

1 **Enhancer-AAVs allow genetic access to oligodendrocytes and diverse populations of  
2 astrocytes across species**

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27 **Abstract:** Proper brain function requires the assembly and function of diverse populations of neurons  
28 and glia. Single cell gene expression studies have mostly focused on characterization of neuronal cell  
29 diversity; however, recent studies have revealed substantial diversity of glial cells, particularly  
30 astrocytes. To better understand glial cell types and their roles in neurobiology, we built a new suite of  
31 adeno-associated viral (AAV)-based genetic tools to enable genetic access to astrocytes and  
32 oligodendrocytes. These oligodendrocyte and astrocyte enhancer-AAVs are highly specific (usually >  
33 95% cell type specificity) with variable expression levels, and our astrocyte enhancer-AAVs show  
34 multiple distinct expression patterns reflecting the spatial distribution of astrocyte cell types. To provide  
35 the best glial-specific functional tools, several enhancer-AAVs were: optimized for higher expression  
36 levels, shown to be functional and specific in rat and macaque, shown to maintain specific activity in  
37 epilepsy where traditional promoters changed activity, and used to drive functional transgenes in  
38 astrocytes including Cre recombinase and acetylcholine-responsive sensor iAChSnFR. The astrocyte-  
39 specific iAChSnFR revealed a clear reward-dependent acetylcholine response in astrocytes of the  
40 nucleus accumbens during reinforcement learning. Together, this collection of glial enhancer-AAVs will  
41 enable characterization of astrocyte and oligodendrocyte populations and their roles across species,  
42 disease states, and behavioral epochs.

## 43 Introduction

44 Glial cell types play critical roles in CNS development, function, and homeostasis<sup>1,2</sup>. Astrocytes provide  
45 trophic support for neurons<sup>3,4</sup>, coordinate regional wiring patterns<sup>5</sup>, respond to and regulate  
46 neurotransmission<sup>6,7</sup>, and drive repair or pathology after traumatic injury<sup>8–10</sup>. Oligodendrocytes form  
47 myelin sheaths<sup>11</sup>, strengthen circuits<sup>12</sup>, secrete critical neurotrophic factors<sup>13</sup>, and contribute to  
48 pathologic disease progression<sup>14,15</sup>. Transcriptomic characterization of glial cells has revealed an array  
49 of astrocyte and oligodendrocyte cell types, often with pronounced regional signatures<sup>18–20</sup>.  
50 Furthermore, species-specific features have been described<sup>21</sup>, although the functional significance of  
51 these differences is unknown. Glial cell types have also been shown to play critical roles in CNS  
52 diseases ranging from epilepsy<sup>22</sup> to neurodegenerative diseases<sup>23</sup> to cancer<sup>24,25</sup>. To understand how  
53 glia differ between cell types, regions, species, and disease states, a set of tools is needed to grant  
54 targeted genetic access to these specific populations across species.

55 Adeno-associated virus (AAV) vectors are exceptionally useful tools for somatic transgenesis across  
56 mammalian species including human<sup>26–31</sup>. Short enhancer or promoter regulatory elements work  
57 effectively in AAV expression cassettes to drive cell-type selective gene expression in the brain or other  
58 organs<sup>32–36</sup>. Recent work has shown that selective AAVs can be rationally designed by using enhancers  
59 identified from epigenetic datasets that are selectively active and fit in an AAV vector<sup>32–36</sup>. Enhancer-  
60 AAVs were recently developed to target different populations of excitatory and inhibitory neurons in the  
61 brain, and some enhancers have shown successful targeting of glial cell populations as well<sup>37,38</sup>.  
62 However, the field largely relies on glial promoters that have some undesirable characteristics, most  
63 notably loss of specificity or change in strength in different contexts as seen for astrocytic *GFAP*  
64 promoter fragments<sup>39–41</sup>. Furthermore, single cell genomics studies have revealed region-specific  
65 astrocyte cell types for which no current tools are available<sup>18–20</sup>.

66 Here we present a collection of enhancer-AAVs that selectively target astrocytes and oligodendrocytes.  
67 Twenty-five astrocyte and 21 oligodendrocyte enhancer AAVs were identified from mouse and human  
68 neocortical epigenetic data that produced reporter expression that was highly specific for the intended  
69 populations, often labeling more than half of the intended cells in the area, and with a wide range of  
70 expression strengths. Multiple astrocyte-targeting vectors exhibited distinct CNS region-specific  
71 expression patterns, whereas oligodendrocyte-selective vectors generally drove expression throughout  
72 the entire CNS. Several enhancer-AAVs maintained selective expression for astrocytes or  
73 oligodendrocytes across rat and macaque. Lastly, several astrocyte tools were adapted to drive  
74 expression of functional transgenes like Cre or the detection of neurotransmitters to reveal the role of  
75 astrocytes in neurobiology. We used astrocyte-selective AAV expressing iAChSnFR<sup>42</sup> to measure the  
76 dynamics of acetylcholine in astrocytes of the nucleus accumbens during reinforcement learning. This  
77 collection of tools opens up new opportunities for selective labeling and functional interrogation of glial  
78 cell types across species and disease states, and could have translational applications via AAV-based  
79 therapeutics<sup>43–45</sup>.

## 80 Results

81 *Generation of astrocyte- and oligodendrocyte-specific enhancer-AAVs.* We identified putative  
82 enhancers specific for astrocytes and oligodendrocytes from single cell/single nucleus assay for

83 transposase-accessible chromatin (sc/snATAC-seq<sup>35,36</sup>) and single nucleus methyl-cytosine sequencing  
84 (snmC-seq) studies from neocortex<sup>46–49</sup>. Thousands of astrocyte- and oligodendrocyte-selective  
85 scATAC-seq peaks were identified previously in both human middle temporal gyrus (MTG) and mouse  
86 primary visual cortex (ViSp), averaging approximately 300-600 bp in size (**Figure 1A**). Additional  
87 ATAC-seq datasets confirmed these peaks<sup>50,51</sup>. Generally, astrocyte and oligodendrocyte candidate  
88 enhancers were accessible in non-neuronal cells but not in neuronal cells across the human  
89 forebrain<sup>35,50</sup> (**Extended Data Figure 1A–F**), and in the corresponding astrocyte or oligodendrocyte  
90 subclasses across the mouse forebrain without strong cell type preferences<sup>51</sup> (**Extended Data Figure**  
91 **1G–J**).

92 We used three strategies to identify putative enhancers for testing: “high specificity”, “high strength”,  
93 and “marker gene” (**Figure 1B–E**). The “high specificity” nomination criteria (gold square or star icons)  
94 required enhancers and their orthologs to show accessibility specifically for both mouse and human  
95 astrocytes or oligodendrocytes but not other cell types<sup>35,36</sup>. In addition, we required that these putative  
96 enhancers not be detected in demethylated genomic regions in both mouse and human neuron  
97 populations<sup>47</sup>. A small number of these “high specificity” enhancers also showed specific demethylation  
98 in bulk human and mouse glial cells<sup>46</sup> (marked by gold star icons). “High strength” putative enhancers  
99 were selected on the basis of strong astrocyte-specific peaks using only mouse scATAC-seq data<sup>36</sup>,  
100 with strength measured by accessibility read count within peaks. Finally, “marker gene” putative  
101 enhancers showed specific and strong accessibility near known astrocyte- and oligodendrocyte-specific  
102 marker genes.

103 We tested putative enhancer function in AAV vectors upstream of a minimal promoter driving the  
104 reporter SYFP2 and evaluated expression throughout the mouse brain after systemic administration of  
105 PHP.eB-serotyped AAVs. Enhancer-AAVs that showed anticipated reporter expression, were further  
106 evaluated for specificity, completeness of expression, and cross-species activity (**Figure 1C** and  
107 **Extended Data Table 1**). All three strategies were effective, with approximately half of the candidates  
108 for both cell types yielding astrocyte or oligodendrocyte expression patterns during primary screening.  
109 Moreover, expression of many of those enhancers was confirmed to be on-target by antibody staining  
110 and/or scRNA-seq (**Figure 1F,G** and see below).

111 *A collection of astrocyte-specific enhancer-AAVs.* We screened 50 candidate astrocyte-specific  
112 enhancer-AAVs, and 25 (50%) of them labeled astrocytes specifically with SYFP2 expression  
113 (**Extended Data Figure 2**). Astrocyte-specific enhancer-AAVs showed a range of expression strengths  
114 and patterns (**Figure 2A–I**) and vectors were categorized based on their labeling as: “Most of the CNS”,  
115 “Regional”, “Scattered”, “Weak” and “Mixed specificities”. “Most of the CNS” astrocyte enhancer-AAVs,  
116 including eHGT\_380h and the human GFAP promoter (GfaABC1D<sup>52</sup>), labeled cells with astrocyte  
117 morphology in both brain and spinal cord (SpC, **Figure 2A–B, G–H**). Other examples in this category  
118 include eHGT\_387m, eHGT\_390h, eHGT\_390m, and the synthetic element ProB12<sup>37</sup> (**Extended Data**  
119 **Figure 2**). “Regional” astrocyte enhancer-AAVs showed regionally restricted expression, such as  
120 eHGT\_385m that labeled astrocytes primarily in the telencephalon (**Figure 2C, I, Extended Data**  
121 **Figure 2**). Other “Regional” enhancer-AAVs labeled astrocytes in subcortical domains but not in the  
122 telencephalon, such as eHGT\_381h and MGT\_E160m (**Extended Data Figure 2**); while eHGT\_375m  
123 only labeled Bergmann glia, specialized astrocytes in the cerebellar cortex (CBX, **Figure 2D**).

124 Interestingly, enhancers MGT\_E120m and MGT\_E160m labeled astrocytes in nearly mutually exclusive  
125 regions (**Extended Data Figure 3**). “Scattered” enhancer-AAVs labeled astrocytes strongly but  
126 sparsely in most brain regions. These enhancer-AAVs include eHGT\_374m (**Figure 2E**) and its  
127 ortholog eHGT\_374h (**Extended Data Figure 2**). Enhancer-AAVs labeled as “Weak” gave astrocyte-  
128 specific patterns with low expression of SYFP (e.g., eHGT\_373m and 386m, **Extended Data Figure 2**).  
129 Last, we designated several enhancer-AAVs as “Mixed specificities” because they labeled astrocytes  
130 and neurons. For example, MGT\_E118m labels many astrocytes strongly and specifically within the  
131 telencephalon, but also labels neurons strongly in non-telencephalic structures like midbrain (MB), deep  
132 cerebellar nuclei (CBN), and globus pallidus, external segment (GPe) (**Figure 2F, Extended Data**  
133 **Figure 2**).

134 We quantified the specificity of many of these astrocyte-specific enhancer-AAVs using multiple  
135 independent techniques. First, we characterized SYFP2-expressing cells with immunohistochemistry  
136 (IHC) for Sox9, a marker of astrocytes throughout the brain<sup>53</sup> (**Figure 2J-O**). Many of the astrocyte-  
137 specific enhancer-AAV vectors show high specificity, which we define as >80% specificity for the target  
138 cell population<sup>35</sup>. Astrocyte-specific enhancer-AAVs are usually >95% specific, and often >99% specific  
139 in VISp for Sox9-expressing astrocytes (**Figure 2P**). Second, we also observed high specificity when  
140 we isolated single SYFP2+ cells by flow cytometry and profiled them by scRNA-seq (**Figure 2P**).  
141 Additionally, we assessed completeness of astrocyte labeling using IHC, and we observed that vectors  
142 scored as “Most of CNS” often label >50% of astrocytes in VISp, but “Weak” or “Scattered” vectors  
143 labeled many fewer astrocytes (**Figure 2P**). “Regional” vectors showed differing completeness across  
144 brain regions as expected (**Figure 2P**). Whole-brain serial two-photon tomography (STPT) of mouse  
145 brain transduced with astrocyte-specific enhancer-AAVs demonstrated distinct astrocyte morphologies  
146 in multiple brain regions (**Figure 2Q**). Thus, our collection of astrocyte-specific enhancer-AAVs are  
147 diverse with regard to the density of labeled cells, expression strength, and regionalization.

148 *A collection of oligodendrocyte-specific enhancer-AAVs.* We screened 43 candidate oligodendrocyte  
149 enhancers, of which 21 (49%) gave oligodendrocyte-specific expression patterns (**Extended Data**  
150 **Figure 4**). Unlike the astrocyte collection, the oligodendrocyte enhancer-AAVs all produced similar  
151 expression patterns throughout the gray matter and white matter tracts without any obvious regional  
152 specificity (**Figure 3A-I**), consistent with the majority of oligodendrocytes in scRNA-seq profiling  
153 studies<sup>20</sup>. Oligodendrocyte-specific enhancer-AAV vectors ranged in expression from strong (for  
154 example eHGT\_410m, eHGT\_641m, eHGT\_395h, and eHGT\_396h **Figure 3A-D**) to moderate (for  
155 example eHGT\_409h, **Figure 3E**, and the Myelin Basic Protein (MBP) promoter<sup>27,54</sup>, **Extended Data**  
156 **Figure 4**) to weak (for example eHGT\_400h, **Figure 3F**). These vectors also labeled oligodendrocytes  
157 throughout the spinal cord (**Figure 3G-I**). We confirmed molecular oligodendrocyte characteristics of  
158 the vector-labeled cells by co-staining with CC1, a marker of oligodendrocytes<sup>55</sup>, which showed most  
159 vectors were highly specific across multiple brain regions (**Figure 3J-O**). Quantification by  
160 immunohistochemistry and scRNA-seq on sorted SYFP2-expressing cells showed >99% specificity and  
161 >45% completeness of labeling in VISp for multiple vectors (**Figure 3P**). STPT demonstrated  
162 myelinating oligodendrocyte morphologies in multiple parts of mouse brain (**Figure 3Q**). This collection  
163 of oligodendrocyte-specific enhancer-AAV vectors shows a diversity of expression strengths, but  
164 appears to label a homogeneous population of oligodendrocytes.

165 *Transcriptomic identities of astrocytes and oligodendrocytes.* To investigate distinctions among  
166 enhancer-AAV-transduced cells, we performed SMARTerV4 scRNA-seq on sorted SYFP2-expressing  
167 cells. We characterized 2040 cells from 47 mice injected with 31 different enhancer-AAVs (1-2 mice per  
168 enhancer-AAV). After removing low-quality single-cell transcriptomes and cells not expressing the  
169 SYFP2 transcript, we focused our analysis on 1946 high-quality single cells. Astrocytes and  
170 oligodendrocytes separated in the UMAP space, as did astrocytes sorted from the distinct brain regions  
171 including VISp, midbrain/hindbrain (MB/HB), and CBX (**Figure 4A**). The molecular distinctions among  
172 regional astrocyte populations agree with findings from recent whole-brain atlases<sup>20</sup>. Indeed, mapping  
173 to a whole-brain taxonomic atlas indicates that, with high confidence, VISp-profiled astrocytes are  
174 predominantly mapped to the *Gja1*- and *Gfap*-expressing cluster “5112 Astro-TE NN\_3”<sup>20</sup>, whereas  
175 MB/HB-profiled astrocytes marked by eHGT\_381h and MGT\_E160m mapped primarily to the *Gja1*-  
176 and *Agt*-expressing cluster “5109 Astro-NT NN\_2”<sup>20</sup>. Likewise, the CBX-profiled Bergmann glia  
177 astrocytes mapped primarily to cluster identity “5102 Bergmann NN” as expected (**Figure 4B-D**). In  
178 contrast, labeled oligodendrocytes largely mapped to *Cldn11*- and *Mog*-expressing and most abundant  
179 oligodendrocyte cluster “5158 MOL NN”<sup>20</sup> regardless of the enhancer used to label them (**Figure 4E**),  
180 confirming that oligodendrocyte enhancer-AAVs label a largely homogeneous population of  
181 oligodendrocytes.

182 To understand the molecular regulation of our astrocyte and oligodendrocyte-selective enhancer-AAVs,  
183 we performed de novo motif detection on a collection of specific and strong astrocyte and  
184 oligodendrocyte enhancers (n = 15 each) using MEME-CHIP<sup>56</sup>. This analysis yielded one motif  
185 occurring in the majority of enhancers in each set (**Figure 4F,G**). These motifs had strong enrichments  
186 as measured by MEME-CHIP E-values less than 0.01, corresponding to the expected number of  
187 equally sized motifs of same or greater log likelihood ratio occurring in a set of random sequences of  
188 equal nucleotide content. We mapped these motifs against known transcription factor (TF) motif  
189 databases<sup>57-59</sup>, which revealed top matches to the Zic family consensus motifs for astrocytes (JASPAR  
190 accession numbers MA0697.2, MA1628.1, and MA1629.1; average of these three shown) and the Sox  
191 family motif for oligodendrocytes (JASPAR accession number MA0442.1 [Sox10 shown], and also  
192 Uniprobe accession numbers UP00030.1 and UP00062.1; **Figure 4F,G**). These analyses suggest that  
193 Zic and Sox family transcription factors might be key determinants of astrocyte versus oligodendrocyte  
194 identity in the CNS<sup>60-63</sup>. Moreover, Zic and Sox gene family members were differentially expressed  
195 between the profiled astrocytes and oligodendrocytes (*Zic5* 32-fold mean difference, non-parametric  
196 Wilcoxon rank-sum test  $W = 577624$ ,  $p < 1e-16$ ; *Sox10* 455-fold mean difference, non-parametric  
197 Wilcoxon rank-sum test  $W = 9838$ ,  $p < 1e-16$ ; **Figure 4H**). These results suggest *Zic5* and *Sox10* play  
198 key roles in determining specificity of these glial enhancer-AAVs.

199 *Regional expression correlates with astrocyte cell type distribution.* Using STPT imaging, we observed  
200 astrocyte-specific enhancer-AAVs to have two distinct expression patterns within the basal ganglia  
201 circuit. Several enhancer-AAVs showed elevated expression in astrocytes of the dorsolateral striatum  
202 and depletion in the globus pallidus (GP; **Figure 4I-K**), and several other enhancer-AAVs drove  
203 stronger transgene expression in astrocytes in the GP compared with those of the lateral striatum  
204 (**Figure 4L-N, Extended Data Figure 3**). To determine if these enhancer-AAV expression patterns  
205 correspond to transcriptomically-defined astrocyte cell types, we evaluated the spatial distributions of all

206 astrocyte cell types in the mouse whole brain taxonomy<sup>20</sup>. Interestingly, two closely related astrocyte  
207 cell types were strongly enriched in the dorsolateral striatum and cortex (“5112 Astro-TE NN\_3” and  
208 “5113 Astro-TE NN\_3”), while another was strongly enriched in the GP (“5109 Astro-NT NN\_2”),  
209 demarcating the same boundaries observed with the collection of astrocyte enhancer-AAVs (**Figure**  
210 **4O-R**).

211 *Measuring and optimizing enhancer strength.* In some cases, enhancer-AAV might not drive sufficient  
212 levels of a transgene to functionally affect the target cell. We sought to boost the expression levels of  
213 some enhancers by assembling concatemers of “core” sequences. These core sequences are  
214 responsible for the selective expression patterns and are often found in the central third of the original  
215 enhancer region identified by snATAC-seq<sup>35</sup> (that is, ~100-200 bp core from ~300-600 bp original  
216 enhancer, **Figure 5A**). We observed that concatenation of the core can substantially increase  
217 expression from the original enhancer, such as eHGT\_387m concatenated to 3xCore1(387m) (**Figure**  
218 **5B,C**), eHGT\_390h concatenated to 3xCore2(390h) (**Figure 5D,E**), or eHGT\_390m concatenated to  
219 3xCore2(390m) (**Figure 5F,G**) while retaining similar expression patterns (**Figure 5H-J**) and cell type  
220 specificity (**Figure 5K**). However, concatenation sometimes resulted in a less dramatic effect on  
221 expression, (e.g., 3xCore(410m) and 3xCore(641m); **Extended Data Figure 4**).

222 We established single-cell measurements of reporter expression to compare enhancer strengths. We  
223 found that single-cell reporter fluorescence by flow cytometry correlated with vector read counts from  
224 scRNA-seq for both astrocytes and oligodendrocytes (astrocyte Pearson correlation coefficient = 0.63, t  
225 = 3.97, df = 24, p < 0.001 by correlation t-test; oligodendrocyte Pearson correlation coefficient = 0.53, t  
226 = 2.82, df = 20, p < 0.05 by correlation t-test; **Figure 5L,M, Extended Data Figure 5**). These  
227 measurements revealed that several concatenated enhancer-AAVs, including 3xCore2(390m) and  
228 3xCore2(390h), drove the strongest expression among the vectors we have tested (**Figure 5L**),  
229 consistent with the microscopy results (**Figure 5B-J**). Conversely, MB/HB (eHGT\_381h and  
230 MGT\_E160m) and Bergmann glia (eHGT\_375m) astrocyte enhancers have among the weakest  
231 expression levels we have tested (**Figure 5L**), likely a consequence of selecting cortical glial  
232 enhancers.

233 *Predictability of enhancer-AAV expression across tissues and disease states.* Recent work suggests  
234 that AAV-mediated transduction and high transgene expression in organs such as the liver and dorsal  
235 root ganglia is associated with toxicity<sup>31,64</sup>. We tested if we could predict off-target activity from  
236 enhancer accessibility profiles in a human body-wide epigenetic dataset<sup>65</sup>. Different astrocyte-specific  
237 enhancers showed either moderate or low accessibility across many body organs (**Figure 6A**). We  
238 assessed off-target transgene expression in liver after intravenous delivery since PHP.eB capsid  
239 transduces the liver<sup>66</sup>. We observed that astrocyte enhancers with moderate levels of accessibility in  
240 liver (eHGT\_381h, 371m, 371h, and 386m) expressed SYFP2 in many hepatocytes, whereas the  
241 enhancers with negligible liver accessibility (eHGT\_387m, 375m, 390h, and 390m) expressed SYFP2  
242 in only few hepatocytes (**Figure 6B-C**). In contrast, the GFAP promoter drives expression in many  
243 hepatocytes, and that is not predictable from any epigenetic or transcriptomic atlases. Finally, we find  
244 one astrocyte enhancer (eHGT\_380h) that is predicted to have negligible liver accessibility but  
245 expresses SYFP2 in many hepatocytes (**Figure 6C**). Thus, the whole-body epigenetic dataset<sup>65</sup>  
246 predicts liver expression from astrocyte enhancer-AAV vectors for 89% (8/9) of vectors tested.

247 *GFAP* expression can change expression in the context of disease or injury<sup>9</sup>, and the synthetic *GFAP*  
248 promoter can change specificity when delivering different transgenes<sup>39–41</sup>, suggesting this might be a  
249 poor tool for genetic access to astrocytes in disease. We compared *GFAP* promoter and one of our  
250 best enhancer-AAVs (eHGT\_390m) in Dravet syndrome model mice since they have strong epilepsy-  
251 associated reactive astrogliosis<sup>67</sup>. We injected SYFP2-expressing enhancer-AAVs into these mice prior  
252 to the Dravet syndrome critical period of high susceptibility to seizures and mortality at P21 and  
253 analyzed tissues at P42 (**Figure 6D**). Significant hippocampal gliosis was seen in *Scn1a*<sup>R613X/+</sup> Dravet  
254 syndrome model mice, revealed by elevated endogenous GFAP immunoreactivity in all hippocampal  
255 layers (**Figure 6E**). Concomitant with this gliosis, the *GFAP* promoter-driven AAV reporter changed its  
256 expression pattern. Normally, this promoter drives moderate levels of astrocyte-specific reporter  
257 expression. However, in the Dravet mice experiencing epilepsy, expression strength in astrocytes was  
258 considerably elevated, and ectopic expression was observed in many dentate gyrus granule cell  
259 neurons (**Figure 6E**). In sharp contrast, the eHGT\_390m enhancer-AAV vector maintains astrocyte  
260 specificity at moderate levels despite the profound reactive gliosis in these diseased animals (**Figure**  
261 **6E**). These results suggest that some enhancer-AAV vectors can provide astrocyte-specific expression  
262 across body organs and across disease states, though this may not be true for all enhancers or disease  
263 states.

264 *Astrocyte-specific AAV-Cre*. AAVs that can selectively drive Cre recombinases are valuable tools for  
265 mouse genetics since the AAV can be delivered somatically for cell type-specific recombination of  
266 floxed alleles. As a proof of principle, we used eHGT\_390m to express a partially disabled R297T  
267 mutant Cre recombinase in *Ai14* reporter mice<sup>68,69</sup> (**Figure 7A-D**). This vector produced 99% astrocyte -  
268 specific recombination in many parts of mouse brain, including medulla, midbrain, hippocampus, and  
269 cortex. Despite the high astrocyte specificity in many brain regions, neurons were labeled in the  
270 thalamus, pontine gray, and the cerebellum (**Extended Data Figure 2**). Thus, the astrocyte-specific  
271 recombination observed in most of the brain will allow this tool to be used in combination with Cre  
272 reporters to better understand the roles of astrocytes in brain biology, but other tools will be required for  
273 drive astrocyte-selective Cre in certain brain regions.

274 *Cross-species genetic access to astrocytes and oligodendrocytes*. We tested whether several glial-  
275 selective enhancer-AAV vectors could maintain specific expression across species. We first tested  
276 conservation in neonatal rats after ICV administration (**Figure 7E**). We found that eHGT\_641m- and  
277 3xCore(410m)-driven AAV vectors labeled rat cortical oligodendrocytes with high specificity (91 and  
278 71% specific, **Figure 7F,G**). We also observed that eHGT\_387m and 390m labeled rat cortical  
279 astrocytes with high specificity (96 and 87% specific, **Figure 7H,I**). In addition, an optimized  
280 3xCore2(390m) vector containing 4X2C miRNA binding sites to prevent any unwanted expression in  
281 excitatory neurons<sup>70</sup> expressed SYFP2 strongly and specifically throughout the rat forebrain (98%  
282 specific, **Figure 7J,K**). Note that astrocyte labeling completeness and spread to caudal brain structures  
283 could not be assessed since ICV administered virus resulted in uneven spread. We also tested some  
284 other vectors in rat which appeared to lose specificity for astrocytes (data not shown). Thus, some but  
285 not all vectors identified in our mouse screen maintained specificity in rats after ICV injection into  
286 neonates.

287 We extended these cross-species tests to non-human primate (NHP), using multisite intraparenchymal  
288 injections (**Figure 7L**). We administered eHGT\_410m AAV vector into motor cortex and observed cell  
289 morphologies of myelinating oligodendrocytes throughout the cortical column (**Figure 7M-N**).  
290 Interestingly, we also observed SYFP2-expressing cells with a different morphology: one to three  
291 processes that spiral around stretches of tubular structures approximately 15-20 microns in diameter  
292 often running perpendicular to the cortical pial surface (**Figure 7O**). These tubular structures have not  
293 yet been defined and were not observed in mouse or rat testing, but both morphological types of  
294 SYFP2-expressing cells co-expressed the oligodendrocyte marker SOX10 by mFISH with high  
295 specificity (94%, **Figure 7P**).  
296 We also injected the somatosensory cortex with the astrocyte-specific eHGT\_390m AAV vector and  
297 observed many SYFP2-expressing cells with astrocyte morphology throughout the cortical column  
298 (**Figure 7Q**; note a small number of large layer 5 pyramidal neurons labeled as well, which we did not  
299 observe in mouse testing). SYFP2-expressing astrocytes co-expressed GFAP either in parenchyma  
300 (**Figure 7R**) or in apposition to a large blood vessel (**Figure 7S**), and some showed fibrous morphology  
301 in white matter (**Extended Data Figure 6**). Enhancer-AAV-labeled astrocytes also expressed the  
302 astrocyte-specific transcript *FGFR3* with high specificity (92%) and about half of the *FGFR3*+ gray  
303 matter astrocytes were labeled through the whole cortical depth near the injection site (51%) (**Figure**  
304 **7T**). These studies suggest that enhancer-AAV vectors provide specific and dependable genetic access  
305 to astrocytes and oligodendrocytes across multiple species and reveal morphological glial features not  
306 observed in the mouse.  
307 *Astrocyte specific sensing of cholinergic signals in the nucleus accumbens during behavior.* We next  
308 asked whether our vectors would drive sufficient expression to obtain functional signals in a cell-type  
309 specific manner. We created a vector driving astrocyte-specific expression of the acetylcholine indicator  
310 iAChSnFR<sup>42</sup> (**Figure 8A,B**), to detect extracellular acetylcholine fluctuations in the nucleus accumbens  
311 (NAc) in an awake and behaving animal using fiber photometry. After stereotaxic injection, we  
312 implanted optical fibers above the injection site to perform fiber photometry. We trained mice to perform  
313 a dynamic foraging reinforcement learning task while we recorded photometry signals to assess bulk  
314 acetylcholine fluctuations in the NAc. In the task, water-restricted mice chose freely between two lick  
315 ports for a water reward after an auditory cue. Reward probabilities of the two lick ports were changed  
316 in a block-design manner, which resulted in both rewarded and unrewarded trials (**Figure 8C**). During  
317 these trials, the astrocyte-specific iAChSnFR vector drove sufficient expression to observe fluorescence  
318 intensity fluctuations (**Figure 8D**) which can be seen to differ during individual rewarded and  
319 unrewarded licks (**Figure 8D**, bottom left). Both rewarded and unrewarded trials showed an increase in  
320 fluorescent signal at the time of choice, followed by a deviation in signal depending on whether the trial  
321 was rewarded (**Figure 8E**). Astrocyte acetylcholine signals decreased more in rewarded trials than in  
322 unrewarded trials (**Figure 8E**). In summary, these results indicate that glial-selective enhancer-AAVs  
323 can be applied to measure functional acetylcholine dynamics in the NAc.

324

325 **Discussion**

326 Flexible and dependable tools to target glial cell populations will be essential to understand their  
327 diverse roles in brain biology. Here we report a collection of astrocyte- and oligodendrocyte-specific  
328 enhancers that can be used in AAV vectors and applied across species. Most of these enhancer-AAVs  
329 generated highly specific labeling of astrocytes or oligodendrocytes, and often substantial  
330 completeness of labeling. Detailed characterization revealed the enhancers showed a range of  
331 expression strengths, and the astrocyte enhancers frequently exhibited regional enrichment and  
332 differences in labeling densities. We demonstrate that this enhancer-AAV toolset can be applied: 1)  
333 across species in mouse, rat and monkey, 2) in epileptic mice where gliosis is occurring without losing  
334 specificity, and 3) to deliver Cre selectively to many astrocyte populations, and 4) to measure circuit  
335 dynamics with a neurotransmitter sensor *in vivo*. As a result, these glial enhancer-AAVs will be useful  
336 for interrogating the roles of these glial cell types in health and disease.

337 *Lesson learned from screening.* Several lessons were learned through the process of screening for  
338 astrocyte and oligodendrocyte enhancer-AAVs. First, multiple selection criteria can identify strong glial  
339 enhancer-AAVs. Excellent functional enhancers were derived from genome-wide peak selection across  
340 mouse and human datasets of distinct epigenetic modalities, peak selection from one mouse dataset  
341 based on peak strength, or peak selection only near marker genes. Second, screening one enhancer-  
342 AAV at a time can be efficient for the identification of useful enhancer-AAVs. Nearly half of the  
343 candidate enhancers proved to be specific for the targeted type, and this created a large and diverse  
344 library of new enhancer-AAVs that labeled astrocytes or oligodendrocytes in different ways. Third, while  
345 enhancers were identified from neocortex at the subclass level, brain-wide patterns match well to whole  
346 brain cell gene expression atlas patterns<sup>20</sup>. Specifically, oligodendrocytes appear homogeneous across  
347 the brain, while astrocytes show prominent regional enrichments and differences in cell density. Fourth,  
348 body-wide specificity of enhancer-AAV expression can be predicted based on body-wide epigenetic  
349 datasets<sup>65</sup>. Thus, off-target expression can be predicted and limited during the enhancer selection  
350 process, and enhancers can be identified that label different cell types throughout the body. These  
351 attributes will make enhancer-AAVs a valuable tool for precision gene therapy where only the cell  
352 population of interest is expressing the therapeutic transgene.

353 *Enhancer-AAVs show diverse expression patterns.* We tested a large collection of astrocyte-selective  
354 enhancer-AAVs and saw a diversity of expression patterns. Some astrocyte enhancer-AAVs  
355 predominantly labeled in telencephalic structures, some in MB/HB structures, and others showed  
356 sparse “Scattered” but uniform expression. Within the forebrain, we also observed multiple enhancer-  
357 AAVs that showed mutually exclusive enriched or depleted expression in dorsolateral striatum or the  
358 globus pallidus. These regional differences were reflected by region-specific astrocyte transcriptomic  
359 profiles in agreement with recent results<sup>20</sup>. The GPe astrocytes express high levels of GABA uptake  
360 gene *Slc6a11*<sup>18,71,72</sup> while striatal and cortical, but not GPe astrocytes express high levels of glutamate  
361 uptake gene *Slc1a2*<sup>18,72,73</sup>. This suggests that the astrocytes help selectively maintain glutamate or  
362 GABA tone depending on the brain structure and astrocyte cell type. The functional roles of astrocytes  
363 in different brain regions will require additional experiments that our collection of enhancer-AAVs may  
364 facilitate. Similarly, it is not yet clear what produces “Scattered” astrocyte reporter expression, and the  
365 answer will await future experiments.

366 *Enhancer-AAVs can be optimized to improve vector function.* Achieving functional levels of transgene  
367 expression is critical for applying enhancer-AAV tools to learn new biology and deliver gene therapies.  
368 We show that two astrocyte enhancers could be optimized through generation of a triple concatenated  
369 core to produce significantly higher levels of transgene expression without sacrificing astrocyte  
370 specificity. One such optimized tool was used to detect acetylcholine activity in the NAc. The NAc has  
371 been implicated in reward-related reinforcement learning and receives inputs from dopaminergic and  
372 serotonergic neurons, in addition to local cholinergic signaling<sup>74–77</sup>. We used enhancer-AAVs to deliver  
373 the acetylcholine indicator iAChSnFR to astrocytes within the NAc, and measured dynamics with fiber  
374 photometry. This experiment showed that our astrocyte enhancer 3xCore2(390m) maintained faithful  
375 astrocyte specificity after direct injection into NAc and expressed sufficient iAChSnFR for sensing of  
376 acetylcholine in live awake animals. It also showed that iAChSnFR expressed by astrocytes could  
377 readily detect acetylcholine dynamics in NAc, and that astrocytes are a good cellular compartment for  
378 the sensor.

379 Different optimization efforts enabled the generation of a functional Cre-AAV that was specific for  
380 astrocytes in most parts of the brain. Cre recombinases can lose specificity when expressed from  
381 enhancer-AAVs, possibly due to low-level expression of this potent enzyme<sup>32</sup>. We were able to  
382 successfully generate an astrocyte-selective Cre AAV using an attenuated Cre recombinase with the  
383 R297T mutation<sup>68,69</sup>, in combination with the eHGT\_390m enhancer. As a result we produced a highly  
384 specific somatic astrocyte Cre, despite some neuronal off-target expression in thalamus, cerebellum,  
385 and a small brainstem nucleus. Similar optimization could be applied to generate Cres for  
386 oligodendrocytes or other cell populations. We anticipate further optimizations to boost expression and  
387 reduce background will be critical to create ideal enhancer-AAV tools.

388 *Enhancers-AAVs can be identified that have conserved specificity from mouse to monkey.* Our data  
389 demonstrate that some astrocyte and oligodendrocyte enhancer-AAVs are active and selective across  
390 species from mouse through monkey. This property will allow these tools to be applied somatically in  
391 multiple organisms besides mice. Testing enhancers in monkeys revealed interesting morphological  
392 differences compared to mouse. Abundant mature oligodendrocytes with large dendritic arbors were  
393 labeled in both mouse and primate tissues with eHGT\_410m, but this enhancer-AAV also labeled  
394 SOX10+ cells wrapping around radial tubes (presumably blood vessels) in primate but not mouse. Also,  
395 the astrocyte enhancer eHGT\_390m labeled abundant protoplasmic astrocytes in the gray matter in  
396 mouse and monkey, but also labeled several large fibrous astrocytes that were not observed in our  
397 mouse experiments. The ability to function across species and label cells that are not obviously  
398 represented in mouse tissue, makes this collection of enhancer-AAVs a powerful toolset to better  
399 understand new biology. It also makes a compelling case that some of these enhancer-AAVs could be  
400 suitable for use in human gene therapy where astrocyte or oligodendrocyte expression selectivity is  
401 required.

402 *Enhancer-AAVs can be identified that appear to be state-independent.* AAVs that drive expression  
403 using promoters can cause gene expression changes in a state dependent fashion. We showed that  
404 the GFAP promoter changed expression strength and specificity in the context of epilepsy-induced  
405 gliosis using an *Scn1a* haploinsufficiency model. This is not surprising since the *GFAP* gene is known  
406 to change in response to disease and injury<sup>8,9</sup>. Enhancer-AAV eHGT-390m, on the other hand, did not

407 show a change of expression. This could be due to it being selected based on cell type identity, and  
408 most properties of cell types have not been seen to change character dramatically in the context of  
409 disease<sup>22,78,79</sup>. Enhancers can also be selected that do not change activity during development, aging,  
410 or disease, in a similar way to avoid selecting enhancers predicted to have activity in off-target tissues.  
411 As epigenetic datasets expand to cover these axes of disease, development, and aging, it will be  
412 feasible to select only putative enhancers with the desired activity profile.

413 *Conclusion.* We have characterized a large collection of enhancer-AAV vectors for targeting astrocytes  
414 and oligodendrocytes. These vectors will provide researchers with the ability to mark and manipulate  
415 these critical cell types in a variety of species, genetic backgrounds, ages, and disease contexts, and  
416 could also enable delivery of therapeutics. Combined with other recently discovered AAV-based  
417 tools<sup>27,28,33–36,66</sup>, our glial-targeting toolbox will help to advance our understanding of the roles of glial  
418 cell types in brain biology, make the complex cellular anatomy of the brain more experimentally  
419 tractable, and advance the development of AAV-based therapeutics for human CNS disorders.

420

421 **Methods**

422 *Epigenetic analysis and enhancer nomination.* We identified candidate astrocyte- and oligodendrocyte-  
423 specific enhancers from cortical epigenetic datasets. We used the following datasets: human middle  
424 temporal gyrus snATAC-seq<sup>35</sup>, mouse primary visual cortex scATAC-seq<sup>36</sup>, human frontal cortex snmC-  
425 seq<sup>47</sup>, mouse frontal cortex snmC-seq<sup>47</sup>, human frontal cortex bulk mC-seq<sup>46</sup>, and mouse frontal cortex  
426 bulk mC-seq<sup>46</sup>. A single cell glial snmC-seq dataset<sup>48,49</sup> became available only after initial identification  
427 of most of the enhancers described in this study. From the single nucleus/cell ATAC-seq datasets, we  
428 aggregated reads according to cell subclass as in the references, and then called peaks using Homer  
429 findPeaks (<http://homer.ucsd.edu/homer/>) with the -region flag, yielding typically tens of thousands of  
430 peaks per subclass, sized approximately 300-600 bp, as previously described<sup>35</sup>. To find differentially  
431 methylated regions (DMRs) we either used the published regions by Luo et al. 2017 in Extended Data  
432 Tables 5 and 6<sup>47</sup>, and aggregated by subclass and then to all neurons using bedtools merge  
433 (<https://bedtools.readthedocs.io/en/latest/>). Alternatively for bulk non-neuronal DMRs we used methylpy  
434 DMRfind with minimum differentially methylated sites set to 1 on the dataset of Lister 2013, as  
435 previously described<sup>35,47</sup>. To convert mouse and human peak or DMR regions to each other's genomic  
436 coordinates for direct intersectional analysis, we used liftOver ([https://genome.ucsc.edu/cgi-  
437 bin/hgLiftOver](https://genome.ucsc.edu/cgi-bin/hgLiftOver)) with minMatch parameter set to 0.6. All peak regions described in this manuscript  
438 successfully liftOver from human to mouse, and vice-versa, except eHGT\_733m which does not have  
439 an obvious human ortholog via liftOver.

440 To automatically identify peaks and DMRs genome-wide that are astrocyte- or oligodendrocyte-specific  
441 within each dataset, we used a series of bedtools intersectBed  
442 (<https://bedtools.readthedocs.io/en/latest/>) operations to filter for regions that are only detected in  
443 astrocytes or oligodendrocytes. For the “high specificity” criteria, we found peaks that were specifically  
444 detected in both human and mouse cortical astrocytes/oligodendrocytes<sup>35,36</sup>, but did not overlap DMRs  
445 from either human or mouse neurons of any subclass<sup>47</sup>, and these candidate enhancers are marked by  
446 gold square icons in Figure 1. These criteria yielded a set of 87 candidate astrocyte-specific enhancers  
447 and 112 candidate oligodendrocyte-specific enhancers, and the top 17 (Astrocyte) or 16  
448 (Oligodendrocyte) candidate enhancers were chosen from this list as ranked by Homer findPeaks  
449 score. Homer findPeaks score is a measure of peak significance relative to local background, not peak  
450 strength. Additionally, a small number of these “high specificity” criteria candidate enhancers also  
451 overlapped with DMRs from both human and mouse non-neuronal cells<sup>46</sup> (3 Astrocyte and 4  
452 Oligodendrocyte), and these are marked by gold star icons in Figure 1 and Extended Data Figure 1.

453 For the “high strength” criteria we found peaks that were specifically detectable in cortical astrocytes,  
454 using mouse scATAC-seq data only<sup>36</sup>, and agnostic to detection in human and methylation datasets.  
455 This analysis yielded 2119 (astrocyte) and 3940 (oligodendrocyte) candidate enhancers, which were  
456 ranked by read counts within the region, and the top 7 candidate enhancers for astrocytes from this list  
457 were chosen. This ranking led to nomination of peaks that are overall stronger and longer, and these  
458 candidate enhancers are marked with a purple triangle in Figure 1B, but accessibility profiles were not  
459 always conserved in human tissue, as shown in Extended Data Figure 1A,C.

460 Some candidate enhancers were identified manually in the vicinity of known astrocyte or  
461 oligodendrocyte marker genes by visual inspection of ATAC-seq read pileups on UCSC browser  
462 (marked as “M” for Marker genes in Figure 1B). Methylation data was not visualized in this manual  
463 nomination process. Importantly we found that both automatic and manual approaches can identify  
464 peaks with high strength and specificity, as shown in Figure 1D-E.

465 Additionally, enhancer MGT\_E160m was initially identified as a candidate enhancer for pericytes in  
466 cortex using the data of Graybuck et al.<sup>36</sup>, but it was found in the course of this study to instead label  
467 mid/hindbrain astrocytes.

468 To model enhancer screening results as a generalized linear model, we confined analysis to 50  
469 screened enhancers where we observed a clear yes/no screening result for both itself and its cross-  
470 species ortholog. These candidate enhancers were eHGT\_371, 372, 373, 375, 377, 379, 380, 382,  
471 383, 384, 388, 393, 394, 398, 399, 401, 406, 407, 408, 374, 376, 390, 395, 409, and 410, both the m  
472 and h orthologs for each. For each of these genomic regions we calculated candidate enhancer  
473 strength (read CPM within either astrocytes or oligodendrocytes), candidate enhancer specificity  
474 (defined as the proportion of astrocyte or oligodendrocyte enhancer strength relative to the summed  
475 strength in all populations, using the data of Mich et al. or Graybuck and Daigle et al.<sup>35,36</sup>), candidate  
476 enhancer length in base pairs, region-segmented PhyloP using the previous method<sup>35</sup>, and tabulated  
477 whether each candidate enhancer’s partner ortholog worked (binary yes [1] or no [0]). We fit a logistic  
478 generalized linear model of testing results from these predictors using glm() in R with the following  
479 command:

480 `glm( Screen_result_01 ~ Length + PhyloP + Specificity + Strength_cpm +`  
481 `Ortholog_result_01, family=binomial(link='logit'), data = data)`

482 The significance of each coefficient to predict the screening result was determined from the coefficients  
483 of the model, using the data as provided in Extended Data Table 1. Although high peak specificity and  
484 strength were important criteria for candidate enhancer identification, these metrics each had little  
485 predictive power to explain success or failure of screening collection testing as evidenced by  
486 coefficients of fit to a logistic linear model (Extended Data Table 1; strength z-value = 1.43, p = 0.15;  
487 specificity [defined as proportional strength within target cell subclass] z-value = -1.20, p = 0.23), similar  
488 to enhancer length (z-value = -0.13, p = 0.89), enhancer sequence conservation measured by PhyloP  
489 (z-value = 0.87, p = 0.38), and the presence of a functional ortholog in testing (z-value = 1.77, p =  
490 0.077), which suggests that there are additional undiscovered elements that determine successes  
491 versus failures in AAV-based enhancer screening. Overall, the null deviance was 67.3 on 49 degrees of  
492 freedom, and the residual deviance was 57.7 on 44 degrees of freedom, again indicating little power of  
493 these features to predict the screening results.

494 *Cloning and packaging enhancer-AAVs.* With candidate enhancers chosen, we next found their  
495 predicted DNA sequence from genomic reference sequence using Bioconductor package Bsgenome<sup>80</sup>.  
496 We extracted the sequence and padded 50 bp to each side of the enhancer to provide room for forward  
497 and reverse primer binding sites that capture the entire enhancer. From these padded sequences, we  
498 used automatic primer design in Geneious to identify primer pairs within the 50 bp pads to specifically  
499 amplify each enhancer, and append a constant 5’ homology arm to each enhancer for automatic

500 Gibson assembly into reporter-AAV plasmid. We amplified the regions from C57Bl/6 tail snip DNA or  
501 from human male genomic DNA (Promega catalog # G1471) using FastPhusion 2x Master Mix  
502 (Thermo Fisher catalog # F548L), and >90% of the PCR reactions were successful on the first try. In  
503 some cases we redesigned primers to attempt a second amplification.

504 We cloned into reporter backbone CN1244 (Addgene plasmid #163493) using the sites MluI/SacI and  
505 the 5' primer homology arms F: TTCCTGCGGCCGCACGCGT and R:  
506 GACTTTATGCCAGCCCCAGCTC, using Infusion kit (Takara catalog # 638949). For some  
507 enhancers we instead cloned into a next generation reporter vector backbone that includes a SYFP2-  
508 P2A-3xFLAG-H2B reporter for detection of cytosolic SYFP2 and nuclear FLAG for simultaneous  
509 expression analysis and snRNA-seq, using the same cut sites and homology arms (see Extended Data  
510 Table 1). We transformed infusion reactions into Mix N' Go (Zymo Research catalog # T3001)  
511 chemically competent Stbl3 E. coli (Thermo Fisher catalog # C737303) and selected on 100 ug/mL  
512 carbenicillin plates. We cultured individual clones at 32 C, verified them by Sanger sequencing,  
513 maxiprepped them with 100 ug/mL ampicillin, and saved them as frozen glycerol stocks at -80°C.

514 We used maxiprep DNA for packaging into PHP.eB AAV particles. For routine enhancer-AAV screening  
515 by intravenous delivery in mouse we generated small-scale crude AAV preps by transfecting 15 ug  
516 maxiprep enhancer-reporter DNA, 15 ug PHP.eB cap plasmid, and 30 ug pHelper plasmid into one 15-  
517 cm dish of confluent HEK-293T cells using PEI-Max (Polysciences Inc. catalog # 24765-1). After  
518 transfection the next day we changed the medium to 1% FBS, and after 5 days the cells and  
519 supernatant were collected, freeze-thawed 3x to release AAV particles, treated with benzonase (1 uL)  
520 for 1 hr to degrade free DNA, then clarified (3000g 10min) and then concentrated to approximately 150  
521 uL by using an Amicon Ultra-15 centrifugal filter unit at 5000g for 30-60 min (NMWL 100 kDa, Sigma  
522 #Z740210-24EA), yielding a titer of approximately 3-5 E13 vg/mL. For large-scale gradient preps for  
523 intraparenchymal injection into NHP or mouse or ICV injection into rat, we transfected 10 15-cm plates  
524 of cells, and also purified preps by iodixanol gradient centrifugation. We assessed viral titer for both  
525 crude and gradient AAV preps by digital droplet PCR on a BioRad QX200 system. All vectors showing  
526 specific expression patterns will be made available through Addgene.

527 *Optimizing enhancer strength through concatemerization.* For some native enhancers that showed  
528 specific expression patterns, we sought to boost their expression levels through concatemerization. To  
529 concatemerized, we segmented the enhancer (typically approximately 400-600 bp) into approximately  
530 thirds with approximately 25 bp of overlaps at the junctions (each a candidate “core” of approximately  
531 200 bp), then designed a tandem array of three cores in series (approximately 600 bp). These synthetic  
532 tandem array sequences were gene synthesized by Azenta/GeneWiz PRIORITYGene synthesis  
533 service with flanking MluI/SacI sites for restriction enzyme digestion and ligation into corresponding  
534 sites in CN1244. We then packaged and tested concatemerized PHP.eB enhancer-AAVs as above.

535 *Mice and injections.* All mouse experimentation was approved by Allen Institute Institutional Animal  
536 Care and Use Committee (IACUC) as part of protocol #2020-2002. In these studies, we purchased  
537 C57Bl/6J mice from The Jackson Laboratory (Stock # 000664). For enhancer screening these C57Bl/6J  
538 mice were injected with AAVs in the retro-orbital sinus at age P21 with 5E11 genome copies of  
539 AAV/PHP.eB viral vectors with brief isoflurane anesthesia. For enhancer validation studies (IHC) mice

540 were injected the same way but between ages P42 to P56. Tissues from mice were harvested at 3 to 4  
541 weeks post injection for analysis. We perfused animals with saline then 4%PFA, and harvested brains  
542 or other tissues and post-fixed in 4%PFA overnight, before rinsing and cryoprotecting in 30% sucrose  
543 solution before sectioning at 30 micron thickness on sliding microtome with a freezing stage. For  
544 enhancer screening we counterstained with DAPI and propidium iodide and mounted in Vectashield  
545 Vybrance, and imaged on either a Nikon Ti-Eclipse or Nikon Ti-Eclipse 2 epifluorescent microscope,  
546 Olympus FV-3000 confocal microscope, or Leica Aperio slide scanner. In some experiments where  
547 noted, we tested enhancer-AAVs after bilateral intracerebroventricular (ICV) injection at age P2 using  
548 the technique of Kim et al.<sup>81</sup> These ICV-injected pups were harvested for tissue analysis at age P21.  
549 For whole brain imaging of expression pattern, we performed sequential blockface imaging of brains  
550 using the TissueCyte 1000 serial two-photon tomography system<sup>82</sup>.

551 For testing in Dravet syndrome model mice, 129S1/SvImJ -*Scn1a*<sup>tm1Dsf/J</sup> mice (strain # 034129) were  
552 purchased from Jackson Laboratories and bred to C57Bl/6J mice to create *Scn1a*<sup>R613X/+</sup> pups on a F1  
553 hybrid C57Bl/6J:129S1/SvImJ background, and these pups were injected retro-orbitally at P21 with  
554 tissue analysis at P42. Additionally, we also tested enhancer-AAV vectors in *CMV-Cre;Scn1a*<sup>A1783V/+</sup>  
555 pups on C57Bl/6J background, which were generated from crossing B6(Cg)-*Scn1a*<sup>tm1.1Dsf/J</sup> male mice  
556 (The Jackson Laboratory, strain #:026133) with homozygous CMV-Cre female mice (*B6.C-Tg(CMV-*  
557 *cre)1Cgn/J*, The Jackson Laboratory, strain # 006054).

558 *Mouse immunohistochemistry (IHC)*. For IHC and ISH, we transcardially perfused mice with ice-cold 25  
559 mL HBSS (Thermo Fisher Scientific # 14175079) containing 0.25 mM EDTA (Thermo Fisher Scientific  
560 # AM9260G), followed by 12 mL of ice-cold 4% paraformaldehyde in 1x PBS, freshly prepared from  
561 16% PFA (Electron Microscopy Sciences #15710). We dissected brains and other tissues from  
562 carcasses and post-fixed them at 4 degrees overnight, and the next morning we rinsed the tissues with  
563 fresh PBS, and then transferred to 30% sucrose solution in PBS for cryoprotection. For sectioning on  
564 Leica CM3050 cryostat, we then embedded tissues in OCT cryo-compound (Tissue-Tek # 4583) at  
565 room temperature at least 3 hours, then froze the blocks on dry ice and stored at -80°C until sectioning  
566 at 25 micron thickness. Alternatively, we sectioned half-brains at 25 µm thickness on frozen 30%  
567 sucrose solution slabs on a sliding microtome (Leica SM2000R) equipped with freezing stage. Sections  
568 were stored at 4 degrees in PBS containing 0.1% sodium azide until analysis.

569 For IHC we used the following antibodies: chicken anti-GFP (Aves # GFP-1010), rabbit anti-Sox9 (Cell  
570 Signaling clone D8G8H, # 82630S), mouse CC1 antibody (Abcam # ab16794), mouse anti-GFAP  
571 (Millipore Sigma clone G-A-5, # G3893), with 5% normal goat serum (Thermo Fisher Scientific #  
572 31872) and 0.1% Triton X-100 (VWR 97062-208) for blocking and permeabilization, and appropriate  
573 Alexa Fluor-conjugated secondary antibodies for detection.

574 *Flow cytometry and single cell transcriptomics*. We prepared cell suspensions for flow cytometry and  
575 single cell RNA-seq from brain tissue as previously described<sup>83</sup>. Briefly, for flow cytometry, we perfused  
576 mice transcardially under anesthesia with ACSF.1. We harvested the brains, embedded in 2% agarose  
577 in PBS, then sliced thick 350 micron sections using a compresstome with blockface imaging, then  
578 picked the sections containing the region of interest (VISp, or mid- and hindbrain, or cerebellar cortex),  
579 and dissected out the regions of interest. We then treated dissected tissues with 30U/mL papain

580 (Worthington LK003176) in ACSF.1 containing 30% trehalose (ACSF.1T) in a dry oven at 35°C for 30  
581 minutes. After papain treatment we quenched digestion with ACSF.1T containing 0.2% BSA, triturated  
582 sequentially using fire-polished glass pipettes with 600, 300, and 150 micron bores, filtered the  
583 released cell suspensions into ACSF.1T containing 1% BSA, centrifuged cells at 100g for 10 min, then  
584 resuspended cells in ACSF.1T containing 0.2% BSA and 1 µg/mL DAPI prior to flow cytometry and  
585 sorting on a FACSaria III (Becton-Dickinson). SYFP2 reporter brightness was measured as the ratio of  
586 positive cell population mean fluorescence intensity, divided by the low mean fluorescence intensity of  
587 autofluorescence in non-expressing cells. This measure of reporter brightness is more consistent than  
588 positive cell population mean fluorescence intensity alone, due to differences in raw signal across days,  
589 cytometers, and cytometer settings.

590 For single cell RNA-seq, we sorted single SYFP2+ cells into tubes and processed them via SMARTer  
591 v4 using the workflow described previously<sup>83</sup>, on 47 enhancer-AAV-injected mice. In each experiment  
592 from one mouse injected with one single enhancer-AAV we sorted and profiled up to 48 cells per  
593 experiment, and each measurement was taken from a distinct individual cell. After retroorbital  
594 injections, enhancer-AAV SYFP2-expressing cells consisted of on average 7% of the positive brain  
595 cells (range 0.1-20.1% of cells, n = 47 experiments). We sequenced single cell-derived SMARTer  
596 libraries at  $659996 \pm 199038$  (mean ± standard deviation) reads per library on an Illumina NovaSeq  
597 instrument at Broad Institute (Cambridge, MA) or on an Illumina NextSeq instrument at Allen Institute  
598 (Seattle, WA). We aligned the libraries to mm10 genome using STAR  
599 (<https://github.com/alexdobin/STAR>), and also aligned them to the synthetic AAV transgene reference  
600 construct using bowtie2 (<https://bowtie-bio.sourceforge.net/bowtie2/index.shtml>). From 2040 initial cells,  
601 we excluded from analysis the libraries with poor library quality metrics, consisting of: firstly low-quantity  
602 or degraded libraries (judged as less than 65% percentage of cDNA library sized greater than 400 bp,  
603 consisting of 71 [3.4%] libraries in this study), and secondly those that lacked AAV transgene-mapping  
604 reads (likely mis-sorted events, 23 [1.1%] of remaining libraries). Applying these filtering criteria yielded  
605 a dataset for analysis of 1946 high-quality AAV transgene-expressing cells, with alignment rates of  $92 \pm$   
606 3% to mm10 genome and  $4654 \pm 1285$  genes detected per cell (mean ± standard deviation). To assess  
607 enhancer specificity within the cortex we mapped the high quality transgene-expressing SMARTer cells  
608 to the SMARTer-based VISp cellular taxonomy generated by Tasic et al. using bootstrapped  
609 hierarchical approximate nearest neighbor mapping<sup>20,83</sup>, and quantified the specificity as the percentage  
610 of positively sorted cells that mapped to the expected cell subclass (astrocytes or oligodendrocytes). To  
611 test for significance of correlation of brightness by flow cytometry with expression levels by scRNA-seq,  
612 we calculated Pearson's product-moment correlation coefficient by cor.test() function in R.

613 To understand different characteristics of different regional astrocyte populations we utilized  
614 scratcch.mapping (<https://github.com/AllenInstitute/scratcch>) from the Allen Institute. To accomplish this  
615 we first transformed these cells by principal component analysis and performed UMAP dimensionality  
616 reduction on the first 40 principal components for visualization using the default scanpy parameters,  
617 which clearly separated oligodendrocytes and regional groupings of astrocytes. For clustering  
618 astrocytes we subset the dataset to astrocytes only, then identified the top 2000 genes ranked by  
619 variance among them, recomputed UMAP projections from these high-variance genes, then performed  
620 Leiden clustering<sup>84</sup> which identified VISp, MB/HB, and CBX astrocyte clusters as expected, and finally

621 identified differential genes among them (differential gene expression threshold false discovery rate  
622 less than 5% and log<sub>2</sub>-fold change greater than 0.5) using scanpy  
623 (<https://scanpy.readthedocs.io/en/stable/>). In doing so we detected two major subgroups of VISp  
624 astrocytes that are distinguished by presence or absence of immediate-early gene markers (for  
625 example, *Fos*, *Fosl2*, *Nr4a1*, *Irs2*, *Pde10a*, and *Pde7b*). This distinction may be an artifact of our cell  
626 dissociation process for scRNA-seq; for the purposes of this study we collapse these cortical astrocyte  
627 clusters. In order to understand the different regional characteristics of astrocyte populations we  
628 mapped cells to whole brain taxonomy we mapped to the best-correlated mean-aggregated taxonomic  
629 cluster<sup>20</sup> with 100 bootstrapped iterations using the top 10% of high-variance genes and omitting a  
630 variable number of genes (10-50%) each round. We interpret the frequency of correct mapping rounds  
631 as the mapping confidence. We also used CELLxGENE for single cell visualization  
632 (<https://github.com/chanzuckerberg/cellxgene>). Spatial transcriptomic analysis was performed as  
633 described in the recent whole brain transcriptomic taxonomy study<sup>20</sup>, and cell type location data was  
634 visualized using Cirrocumulus (<https://cirrocumulus.readthedocs.io/en/latest/index.html>).

635 For determination of *Zic5* and *Sox10* differential gene expression between astrocytes and  
636 oligodendrocytes, we used two-sided ANOVA on expression measurements from individually profiled  
637 cells from all the experiments with no exclusion, and no covariates were tested. Testing for normality by  
638 the Shapiro-Wilk test revealed that *Zic5* and *Sox10* expression are not normally distributed (*Zic5* W =  
639 0.539, p-value < 2.2e-16; *Sox10* W = 0.958, p-value = 5.1e-16), so we used a non-parametric Wilcoxon  
640 rank-sum test. No significance thresholds adjustments were made for multiple comparisons since only  
641 one comparison was performed. For the comparison of *Sox10* versus *Zic5* expression (mean counts  
642 per million +/- standard deviation, n cells): astrocyte *Zic5* expression  $32 \pm 71$ , n = 864; astrocyte *Sox10*  
643 expression  $0.3 \pm 5$ , n = 864; oligodendrocyte *Zic5* expression  $0.6 \pm 6$ , n = 964, oligodendrocyte *Sox10*  
644 expression  $456 \pm 276$ , n = 964.

645 *Motif analysis.* We performed de novo motif discovery from the sets of astrocyte and oligodendrocyte  
646 enhancers that showed specific and strong expression patterns, excluding those enhancers scored as  
647 weak. For astrocytes this list consisted of: eHGT\_375m, eHGT\_376h, eHGT\_376m, eHGT\_377m,  
648 eHGT\_380h, eHGT\_381h, eHGT\_385h, eHGT\_385m, eHGT\_380h, eHGT\_390h, eHGT\_390m,  
649 MGT\_E120m, MGT\_E122m, MGT\_E160m, and ProB12. For oligodendrocytes this list consisted of:  
650 eHGT\_361h, eHGT\_395h, eHGT\_395m, eHGT\_396h, eHGT\_397m, eHGT\_398h, eHGT\_400m,  
651 eHGT\_401h, eHGT\_403h, eHGT\_407h, eHGT\_409h, eHGT\_409m, eHGT\_410h, eHGT\_410m, and  
652 eHGT\_641m. We used MEME-CHIP<sup>56</sup> to identify recurrent de novo motifs in these sets of sequences,  
653 using the parameter -meme-maxw 12, and comparing to a background set of random sequence with  
654 the same nucleotide content. This analysis revealed one strong motif in each set of sequences, as  
655 measured by its E-value, which is an estimate of the number of motifs expected by chance to have as  
656 strong a log likelihood ratio as itself within the given sequences. These de novo motifs where then  
657 mapped to known sequences using TomTom<sup>56</sup> which revealed several possible matches to known  
658 motifs at significant p-values, but the strongest motif match (lowest p-value) in each case is shown. In  
659 the case of the Zic family transcription factors, for simplicity we averaged together the highly correlated  
660 strongest hits in the Zic family (JASPAR accession numbers MA0697.2, MA1628.1, and MA1629.1  
661 covering Zic1, Zic2, and Zic3), since Zic5 itself is not present in databases. In the case of Sox10, the

662 highly correlated Sox family members Sox4 and Sox11 (Uniprobe accession numbers UP00062.1 and  
663 UP00030.1) showed slightly stronger motif match p-values than Sox10 (JASPAR accession number  
664 MA0442.1), but these were excluded from analysis due to lack of expression in almost all  
665 oligodendrocytes as observed by Tasic et al.<sup>83</sup> and in this study.

666 *Enhancer-AAV testing in rat.* The Allen Institute Institutional Animal Care and Use Committee (IACUC)  
667 approved the following in vivo testing experiments in rat under protocol 2010. We procured timed-  
668 pregnant female Sprague-Dawley rats from Charles River laboratories. We tattooed and injected ice-  
669 anesthetized neonatal pups at P1 with 1.5e11 viral genomes of enhancer-AAV virus, diluted with 1X  
670 PBS to a total volume of 10 µL, unilaterally into the forebrain lateral ventricle (ICV delivery) with a 31-  
671 gauge, 4 point, 12° bevel 1 inch needle (custom ordered from Hamilton) and 25 µL capacity removable  
672 needle syringe (Hamilton, 7636-01). Between injections we washed the needle and syringe with 100%  
673 ethanol, and then nuclease-free water. We targeted the ICV space at 2 mm posterior to bregma, 2 mm  
674 lateral to the anterior-posterior midline, and at a depth of 2 mm perpendicular to the surface of the skull.  
675 We injected into the ventricle slowly over approximately 30 seconds. After injection, we held the needle in  
676 place for approximately 10 seconds to prevent viral leakage, then slowly withdrew the needle at the  
677 same relative angle as injection and then placed the animal onto a prewarmed heating pad in a clean  
678 cage. We sacrificed pups at 18 days post injection, prior to weaning, and transcardially perfused with  
679 1X PBS and then 4% PFA in PBS. We hemisected each brain and cryoprotected in 30% sucrose in  
680 deionized water for a minimum of 24 hours before sectioning. We sectioned each brain at 30 µm  
681 thickness using a sliding microtome (Leica part number SM2000R) on a leveled mount of Tissue-Tek®  
682 O.C.T. Compound, collecting 3 sagittal planes separated by approximately 500 µm. We counterstained  
683 sections with 1 µg/mL DAPI and 2.5 µg/mL propidium iodide (Thermo catalog # P1304MP) overnight at  
684 4°C and mounted in VECTASHIELD® HardSet™ Antifade Mounting Medium prior to imaging by  
685 epifluorescence.

686 *NHP enhancer-AAV testing.* NHP animals were housed and injected at the Washington National  
687 Primate Center according to NIH guidelines and as approved by the University of Washington Animal  
688 Care and Use Committee under UW IACUC protocol #41-6701. These animals received several  
689 intraparenchymal injections under general anesthesia at spatially distinct sites located at least ~1cm  
690 apart throughout the brain. During injection, over the course of 10 minutes we expelled a total of  
691 approximately 1e11 gc iodixanol gradient-purified PHP.eB-packaged viral vectors in a total volume of 5  
692 uL at 10 depths ranging from 200 to 2000 microns deep in the animals. After injection the animal rested  
693 for 10 minutes between injections. These numbers are approximate and timing, volume, and depths,  
694 may be adjusted according to animal anatomy and surgical considerations. The experiments described  
695 here result from two injection sites in one male *Macaca nemestrina* animal. We harvested tissue from  
696 this animal after necropsy at 113 days post injection.

697 After locating the injection sites and cutting out tissue blocks about 1-2cm on each side surrounding the  
698 injection sites, we fixed these tissue blocks in 4% PFA for 24 hrs. Then we rinsed the blocks with PBS,  
699 cut 350 µm thick slices on the sliding microtome, and postfixed the slices in 4% PFA for 2 hours at  
700 room temperature (RT), washed three times in PBS for 10 min each, then transferred to 70% EtOH at  
701 4°C for a minimum of 12 hours, and up to 30 days.

702 For ISH analysis we first incubated the slices in 8% SDS in PBS at RT for two hours with agitation, then  
703 washed the slices at RT with 5X sodium chloride sodium citrate (SSC) for three hours, exchanging with  
704 fresh 5X SSC every hour. Next we performed HCR v3.0 using reagents and a modified protocol from  
705 Molecular Technologies and Molecular Instruments<sup>85</sup>. We first incubated slices in pre-warmed 30%  
706 probe hybridization buffer (30% formamide, 5X sodium chloride sodium citrate (SSC), 9 mM citric acid  
707 pH 6.0, 0.1% Tween 20, 50 µg/mL heparin, 1X Denhardt's solution, 10% dextran sulfate) at 37°C for 5  
708 min. Then we exchanged hybridization buffer for hybridization buffer containing probes added at a  
709 concentration of 2 nM. Molecular Instruments designed the probes using the following accession  
710 numbers: SLC17A7 – XM\_011768126.1, GAD1 – XM\_011744029.1, FGFR3 – XM\_011744842.2,  
711 SOX10 – XM\_011712410.2. Hybridization proceeded overnight at 37°C, and afterwards we washed the  
712 tissue thrice with 5X SSC for 10 minutes each (total 30 minutes), then 30% probe wash buffer (30%  
713 formamide, 5X SSC, 9 mM citric acid pH 6.0, 0.1% Tween 20, 50 µg/mL heparin) for one hour at 37°C.  
714 Then we exchanged probe wash buffer with 5X SSC, then amplification buffer (5X SSC, 0.1% Tween  
715 20, 10% dextran sulfate) for 5 min at room temperature. Meanwhile we pooled even and odd  
716 amplification hairpins for each of the three genes and snap-cooled them by heating to 95°C for 90  
717 seconds then cooling to room temperature for 30 min, and afterwards we added the snap-cooled  
718 hairpins to amplification buffer at a final concentration of 60 nM, and finally centrifuged at 18000g for 1  
719 minute. Then we incubated tissue slices in amplification solution containing amplification hairpins for 4  
720 hours at room temperature, followed by staining in DAPI (10µg/mL in 2X SSC) for 1 hour at room  
721 temperature, and finally washing twice for 30 min in 2X SSC at room temperature before imaging. We  
722 prepared a fresh aliquot of 67% 2,2'-Thiodiethanol (TDE) solution for use as a clearing and immersion  
723 fluid by mixing ≥99% TDE (Sigma-Aldrich) with deionized water to create a 67% TDE solution with a  
724 refractive index of 1.46. We transferred slices to 67% TDE and allowed them to equilibrate for at least 1  
725 hour at room temperature prior to imaging on a confocal microscope (Olympus FV-3000).

726 *Stereotaxic injection and fiber implant surgery.* Virus injection and optic fiber implantation surgery was  
727 performed in C57BL/6J mice (The Jackson Laboratory, #000664) at around P60. Mice were  
728 anesthetized with isoflurane and monitored throughout the surgery using breathing rate and tail pinch.  
729 The skin above the skull surface was removed to make room for the fiber implant and headframe. After  
730 leveling the skull, a craniotomy was drilled above the injection and fiber coordinates (AP: 1.2 mm, ML: -  
731 1.3 mm, DV: 4.1 mm). First, a glass pipette positioned at the injection coordinates was lowered through  
732 the craniotomy and virus injection was performed (100 nl, titer: 4E13). Once the injection was complete,  
733 the pipette was slowly raised, and the optic fiber probe was position at the same AP and ML  
734 coordinates as the injection. The tip of the fiber was then lowered to 100 µm above the injection site  
735 and glued in place where the base of the fiber ferrule contacts the skull. A custom headframe was then  
736 glued to the skull to allow head-fixed behavior and imaging. After surgery, the mouse was returned to  
737 the home cage and allowed to recover for at least two weeks prior to start of water restriction for  
738 behavior and imaging.

739 *Dynamic foraging reinforcement learning task.* Water-restricted and head-restrained mice were trained  
740 to perform a reinforcement learning task where they freely choose between two lick ports that delivered  
741 a water reward with nonstationary probabilities. This is a variation on the task described in Bari et al.<sup>86</sup>.  
742 The base reward probability of both lick ports summed to 0.6 where the probabilities of the two lick

743 ports were selected from two sets of ratios (0.53/0.07, 0.51/0.09). Block lengths that corresponded to  
744 each ratio lasted for about 30 trials (min trials per block: 40, max trials per block: 60). Each trial began  
745 with an auditory “go cue” that signaled the start of a trial. The mouse was free to choose between the  
746 left or right lick port immediately after the “go cue”. The trials were separated by a variable inter-trial-  
747 interval (range between 1-7 seconds). The data shown in this study was from a two-hour behavior  
748 session that consisted of 438 trials (170 rewarded trials).

749 *Fiber photometry and analysis.* Fiber photometry was performed using a commercially available  
750 photometry system (Neurophotometrics LLC, FP3002). A 470 nm LED was used to excite the  
751 iAChSnFR fluorophore, Venus, and the emitted fluorescence signals were collected using a CMOS  
752 camera. The 470 nm excitation was interleaved with a 415 nm LED as an isosbestic control to remove  
753 motion artifacts. Bonsai acquisition software was used to record the photometry signals as well as the  
754 behavior trigger signals events (go-cue, left and/or right lick choices, reward/no reward) for offline  
755 alignment of imaging data to behavioral events. Prior to start of acquisition, an ROI was drawn over the  
756 fiber image seen on the camera, and fluorescence intensity within this ROI was averaged for real-time  
757 signal visualization and offline analysis. First, the fiber photometry acquisition was started, following  
758 which the behavior task was initialized. Photometry signals were analyzed using custom python scripts.  
759 First, the fluorescence signal was detrended for photobleaching using a fourth order polynomial  
760 function and then corrected for motion using the control signal from the 415 nm excitation using  
761 standard photometry analysis techniques<sup>87</sup>. Acetylcholine signal changes were calculated as a change  
762 in fluorescence intensity over the mean fluorescence ( $\Delta F/F$  as a percentage). The photometry signals  
763 were then aligned to behavior events using simultaneously acquired TTL readouts of behavior events  
764 (go-cue, left and/or right lick choices, reward/no reward) using a NI USB card. These behavior events  
765 were then used to calculate trial averaged traces of rewarded and unrewarded signals.

766

767 **Data Availability:**

768 All AAV viral vector plasmids will be made freely available for research use at Addgene ([addgene.org/](http://addgene.org/)).  
769 Mouse scRNA-seq generated from this study will be made available at GEO with the accession number  
770 GSE235987 (<https://www.ncbi.nlm.nih.gov/geo/>). Mouse serial two photon tomography datasets will be  
771 made available through the Brain Imaging Library (<https://www.brainimagelibrary.org/>). All other data  
772 will be made available upon request.

773 **Ethics Declarations:**

774 Competing interests

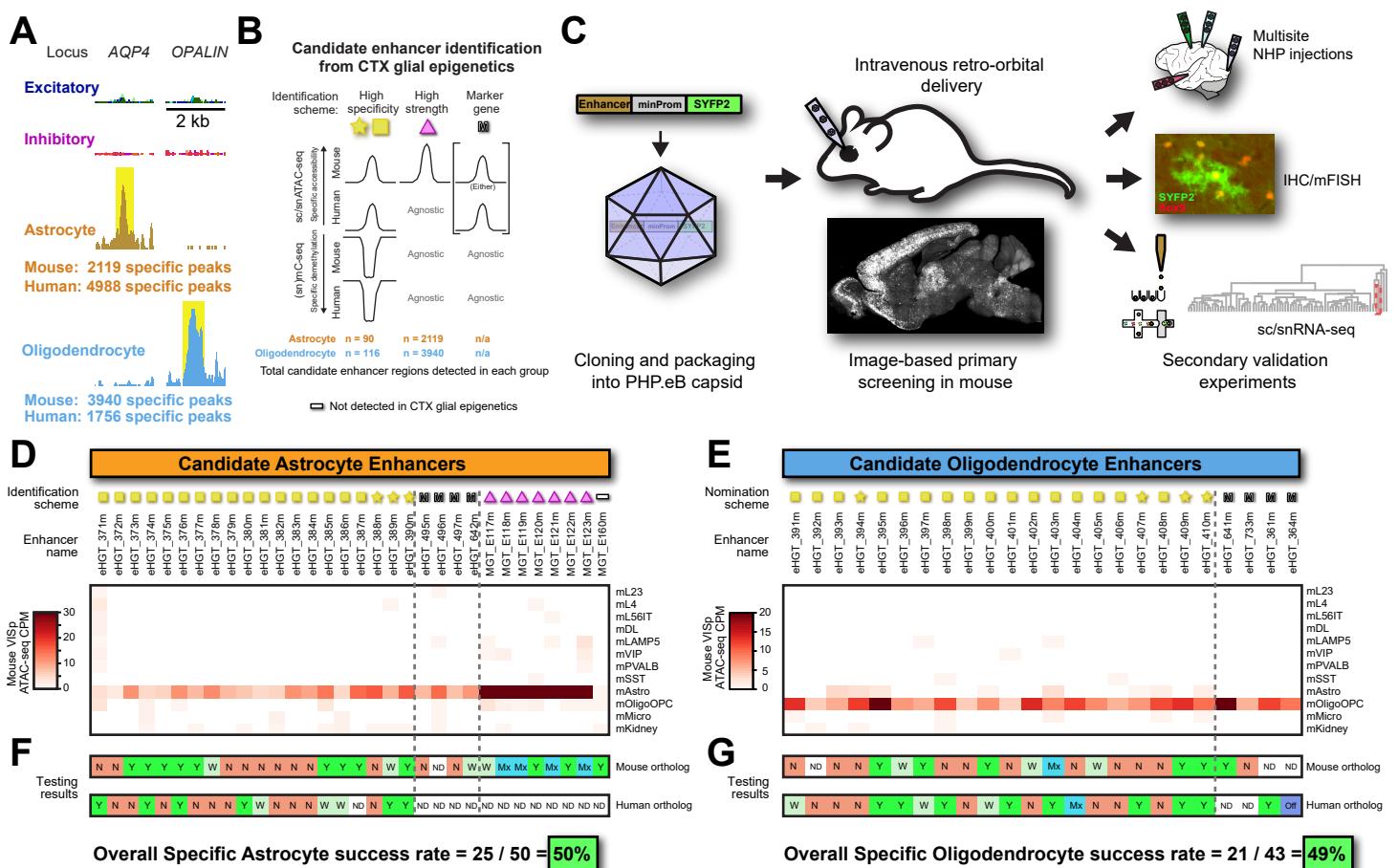
775 Several authors including ESL, JTT, JKM, RAM, XOA, BT and BPL are inventors on one PCT stage  
776 patent application (PCT\_US2021\_024525) and one provisional patent covering vectors described in  
777 this manuscript. BPL is a scientific advisor for Patch Bioscience.

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789 **Figures and Figure Legends**

790



791 **Figure 1: Astrocyte and oligodendrocyte enhancer discovery from single cell epigenetics.**

792  
793 (A) Example astrocyte- and oligodendrocyte-specific peaks near the loci of astrocyte-specific gene  
794 AQP4 and oligodendrocyte-specific gene OPALIN, identified in human MTG snATAC-seq data<sup>35</sup>.

795 (B) Differing approaches to identify candidate enhancers. Specific accessibility peaks are depicted as  
796 peaks, and specifically demethylated regions are depicted as troughs. Schemes not utilizing a particular  
797 data modality are shown as “Agnostic”. Marker gene selection criteria can use accessibility from either  
798 mouse or human. Icons represent identification schemes; gold star candidate enhancers undergo more  
799 stringent criteria than those with gold squares (see Methods for details).

800 (C) Workflow for enhancer cloning, packaging, screening, and validation. Enhancers are cloned into a  
801 pAAV plasmid upstream of a minimal human beta-globin promoter and SYFP2 reporter, and plasmids  
802 are packaged into PHP.eB AAVs. Enhancer-AAVs are injected intravenously into retro-orbital sinus,  
803 and expression is assessed by imaging. Promising enhancer-AAVs then go on to secondary validation  
804 experiments consisting of cross-species validation, molecular characterization by IHC and/or  
805 multiplexed FISH, and flow cytometry for single cell RNA-seq.

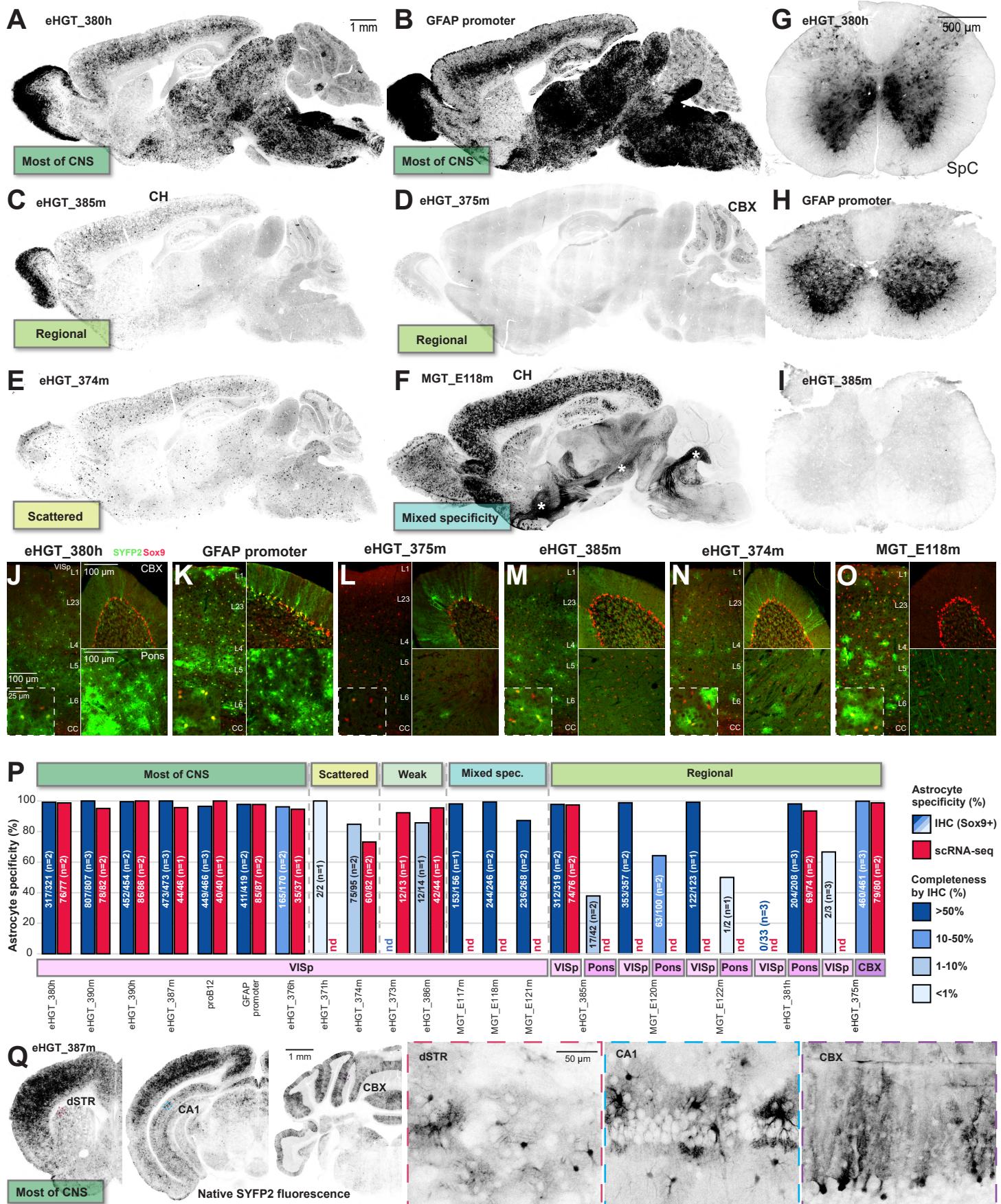
806 (D-E) Accessibility profiles of candidate mouse astrocyte-specific (D) and oligodendrocyte-specific  
807 enhancers (E). For each genomic region we show their peak nomination scheme matching to Figure  
808 1B, enhancer name, and enhancer accessibility profile transformed to CPM in mouse V1Sp scATAC-  
809 seq dataset<sup>36</sup>.

810 (F-G) Summarized screening results. Overall, we observed high success rates of tested enhancer-  
811 AAVs giving specific astrocyte or oligodendrocyte expression patterns. Testing result bar: Y = yes,  
812 enhancer-AAV gives strong or moderate on-target expression pattern; N = no, enhancer-AAV fails to  
813 express; W = weak on-target expression pattern; Mx = mixed expression pattern consisting of on-target  
814 cells plus unwanted neuronal populations; Off = off-target expression pattern; ND = no data. Note both  
815 enhancers giving strong/moderate (“Y”) and weak (“W”) specific expression are grouped here for  
816 overall success rate analysis.

817

818

819



820     **Figure 2: A collection of astrocyte-specific enhancer-AAV vectors with varying regional**  
821     **specificities and expression densities.**

822     (A-B) Astrocyte-specific enhancer-AAVs marking many astrocytes throughout most of the CNS.  
823     eHGT\_380h (A) and GFAP promoter (B) mark many astrocytes throughout gray matter in FB, MB, HB,  
824     and CBX.

825     (C-D) Astrocyte-specific enhancer-AAVs marking many astrocytes in isolated regions of the brain. (C)  
826     eHGT\_375m specifically labels cerebellar (CBX) Bergmann glia but not FB, MB, or HB. (D)  
827     eHGT\_385m labels astrocytes in cerebrum (CH) but not in MB, HB, or CBX.

828     (E) Astrocyte-specific enhancer-AAV eHGT\_374m marking scattered astrocytes. These scattered  
829     astrocytes are located throughout FB, MB, HB, and CBX.

830     (F) Mixed specificities from astrocyte enhancer MGT\_E118m. MGT\_E118m labels astrocytes in the  
831     cerebrum (CH) but also off-target neuron populations in deep cerebellar nuclei, midbrain, and globus  
832     pallidus, external segment (marked by asterisks).

833     (G-I) Astrocyte-specific enhancer-AAVs labeling astrocytes in lumbar SpC. eHGT\_380h (G) and GFAP  
834     promoter (H) label many astrocytes in SpC gray matter, but eHGT\_385m (I) does not label SpC  
835     astrocytes.

836     (J-O) Positive confirmation of molecular astrocyte identity across brain regions. SYFP2+ astrocytes are  
837     colabeled with anti-Sox9 immunoreactivity in VIsP, CBX, and Pons.

838     (P) Quantification of specificity for astrocytes by astrocyte enhancer-AAVs. Specificity and  
839     completeness for astrocyte labeling by enhancer-AAVs was quantified by costaining with anti-Sox9  
840     antibody in VIsP, Pons, and CBX. Specificity is defined as the number of SYFP2+Sox9+ / total SYFP2+  
841     cells x 100%. Completeness is defined as the number of SYFP2+Sox9+ / total Sox9+ cells x 100%.  
842     Brains from one to three mice per condition were analyzed, with range 131-827 cells counted (median  
843     311) per brain region analyzed. eHGT\_375m-labeling was only quantified in the Purkinje cell layer of  
844     CBX, not in the granule or molecular layers. Specificity was also quantified by scRNA-seq, defined as  
845     the percentage of sorted SYFP2+ cells mapping as astrocytes within the VIsP molecular taxonomy<sup>83</sup>.  
846     Overall, specificity is high for many astrocyte-specific vectors, with “Scattered” and “Weak” vectors  
847     showing low completeness, and “Regional” vectors showing more completeness in certain regions.

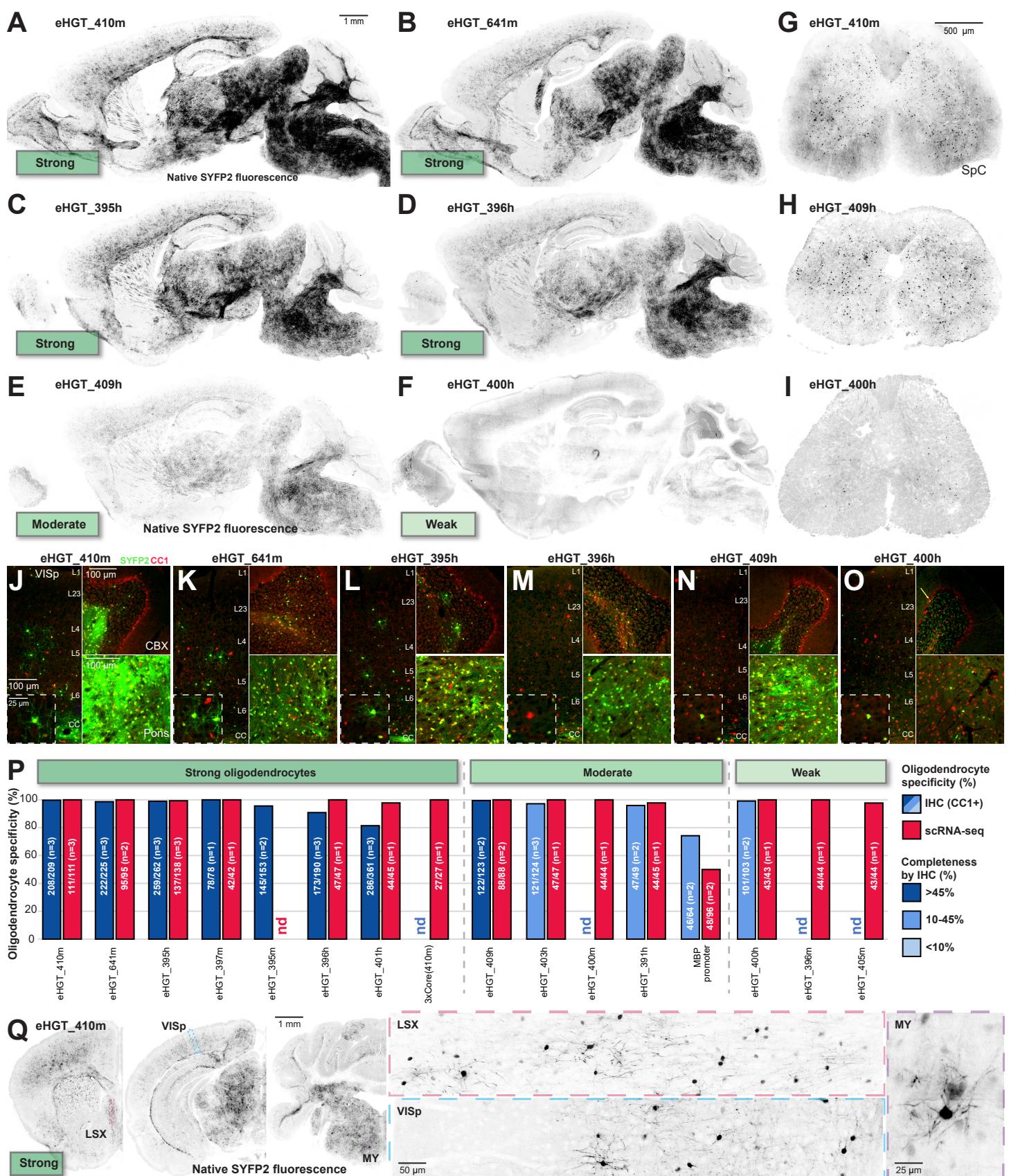
848     (Q) Distinct astrocyte morphologies throughout the brain with eHGT\_387m enhancer-AAV targeting  
849     “Most of CNS”. Images were acquired on a serial blockface imaging platform (TissueCyte).

850     Abbreviations: CH cerebrum, dSTR dorsal striatum, CA1 cornu ammonis 1, CBX cerebellar cortex, SpC  
851     spinal cord, VIsP primary visual cortex.

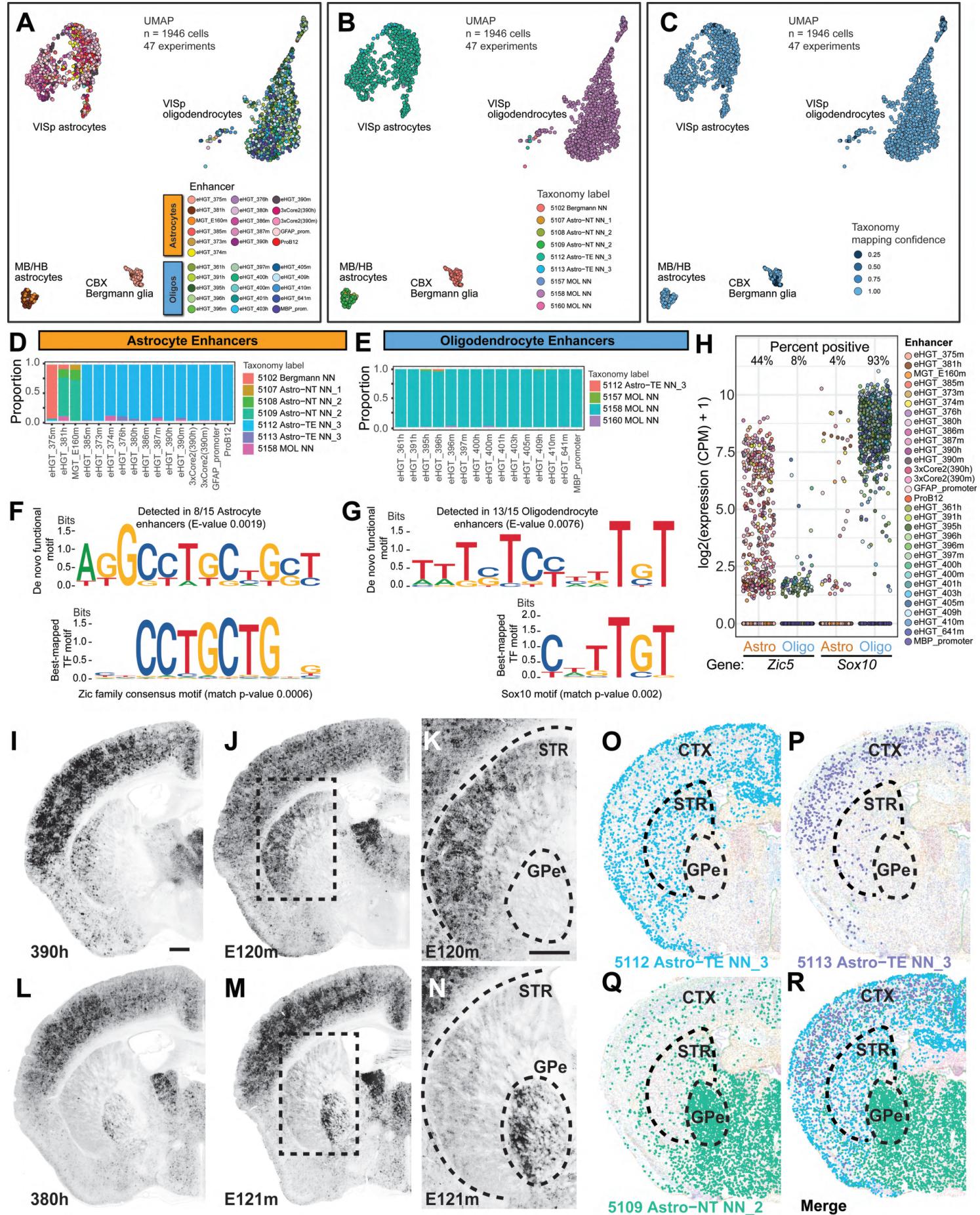
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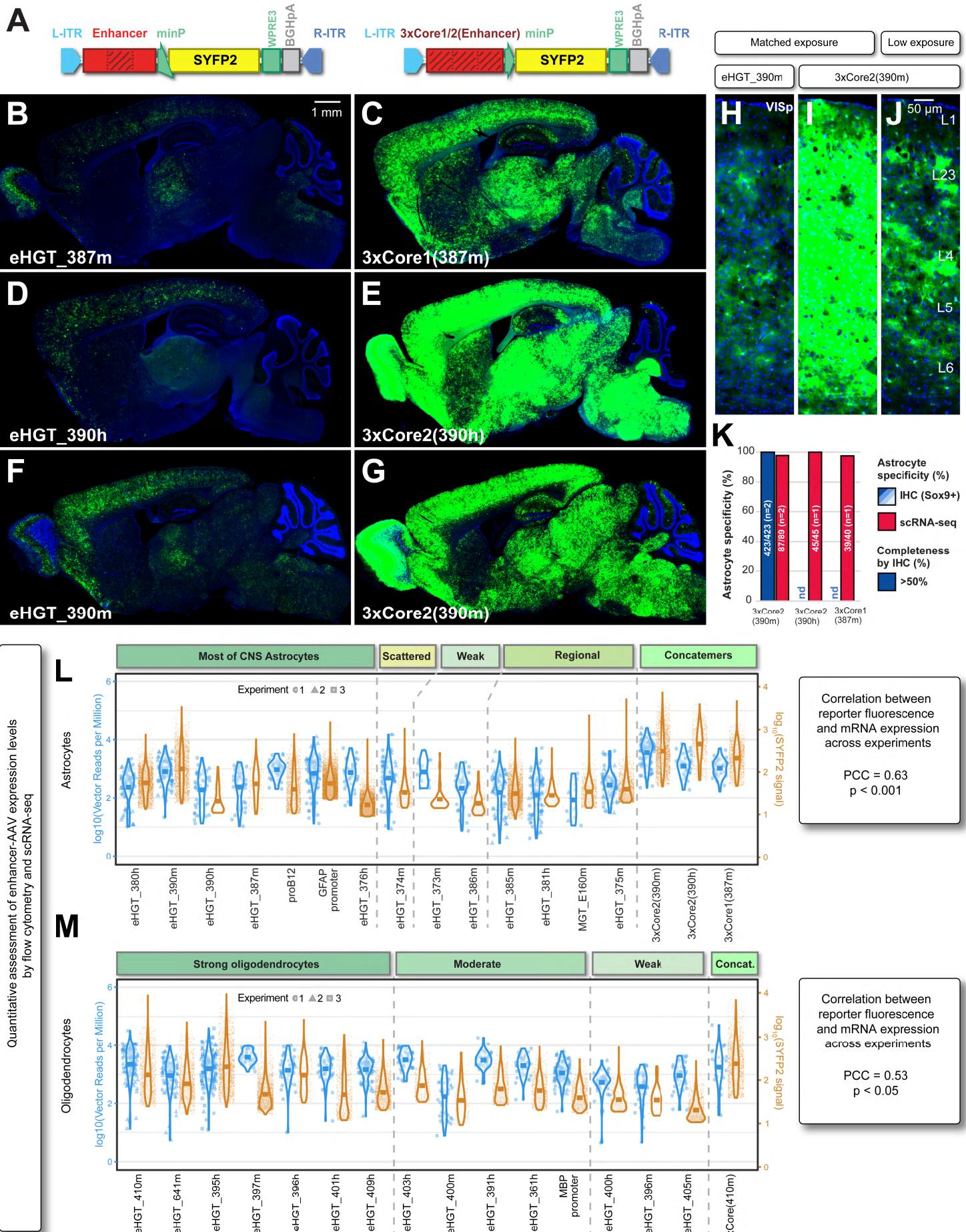
854



855     **Figure 3: A collection of oligodendrocyte-specific enhancer-AAV vectors with varying levels of**  
856     **expression.**  
857  
858     (A-F) Oligodendrocyte enhancer-AAVs marking many oligodendrocytes throughout most of the CNS.  
859     eHGT\_410m (A), eHGT\_641m (B), eHGT\_395h (C), eHGT\_396h (D), eHGT\_409h (E), and  
860     eHGT\_400h (F) label many oligodendrocytes throughout FB, MB, HB, and CBX, but at differing  
861     expression levels.  
862     (G-I) Oligodendrocyte enhancer-AAVs marking oligodendrocytes in lumbar SpC. eHGT\_410m (G),  
863     eHGT\_409h (H), and eHGT\_400h (I) mark oligodendrocytes in gray and white matter of SpC, but at  
864     different intensities.  
865     (J-O) Positive confirmation of molecular oligodendrocyte identity across brain regions. SYFP2+  
866     oligodendrocytes are colabeled with CC1 immunoreactivity in VISp, CBX, and Pons.  
867     (P) Quantification of specificity for oligodendrocytes by oligodendrocyte enhancer-AAVs. Specificity and  
868     completeness for oligodendrocyte labeling by enhancer-AAVs was quantified by costaining with CC1  
869     antibody in VISp, Pons, and CBX. Specificity is defined as the number of SYFP2+CC1+ / total SYFP2+  
870     cells x 100%. Completeness is defined as the number of SYFP2+CC1+ / total CC1+ cells x 100%.  
871     Brains from one to three mice per condition were analyzed, with range 101-332 cells counted (median  
872     147) per brain region analyzed. Specificity was also quantified by scRNA-seq, defined as the  
873     percentage of sorted SYFP2+ cells mapping as oligodendrocytes within the VISp molecular  
874     taxonomy<sup>83</sup>. Overall, specificity is high for many oligodendrocyte-specific vectors, with “Weak” vectors  
875     showing low completeness.  
876     (Q) Myelinating oligodendrocyte morphologies throughout the brain with eHGT\_410m. Sections were  
877     visualized with serial blockface imaging on the Tissuecyte platform.  
878     Abbreviations: SpC spinal cord, VISp primary visual cortex, CBX cerebellar cortex, LSX lateral septal  
879     complex, MY medulla.  
880  
881



882 **Figure 4: Transcriptomic identities of prospectively targeted astrocytes and oligodendrocytes.**  
883  
884 (A-C) Groups of transcriptomically profiled single cells, as visualized by UMAP. Single cells labeled by  
885 various astrocyte- and oligodendrocyte-specific enhancer-AAVs ( $n = 1946$  quality-filtered cells) were  
886 profiled from 47 brains in 47 independent experiments by SMARTerV4<sup>83</sup>. Libraries were aligned to  
887 mm10 and transformed into UMAP space for visualization, with coloring by enhancer (A), mapped  
888 taxonomic cell type cluster (B), and taxonomic mapping confidence (C). Overall CTX astrocytes group  
889 away from CTX oligodendrocytes as expected, and MB/HB astrocytes and Bergmann glia astrocytes  
890 group away from CTX astrocytes, consistent with recent results<sup>20</sup>. Note that eHGT\_381h- and  
891 MGT\_E160m-labeled astrocytes were dissected from MB/HB region, and eHGT\_375m-labeled  
892 Bergmann glia were dissected from CBX region, but the remainder of the cells were dissected from  
893 VISp.  
894  
895 (D-E) Quantifications of taxonomic cell type cluster mapping by enhancer vector. Prospectively labeled  
896 astrocytes from all enhancer-AAV vectors dissected from VISp predominantly map to cluster “5112  
897 Astro-TE NN\_3”, whereas those from MB/HB dissections (eHGT\_381h and MGT\_E160m)  
898 predominantly map to cluster “5109 Astro-NT NN\_2”, and eHGT\_375m-labeled astrocytes from CBX  
899 dissections predominantly map to cluster “5102 Bergmann NN”. In contrast, all prospectively labeled  
900 oligodendrocytes predominantly map to cluster “5158 MOL NN”. Cluster identities are from a recent  
whole mouse brain taxonomy study<sup>20</sup>.  
901  
902 (F-H) De novo motif detection in astrocyte- and oligodendrocyte-specific enhancer sequences using  
903 MEME-CHIP<sup>56</sup> identifies one strong consensus motif in each set of sequences (top). These de novo  
904 motifs were mapped against databases of known TF motifs using TomTom (bottom), which identified  
905 the top hits as the Zic family consensus motifs for astrocytes, and Sox family motif for oligodendrocytes  
906 (Sox10 shown). These TFs (Zic5 and Sox10) show highly specific expression differences between  
astrocytes and oligodendrocytes from prospective scRNA-seq profiling (H).  
907  
908 (I-N) Intrinsic SYFP2 expression from the indicated enhancer-AAVs after retro-orbital administration.  
909 Images were generated by STPT. Boxes in I and L correspond to K and N, respectively. Scale in I and  
K is 500  $\mu\text{m}$ .  
910  
911 (O-R) MERFISH data showing the distribution of three astrocyte cell types revealed by single cell gene  
expression from the whole mouse brain<sup>20</sup>.  
912 Abbreviations: CTX cerebral cortex, STR striatum, GPe globus pallidus, external segment.  
913  
914



915     **Figure 5: Optimizing astrocyte and oligodendrocyte enhancer strength.**

916  
917     (A) Native Enhancer and for 3xCore2(Enhancer) vector designs. The central approximate third of the  
918     enhancer (the “Core2” element) is marked by dark hatches, and this element is triply concatemerized in  
919     the 3xCore2(Enhancer) vector. Alternatively, the first or third segment (“Core1” or “Core3”) may be  
920     concatemerized (determined empirically).

921     (B-J) Dramatic increase in expression levels while maintaining specificity using 3xCore1/2(Enhancer)  
922     vector designs. Brains from mice injected with the Enhancer or 3xCore1/2(Enhancer) vectors were  
923     processed and imaged in parallel in these experiments. (H-J) Zoom in view of eHGT\_390m- and  
924     3xCore2(390m)-injected mouse VISp shows high specificity for morphological astrocytes throughout  
925     cortical layers in both cases.

926     (K) Quantification of specificity for astrocytes by concatemer astrocyte enhancer-AAVs within VISp by  
927     IHC and scRNA-seq as described in Figure 2P.

928     (L-M) Direct correlated quantification of enhancer strength by flow cytometry and scRNA-seq, for both  
929     astrocyte- (L) and oligodendrocyte-specific (M) enhancer-AAVs. The left (blue) y-axis represents the  
930     log-transformed vector transgene reads per million in individual sorted scRNA-seq-profiled cells. The  
931     right (brown) y-axis represents the log-transformed SYFP2 signal intensity of positively gated vector-  
932     expressing cells observed on the flow cytometer, quantified as the fold signal of positive cells  
933     normalized to non-expressing cell autofluorescence (taken as background). Points represent individual  
934     cells observed by scRNA-seq and by flow cytometry, visualized also as violins, and with the horizontal  
935     bar representing mean expression levels across all cells expressing that enhancer-AAV, across one to  
936     three replicate experiments per vector. Across all experiments, we observe significant correlation  
937     between mean expression intensity at the RNA level by scRNA-seq, and mean SYFP2 reporter  
938     expression by signal intensity (astrocytes: n = 26 experiments, Pearson correlation coefficient [PCC]  
939     0.63, t = 3.97, df = 24, p = 0.00057; oligodendrocyte n = 22 experiments, PCC 0.53, t = 2.82, df = 20, p  
940     = 0.011). Furthermore, 3xCore astrocyte enhancers are among the strongest enhancers we have  
941     characterized, typically several fold stronger than their native counterparts.

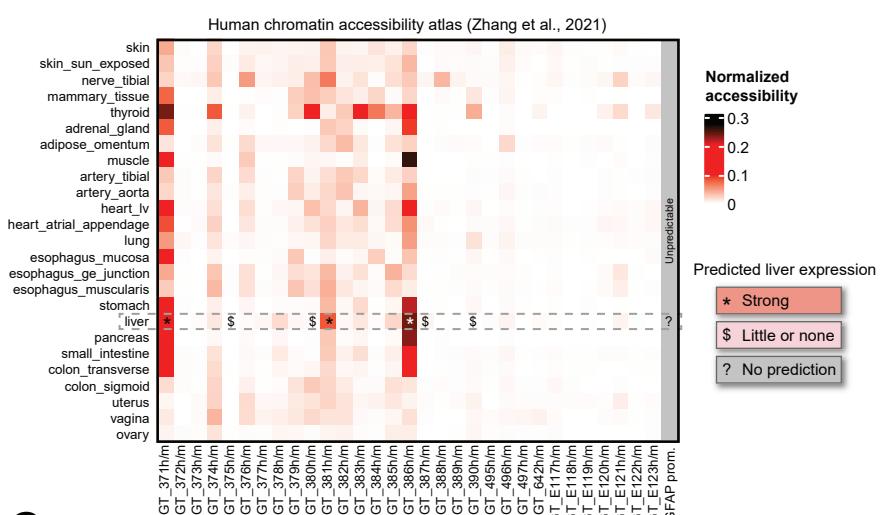
942     Abbreviations: VISp primary visual cortex.

943

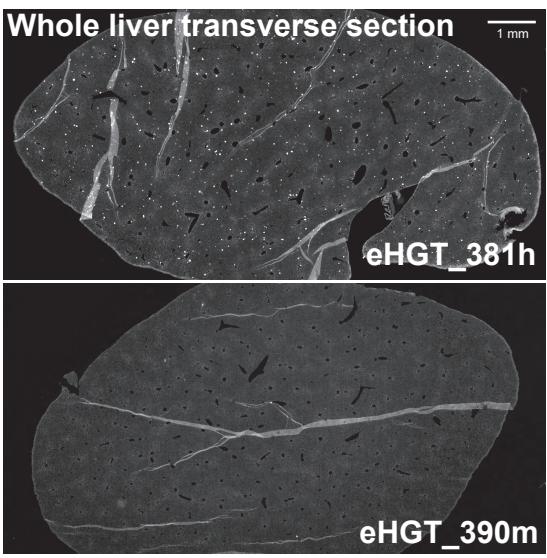
944

**A**

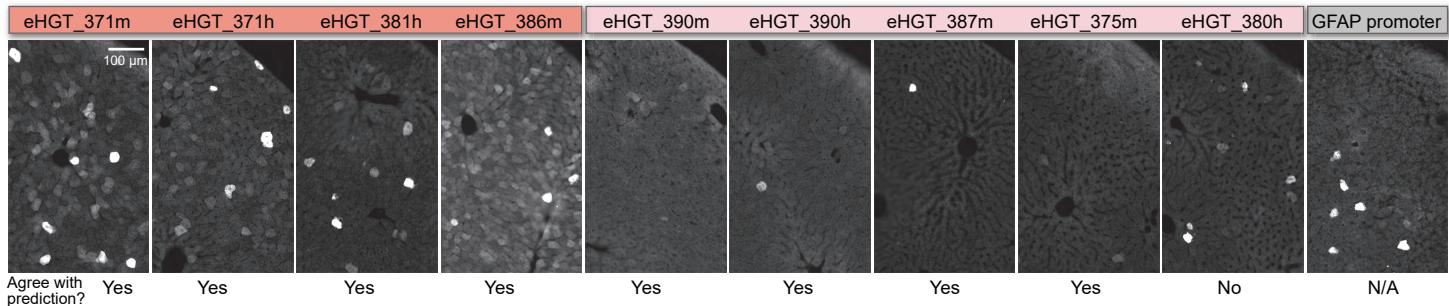
Predictability of enhancer-AAV expression across body organs



**B**



**C**



**D**

Predictability of enhancer-AAV expression across disease status

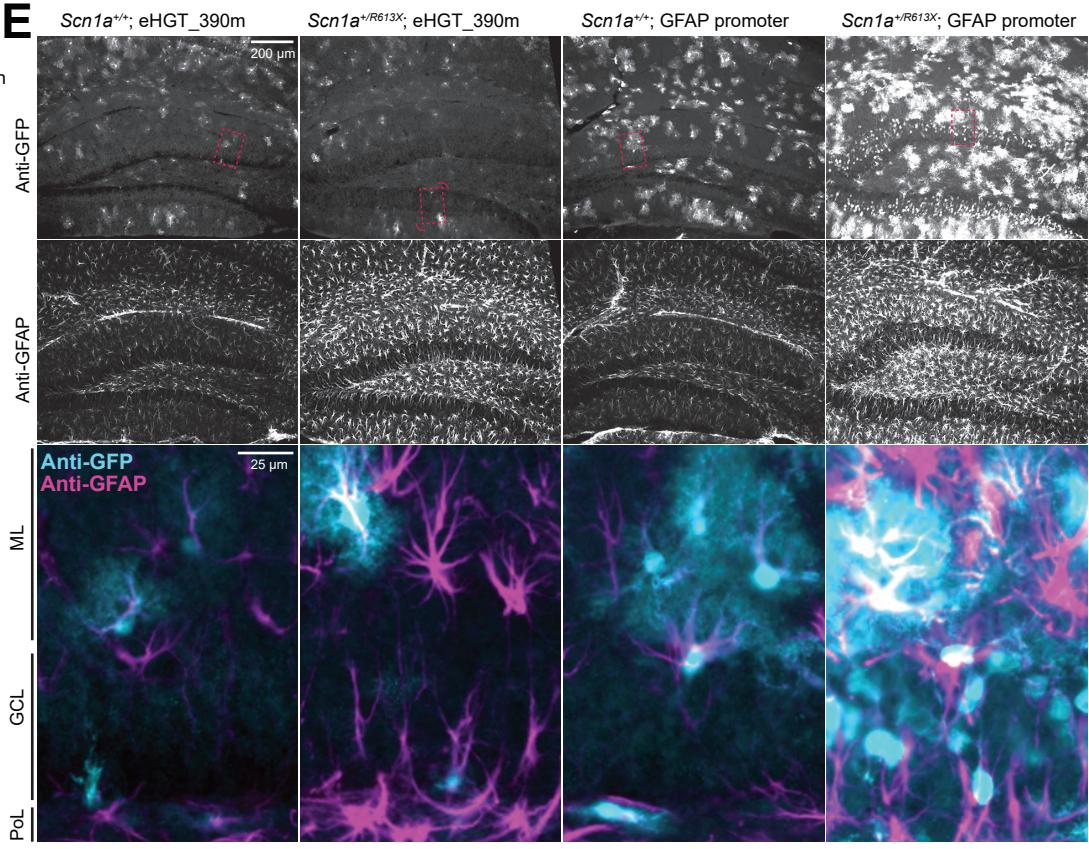
Astrocyte enhancer-AAVs delivered at P21 via intravenous retro-orbital delivery



*Scn1a<sup>+/+</sup>* or littermate controls

Expression during Dravet critical period (P21-P35)

Tissue analysis at P42



945     **Figure 6: Predictability of astrocyte enhancer-AAV expression patterns across body organs and**  
946     **across disease states.**

947  
948     (A) Accessibility profiles of astrocyte-specific enhancers in the human whole-body accessibility atlas<sup>65</sup>.  
949     Single-cell profiles were grouped within each tissue into pseudo-bulk aggregates, then normalized  
950     according to the signal (reads in peaks) within the dataset. Accessibility profiles are likely to predict  
951     enhancer activities within each tissue. Focusing on liver, some astrocyte-specific enhancers are  
952     predicted to have high expression, and some are predicted to have very little or no expression. In  
953     contrast, accessibility atlases do not predict expression of *GFAP* promoter across tissues.

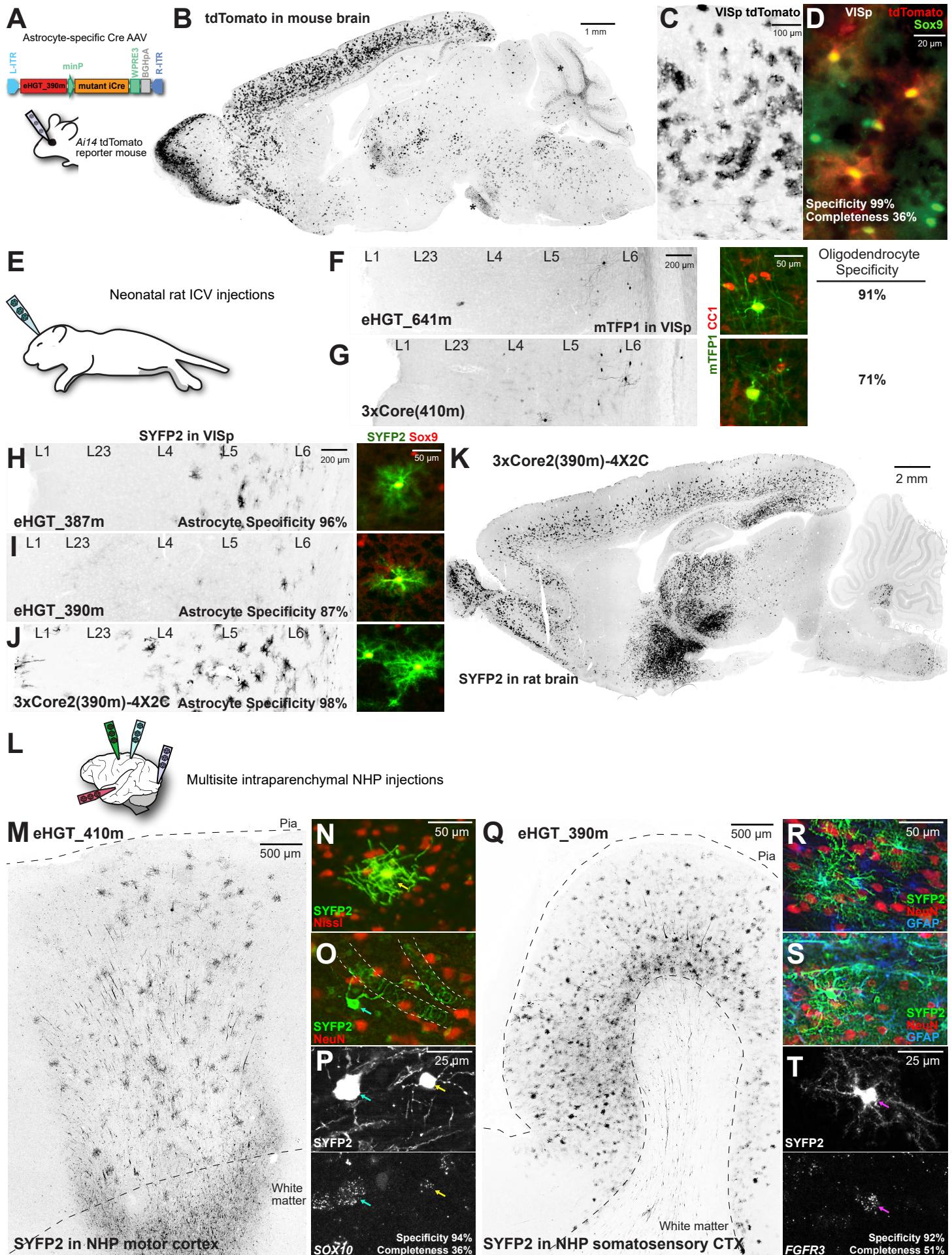
954     (B) Whole livers from mice injected intravenously with eHGT\_381h- and eHGT\_390m-enhancer-AAV  
955     vectors, stained with anti-GFP antibody. eHGT\_381h has high liver accessibility, is predicted to have  
956     high liver expression, and shows many strong SYFP2-expressing hepatocytes throughout the liver as  
957     predicted. In contrast, eHGT\_390m has very little liver accessibility and so is predicted to have little  
958     liver expression, and in fact shows few positive SYFP2-expressing hepatocytes as predicted.

959     (C) Agreement between liver expression predictions and liver expression measurements across several  
960     astrocyte-specific enhancer-AAV vectors. eHGT\_371m, 371h, 381h, and 386m all show many SYFP2-  
961     expressing hepatocytes as predicted. eHGT\_390m, 390h, 375m, and 387m show few weak SYFP2-  
962     expressing hepatocytes as predicted. *GFAP* promoter shows many expressing hepatocytes, which was  
963     not predictable from the accessibility atlases. eHGT\_380h shows many SYFP2-expressing astrocytes,  
964     in contrast to the epigenetic prediction. Liver images in B and C represent one to two mice analyzed for  
965     each vector.

966     (D-E) Testing fidelity of enhancer-AAV expression across disease states. We used a Dravet syndrome  
967     model *Scn1a*<sup>R613X/+</sup> mouse to induce epilepsy-associated hippocampal gliosis, injected enhancer-AAVs  
968     prior to the critical period, and analyzed tissue for expression patterns after the critical period (D). We  
969     assessed hippocampal gliosis with anti-GFAP antibody and enhancer-AAV expression with anti-GFP  
970     antibody (E). eHGT\_390m maintained specific expression and similar levels in hippocampal astrocytes  
971     regardless of epileptic gliosis. In contrast, *GFAP* promoter expression strongly increased in gliotic  
972     astrocytes, and also was observed in dentate gyrus granule cells. Red dashed rectangles indicate the  
973     position of the expanded zoomed view, and the curved arrows indicate a rotated view.

974     Abbreviations: ML molecular layer, GCL granule cell layer, PoL polymorphic layer.

975  
976



977 **Figure 7: Genetic targeting of astrocytes and oligodendrocytes with functional transgenes and**  
978 **across species.**

979

980 (A) Design and testing of an astrocyte-specific mutant Cre-expressing enhancer-AAV.

981 (B-D) Specific recombination in astrocytes. Ai14 reporter recombination is observed in multiple parts of  
982 mouse brain (B), except for a few regions with non-astrocyte recombination including thalamus, pontine  
983 gray, and cerebellar granule layer (marked by asterisks). Recombination within cortex is highly specific  
984 (C-D).

985 (E) Testing enhancer-AAV vectors by neonatal rat ICV injections.

986 (F-G) Validation of oligodendrocyte-specific enhancer-AAV vectors in rat. eHGT\_410m and 641m show  
987 specific expression in CC1+ VISp oligodendrocytes.

988 (H-J) Validation of astrocyte-specific enhancer-AAV vectors in rat. eHGT\_387m, 390m, and  
989 3xCore2(390m) show specific expression in Sox9+ VISp astrocytes. 3xCore2(390m) vector also  
990 incorporates 4X2C 3'UTR miRNA binding sites to prevent any off-target labeling in excitatory neurons<sup>70</sup>.

991 (K) 3xCore2(390m) with 4X2C 3'UTR miRNA binding sites<sup>70</sup> achieves widespread expression  
992 throughout the rat forebrain.

993 (L) Multiple stereotactic intraparenchymal injections into NHP brain.

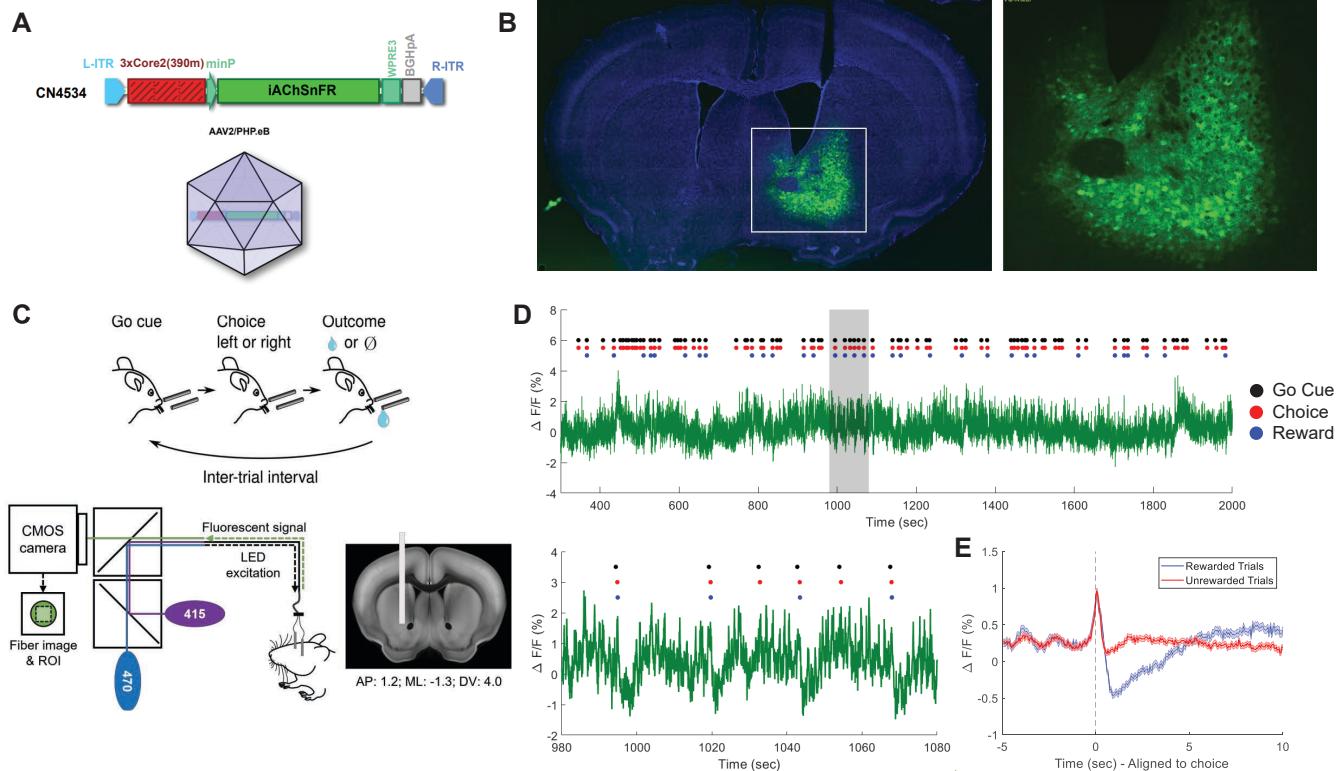
994 (M-P) Prospective labeling of NHP oligodendrocytes in vivo. eHGT\_410m enhancer-AAV vector gives  
995 widespread labeling of oligodendrocytes throughout the depth of motor cortex (M). Most labeled NHP  
996 oligodendrocytes exhibit multipolar ramified morphology indicative of local axon myelination (N). Some  
997 labeled oligodendrocytes exhibit morphologies suggesting wrapping around wider tubular structures  
998 highlighted with dashed white lines (O). SYFP2-expressing cells of both morphological types express  
999 the oligodendrocyte/OPC marker SOX10 with high specificity (P).

1000 (Q-T) Prospective labeling of NHP astrocytes in vivo. eHGT\_390m enhancer-AAV vector gives  
1001 widespread labeling of astrocytes throughout the depth of somatosensory cortex (Q). A few large L5ET  
1002 neurons are also labeled. Labeled astrocytes show the expected bushy morphology and GFAP  
1003 immunoreactivity of astrocytes in parenchyma (R) and sometimes reside near walls of large-diameter  
1004 tubular structures (S). SYFP2-expressing astrocytes express the astrocyte marker FGFR3 with high  
1005 specificity (T).

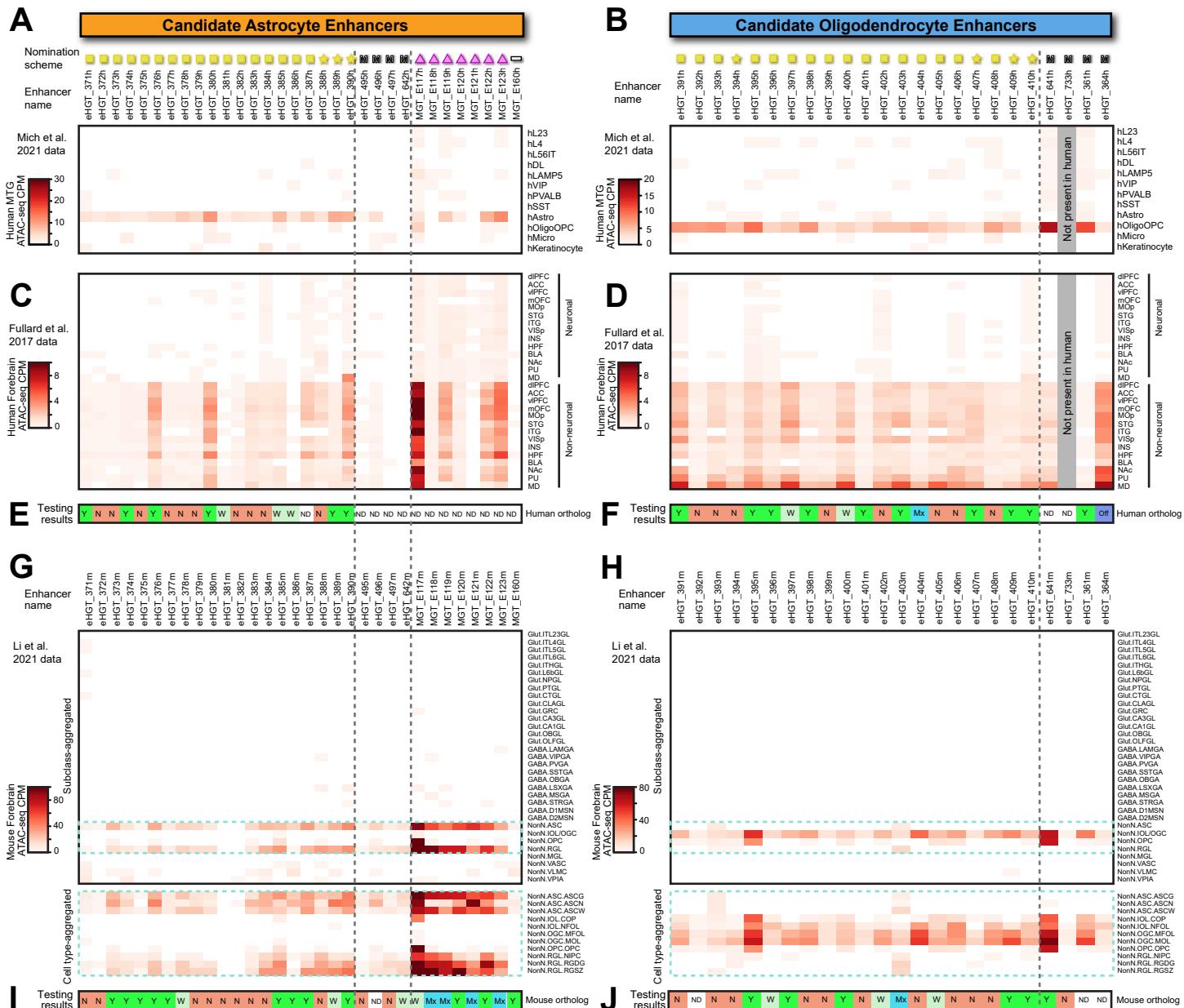
1006 Abbreviations: VISp primary visual cortex.

1007

1008



- 1009     **Figure 8: Astrocyte specific sensing of cholinergic signals in the nucleus accumbens during**  
1010     **behavior.**
- 1011  
1012     (A) 3xCore2(390m) driving expression of iAChSnFR. Enhancer vector is cloned into a pAAV plasmid  
1013     and packaged into PHP.eB AAVs.
- 1014     (B) Coronal section showing stereotaxic injection of enhancer virus expressing iAChSnFR in the  
1015     nucleus accumbens (injection coordinates: AP: 1.2, ML: 1.3, DV: 4.1).
- 1016     (C) Behavior and imaging experiment setup. Top: dynamic foraging behavior task schematic. Bottom:  
1017     Fiber photometry instrumentation schematic and fiber location in a coronal section.
- 1018     (D) Fiber photometry signals of acetylcholine fluctuations during task performance. Top: ~30 min  
1019     segment of a 2-hour session of dynamic foraging. Black dots represent the auditory cue, red dots  
1020     represent time of first lick, blue dots represent water reward delivery. Bottom: 100 second (980-1080  
1021     seconds) zoom in on above session with 6 individual trials (4 rewarded and 2 unrewarded trials).
- 1022     (E) Trial-averaged signals of rewarded and unrewarded trials aligned to time of first lick (mean±sem).
- 1023  
1024



1025   **Extended Data Figure 1: Epigenetic characterization of candidate enhancers in additional**  
1026   **chromatin accessibility datasets.**

1027  
1028   (A-D) Accessibility profiles of all tested candidate human astrocyte-specific (A,C) and human  
1029   oligodendrocyte-specific (B, D) enhancers. Human enhancer regions are characterized in the datasets  
1030   of Mich et al.<sup>35</sup> (A-B), who performed snATAC-seq on neurosurgical MTG samples, and of Fullard et  
1031   al.<sup>88</sup> (C-D), who performed bulk ATAC-seq on neuronal (sorted NeuN<sup>+</sup>) and non-neuronal (sorted NeuN<sup>-</sup>)  
1032   nuclei from dissections spanning multiple regions of human postmortem forebrain. Overall, many  
1033   candidate astrocyte- and oligodendrocyte-specific enhancers show accessibility specific to non-  
1034   neuronal cells across much of the human forebrain.

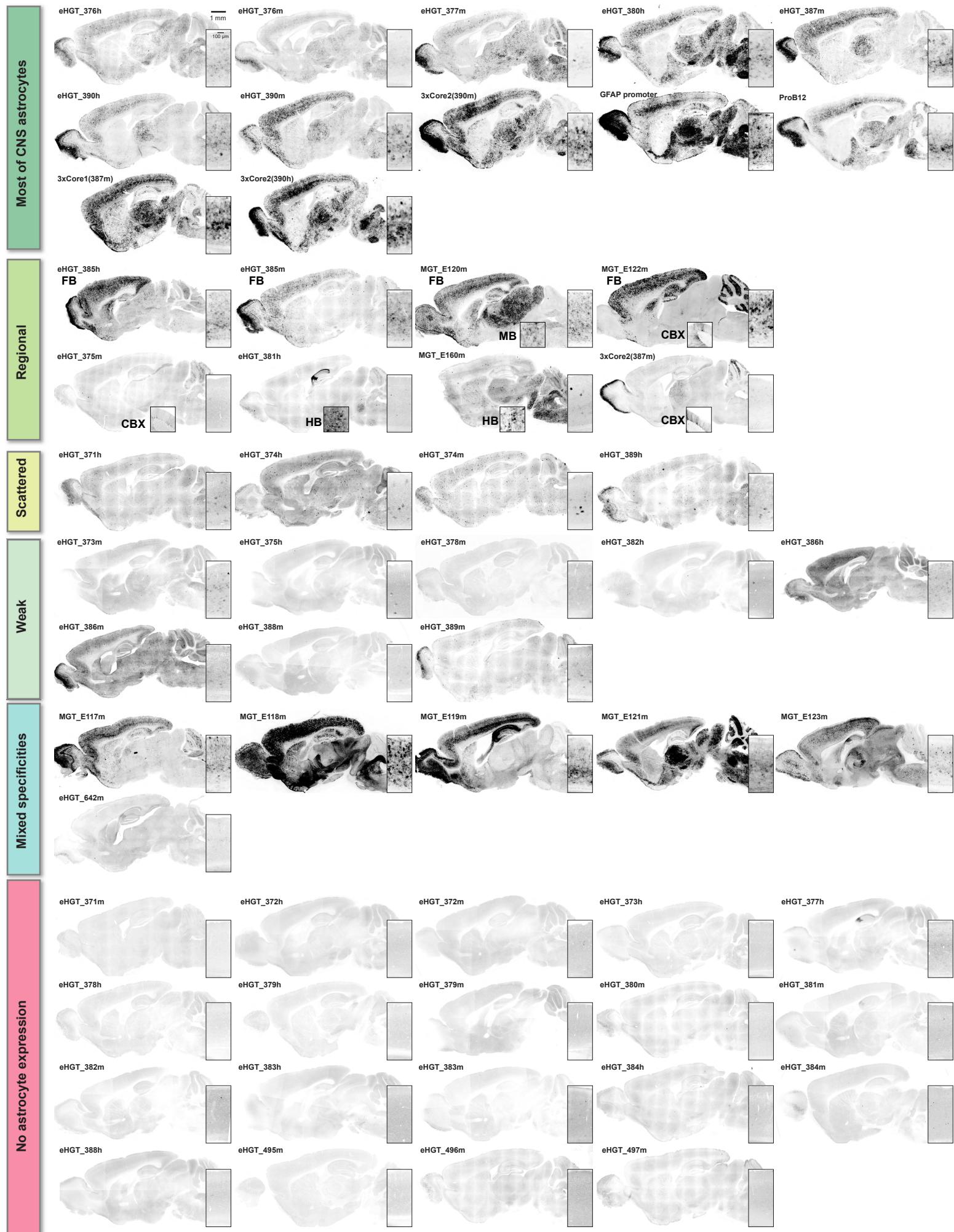
1035   (E-F) Screening results from testing human candidate enhancers (same as Figure 1F-G, provided  
1036   again for visualization). Testing result bar: Y = yes, enhancer-AAV gives strong or moderate on-target  
1037   expression pattern; N = no, enhancer-AAV fails to express; W = weak on-target expression pattern; Mx  
1038   = mixed specificities consisting of on-target cells plus unwanted neuronal populations; Off = off-target  
1039   expression pattern, ND = no data.

1040   (G-H) Accessibility profiles for all tested candidate mouse astrocyte-specific (G) and oligodendrocyte-  
1041   specific enhancers (H). Mouse enhancer regions are characterized in the dataset of Li et al.<sup>48</sup>, who  
1042   performed droplet-based snATAC-seq on many regions spanning the full mouse cerebrum.

1043   (I-J) Screening results from testing mouse candidate enhancers (same as Figure 1F-G, provided again  
1044   for visualization).

1045

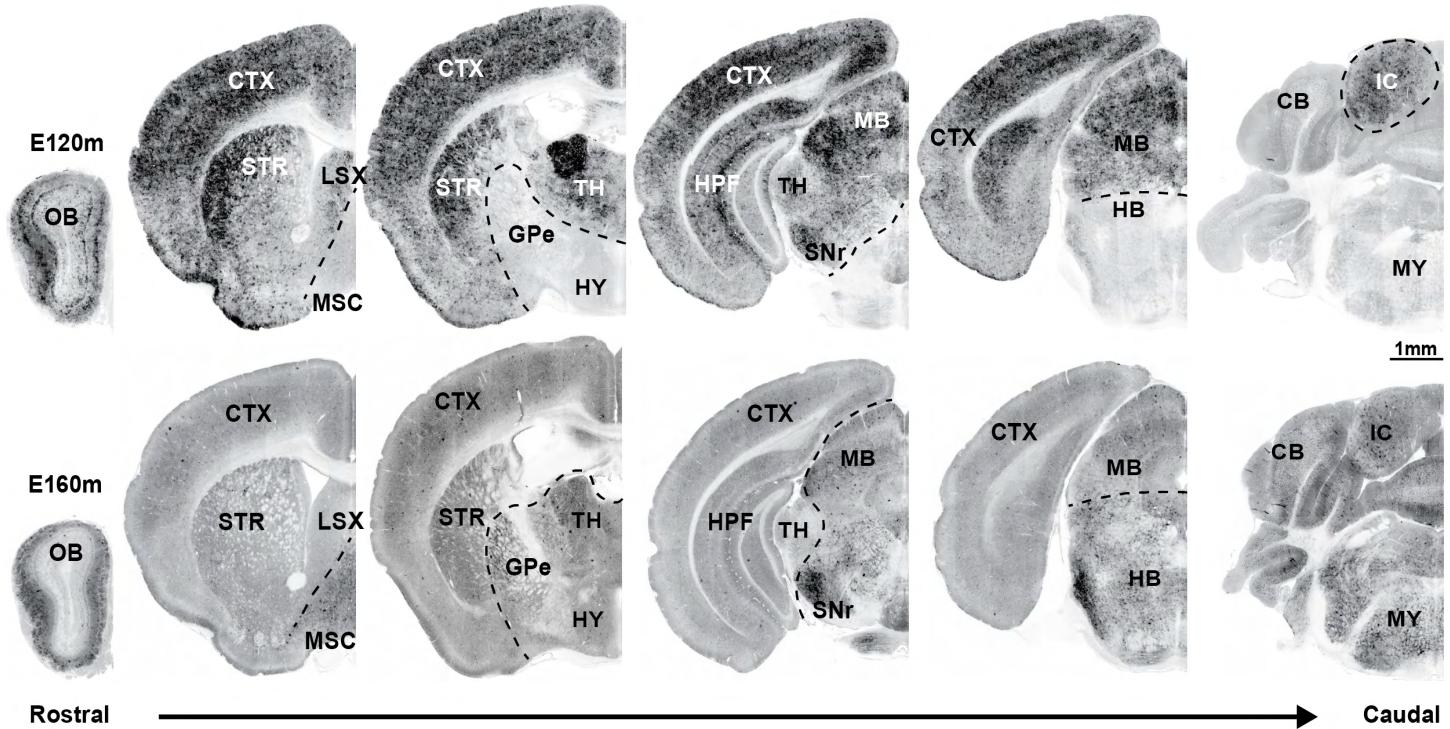
1046



1047   **Extended Data Figure 2: Full screening results of all candidate enhancer-AAVs targeting**  
1048   **astrocytes.**

1049   We injected mice with the indicated enhancer-AAV vectors between P42 and P56, then after 3-4 weeks  
1050   we harvested brains, sliced them on a sliding microtome with freezing stage at 30 µm thickness, co-  
1051   stained the sections with DAPI, then mounted them with Vectashield Vybrance. Insets show a full  
1052   cortical column from VISp (primary visual cortex), and in some cases also the labeling in MB (midbrain)  
1053   or HB (hindbrain) or CBX (cerebellar cortex) is also shown. Astrocyte-specific enhancer-AAV vectors  
1054   are broadly grouped by expression pattern into the following categories: “Most of CNS astrocytes”,  
1055   “Regional” meaning present at medium-to-high levels in one or more broad brain regions but not all,  
1056   “Scattered” meaning a few astrocytes are strongly labeled throughout the brain, “Weak” meaning many  
1057   astrocytes throughout the brain are labeled at low level, “Mixed specificities” meaning one or more off-  
1058   target neuron populations are also labeled in addition to astrocytes, and “No astrocyte expression”  
1059   meaning failure to detect any clear astrocytes in these whole-brain sagittal images. These screening  
1060   images were taken on multiple different microscopes, so the absolute levels of expression are difficult  
1061   to compare directly across brains.

1062  
1063



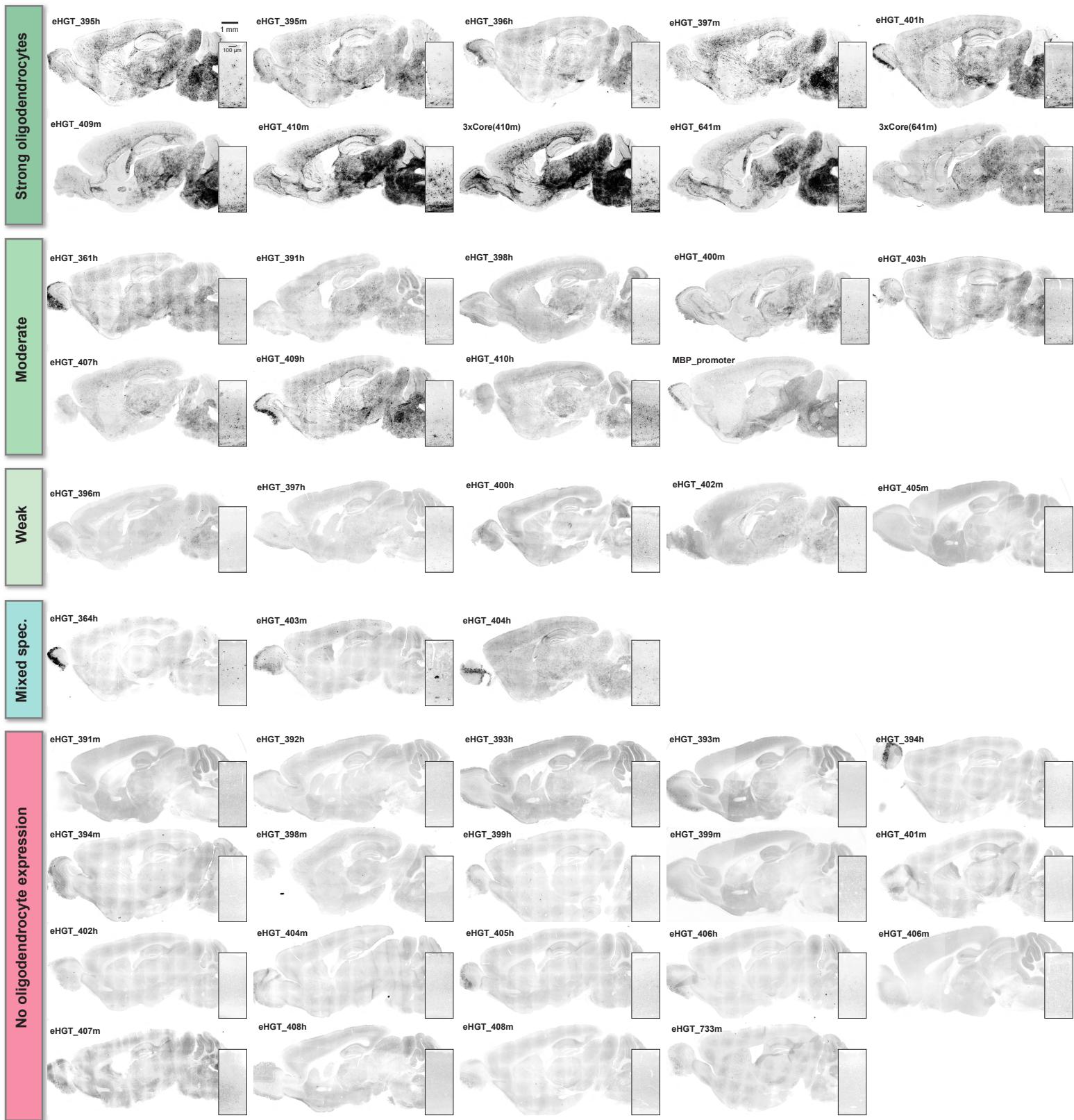
1064   **Extended Data Figure 3: Distinct astrocyte-specific expression domains of MGT\_E120m and**  
1065   **MGT\_E160m.**

1066  
1067   We injected mice with the indicated astrocyte-specific SYFP2-expressing enhancer-AAVs and  
1068   performed whole-brain blockface imaging using the TissueCyte platform<sup>82</sup>. These vectors display  
1069   largely non-overlapping zones of astrocyte expression: E120m is expressed in astrocytes within  
1070   multiple forebrain structures including CTX, STR, OB, LSX, HPF, and TH, as well as MB, whereas  
1071   E160m is expressed in MB, CBX, and HB structures as well as complementary forebrain structures  
1072   including HY, MSC, and GPe, and OB. In the OB E120m is expressed in astrocytes within the granule  
1073   cell layer, internal plexiform layer, and periglomerular cell layer, whereas E160m is expressed in a  
1074   complementary pattern of astrocytes within the external plexiform layer.

1075

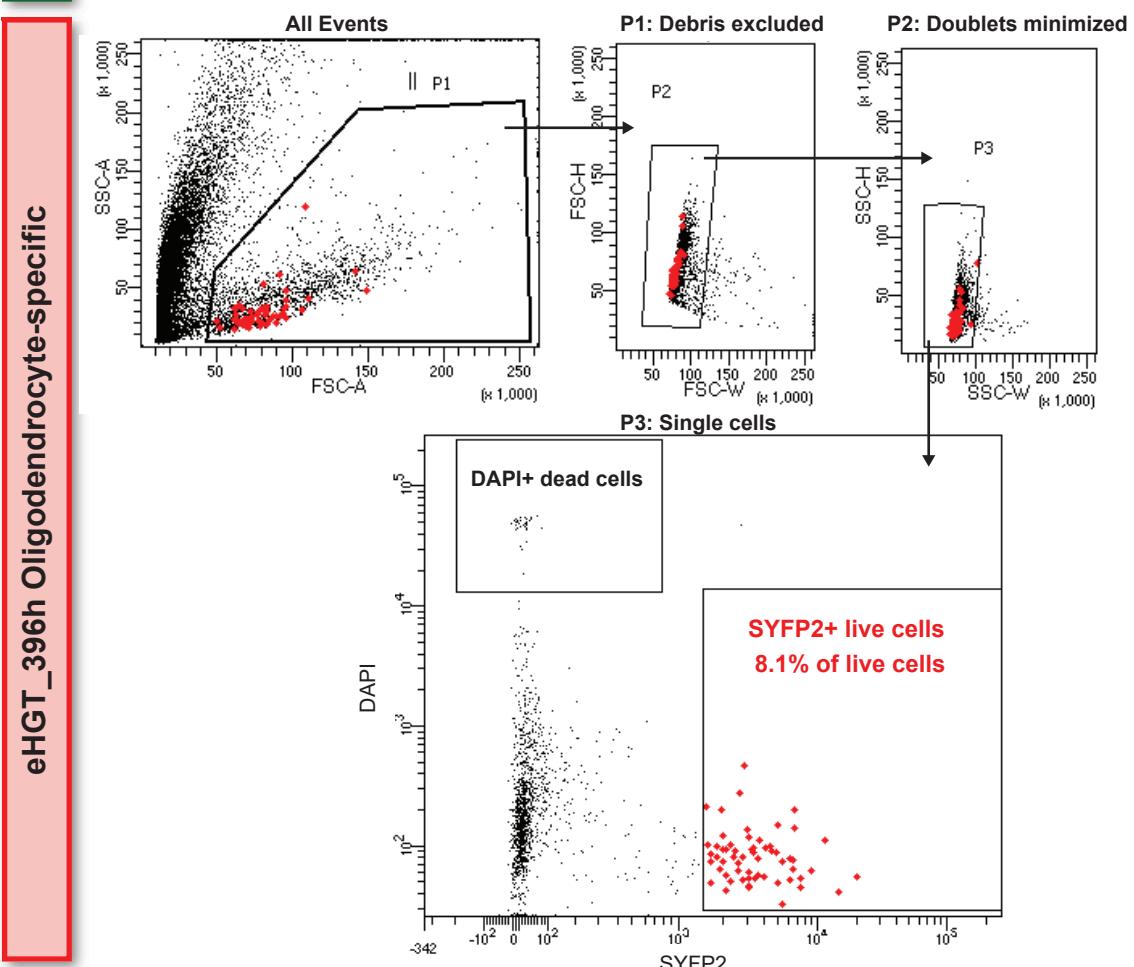
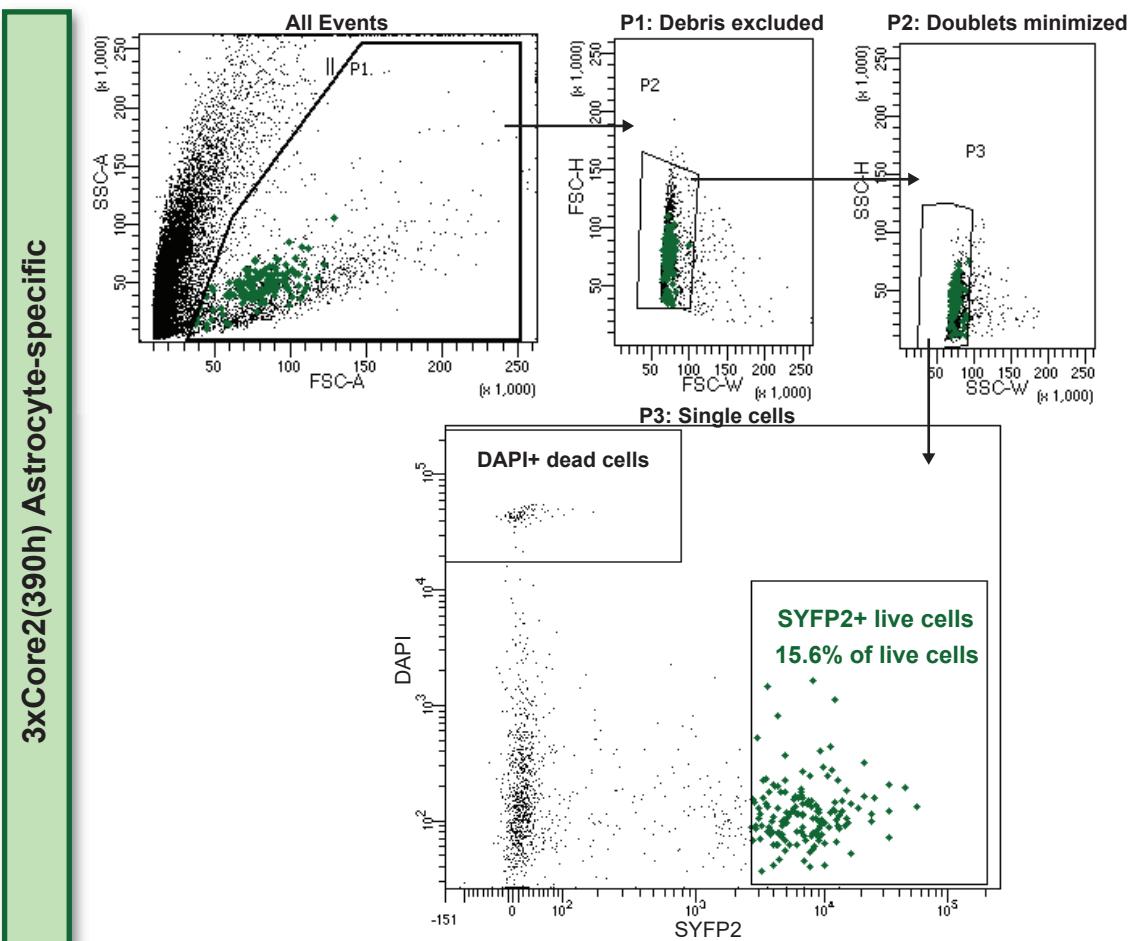
1076   Abbreviations: CTX cerebral cortex, STR striatum, OB olfactory bulb, LSX lateral septal complex, HPF  
1077   hippocampal formation, TH thalamus, MB midbrain, CBX cerebellar cortex, HB hindbrain, HY  
1078   hypothalamus, MSC medial septal complex, GPe globus pallidus, external layer.

1079



1080   **Extended Data Figure 4: Full screening results of all candidate enhancer-AAVs targeting**  
1081   **oligodendrocytes.**

1082   We injected mice with the indicated enhancer-AAV vectors between P42 and P56, then after 3-4 weeks  
1083   we harvested brains, sliced them on a sliding microtome with freezing stage at 30 µm thickness, co-  
1084   stained the sections with DAPI, and mounted them with Vectashield Vybrance. Oligodendrocyte-  
1085   specific enhancer-AAV vectors are broadly grouped by expression pattern into the following categories:  
1086   “Strong oligodendrocytes”, “Weak” meaning many oligodendrocytes throughout the brain are labeled at  
1087   low level, “Mixed specificities” meaning several off-target neuron or astrocyte populations are also  
1088   present in addition to oligodendrocytes, and “No oligodendrocyte expression” meaning failure to detect  
1089   any clear oligodendrocytes in these whole-brain sagittal images. These screening images were taken  
1090   on multiple different microscopes, so the absolute levels of expression are difficult to compare directly  
1091   across injections.  
1092



1093 **Extended Data Figure 5: Sorting enhancer-AAV-labeled astrocytes and oligodendrocytes.**

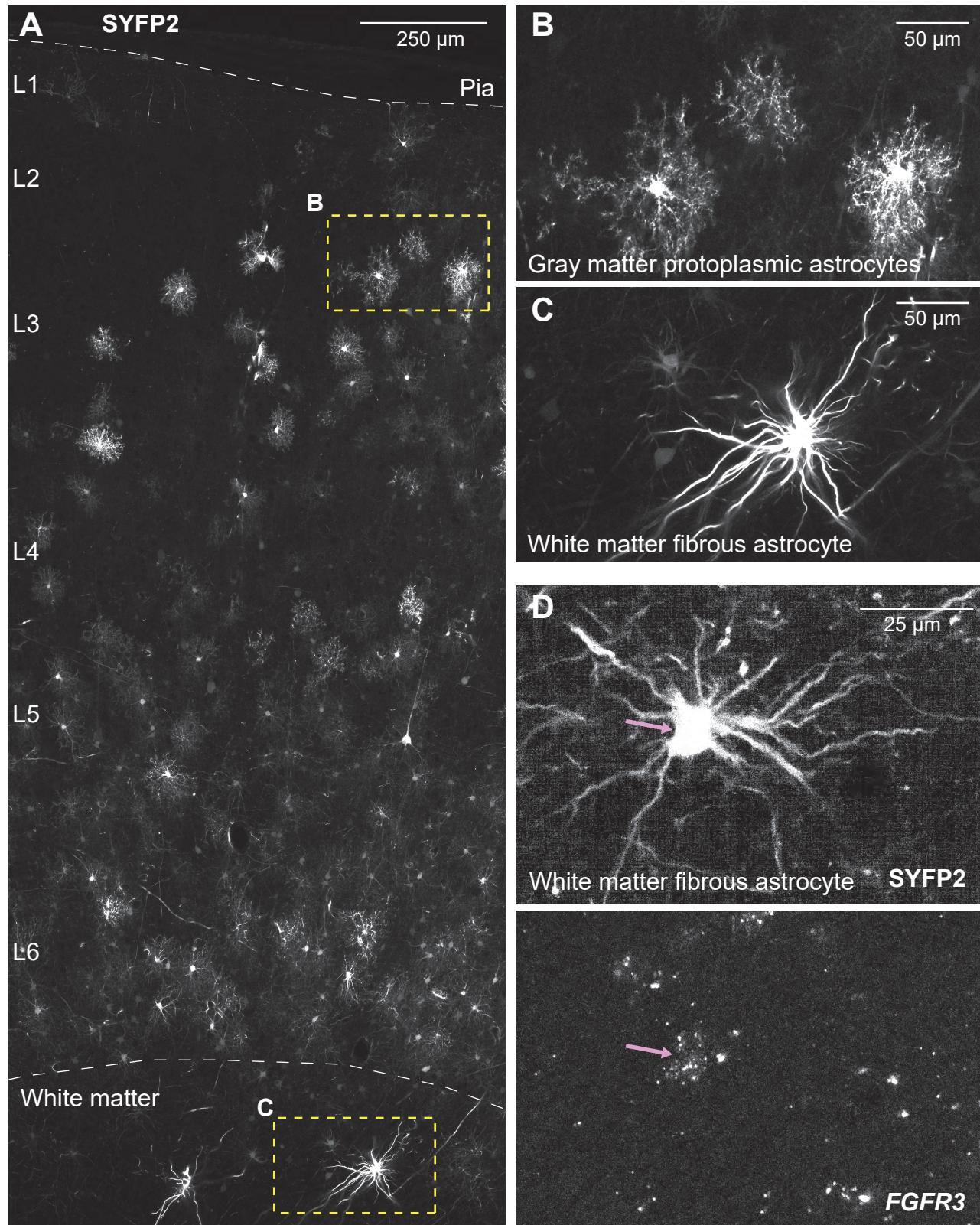
1094

1095 Example gating strategies for sorting 3xCore2(390h)-labeled astrocytes and eHGT\_396h-labeled  
1096 oligodendrocytes from mouse VISp.

1097

1098

## eHGT\_390m in NHP somatosensory cortex



1099   **Extended Data Figure 6: Diverse morphologies of NHP astrocytes labeled by enhancer-AAVs.**

1100

1101   (A-C) Labeling of both gray matter protoplasmic astrocytes and white matter fibrous astrocytes by  
1102   eHGT\_390m enhancer-AAV. We show full cortical column of a somatosensory cortex injection site in A,  
1103   with expanded insets to show protoplasmic astrocytes in gray matter (B) and fibrous astrocytes in white  
1104   matter (C).

1105   (D) Confirmation of astrocyte identity by mFISH. Fibrous astrocytes in white matter express the  
1106   astrocyte marker *FGFR3*, similar to gray matter protoplasmic astrocytes (**Figure 7T**).

1107

Enhancer name	Identification scheme	Genome	Chr	Start	Stop	Enhancer_group	PhyloP	Length	Specificity	Strength (CPM)	Screen result	Expression pattern	Included in GLM model
eHGT_361h	Marker_gene	hg38	chrX	103780509	103781455	Human_Oligo	1.823	855	0.635	11.72	Yes	Oligodendrocytes_moderate	No
eHGT_361m	Marker_gene	mm10	chrX	136826512	136827460	Mouse_Oligo	1.488	807	0.879	12.03	No_data	No_data	No
eHGT_364h	Marker_gene	hg38	chr21	33051808	33052323	Human_Oligo	2.5	458	0.614	2.29	Mixed_spe	Mixed_specificity_oligodendrocy	No
eHGT_364m	Marker_gene	mm10	chr16	91246204	91246699	Mouse_Oligo	1.99	444	0.945	9.54	No_data	No_data	No
eHGT_371h	High_specificity	hg38	chr3	13485182	13485473	Human_Astro	0.967	269	0.454	3.81	Yes	Scattered_astrocytes	Yes
eHGT_371m	High_specificity	mm10	chr6	91160424	91160736	Mouse_Astro	0.87	241	0.216	4.28	No	No_astrocyte_expression	Yes
eHGT_372h	High_specificity	hg38	chr7	121914818	121915080	Human_Astro	0.33	216	0.668	2.6	No	No_astrocyte_expression	Yes
eHGT_372m	High_specificity	mm10	chr6	22910076	22910291	Mouse_Astro	0.194	187	0.742	2.85	No	No_astrocyte_expression	Yes
eHGT_373h	High_specificity	hg38	chr5	97134713	97135080	Human_Astro	0.537	329	0.72	3.81	No	No_astrocyte_expression	Yes
eHGT_373m	High_specificity	mm10	chr17	17409626	17409965	Mouse_Astro	0.531	319	0.909	11.78	Yes	Most_astrocytes_weak	Yes
eHGT_374h	High_specificity	hg38	chr15	64845370	64845672	Human_Astro	1.618	266	0.424	2.86	Yes	Scattered_astrocytes	Yes
eHGT_374m	High_specificity	mm10	chr9	65576810	65577101	Mouse_Astro	1.091	259	0.542	5.35	Yes	Scattered_astrocytes	Yes
eHGT_375h	High_specificity	hg38	chr13	99961720	99962046	Human_Astro	4.977	268	0.659	2.51	No	No_astrocyte_expression	Yes
eHGT_375m	High_specificity	mm10	chr14	122455334	122455655	Mouse_Astro	3.82	268	0.867	5.71	Yes	Bergmann_glia_astrocytes	Yes
eHGT_376h	High_specificity	hg38	chr3	188076136	188076690	Human_Astro	1.341	457	0.646	4.24	Yes	Most_astrocytes_strong	Yes
eHGT_376m	High_specificity	mm10	chr16	24326000	24326508	Mouse_Astro	1.083	435	0.865	11.78	Yes	Most_astrocytes_strong	Yes
eHGT_377h	High_specificity	hg38	chr10	80406003	80406485	Human_Astro	0.687	453	0.782	4.41	No	No_astrocyte_expression	Yes
eHGT_377m	High_specificity	mm10	chr14	41015471	41016233	Mouse_Astro	0.541	747	0.656	7.85	Yes	Most_astrocytes_strong	Yes
eHGT_378h	High_specificity	hg38	chr11	120182373	120182964	Human_Astro	2.312	559	0.681	6.32	No	No_astrocyte_expression	No
eHGT_378m	High_specificity	mm10	chr9	43268975	43269581	Mouse_Astro	1.699	559	0.911	12.49	Weak	Most_astrocytes_weak	No
eHGT_379h	High_specificity	hg38	chr3	64837170	64837760	Human_Astro	0.151	496	0.659	4.93	No	No_astrocyte_expression	Yes
eHGT_379m	High_specificity	mm10	chr6	93093752	93094309	Mouse_Astro	0.216	460	0.939	8.21	No	No_astrocyte_expression	Yes
eHGT_380h	High_specificity	hg38	chr6	44045566	44046145	Human_Astro	0.591	527	0.633	10.82	Yes	Most_astrocytes_strong	Yes
eHGT_380m	High_specificity	mm10	chr17	45765782	45766284	Mouse_Astro	0.536	479	0.727	7.14	No	No_astrocyte_expression	Yes
eHGT_381h	High_specificity	hg38	chr7	131400918	131401149	Human_Astro	2.166	173	0.4	1.9	Weak	Mid/hindbrain_astrocytes	No
eHGT_381m	High_specificity	mm10	chr6	31434638	31434879	Mouse_Astro	1.8	172	0.927	5.35	No	No_astrocyte_expression	No
eHGT_382h	High_specificity	hg38	chr10	113047655	113048169	Human_Astro	0.544	435	0.685	3.98	No	No_astrocyte_expression	Yes
eHGT_382m	High_specificity	mm10	chr19	55825471	55825949	Mouse_Astro	0.439	424	0.694	5.71	No	No_astrocyte_expression	Yes
eHGT_383h	High_specificity	hg38	chr2	220959419	220959789	Human_Astro	1.026	312	0.858	3.38	No	No_astrocyte_expression	Yes
eHGT_383m	High_specificity	mm10	chr1	76942759	76943103	Mouse_Astro	0.81	315	0.902	11.42	No	No_astrocyte_expression	Yes
eHGT_384h	High_specificity	hg38	chr6	121897357	121897797	Human_Astro	1.695	351	0.342	2.77	No	No_astrocyte_expression	Yes
eHGT_384m	High_specificity	mm10	chr10	56893293	56893720	Mouse_Astro	1.576	366	0.846	9.64	No	No_astrocyte_expression	Yes
eHGT_385h	High_specificity	hg38	chr9	123741938	123742543	Human_Astro	6.233	563	0.655	7.88	Weak	Telencephalon_astrocytes	No
eHGT_385m	High_specificity	mm10	chr2	38111566	38112219	Mouse_Astro	4.798	572	0.714	14.27	Yes	Telencephalon_astrocytes	No
eHGT_386h	High_specificity	hg38	chr1	15736012	15736372	Human_Astro	0.595	287	0.417	2.51	Weak	Most_astrocytes_weak	No
eHGT_386m	High_specificity	mm10	chr4	141623748	141624075	Mouse_Astro	1.042	231	0.527	5	Yes	Most_astrocytes_weak	No
eHGT_387h	High_specificity	hg38	chr1	219881964	219882484	Human_Astro	0.601	467	0.729	6.06	No_data	No_data	No
eHGT_387m	High_specificity	mm10	chr1	185493675	185494167	Mouse_Astro	0.633	425	0.959	15.35	Yes	Most_astrocytes_strong	No
eHGT_388h	High_specificity	hg38	chr12	20657459	20657845	Human_Astro	2.912	369	0.754	3.98	No	No_astrocyte_expression	Yes
eHGT_388m	High_specificity	mm10	chr6	141495577	141496010	Mouse_Astro	2.283	362	0.879	16.77	No	No_astrocyte_expression	Yes
eHGT_389h	High_specificity	hg38	chr20	63252174	63252906	Human_Astro	0.003	694	0.648	9.52	Yes	Scattered_astrocytes	No
eHGT_389m	High_specificity	mm10	chr2	180952658	180953361	Mouse_Astro	0.351	667	0.695	8.56	Weak	Most_astrocytes_weak	No
eHGT_390h	High_specificity	hg38	chr7	42152720	42153410	Human_Astro	7.463	634	0.699	8.91	Yes	Most_astrocytes_strong	Yes
eHGT_390m	High_specificity	mm10	chr13	15543638	15544333	Mouse_Astro	5.544	639	0.869	16.42	Yes	Most_astrocytes_strong	Yes
eHGT_391h	High_specificity	hg38	chr11	729607	730042	Human_Oligo	-0.132	419	0.688	7.49	Weak	Oligodendrocytes_moderate	No
eHGT_391m	High_specificity	mm10	chr7	141365022	141365541	Mouse_Oligo	0.135	474	0.901	13.2	No	No_oligodendrocyte_expression	No
eHGT_392h	High_specificity	hg38	chr4	48787039	48787541	Human_Oligo	1.369	431	0.776	6.88	No	No_oligodendrocyte_expression	No
eHGT_392m	High_specificity	mm10	chr5	73264044	73264568	Mouse_Oligo	1.225	448	0.832	4.25	No_data	No_data	No
eHGT_393h	High_specificity	hg38	chr18	66603993	66604511	Human_Oligo	2.036	451	0.755	7.23	No	No_oligodendrocyte_expression	Yes
eHGT_393m	High_specificity	mm10	chr1	110977271	110977865	Mouse_Oligo	1.391	548	0.593	6.16	No	No_oligodendrocyte_expression	Yes
eHGT_394h	High_specificity	hg38	chr3	171776470	171776980	Human_Oligo	0.518	444	0.782	5.2	No	No_oligodendrocyte_expression	Yes
eHGT_394m	High_specificity	mm10	chr3	27977667	27978413	Mouse_Oligo	0.254	661	0.738	12.62	No	No_oligodendrocyte_expression	Yes
eHGT_395h	High_specificity	hg38	chr9	128374105	128374728	Human_Oligo	2.782	595	0.742	9.96	Yes	Oligodendrocytes_strong	Yes
eHGT_395m	High_specificity	mm10	chr2	29830874	29831537	Mouse_Oligo	2.002	632	0.881	24.5	Yes	Oligodendrocytes_strong	Yes
eHGT_396h	High_specificity	hg38	chr11	117295457	117295798	Human_Oligo	1.384	302	0.561	3.88	Yes	Oligodendrocytes_strong	No

ehGT_396m	High_specificity	mm10	chr9	45854220	45854539	Mouse_Oligo	1.185	288	0.824	7.33	Weak	Oligodendrocytes_weak	No
ehGT_397h	High_specificity	hg38	chr15	44188767	44189188	Human_Oligo	1.986	396	0.591	4.67	Weak	Oligodendrocytes_weak	No
ehGT_397m	High_specificity	mm10	chr2	121799010	121799455	Mouse_Oligo	1.661	385	0.759	6.16	Yes	Oligodendrocytes_strong	No
ehGT_398h	High_specificity	hg38	chr16	57270420	57270919	Human_Oligo	1.042	475	0.793	7.49	Yes	Oligodendrocytes_moderate	Yes
ehGT_398m	High_specificity	mm10	chr8	94684265	94684766	Mouse_Oligo	1.199	446	0.806	11.74	No	No_oligodendrocyte_expression	Yes
ehGT_399h	High_specificity	hg38	chr2	36902653	36902934	Human_Oligo	4.206	253	0.628	2.73	No	No_oligodendrocyte_expression	Yes
ehGT_399m	High_specificity	mm10	chr17	78684296	78684576	Mouse_Oligo	3.818	249	0.885	4.11	No	No_oligodendrocyte_expression	Yes
ehGT_400h	High_specificity	hg38	chr4	114636222	114636638	Human_Oligo	2.948	372	0.605	3.88	Weak	Oligodendrocytes_weak	No
ehGT_400m	High_specificity	mm10	chr3	125898885	125899302	Mouse_Oligo	2.414	373	0.819	7.63	Yes	Oligodendrocytes_moderate	No
ehGT_401h	High_specificity	hg38	chr1	44592226	44592529	Human_Oligo	1.081	256	0.55	3.17	Yes	Oligodendrocytes_strong	Yes
ehGT_401m	High_specificity	mm10	chr4	117326998	117327312	Mouse_Oligo	1.024	271	0.778	3.96	No	No_oligodendrocyte_expression	Yes
ehGT_402h	High_specificity	hg38	chr17	75697915	75698299	Human_Oligo	0.976	320	0.626	4.67	No	No_oligodendrocyte_expression	No
ehGT_402m	High_specificity	mm10	chr11	115956504	115956883	Mouse_Oligo	0.956	326	0.919	13.94	Weak	Oligodendrocytes_weak	No
ehGT_403h	High_specificity	hg38	chr11	67410338	67410731	Human_Oligo	0.524	382	0.588	4.5	Yes	Oligodendrocytes_moderate	No
ehGT_403m	High_specificity	mm10	chr19	4183762	4184154	Mouse_Oligo	1.019	346	0.586	8.51	Mixed_spe	Mixed_specificity_oligodendrocy	No
ehGT_404h	High_specificity	hg38	chr2	163943542	163943945	Human_Oligo	1.738	370	0.721	5.2	Mixed_spe	Mixed_specificity_oligodendrocy	No
ehGT_404m	High_specificity	mm10	chr2	64325556	64326248	Mouse_Oligo	1.562	599	0.933	12.18	No	No_oligodendrocyte_expression	No
ehGT_405h	High_specificity	hg38	chr3	185175495	185175886	Human_Oligo	0.607	362	0.49	4.05	No	No_oligodendrocyte_expression	No
ehGT_405m	High_specificity	mm10	chr16	21724337	21724697	Mouse_Oligo	0.444	335	0.91	8.36	Weak	Oligodendrocytes_weak	No
ehGT_406h	High_specificity	hg38	chr2	88735239	88735593	Human_Oligo	-0.058	304	0.518	3.26	No	No_oligodendrocyte_expression	Yes
ehGT_406m	High_specificity	mm10	chr6	70774375	70774694	Mouse_Oligo	0.091	256	0.696	5.87	No	No_oligodendrocyte_expression	Yes
ehGT_407h	High_specificity	hg38	chr6	46098744	46099007	Human_Oligo	0.581	253	0.614	2.47	Yes	Oligodendrocytes_moderate	Yes
ehGT_407m	High_specificity	mm10	chr17	44121834	44122162	Mouse_Oligo	0.545	271	0.847	7.48	No	No_oligodendrocyte_expression	Yes
ehGT_408h	High_specificity	hg38	chr8	60867028	60867631	Human_Oligo	3.002	574	0.53	6.61	No	No_oligodendrocyte_expression	Yes
ehGT_408m	High_specificity	mm10	chr4	8867749	8868420	Mouse_Oligo	2.361	596	0.769	11.88	No	No_oligodendrocyte_expression	Yes
ehGT_409h	High_specificity	hg38	chr14	67539037	67539541	Human_Oligo	2.042	452	0.564	4.5	Yes	Oligodendrocytes_moderate	Yes
ehGT_409m	High_specificity	mm10	chr12	79035271	79035759	Mouse_Oligo	1.603	437	0.908	13.5	Yes	Oligodendrocytes_strong	Yes
ehGT_410h	High_specificity	hg38	chr9	81717541	81717791	Human_Oligo	2.177	202	0.579	1.41	Yes	Oligodendrocytes_moderate	Yes
ehGT_410m	High_specificity	mm10	chr4	72233773	72234067	Mouse_Oligo	1.838	203	0.817	9.68	Yes	Oligodendrocytes_strong	Yes
ehGT_495h	Marker_gene	hg38	chr19	33217923	33218346	Human_Astro	1.481	364	0.302	0.95	No_data	No_data	No
ehGT_495m	Marker_gene	mm10	chr7	35192340	35192802	Mouse_Astro	0.977	396	0.818	7.85	No	No_astrocyte_expression	No
ehGT_496h	Marker_gene	hg38	chr19	33225554	33226354	Human_Astro	1.18	749	0.516	4.76	No_data	No_data	No
ehGT_496m	Marker_gene	mm10	chr7	35185913	35186648	Mouse_Astro	0.955	686	0.734	15.35	No_data	No_data	No
ehGT_497h	Marker_gene	hg38	chr3	55237236	55237718	Human_Astro	1.648	437	0.037	0.09	No_data	No_data	No
ehGT_497m	Marker_gene	mm10	chr14	28781601	28782057	Mouse_Astro	1.444	410	0.966	7.49	No	No_astrocyte_expression	No
ehGT_641h	Marker_gene	hg38	chr21	33116993	33117641	Human_Oligo	4.073	557	0.755	16.66	No_data	No_data	No
ehGT_641m	Marker_gene	mm10	chr16	91305334	91305956	Mouse_Oligo	3.233	529	0.92	23.32	Yes	Oligodendrocytes_strong	No
ehGT_642h	Marker_gene	hg38	chr17	44539855	44540164	Human_Astro	2.026	230	0.463	0.26	No_data	No_data	No
ehGT_642m	Marker_gene	mm10	chr11	102579463	102579768	Mouse_Astro	1.655	228	0.86	11.06	Mixed_spe	Non-specific_astrocyes	No
ehGT_733m	Marker_gene	mm10	chr2	29273794	29274249	Mouse_Oligo	0.127	366	0.876	5.72	No	No_oligodendrocyte_expression	No
MGT_E117h	High_strength	hg38	chr7	23230417	23231661	Human_Astro	1.333	1213	0.197	4.15	No_data	No_data	No
MGT_E117m	High_strength	mm10	chr6	49020855	49022070	Mouse_Astro	1.202	1174	0.852	49.25	Mixed_spe	Non-specific_astrocyes	No
MGT_E118h	High_strength	hg38	chr13	25910516	25911691	Human_Astro	3.328	1146	0.062	0.26	No_data	No_data	No
MGT_E118m	High_strength	mm10	chr14	59736768	59737951	Mouse_Astro	2.384	1143	0.889	47.11	Mixed_spe	Mixed_specificity_astrocyes	No
MGT_E119h	High_strength	hg38	chr18	59386813	59388237	Human_Astro	4.406	1421	0.532	6.58	No_data	No_data	No
MGT_E119m	High_strength	mm10	chr18	66022617	66024006	Mouse_Astro	3.435	1386	0.926	38.9	Mixed_spe	Mixed_specificity_astrocyes	No
MGT_E120h	High_strength	hg38	chr2	134072857	134074292	Human_Astro	0.875	1436	0.333	2.16	No_data	No_data	No
MGT_E120m	High_strength	mm10	chr1	127159651	127161393	Mouse_Astro	0.697	1661	0.896	37.83	Yes	Forebrain_midbrain_astrocyes	No
MGT_E121h	High_strength	hg38	chr17	68641683	68642415	Human_Astro	0.531	733	0.139	0.17	No_data	No_data	No
MGT_E121m	High_strength	mm10	chr11	109753663	109754656	Mouse_Astro	0.322	994	0.911	38.9	Mixed_spe	Mixed_specificity_astrocyes	No
MGT_E122h	High_strength	hg38	chr4	175988170	175989315	Human_Astro	1.134	1121	0.675	8.65	No_data	No_data	No
MGT_E122m	High_strength	mm10	chr8	54791804	54792949	Mouse_Astro	1.078	1117	0.922	37.47	Yes	Forebrain_cerebellum_astrocye	No
MGT_E123h	High_strength	hg38	chr14	80206581	80208078	Human_Astro	2.747	1497	0.701	12.12	No_data	No_data	No
MGT_E123m	High_strength	mm10	chr12	90733627	90735117	Mouse_Astro	2.427	1490	0.808	36.76	Mixed_spe	Mixed_specificity_astrocyes	No
MGT_E160h	None	hg38	chr2	48184996	48185538	Human_Astro	0.314	538	0	0	No_data	No_data	No
MGT_E160m	None	mm10	chr17	88297369	88297914	Mouse_Astro	0.347	516	0.282	1.07	Yes	Mid/hindbrain_astrocyes	No

1111   **Extended Data Table 1: Genomic coordinates, sequence characterization, and mouse screening**  
1112   **results of all tested astrocyte and oligodendrocyte enhancers.**

1113   Calculations of parameters are as described in Methods section.

1114

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