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University of  
Zurich<sup>UZH</sup>

# Enhancer AAVs for enhanced cell-type specificity

Technical Journal Club

# Today's Papers

## Neuron

### NeuroResource

## An enhancer-AAV toolbox to target and manipulate distinct interneuron subtypes

### Highlights

- We identified enhancers to target GABAergic and cholinergic cell types in the brain
- When delivered using AAVs, these tools can specifically target cell types of interest
- Target cells can be efficiently labeled and manipulated in mice and non-human primates
- These tools can be used to study the role of target cell types for brain function

### Authors

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### In brief

Furlanis et al. developed rAAV-enhancer tools to target the main types and subtypes of GABAergic cortical neurons and cholinergic striatal cells in both mice and non-human primates. Through careful anatomical, morphological, and electrophysiological characterization across multiple conditions, they have validated that these tools are effective for targeting and manipulating these cell types in the brain.

## Technical and biological sources of noise confound multiplexed enhancer AAV screening

### Authors

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### Keywords

AAV, enhancer, multiplex screen, single cell RNA-sequencing, background noise

# An enhancer-AAV toolbox to target and manipulate distinct interneuron subtypes

## Neuron

NeuroResource

### An enhancer-AAV toolbox to target and manipulate distinct interneuron subtypes

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- We identified enhancers to target GABAergic and cholinergic cell types in the brain
- When delivered using AAVs, these tools can specifically target cell types of interest
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#### In brief

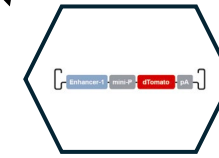
Furlanis et al. developed rAAV-enhancer tools to target the main types and subtypes of GABAergic cortical neurons and cholinergic striatal cells in both mice and non-human primates. Through careful anatomical, morphological, and electrophysiological characterization across multiple conditions, they have validated that these tools are effective for targeting and manipulating these cell types in the brain.

- 30 – 120 subtypes of interneurons.
- **Four major classes:**
  - Parvalbumin (PV)
  - Somatostatin (SST)
  - Vasoactive intestinal peptide (VIP)
  - Lysosomal-associated membrane protein 5 (LAMP5).
- **Aim:** Identify enhancers that work in the context of AAVs to target each of the major cardinal classes and the most prominent subclasses within each of these cardinal divisions.
- **Overall goal** of providing a toolset to explore interneuron function and connectivity.

# Enhancer AAVs – the concept?

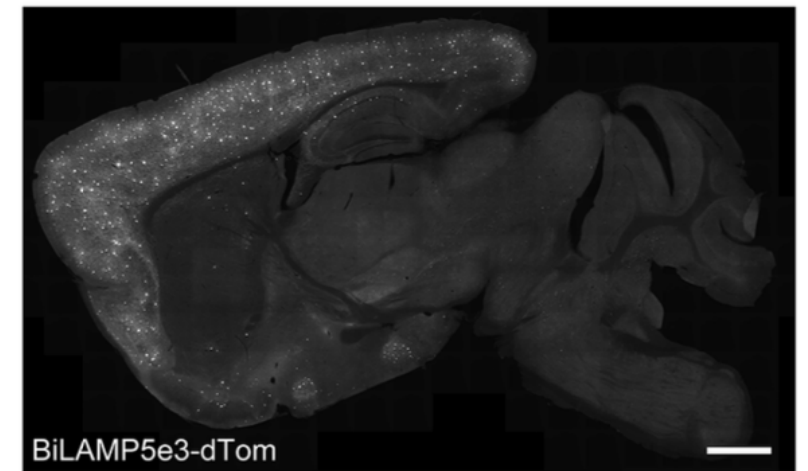


**AAV cassette design**



**AAV packaging**

- **Enhancers:** short regions of DNA that increase the transcription of a target gene by serving as binding sites for transcription factors and coactivators.
- Packaging gene of interest into AAVs downstream of an enhancer & promoter.
- Mini promoter necessary due to size constraints.
- Expression driven in enhancer specific cell types only.



**Enhanced specificity for target cells**

# Two important terms before we start

## Specificity

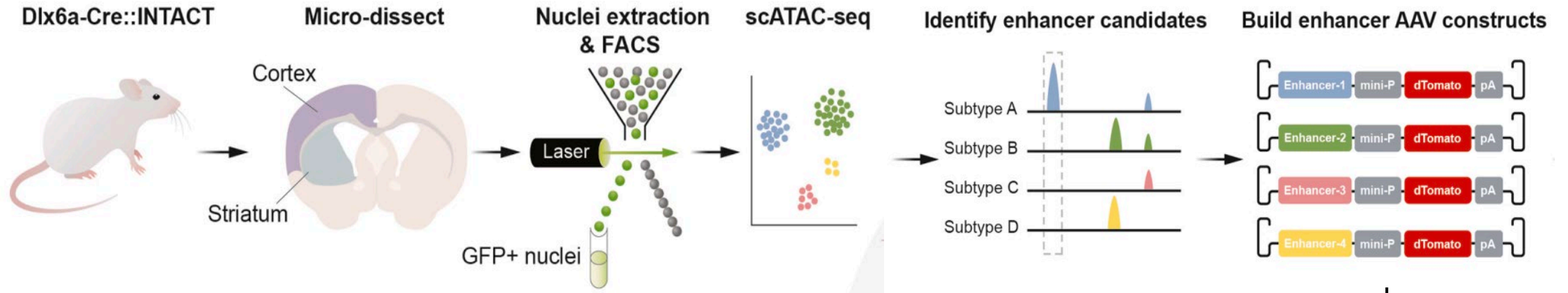
**Specificity** for the intended target population.  
*(Percentage of colocalization of dTomato-positive cells labeled by the AAV enhancer tool, and cell-type-specific markers of interest)*

## Sensitivity

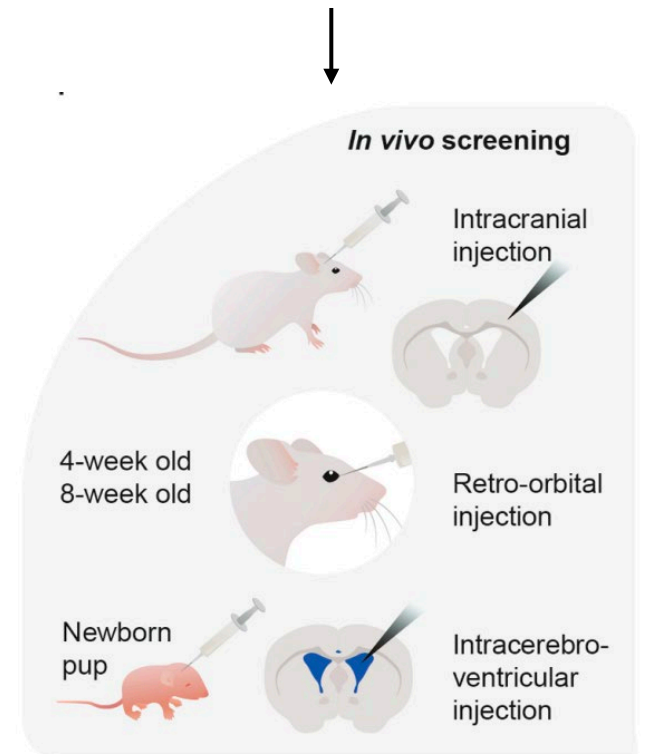
**Efficiency** of a given enhancer in targeting all the cells of a given population, in a given area, and under the conditions used.  
*(number of dTom+ marker+ / number of marker+)*

# Enhancer sequence identification workflow

A



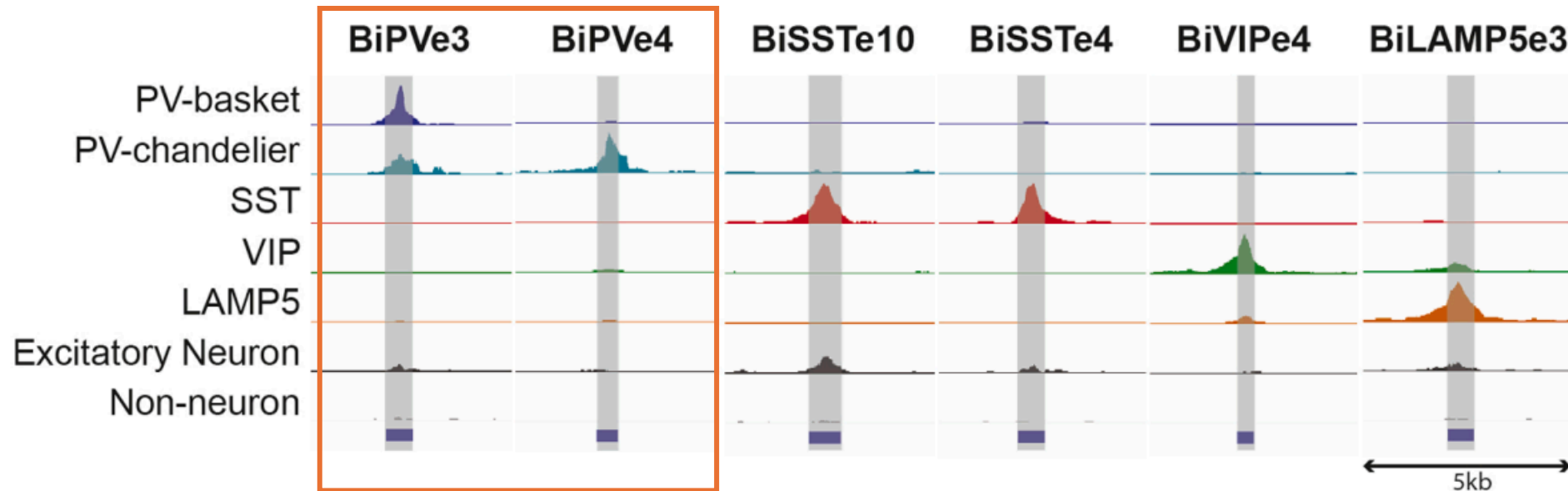
- Mouse strain – tags nuclei of GABAergic neurons (Dlx6a marker) with GFP – enriched by sorting.
- Sorted nuclei from the Cortex and Striatum (GFP for GABAergic neurons).
- single cell ATAC seq (Assay for Transposase Accessible Chromatin) used to ID regions of open/accessible DNA which are often enhancer regions.
- Single cell basis to identify open chromatin sequences specific to the individual subclasses of GABAergic neurons.





# Enhancer-based viral targeting of PV neuron subtypes in mice

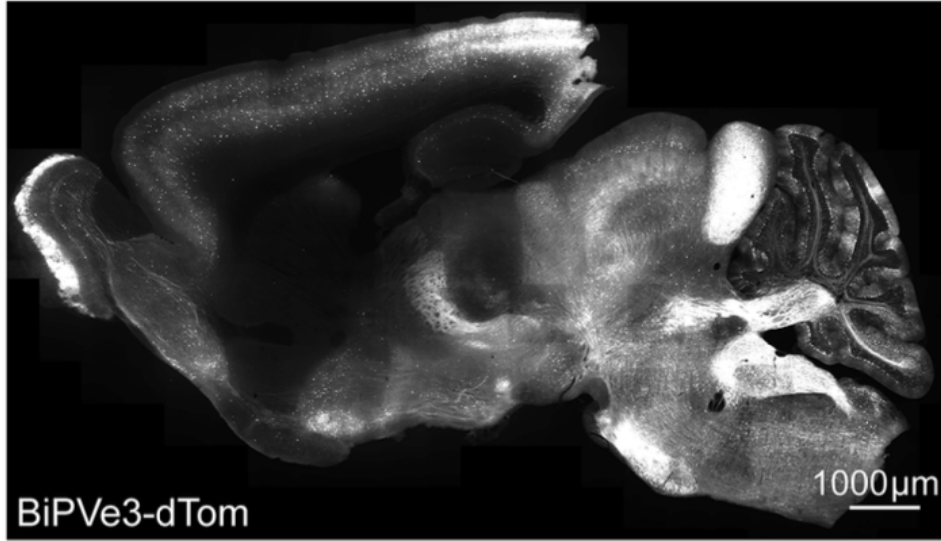
- **PV-positive neurons** – originate from the medial ganglionic eminence (MGE).
- **Two major PV subclasses,**
  - Basket cells
  - Chandelier cells
- Two enhancers identified in initial screen for further characterization – enhancer chromatin is accessible only/mostly in PV-basket or PV-Chandelier cells.



# Enhancer-based viral targeting of PV neuron subtypes in mice

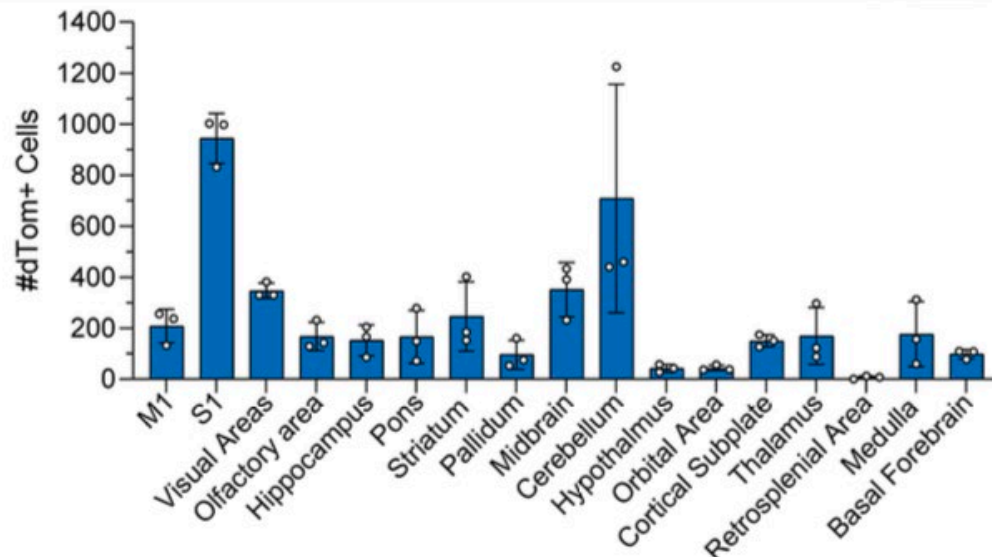
## BiPVe3 enhancer - intended target: basket cells

A

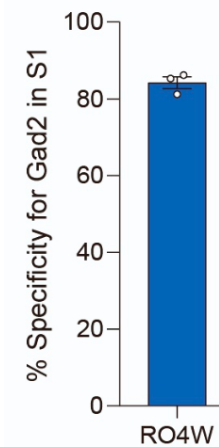


- Retro-orbitally injected BiPVe3 or BiPVe4 dTomato rAAVs into 4-week-old mice
- dTomato reporter expression across the whole brain.
- Strong activity in putative basket cells across all major cortical areas.

C



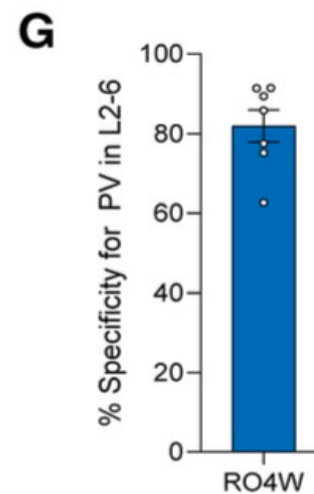
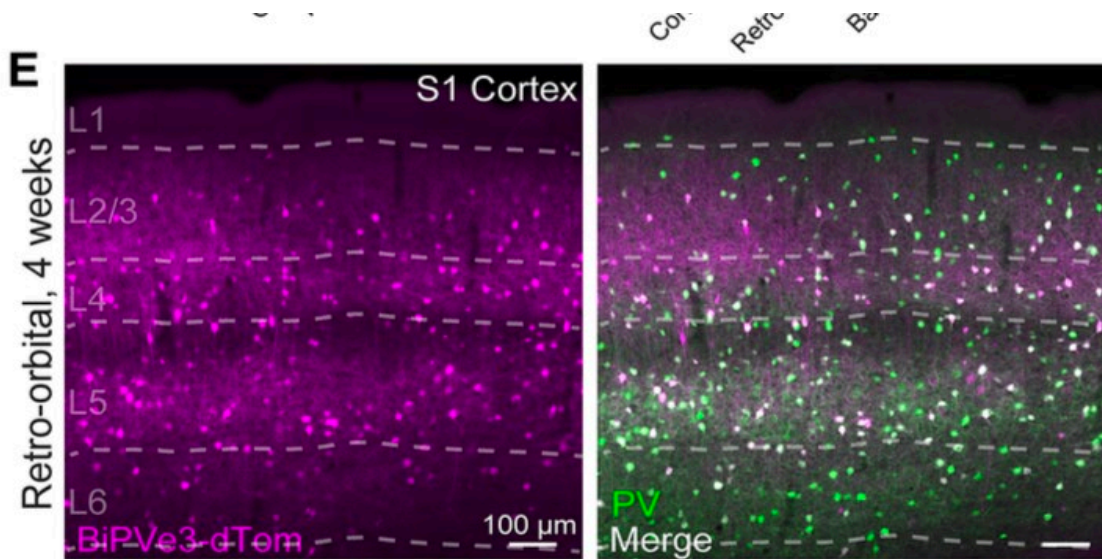
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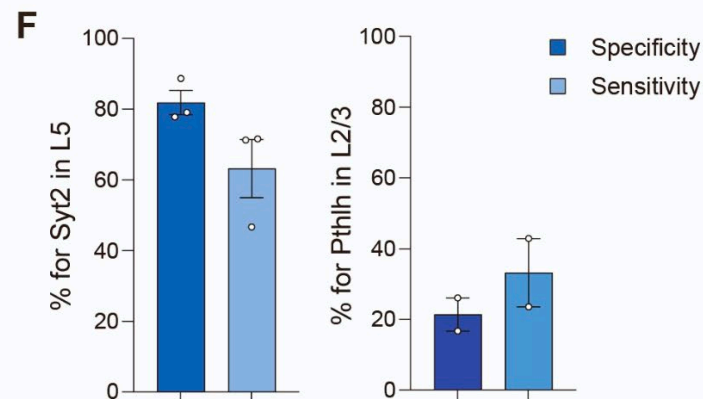
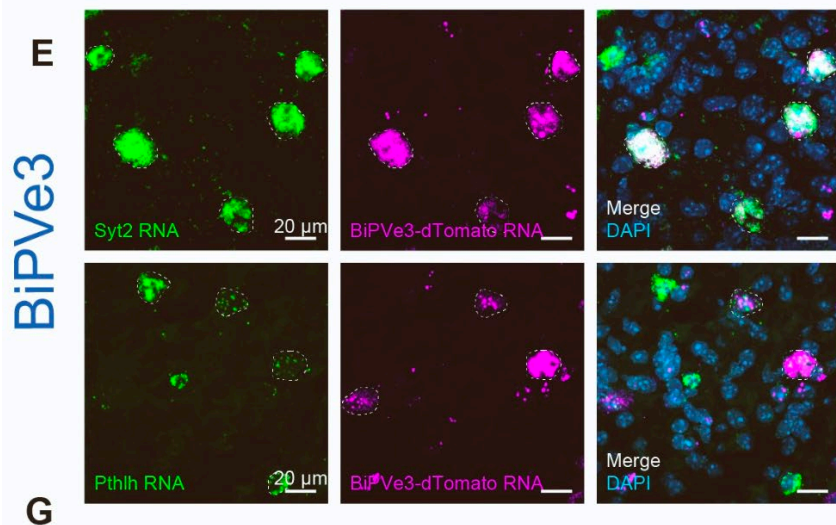
- GAD2: pan-GABAergic neuron marker.
- 81% specificity for marking GABAergic neurons.
- i.e. broadly specific for GABAergic neurons – a good start.

# Enhancer-based viral targeting of PV neuron subtypes in mice

## BiPVe3 enhancer - intended target: basket cells



- Labelled S1 cortical layers all besides L1 & low-density labelling in L2/3 – as expected.
- Good colocalization with PV neuron marker parvalbumin (Pvalb).
- Specificity of 81 % for BiPVe3 = good targeting & specificity for GABAergic interneurons of PV neuron subtype

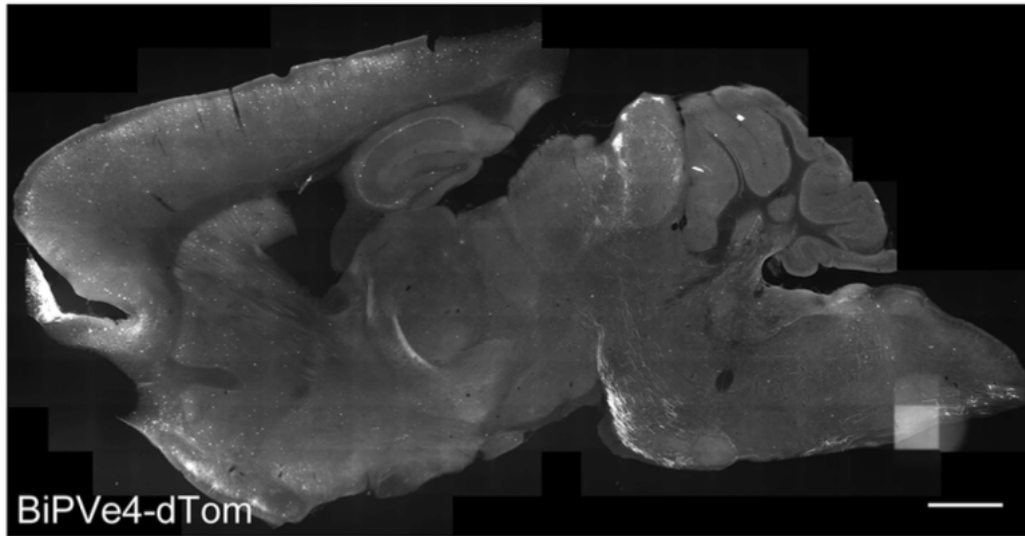


- **Syt2**: marker enriched in basket cells. **Pthlh**: marker enriched in chandelier cells.
- **High specificity and sensitivity for Syt2+ basket cells** but not chandelier cells – as intended.

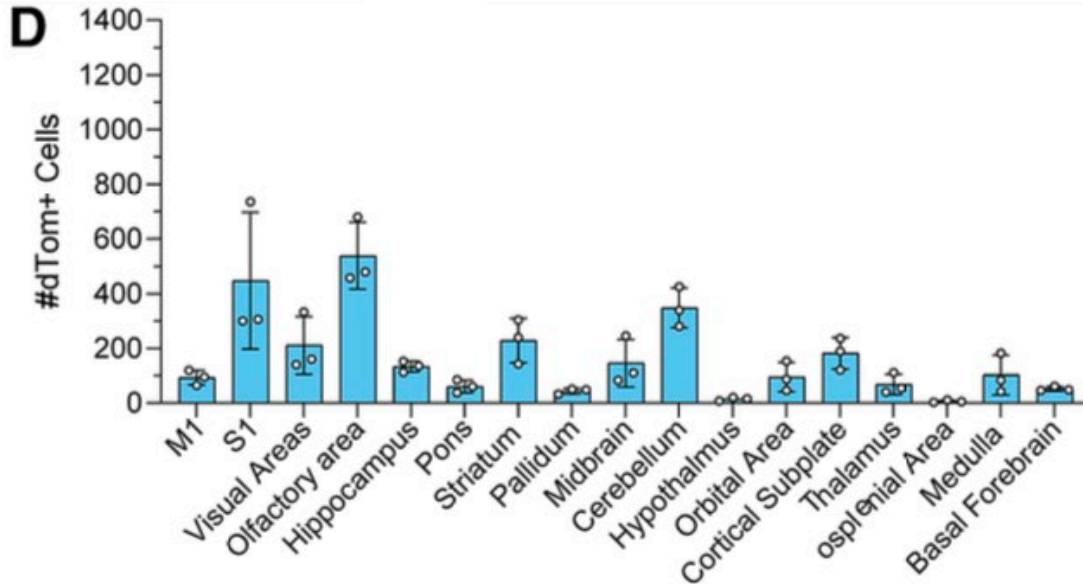
# Enhancer-based viral targeting of PV neuron subtypes in mice

## BiPVe4 enhancer – intended target: chandelier cells

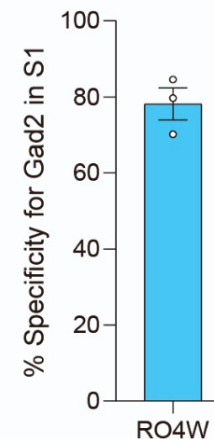
B



- Retro-orbitally injected dTomato rAAVs into 4-week-old mice
- dTomato reporter expression across the whole brain.
- Strong activity in putative basket cells across all major cortical areas.



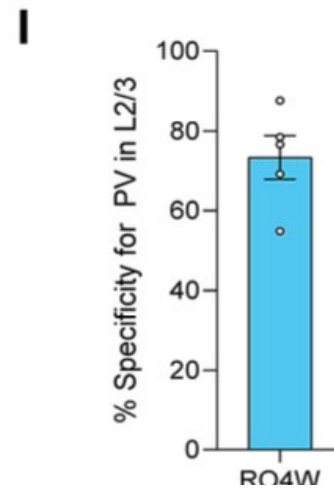
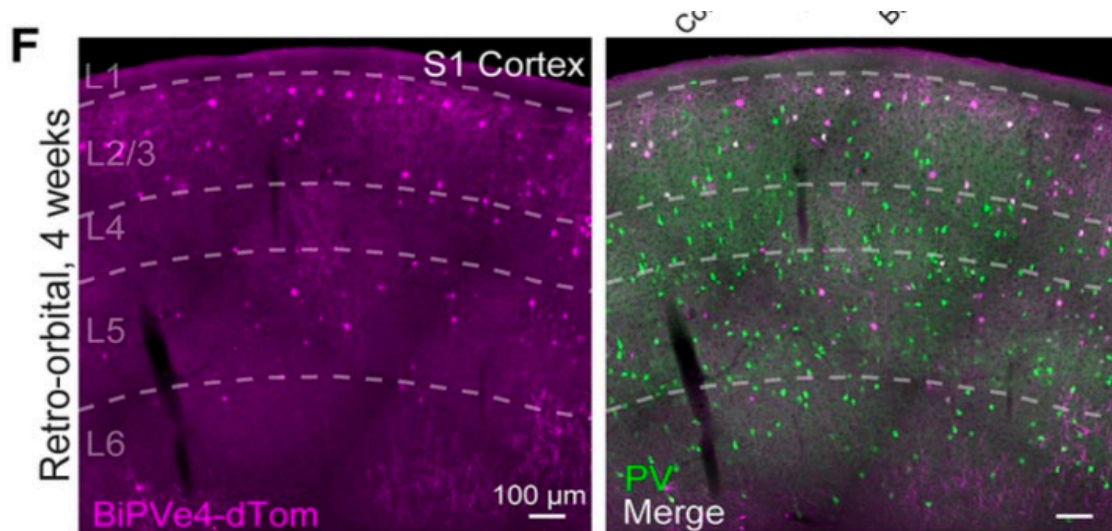
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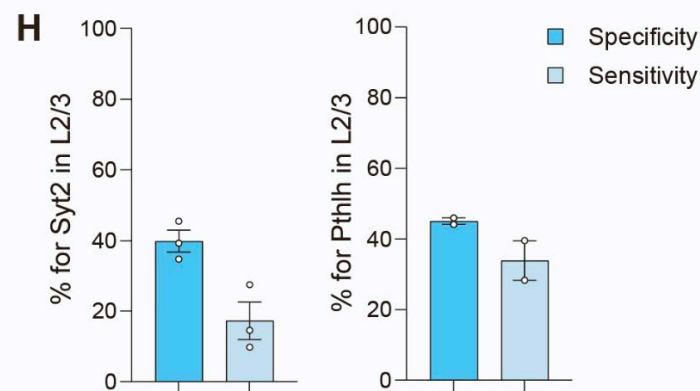
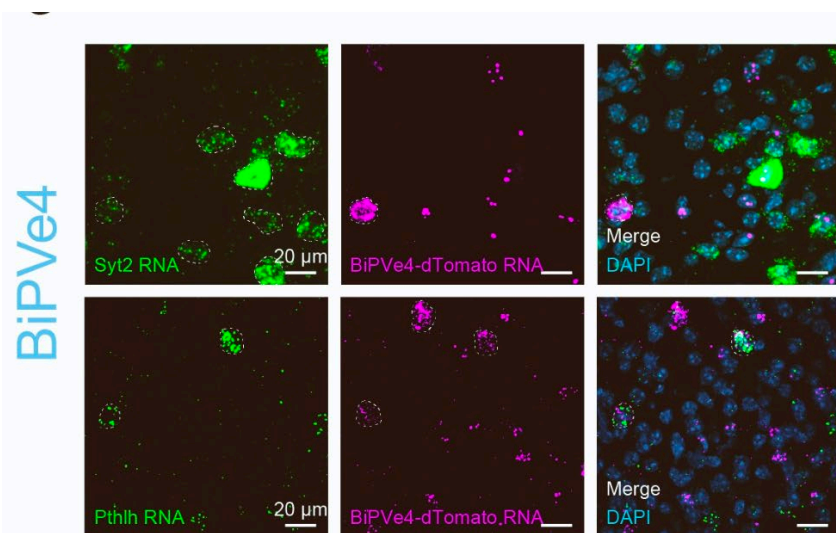
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# Enhancer-based viral targeting of PV neuron subtypes in mice

## BiPVe4 enhancer – intended target: chandelier cells



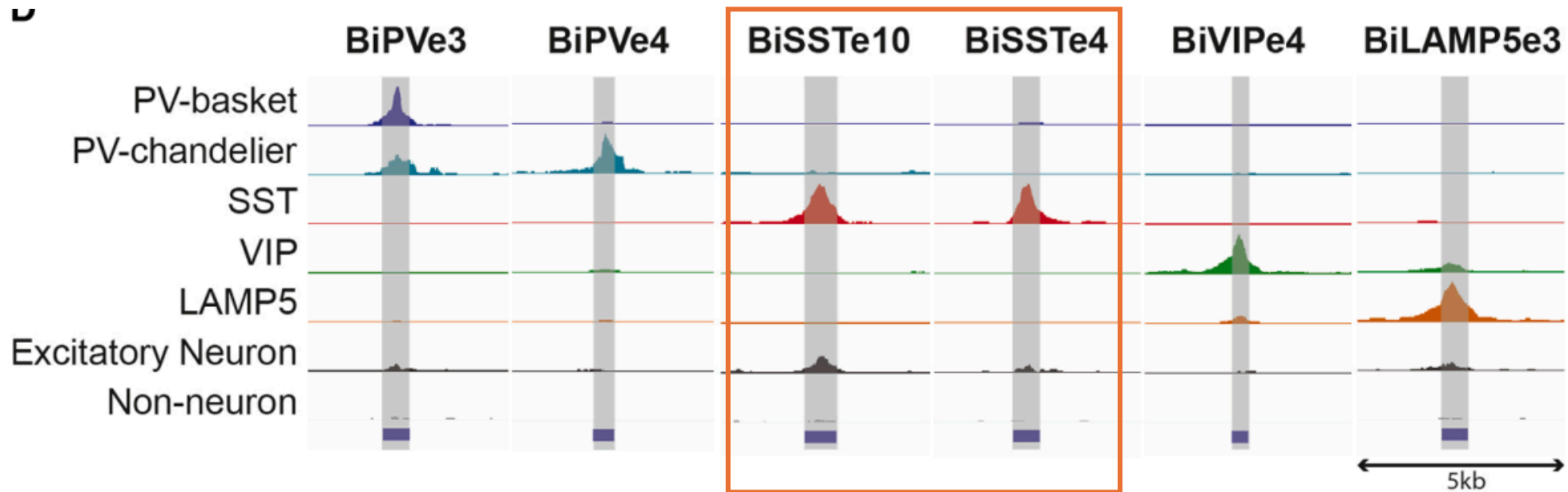
- Labelling largely restricted to L2/3 – as expected.
- Good colocalization with PV neuron marker parvalbumin (Pvalb).
- Specificity of 81 % for BiPVe4 = also good targeting & specificity for GABAergic interneurons of PV neuron subtype.



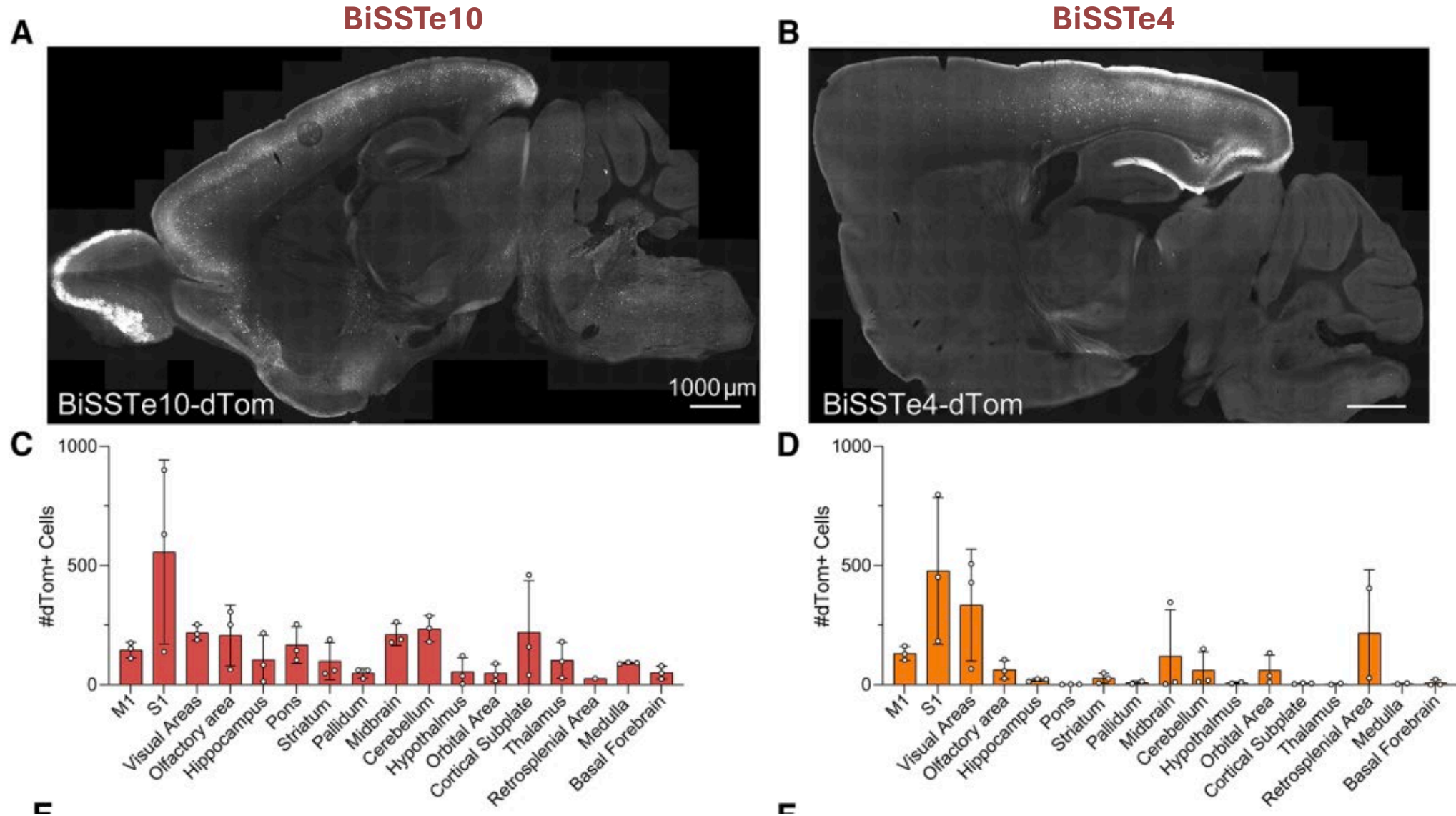
- **Syt2**: marker enriched in basket cells. **Pthlh**: marker enriched in chandelier cells.
- Not so specific for chandelier cells.
- Possible unspecific labeling of basket cells by the enhancer BiPVe4.

# Enhancer-based viral targeting of SST neuron subtypes in mice

- SST Neurons = abundant and heterogenous
- Originate from the medial ganglionic eminence (MGE).
- 9 subtypes but 2 main classes
- Non-Martinotti – project axons locally in L4/5.
- Martinotti – project axons that ramify in L1.
- Two enhancers identified in initial screen for further characterization.



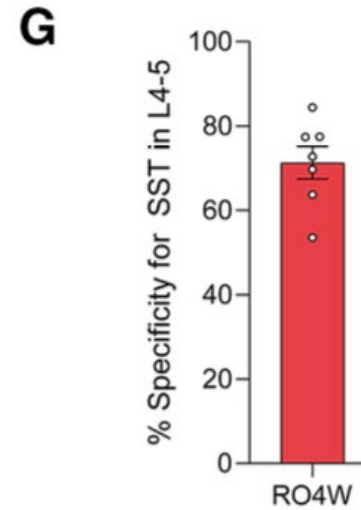
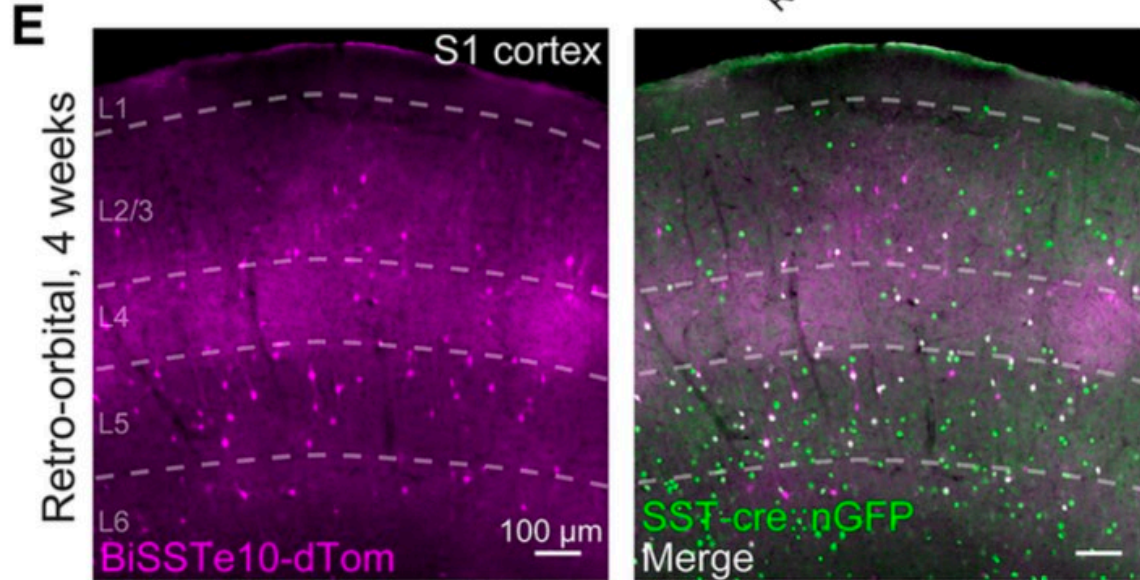
# Enhancer-based viral targeting of SST neuron subtypes in mice



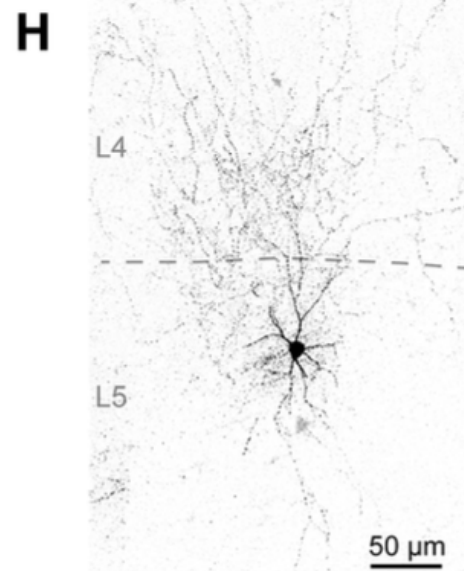
- BiSSTe10 and BiSSTe4 labelled all the major cortical areas & sparsely labelled other regions.

# Enhancer-based viral targeting of SST neuron subtypes in mice

## BiSSTe10 enhancer



- colocalization of dTomato labelled cells with SST marker in S1.
- High degree of colocalization with the SST marker in L4/5 (71%) - lower specificity across other cortical layers as expected.
- Heavy innervation of BiSSTe10-dTomato cells to L4.

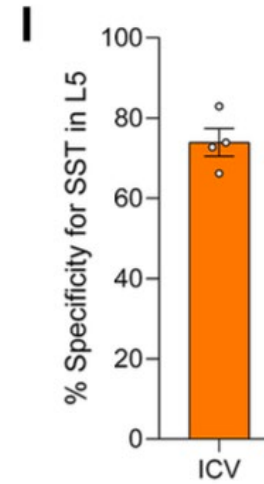
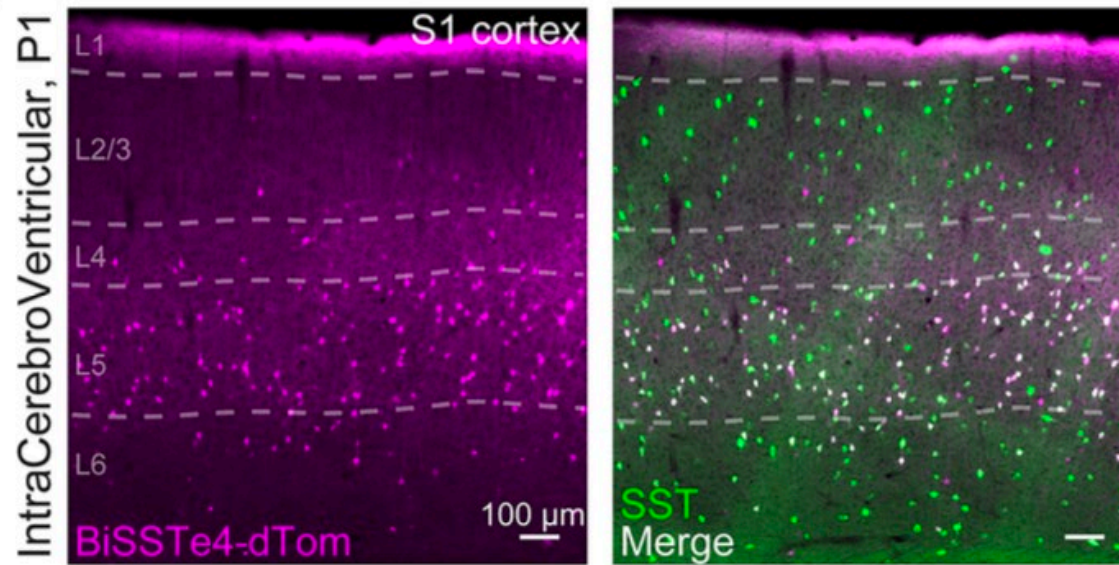


- Biocytin filling of BiSSTe10 labelled cells.
- Processes heavily innervating L4.
- Characteristic of **non-Martinotti SST neurons**.
- **Preferential targeting of Non-Martinotti neurons by BiSSTe4.**

# Enhancer-based viral targeting of SST neuron subtypes in mice

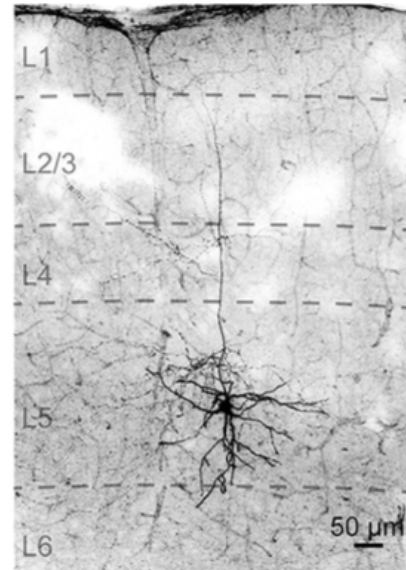
## BiSSTe4 enhancer

F



- colocalization of dTomato labelled cells with SST marker in S1.
- High degree of colocalization with the SST marker in L5 (73%) – as expected. Low in other layers.
- Axons projected to L1

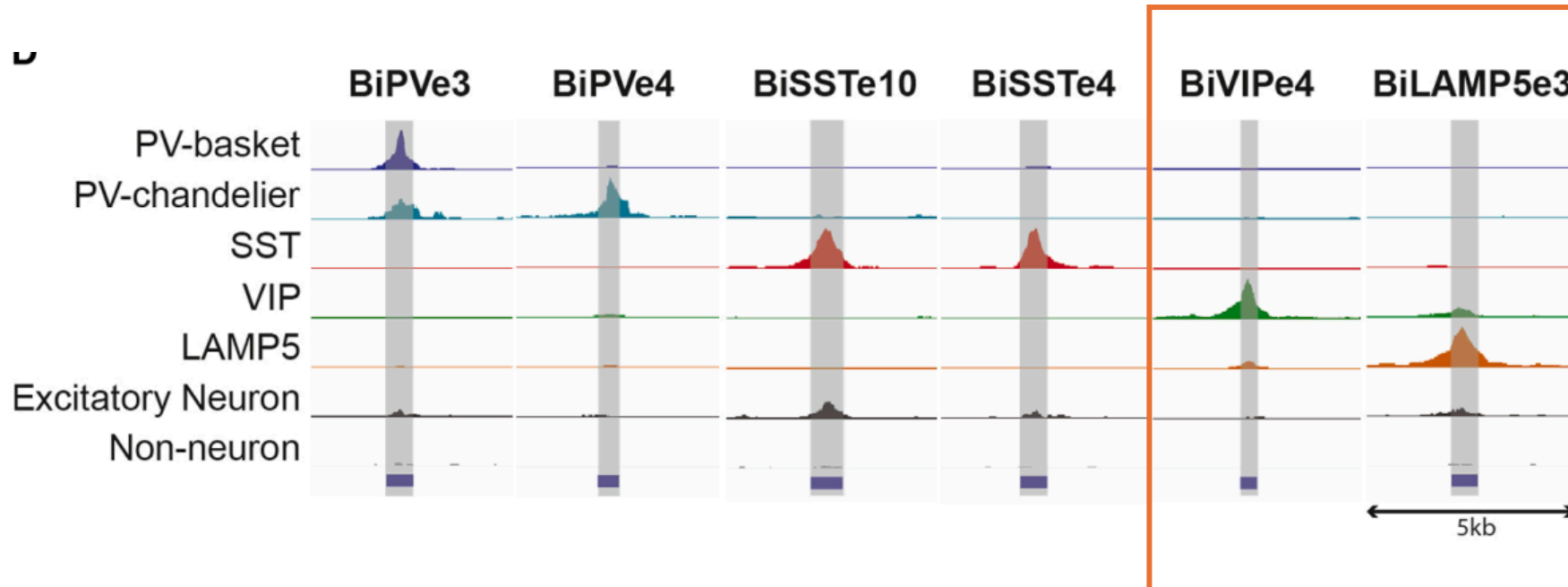
J



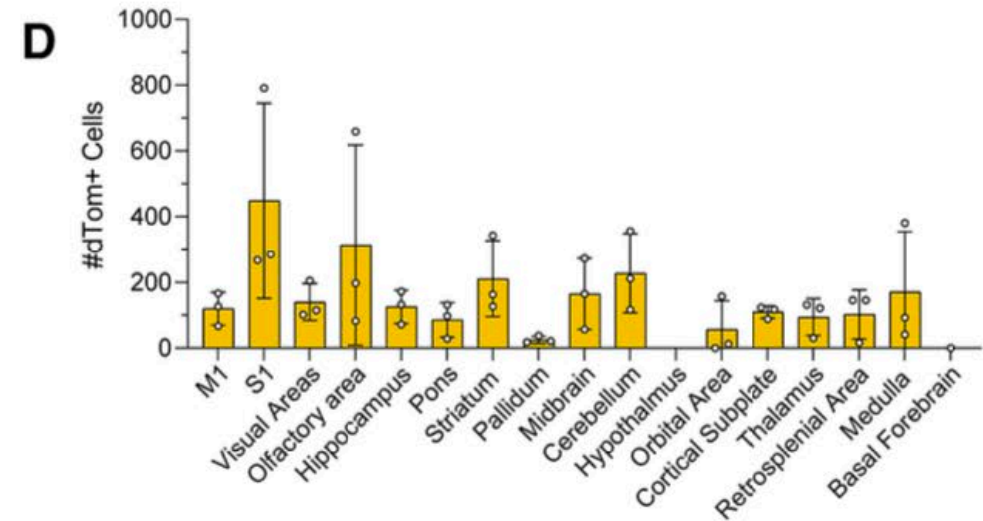
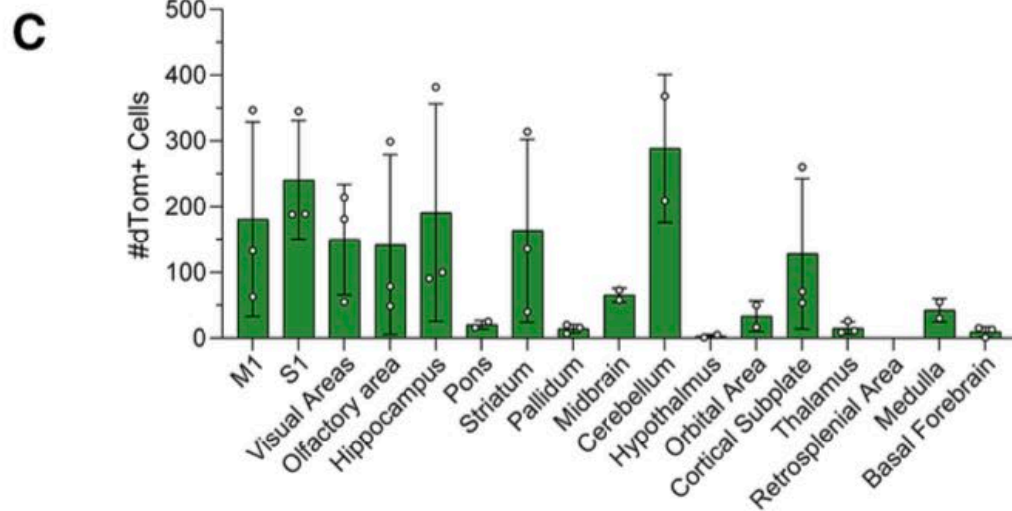
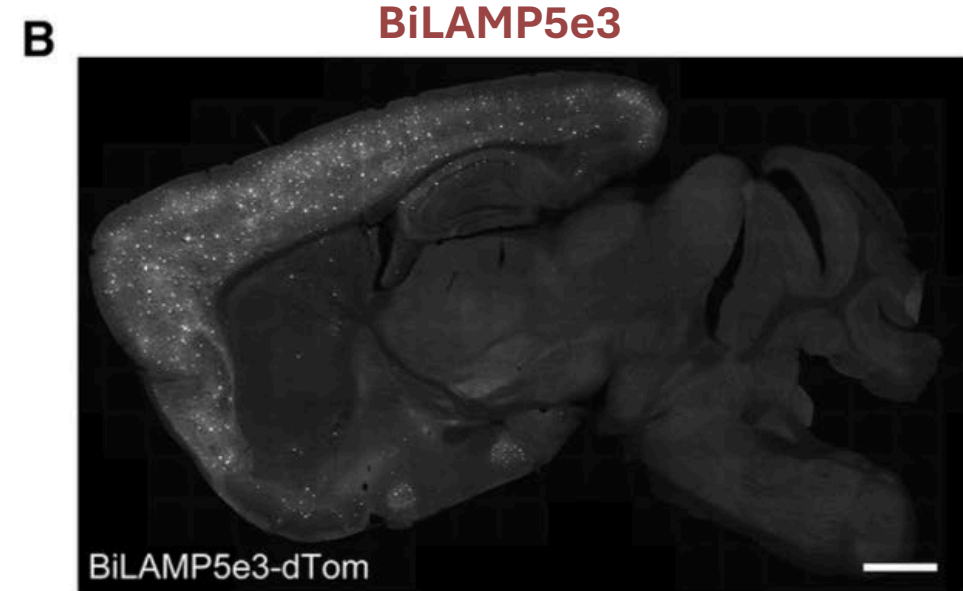
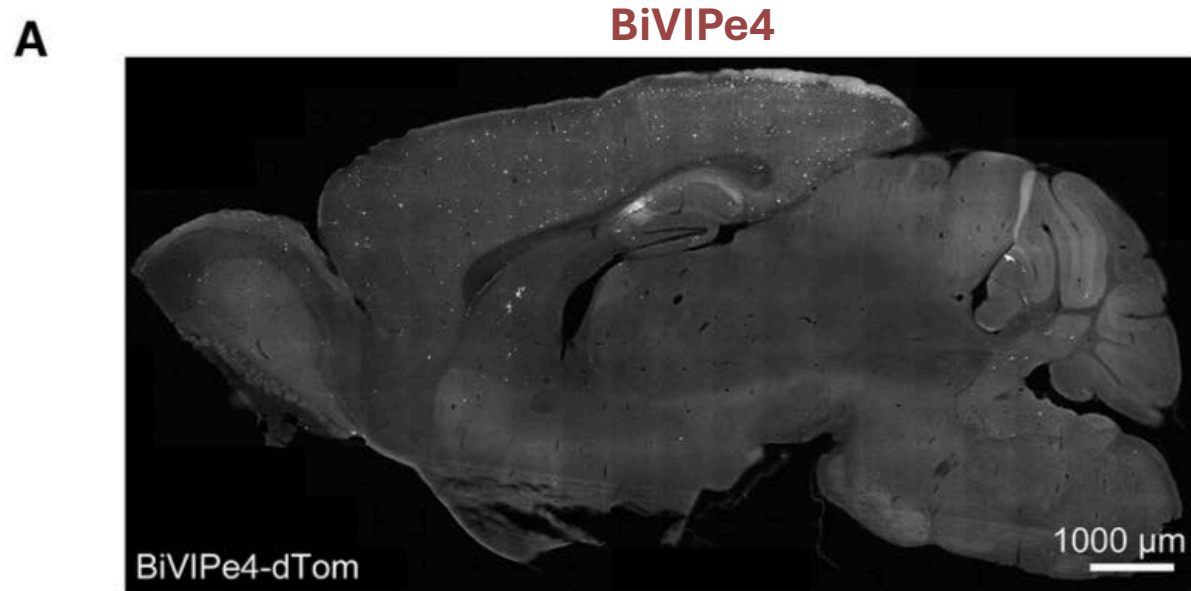
- Biocytin filling of BiSSTe4 labelled cells.
- Axons project from L5 to L1.
- Characteristic of **Martinotti SST neurons**.
- **Preferential targeting of Martinotti neurons by BiSSTe4.**

# Enhancer-based viral targeting of CGE-derived cortical interneurons in mice

- Originate from the caudal ganglionic eminence (CGE).
- Two classes:
  - **VIP neurons** - inhibit other GABAergic neurons
  - **LAMP5 neurons** - provide long-lasting inhibition to pyramidal neurons
- Top enhancer selected for each class from initial screen:



# Enhancer-based viral targeting of CGE-derived cortical interneurons in mice

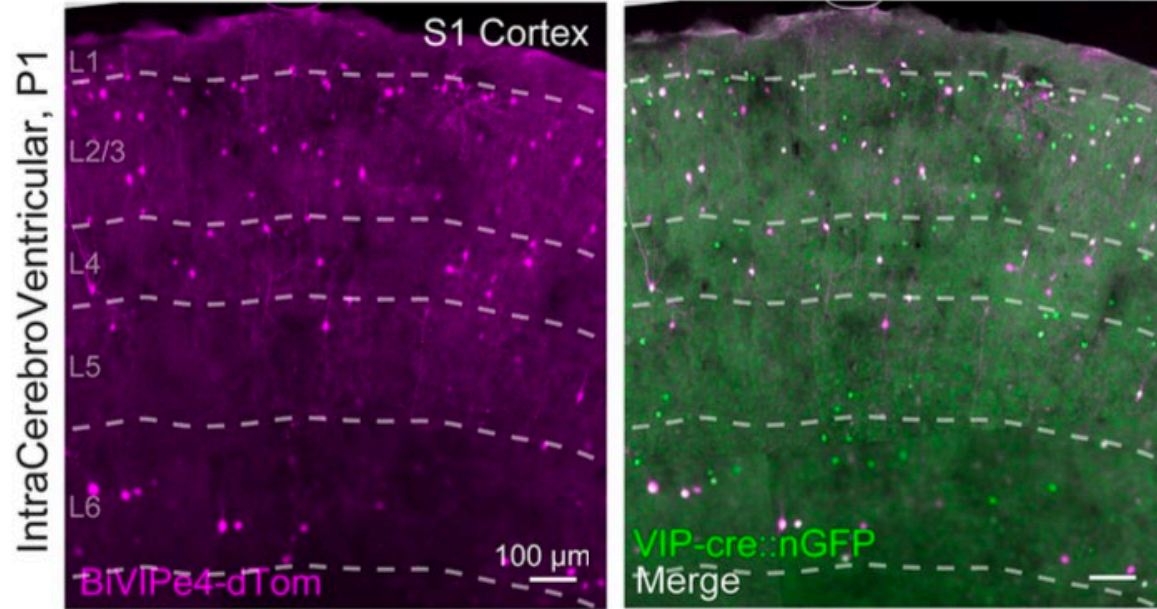


- Higher accumulation in superficial cortical layers.
- Sparse cell labeling could be observed also in areas such as the hippocampus, midbrain, medulla and striatum.

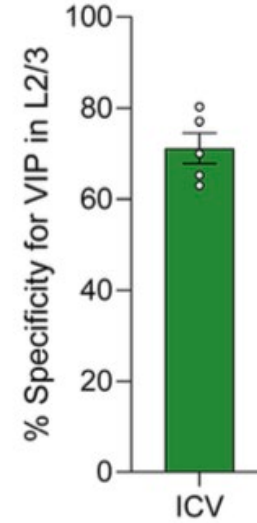
# Enhancer-based viral targeting of CGE-derived cortical interneurons in mice

## BiVIPe4 enhancer

**E**



**G**



- Labelling mostly in superficial cortical layers.
- High degree of colocalization with the VIP marker in L2/3.

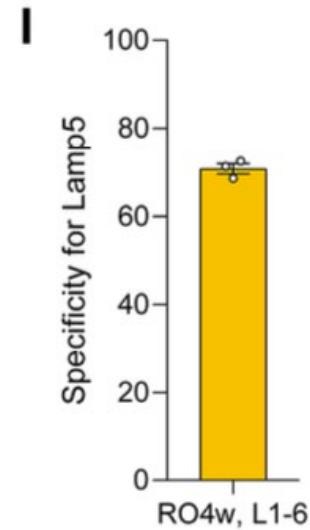
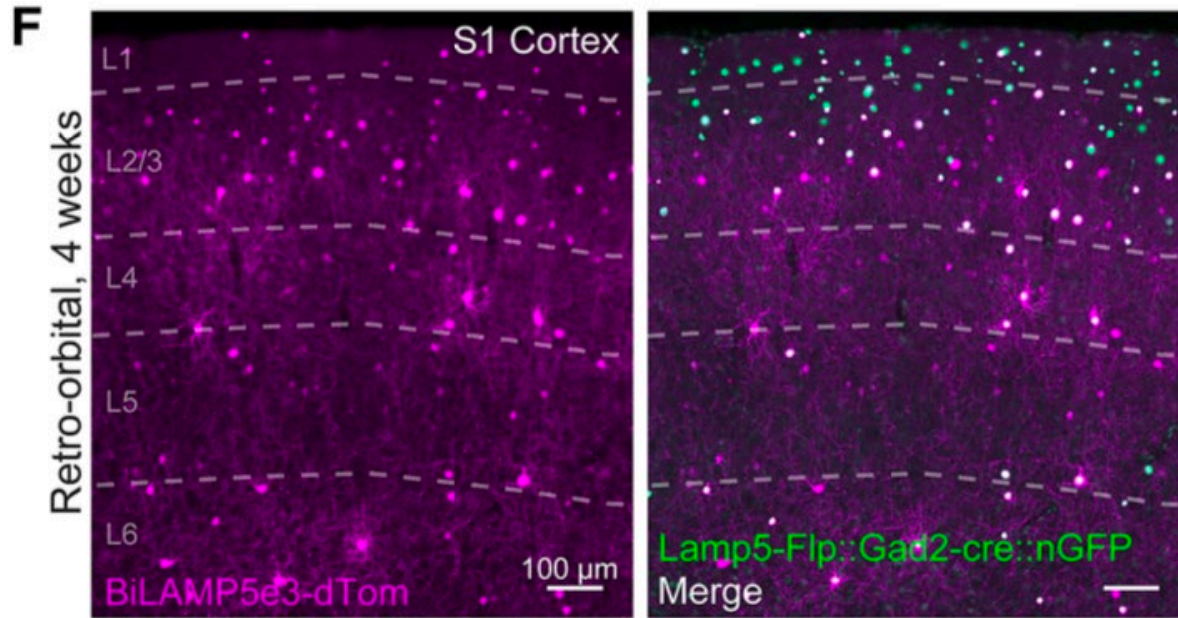
**H**



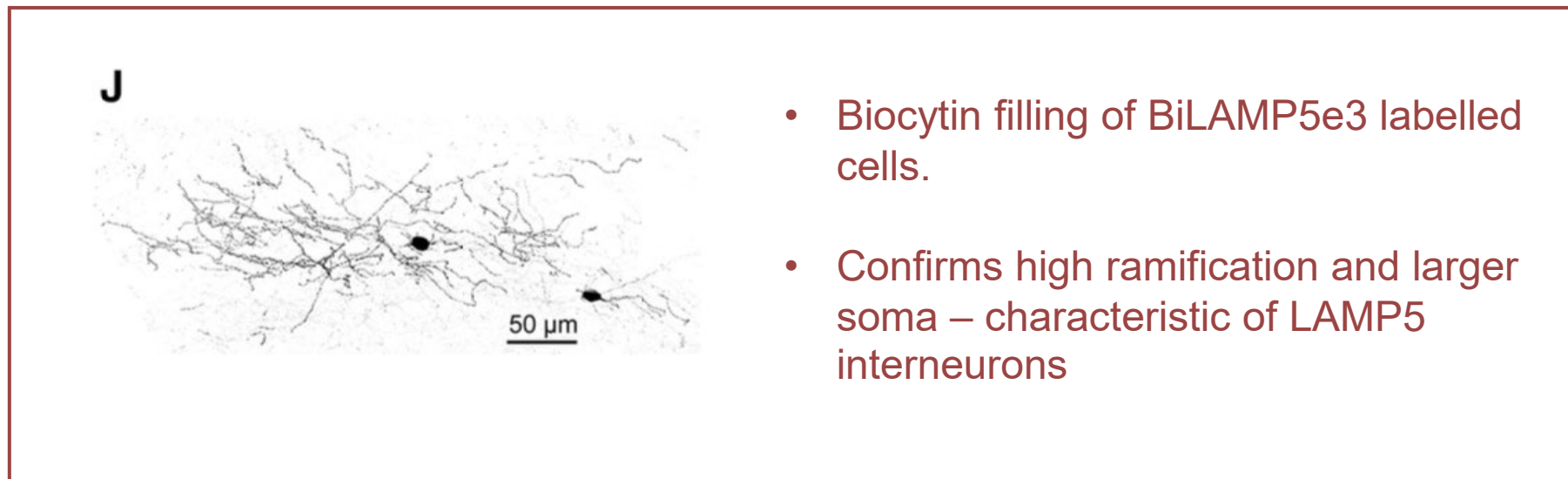
- Biocytin filling of BiVIPe4 labelled cells.
- Small soma - characteristic of VIP neurons.

# Enhancer-based viral targeting of CGE-derived cortical interneurons in mice

## BiLAMP5e3 enhancer

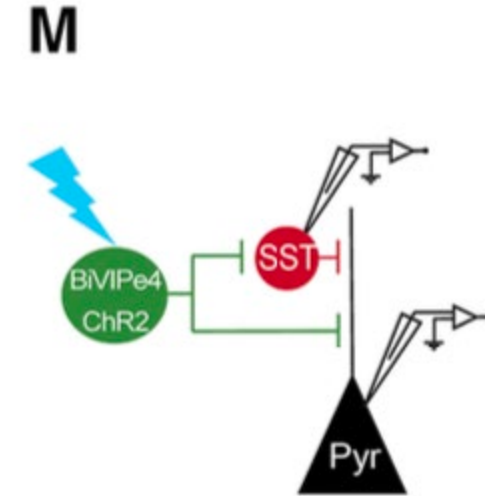


- Labelling mostly in superficial cortical layers.
- High degree of colocalization with the LAMP5 marker.
- highly ramified and larger somas - more typical of LAMP5 interneurons



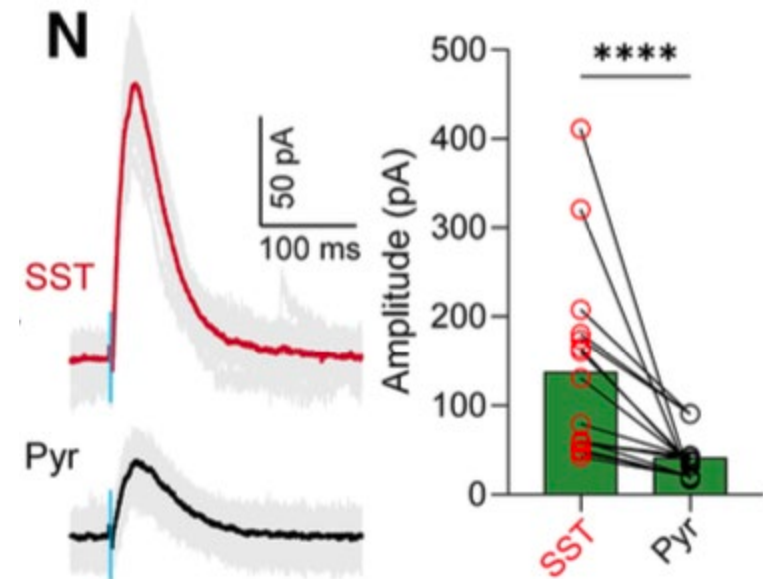
# Functional validation of VIP interneuron targeting by enhancer AAVs in mouse brain slices

- Known that VIP neurons strongly inhibit SST neurons.
- Tested if stimulation of VIP neurons induce inhibitory currents in SST neurons.
- AAV-BiVIPe4-ChR2 used to drive optogenetic activation VIP neurons.
- Blue light activates ChR2 VIP neurons only.
- GFP-labelled SST interneurons.
- Patch-clamped SST and Pyramidal neurons.



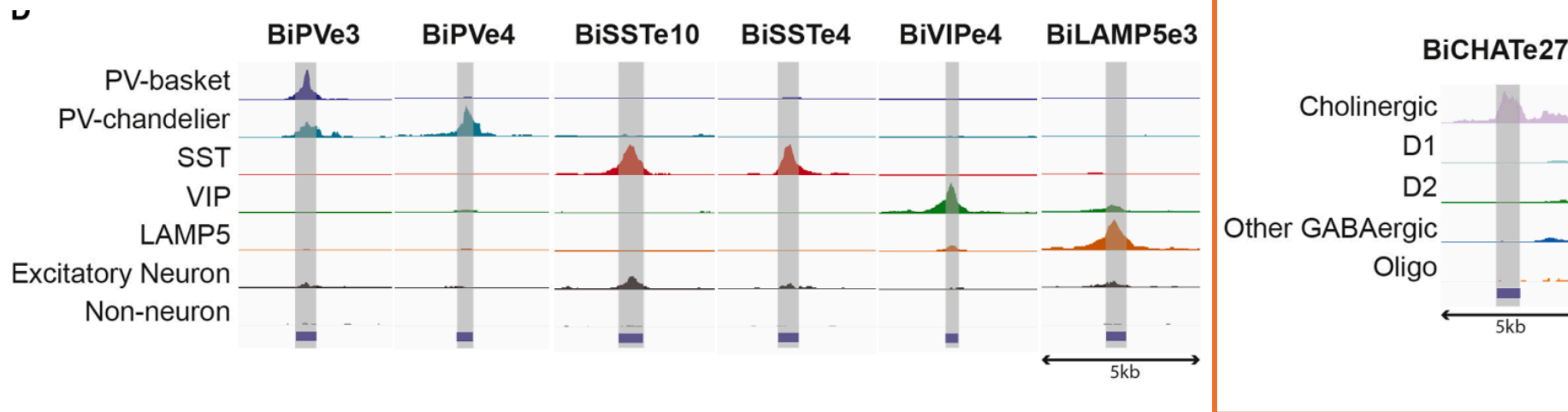
- VIP neurons are GABAergic release GABA onto postsynaptic SST neurons.
- Large inhibitory post synaptic currents in SST neurons.
- Pyramidal neurons weakly inhibited.

**Functionally validates enhancer targeting of VIP neurons.**

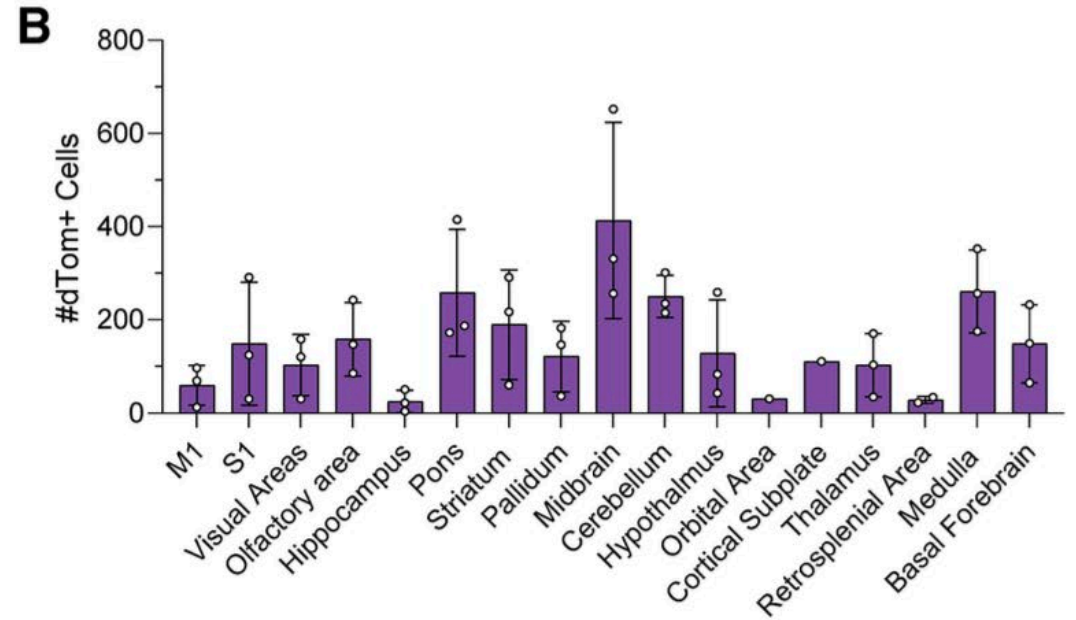
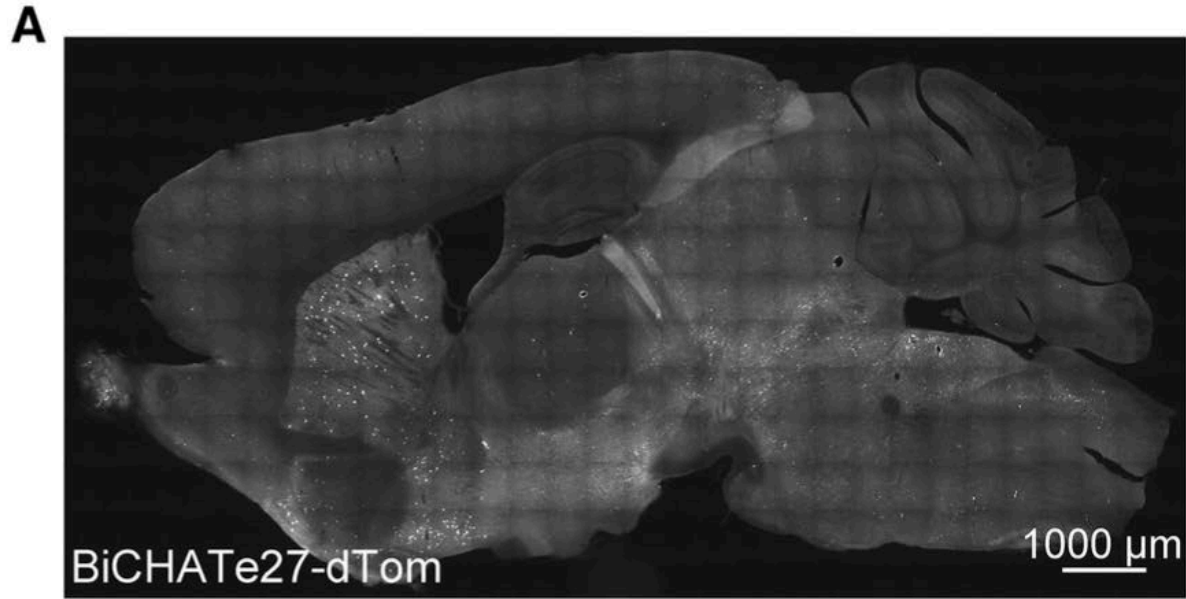


# Enhancer-based viral targeting of CHAT-positive neurons in mice

- Cholinergic (choline acetyltransferase [Chat]) neurons
- Acetylcholine = primary neurotransmitter
- Crucial role in a variety of functions including motor control, memory, and attention.
- Isolated GFP+ nuclei from the striatum of *Dlx5a-Cre::INTACT* mice and performed single-cell ATAC-seq experiments.
- Once candidate identified from screening:



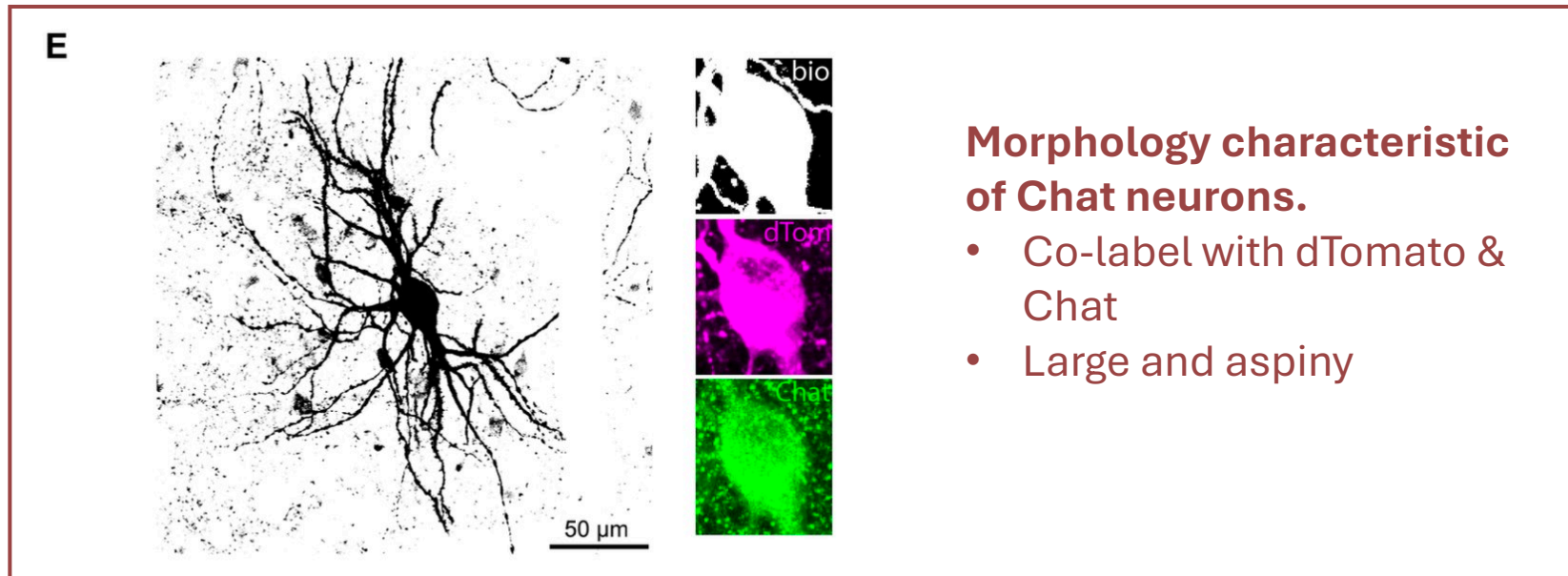
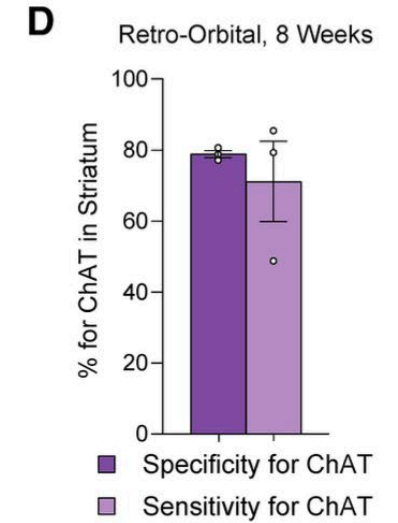
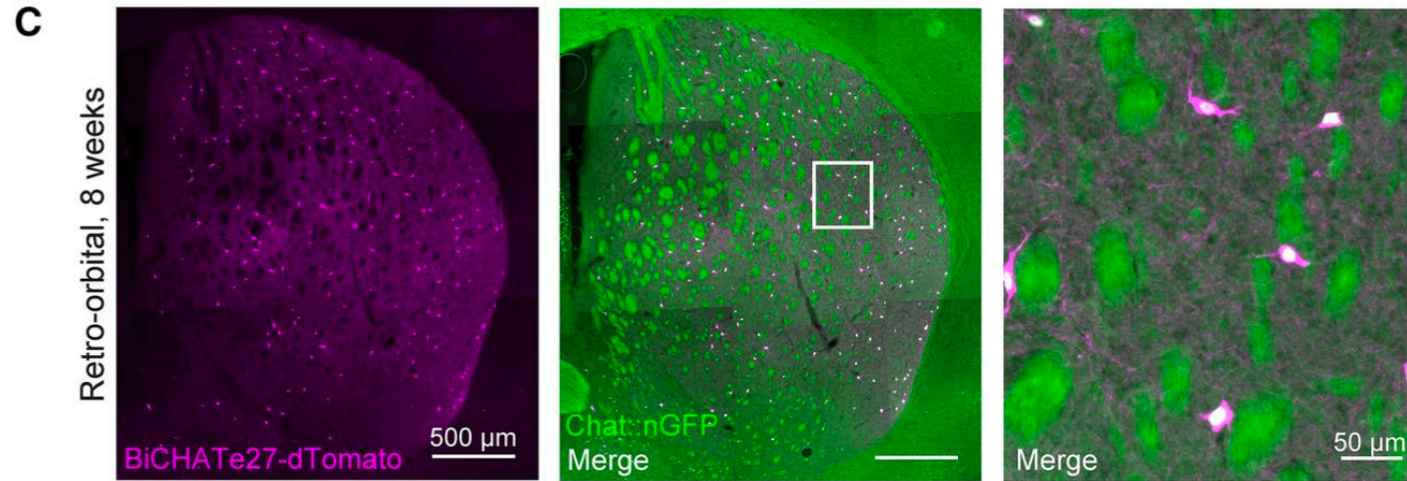
# Enhancer-based viral targeting of CHAT-positive neurons in mice



- Mostly labelled the striatal regions as expected.

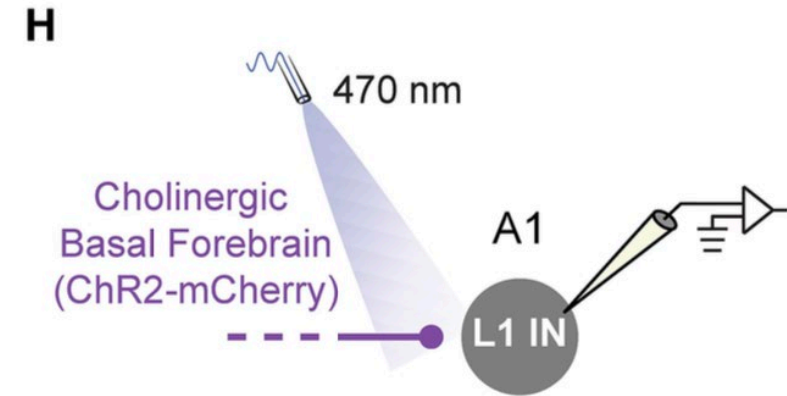
# Enhancer-based viral targeting of CHAT-positive neurons in mice

- Colocalization of BiCHATe27-dTomato cells in the caudoputamen with the cholinergic marker ChAT.
- High specificity and sensitivity for ChAT.

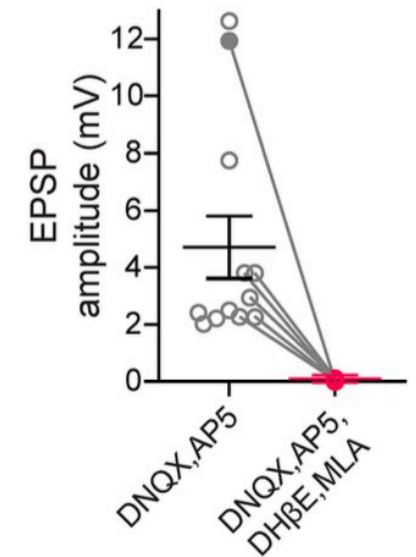
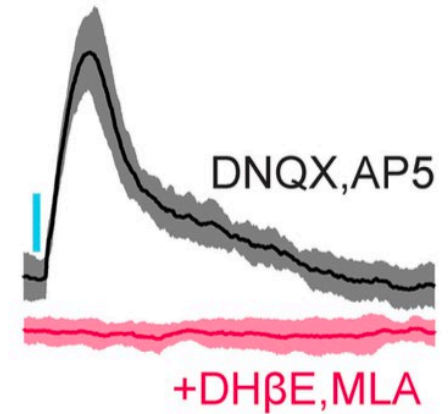


# Enhancer-based viral targeting of CHAT-positive neurons in mice

- Chat neurons known to project from L1 of auditory cortex & excite interneurons
- Tested if stimulation of Chat+ neurons induce excitatory currents in L1 interneurons
- AAV- BiCHATE27-ChR2-mCherry used to drive optogenetic activation in Chat neurons.
- Blue light activates ChR2<sup>+</sup> VIP neurons only.
- Measured **excitatory postsynaptic potentials (EPSPs)** in the recorded L1 neurons.

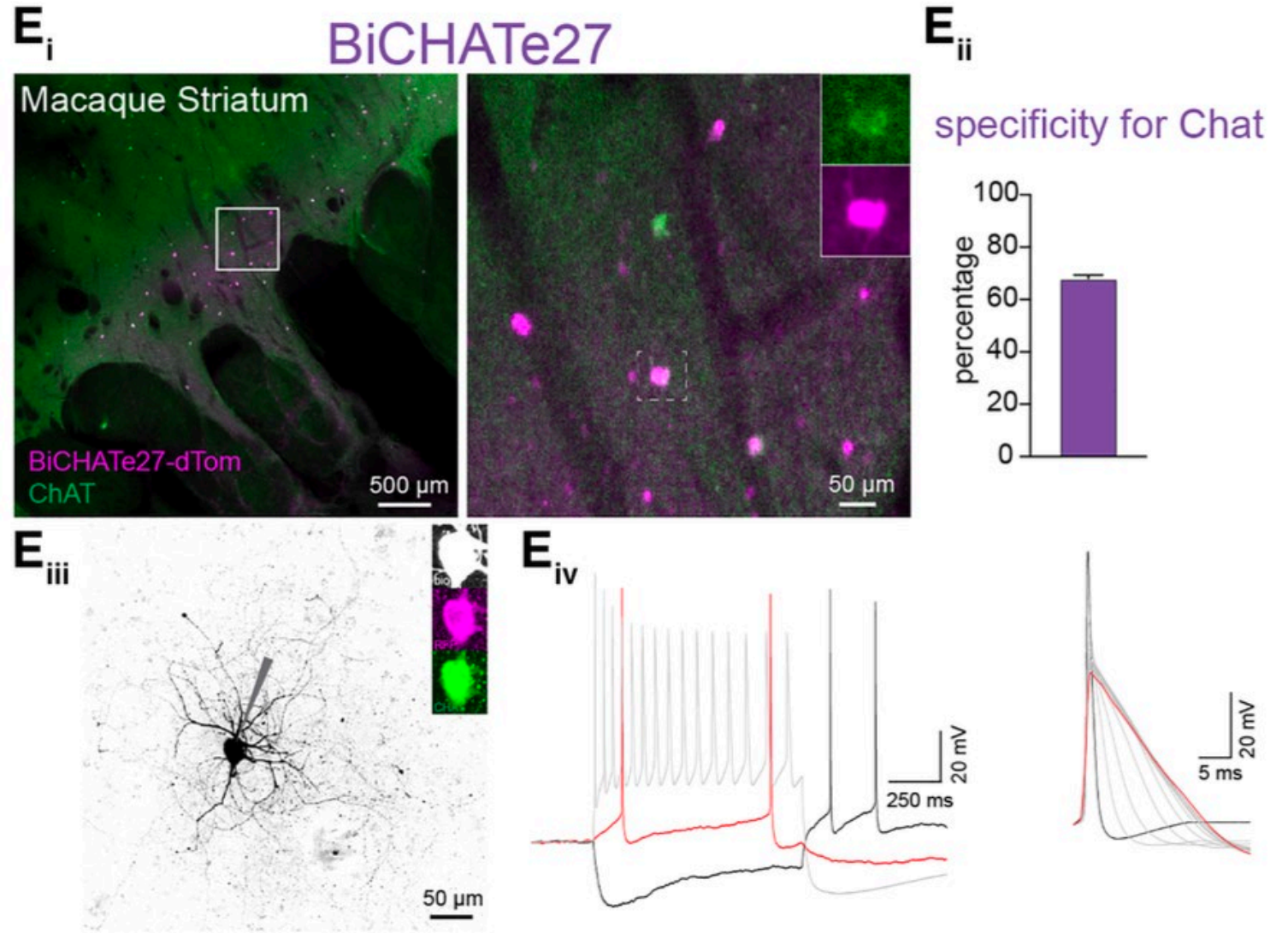


- EPSPs **persisted** in the presence of DNQX & AP5 therefore not mediated by glutamatergic neurons.
- **Nicotinic ACh receptor (nAChR) blockers prevent EPSPs – currents mediated by activation of Chat neurons**
- **Functionally validates enhancer targeting of Chat+ neurons.**



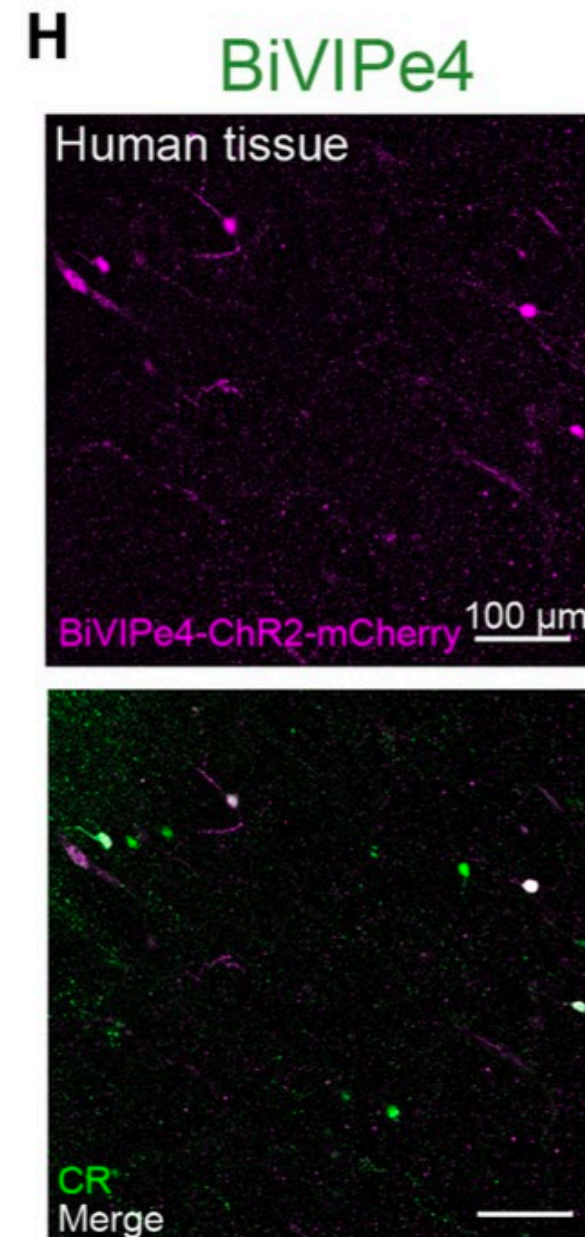
# Validation of enhancer-based viral tools in NHPs

- AAV- BiCHATe27-dTom delivered intracranially into rhesus macaque striatum.
- Large and aspiny morphology.
- Tonic AP firing at rest with relatively wide half- widths.
- Other enhancers behaved similarly with specificities ranging from ~40 to 90 %.



# Validation of enhancer-based viral tools in human tissue

- Surgically resected human tissue collected from the temporal lobe.
- Sectioned and incubated with AAV-BiVIPe4-ChR2-mCherry expression cassette.
- specificity of BiVIPe4 evaluated six days later using CR as a marker for human VIP interneurons.
- Some co-expression of mCherry and CR suggests BiVIPe4 enhancer remains VIP specific in human tissue.



# Potential clinical and commercial pitfalls

- Changing the **capsid may alter specificity**. Only AAV-PHP.eB used here.
- Changing AAV genome composition changes encapsidated **impurity profile**.
- **Manufacturing challenges** – titer of these viruses was not referenced.
- AAV genome **size limits**.

## Stowaways in the cargo: Contaminating nucleic acids in rAAV preparations for gene therapy

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NEWS | 25 September 2025

## Huntington's disease treated for first time using gene therapy

Preliminary results from a small trial offer the clearest evidence yet that the brain disease's progression can be slowed.

## Preventing packaging of translatable P5-associated DNA contaminants in recombinant AAV vector preps

Mark A. Brimble,<sup>1,2</sup> Pei-Hsin Cheng,<sup>1</sup> Stephen M. Winston,<sup>1,3</sup> Isaiah L. Reeves,<sup>1,3</sup> Aisha Souquette,<sup>4</sup> Yunyu Spence,<sup>1</sup> Junfang Zhou,<sup>1</sup> Yong-Dong Wang,<sup>5</sup> Christopher L. Morton,<sup>1</sup> Marcus Valentine,<sup>6</sup> Paul G. Thomas,<sup>4</sup> Amit C. Nathwani,<sup>2,7</sup> John T. Gray,<sup>8</sup> and Andrew M. Davidoff<sup>1,3</sup>

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# Technical and biological sources of noise confound multiplexed enhancer AAV screening

## Technical and biological sources of noise confound multiplexed enhancer AAV screening

### Authors

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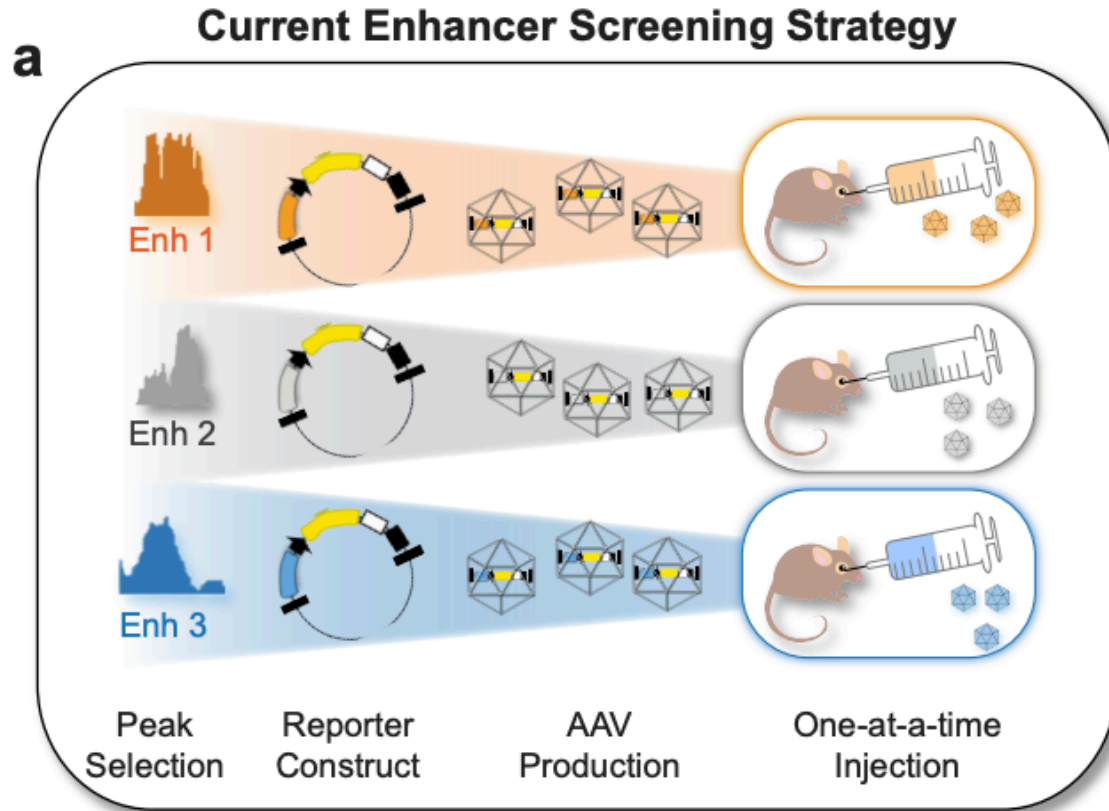
Correspondence: Avery C. Hunker, [avery.hunker@alleninstitute.org](mailto:avery.hunker@alleninstitute.org)

### Keywords

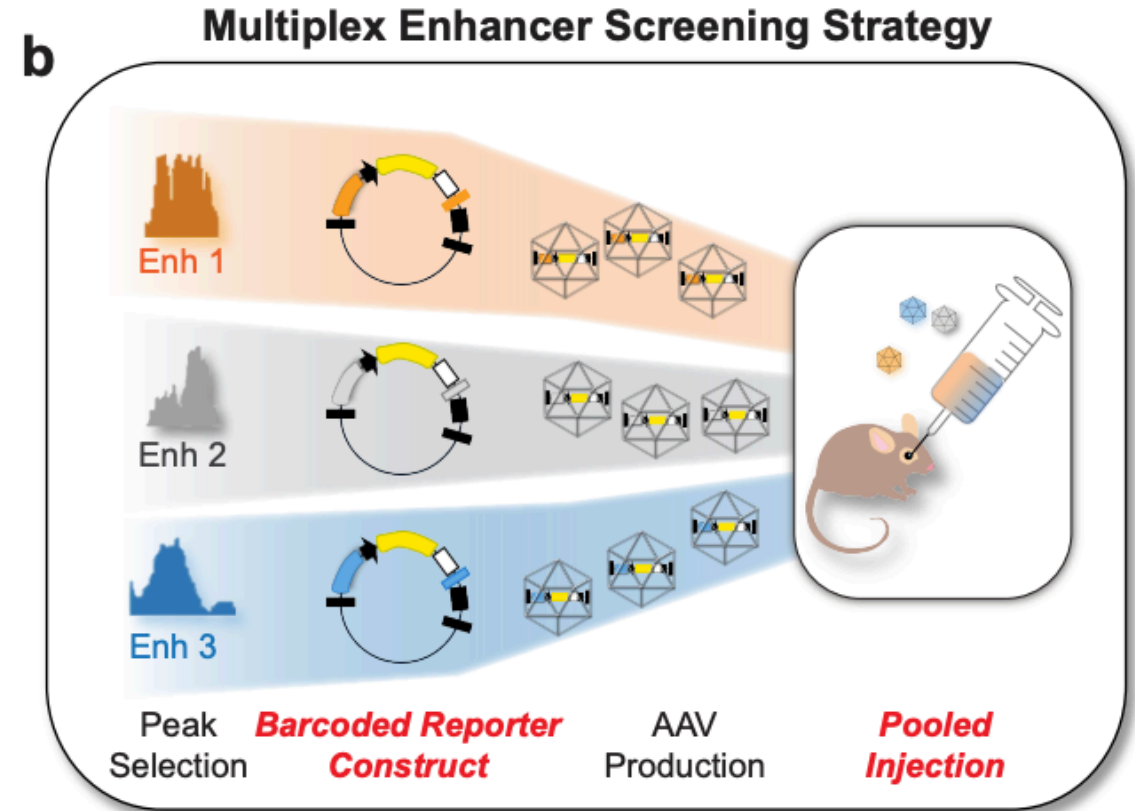
AAV, enhancer, multiplex screen, single cell RNA-sequencing, background noise

- Barcoded multiplex strategy for screening enhancer AAVs.
- Cell type resolution using single cell 33 RNA sequencing and taxonomy mapping.
- Proof-of-concept study using a small 34 pool of validated enhancer AAVs
- Technical & biological issues or “noise” stalled the project and resulted in this “troubleshooting” manuscript.

# One-at-a-time vs multiplexed enhancer screening



- Similar strategy to first paper.
- One-at-a-time analysis of cell-type-specificity.
- Time consuming & laborious.

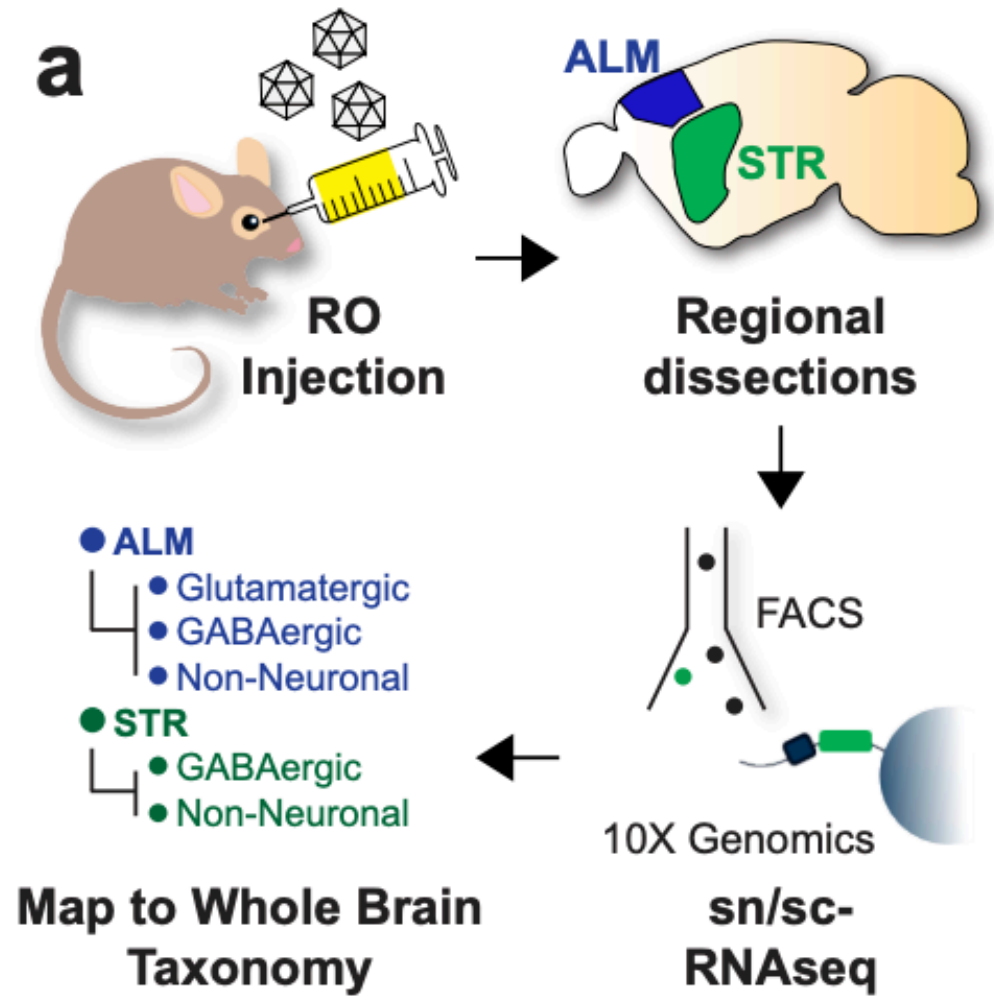


- Barcode & pool enhancer constructs.
- Pooled injection.
- Simultaneous analysis of cell-type-specificity.

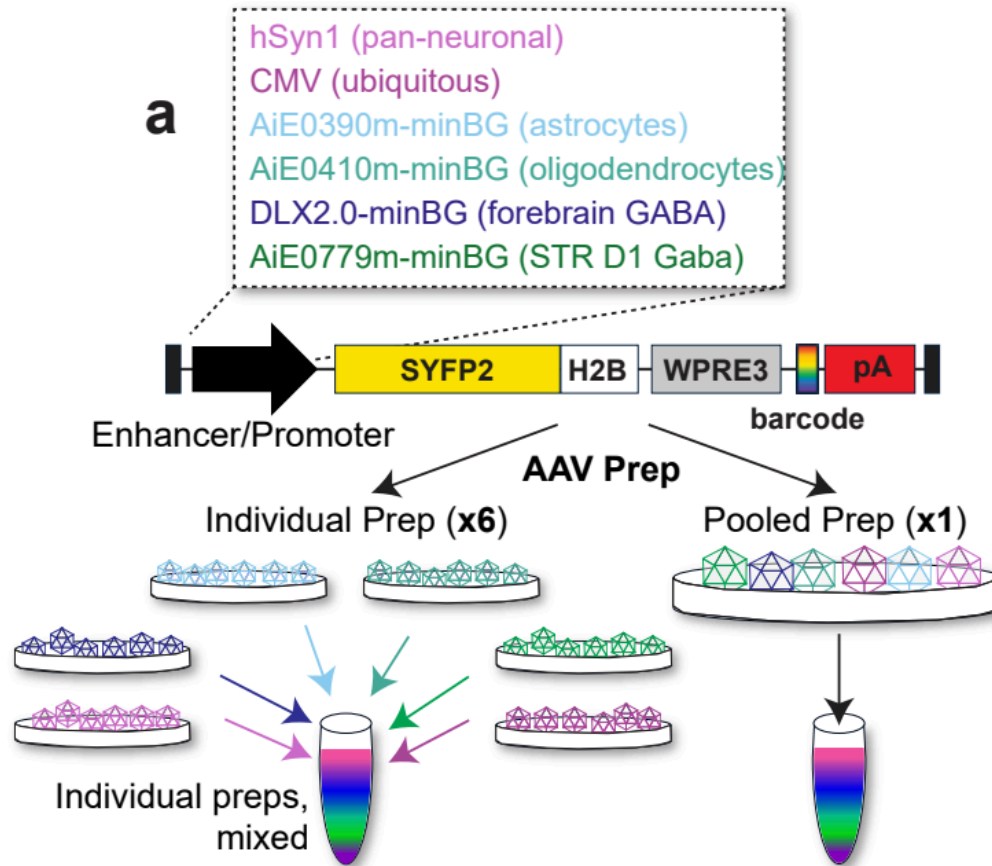
# Multiplexed enhancer screening workflow



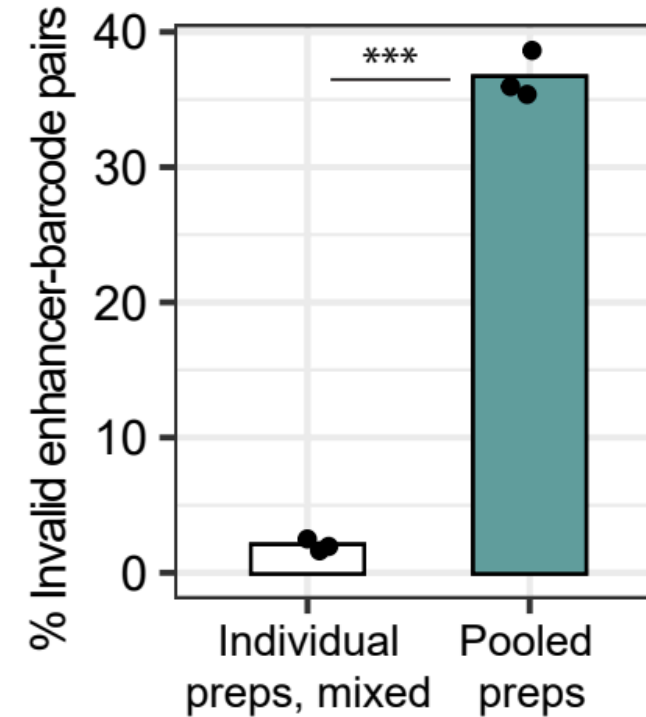
- Enhancers packaged into AAVs with SYFP2-H2B nuclear reporter and barcode.
- Extraction of antero-lateral motor cortex (ALM) & Striatum for nuclei extraction and sorting.
- Cell type AAV expression determined by mapping RNA sequencing reads to a mouse whole brain taxonomy.



# Pooled enhancer AAV packaging causes enhancer-barcode mismatches



## **f** AAV Packaging Recombination



- Pooled or arrayed packaging of 6 known enhancer AAVs with known barcode-enhancer combinations

- Long read seq finds ~35 % of enhancer AAVs from pooled preps had incorrect barcode-enhancer combinations

# Conclusions

- Enhancer AAVs can improve transgene expression specificity in neuronal subtypes.
- scATAC-seq can be used to identify novel enhancer sequences.
- Enhancer sequences identified in mice appear to NHP and humans.
- Barcode-Enhancer mismatches in pooled Enhancer AAV preps mean enhancers must be screened using a one-at-a-time approach.
- Arrayed packaging and post-pooling solves mismatch issues.
- Improved methods are required to enable high-throughput Enhancer AAV screening.