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 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 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 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. Thus, these viral vectors represent promising tools for studying GABA and PV cell function and connectivity in the primate cortex.

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)- 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.

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 recombinant adeno-associated viral vectors (AAV) that restrict gene expression to GABAergic neurons under the *mDlx* enhancer (Dimidschstein et al., 2016) or *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).

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 four of these 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, three serotypes of an AAV that restricts gene expression to GABAergic neurons under the *h56D* promoter (Mehta et al., 2019), and one serotype of an AAV that restricts gene expression to PV cells under the E2 regulatory element (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-specific 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 GABA-virus 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 PV-AAV shows up to 98% specificity and 86-90% coverage. 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 GABA-specific AAV, specifically pAAV-h56D-tdTomato (Mehta et al., 2019), 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 an AAV-PHP.eB-S5E2.tdTomato (Vormstein-Schneider et al., 2020). 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 AAV-PHP.eB-S5E2.tdTomato. We report results from 10 viral injections (3 injections of AAV-h56D-tdTomato, 7 injections of AAV-PHP.eB-S5E2.tdTomato; see Methods) made in 4 marmoset monkeys. 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 2x100µm-wide regions of interest (ROIs) spanning all V1 layers for a total of 6 ROIs in 3 tissue sections. 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 (**Fig. 2B**), in each cortical layer. Both measures revealed that both GABA+ and PV+ cells peak in layers (L) 2/3 and 4C. There was no significant difference in the percent or density of GABA+ cells in L 2/3 (33%±2, and 1,294 cells/mm^2±74.4, respectively) vs. L 4C (33.4%±3.1, and 1,052 cells/mm^2±42.2, respectively), as determined by a Bonferroni-corrected Kruskall-Wallis test (p=1, n=6 ROIs across 3 sections, L2/3 vs L4C in **Fig. 2A**) or by a Bonferroni-corrected ANOVA test (p=1, 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 for both comparisons, n=6 ROIs). However, density of PV+ cells in L4C was significantly higher than in all remaining layers ($p<0.05$ 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 ($p<0.001$ for L2/3 vs. L1 and 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 ($p<0.05$ vs. L4A/B and L6, $p<0.01$ vs. L1 and 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=<0.0001). 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 three injection sites each of a different serotype (9,7,1) of the GABA-specific AAV-h56D-tdT (we refer to this virus as GABA-AAV). 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.

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, 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 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, AAV9 should be the serotype of choice for marmoset studies of GABAergic neurons requiring highest specificity and coverage across all layers, but AAV7 could be the serotype of 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 PV-specific AAV-PHP.eB-S5E2-tdT (we refer to this virus as PV-AAV) following injections of different viral volumes ranging from 100 nl to 600nl. **Figure 5** shows fluorescent images of two example injection sites of the PV-AAV resulting from injection volumes of 100 nl (**Fig. 5A**) and 300 nl (**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 300-600nl volumes were pooled as injections >300nl produced similar results. 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 each injection volume is quantified in **Figure 6B**. The degree of viral specificity was high at all volumes, but depended slightly on injection volume. Overall, across all layers, volumes of ~200nl showed the highest specificity (94.7%±1.6), which differed significantly from the specificity resulting from volumes >300nl (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 >300nl resulted in slightly lower specificity than smaller volumes in L4C (76.6%±5.6 for >300nl volumes vs 95.2%±1.7 and 94.9%±3 for 100nl and 180nl volumes, respectively) and L5 (80.1±5.8 for >300nl vs 97.9%±2.1 and 97%±1.9 for 100 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**. Coverage of the PV-AAV 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 for injection volumes >300nl to 81.6%±1.8 for injection volumes of 100nl.

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 farther from 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 GABA-AAV injection sites, and 28 ROIs across 14 sections for PV-AAV injection sites) and at sites located several millimeters away from 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 GABA-AAV (**Fig. 7A-D**) and PV-AAV (**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 GABA-AAV 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 PV-AAV, 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 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. In this study, we have characterized several AAV vectors designed to restrict expression to GABAergic cells or their PV subtypes, and have identified two 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 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. 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 finding 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), and 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). 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 compared laminar distribution, specificity and coverage resulting from different volume injections of the AAV-PHP.eB-S5E2 vector. Injections of about 200nl volume resulted in higher specificity (95% across layers) and coverage (81% across all layers) than obtained with injection volumes larger than 300nl (specificity 82% and coverage 78% across all layers). This mild dose-dependent alteration of specificity could depend on some off-target expression reaching above detection levels at higher doses. Thus, injection volumes ≤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 GABA-AAV injection site, while reduction in PV expression was more marked at the PV-AAV 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. 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. Notably, viral-induced downregulation of gene expression in host cells, including of inhibitory neuron marker genes such as *PV*, has been documented for 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

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 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, 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. 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 (titer 8.3E+09 vg/µl), 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: 600 nl (1 injection), 300 nl (2 injections), 180 nl (2 injections) and 90-100 nl (2 injections). The GABA-AAVs 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.

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), the brain was sunk in a 20% glycerol solution and frozen at -70˚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. 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. 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.

ACKNOWLEDGMENTS

We thank Kesi Sainsbury for histological assistance. This work was supported primarily by a grant from the National Institute of Health (NIH) to A.A. (R01 EY031959). Other grants were provided by the NIH (R01 EY026812), the National Science Foundation (IOS 1755431) and the Mary Boesche endowed Professorship, to A.A; an unrestricted grant from Research to Prevent Blindness, Inc. and a core grant from the NIH (P30 EY014800) to the Department of Ophthalmology, University of Utah.

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.

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FIGURES LEGENDS

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**: V1 region 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.

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 absolute density of GABA+ and PV+ cells in each layer. **(C)** Mean absolute 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 absolute 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) Top: tdT expression (*red*) across V1 layers following injection of an identical volume of AAV-h56D-tdT serotype 9 (A), serotype 7 (B) and serotype 1 (C). *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* in each panel is the region shown at higher magnification in each respective bottom panels. Scale bar: 500µm (valid for A-C). **Bottom:** Higher magnification of the V1 region inside the yellow box in each respective top 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).

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 100 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 300nl.

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 100nl, 8 for180 nl, 12 for 300-600nl PV-AAV injection volumes and 6 for PV-IHC), and *asterisks* indicate statistically significant differences.

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.

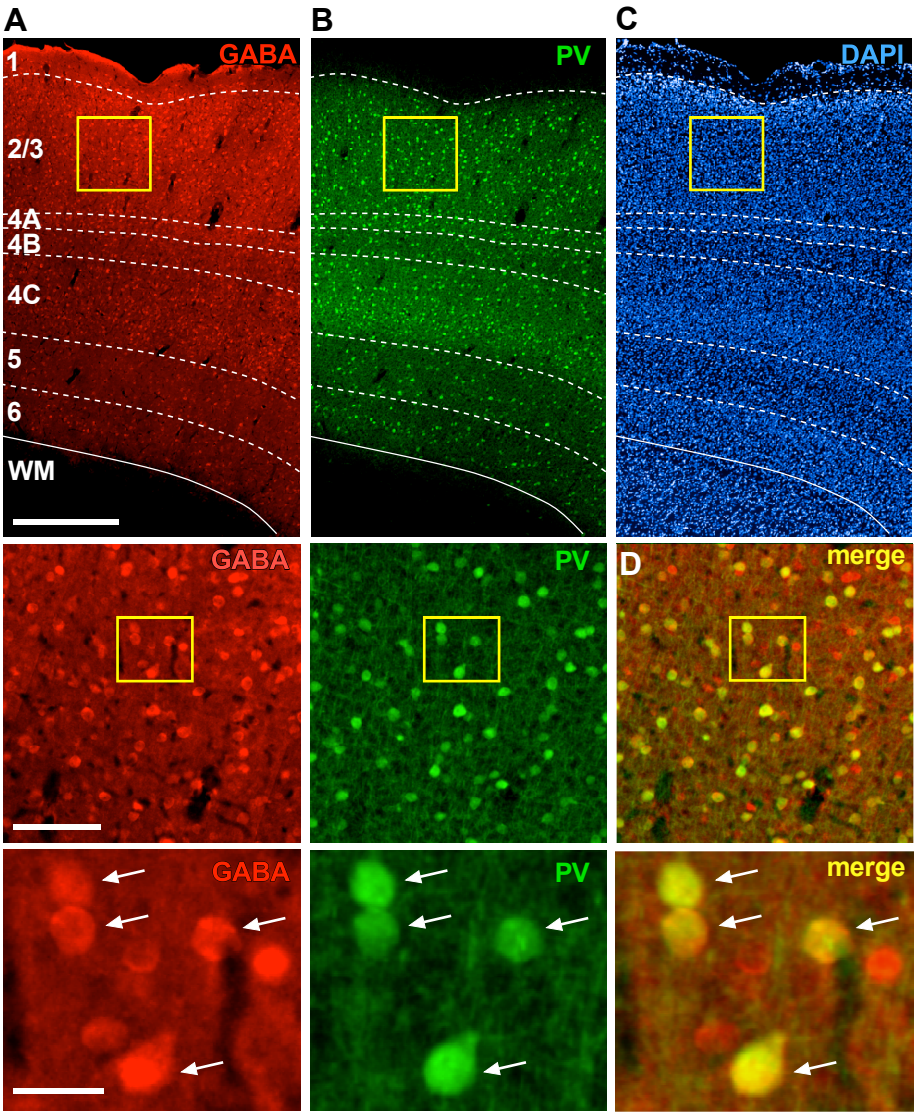


Figure 1

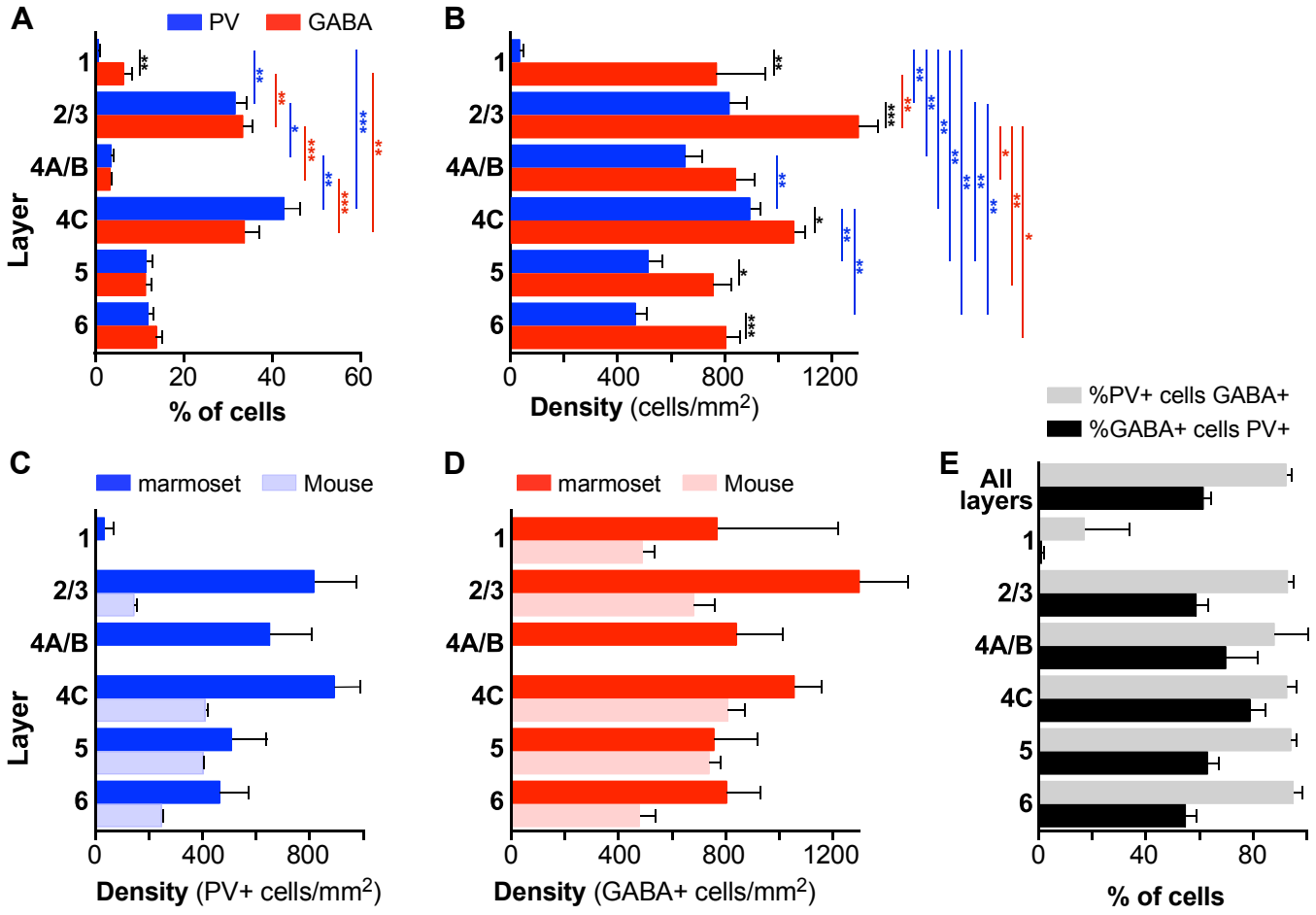

A
PV
GABA
Layer
1
2/3
4A/B
4C
5
6
0
20
40
60
% of cells
B
0
400
800
1200
Density (cells/mm2)
C
marmoset
Mouse
Density (PV+ cells/mm2)
D
marmoset
Mouse
Density (GABA+ cells/mm2)
E
%PV+ cells GABA+
%GABA+ cells PV+
All layers
0
40
80
% of cells

Figure 2

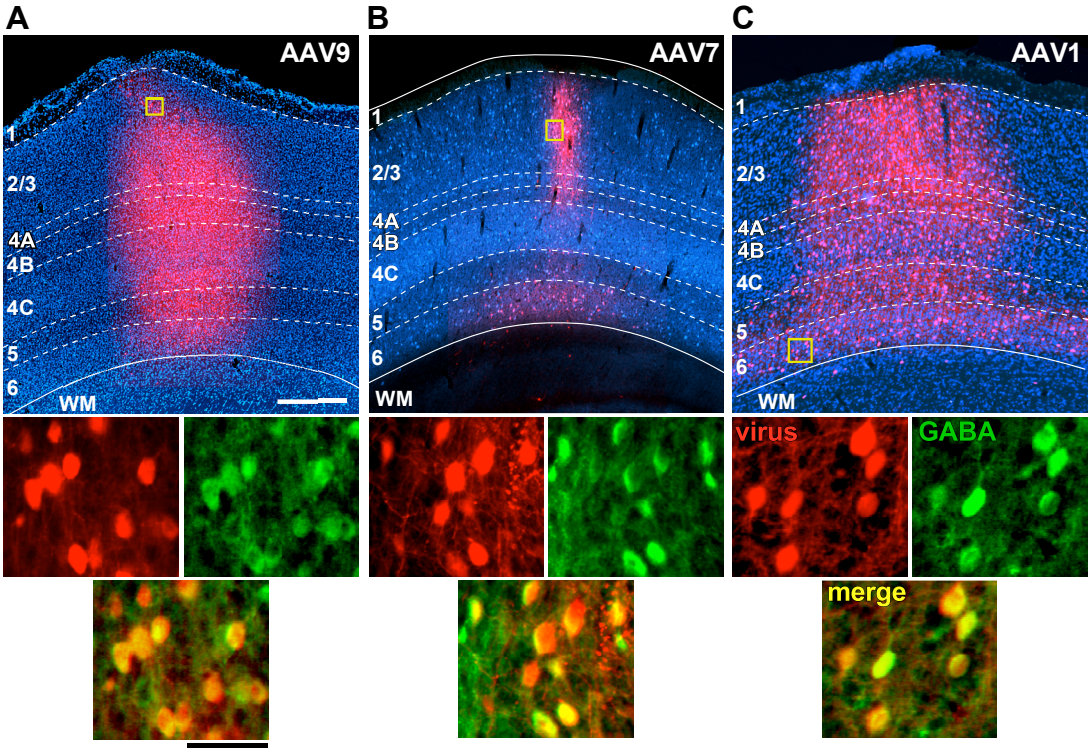


Figure 3

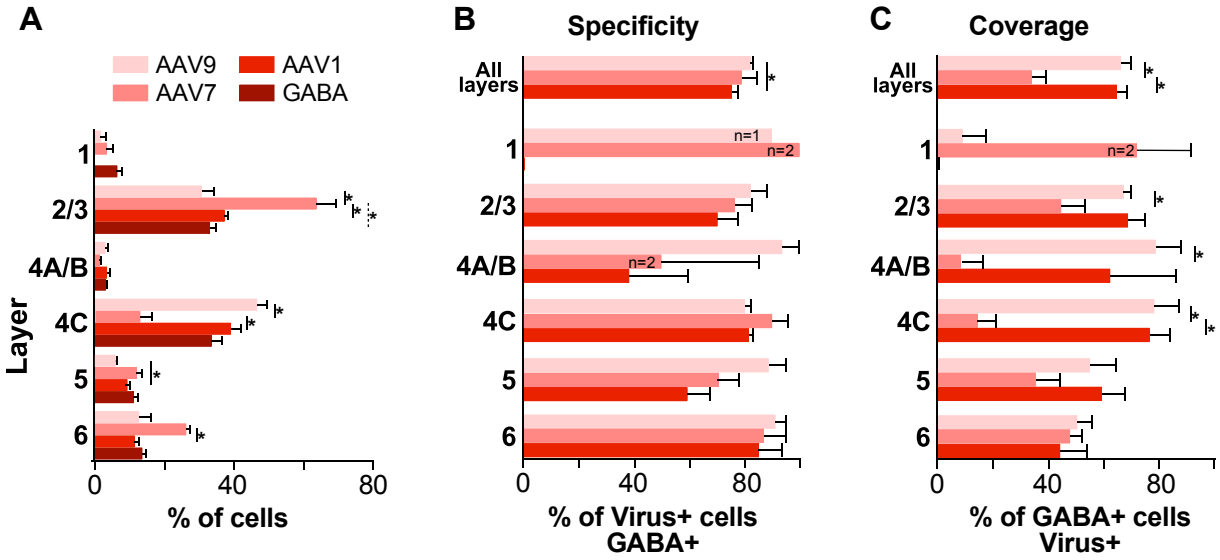

A
AAV9
AAV7
AAV1
GABA
Layer
1
2/3
4A/B
4C
5
6
0
40
80
% of cells
B
Specificity
All layers
n=1
n=2
% of Virus+ cells GABA+
C
Coverage
% of GABA+ cells Virus+

Figure 4

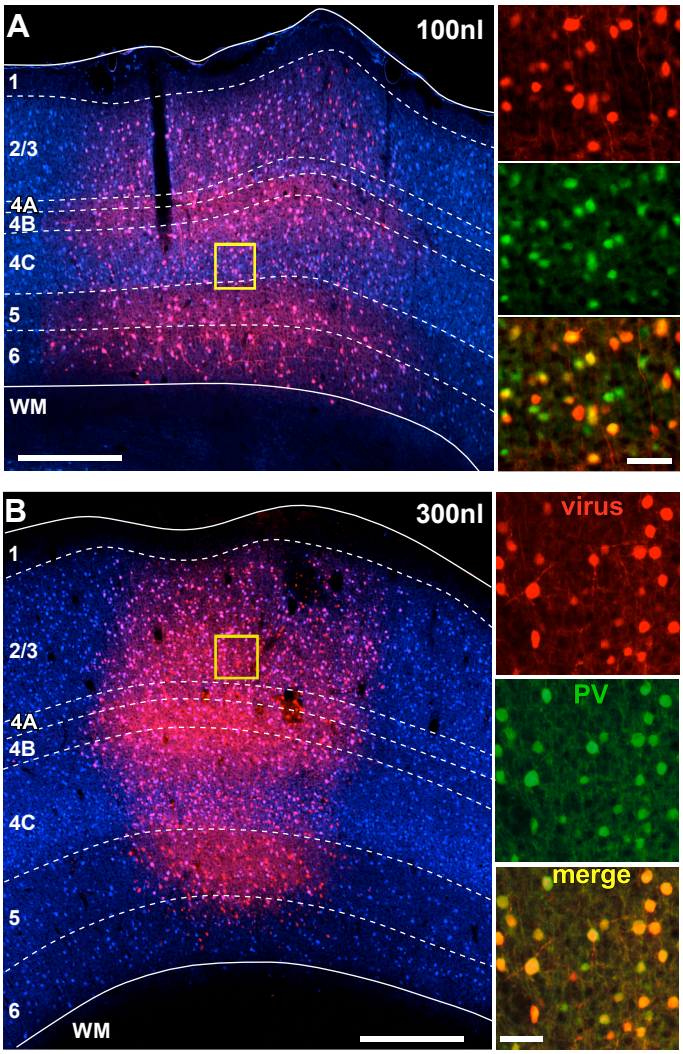

A
100nl
1
2/3
4A
4B
4C
5
6
WM
B
300nl
virus
PV
merge
1
2/3
4A
4B
4C
5
6
WM

Figure 5

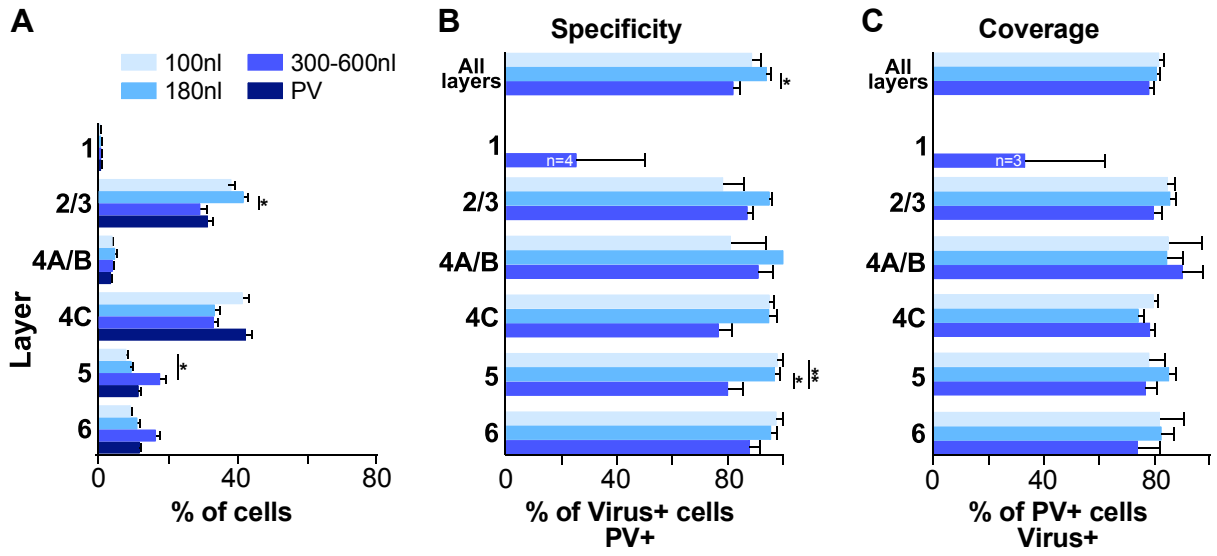

A
100nl
180nl
300-600nl
PV
Layer
1
2/3
4A/B
4C
5
6
*
0
40
80
% of cells
B
Specificity
All layers
n=4
*
**
% of Virus+ cells PV+
C
Coverage
n=3
% of PV+ cells Virus+

Figure 6

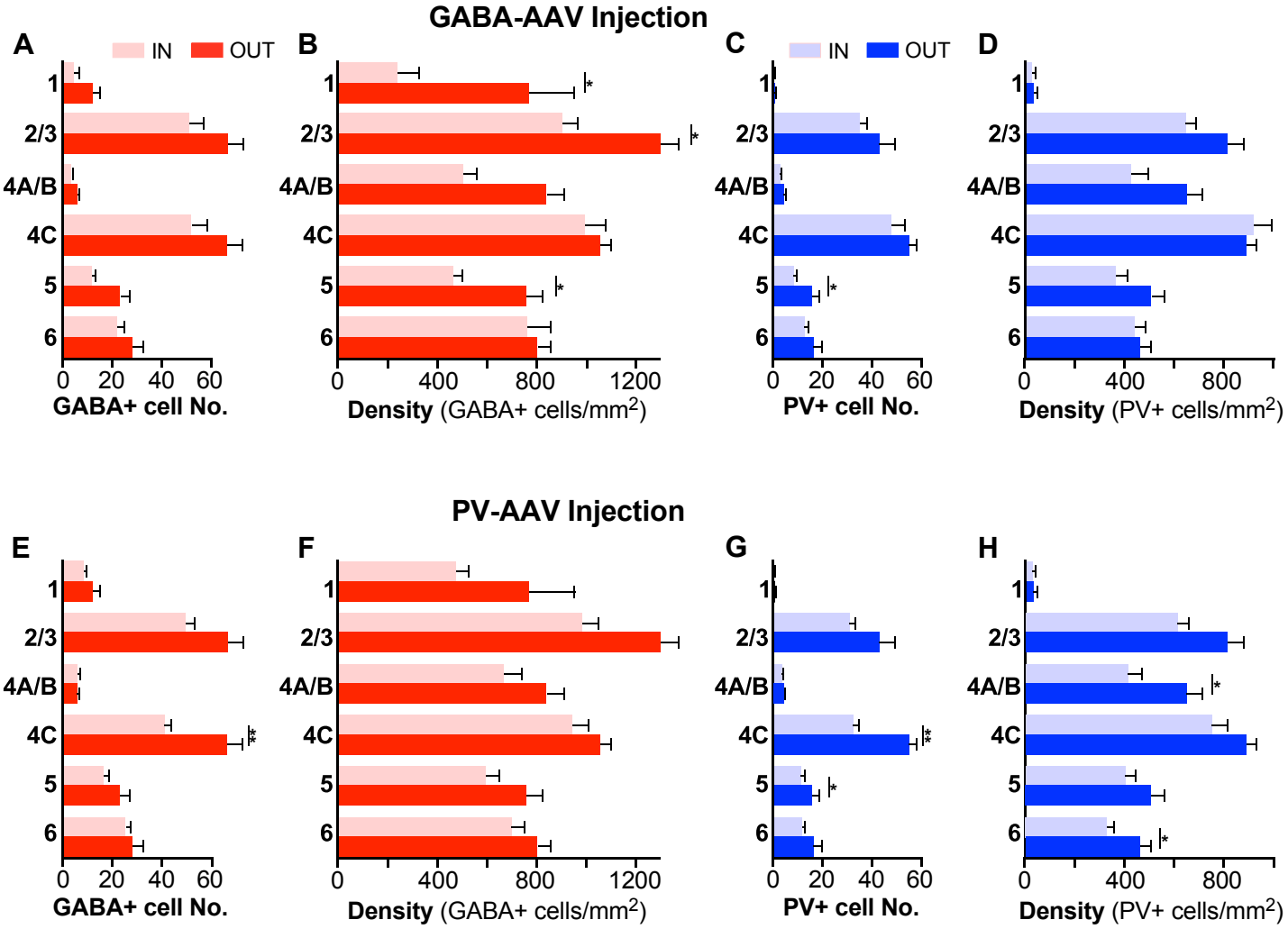
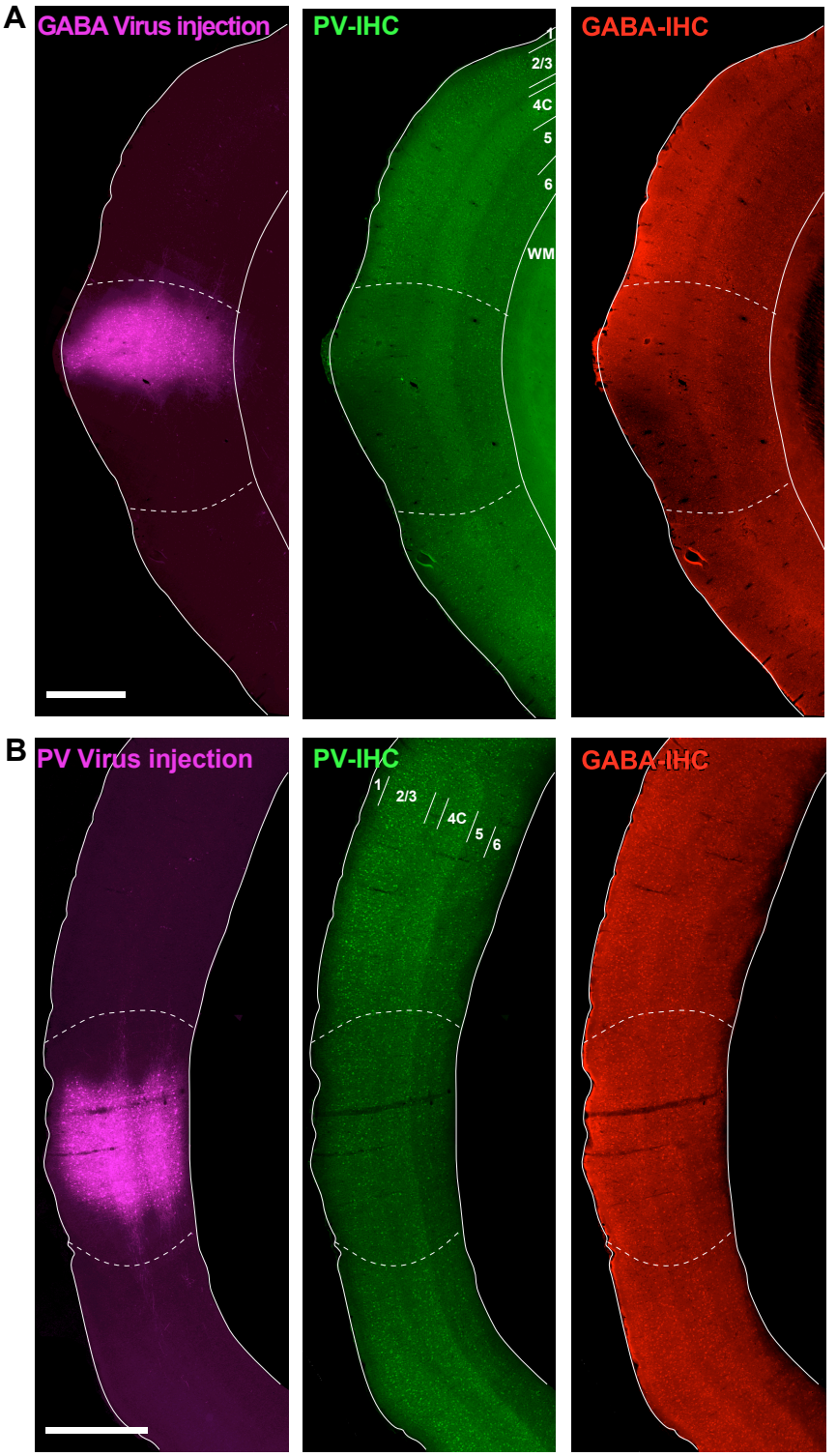

GABA-AAV Injection
A
IN
OUT
1
2/3
4A/B
4C
5
6
0
20
40
60
GABA+ cell No.
B
0
400
800
1200
Density (GABA+ cells/mm2)
C
PV+ cell No.
D
Density (PV+ cells/mm2)
PV-AAV Injection
E
F
G
H

Figure 7



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.