Neuronal circuits and activity

Mapping and functionally controlling neuronal circuits
&
Characterization of cell state-related changes of neuronal activity

Technical journal club

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18.01.2022

Paper #1

ARTICLES
https://doi.org/10.1038/s41592-021-01319-9

nature methods

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Anterograde transneuronal tracing and genetic control with engineered yellow fever vaccine YFV-17D

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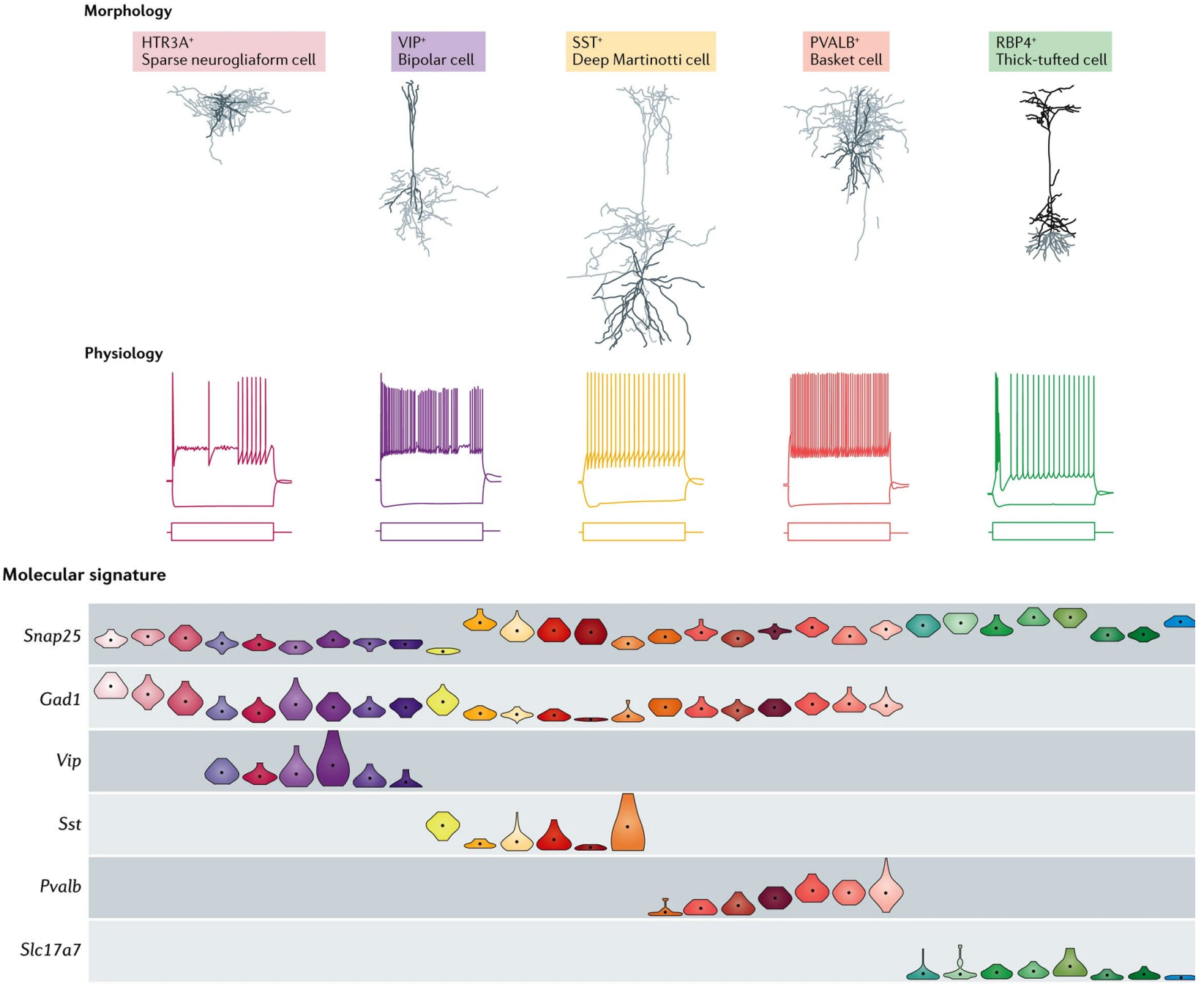
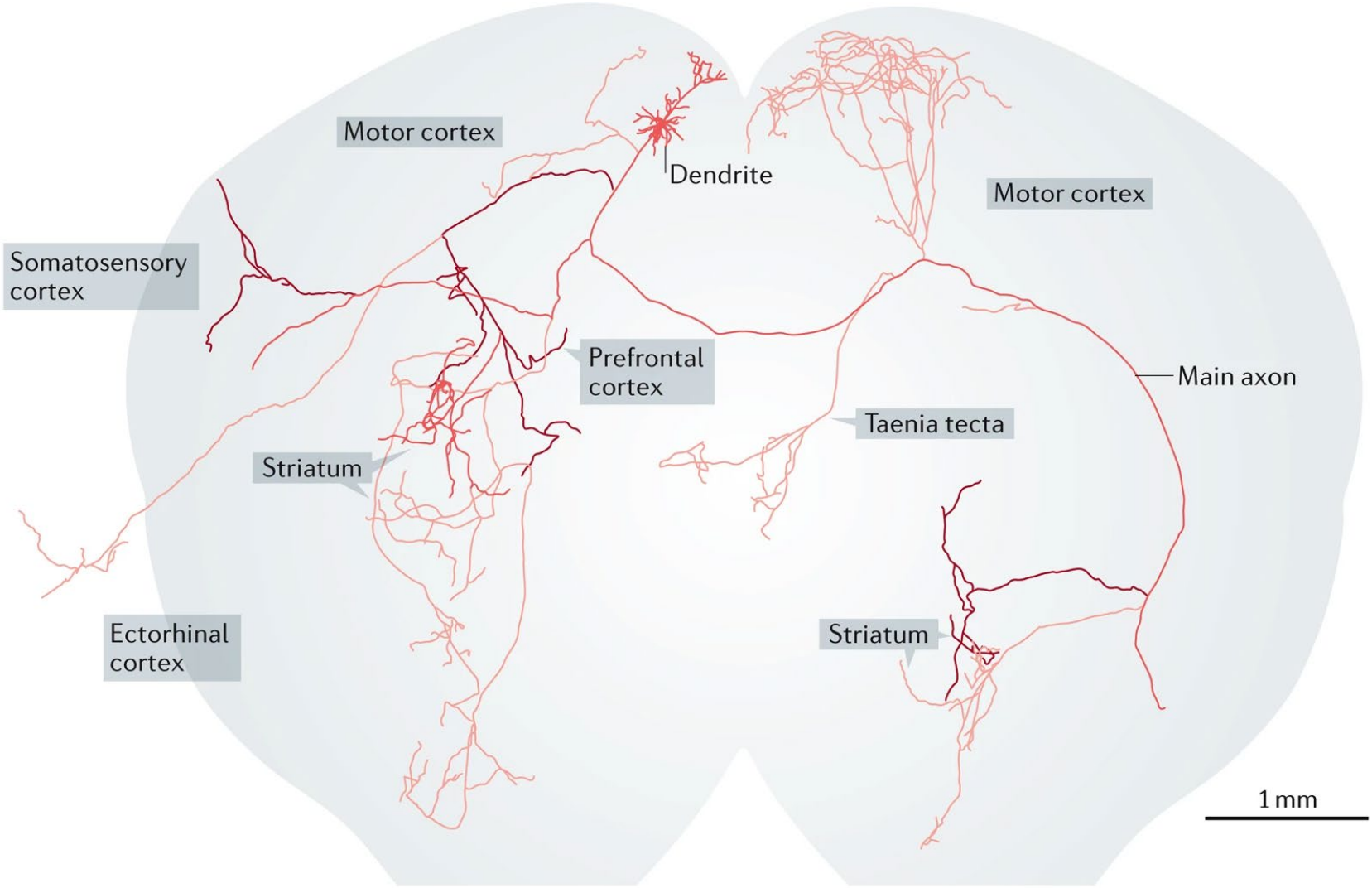
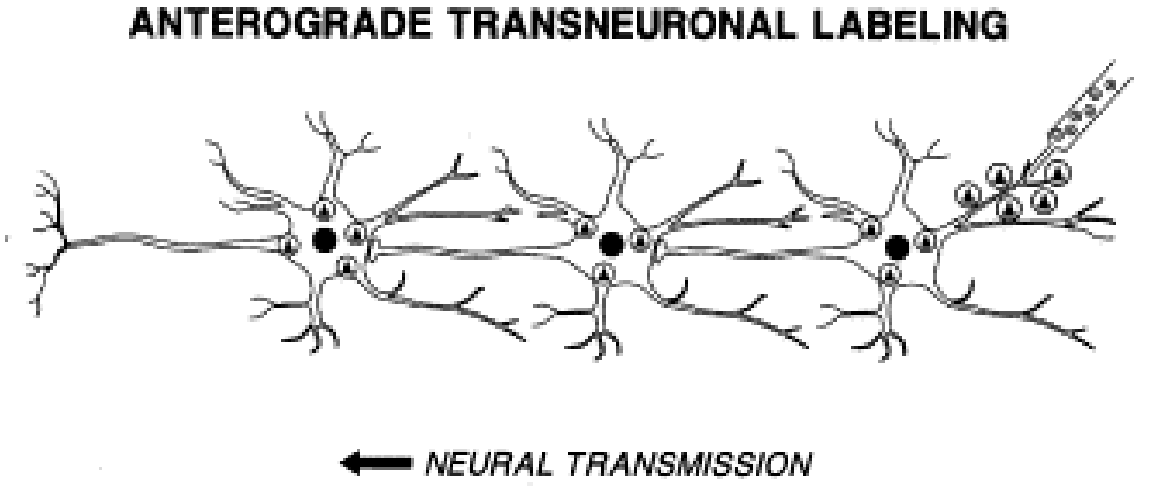
Neuronal circuits



Neuronal circuits

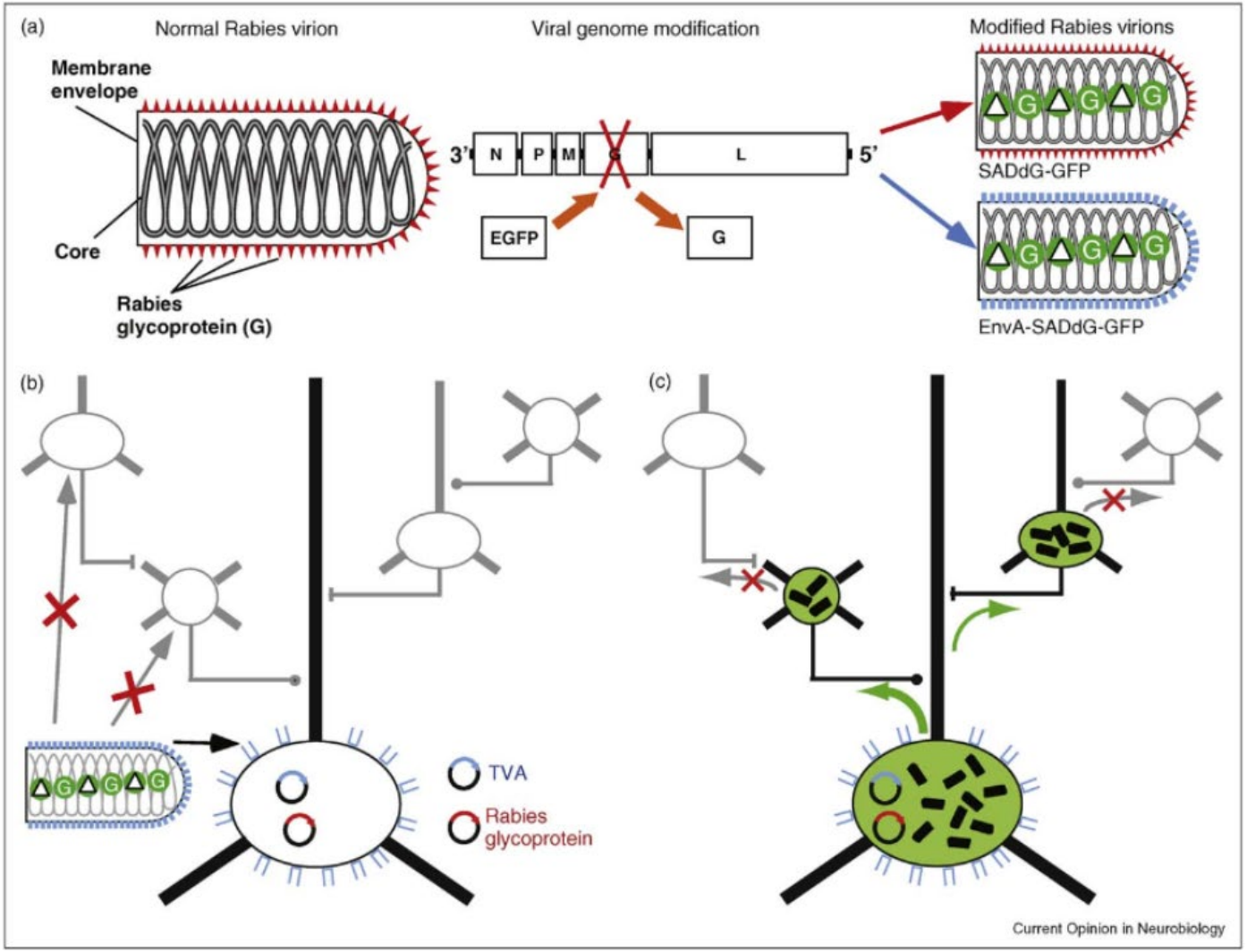


Tracing


ANTEROGRADE TRANSNEURONAL LABELING
NEURAL TRANSMISSION
RETROGRADE TRANSNEURONAL LABELING
1°
2°
3°

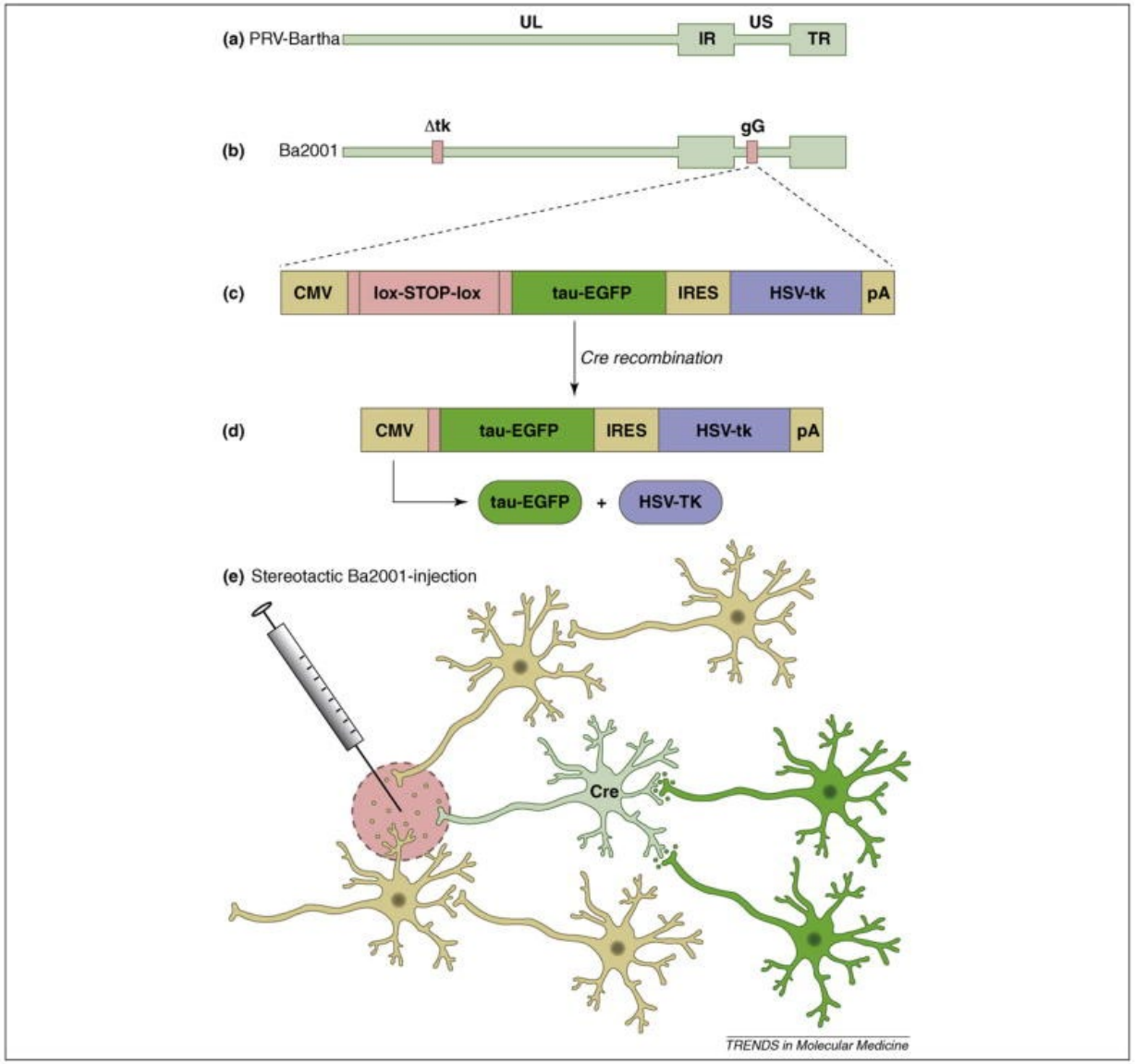
Old methods

Trans-neuronal circuit tracing with neurotropic viruses



Old methods

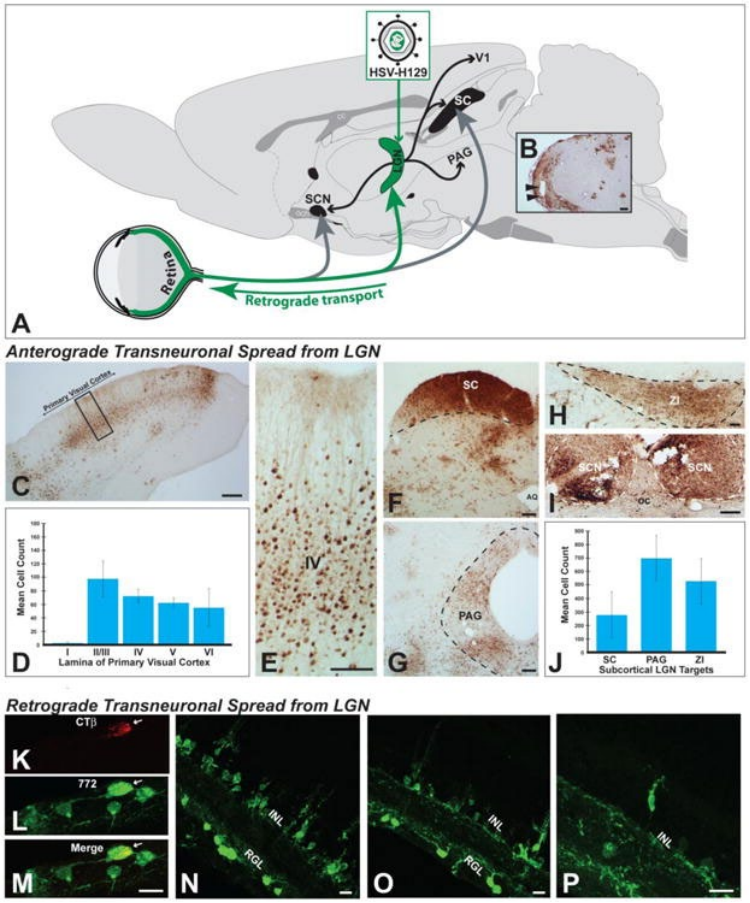
Retrograde tracing from molecularly defined neurons

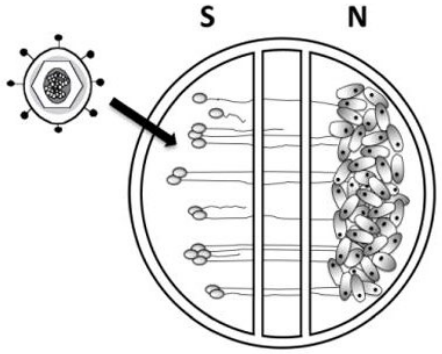


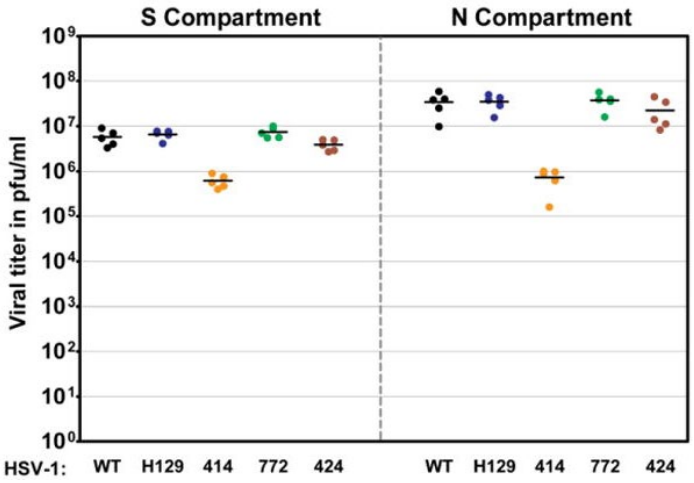
An **anterograde virus** propagating from presynaptic neurons to postsynaptic neurons will be crucial for revealing synaptic outputs, and to targeting postsynaptic neurons in functional studies.

Ideal anterograde virus yet to be discovered

- Just a few viruses can spread anterogradely.
- It has gained broader applications with recent modifications, but still has a few limitations.
- It shows retrograde axonal uptake at the injection site and a delayed retrograde transneuronal transport.







Ideal anterograde virus yet to be discovered

- H129 strain of HSV1, as human pathogen, it is highly toxic to neurons.

- Besides H129, pseudotyped vesicular stomatitis virus spreads anterogradely, but its efficiency and toxicity have not been extensively examined.

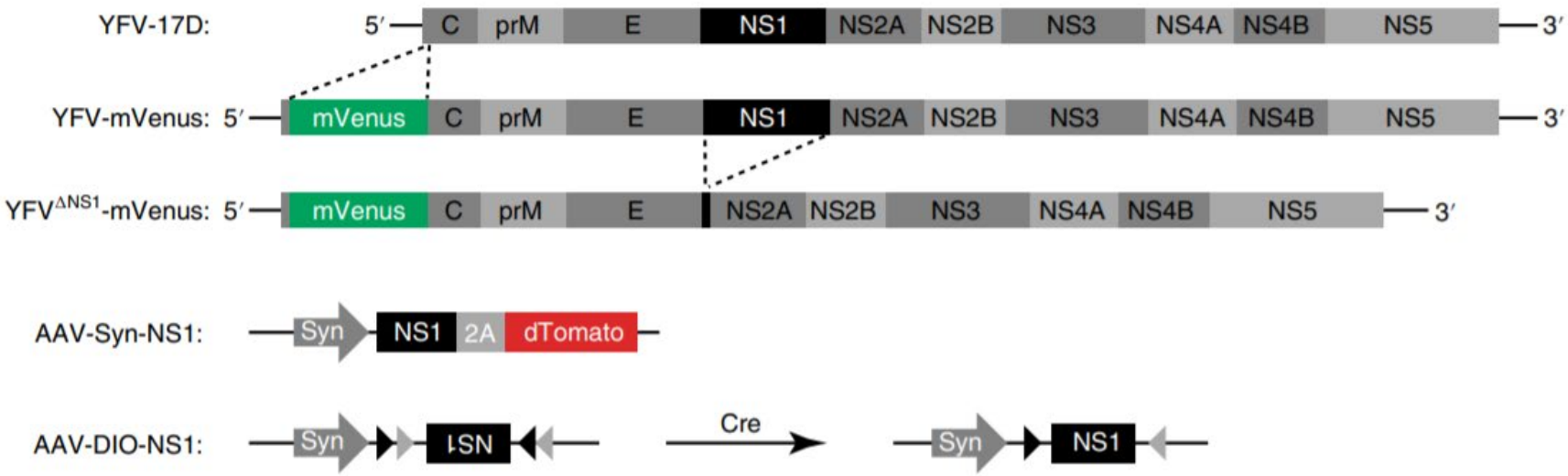
- Certain serotypes of adeno-associated viruses (AAVs) also spread anterogradely, but they show retrograde uptake as well.

Development of an **anterograde** viral system based on a live attenuated vaccine for yellow fever—YFV-17D

Results

Controlled anterograde transneuronal spreading of YFV-17D

- YFV-17D is a positive-sense single-stranded RNA virus.
- It encodes one open reading frame whose gene product is post-translationally cleaved into ten proteins



Results

Controlled anterograde transneuronal spreading of YFV-17D

- They injected YFV-mVenus into the PFC.
- Six days later, they observed in the striatum mVenus-positive neurons that were surrounded and contacted by axons originating from the PFC.
- At a later time point, they found that YFV-mVenus had continued to travel from the striatum to substantia nigra.

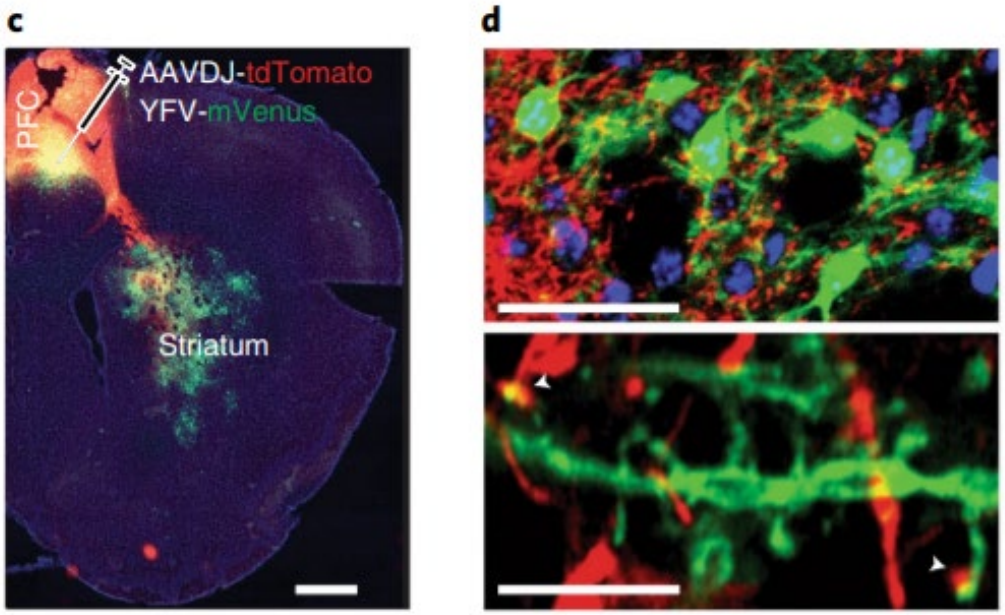
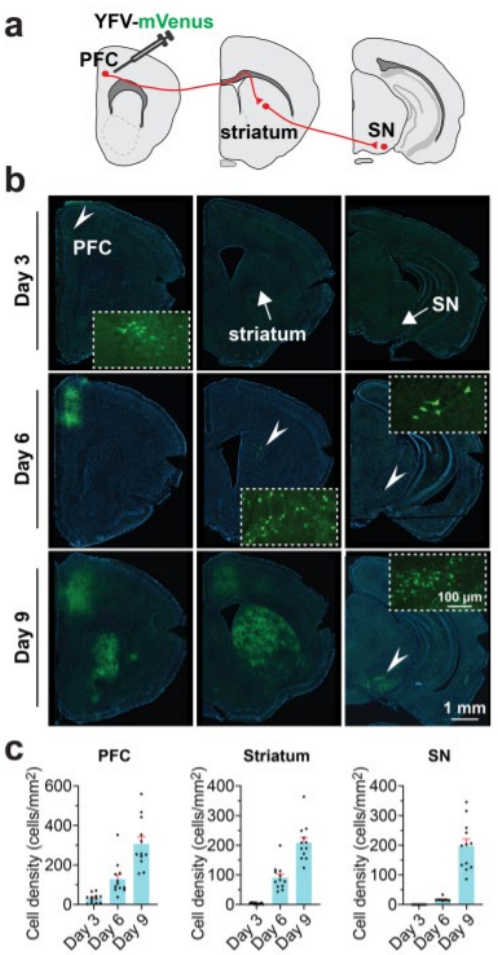


Figure 1



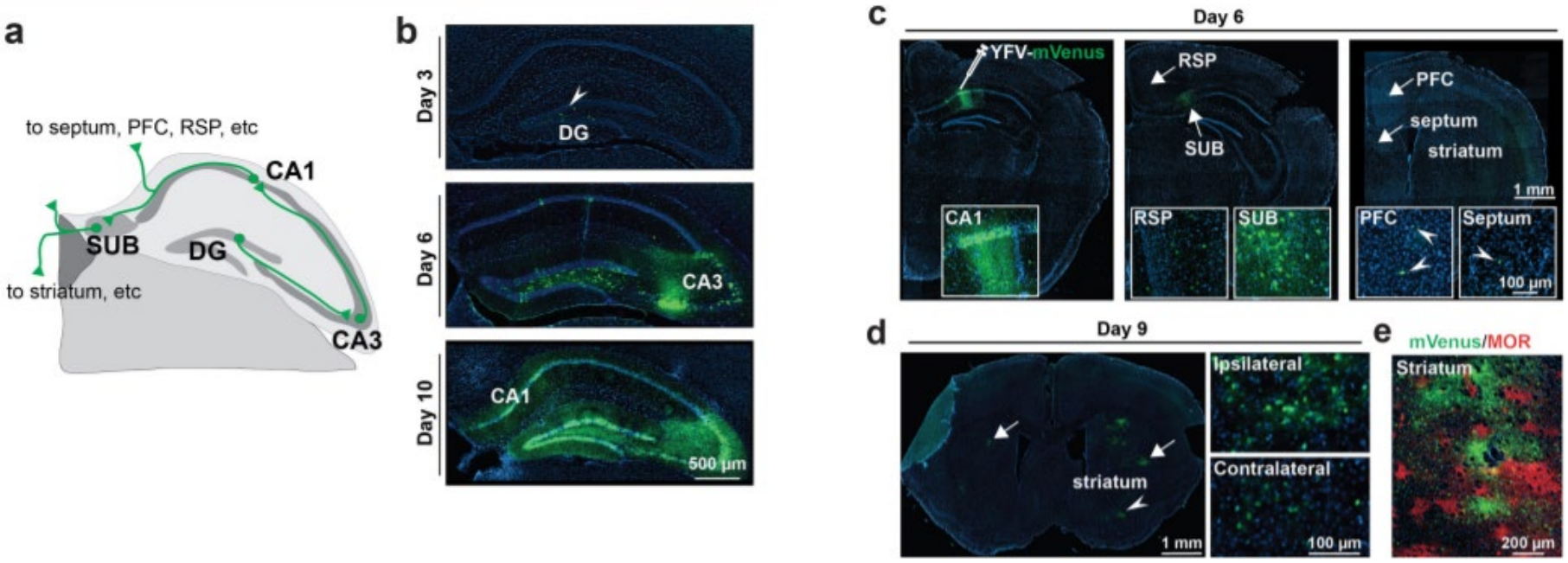
Extended data Figure 1

Results

Controlled anterograde trans-neuronal spreading of YFV-17D

- Similarly, YFV-mVenus spreads along the dentate gyrus–CA3–CA1–subiculum pathway

→ indicating that YFV-mVenus propagates along neuronal circuits in anterograde direction.

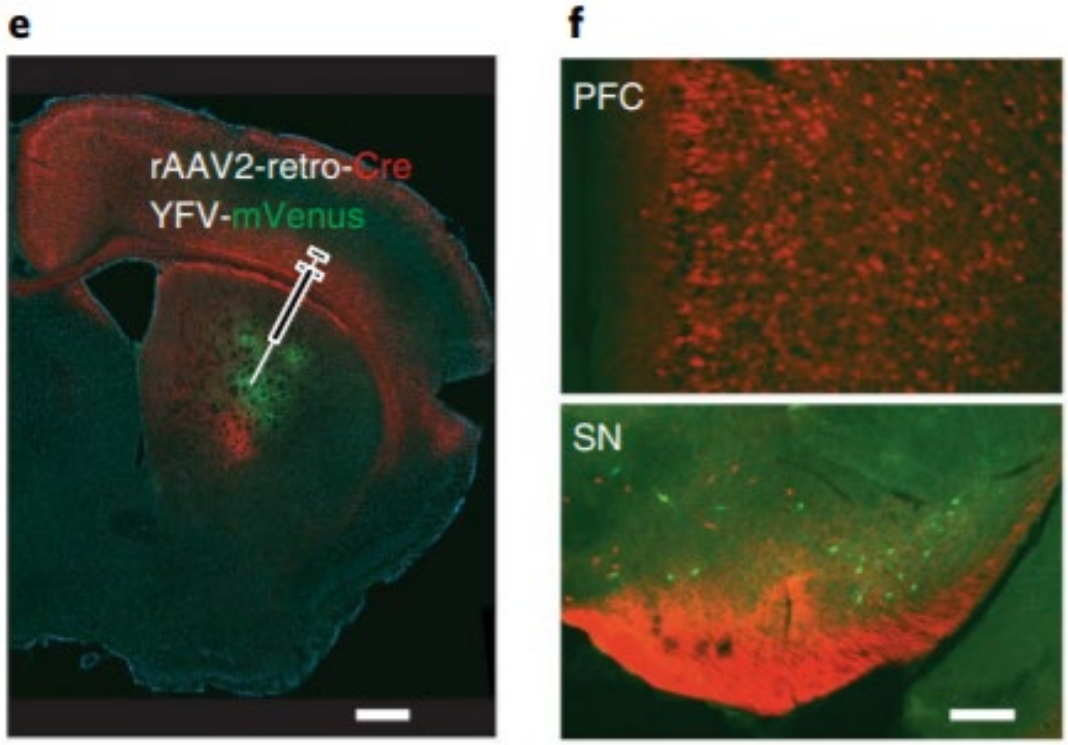


Extended data Figure 2

Results

Controlled anterograde transneuronal spreading of YFV-17D

- To test whether YFV-mVenus also travels in retrograde direction, they injected rAAV2-retro carrying Cre (rAAV2-retro-Cre) into the striatum of tdTomato reporter mice (Ai9 mice).
- Then injected YFV-mVenus into the same locus 2 weeks later.



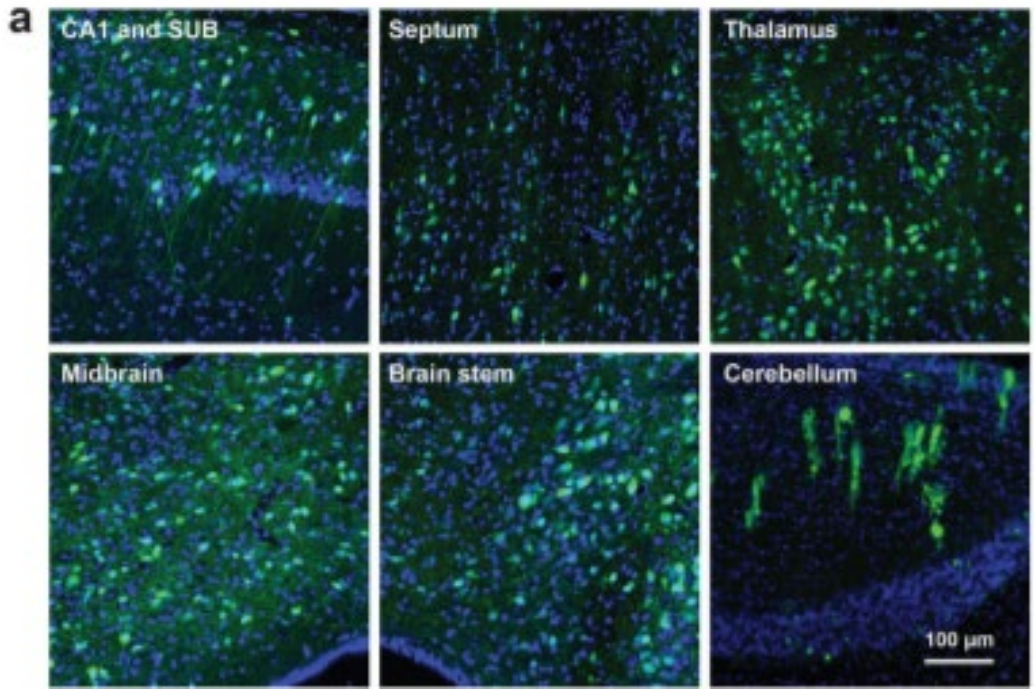
- Observed tdTomato-positive neurons in the PFC and substantia nigra, illustrating that rAAV2-retro retrogradely infected neurons projecting to the striatum.
- mVenus-positive neurons in the substantia nigra, but not in the PFC, showing that **YFV-mVenus** moves **only in anterograde direction** at this time point.

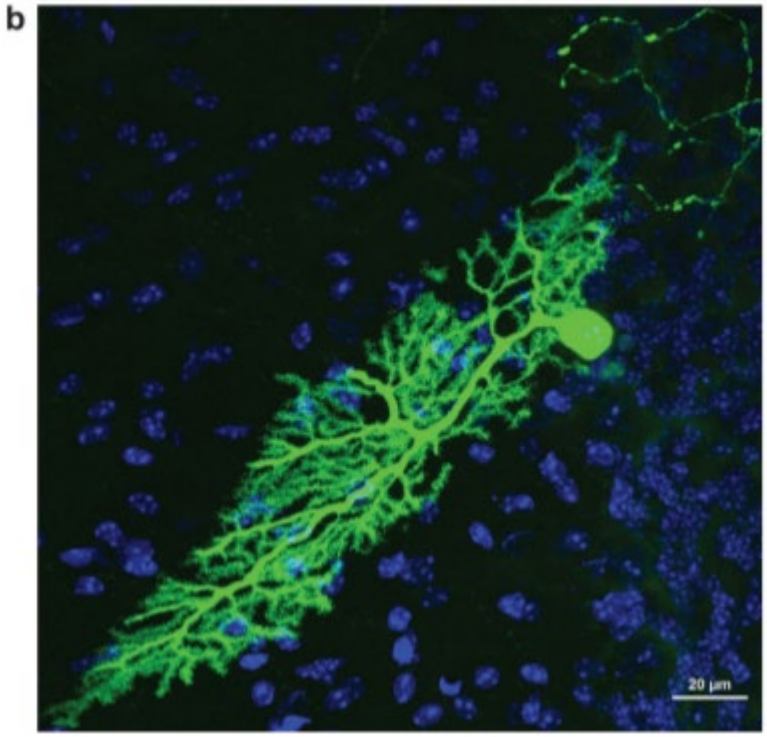
Figure 1

Results

Broad tropism for neurons

- YFV-17D showed a broad tropism for neurons





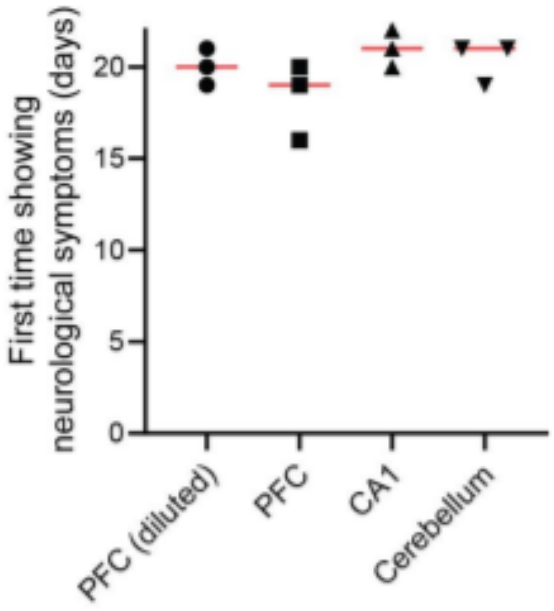
Extended Data Fig. 7

Results

Broad tropism for neurons

Mice receiving YFV-mVenus started to show neurological symptoms 16–22 days after the injection

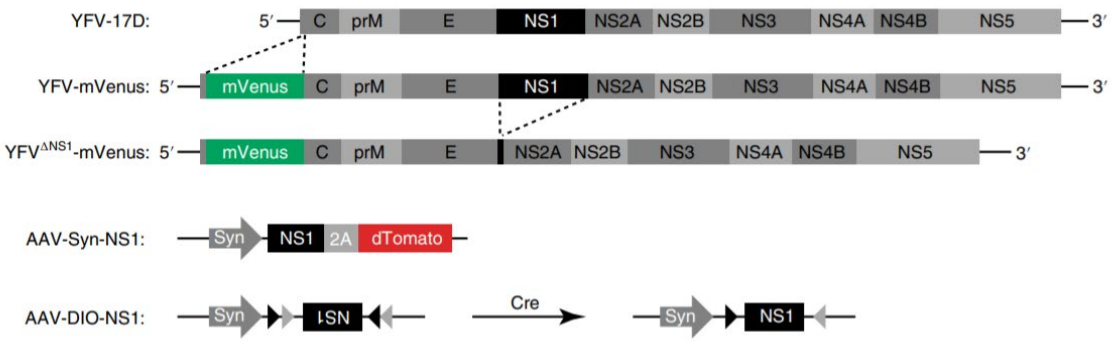
→ which appeared later compared to some other transneuronal viruses, suggesting a lower level of neuronal toxicity.

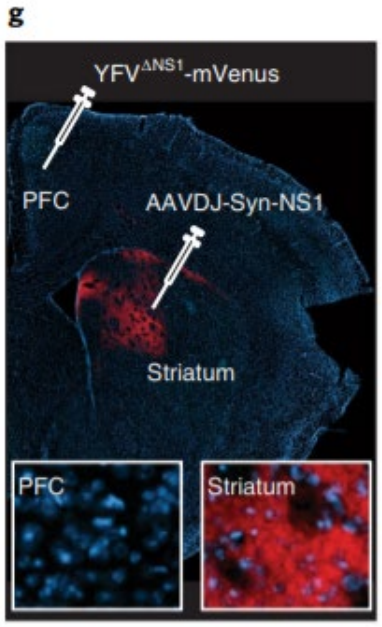


Extended Data Fig. 7

Results

They injected AAVDJ-Syn-NS1 into the striatum, and YFVΔNS1-mVenus into the PFC

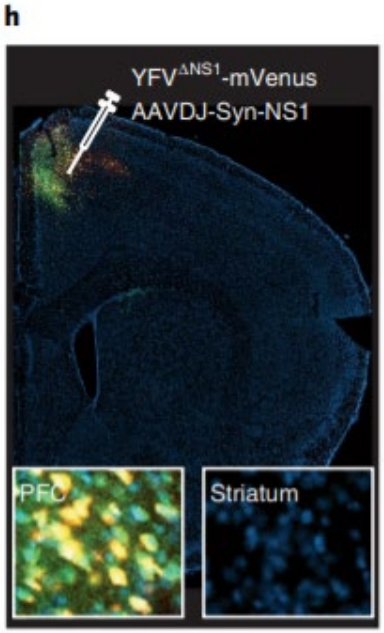




Red fluorescence appeared in the striatum, indicating expression of NS1 and dTomato.

They did not detect mVenus.

→ Without NS1 being supplied in the PFC, YFVΔNS1-mVenus was insufficient to replicate and express its genes to a detectable level.



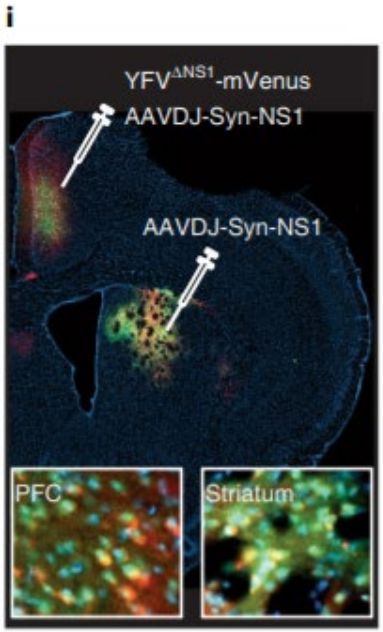
Both dTomato and mVenus appeared in the PFC
→ showing that NS1 expression enabled YFVΔNS1-mVenus replication and mVenus expression.

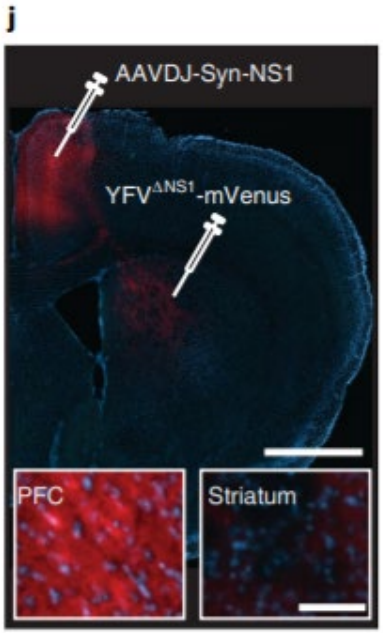
→ YFVΔNS1-mVenus by itself was not sufficient to express mVenus to a detectable level in postsynaptic neurons

Figure 1

Results

They injected AAVDJ-Syn-NS1 into both the PFC and striatum
&
injected YFVΔNS1-mVenus into the PFC





mVenus-positive neurons showed up in both the PFC and the striatum.

Demonstrating that:

- YFVΔNS1 replication in the PFC led to transneuronal transport to the striatum
- NS1 in the striatal neurons enabled YFVΔNS1 replication and mVenus expression at a detectable level

These results demonstrate that NS1 can control the spreading of YFVΔNS1.

They did not detect mVenus-positive neurons in the PFC or striatum, suggesting that YFVΔNS1-mVenus was not subject to retrograde axonal uptake.

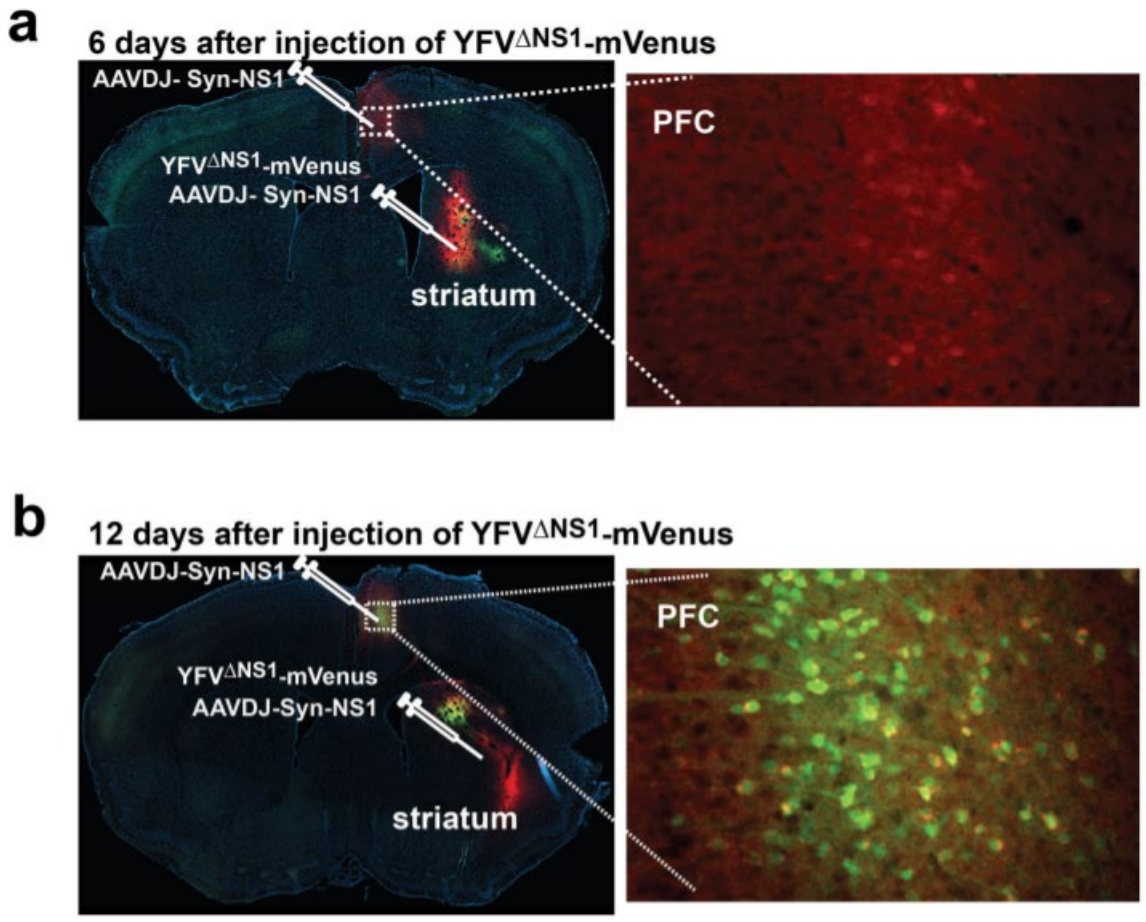
This result suggests that YFVΔNS1 may have low affinity to axonal terminals or may not be efficiently transported back to the soma

Figure 1

Results

Exclusively anterograde??

They injected AAVDJ-NS1 into both the PFC and striatum and 2weeks later injected YFVΔNS1-mVenus into the striatum.



After another 6 or 7days, they did not detect any mVenus-positive neurons in the PFC, consistent with anterograde-only transport of YFV-mVenus at this time point.

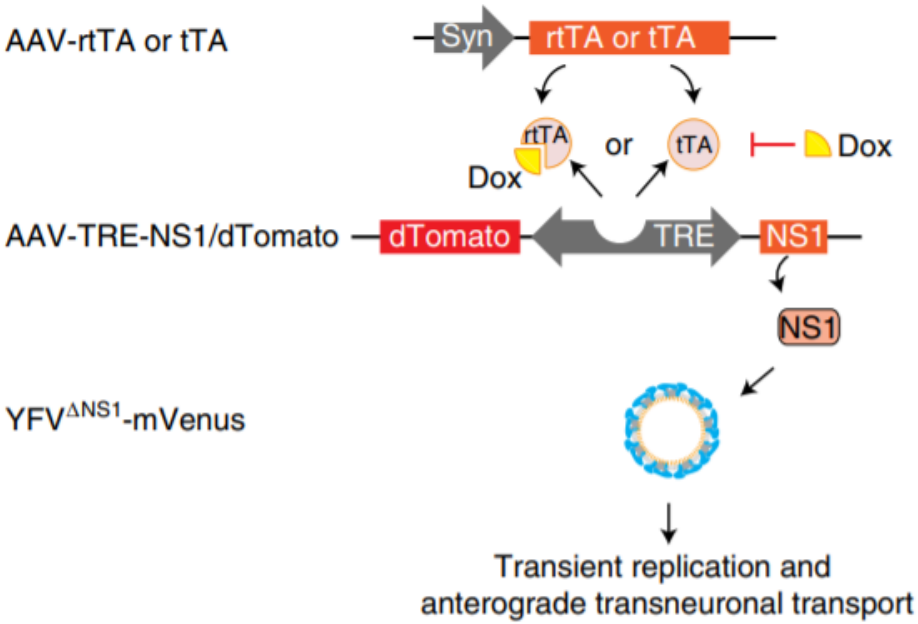
However, 12days after injection, mVenus-positive neurons appeared in the PFC, suggesting a temporally delayed retrograde transport of YFVΔNS1-mVenus.

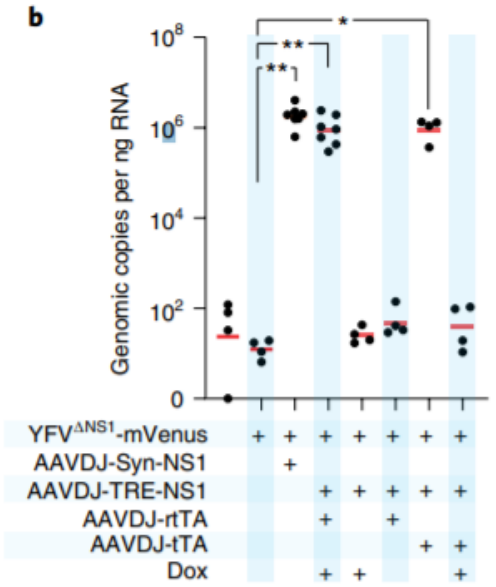
Figure 1

Anterograde-only tracing by inducible replication of YFVΔNS1

Results

Construction of an inducible system to control YFVΔNS1 replication





We made two versions of AAV-TRE-NS1:

- AAV-TRE-NS1/dTomato encodes a fluorescent protein
- AAV-TRE-NS1/NF does not.

To validate this system, they injected into the PFC different combinations of AAVs, and 2weeks later, YFVΔNS1-mVenus.

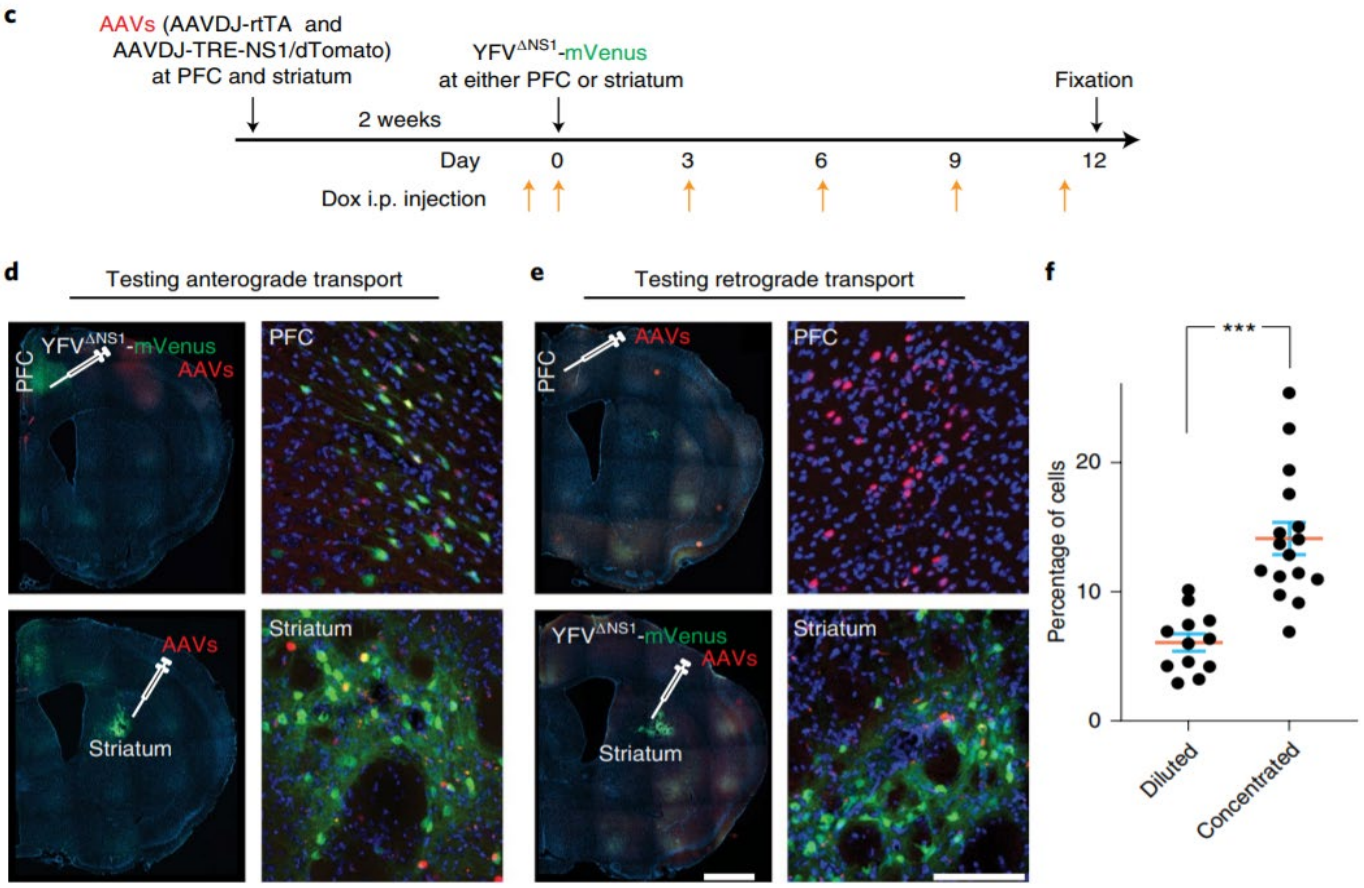
The results demonstrate that rtTA or tTA and Dox can control the replication of YFVΔNS1-mVenus.

Figure 2

Results

Construction of an inducible system to control YFVΔNS1 replication

Achievement of anterograde-only tracing with this system



mVenus-positive neurons appeared in both the PFC and striatum
→ indicating anterograde transport of YFVΔNS1-mVenus

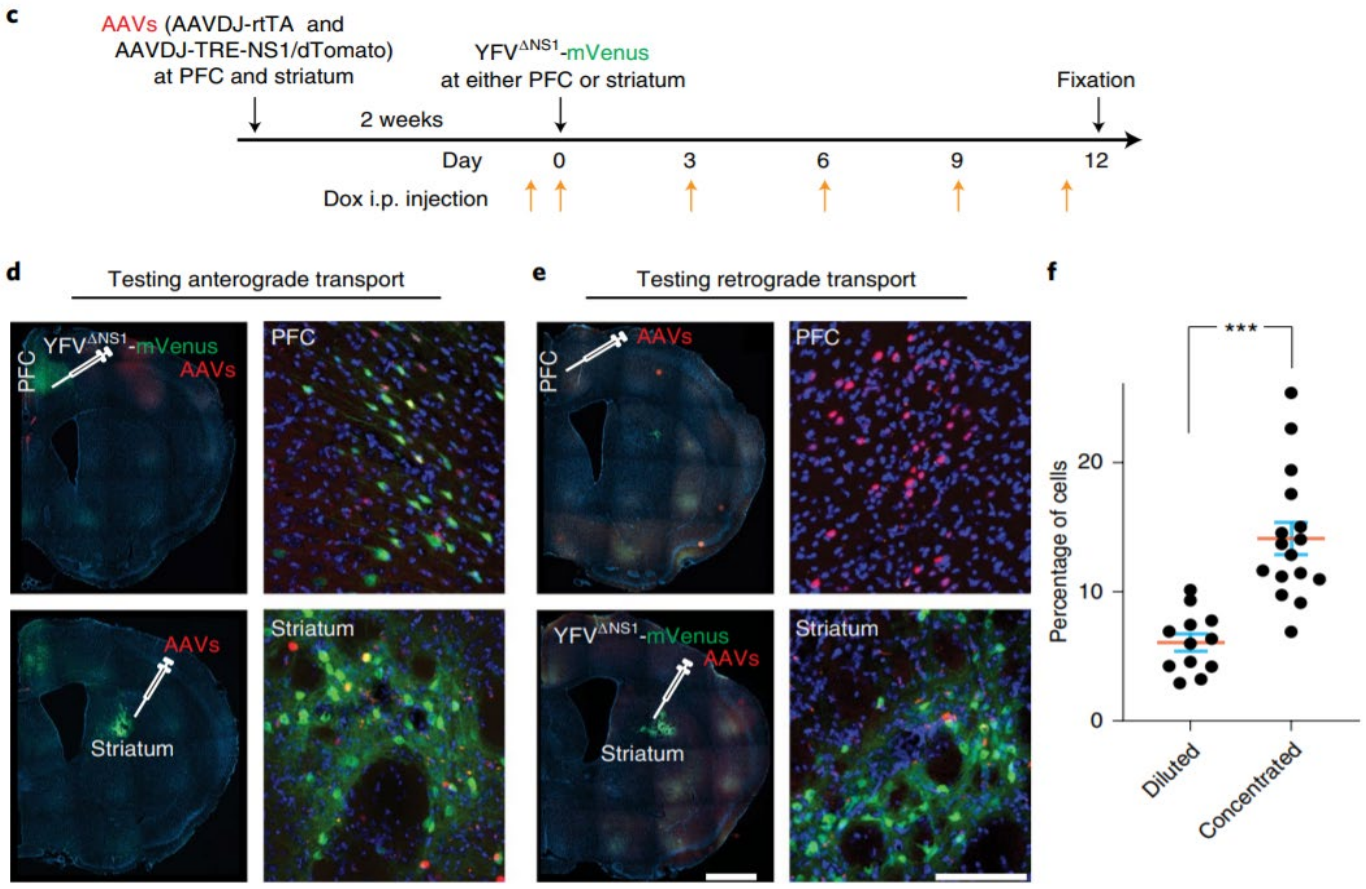
mVenus-positive neurons only in the striatum but not PFC
→ suggesting no retrograde transport of YFVΔNS1-mVenus

Figure 2

Results

Construction of an inducible system to control YFVΔNS1 replication

Achievement of anterograde-only tracing with this system



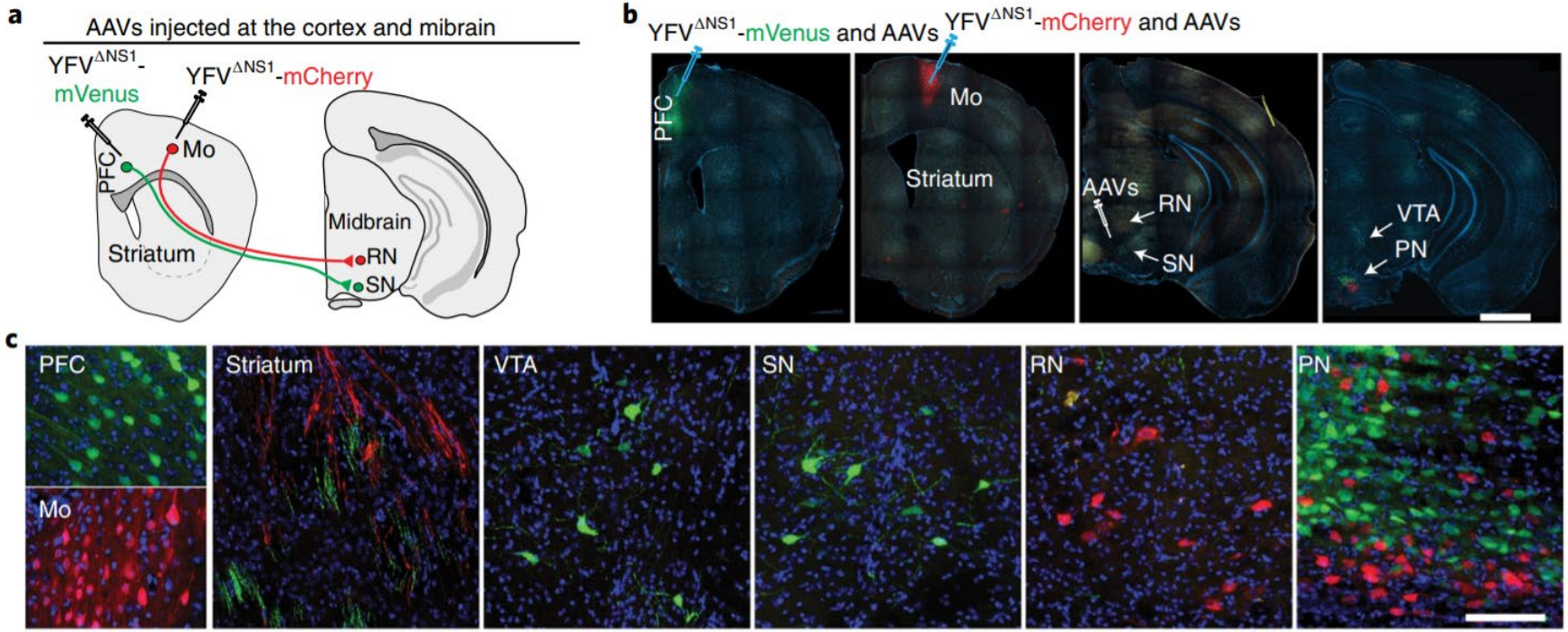
By temporally restricting NS1 expression they could achieve anterograde-only transport of YFVΔNS1-mVenus

Figure 2

Dual fluorescence tracing of parallel circuits

Results

To uncover how neural circuits diverge or converge, they generated YFVΔNS1-mCherry



Traced the pathways from two cortical regions—the PFC and motor cortex (Mo)—to the midbrain with YFVΔNS1-mVenus and YFVΔNS1-mCherry, with NS1 provided in the PFC, Mo and midbrain.

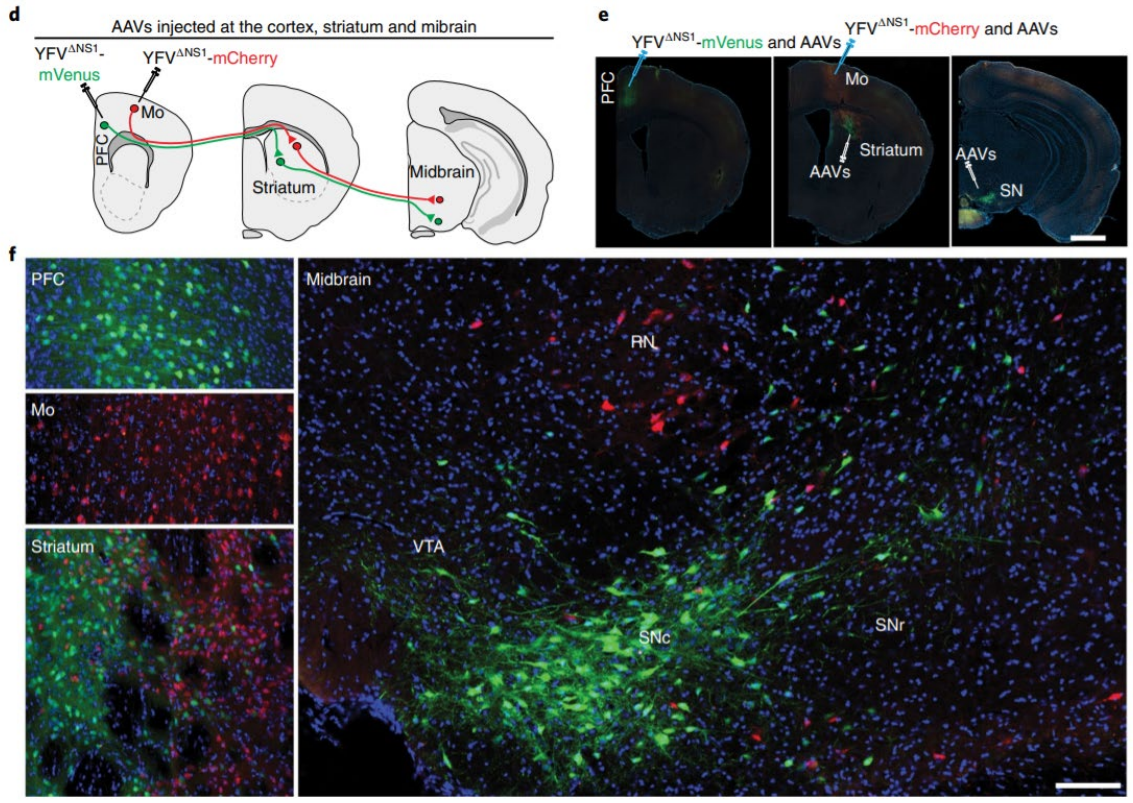
mVenus- or mCherry-positive axons from the PFC or Mo, respectively, passed the dorsal striatum and then diverged to different nuclei in the midbrain.

Figure 3

Results

Can the YFVΔNS1 system trace polysynaptic pathways as well??

NS1 was provided in the striatum in addition to PFC, Mo and midbrain



- mVenus- or mCherry-positive neurons:

- mVenus concentrated in the dorsomedial striatum
- mCherry in the dorsolateral striatum

- In the midbrain, mVenus-positive neurons were densely distributed in the substantia nigra and ventral tegmental area (VTA):

→ more substantia nigra and VTA neurons receive indirect PFC innervation via the striatum than those receiving direct PFC innervation.

→ mCherry-positive neurons were concentrated in the red nucleus.

Separate cortex–striatum–midbrain pathways

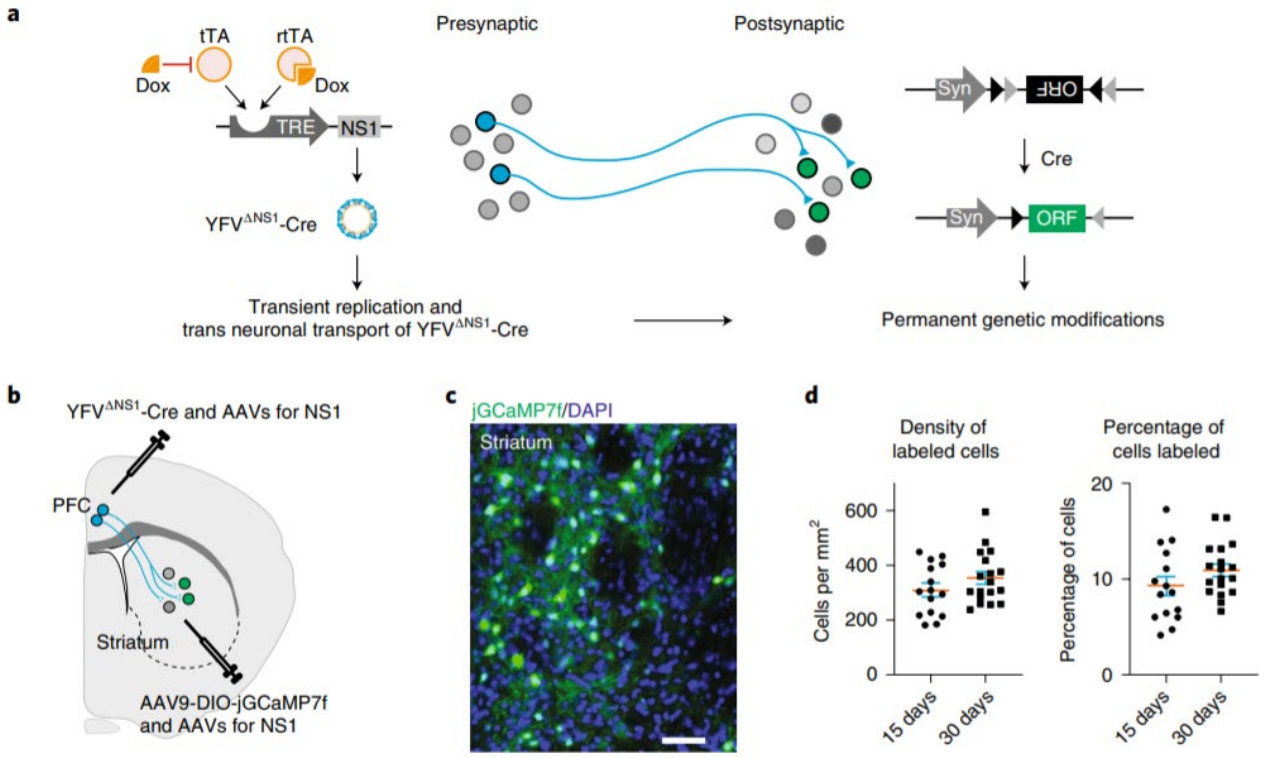
Figure 3

Transneuronal genetic control by YFVΔNS1-Cre

Results

- **They created YFVΔNS1-Cre to functionally manipulate postsynaptic neurons.**

- **YFVΔNS1-Cre can be induced to replicate transiently and make permanent genetic modifications in postsynaptic neurons.**



The transient viral replication minimizes neuronal toxicity caused by unrestricted viral replication

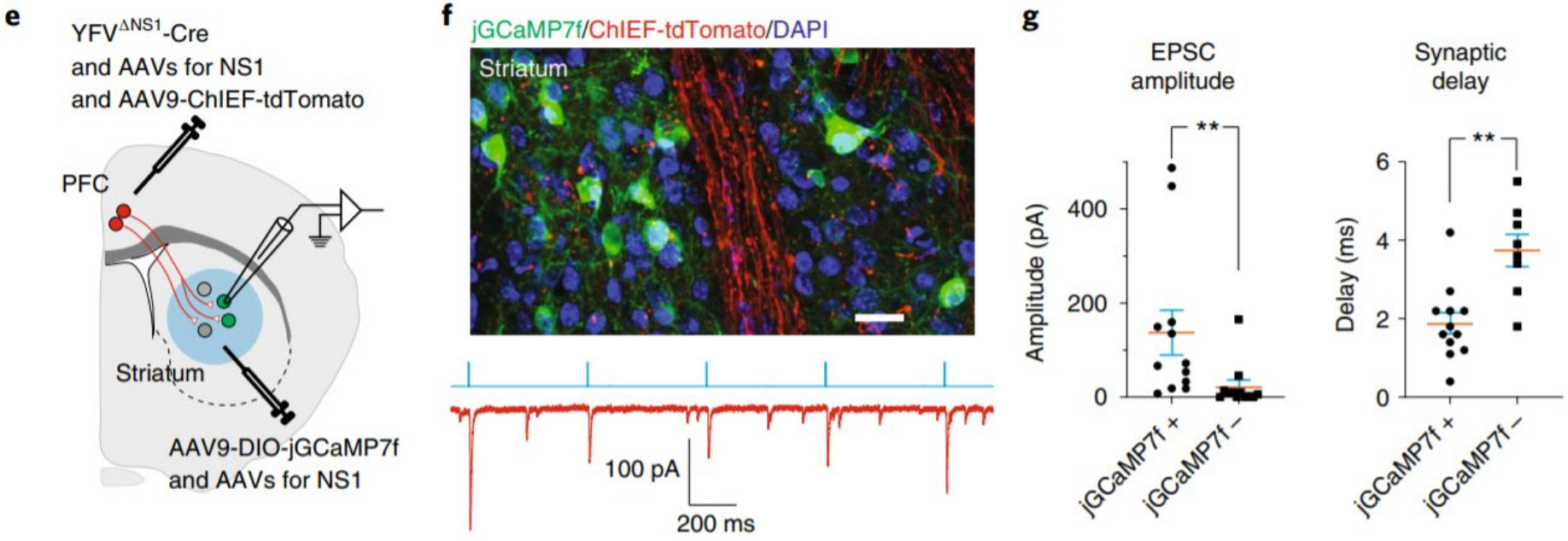
- They found jGCaMP7f-positive neurons in the striatum → showing that YFVΔNS1-Cre can modify genes in postsynaptic neurons.

- The density of jGCaMP7f-positive neurons did not change from 15 to 30days (Fig. 4d), suggesting low neuronal toxicity of this system.

Figure 4

Results

They conducted electrophysiological recordings to verify synaptic connections between the PFC starter neurons and the traced striatal neurons.



- In all of the jGCaMP7f-positive striatal cells recorded, optogenetic stimulation of the axonal terminals originating from PFC elicited postsynaptic currents.

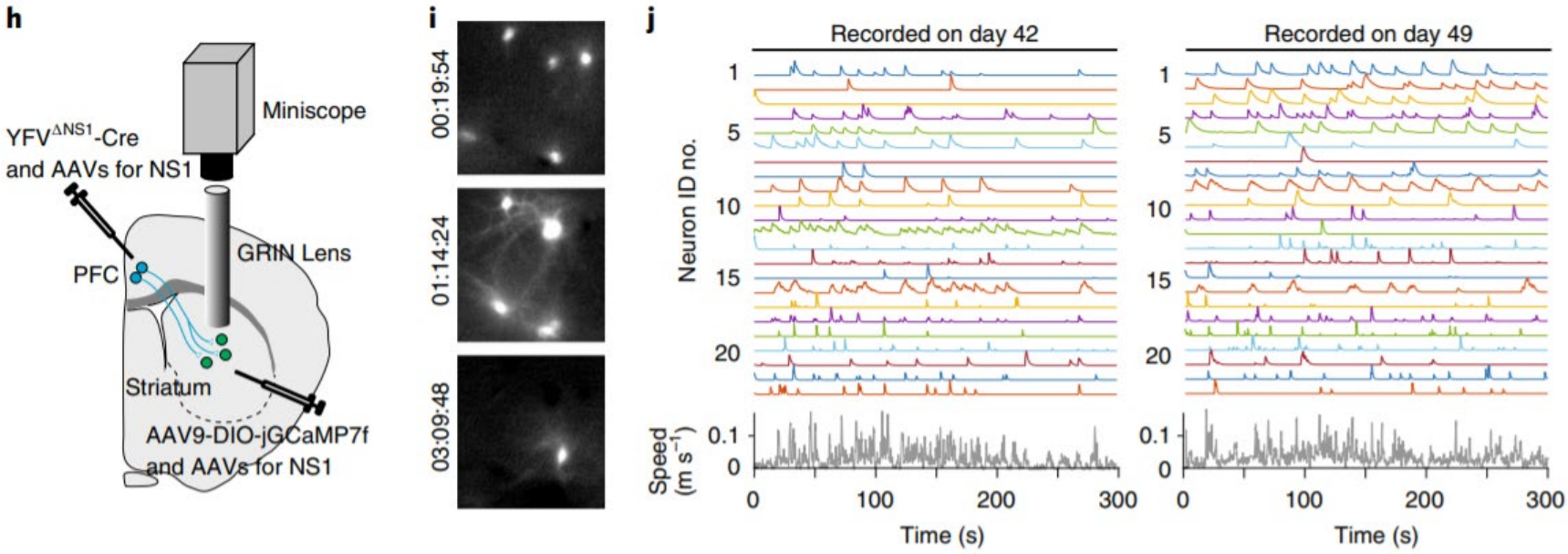
- In 11 of the 12 jGCaMP7f-positive cells the synaptic latencies were in the range of 0.3 to 2.2ms, suggesting monosynaptic connections.

Figure 4

Results

Can YFVΔNS1-Cre help to monitor long-term neuronal activity??

They injected YFVΔNS1-Cre into the PFC to turn on the expression of jGCaMP7f in the striatum and conducted calcium imaging in freely moving mice with a miniaturized microscope through a lens implanted in the striatum.



They detected robust neuronal calcium signals.

Most of the neurons identified at 6weeks were detected again at 7weeks.

→ **indicating that YFVΔNS1-Cre can mediate anterograde transneuronal genetic manipulations.**
→ **the transient activation of YFVΔNS1-Cre can lead to long-term expression of reporter genes.**

Figure 4

Results

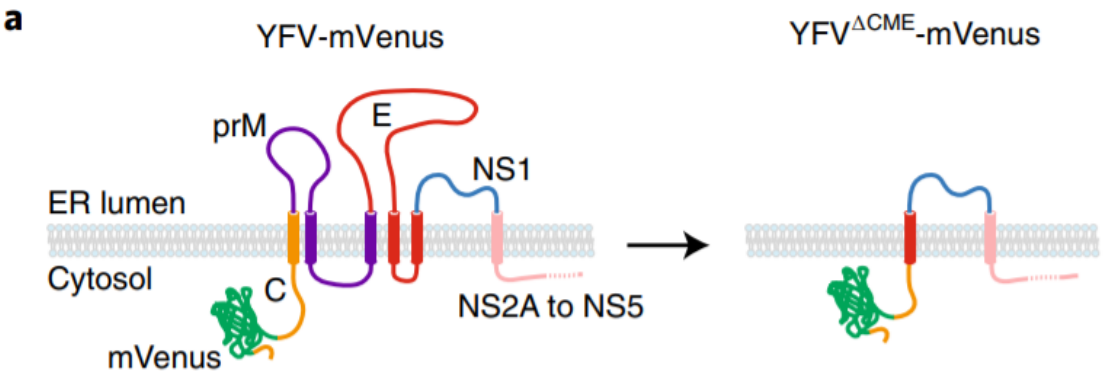
Can YFVΔNS1-Cre help to monitor long-term neuronal activity??

Mapping monosynaptic projectomes with YFV$^{\Delta CME}$

Results

Tracing the whole-brain projection patterns of defined neurons (projectomes)

a) Constructed the packaging-deficient YFVΔCME by removing the structural proteins C, prM and E.



b) They tested this system in vivo and compared it to AAV-SynaptoTAG2.

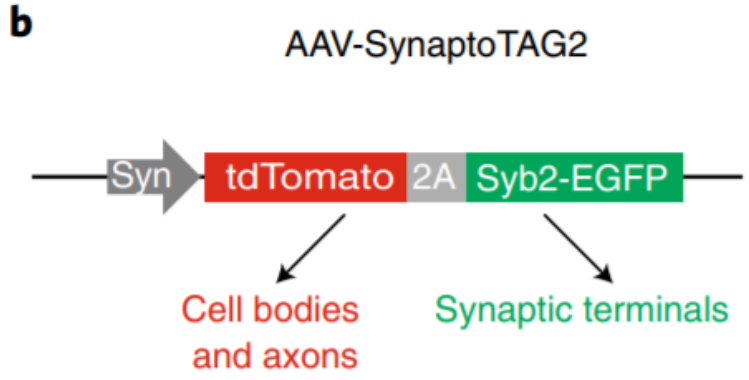


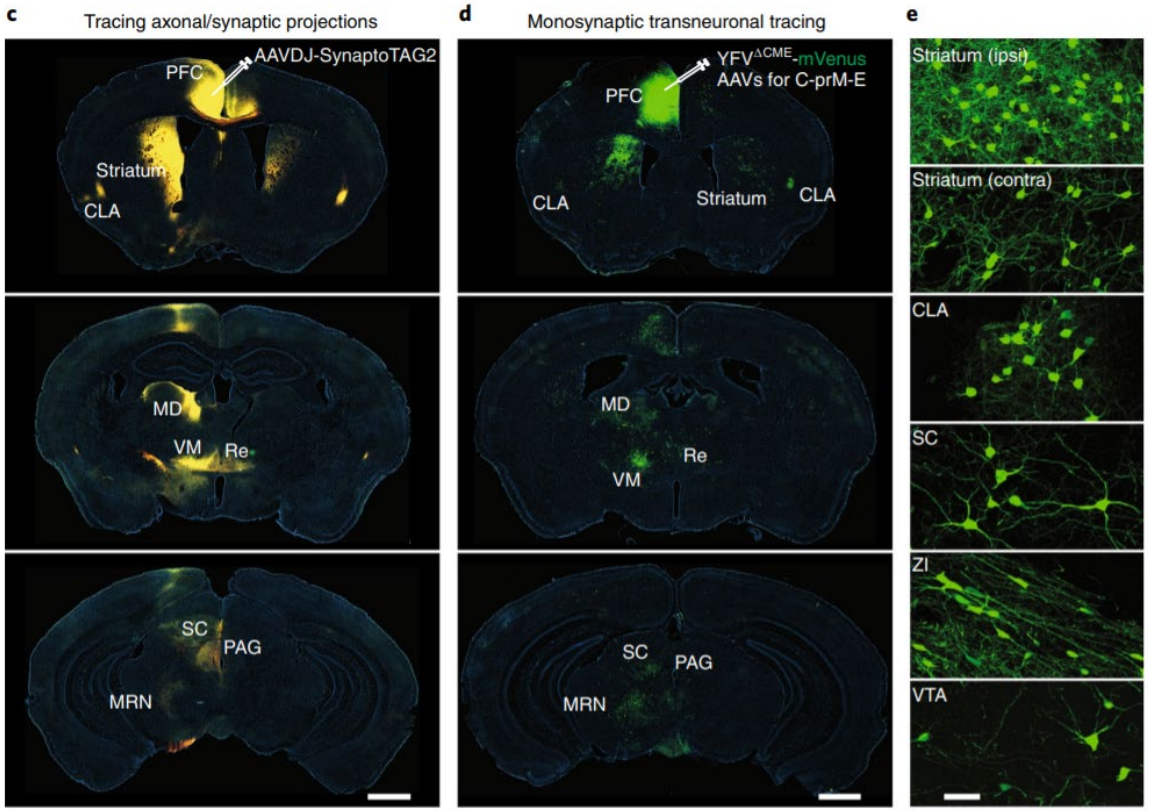
Figure 5

Results

Tracing the whole-brain projection patterns of defined neurons (projectomes)

c) EGFP-positive synaptic terminals in the brain regions downstream of the PFC.

d) We detected mVenus-positive neurons in the brain regions innervated by PFC neurons.



YFVΔCME can replicate in the infected neurons.

Therefore, neuronal projection patterns should be analyzed shortly after injection (in 7–9days).

Figure 5

Monosynaptic transneuronal genetic control with YFVΔCMENS1

Results

Combine the advantages of YFV∆NS1-Cre (minimal neuronal toxicity) and YFV∆CME (monosynaptic spreading)

a) They constructed YFV∆CMENS1-Cre by removing C-prM-E and NS1.

→ When both C-prM-E and NS1 are expressed in starter cells, YFV∆CMENS1-Cre can replicate in the starter cells and propagate to postsynaptic neurons.

b) NS1 provided in the postsynaptic neurons enables the replication of YFV genome to enhance the expression of Cre, but does not allow YFV to spread.

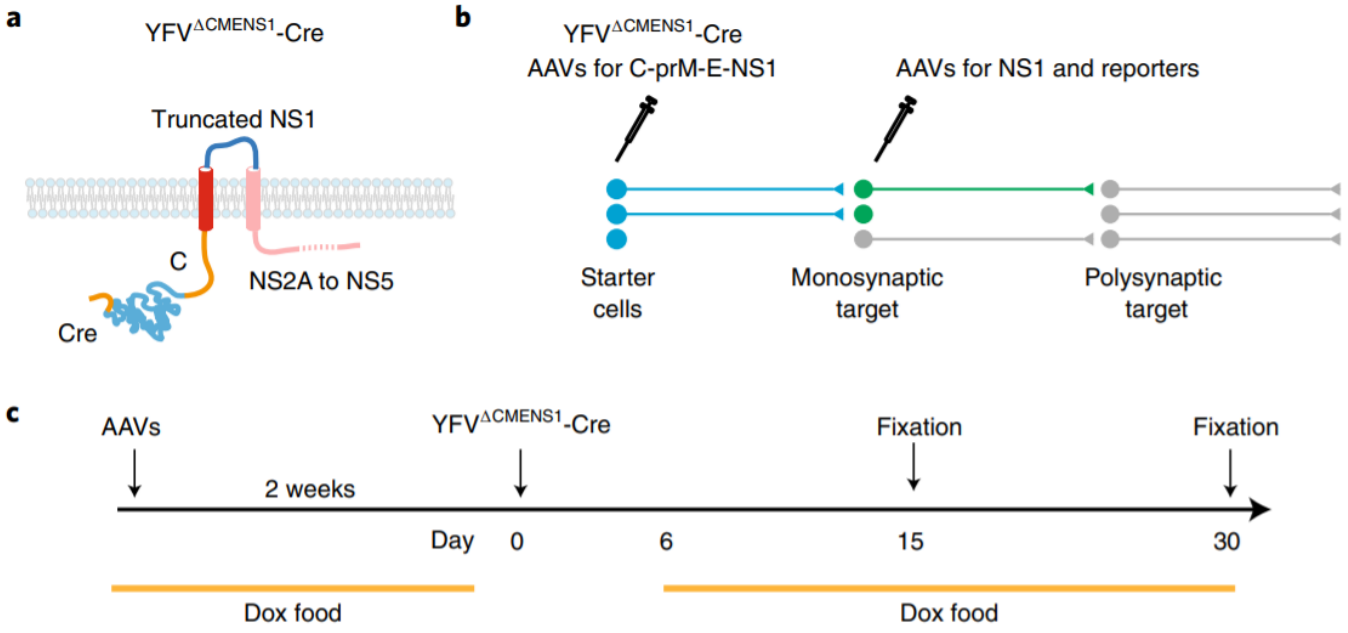


Figure 6

Results

Combine the advantages of YFV∆NS1-Cre (minimal neuronal toxicity) and YFV∆CME (monosynaptic spreading)

(d-f) Tested in the PFC-striatum pathway, YFV∆CMENS1-Cre induced genetic modifications transneuronally to turn on the expression of reporters in postsynaptic neurons in the striatum.

g) The density of the traced striatal neurons did not change from 15 to 30 days, suggesting that YFV∆CMENS1-Cre did not cause neuronal loss (Fig. 6g).

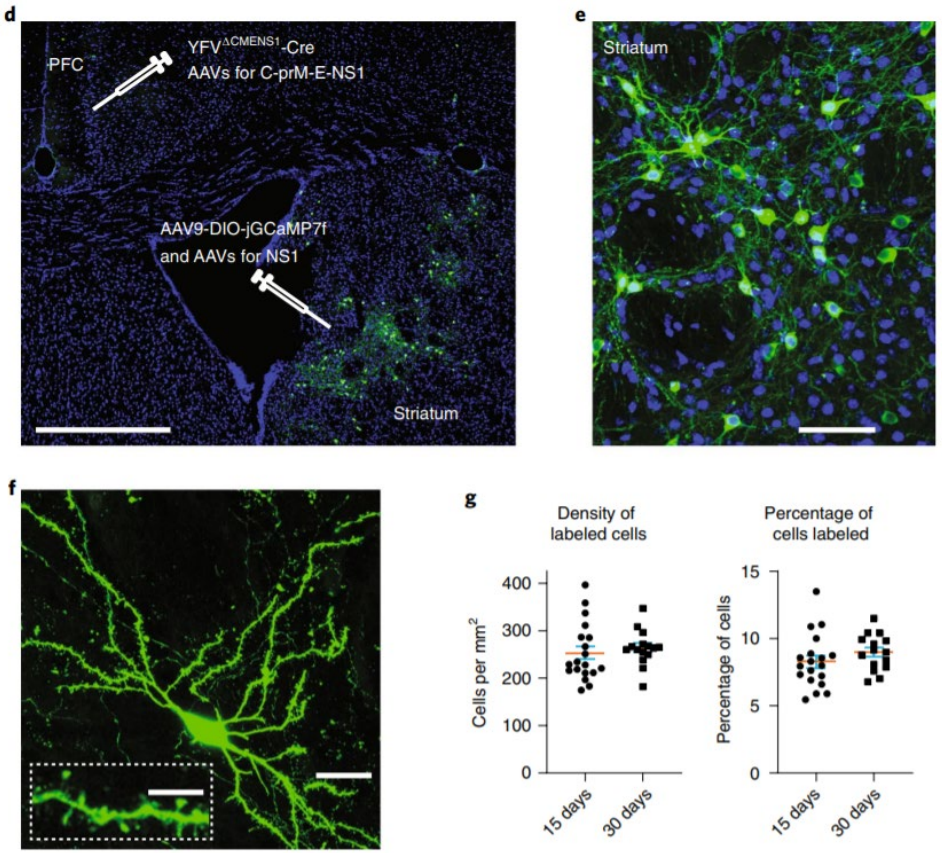


Figure 6

Discussion

- Transneuronal viruses are useful tools because they can both carry various genes across synapses and self-amplify the signal.

- They identified YFV-17D as a primarily anterograde transneuronal virus and used two transcomplementation strategies to improve its performance.

- Deletion of the NS1 gene in YFVΔNS1 targeted the viral replication process, and achieved **anterograde-only tracing** and **minimized neuronal toxicity.**

- Since NS1 is needed in postsynaptic neurons for efficient tracing or genetic manipulation, this system is ideal for **studying the interactions between two chosen brain regions** or two groups of neurons.

- Deletion of the genes for structural proteins in YFVΔCME is ideal **for mapping monosynaptic projections** at the whole-brain scale. → However, neuronal toxicity may limit its applications in functional analyses.

- Combination of strategies by generating YFVΔCMENS1-Cre to achieve monosynaptic transneuronal genetic modifications.

- Synapse specificity of YFV17D in transneuronal transport.

The engineered YFV-17D will be developed into tools for broader applications in research or even in clinical treatments of neuropsychiatric disorders

Paper #2

ARTICLES
https://doi.org/10.1038/s41592-021-01278-1

nature methods

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Joint single-cell measurements of nuclear proteins and RNA in vivo

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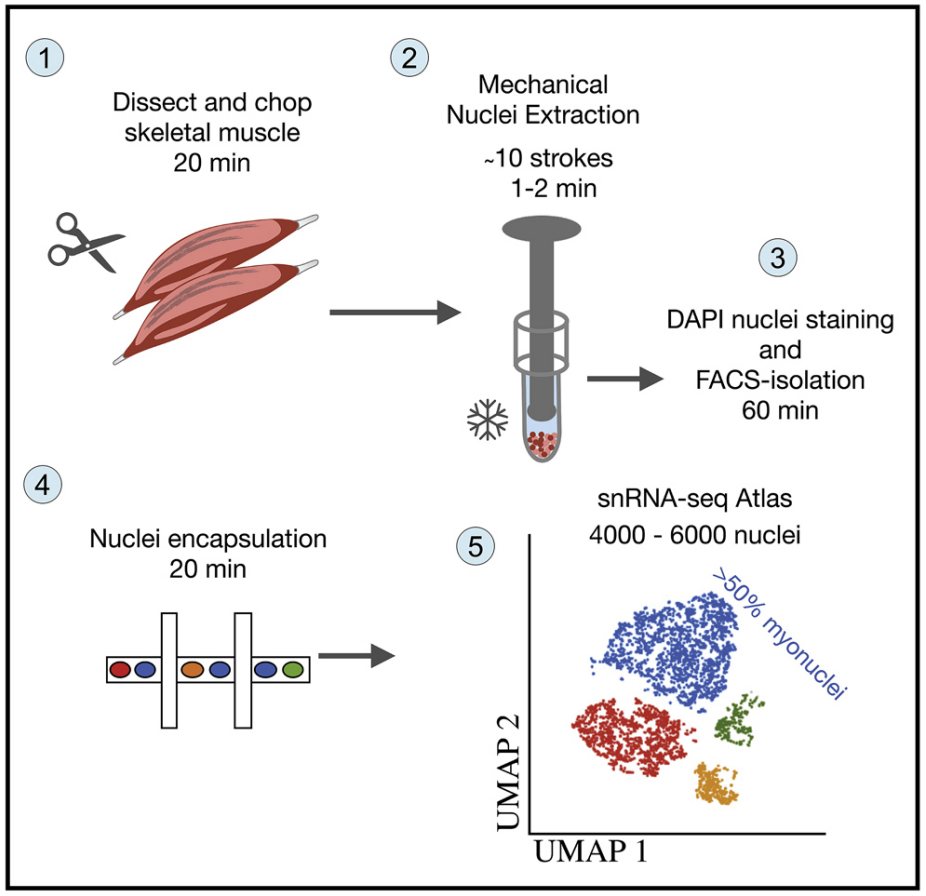
Single-nucleus (sn)RNA-seq

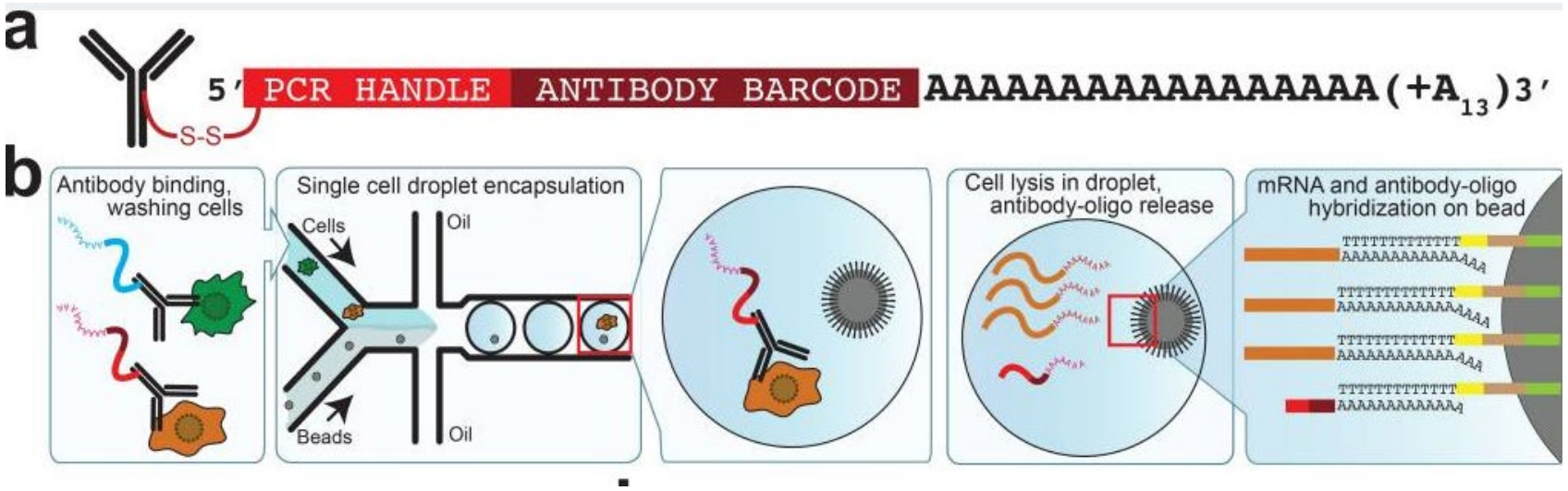
The nucleus is also a key site of gene regulation by a wide array of proteins, the presence and levels of which shape gene expression.

→ Nuclear concentrations of TFs are mechanistic determinants of gene expression that influence the dynamics of TF target binding



Simultaneously measuring quantitative protein levels and the transcriptome inside individual nuclei enables integrating rich phenotypic and genomic information in tissues.

Methods to jointly measure surface protein levels and RNA at single-cell resolution



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CITE-seq enables simultaneous detection of single cell transcriptomes and protein markers

These methods are less suited for non-immune cells and solid tissues where dissociation disrupts the integrity of cellular membranes

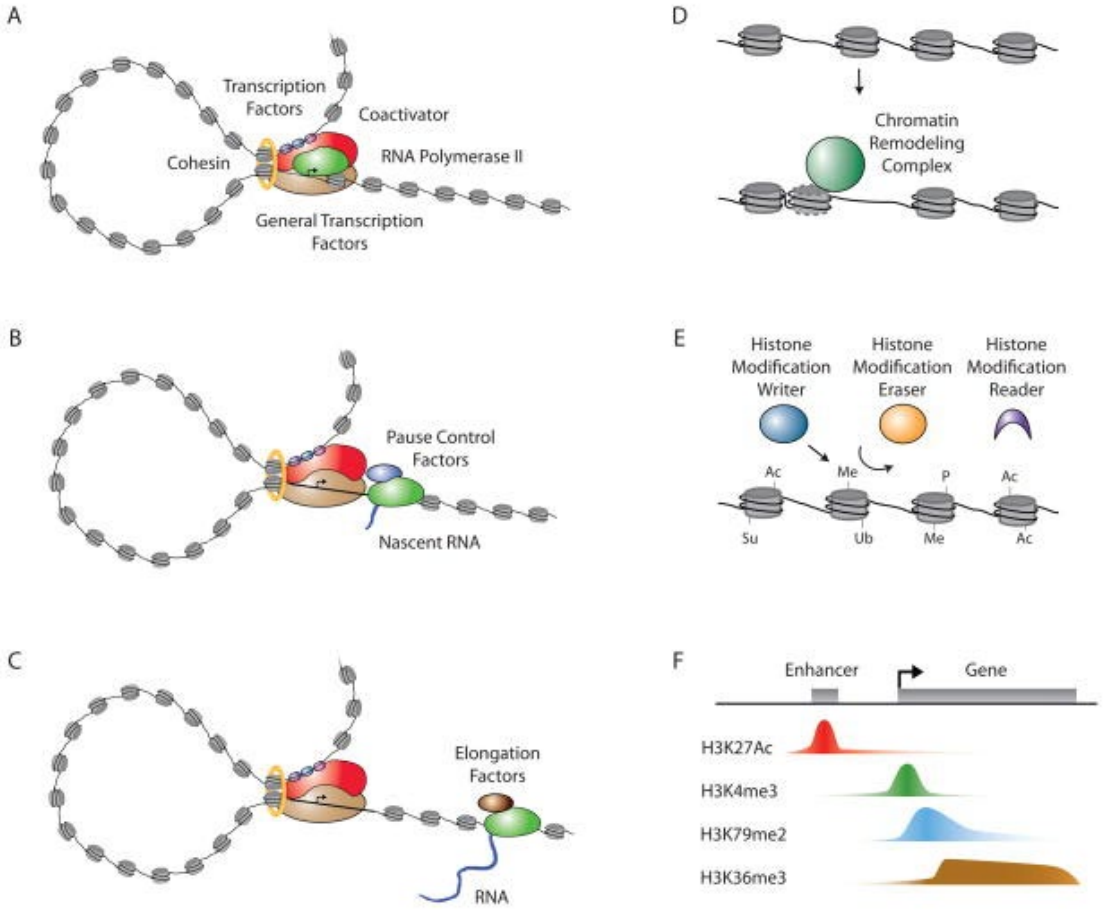
<u>Challenge to quantitatively measure protein levels with the transcriptome in individual nuclei in solid tissue</u>

(as DNA-conjugated antibodies are ‘sticky’ inside the nucleus due to ubiquitous nonspecific binding)

Important aspects

Aberrations in nuclear levels of specific TFs can be hallmarks of disease and can even be used to predict patient outcomes.

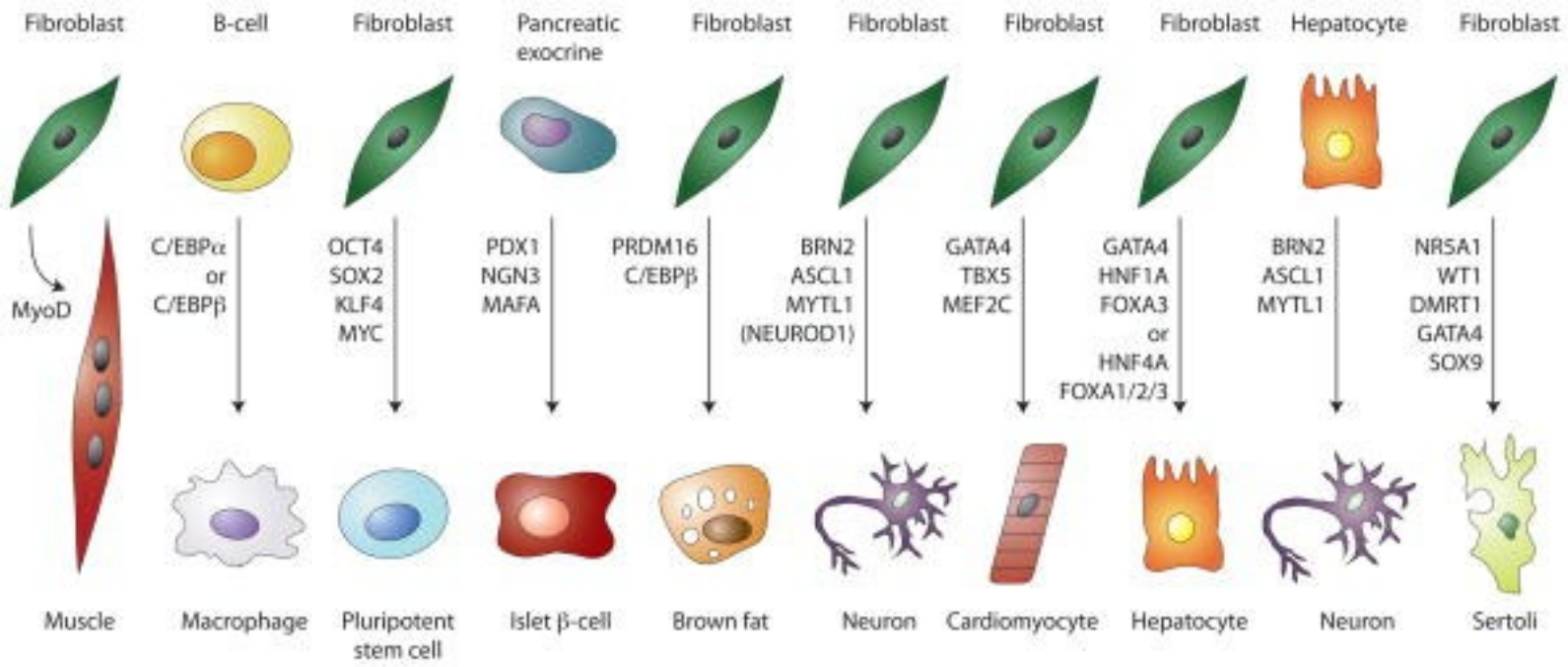
Simultaneously measuring nuclear proteins and the transcriptome in single cells would enable relating levels of nuclear proteins and newly transcribed RNA to:
→ reveal genes and pathways involved in cell state changes
→ and how gene networks regulated by TFs vary across contexts and in disease.



Important aspects

Master transcriptional regulators and reprogramming factors Transcription factors:

- dominant roles in the control of specific cell states
- capable of reprogramming cell states when ectopically expressed in various cell types

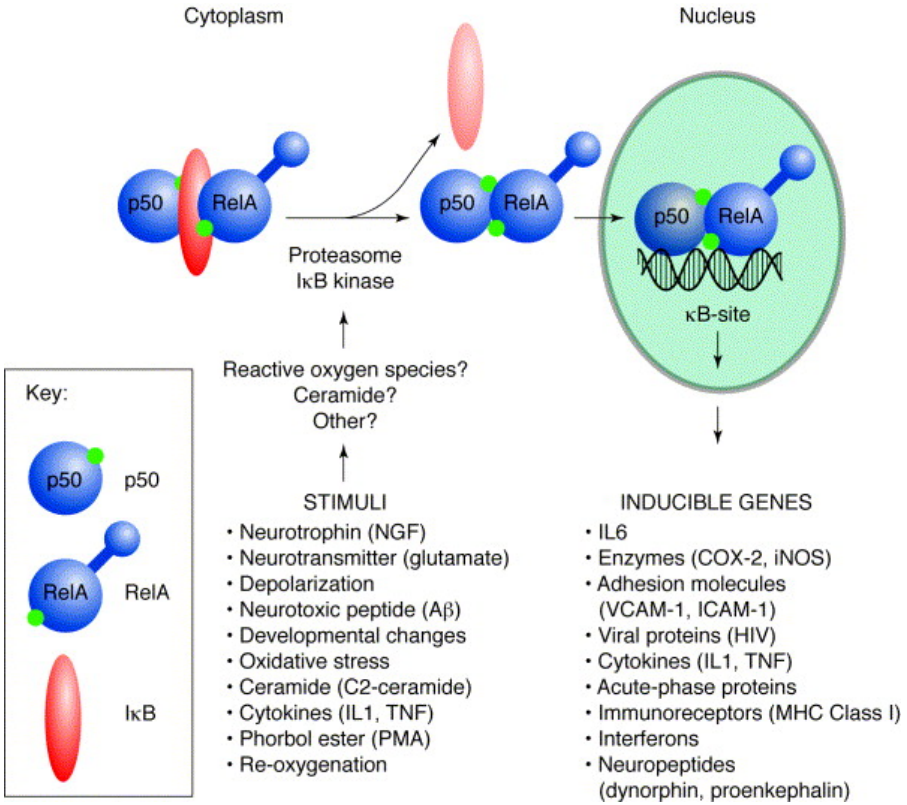


Important aspects

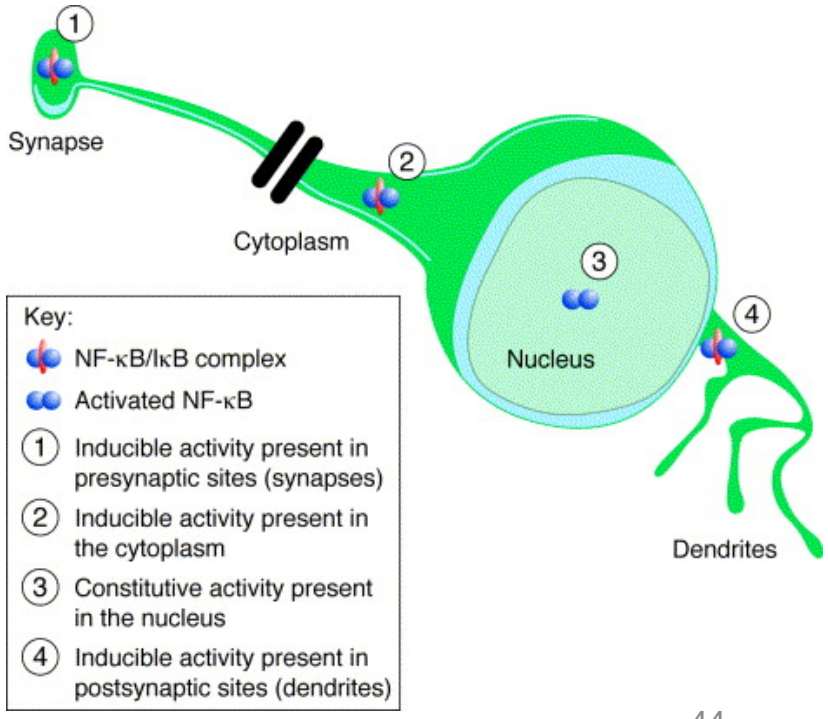
The activity-regulated TF complexes nuclear factor (NF)-κB and activator protein (AP)-1 and their components **p65** and **c-Fos** transiently localize to the nucleus downstream of signal transduction

→they regulate diverse pathways related to inflammation, oncogenesis, apoptosis, cell proliferation and synaptic remodeling

Diverse stimuli activate NF-kB in brain cells, leading to the expression of a range of genes.



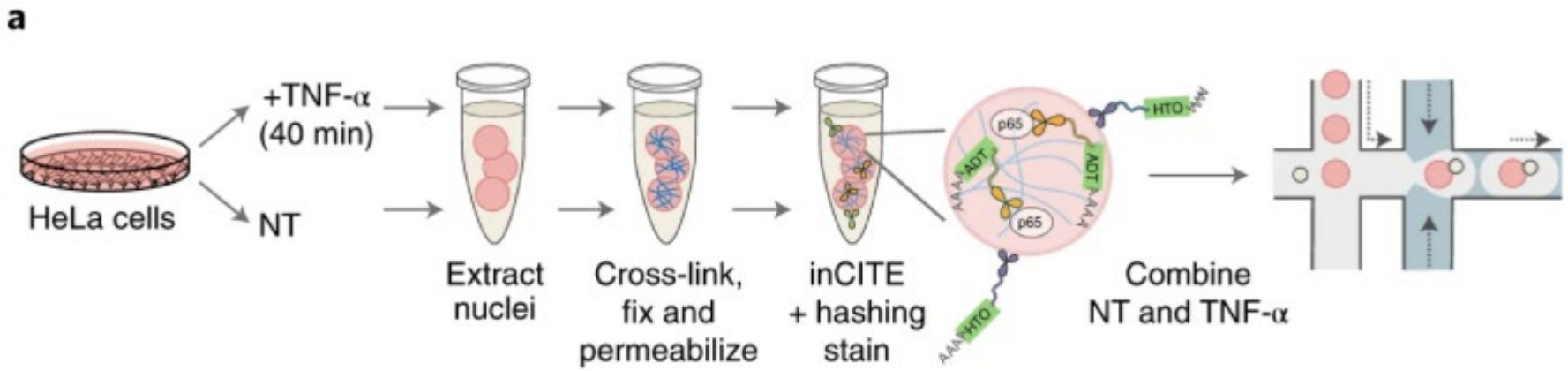
Inducible and constitutive NF-kB occur in neurons.



Current methods to monitor nuclear TF levels and gene expression

1. Live cell imaging
2. In situ measurement of protein and RNA levels in tissue by staining and hybridization
3. Cell sorting and profiling based on fluorescent reporters.

Limited in their ability to relate changes in protein localization to their genome-wide impacts on transcription.



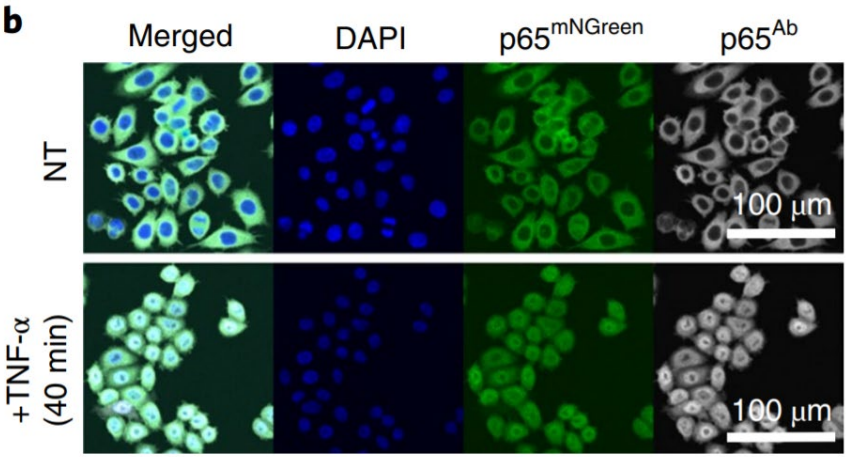
- **inCITE-seq**, a method that enables multiplexed and quantitative intranuclear protein measurements using DNA-conjugated antibodies coupled with RNA-seq on a droplet-based profiling platform.

- inCITE-seq for **profiling the response to environmental stimuli in cells and tissues**

Figure 1a

Results

inCITE-seq detects nuclear translocation of a TF induced by an extracellular signal



Flow cytometry of HeLa nuclei stained with p65inCITE-Ab followed by Alexa Fluor 647 secondary antibody (x axis) sampled from NT (blue) or 40 min after TNF-α treatment (red)

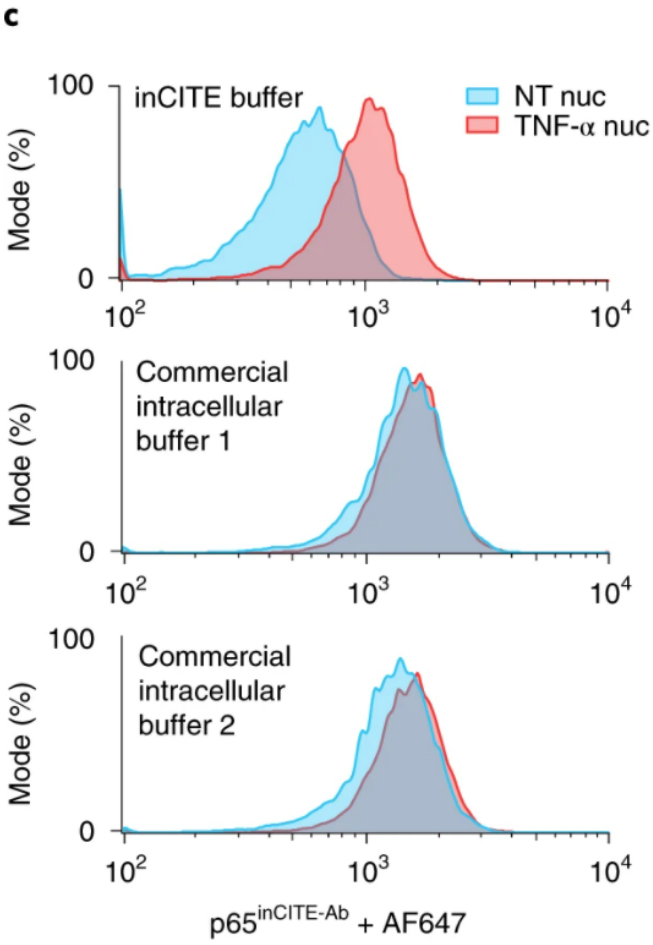


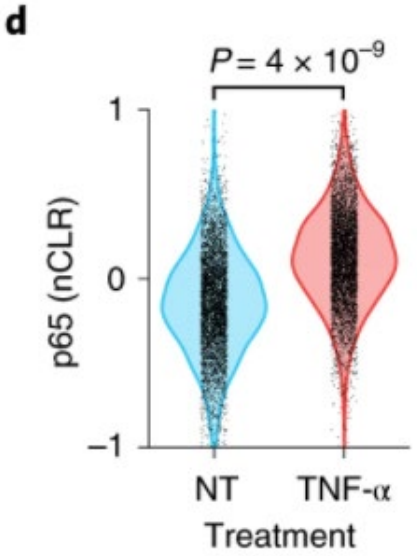
Figure 1

Results

inCITE-seq detects nuclear translocation of a TF induced by an extracellular signal

inCITE-seq to profile 10,014 single nuclei from NT and TNF-α-treated HeLa cells that were stained with **p65 inCITE-Ab** (without sorting) and barcoded for multiplexing with nucleus hashing.

Antibody levels estimated as counts of antibody-derived tags (ADTs) were normalized by:
- counts of the nucleus hashtag (hashtag oligonucleotides (HTOs)) to yield nuclear ADT (nADT) units in order to account for differences in poly-dT capture on beads.



Sequencing-derived levels of nuclear p65 differed significantly across NT and TNF-α-treated populations

Quantitative protein detection by inCITE-seq can distinguish altered cell state due to treatment.

Figure 1

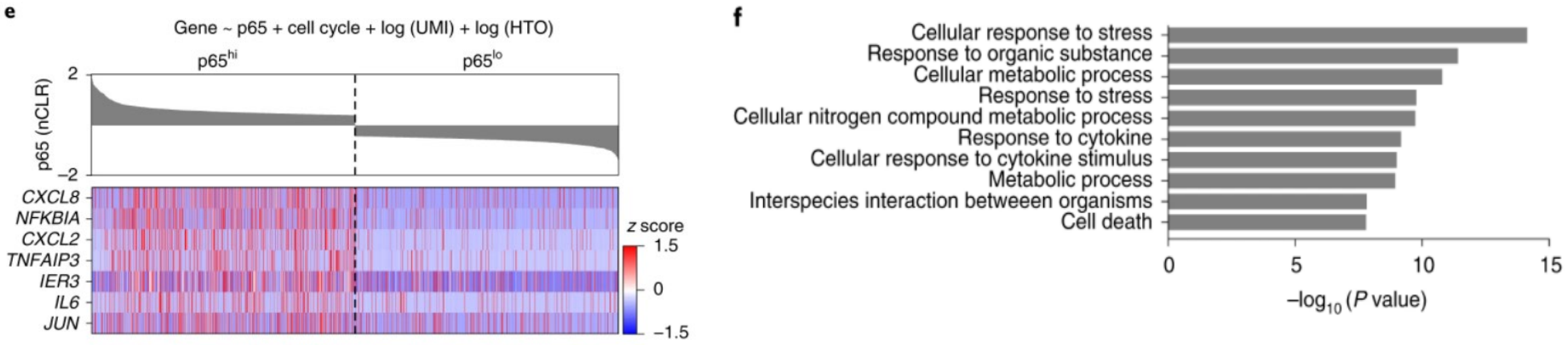
Results

Relating genome-wide expression to p65 protein levels

Identify genes for which RNA expression was associated with p65 levels:

used a linear model to fit each gene's expression as a function of continuous p65 levels.

→ identified 142 genes positively associated with p65 levels
→ These genes included well-known NF-κB targets CXCL8, NFKBIA and TNFAIP3 (Fig. 1e), and were enriched for pathways such as cytokine response



It supports prior studies showing fast mRNA degradation, but slow protein-degradation kinetics→ importance of obtaining protein measurements.

inCITE-seq accurately quantifies nuclear protein and RNA levels that can be integrated to identify putative targets of a TF.

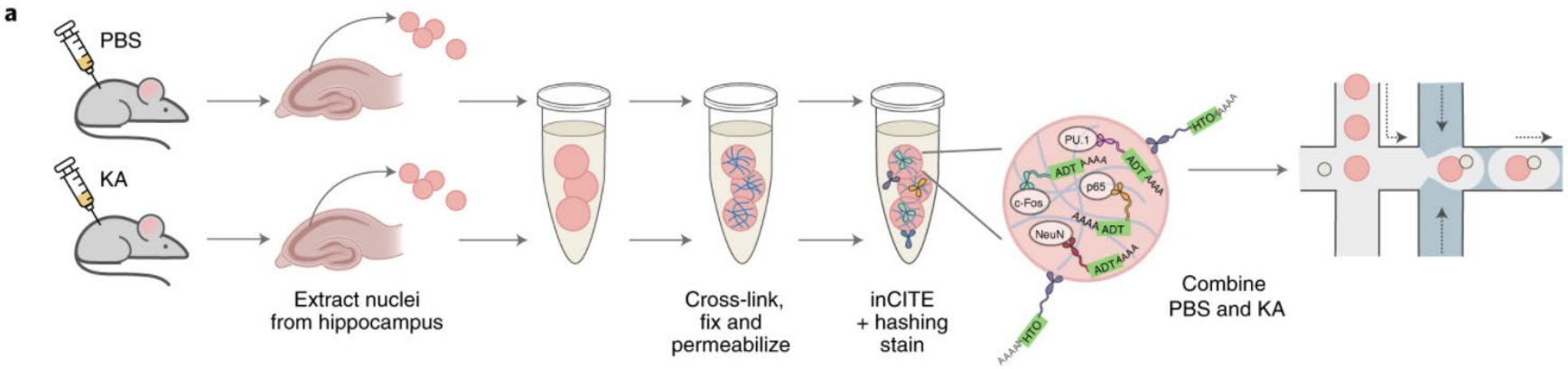
Figure 1

Results

inCITE-seq profiling of the mouse brain after in vivo induction of seizure

In vivo inCITE-seq to characterize the mouse hippocampus during the early response to seizure:

inCITE-seq to profile single nuclei from the hippocampus 2 h after KA treatment with multiplexed measurements of the proteins p65, c-Fos, NeuN and PU.1



→ NF-κB and AP-1 are involved in neuroinflammation and oxidative stress, affecting multiple cell types and pathways.

- Seizures were induced by treatment with kainic acid (KA), a glutamatergic agonist used in models of temporal lobe epilepsy.
- They characterize how the components p65 and c-Fos of the complexes NF-κB and AP-1 are linked to gene expression in early response to seizure.

Figure 2

Results

RNA profiles from inCITE-seq reveal key cell subsets of the mouse hippocampus

Unsupervised clustering with variable genes identified jointly across 37,767 high-quality nuclei showed 16 well-delineated clusters.

Robust mixing across batches and treatment, and well annotated post hoc using known cell type markers.

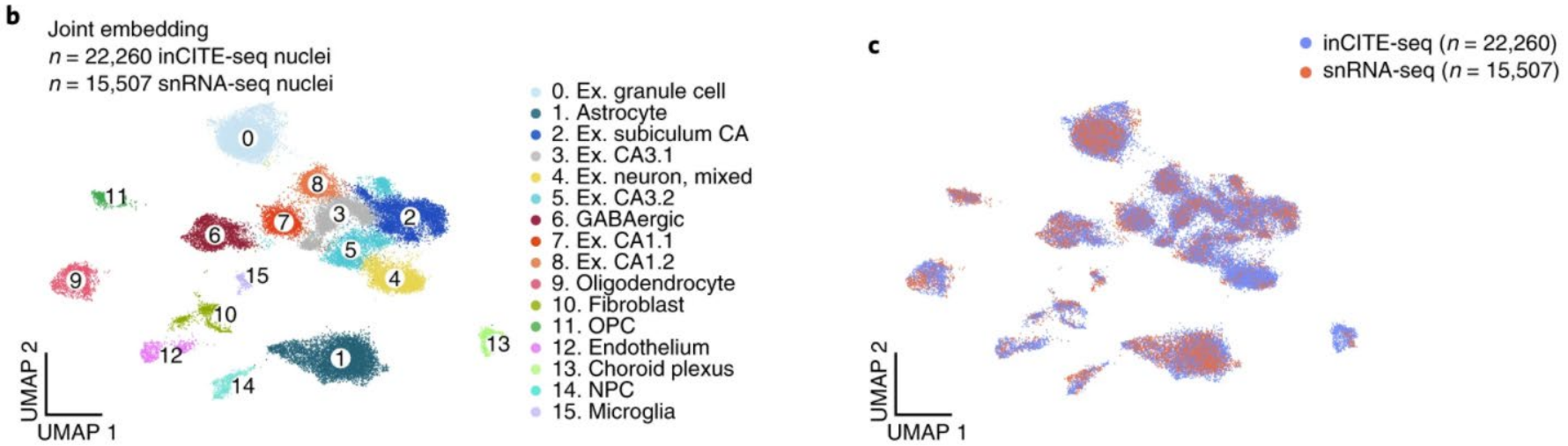
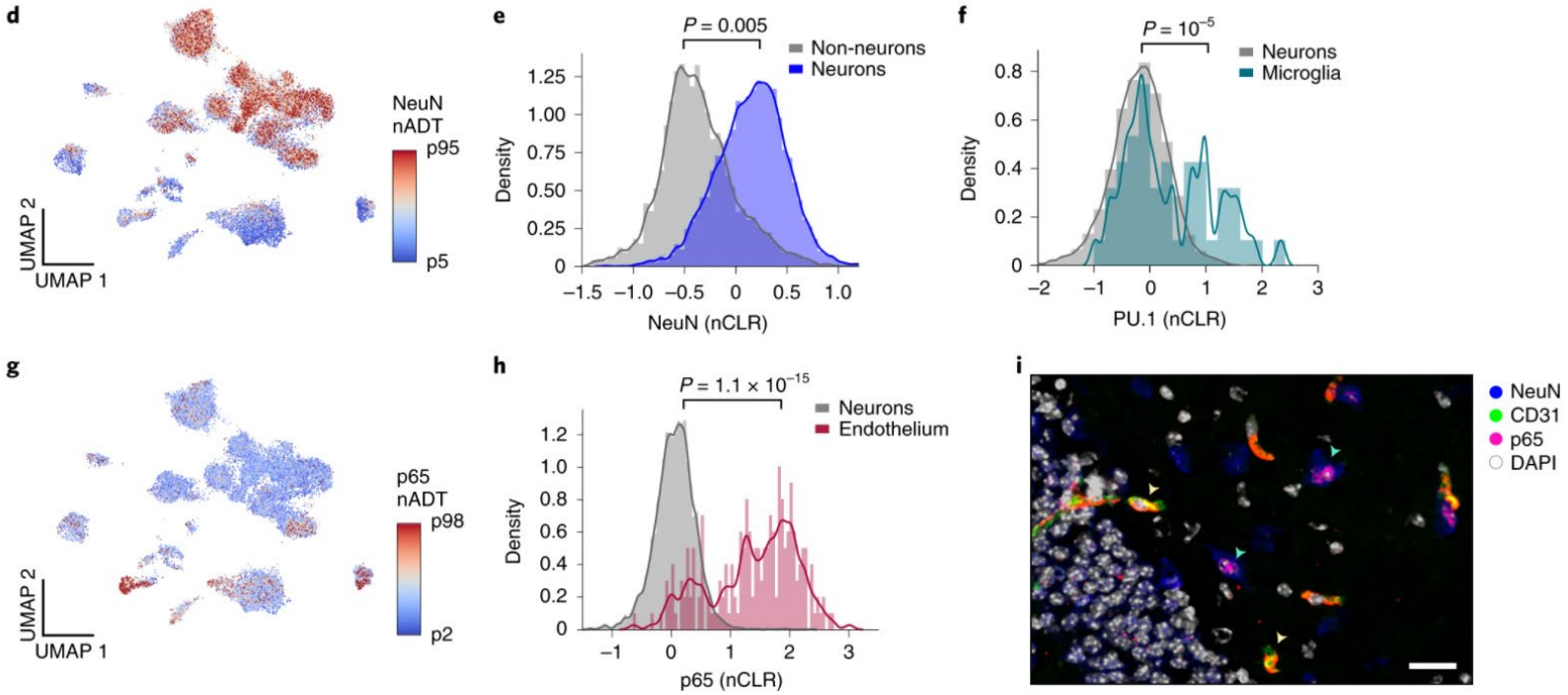


Figure 2

Results

Protein levels match cell type-specific and condition-specific expression in RNA-based clusters

Nuclear protein levels measured by inCITE-seq differed across RNA-defined cell types as expected.



- NeuN levels were elevated in neuronal clusters as expected
- Levels of PU.1, the microglial marker and a lineage-specifying TF, were significantly higher in microglia than those in neurons.
- Expression of p65 was enriched in endothelial nuclei

Figure 2

Results

Protein levels match cell type-specific and condition-specific expression in RNA-based clusters

a) Widespread expression of inCITE-seq-derived nuclear c-Fos.
b) Significant upregulation in neurons after KA treatment.
c) Subsets of neurons differed in c-Fos levels, such that nuclei from cornu ammonis (CA) neurons had lower levels than those from granule cells of the dentate gyrus (DG).
d) By contrast, p65 levels did not change after KA treatment at this time scale.
e) These patterns were confirmed by immunofluorescence, showing that c-Fos is expressed in multiple neuronal types, including DG granule cells, CA neurons and somatostatin (SST+) interneurons, with higher c-Fos intensity in granule cells than that in CA neurons.

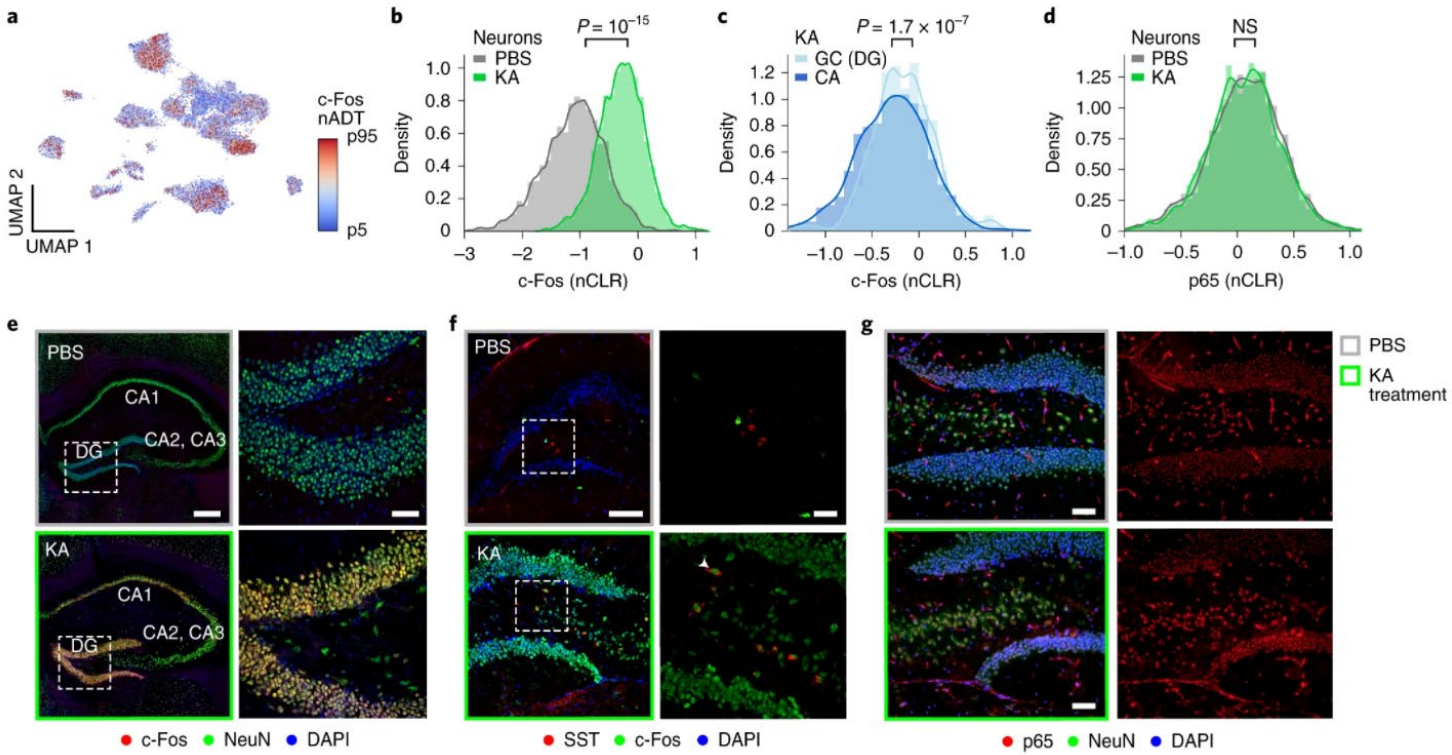


Figure 3

Results

Protein levels match cell type-specific and condition-specific expression in RNA-based clusters

Overall, inCITE-seq quantitatively measured nuclear protein levels that reflected diverse levels of activity-regulated TFs across cell types and treatment.

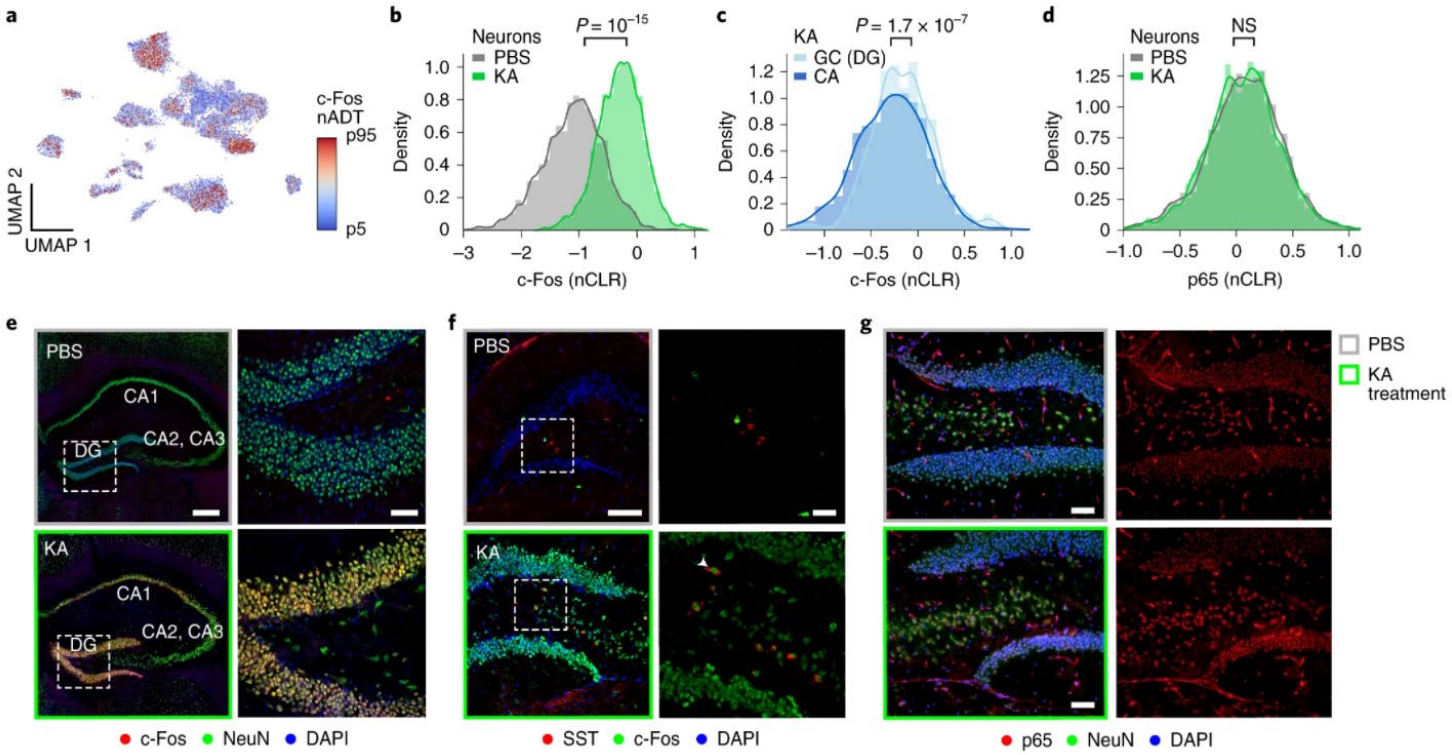


Figure 3

Results

Relating protein and mRNA levels of inCITE target genes

Compared transcript and protein levels across nuclei with ‘high’ or ‘low’ protein levels and assessed how treatment impacted their relationship.

- Transcript levels of the activity-regulated gene Fos were elevated in populations with high protein levels (c-Fos-hi) compared to those in c-Fos-lo populations, but only after PBS treatment

→ whereas Fos mRNA was highly expressed after KA treatment regardless of c-Fos protein levels.

- By contrast, NeuN and its encoding transcript Rbfox3 were inversely.

- Rela mRNA levels did not differ across p65 protein levels, and Spi1 transcripts (encoding PU.1) were not detected.

→ underscoring the importance of protein measurements as a complement to RNA measurements, particularly for TFs for which the corresponding RNA is often lowly expressed.

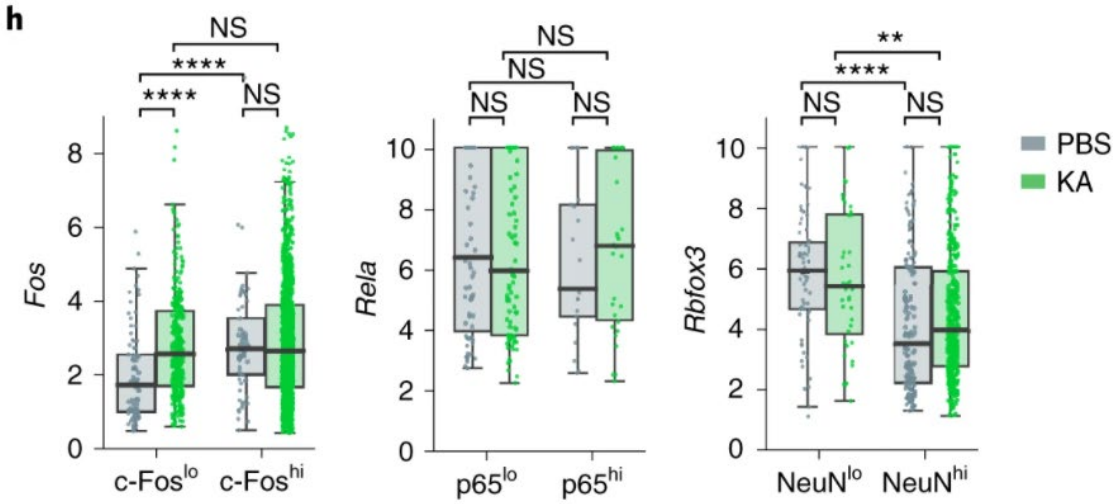


Figure 3

Results

Modeling genome-wide association with each protein recovers known TF targets

Inferred the assumed genome-wide impact of each TF on gene expression based on nuclear protein levels.

- As concentrations of TFs in the nucleus shape gene expression, they modeled gene expression as a function of protein levels.

To first identify global impacts of TFs, we modeled each gene's RNA as a linear combination of the four proteins (c-Fos, p65, PU.1, NeuN) after regressing out contributions of cell type (cluster), treatment and their interaction to account for collinearity.

Genes significantly associated with each of the three TFs were interpreted as putative TF-regulated genes.

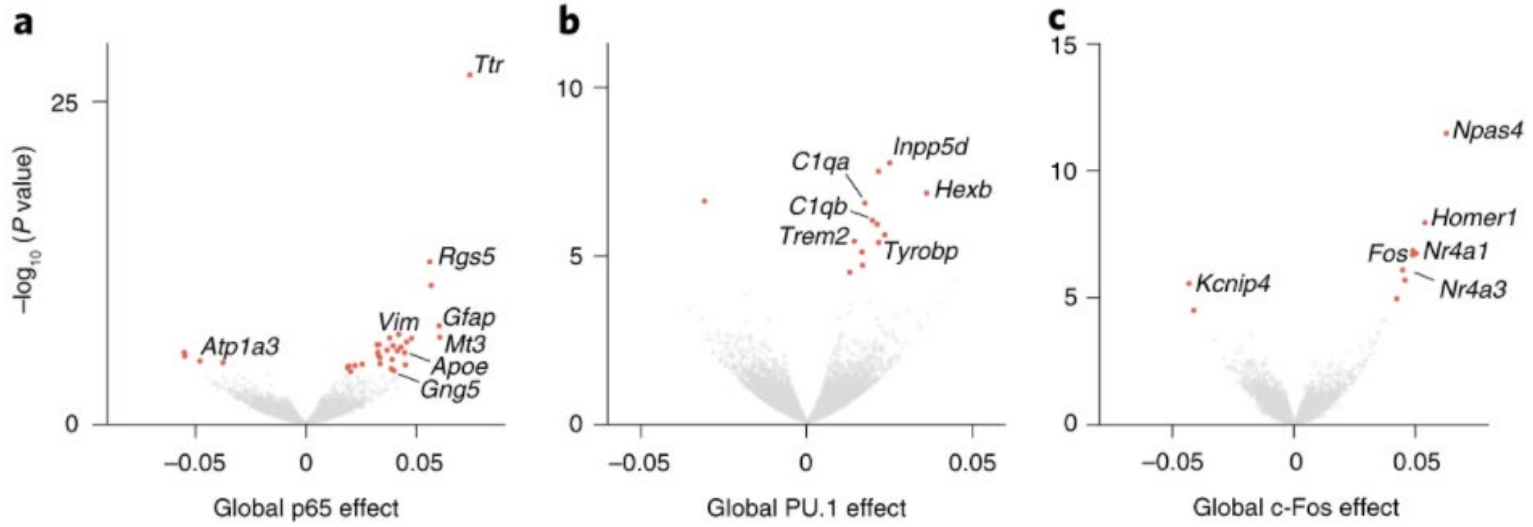
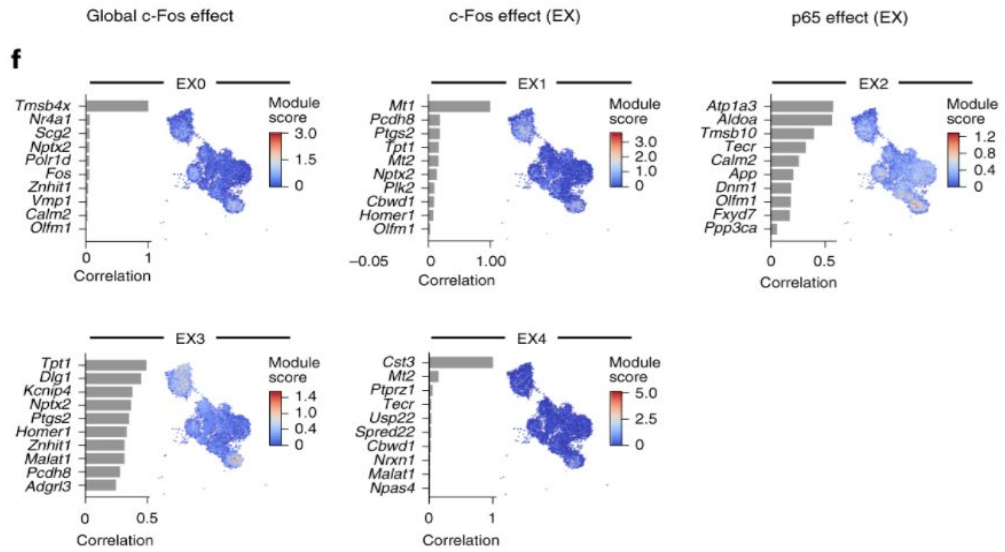
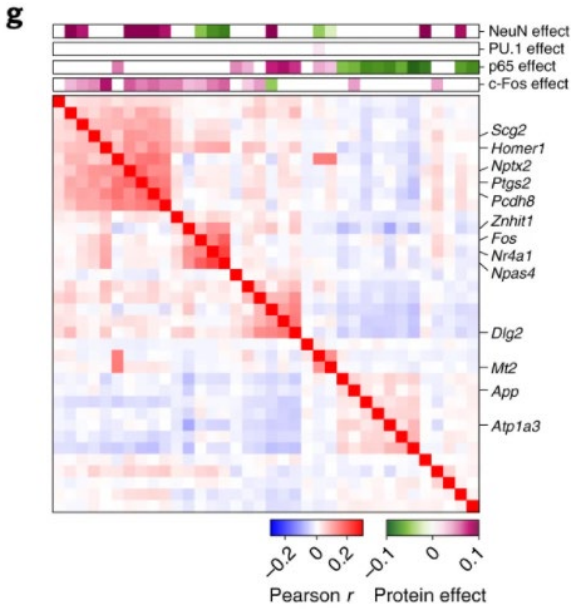


Figure 4

Results

Modeling genome-wide association with each protein recovers known TF targets





Gene programs that are normally identified from expression alone, align with protein effects?

Using non-negative matrix factorization (NMF) on RNA profiles alone, they identified five gene programs in EX neurons

Coexpression patterns of the top ten genes of each program revealed that each program also coincided with the different types of TF effects.

Figure 4

Results

Modeling genome-wide association with each protein recovers known TF targets

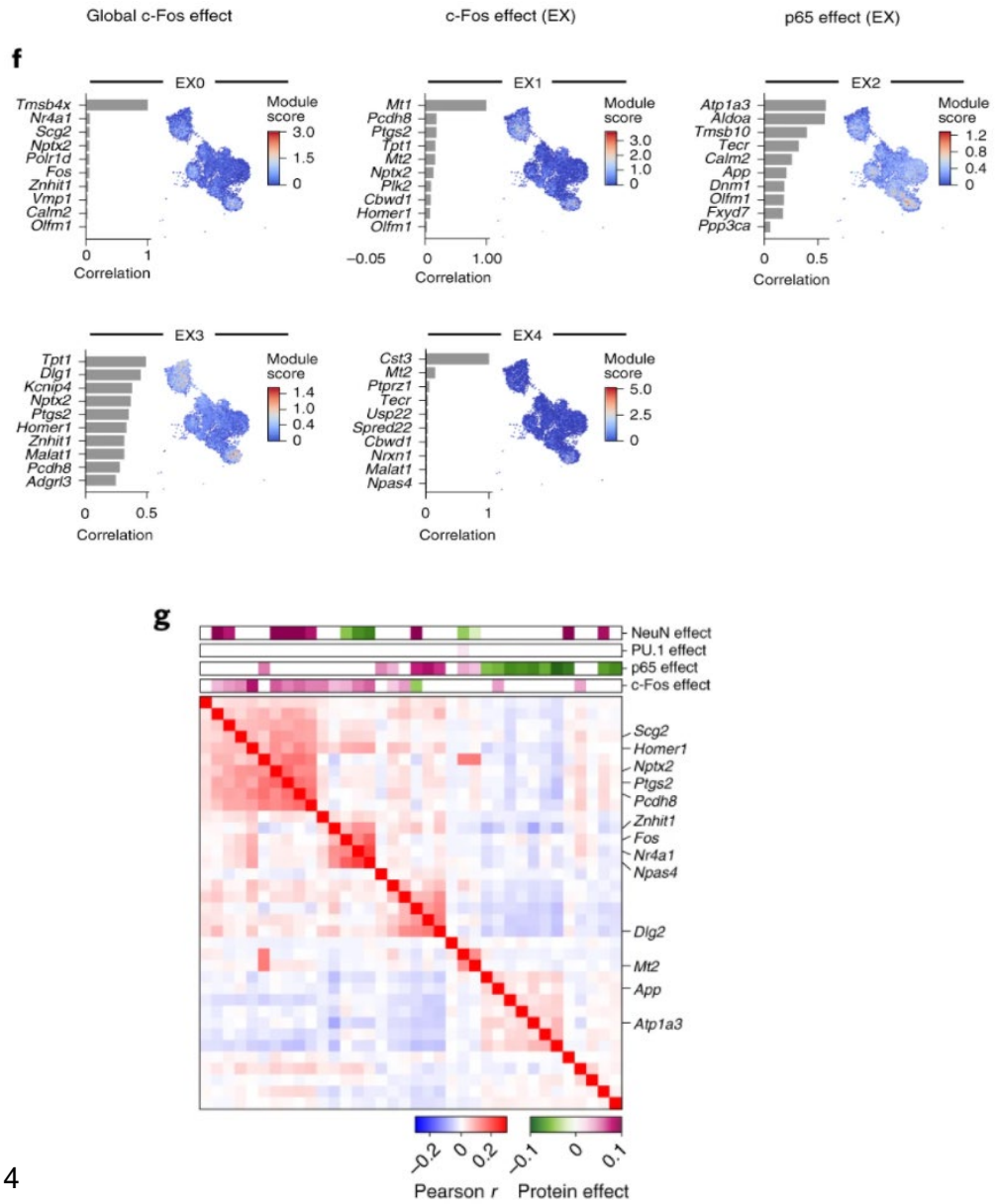


Figure 4

Their approach allows quantifying the associations between TFs and gene expression modules or pathways.

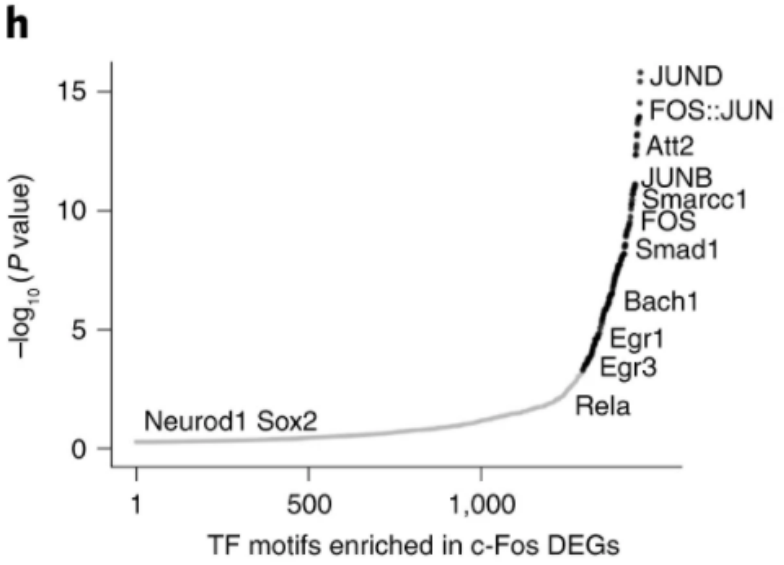
Unrevealing contributions of TF combinations, which can increase the interpretability of gene expression programs.

Results

Modeling genome-wide association with each protein recovers known TF targets

- **Assess whether each TF could play a direct or indirect role in regulating their associated DEGs**
- **Identify other TFs that may be involved in co-regulation**

They analyzed the cis regulatory TF motifs enriched in the enhancer regions of c-Fos-associated and p65-associated genes.



Using enhancers defined by differentially accessible regions (DARs) that were profiled in the hippocampus of saline- or KA-treated mice, we identified TF motifs significantly enriched each gene set.

Observed TF motifs included components of the AP-1 complex (Fos, JunB and JunD), activity-regulated TFs Egr1 and Egr3, and Atf2 which is phosphorylated by c-Jun in KA-induced seizure models.

Notably, motifs of NF-κB components (encoded by Rel, Rela, Relb, Nfkb1, Nfkb2) were not significant. This suggests that TF-associated genes in their analysis may also reflect indirect effects of each TF, or that direct effects occur at a different time scale in the case of p65, or depend on the direction of the TF effect (that is, downregulated versus upregulated).

Figure 4

Results

Inferred TF impact on genes depends on treatment context and cell type

Is TF impact on genome-wide expression depended on the treatment context?

To assess the global treatment impact, they modeled RNA levels as a linear combination of the four proteins separately within each treatment (PBS or KA) and compared their effects on each gene across treatments.

- The impact of p65 on gene expression was largely consistent across PBS and KA treatments.
- By contrast, c-Fos effects varied by treatment.
- Genes associated with c-Fos only upon KA treatment included Ptgs2 (encoding cyclooxygenase 2, COX-2), a responder to oxidative stress after traumatic brain injury.
- Cell type-specific TF effects upon KA treatment can be identified.

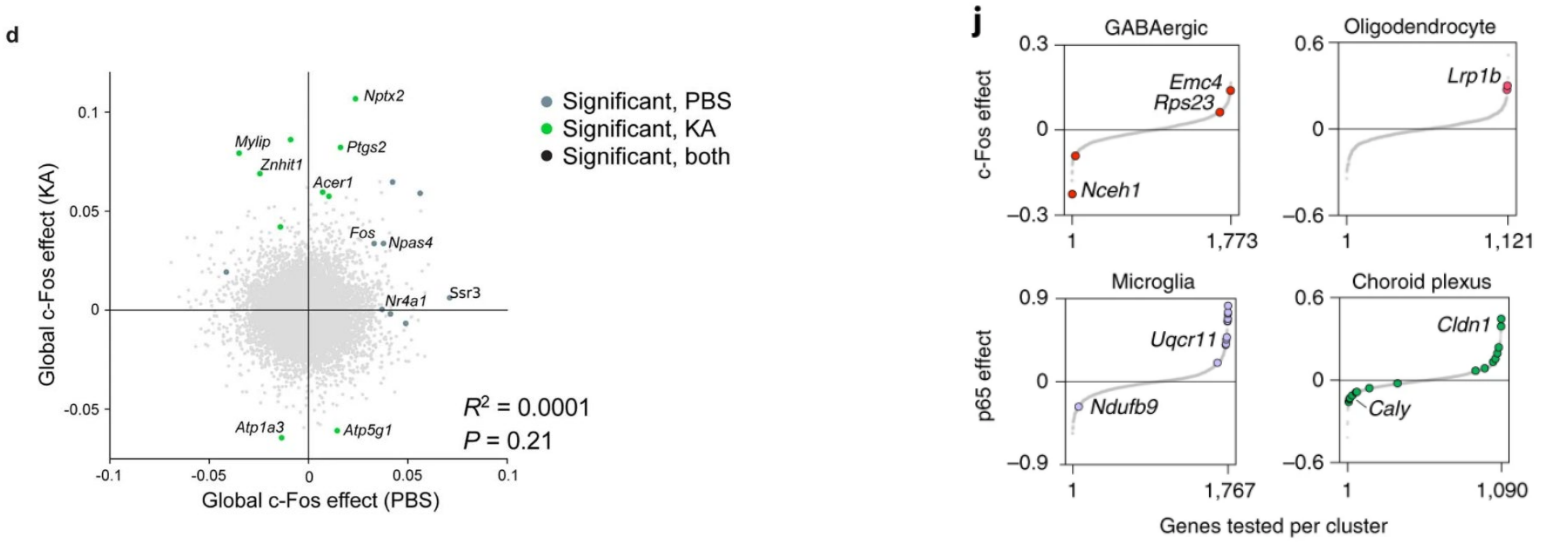


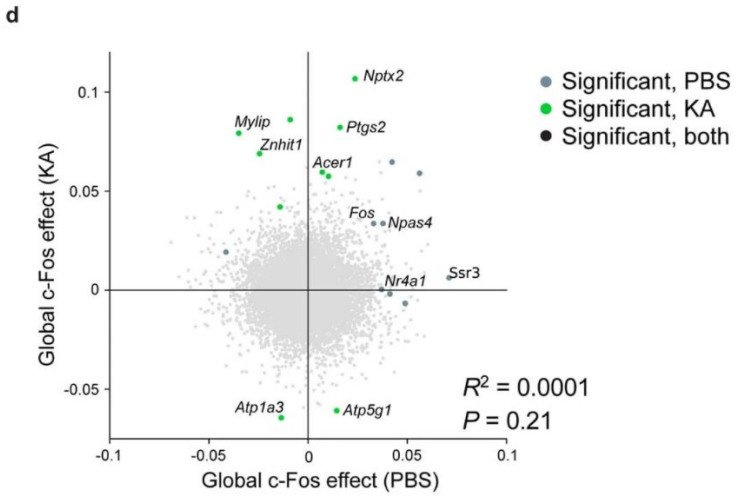
Figure 4 and Extended data figure 9

Results

Inferred TF impact on genes depends on treatment context and cell type

Is TF impact on genome-wide expression depended on the treatment context?

Direct or indirect regulatory impact of TFs on individual genes can depend on cell type and environmental context.



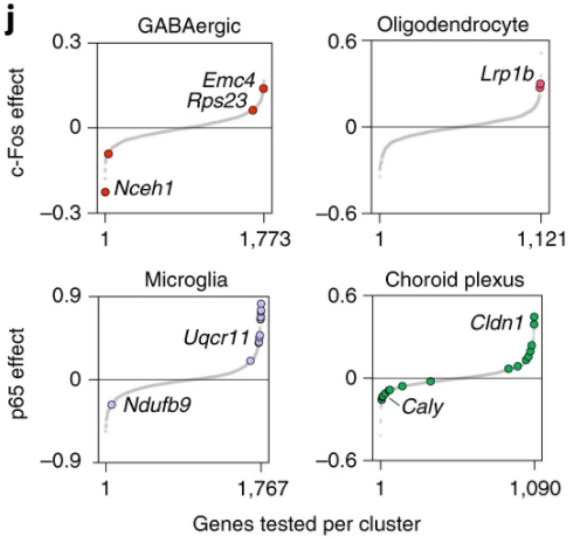


Figure 4 and Extended data figure 9

Discussion

- inCITE-seq reliably measures **quantitative protein and RNA levels in individual nuclei**.
- Nuclear protein levels can be used as an interpretable and mechanistic link **bridging regulatory proteins and their genome-wide effects**, and enable cell type-specific studies of signaling pathways in complex tissues in vivo.
- While numerous multimodal methods measure intracellular protein targets, **proteins in the cytoplasm carry different information than those in the nucleus**, as in the case of regulatory proteins and translocating TFs.
- Furthermore, nucleus-based multimodal profiling surmounts key technical challenges to enable **characterization of cells from solid tissues** that are either difficult to dissociate or archived in frozen form, especially clinical specimens from human disease studies such as cancer and neurodegeneration.
- inCITE-seq is particularly well suited for **studying proteins in pathways that are affected by cellular dissociation protocols**, for example, the activity-regulated TF c-Fos.

Discussion

- For refined **understanding of gene regulation in tissues during dynamic response**, inCITE-seq can be applied with antibodies targeting phosphorylated forms of TFs in samples collected across time.

- Future studies can **combine inCITE-seq with other modalities**, such as metabolic labeling and joint RNA and chromatin accessibility profiles.

- By **measuring TFs simultaneously**, inCITE-seq opens the way to decipher **complex phenotypes and regulatory mechanisms in development** when TF combinations are key to defining cell type diversity or to disentangle interacting pathways.

- Multiplexed profiling of signaling proteins will **enable deciphering changes in activity states**, which could be used to recover the impact of ligands acting on multiple receptors across cell types in tissues.

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Thank you for your attention!