inside (*pink*; n=28 ROIs across 14 sections) and outside (*red*; n=6 ROIs across 3 sections) the PV-AAV injection sites. **(G,H)** Number (G) and density (H) of PV+ cells inside (*light blue*; n=28 ROIs across 14 sections) and outside (*dark blue;* n=6 ROIs across 3 sections) the PV-AAV injection sites. Error bars: s.e.m. *Asterisks:* statistically significant comparisons. IN each panel, statistical comparisons across layers were performed using the Bonferroni-corrected Kruskall-Wallis or independent-samples Median tests; comparisons between total IN and OUT populations in each panel were performed using the Mann-Whitney U test.

expression in GABAergic cells with up to 91-94% specificity and 80% coverage, depending on layer, and the PV-specific AAV-PHP.eB-S5E2 (Vormstein-Schneider et al., 2020) induces transgene expression in PV cells with up to 98% specificity and 86-90% coverage, also depending on layer. We conclude that these two viral vector types provide useful tools to study inhibitory neuron connectivity and function in primate cortex.

Many recent studies have investigated the connectivity and function of distinct classes of inhibitory neurons in mouse V1 and other cortical areas (Tremblay et al., 2016; Wood et al., 2017; Shin and Adesnik, 2023). In contrast, similar studies in the primate have been missing due to the lack of tools to selectively express transgenes in specific cell types and the difficulty of performing genetic manipulation in this species. It is important to study inhibitory neuron function in the primate, because it is unclear whether findings in mice apply to higher species, and inhibitory neuron dysfunction in humans has been implicated in several neurological and psychiatric disorders (Marin, 2012; Goldberg and Coulter, 2013; Lewis, 2014). While the basic inhibitory neuron subtypes seem to exist across most mammalian species studied (DeFelipe, 2002) species differences may exist, particularly given the evolutionary distance between mouse and primate. Indeed, species differences have been reported in marker expression patterns (Hof et al., 1999), in the proportion of cortical GABAergic neurons (24-30% in primates vs. 15% in rodents) (Hendry et al., 1987; Beaulieu, 1993), in the proportion of PV neurons (74% in macaque V1 vs. 30-40% in mouse) (van Brederode et al., 1990; DeFelipe et al., 1999; Xu et al., 2010), and in the abundance of the various subtypes (Krienen et al., 2020). Here, we found that PV cells in marmoset V1 across all layers represent on average 61% of all GABAergic cells, and up to 79% in V1 L4C. These percentages are lower than previously reported for macaque V1 by Van Brederode et al. (1990) (74% across all layers and nearly 100% in L4C), but higher than recently reported by Kelly et al. (2019) (52% across all V1 layers, up to 80% in L4C). We also found differences in the V1 laminar expression of both GABA+ and PV+ cells between mouse and marmoset. Specifically, GABA+ and PV+ expression peaks in L2/3 and 4C in marmoset V1, but in L4 and 5 in mouse V1. Similar differences between mouse and primate V1 in the laminar distribution of PV cells were reported previously (Kooijmans et al., 2020; Medalla et al., 2023). Our results on the laminar distribution of PV and GABA immunoreactivity is consistent with previous qualitative and quantitative studies in macaque V1 (Hendry et al., 1989; Blumcke et al., 1990; DeFelipe et al., 1999; Disney and Aoki, 2008; Kelly et al., 2019; Kooijmans et al., 2020; Medalla et al., 2023), and with a quantitative study in marmoset V1(Goodchild and Martin, 1998).

We compared laminar distribution, specificity and coverage of three different serotypes of the AAV-h56D vector. Serotypes 9 and 7 showed slightly greater specificity than serotype 1, and the specificity of AAV9 was more consistent across layers than the specificity of serotypes 7 and 1, which instead varied somewhat across layers. Serotypes 9 and 1 showed greater coverage than serotype 7. Thus, serotype 9 is a better choice when high specificity and coverage across all layers are required. Serotype 7, instead, showed high specificity (80%) but low coverage (34%), except in layer 6 (48%) and L2/3 (45%), therefore, this serotype may be desirable to restrict transgene expression to L6 or 2/3 GABAergic cells. We note that these differences among serotypes should be interpreted with caution as they are based on a single injection per serotype. Despite this, our results demonstrate sufficiently high efficiency and specificity of transgene expression in GABA cells using the h56D promoter, at least with two of the 3 AAV serotypes we tested, warranting their use in the non-human primate.

We compared laminar distribution, specificity and coverage resulting from different volume injections of the AAV-PHP.eB-S5E2 vector. Injections of 180nl volume resulted in higher specificity (95% across layers) and coverage (81% across all layers) than obtained with injection volumes equal to or larger than 315nl (specificity 82% and coverage 78% across all layers), although coverage did not differ significantly across volumes. This mild dose-dependent alteration of specificity could depend on some off-target expression reaching above detection levels at higher

doses. Thus, injection volumes of 150-300nl are recommended for studying PV neuron function and connectivity using this viral vector. Alternatively, or in addition, an apparent dose-dependent reduction in specificity may result from viral-induced suppression of PV immunoreactivity, which could be more pronounced for larger volume injections. Indeed, we found that both GABA- and PV-specific AAVs slightly, but significantly, reduced both GABA and PV immunoreactivity at the site of the viral injection, but GABA expression was more reduced at the AAV-h56D injection site, while reduction in PV expression was more marked at the AAV-S5E2 injection site. This reduction in GABA and PV immunoreactivity at the viral injected sites most likely affected our measurements of specificity, suggesting that specificity for the viruses tested in this study is even higher than revealed by our counts. However, this reduced immunoreactivity raises concerns about the virus or the high level of reporter protein possibly harming the cell physiology. Our data does not allow us to assess the origin of the reduced GABA and PV immunoreactivity. Qualitative observations did not reveal structural damage at the site of the viral injections to suggest cell death. Moreover, we have been able to record the electrophysiological responses of V1 neurons in which opsin protein expression was induced via injections of these viruses (Vafa et al., 2024). Notably, viral-induced downregulation of gene expression in host cells, including of inhibitory neuron marker genes such as *PV*, has been documented for other viruses, such as rabies virus (Prosniak et al., 2001; Zhao et al., 2011; Patino et al., 2022). As such, it is possible that subtle alteration of the cortical circuit upon parenchymal injection of viruses (including AAVs) leads to alteration of activity-dependent expression of *PV* and *GABA*.

Methods

Experimental Design

Enhancer-AAV vectors carrying the gene for the reporter protein tdTomato (tdT) were injected in area V1 of marmoset monkeys. After an appropriate survival time, the animals were euthanized. The brains were processed for histology and immunohistochemistry (IHC) to identify GABA+ and PV+ cells and cortical layers. The laminar distribution of GABA+ and PV+ cells, and of viral-mediated tdT expression was analyzed quantitatively.

Animals

Four female marmosets between the ages of 2 and 8 years old (weight about 500gr), obtained from the University of Utah in-house colony, were used in this study. All procedures were approved by the University of Utah Institutional Animal Care and Use Committee and conformed to the ethical guidelines set forth by the USDA and NIH.

Surgical Procedures

Animals were pre-anesthetized with alfaxalone (10mg/kg, i.m.) and midazolam (0.1mg/kg, i.m.) and an IV catheter was placed in either the saphenous or tail vein. To maintain proper hydration Lactated Ringers solution was continuously infused at 2-4 cc/kg/hr. The animal was then intubated with an endotracheal tube, placed in a stereotaxic apparatus, and artificially ventilated. Anesthesia was maintained with isoflurane (0.5-2.5%) in 100% oxygen. Throughout the experiment, end-tidal CO2, ECG, blood oxygenation, and rectal temperature were monitored continuously.

Under aseptic conditions the scalp was incised and several small (~2mm) craniotomies and durotomies were made over dorsal V1. A single injection of a viral vector was made into each craniotomy. On completion of the injections, each craniotomy was filled with Gelfoam and sealed with dental cement, the skin was sutured, and the animal was recovered from anesthesia. Animals survived 3-4 weeks (one animal survived 2 weeks) post-injections (**Supplementary Table 1**), to

allow for viral expression, and were sacrificed with Beuthanasia (0.22 ml/kg, i.p.) and perfused transcardially with saline for 2-3 minutes, followed by 4% paraformaldehyde in 0.1 M phosphate buffer for 15-20 minutes.

Injection of Viral Vectors

A total of 10 viral injections were made in 4 marmosets (**Supplementary Table 1**). Each of two animals received 1 injection, and one animal 5 injections (3 in one hemisphere and 2 in the other hemisphere) of AAV-PHP.eB-S5E2.tdTomato, obtained from the Dimidschstein laboratory (Vormstein-Schneider et al., 2020). The fourth animal received 3 injections, each of a different AAV serotype (1, 7, and 9) of the AAV-h56D-tdTomato (Mehta et al., 2019), obtained from the Zemelman laboratory (UT Austin). Viral vectors were loaded in glass micropipettes (tip diameter 30-45 µm) and pressure injected using a PicoPump (World Precision Instruments). To ensure viral infection of all cortical layers, each injection was made at 3 depths within the cortical column: 1.2-1.5 mm from the cortical surface (deep), 0.8-1.0 mm (middle), and 0.4-0.6 mm (superficial). After injecting at each depth, the pipette was left in place for 2-4 minutes before being retracted to the next depth, and for 5 minutes before being fully retracted from the brain. The PV-specific AAV was injected at 4 different total volumes: 585 nl (1 injection), 315 nl (2 injections), 180 nl (2 injections) and 90-105 nl (2 injections). The AAV-h56D vectors were each injected at a total volume of 600 nl. One third of each total volume per injection was slowly (6-15 nl/min) injected at each of the 3 depths. For animals that received multiple injections in the same hemisphere, injections were spaced at least 3 mm apart to ensure no overlap. Viral titers and volumes of each injection as well as post-injection survival times for each case are reported in **Supplementary Table 1**.

Viral Preparation

AAV-PHP.eB-S5E2.tdT

Details about AAV-PHP.eB-S5E2.tdTomato cloning and production are provided in the original publication (Vormstein-Schneider et al., 2020). Briefly, the E2 enhancer sequence was amplified from mouse genomic DNA using the primer aatctaacatggctgctata and caattgctcagagttatttt (618 bp). Enhancer, reporter and effector cloning was performed using the Gibson Cloning Assembly Kit (New England BioLabs, catalog no. NEB-E5510S) following standard procedures. Specifically, for AAV-E2-SYP-dTomato, we amplified the SYP–tdTomato coding sequence from the plasmid Addgene no. 34881. The rAAVs were produced using standard production methods. Polyethylenimine was used for transfection and OptiPrep gradient (Sigma) was used for viral particle purification. Titer was estimated by quantitative PCR with primers for the WPRE sequence. The batch used in this study had a titer of 8.3×10^{12} viral genomes/ml.

AAV-h56D.tdT

Details about AAV-h56D.tdTomato cloning and production are provided in the original publication (Mehta et al., 2019). Briefly, Viruses were assembled using a modified helper-free system (Stratagene) as the indicated serotypes (rep/cap). Viruses were purified on sequential cesium gradients according to published methods (Grieger et al., 2006). Titers were measured using a payload-independent qPCR technique (Aurnhammer et al., 2012). Typical titers were 1×10^{13} 1×10^{14} viral genomes/ml.

Histology and Immunohistochemistry

Area V1 was dissected away from the rest of the visual cortex. The block was postfixed for 3-12 hours in 4% paraformaldehyde, sunk in 30% sucrose for cryoprotection, and frozen sectioned in the parasagittal plane at 40µm thickness. In one case (MM423, which received a 315nl injection of AAV-PHP.eB-S5E2.tdTomato), the brain was sunk in a 20% glycerol solution and frozen at −80°C for 6 months prior to being sectioned. To locate the viral injection sites, a 1:5 series of tissue sections

were wet-mounted and observed under microscopic fluorescent illumination. Sections containing each injection site had their coverslips removed, and fluorescent immunohistochemistry (IHC) was performed on free-floating sections to reveal both GABA+ and PV+ neurons. No IHC was performed to enhance reporter proteins signals as these were sufficiently bright. GABA and PV IHC was performed by incubating sections for 3 days at 4°C in primary antibody, followed by 12-hour incubation at room temperature in secondary antibody. The primary and secondary antibodies used for GABA-IHC were a rabbit anti-GABA antibody (1:200; Sigma Aldrich, Burlington, MA; RRID:AB_477652) and an Alexa Fluor® 647 AffiniPure™ Donkey Anti-Rabbit IgG (H+L) (1:200; Jackson ImmunoResearch Laboratoris Inc., West Grove, PA; RRID:AB_2492288), respectively. The primary and secondary antibodies used for PV-IHC were a guinea pig anti-parvalbumin antibody (1:1000; Swant, Burgdorf, Switzerland; RRID:AB_2665495) and an Alexa Fluor® 488 AffiniPure™ Donkey Anti-Guinea Pig IgG (H+L) (1:200; Jackson ImmunoResearch Laboratories Inc.; RRID:AB_2340472), respectively. The sections were then mounted and coverslipped with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, Newark, CA).

Data Analysis

Multi-channel wide-field fluorescent images of V1 tissue sections containing an injection site spanning all layers were acquired at 5-7 depths in the z plane using a Zeiss AxioImager Z2 fluorescent microscope equipped with a 10x objective. Images were stitched, rotated, and cropped as necessary using Zen Blue software (Carl Zeiss AG) and loaded into Neurolucida software (MBF Bioscience) for data quantification. To quantify inhibitory neurons that expressed the viral-mediated reporter protein tdTomato, GABA+ and PV+ neurons revealed by IHC at the viral injection sites (i.e. the data shown in **Figs. 4**,**6**, and the "IN" data in **Fig. 7**), we counted single, double- and triple-labeled cells across two 100µm-wide ROIs extending through all layers at the injection site on each channel, yielding a total of 4 ROIs across 2 tissue sections being counted and analyzed for each viral injection site. All ROIs used for counts were positioned at the center of the viral expression region in sections where the latter encompassed all cortical layers. To quantify the normal distribution of GABA+ and PV+ immunoreactivity in control tissue (i.e., the data shown in **Figs. 2**, and the "OUT" data in **Fig. 7**) we counted single and double-labeled cells across two 100µm-wide ROI's extending through all layers in each tissue section for a total of 6 ROIs across 3 sections. The ROIs for this analysis were selected to be millimeters away from the V1 region containing the viral injection sites. Cell counting was performed by two undergraduate researchers (AI, PB) and reviewed for accuracy by senior lab members (FF, AA). Cortical layer boundaries were determined using DAPI staining or PV-IHC (after confirming the layer boundaries based on PV-IHC matched those seen in DAPI). Data collected in Neurolucida were exported to Excel (Microsoft) and SPSS (IBM) software for quantitative and statistical analyses.

Statistical Analysis

To compare cell counts and neuronal densities across different viral serotypes (for the GABA-AAVs), different viral volumes (for the PV-AAV), or different cortical layers, we used an ANOVA test, when the data were normally distributed, and either the non-parametric Independent Samples Kruskall-Wallis test, an Independent Samples Median test or the Mann-Whitney U test for data that were not normally distributed, unless otherwise indicated in the Results section. All multiple comparisons were Bonferroni corrected.

Data availability statement

The data presented here will be provided upon reasonable request to the corresponding authors. Source data for the figures are provided with the paper.

Acknowledgements

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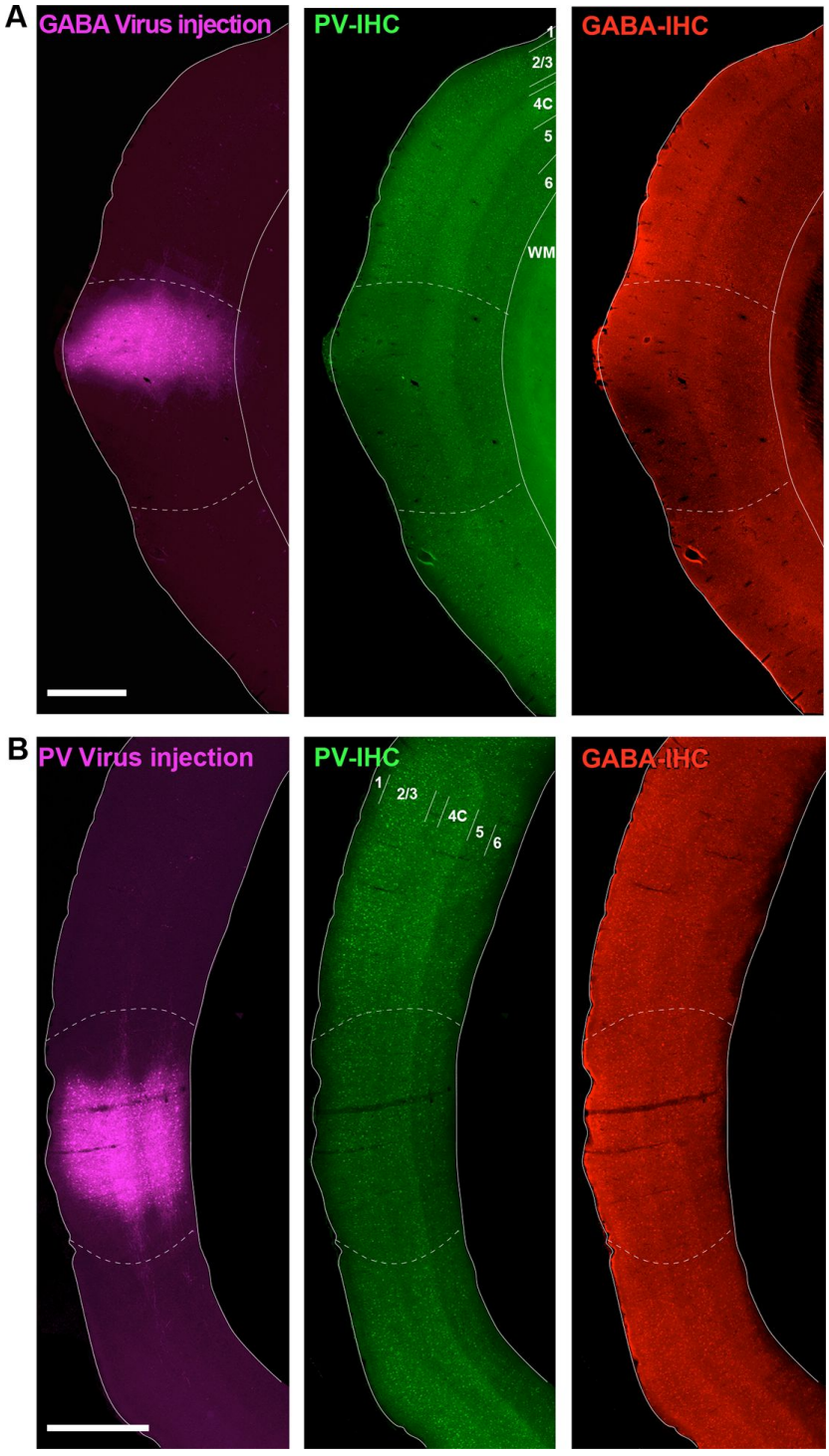
Author contributions

Conceptualization: F.F., J.B., J.D., A.A. Investigation: F.F., J.B., A.A. Data Analysis: F.F., J.B., A.I., D.P.B. Virus production: B.V.Z., J.D. Writing-Original Draft: F.F., A.A. Writing– Review/Editing: all authors. Visualization: F.F., A.A. Supervision & Funding Acquisition: A.A.

Competing interests statement

The authors declare no competing interests.



Supplementary Figure 1

Reduced GABA and PV immunoreactivity at the viral injection site. (A) Left: Epifluorescent image of an example GABA-AAV injection site in V1. Middle: Same section imaged under the green channel to reveal PV-IHC. Right: Same section imaged under the red channel to reveal GABA-IHC. In all panels, *solid white contours* mark the top and bottom of the cortex, *dashed contours* outline the region of reduced immunoreactivity. Cortical layers are indicated in the middle panel. (B) Same as in (A) but for an example PV-AAV injected sited. Scale bars in (A,B): 1 mm.

AAV-h56D vectors injection parameters

Case No.	Deep Inj.		Middle Inj.		Superficial Inj.		Total Volume (nl)	Viral Titer (gc/ml)	Survival Time (days)
Serotype	Depth (mm)	Volume (nl)	Depth (mm)	Volume (nl)	Depth (mm)	Volume (nl)			
MM404RH *AAV1*	1.5	200	1.0	200	0.5	200	600nL	1.0E14	28
MM404RH *AAV7*	1.5	200	1.0	200	0.5	200	600nL	1.0E13	28
MM404RH *AAV9*	1.5	200	1.0	200	0.5	200	600nL	1.0E13	28

AAV-PHP.eB-S5E2 vector injection parameters

Case No.	Deep Inj.		Middle Inj.		Superficial Inj.		Total Volume (nl)	Viral Titer (gc/ml)	Survival Time (days)
	Depth (mm)	Volume (nl)	Depth (mm)	Volume (nl)	Depth (mm)	Volume (nl)			
MM417RH	1.3	195	0.8	195	0.6	195	585	8.3E12	12
MM423LH	1.2	105	0.8	105	0.4	105	315	8.3E12	21
MM430LH	1.2	105	0.8	105	0.4	105	315	8.3E12	21
MM430RH	1.2	60	0.8	60	0.4	60	180	8.3E12	21
MM430LH	1.2	60	0.8	60	0.4	60	180	8.3E12	21
MM430RH	1.2	35	0.8	35	0.4	35	105	8.3E12	21
MM430LH	1.2	30	0.8	30	0.4	30	90	8.3E12	21

Supplementary Table 1.

Animals and Viral Injections

Case No.	Tot volume (nl)	Specificity (mean±sem%)	Coverage (mean±sem%)	Total cell count (n)
MM417RH	585	0.79±0.058	0.79±0.024	283
MM423LH	315	0.76±0.064	0.82±0.017	313
MM430LH	315	0.89±0.023	0.70±0.032	459
MM430RH	180	0.95±0.008	0.81±0.005	369
MM430LH	180	0.94±0.033	0.80±0.020	298
MM430RH	105	0.97±0.008	0.83±0.009	276
MM430LH	90	0.80±0.031	0.80±0.036	280

Supplementary Table 2.

AAV-PHP.eB-S5E2-tdT specificity and coverage: individual injections

References

Ascoli GA, Alonso-Nanclares L, Anderson SA, Barrionuevo G (2008) **Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex** *Nat Rev Neurosci* **9**:557–568

Aurnhammer C *et al.* (2012) **Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences** *Hum Gene Ther Methods* **23**:18–28

Beaulieu C (1993) **Numerical data on neocortical neurons in adult rat, with special reference to the GABA population** *Brain Res* **609**:284–292

Blumcke I, Hof PR, Morrison JH, Celio MR (1990) **Distribution of parvalbumin immunoreactivity in the visual cortex of Old World monkeys and humans** *J Comp Neurol* **301**:417–432

Burkhalter A (2008) **Many specialists for suppressing cortical excitation** *Front Neurosci* **2**:155–167

Cheah CS, Yu FH, Westenbroek RE, Kalume FK, Oakley JC, Potter GB, Rubenstein JL, Catterall WA (2012) **Specific deletion of NaV1.1 sodium channels in inhibitory interneurons causes seizures and premature death in a mouse model of Dravet syndrome** *Proc Natl Acad Sci U S A* **109**:14646–14651

DeFelipe J (2002) **Cortical interneurons: from Cajal to 2001** *Prog Brain Res* **136**:215–238

DeFelipe J, Gonzalez-Albo MC, Del Rio MR, Elston GN (1999) **Distribution and patterns of connectivity of interneurons containing calbindin, calretinin, and parvalbumin in visual areas of the occipital and temporal lobes of the macaque monkey** *J Comp Neurol* **412**:515–526

Dimidschstein J, et al. (2016) **A viral strategy for targeting and manipulating interneurons across vertebrate species** *Nat Neurosci* **19**:1743–1749

Disney AA, Aoki C (2008) **Muscarinic acetylcholine receptors in macaque V1 are most frequently expressed by parvalbumin-immunoreactive neurons** *J Comp Neurol* **507**:1748–1762

Ferster D, Miller KD (2000) **Neural mechanisms of orientation selectivity in the visual cortex** *Ann Rev Neurosci* **23**:441–471

Goldberg EM, Coulter DA (2013) **Mechanisms of epileptogenesis: a convergence on neural circuit dysfunction** *Nat Rev Neurosci* **14**:337–349

Goodchild AK, Martin PR (1998) **The distribution of calcium-binding proteins in the lateral geniculate nucleus and visual cortex of a New World monkey, the marmoset, Callithrix jacchus** *Vis Neurosci* **15**:625–642

Grieger JC, Snowdy S, Samulski RJ (2006) **Separate basic region motifs within the adeno-associated virus capsid proteins are essential for infectivity and assembly** *J Virol* **80**:5199–5210

Hendry SH, Schwark HD, Jones EG, Yan J (1987) **Numbers and proportions of GABA-immunoreactive neurons in different areas of monkey cerebral cortex** *J Neurosci* **7**:1503–1519

Hendry SH, Jones EG, Emson PC, Lawson DE, Heizmann CW, Streit P (1989) **Two classes of cortical GABA neurons defined by differential calcium binding protein immunoreactivities** *Exp Brain Res* **76**:467–472

Hof PR Glezer, Conde F II, Flagg RA, Rubin MB, Nimchinsky EA, Vogt Weisenhorn DM (1999) **Cellular distribution of the calcium-binding proteins parvalbumin, calbindin, and calretinin in the neocortex of mammals: phylogenetic and developmental patterns** *J Chem Neuroanat* **16**:77–116

Kelly JG, Garcia-Marin V, Rudy B, Hawken MJ (2019) **Densities and Laminar Distributions of Kv3.1b-, PV-, GABA-, and SMI-32-Immunoreactive Neurons in Macaque Area V1** *Cereb Cortex* **29**:1921–1937

Kooijmans RN, Sierhuis W, Self MW, Roelfsema PR (2020) **A Quantitative Comparison of Inhibitory Interneuron Size and Distribution between Mouse and Macaque V1** *Using Calcium-Binding Proteins. Cereb Cortex Commun* **1**

Krienen FM, et al. (2020) **Innovations present in the primate interneuron repertoire** *Nature* **586**:262–269

Kubota Y, Karube F, Nomura M, Kawaguchi Y (2016) **The diversity of cortical inhibitory synapses** *Front Neural Circuits* **10**

Lewis DA (2014) **Inhibitory neurons in human cortical circuits: substrate for cognitive dysfunction in schizophrenia** *Curr Opin Neurobiol* **26**:22–26

Marin O (2012) **Interneuron dysfunction in psychiatric disorders** *Nat Rev Neurosci* **13**:107–120

Medalla M, Mo B, Nasar R, Zhou Y, Park J, Luebke JI (2023) **Comparative features of calretinin, calbindin, and parvalbumin expressing interneurons in mouse and monkey primary visual and frontal cortices** *J Comp Neurol* **531**:1934–1962

Mehta P *et al.* (2019) **Functional Access to Neuron Subclasses in Rodent and Primate Forebrain** *Cell Rep* **26**:2818–2832

Mich JK, et al. (2021) **Functional enhancer elements drive subclass-selective expression from mouse to primate neocortex** *Cell Rep* **34**

Mukherjee A, Carvalho F, Eliez S, Caroni P (2019) **Long-Lasting Rescue of Network and Cognitive Dysfunction in a Genetic Schizophrenia Model** *Cell* **178**:1387–1402

Patino M, Lagos WN, Patne NS, Tasic B, Zeng H, Callaway EM (2022) **Single-cell transcriptomic classification of rabies-infected cortical neurons** *Proc Natl Acad Sci U S A* **119**

Prosniak M, Hooper DC, Dietzschold B, Koprowski H (2001) **Effect of rabies virus infection on gene expression in mouse brain** *Proc Natl Acad Sci U S A* **98**:2758–2763

Rudy B, Fishell G, Lee S, Hjerling-Leffler J (2011) **Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons** *Dev Neurobiol* **71**:45–61

Shapley R, Hawken M, Ringach DL (2003) **Dynamics of orientation selectivity in the primary visual cortex and the importance of cortical inhibition** *Neuron* **38**:689–699

Shin H, Adesnik H, Usrey WM, M SS (2023) **Functional roles of cortical inhibitory interneurons** *The Cerebral Cortex and Thalamus* :72–80

Tremblay R, Lee S, Rudy B (2016) **GABAergic Interneurons in the Neocortex: From Cellular Properties to Circuits** *Neuron* **91**:260–292

Vafa A, Clark AM, Federer F, Angelucci A (2024) **Computational function of parvalbumin-expressing inhibitory neurons in the primate primary visual cortex (V1)** *Soc Neurosci Abstr Online*

van Brederode JFM, Mulligan KA, Hendrickson AE (1990) **Calcium-binding proteins as markers for subpopulation of GABAergic neurons in monkey striate cortex** *J Comp Neurol* **298**:1–22

Verret L *et al.* (2012) **Inhibitory interneuron deficit links altered network activity and cognitive dysfunction in Alzheimer model** *Cell* **149**:708–721

Vormstein-Schneider DC, et al. (2020) **Viral manipulation of functionally distinct interneurons in mice, non-human primates and humans** *Nat Neurosci* **23**:1629–1636

Wood KC, Blackwell JM, Geffen MN (2017) **Cortical inhibitory interneurons control sensory processing** *Curr Opin Neurobiol* **46**:200–207

Xu X, Roby KD, Callaway EM (2010) **Immunochemical characterization of inhibitory mouse cortical neurons: three chemically distinct classes of inhibitory cells** *J Comp Neurol* **518**:389–404

Zhao P *et al.* (2011) **Innate immune response gene expression profiles in central nervous system of mice infected with rabies virus** *Comp Immunol Microbiol Infect Dis* **34**:503–512

Editors

Reviewer #1 (Public Review):

Summary:

Federer et al. tested AAVs designed to target GABAergic cells and parvalbumin-expressing cells in marmoset V1. Several new results were obtained. First, AAV-h56D targeted GABAergic cells with high specificity but in ways that varied across serotype and layer. Second, AAV-PHP.eB.S5E2 targeted parvalbumin-expressing neurons with similarly high specificity. Third, immunohistochemical GABA and PV signals were attenuated near viral injection sites.

A strength of this study is the analysis of marker gene expression at AAV injection sites. Some endogenous genes are difficult to detect following AAV injections, which is an important observation. A second contribution is the demonstration that AAV-S5E2 drives transgene expression selectively in parvalbumin-expressing neurons when vectors are delivered intraparenchymally (the study introducing AAV-S5E2 used intravenous injections).

A weakness of this study is that the data set is small. Which of the results would hold up had a larger number of injections been made into a larger number of marmosets remains unclear.

A major goal of this study was to quantify the specificity and coverage of AAV-h56D and AAV-S5E2 vectors in marmoset cortex. This goal was achieved. This report provides a valuable guide for other investigators using these tools. It also provides a rigorous survey of the laminar distributions of GABA+ and PV+ neurons in marmoset V1 which has value independent of the viral injections.

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Reviewer #3 (Public Review):
Summary:

Federer et al. describe the laminar profiles of GABA+ and of PV+ neurons in marmoset V1. They also report on the selectivity and efficiency of expression of a PV-selective enhancer (S5E2) across laminae. Three further viruses were tested, with a view to characterizing the expression profiles of a GABA-selective enhancer (h56d), but these results are preliminary.

Strengths:

The derivation of cell-type specific enhancers is key for translating the types of circuit analyses that can be performed in mice - which rely on germline modifications for access to cell-type specific manipulation - in higher-order mammals. Federer et al. further validate the utility of S5E2 as a PV-selective enhancer in NHPs.

Additionally, the authors characterize the laminar distribution pattern of GABA+ and PV+ cells in V1. This survey may prove valuable to researchers seeking to understand and manipulate the microcircuitry mediating the excitation-inhibition balance in this region of the marmoset brain.

Weaknesses:

Enhancer/promoter specificity and efficiency cannot be directly compared, because they were packaged in different serotypes of AAV.

The three different serotypes of AAV expressing reporter under the h56D promoter were only tested once each, and all in the same animal. There are many variables that can contribute to the success (or failure) of a viral injection, so observations with an n=1 cannot be considered reliable.

Added after revision:

The revisions satisfy my concerns. In particular, the new language to qualify the strength of evidence relating to the interpretation of the data relating to the 3 different serotypes of virus used to test h56D is appropriate.

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Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *Federer et al. tested AAVs designed to target GABAergic cells and parvalbumin-expressing cells in marmoset V1. Several new results were obtained. First, AAV-h56D targeted GABAergic cells with >90% specificity, and this varied with serotype and layer. Second, AAV-PHP.eB.S5E2 targeted parvalbumin-expressing neurons with up to 98% specificity. Third, the immunohistochemical detection of GABA and PV was attenuated near viral injection sites.*
>
> *Strengths:*
>
> *Vormstein-Schneider et al. (2020) tested their AAV-S5E2 vector in marmosets by intravenous injection. The data presented in this manuscript are valuable in part because they show the transduction pattern produced by intraparenchymal injections, which are more conventional and efficient.*

Our manuscript additionally provides detailed information on the laminar specificity and coverage of these viral vectors, which was not investigated in the original studies.

> *Weaknesses:*
>
> *The conclusions regarding the effects of serotype are based on data from single injection tracks in a single animal. I understand that ethical and financial constraints preclude high throughput testing, but these limitations do not change what can be inferred from the measurements. The text asserts that "...serotype 9 is a better choice when high specificity and coverage across all layers are required". The data presented are consistent with this idea but do not make a strong case for it.*

We are aware of the limitations of our results on the AAV-h56D. We agree with the Reviewer that a single injection per serotype does not allow us to make strong statements about differences between the 3 serotypes. Therefore, in the revised version of the manuscript we have tempered our claims about such differences and use more caution in the interpretation of these data (Results p. 6 and Discussion p.10). Despite this weakness, we feel that these data still demonstrate high efficiency and specificity across cortical layers of transgene expression in GABA cells using the h56D promoter, at least with two of the 3 AAV serotypes we tested. We feel that in itself this is sufficiently useful information for the primate community, worthy of being reported. Due to cost, time and ethical considerations related to the use of primates, we chose not to perform additional experiments to determine precise differences among serotypes. Thus, for example, while it is possible that had we replicated these experiments, serotype 7 could have proven equally efficient and specific as the other two serotypes, we felt answering this question did not warrant additional experiments in this precious species.

> *A related criticism extends to the analysis of Injection volume on viral specificity. Some replication was performed here, but reliability across injections was not reported. My understanding is that individual ROIs were treated as independent observations. These are not biological replicates (arguably, neither are multiple injection tracks in a single animal, but they are certainly closer). Idiosyncrasies between animals or injections (e.g., if one injection happened to hit one layer more than another) could have substantial impacts on the measurements. It remains unclear which results regarding injection volume or serotype would hold up had a large number of injections been made into a large number of marmosets.*

For the AAV-S5E2, we made a total of 7 injections (at least 2 at each volume), all of which, irrespective of volume, resulted in high specificity and efficiency for PV interneurons. Our conclusion is that larger volumes are slightly less specific, but the differences are minimal and do not warrant additional injections. Additionally, we kept all the other parameters across animals constant (see new Supplementary Table 1), all of our injections involved all cortical layers, and the ROIs we selected for counts encompassed reporter protein expression across all layers. To provide a better sense of the reliability of the results across injections, in the revised version of the manuscript we now provide results for each of the AAV-S5E2 injection case separately in a new Supplementary Table 2. The results in this table indicate the results are indeed rather consistent across cases with slightly greater specificity for injection volumes in the range of 105-180 nl.

> ***Reviewer #2 (Public Review):***
>
> *This is a straightforward manuscript assessing the specificity and efficiency of transgene expression in marmoset primary visual cortex (V1), for 4 different AAV vectors known to target transgene expression to either inhibitory cortical neurons (3 serotypes of AAV-h56D-tdTomato) or parvalbumin (PV)+ inhibitory cortical neurons in mice. Vectors are injected into the marmoset cortex and then postmortem tissue is analyzed following antibody labeling against GABA and PV. It is reported that: "in marmoset V1 AAV-h56D induces transgene expression in GABAergic cells with up to 91-94% specificity and 80% efficiency, depending on viral serotype and cortical layer. AAV-PHP.eB-S5E2 induces transgene expression in PV cells across all cortical layers with up to 98% specificity and 86-90% efficiency."*
>
> *These claims are largely supported but slightly exaggerated relative to the actual values in the results presented. In particular, the overall efficiency for the best h56D vectors described in the results is: "Overall, across all layers, AAV9 and AAV1 showed significantly higher coverage (66.1{plus minus}3.9 and 64.9%{plus minus}3.7)". The highest coverage observed is just in middle layers and is also less than 80%: "(AAV9: 78.5%{plus minus}9.1; AAV1: 76.9%{plus minus}7.4)".*

In the abstract, we indeed summarize the overall data and round up the decimals, and state that these percentages are upper bound but that they vary by serotype and layer while in the Results we report the detailed counts with decimals. To clarify this, in the revised version of the Abstract we have changed 80% to 79% and emphasize even more clearly the dependence on serotype and layer. We have amended this sentence of the Abstract as follows: “We show that in marmoset V1 AAV-h56D induces transgene expression in GABAergic cells with up to 91-94% specificity and 79% efficiency, but this depends on viral serotype and cortical layer.”

> *For the AAV-PHP.eB-S5E2 the efficiency reported in the abstract (“86-90%) is also slightly exaggerated relative to the results: “Overall, across all layers coverage ranged from 78% {plus minus}1.9 for injection volumes >300nl to 81.6%{plus minus}1.8 for injection volumes of 100nl.”*

Indeed, the numbers in the Abstract are upper bounds, for example efficiency in L4A/B with S5E2 reaches 90%. To further clarify this important point, in the revised abstract we now state ”AAV-PHP.eB-S5E2 induces transgene expression in PV cells across all cortical layers with up to 98% specificity and 86-90% efficiency, depending on layer”.

> *These data will be useful to others who might be interested in targeting transgene expression in these cell types in monkeys. Suggestions for improvement are to include more details about the vectors injected and to delete some comments about results that are not documented based on vectors that are not described (see below).*
>
> *Major comments:*
>
> *Details provided about the AAV vectors used with the h56D enhancer are not sufficient to allow assessment of their potential utility relative to the results presented. All that is provided is: "The fourth animal received 3 injections, each of a different AAV serotype (1, 7, and 9) of the AAV-h56D-tdTomato (Mehta et al., 2019), obtained from the Zemelman laboratory (UT Austin)." At a minimum, it is necessary to provide the titers of each of the vectors. It would also be helpful to provide more information about viral preparation for both these vectors and the AAVPHP.eB-S5E2.tdTomato. Notably, what purification methods were used, and what specific methods were used to measure the titers?*

We thank the Reviewer for this comment. In the revised version of the manuscript, we now provide a new Supplementary Table 1 with titers and other information for each viral vector injection. We also provide information regarding viral preparation in a new sections in the Methods entitled “ Viral Preparation” (p12).

> *The first paragraph of the results includes brief anecdotal claims without any data to support them and without any details about the relevant vectors that would allow any data that might have been collected to be critically assessed. These statements should be deleted. Specifically, delete: “as well as 3 different kinds of PV-specific AAVs, specifically a mixture of AAV1-PaqR4-Flp and AAV1-h56D-mCherry-FRT (Mehta et al., 2019), an AAV1-PV1-ChR2-eYFP (donated by G. Horwitz, University of Washington),” and delete “Here we report results only from those vectors that were deemed to be most promising for use in primate cortex, based on infectivity and specificity. These were the 3 serotypes of the GABA-specific pAAV-h56D-tdTomato, and the PV-specific AAVPHP.eB-S5E2.tdTomato.” These tools might in fact be just as useful or even better than what is actually tested and reported here, but maybe the viral titer was too low to expect any expression.*

These data are indeed anecdotal, but we felt this could be useful information, potentially preventing other primate labs from wasting resources, animals and time, particularly, as some of these vectors have been reported to be selective and efficient in primate cortex, which we have not been able to confirm. We made several injections in several animals of those vectors that failed either to infect a sufficient number of cells or turned out to be poorly specific. Therefore, the negative results have been consistent in our hands. But we agree with the Reviewer that our negative results could have depended on factors such as titer. In the revised version of the manuscript, following the reviewer’s suggestion, we have deleted this information.

> *Based on the description in the Methods it seems that no antibody labeling against TdTomato was used to amplify the detection of the transgenes expressed from the AAV vectors. It should be verified that this is the case - a statement could be added to the Methods.*

That is indeed the case. We used no immunohistochemistry to enhance the reporter proteins as this was unnecessary. The native/ non-amplified tdT signal was strong. This is now stated in the methods (p.12).

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *Federer et al. describe the laminar profiles of GABA+ and of PV+ neurons in marmoset V1. They also report on the selectivity and efficiency of expression of a PV-selective enhancer (S5E2). Three further viruses were tested, with a view to characterizing the expression profiles of a GABA-selective enhancer (h56d), but these results are preliminary.*
>
> *Strengths:*
>
> *The derivation of cell-type specific enhancers is key for translating the types of circuit analyses that can be performed in mice - which rely on germline modifications for access to cell-type specific manipulation - in higher-order mammals. Federer et al. further validate the utility of S5E2 as a PV-selective enhancer in NHPs.*
>
> *Additionally, the authors characterize the laminar distribution pattern of GABA+ and PV+ cells in V1. This survey may prove valuable to researchers seeking to understand and manipulate the microcircuitry mediating the excitation-inhibition balance in this region of the marmoset brain.*
>
> *Weaknesses:*
>
> *Enhancer/promoter specificity and efficiency cannot be directly compared, because they were packaged in different serotypes of AAV.*
>
> *The three different serotypes of AAV expressing reporter under the h56D promoter were only tested once each, and all in the same animal. There are many variables that can contribute to the success (or failure) of a viral injection, so observations with an n=1 cannot be considered reliable.*

This is an important point that was also brough up by Reviewer 1, which we have addressed in our reply-to-Reviewer 1. For clarity and convenience, below we copy our response to Reviewer 1.

“We are aware of the limitations of our results on the AAV-h56D. We agree with the Reviewer that a single injection per serotype does not allow us to make strong statements about differences between the 3 serotypes. Therefore, in the revised version of the manuscript we will temper our claims about such differences and use more caution in the interpretation of these data. Despite this weakness, we feel that these data still demonstrate high efficiency and specificity across cortical layers of transgene expression in GABA cells using the h56D promoter, at least with two of the 3 AAV serotypes we tested. We feel that in itself this is sufficiently useful information for the primate community, worthy of being reported. Due to cost, time and ethical considerations related to the use of primates, we chose not to perform additional experiments to determine precise differences among serotypes. Thus, for example, while it is possible that had we replicated these experiments, serotype 7 would have proven equally efficient and specific as the other two serotypes, we felt answering this question did not warrant additional experiments in this precious species.”

> *The language used throughout conflates the cell-type specificity conferred by the regulatory elements with that conferred by the serotype of the virus.*

Authors' reply. In the revised version of the manuscript, we have corrected ambiguous language throughout.

> ***Recommendations for the authors***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *My Public Review comments can be addressed by dialing down the interpretation of the data or providing appropriate caveats in the presentation of the relevant results and their discussion.*

We have done so. See text additions on p. 6 of the Results and p.10 of the Discussion.

> *Minor comments:*
>
> *92% of PV+ neurons in the marmoset cortex were GABAergic. Can the authors speculate on the identity of the 8% PV+/GABA- neurons (e.g., on the basis of morphology)? Are they likely excitatory? Are they more likely to represent failures of GABA staining?*

We do not know what the other 8% of PV+/GABA- neurons are because we did not perform any other kind of IHC staining. Our best guess is that at least to some extent these represent failures of GABA staining, which is always challenging to perform in primate cortex. However, in mouse PV expression has been demonstrated in a minority of excitatory neurons.

> *"Coverage of the PV-AAV was high, did not depend on injection volume.." The fact that the coverage did not depend on injection volume presumably depends, at least in part, on how ROIs were selected. Surely different volumes of injection transduce different numbers of neurons at different distances from the injection track. This should be clarified.*

The ROIs were selected at the center of the injected site/expression core from sections in which the expression region encompassed all cortical layers. Of course, larger volumes of injection resulted in larger transduced regions and therefore overall larger number of transduced neurons, but we counted cells only withing 100 μm wide ROIs at the center of the injection and the percent of transduced PV cells in this core region did not vary significantly across volumes. We have clarified the methods of ROI selection (see Methods pp. 13).

> *Figure 2. What is meant by "absolute" in the legend for Figure 2? (How does "mean absolute density" differ from "mean density?")*

We meant not relative, but this is obvious from the units, so we have removed the word "absolute" in the legend.

> *Some non-significant p-values are indicated by "p>0.05" whereas others are given precisely (e.g., p = 1). Please provide precise p-values throughout. Also, the p-value from a surprisingly large number of comparisons in the first section of the results is "1". Is this due to rounding? Is it possible to get significance in a Bonferroni-corrected Kruskal-Wallis test with only 6 observations per condition?*

We now report exact p values throughout the manuscript (with a couple of exceptions where, in order to avoid reporting a large number of p values which interrupts the flow of the manuscript) we provide the upper bound value and state all those comparisons were below

that value). The minimum sample size for Kruskall Wallis is 5, for each group being compared, and we our sample is 6 per group.

> *Figure 3: The density of tdTomato-expressing cells appears to be greater at the AAV9 injection site than at the AAV1 injection site in the example sections shown. Might some of the differences between serotypes be due to this difference? I would imagine that resolving individual cells with certainty becomes more difficult as the amount of tdTomato expression increases.*

There was an error in the scale bar of Fig. 3C, so that the AAV1 injection site was shown at higher magnification than indicated by the wrong scale bar. Hence the density of tdTomato appeared lower than it is. Moreover, the tdT expression region shown in Fig. 3A is a merge of two sections, while it is only from a single section in panels B and C, leading to the impression of higher density of infected cells in panel A. The pipette used for the injection in panel A was not inserted perfectly vertical to the cortical surface, resulting in an injection site that did not span all layers in a single section; thus, to demonstrate that the injection indeed encompassed all layers (and that the virus infected cells in all layers), we collapsed label from two sections. We have now corrected the magnification of panel C so that it matches the scale bar in panel A, and specify in the figure legend that panel A label is from two sections.

> *Text regarding Figure 3: The term "injection sizes" is confusing. I think it is intended to mean "the area over which tdTomato-expressing cells were found" but this should be clarified.*

Throughout the manuscript, we have changed the term injection site to "viral-expression region".

> *Figure 3: What were the titers of the three AAV-h56D vectors?*

Titers are now reported in the new Supplementary Table 1.

> *Figure 3: The yellow box in Figure 3C is slightly larger than the yellow boxes in 3A and 3B. Is this an error or should the inset of Figure 3 have a scale bar that differs from the 50 µm scale bar in 3A?*

There were indeed errors in scale bars in this figure, which we have now corrected. Now all boxes have the same scale bar.

> *Was MM423 one of the animals that received the AAV-h56D injections or one of the three that received AAV-S5E2 injection?*

This is an animal that received a 315nl injection of AAV-PHP.eB-S5E2.tdTomato. This is now specified in the Methods (see p. 12) and in the new Supplementary Table 1.

> *Please provide raw cell counts and post-injection survival times for each animal.*

We now provide this information in Supplementary Tables 1 and 2.

> *How were the different injection volumes of the AAV-S5E2 virus arranged by animal? Which volume of the AAV-S5E2 virus was injected into the two animals who received single injections?*

We now provide this information in Supplementary Table 1.

> *Figure 6A: the point is made in the text that "[the distribution of tdT+ and PV+ neurons] did not differ significantly... peaking in L2/3 and 4C " Is the fact that the number of tdT+ and PV+ peak in layers 2/3 and 4C a consequence of these layers being thicker than the others? If so, this statement seems trivial.*

No, and this is the reason why we measured density in addition to percent of cells across layers in Figure 2. Figure 2B shows that even when measuring density, therefore normalizing by area, GABA+ and PV+ cell density still peaks in L2/3 and 4. Thus, these peaks do not simply reflect the greater thickness of these layers.

> *Do the authors have permission to use data from Xu et al. 2010?*

Yes, we do.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Minor comments:*
>
> *"Viral strategies to restrict gene expression to PV neurons have also been recently developed (Mehta et al., 2019; Vormstein-Schneider et al., 2020)." Mich et al. should also be cited here. Cell Rep. 2021;34(13):108754.*

We thank the reviewer for pointing out this missing references. This is now cited.

> *"GABA density in L4C did not differ from any other layers, but the percent of GABA+ cells in L4C was significantly higher than in L1 (p=0.009) and 4A/B (p=<0.0001)." This and other similar observations depend on calculating the percentage of cells relative to the total number of DAPI-labeled cells in each layer. Since it is apparent that there must be considerable variability between layers, it would be helpful to add a histogram showing the densities of all DAPI-labeled cells for each layer.*

This is not how we calculated density. Density, as now clarified in the Results on p. 4, was defined as the number of cells per unit area. Counts in each layer were divided by each layers' counting area. This corrects for differences in number of total labeled cells per layer. Therefore, reporting DAPI density is not necessary (we did not count DAPI cell density per layer).

> *"Identical injection volumes of each serotype, delivered at 3 different cortical depths (see Methods), resulted in different injection sizes, suggesting the different serotypes have different capacity of infecting cortical neurons. AAV7 produced the smallest injection site, which additionally was biased to the superficial and deep layers, with only few cells expressing tdT in the middle layers (Fig. 3B). AAV9 (Fig. 3A) and AAV1 (Fig. 3C) resulted in larger injection sites and infected all cortical layers." Differences noted here might reflect either differences related to the AAV serotype or to differences in titers. Please add details about titers for each vector and add comments as appropriate. Another interpretation would be that there are differences in viral spread within the tissue.*

We have now added Supplementary Table 1 which reports titers in addition to other information about injections. The titers and volumes used for AAV9 and AAV7 were identical, while the titer for AAV1 was higher. Therefore, the differences in infectivity, particularly the much smaller expression region obtained with AAV7 cannot be attributed to titer. Likely this is due to differences in tropism and/or viral spread among serotypes. This is now discussed (see Results p. 5bottom and 6 top).

> *"Recently, several viral vectors have been identified that selectively and efficiently restrict gene expression to GABAergic neurons and their subtypes across several species, but a thorough validation and characterization of these vectors in primate cortex has lacked." Is this really a fair statement, or is the characterization presented here also lacking? Methods used by others for quantifying specificity and efficiency are essentially the same as used here. See for example Mich et al. (which is not cited).*

The original validation in primates of the vectors examined in our study was based on small tissue samples and did not examine the laminar expression profile of transgene expression induced by these enhancer-AAVs. For example, the validation of the h56D-AAV in marmoset cortex in the original paper by Mehta et al (2019) was performed on a tissue biopsy with no knowledge of which cortical layers were included in the tissue sample. The only study that shows laminar expression in primate cortex (Mich et al., which is now cited), only shows qualitative images of viral expression across layers, reporting total specificity and coverage pooled across samples; moreover, the study by Mich et al. deals with different PV-specific enhancers than the ones characterized in our study. Unlike any of the previous studies, here we have quantified specificity and coverage across layers.

> *"Specifically, we have shown that the GABA-specific AAV9-h56D (Mehta et al., 2019) induces transgene expression in GABAergic cells with up to 91-94% specificity and 80% coverage, and the PV-specific AAV-PHP.eB-S5E2 (Vormstein-Schneider et al., 2020) induces transgene expression in PV cells with up to 98% specificity and 86-90% coverage." These statements in the discussion repeat the somewhat exaggerated coverage numbers noted above for the Abstract.*

The averages across all layers are reported in the Results. The Discussion, abstract and discussion report upper limits, and this is made clear by stating “up to”, and now we have also added “depending on layer”.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *Abstract:*
>
> *• Ln 2: Can you be more specific about what you mean by the 'various functions of inhibition'? e.g. do you mean 'the various inhibitory influences on the local microcircuit' or similar?*

These are listed in the introduction to the paper but there is no space in the abstract to do so. Now the sentence reads: “various computational functions of…”.

> *• Ln 5: 'has' to 'is'/'has been'.*

The grammar here is correct “has derived”.

> *• Ln 6: humans are primates! Maybe change this to 'nonhuman primates'?*

We have added “non-human”

> *• Ln n-1: 'viral vectors represent' -> 'viral vectors are'.*

We have changed it to “are”

> *Intro:*

> *• Many readers may expect 'VIP' to be listed as the third major sub-class of interneurons. Could you note that the 5HT3a receptor-expressing group includes VIP cells?*

Done (p.3).

> *• "Understanding cortical inhibitory neuron function in the primate is critical for understanding cortical function and dysfunction in the model system closest to humans" - this seems close to being circular logic (not quite, but close). Could you modify this sentence to reflect why understanding cortical function and dysfunction in NHP may be of interest?*

This sentence now reads (p.3):" Understanding cortical inhibitory neuron function in the primate is critical for understanding cortical function and dysfunction in the model system closest to humans, where cortical inhibitory neuron dysfunction has been implicated in many neurological and psychiatric disorders, such as epilepsy, schizophrenia and Alzheimer's disease (Cheah et al., 2012; Verret et al., 2012; Mukherjee et al., 2019)". We also note that this was already stated in the previous version of the paper but in the Discussion section which read (and still reads on p. 9 2nd paragraph): "It is important to study inhibitory neuron function in the primate, because it is unclear whether findings in mice apply to higher species, and inhibitory neuron dysfunction in humans has been implicated in several neurological and psychiatric disorders (Marin, 2012; Goldberg and Coulter, 2013; Lewis, 2014).".

> *• "In particular, two recent studies have developed recombinant adeno-associated viral vectors (AAV) that restrict gene expression to GABAergic neurons". This sentence places the emphasis on the wrong component of the technology. The fact that AAV was used is irrelevant; these constructs could equally have been packaged in a lenti, CAV, HSV, rabies, etc. The emphasis should be on the recently developed regulatory elements (the enhancers/promoters).*
>
> *Same problem with the following excerpts; this text implies that the serotype/vector confers cell-type selectivity, but the results presented do not support this assertion (the promoter/enhancer is what confers the selectivity).*
>
> *• "specifically, three serotypes of an AAV that restricts gene expression to GABAergic neurons".*
>
> *• "one serotype of an AAV that restricts gene expression to PV cells".*
>
> *• "GABA- and PV-specific AAVs".*
>
> *• "GABA-specific AAV" (in results).*
>
> *• "PV-specific AAVs".*
>
> *• "In this study, we have characterized several AAV vectors designed to restrict expression to GABAergic cells" (in discussion).*
>
> *• "GABA-virus". GABA is a NT, not a virus.*

We have modified the language in all these sections and throughout the manuscript.

> *Results:*
>
> *• Enhancer specificity and efficiency cannot be directly compared, because they were packaged in different serotypes of AAV.*

We agree, and in fact we are not making comparisons between different enhancers (i.e., S5E2 and h56D).

> *The three different serotypes of AAV expressing reporter under the h56D promoter were only tested once each, and all in the same animal. There are many variables that can contribute to the success (or failure) of a viral injection, so observations with an n=1 cannot be considered reliable.*
>
> *The authors need to either: (1) replicate the h56D virus injections in (at least) a second animal, or (2) rewrite the paper to focus on the AAV.PhP mDlx virus alone - for which they have adequate data - and mention the h56D data as an anecdotal result, with clear warnings about the preliminary nature of the observations due to lack of replication.*

We agree about the lack of sufficient data to make strong statements about the differences between serotypes for the h56D-AAV. In the revised version of the manuscript, following the Reviewers' suggestion, we have chosen to temper our claims about differences between serotypes for the h56D enhancer and use more caution in the interpretation of these data. We feel that these data still demonstrate sufficiently high efficiency and specificity across cortical layers of transgene expression in GABA cells using the h56D promoter, at least with two of the 3 AAV serotypes we tested, to warrant their use in primates. Due to cost, time and ethical considerations related to the use of primates, we chose not to perform additional experiments to determine precise differences among serotypes. Thus, for example, while it is possible that had we replicated these experiments, serotype 7 could have proven equally efficient and specific as the other two serotypes, we felt answering this question did not warrant additional experiments in this precious species. Our edits in regard to this point can be found in the Results on p. 6 and Discussion on p. 10.

> *• Did the authors compare h56D vs mDlx? This would be a useful and interesting comparison.*

We did not.

> *• 3 tissue sections were used for analysis. How were these selected? Did the authors use a stereological approach?*

For the analysis in Fig. 2, the 3 sections were randomly selected and for the positioning of the ROIs we selected a region in dorsal V1 anterior to the posterior pole (to avoid laminar distortions due to the curvature of the brain). This is now specified (see p. 4).

> *• "both GABA+ and PV+ cells peak in layers" revise for clarity (e.g., the counts peak).*

In now reads "GABA+ and PV+ cell percent and density" (see p.4).

> *• "we refer to this virus as GABA-AAV" these are 3 different viruses!*

The idea here was to use an abbreviation instead of using the full viral name every single time. Clearly the reviewer does not like this, so we have removed this convention throughout the paper and now specify the entire viral name each time.

> *• "Identical injection volumes of each serotype, delivered at 3 different cortical depths (see Methods), resulted in different injection sizes". Do you mean 'resulted in different volumes of expression'?*

Yes. We have now rephrased this as follows: "…resulted in viral expression regions that differed in both size as well as laminar distribution" (p.5).

> • *"suggesting the different serotypes have different capacity of infecting cortical neurons". You can't draw any firm conclusions from a single injection. The rest of this section of the results, along with the whole of Figure 4, and Figure 7a-d, is in danger of being misleading. Please remove. The best you can do here is to say 'we injected 3 different viruses that express reporter under the h56D promoter. The results are shown in Figure 3, but these are anecdotal, as only a single injection of each virus was performed'. You could then note in the discussion to what extent these results are consistent with the existing literature (e.g., AAV9 often produces good coverage in NHP – anterograde and retrograde, AAV1 also works well in the CNS, although generally doesn't infect as aggressively as AAV9. I'm not familiar with any attempts to use AAV7).*

With respect to Fig. 4, our approach in the revised version is detailed above. For convenience we copy it below here. With respect to Fig 7A-D, we feel the results are more robust as the data from the 3 serotypes here were pooled together, as the 3 serotype similarly downregulated GABA and PV expression at the injection site, and we do not make any statement about differences among serotypes for the data shown in Fig. 7A-D.

"In the revised version of the manuscript, following the Reviewer 's suggestion, we have chosen to temper our claims about differences between serotypes for the h56D enhancer and use more caution in the interpretation of these data (see revised text in the Results on p. 6 and in the Discussion on p. 10). We feel that these data still demonstrate sufficiently high efficiency and specificity across cortical layers of transgene expression in GABA cells using the h56D promoter, at least with two of the 3 AAV serotypes we tested, to warrant their use in primates. Due to cost, time and ethical considerations related to the use of primates, we chose not to perform additional experiments to determine precise differences among serotypes. Thus, for example, while it is possible that had we replicated these experiments, serotype 7 could have proven equally efficient and specific as the other two serotypes, we felt answering this question did not warrant additional experiments in this precious species."

> • *Figure 3: why the large variation in tissue quality? Are the 3 upper images taken at the same magnification? If not, they need different scale bars. The cells in A (upper row) look much smaller than those in B and C, and the size of the 'inset' box varies.*

We thank the reviewer for noticing this. We discovered an error in the scale bar of Fig. 3C, so that the AAV1 injection site was shown at higher magnification than indicated by the wrong scale bar. We have now corrected the error in scale bars. We have also fixed the different box sizes.

> • *"Overall, across all layers coverage ranged from 78%{plus minus}1.9 for injection volumes >300nl to 81.6%{plus minus}1.8 for injection volumes of 100nl." Coverage didn't differ between layers, so revise this to: "Overall, across all layers coverage ranged from 78% to 81.6%." or give an overall mean (~80%).*

We have corrected the sentence as suggested by the Reviewer (see p. 8 first paragraph).

> • *"extending farther from the borders" -> "extending beyond the borders".*

We have corrected the sentence as suggested by the Reviewer (see p. 8).

> • *"The reduced GABA and PV immunoreactivity caused by the viruses implies that the specificity of the viruses we have validated in this study is likely higher than estimated".*

> *Yes, but for balance you should also note that they may harm the physiology of the cell.*

We have added a sentence acknowledging this to the Discussion. Specifically, on p. 10, we now state: “However, this reduced immunoreactivity raises concerns about the virus or high levels of reporter protein possibly harming the cell physiology.”

> *Discussion:*
>
> *• "but a thorough validation and characterization of these vectors in primate cortex has lacked" better to say "has been limited", because Dimidschstein 2016 (marmoset V1) and Vormstein-schneider 2020 (macaque S1 and PFC) both reported expression in NHP.*

We have added the following sentence to this paragraph of the Discussion. “In particular, previous studies have not characterized the specificity and coverage of these vectors across cortical layers.”(see p. 8).

> *• "whether finding in mice" -> 'whether findings in mice'.*

Corrected, thanks.

> *• The discussion re: species differences is missing reference to Kreinen 2020 (10.1038/s41586-020-2781-z).*

This reference has been added. Thanks.

> *• “Injections of about 200nl volume resulted in higher specificity (95% across layers) and coverage” – this is misleading. The coverage was not statistically different among injection volumes.*

We have added the following sentence: ”although coverage did not differ significantly across volumes.” (see p. 10).

> *• "it is possible that subtle alteration of the cortical circuit upon parenchymal injection of viruses (including AAVs) leads to alteration of activity-dependent expression of PV and GABA." Or (and I would argue, more likely) the expression of large quantities of your big reporter protein compromised the function of the cell, leading to reduced expression of native proteins. You don't mention any IHC to amplify the RFP signal, so I'm assuming that your images are of direct expression. If so, you are expressing A LOT of reporter protein.*

We have added a sentence acknowledging this to the Discussion. Specifically, on p. 10, we now state: “However, this reduced immunoreactivity raises concerns about the virus or high levels of reporter protein possibly harming the cell physiology.”

> *Methods:*
>
> *• It's difficult to piece together which viruses were injected in which monkeys, at what volumes, and at what titer. Please compile this info into a table for ease of reference (including any other relevant parameters).*

We now provide a Supplementary Table 1.

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