Cell type-specific biotin labeling in vivo resolves regional neuronal and astrocyte proteomic differences in mouse brain

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Proteomic profiling of brain cell types using isolation-based strategies pose limitations in resolving cellular phenotypes representative of their native state. We describe a mouse line for cell type-specific expression of biotin ligase TurboID, for in vivo biotinylation of proteins. Using adenoviral and transgenic approaches to label neurons, we show robust protein biotinylation in neuronal soma and axons throughout the brain, allowing quantitation of over 2000 neuron-derived proteins spanning synaptic proteins, transporters, ion channels and disease-relevant druggable targets. Next, we contrast Camk2a-neuron and Aldh1l1-astrocyte proteomes and identify brain region-specific proteomic differences within both cell types, some of which might potentially underlie the selective vulnerability to neurological diseases. Leveraging the cellular specificity of proteomic labeling, we apply an antibody-based approach to uncover differences in neuron and astrocyte-derived signaling phospho-proteins and cytokines. This approach will facilitate the characterization of cell-type specific proteomes in a diverse number of tissues under both physiological and pathological states.
The brain is comprised of distinct cell types including neurons, glia (astrocytes, oligodendrocytes, microglia), and vascular cells (endothelial cells and pericytes). Brain cell types demonstrate complex and nuanced changes in their molecular composition (DNA, mRNA, protein) during physiological processes in development, aging, and in pathologic states. Recent advances in bulk and single cell transcriptomic profiling of healthy and pathogenic states of mouse and human brain have provided numerous new insights into diverse molecular signatures adopted by brain cell types. Specifically, they have highlighted several causal neuronal and glial cellular responses in neurological diseases; however, transcriptomic findings only modestly correlate with protein-level (proteomic) changes. Proteins also undergo post-translational modifications that impact their function and expression profiles quantitatively, temporally, and spatially; a feature not reflected by transcript abundance. Therefore, proteomic characterization of distinct brain cell types can provide important insights into cellular mechanisms of development, aging and neuropathology.

Proteomic profiling of brain cell types requires isolation of intact cells from fresh unfrozen brain, a pre-requisite that is not necessary for single-nucleus transcriptomic studies of intact nuclei from frozen brain. There are numerous methods by which relatively pure, live cells of interest can be isolated with minimal contamination and high sensitivity. For instance, magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS) have been used to isolate one or more brain cell type for mass proteomic labeling. In vivo proteomic labeling has been achieved by proximity-dependent protein labeling approaches using an engineered biotin ligase, such as TurboID, or ascorbate peroxidase, APEX, where tagging (BONCAT) where biotinylated proteins from total brain homogenates and captured axons in the mouse brain without histological or electrophysiological abnormalities. We successfully enriched these biotinylated proteins from total brain homogenates and captured neuronal and astrocyte proteomes (>2,000 proteins in each cell type) by MS and identified >200 proteins that differentiated neurons and astrocytes. Furthermore, we were able to resolve unique proteomic signatures of Camk2a neurons and Aldh1l1 astrocytes that are brain region-specific and indicative of distinct cellular functions and disease-vulnerability. Next, we complemented our untargeted proteomic approach with a targeted measurement of biotinylated phospho-proteins via immunoassays from key cellular signaling pathways (MAPK and Akt/mTOR) as well as biotinylated cytokines that are often below MS detection limits. This enabled us to quantify neuron-derived signaling phosphoproteins and cytokines, demonstrating regional expression patterns in the brain. Lastly, we extended this approach to contrast signaling phosphoprotein profiles of neurons and astrocytes, revealing relatively increased activation of MAPK signaling in neurons. The Rosa26 TurboID mouse, our validated approaches for cell type-specific in vivo proteomic labeling, and optimized workflow for proteomic characterization represent a highly promising approach for global cellular proteomics of desired cell types in their native state in vivo without the need for cell type isolation. Critically, these neuron- and astrocyte-specific proteomic data provide a resource of native-state proteomes of brain cell types.

Results
Hippocampal pan-neuronal proteomics using an adeno-associated viral (AAV) strategy. We first developed and validated a targeting vector designed to insert TurboID into the Rosa26 locus in mice and then generated the Rosa26 TurboID mouse line (Fig. 1a and Supplementary Fig. 1a–c). In a cohort of 3-month-old Rosa26 TurboID mice (Fig. 1a) and wild-type (WT) littermate controls, we stereotaxically injected AAV9 carrying the Cre recombinase gene under the hSyn promoter (AAV9-hSyn-Cre) into bilateral hippocampi for pan-neuronal TurboID expression (Fig. 1b). Un-injected WT mice also served as negative
controls (Fig. 1b). Following 4 weeks, mice then received biotin supplementation in water (37.5 mg/L) for 2 weeks with no observable adverse effects (e.g., weight and locomotor activity). Immunofluorescent imaging of hippocampal regions confirmed neuronal (NeuN) biotinylation (detected by streptavidin Alexa 488) in cell bodies, axons, and synapses in majority of hippocampal CA2/3 neurons (Fig. 1c). Western blot analysis of forebrain lysates from mice confirmed TurboID expression (based on V5 detection) and robust biotinylation of proteins in the Rosa26TurboID/wt/hSyn mice as compared to minimal endogenous biotinylation observed in control mice (Fig. 1d).

Biotinylated proteins were enriched from forebrain lysates using streptavidin beads. Enrichment was confirmed by eluting a small fraction of bound proteins for confirmatory Western blot and silver stain studies, which showed maximum biotinylated protein yield in the Rosa26TurboID/wt/hSyn mice (Fig. 1e). After label-free quantitation via mass spectrometry (LFQ-MS) of enriched biotinylated proteins, we quantified 2307 proteins. We first compared Rosa26TurboID/wt/hSyn mice with all control mice and identified 2,143 proteins with a significant \( p \leq 0.05 \geq 2 \)-fold enrichment, while only 5 proteins with \( \geq 2 \)-fold enrichment in the control proteome were identified (Fig. 1f, Supplementary Data 1), which included endogenously biotinylated carboxylases and keratins that were enriched by streptavidin binding. By including phosphorylation of Ser/Thr/Tyr residues in the searches, we identified 147 phospho-peptides from 110 unique proteins of which 55 showed at least 2-fold higher levels in the labeled neuronal proteome (e.g., Map2, Ncam1, Mapt, Map1b, Amph, etc.).
Development and validation of the Rosa26<sup>TurboID/wt</sup> mouse line for cell type-specific proteomics. 

**a** Genetic strategy for targeting TurboID (V5-TurboID-NESS) to the Rosa26 locus. **b** Schematic of AAV studies to direct Cre recombinase expression in hippocampal neurons (n = 3 WT mice with no injections, n = 3 WT mice received AAV9-hSyn-Cre, n = 3 Rosa26<sup>TurboID/wt</sup> mice received AAV9-hSyn-Cre). **c** Representative immunofluorescence images of CA2/3 of the hippocampus from WT/hSyn (n = 3 mice) and Rosa26<sup>TurboID/wt</sup>/hSyn (n = 3 mice) showing biotinylation (green: streptavidin Alexa488) in relation to neuronal nuclei (magenta: NeuN). **d** Western blot of brain lysates (representative animal from n = 3 mice/group) probed with streptavidin fluorophore, anti-V5, and anti-Gapdh antibodies. Rosa26<sup>TurboID/wt</sup>/hSyn brain showed biotinylated proteins of different molecular weights as compared to few endogenously biotinylated proteins in the two control groups. Right: Densitometry confirming significant increase in biotinylation signal in Rosa26<sup>TurboID/wt</sup>/hSyn brains (one-way ANOVA, **p** = 0.0001; data represented as mean ± SEM). **e** Western blot (left) and silver stain (right) of enriched biotinylated proteins after streptavidin-pulldown and release of biotinylated proteins from 10% of streptavidin beads (representative images from n = 3 mice/group). As compared to minimal protein enriched in the two control groups, several biotinylated proteins were enriched from Rosa26<sup>TurboID/wt</sup>/hSyn brain. **f** Volcano plots showing differentially enriched proteins comparing Rosa26<sup>TurboID/wt</sup>/hSyn (n = 3) and control mice (n = 3 WT and n = 3 WT/hSyn). Orange symbols (two-sided T-test unadjusted p ≤ 0.05 and ≥ 2-fold change) represent biotinylated proteins enriched in the Rosa26<sup>TurboID/wt</sup>/hSyn brain and examples of neuron-specific proteins are highlighted, in addition to TurboID. Blue symbols represent endogenously biotinylated carboxylases enriched in the control brains. For group wise comparisons, see Supplementary Fig. 1. **g** Results from GSEA of ≥ 2-fold biotinylated neuronal proteins (orange symbols from panel f), as compared to reference list<sup>5</sup> (mouse brain: n = 7736) showed enrichment of neuronal and synaptic proteins confirming neuron-specific labeling. **h** Graphical representation of the number of proteins within various cellular compartments determined from GSEA. For related M5 data and additional analyses, see Supplementary Data 1, 3, 7, and 8. Source data are provided as a Source Data file.

Supplementary Data 2), highlighting the ability of CIBOP to capture abundant phosphorylated proteoforms even without phosphopeptide enrichment.

Gene set enrichment analyses (GSEA) of the 2143 enriched biotinylated proteins revealed enrichment of neuronal and synaptic proteins involved in neuron projection and synapse organization, confirming neuron-specificity of labeling (Fig. 1g, h, Supplementary Data 3). GSEA also showed labeling of proteins involved in a diverse set of molecular functions such as cytoskeletal protein binding, substrate-specific transporter activity, and calmodulin binding. Specifically, we observed biotinylation of 54 cell surface proteins, 63 transmembrane transporters, and 45 ion channel subunits (7 calcium, 9 glutamate, 6 GABA, 13 potassium, 1 sodium, 4 anionic) (Supplementary Data 4). The biotinylated proteome was enriched in cytoplasmic, membrane, synaptic, and cytoskeletal proteins, which are representative of the whole cell proteome rather than a bias to a specific subcellular compartment (Fig. 1h, Supplementary Data 3). In comparison to bulk brain proteomic data from the AAV cohort, our pan-neuronal enriched proteome identified 354 neuron-derived proteins (Supplementary Data 1) that were not previously quantified at the whole brain level. GSEA of these 354 neuron-derived proteins showed that they were predominantly membrane proteins involved in a variety of molecular functions such as serotonin receptor, potassium channel, glutamate receptor, and protein kinase activity (Supplementary Data 6). Within the 354 proteins, we identified 33 druggable targets of disease relevance (Supplementary Data 1), including solute transporters, GPCRs (e.g., GRM1), lipid metabolic proteins (e.g., GABA2), and signaling proteins including AKT1 which is a key member of the IGFI/PI3K/AKT1/mTOR axis that is relevant to synaptic functioning and memory<sup>31</sup>. We also observed all pairwise comparisons between labeled and control mice and obtained nearly identical GSEA results as above (Supplementary Fig. 1d, e and Supplementary Data 1, 7, 8).

In sum, AAV-CIBOP resulted in robust pan-neuronal labeling of proteins in the hippocampus by TurboID in vivo. Proteomic analysis of biotinylated proteins confirmed neuronal enrichment and representation of proteins within a diverse number of molecular functions from various cellular compartments, including numerous synaptic proteins, transmembrane proteins, and several druggable targets which are otherwise challenging to sample in the native state of neurons in adult mouse brain.

Camk2a-neuronal protein biotinylation in adult mouse brain using a transgenic strategy. We next employed a transgenic approach to express TurboID within Camk2a neurons by breeding Rosa26<sup>TurboID/wt</sup> mice with Camk2a-Cre<sup>ERT2</sup> and inducing Cre recombinase expression by intraperitoneal tamoxifen. Camk2a (Ca<sup>2+</sup>-calmodulin-activated protein kinase 2A) is an abundant serine-threonine kinase highly expressed by excitatory neurons, particularly in the synapse, where it regulates synaptic transmission, excitability, and long-term potentiation. Camk2a was chosen based on extensive validation, specificity, and non-leakiness of available Camk2a-Cre<sup>ERT2</sup> driver lines and the well-characterized expression patterns of Camk2a across brain regions<sup>26,27</sup>. Rosa26<sup>TurboID/wt</sup>/Camk2a and littermate controls received tamoxifen at 6 weeks of age, allowed 3 weeks for recombination, and followed by biotin supplementation for 2 weeks (Fig. 2a). There were no associated phenotypic changes or observable adverse effects during biotin supplementation (e.g., weight and locomotor activity). Western blot analysis of lysates from different brain regions (cortex, hippocampus, striatum/thalamus, pons/medulla, and cerebellum) confirmed robust biotinylation of proteins in the Rosa26<sup>TurboID/wt</sup>/Camk2a mice as compared to minimal endogenous biotinylation observed in control mice (Fig. 2b). Qualitatively, the highest level of labeling was observed in the cortex, hippocampus, and striatum/thalamus regions as compared to cerebellum and pons/medulla, consistent with known Camk2a expression patterns<sup>32</sup>. We also confirmed TurboID protein expression via detection of V5 in Rosa26<sup>TurboID/wt</sup>/Camk2a brain regions only, which followed a similar pattern to level of biotinylation (Fig. 2b).

Immunofluorescent imaging of the whole brain displayed wide-spread biotinylation in Rosa26<sup>TurboID/wt</sup>/Camk2a brains as compared to control brains (Fig. 2c). Furthermore, biotinylation was observed predominantly within cell bodies and axonal projections in all brain regions sampled for proteomic analysis (Fig. 2c). Map2 and streptavidin co-immunofluorescence confirmed neuronal labeling throughout the hippocampus (Fig. 2d), as well as the cortex (Supplementary Fig. 2a), pons/medulla (Supplementary Fig. 2b), striatum/thalamus (Supplementary Fig. 2c), and cerebellum (Supplementary Fig. 2d). Importantly, biotinylation was not observed in astrocytes (Supplementary Fig. 3a) or microglia (Supplementary Fig. 3b), and there was no evidence of reactive gliosis in Rosa26<sup>TurboID/wt</sup>/Camk2a mice compared to control mice (Supplementary Fig. 3).

To determine whether TurboID expression and protein biotinylation impact Camk2a neuronal function, we performed electrophysiological studies on 3-month-old Rosa26<sup>TurboID/wt</sup>/Camk2a mice and littermate controls that received tamoxifen and biotin supplementation (Fig. 2e). Somatic whole-cell recordings from pyramidal neurons in the CA3c region of the hippocampus,
a region that displayed robust biotinylation (Supplementary Fig. 4a), showed no significant differences in mean action potential firing between control and Rosa26TurboID/wt/Camk2a mice (Fig. 2f). Furthermore, we did not observe significant differences in various passive and active parameters measured (Supplementary Fig. 4b). In summary, these findings validate the Tg-CIBOP approach and confirm lack of any electrophysiological perturbations in Camk2a neurons despite robust proteomic biotinylation as well as the lack of phenotypic changes in the mouse.

Proteomic analysis reveals unique Camk2a neuron brain region signatures. After confirming biotinylation of proteins by Western blot and immunofluorescence imaging in Rosa26TurboID/wt/Camk2a brains, we enriched biotinylated proteins from cortex, hippocampus, striatum, and cerebellum.
Fig. 3 Camk2a neurons exhibit region-specific proteomic differences in adult mouse brain. 
a Experimental outline for LFQ-MS studies performed on biotinylated proteins enriched using streptavidin beads from Rosa26TurboID/wt/Camk2a-CreERT2 and littermate control (Camk2a-CreERT2) mice (n = 2 mice per experimental group, five regions per mouse with 2 technical replicates per region). Asterisk (*) indicates technical replicates for each brain region from Rosa26TurboID/wt/Camk2a-CreERT2 mice. 
b Principal Component Analysis (PCA) of MS data after normalization to TurboID abundance in each sample/brain region. PCA identified distinct clusters based on region except for hippocampal and cortical regions clustering together. Three PCs explained 30%, 14% and 13% of variance, respectively. 
c Clustering representation of protein abundance data of core groups of proteins most highly expressed in specific brain regions with at least 4-fold higher levels in a specific region compared to all other regions (p ≤ 0.05). STRING analysis identified networks of known direct (protein-protein) and indirect (functional) interactions within core regional protein signatures. 
d Heatmap representation, based on enrichment Z-scores, of KEGG pathways, Pathway Commons, and diseases from the Comparative Toxicogenomics Database (CTD) enriched in core regional proteins. For related MS data and additional analyses, see Supplementary Data 9, 11, 12, and 13. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.
114 neuron-derived proteins that were not previously quantified at the whole brain level (Supplementary Data 9).

Principal component analysis (PCA) of Rosa26-TgTurboD/cre Camk2a biotinylated protein abundance data showed that nearly 60% of variance was explained by 3 principal components (PCs), of which PC1 explained 30% while PC2 explained 14% and PC3 explained 13% of variance (Fig. 3b). Notably, PCA identified four distinct brain regional proteomic clusters: cortex and hippocampus, striatum/thalamus, pons/medulla, and cerebellum (Fig. 3b).

We identified core groups of highly expressed region-specific proteomic signatures of Camk2a neurons (≥ 4-fold change and p ≤ 0.05, Fig. 3c, Supplementary Data 11). The cortex/hippocampus contained 83 signature proteins while cerebellum contained 138, pons/medulla contained 128, and striatum/thalamus contained 6. We also identified networks of known direct (protein–protein) and indirect (functional) interactions (STRING) within these core regional protein signatures (Fig. 3c).

GSEA of core protein signatures revealed distinct molecular and biological features of Camk2a neurons (Supplementary Data 12). The proteomic signature of cortical and hippocampal neurons was indicative of glutamatergic synaptic transmission, calcium signaling, synaptic plasticity, and more specifically, E-cadherin and syndecan-4-mediated signaling and RhoA activity (Fig. 3d). Cortical and hippocampal neuronal signature proteins were enriched in genes associated with epilepsy, mood and psychotic disorders, substance abuse, and hypoxic brain injury (Fig. 3d). The cerebellar Camk2a proteomic signature was indicative of increased MAPK signaling and mRNA processing (splicing, snRNP assembly) as well as genes related to axatia, spinal cord disease, and hereditary eye diseases (e.g., retinitis pigmentosa) (Fig. 3d). Unlike cortical and cerebellar neurons, Camk2a neurons from the pons/medulla showed increased levels of proteins involved in amino acid, fatty acid, and carbohydrate metabolism (Fig. 3d), supportive of elevated metabolic activity in these neurons and axons. Consistent with the abundant metabolic protein signature of pons/medullary Camk2a neurons and the axonal predominance in this region, this regional proteomic signature was enriched in gene symbols related to inborn errors of metabolism, leukoencephalopathies, and polynuropathies (Fig. 3d). Since ion channels determine the electrophysiological properties of neurons, we also identified distinct ion channel subunits that showed regional specificity. In the cortex and hippocampus, core ion channel proteins included voltage gated calcium channel subunits Cacna1a, Cacna2d3, and NMDA receptor subunit Grin1. Cerebellar Camk2a neuronal signatures were characterized by higher levels of voltage-gated calcium channel subunits Cacna1a, Cacna2d, Caeng2, chloride channel Clyc1, and several potassium channels including Kcncl, Kcncl3, Kcnd2, Kcni1p. In contrast, pons/medulla Camk2a neurons were characterized by higher expression of potassium channels Hcn2, Hcn2 and Kcnj10. Similar to the AAV-CIBOP results, we identified several (n = 52) druggable target proteins that exhibited region-specific patterns (Supplementary Data 11). Drug targets with high levels in cortical Camk2a neurons included ion channel Gria3, a regulator of neurogenesis (Ntrk2), and lipid metabolic protein Plcb1. In contrast, drug targets in the cerebellum included mRNA transport and mRNA processing proteins (e.g., Snrnp200) and the phospholipase Plcb4.

Region specific proteins were further searched against curated lists of gene-disease associations (DisGeNet) to define regional gene-disease associations (Supplementary Data 13). Cortex and hippocampal Camk2a neuron proteins enriched in glutamatergic transmission proteins and long-term potentiation (e.g., Lingo1, Homer1, Gria1) were associated with mental health disorders (schizophrenia, bipolar disease, depression, autism), neurodevelopmental disorders, essential tremor, and primary epileptic disorders (e.g., West syndrome). Pons/medulla enriched proteins were associated with dystonia, Parkinson’s disease, polyneuropathies, and inborn errors in metabolism consistent with the high metabolic protein levels in this region. Cerebellar Camk2a neuronal proteins enriched in mRNA processing and splicing proteins were linked to ataxias, primary lateral sclerosis, and psychiatric disorders, suggesting that splicing dysregulation in cerebellar Purkinje neurons may underlie pathogenesis of these conditions.

Since the above analyses were targeted towards proteins with region-specific enrichment patterns, we also analyzed our data in an unbiased manner (K-means clustering). As a result, we identified five clusters of proteins that showed regional patterns of protein expression (Supplementary Fig. 6a, Supplementary Data 14) consistent with region-specific analyses described above. GSEA of the clusters revealed that cortical and hippocampal Camk2a neurons expressed higher levels of proteins involved in GABA signaling and glutamate signaling pathways, neuron development, and synaptic function (Cluster 2 & 4, Supplementary Fig. 6b, Supplementary Data 15) while the cerebellar Camk2a neuronal proteome showed higher levels of proteins involved in hydrolyase activity and translation initiation factor 3 (Cluster 5, Supplementary Fig. 6b, Supplementary Data 15). The pons/medulla Camk2a proteome showed a unique signature of pigment granule as well as tau-kinase activity related proteins. GSEA also indicated regional metabolic differences in Camk2a neurons, with over-representation of glycolytic, amino acid, and fatty acid metabolic proteins in the pons/medulla (Cluster 1 & 3, Supplementary Fig. 6b, Supplementary Data 15).

We also quantified the whole brain regional proteomes (background) from Rosa26-TgTurboD/cre Camk2a and control mice. A total of 3969 proteins were quantified and displayed regional enrichment (Supplementary Fig. 7a, Supplementary Data 16). Among the 3969 proteins, 1872 were also identified in the Camk2a-enriched proteome with 201 proteins being exclusive to the Camk2a-enriched proteome (Supplementary Fig. 7b). Using definitions of core regional protein markers (≥4-fold enrichment in the specific region over other regions and p ≤ 0.05), 1110 proteins were identified as core-markers in the Camk2a-enriched proteome, of which only 343 were also identified as core markers in the background proteome (Supplementary Fig. 7c). When we assessed the degree of overlap between each region, we found that the Camk2a-enriched proteome contained 549 regionally enriched proteins in the cortex/hippocampus, of which 419 (76.3%) were also regionally enriched in the background proteome (Supplementary Fig. 7d). In contrast, lower levels of overlap were observed in other regions - 9.6% in the striatum/thalamus, 58% in the pons/medulla, and 59% in the cerebellum (Supplementary Fig. 7e–g). This shows that the Camk2a-enriched proteome identifies twice as many core-regional proteomic differences within Camk2a neurons that were not captured by the background proteome.

In sum, Tg-CIBOP allowed us to comprehensively characterize the proteome of Camk2a neurons in the adult mouse brain and resolve regional differences in proteomic composition of Camk2a neurons, which was not easily attainable with the AAV approach or from mouse post-natal or embryonic neuronal culture systems. Importantly, we uncovered potential links between regional proteomic characteristics of Camk2a neurons and disease vulnerability.
and signaling proteins without prior enrichment. Thus, we extended our studies to measure specific biotinylated cytokines and phospho-proteins involved in cellular signaling cascades (e.g., MAPK and Akt/mTOR) using an antibody-based approach. We adapted the multiplexed Luminex sandwich ELISA approach (Fig. 4a) to directly measure biotinylated phospho-proteins from the MAPK and Akt/mTOR pathways and an array of 32 inflammatory cytokines in brain tissues from both Rosa26TurboID/wt AAV and transgenic cohorts (Fig. 4b). First, we captured the proteins of interest onto beads using target-specific antibodies, then a second biotinylated detection antibody followed by a streptavidin fluorophore and fluorescence quantitation (Fig. 4a, standard assay). In parallel, we adapted this assay to exclude the second biotinylated detection antibody to directly detect neuronal biotinylated proteins via streptavidin fluorophore (Fig. 4a, adapted assay). This provided an estimate of total (standard assay) and neuron-derived...
phospho-proteins measured using standard and adapted assays, from control and labeled mice (also see Supplementary Data 17). Data shown as mean ± SEM. Derived cytokines or phospho-proteins have similar fluorescence values in both standard and adapted assays, while predominantly non-neuronal cytokines or phospho-proteins have a markedly lower adapted assay readout as compared to the standard assay, determined by pairwise comparison between control and labeled mice (also see Supplementary Data 17). Data shown as mean ± SEM. 

(aapt assay) levels of the target protein without the need for enrichment of biotinylated proteins. In the AAV cohort, the standard Luminex assay showed that AAV9-hSyn-Cre increased levels of signaling phospho-proteins (pAkt, pPTEN) and inflammatory cytokines (IL-2, IL-13, IL-17, IP-10, KC, and MIG) indicating an inflammatory response related to AAV9 injection (Supplementary Data 17). Given this effect of AAV9, we subsequently compared Rosa26TurbolID/wt/hSyn mice to WT/hSyn mice using the standard assay and found significant elevation of 10 phospho-proteins (e.g., pMSK1, pStat1, pMek1) and 9 cytokines (e.g., VEGF, MIG, IL7) in labeled mice. This increased phospho-protein and cytokine response in Rosa26TurbolID/wt/hSyn mice could be due to an inflammatory response or due to additive increased signal of biotinylated target proteins in the assay. Despite these differences between groups using the standard assay, the adapted Luminex approach detected 9-fold higher signal in the Rosa26TurbolID/wt/hSyn brains compared to minimal signal in the control brains, confirming that the elevated signal was indeed due to biotinylation by TurbolID. By comparing the standard assay signal (total analyte abundance) with the adapted assay signal (biotinylated neuron-derived signal) in the Rosa26TurbolID/wt/hSyn brain lysates, we found that majority of cytokines, with the exceptions of IL-10, IL-2, TNF-α, IL-1α, and RANTES (Fig. 4c, underlined proteins), had adapted assay signals less than 50% of the standard assay signals, suggesting their predominant origin from non-neuronal cells. We also quantified several MAPK phosphoproteins (Fig. 4d, underlined proteins, e.g., p-p38, pMek1, and pErk1/2) and Akt/mTOR phosphoproteins (Fig. 4e, underlined proteins, e.g., pGSK-α and -β, p-mTOR), and observed that >50% of the standard assay signal for these analytes, was accounted for by the adapted signal, indicating their predominant origin from non-neuronal cells.

We also applied the adapted Luminex approach to investigate regional differences in neuron-derived signaling proteins and cytokines in Rosa26TurbolID/wt/Camk2a mice. After accounting for background, adapted Luminex assay signals were negligible in the control mice and were subtracted (Supplementary Data 18). Highest signal intensities in the adapted assay across all regions were observed for 25 of 32 cytokines (Top 5: IL-2, IL-1α, IL-9, IP-10, and VEGF) and phospho-proteins from both Akt/mTOR (11 of 11 analytes) and MAPK (10 of 10) signaling pathways. Similar to the results from our AAV cohort, phosphoproteins from signaling pathways were more robustly biotinylated as compared to cytokines (Supplementary Data 18). A combined PCA of fluorescent intensities from adapted Luminex assays for MAPK, Akt/mTOR, and cytokine panels revealed four distinct clusters associated with brain regions of Rosa26TurbolID/wt/Camk2a mice (Fig. 4f). Specifically, we identified unique patterns of neuron-derived cytokine levels that distinguished the cerebellum from the cortex and pons/medulla after adjusting for TurbolID levels (Fig. 4g). Intriguingly, cortical Camk2a neuron-derived signature included low levels of cytokines but high levels of phospho-proteins from the Akt/mTOR pathway (Fig. 4h). In contrast, cerebellar Camk2a neuronal signature displayed higher level of cytokines and low levels of Akt/mTOR and MAPK phospho-proteins, while MAPK phospho-proteins appear to be enriched in the striatum/thalamus. An integrated t-distributed stochastic neighbor embedding (tsNE) analysis of core regional proteomic signatures derived from LFQ-MS studies and from Luminex data confirmed clustering of Akt/mTOR signaling with cortex-specific proteins while elevated cytokines clustered with the cerebellar proteomic signature and MAPK clustered with striatal/thalamic proteomic signature. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. Source data are provided as a Source Data file.

Astrocye protein biotinylation reveals region-specific proteomic signatures and differences between Camk2a neurons in adult mouse brain. Based on our successful application of TgCIBOP to Camk2a neurons, we extended this approach to Aldh1l1 astrocytes and contrasted the proteomic profiles of the two cell types in their native state. To achieve astrocyte-specific proteomic labeling, we bred Rosa26TurbolID/wt/mice with Aldh1l1-CreERT2 mice, a well-validated inducible Cre mouse line, to target mature astrocytes in the brain and applied the same tamoxifen and biotinylation paradigms as above (Fig. 5a). Rosa26TurbolID/wt/Aldh1l1, Rosa26TurbolID/wt/Camk2a (independent of those used in Figs. 2–3), and littermate Rosa26TurbolID/wt control mice were used for experiments. After 2 weeks of biotinylation, brain regions (cortex, hippocampus, striatum/thalamus, pons/medulla, cerebellum, and spinal cord) were dissected from one hemisphere for proteomic studies, while the other hemisphere was used for immunohistochemical studies. Western blot analysis of lysates from different brain regions of Rosa26TurbolID/wt/Aldh1l1 and
Rosa26TurboID/wt/Camk2a mice confirmed biotinylation of proteins with distinct patterns of biotinylation between the two mouse lines, with few endogenously biotinylated proteins observed in control mice (Fig. 5b). Qualitatively, Rosa26TurboID/wt/Camk2a brain regions displayed a higher degree of labeling compared to Rosa26TurboID/wt/Aldh111 brain regions. We also confirmed TurboID protein expression via detection of V5 (Fig. 5b). Immunofluorescent imaging of brain sections from...
Fig. 5 Astrocyte protein biotinylation reveals region-specific proteomic signatures and differences between Camk2a neurons. a Study design for neuron-specific and astrocyte-specific proteomic biotinylation. Rosa26 TurboID/wt, Rosa26 TurboID/wt/Camk2a-CreERT2, Rosa26 TurboID/wt/Aldh111-CreERT2 mice received tamoxifen intraperitoneally for 5 days. After 4 weeks, mice received bacterin water for 2 weeks. b Representative Western blots from brain region lysates (n = 2 mice/group), probed for biotin (streptavidin Alexa488), V5 and Gapdh are shown. c Representative images (n = 2 mice/group) showing biotinylation in the hippocampus. d Representative immunofluorescence images (n = 2 mice/group) showing overlap between astrocytic biotinylation (streptavidin Alexa488), Gfap, and Ndrg2 in the hippocampus region from Rosa26 TurboID/wt/Aldh111 mice. e Representative immunofluorescence images (n = 2 mice/group) showing no overlap between biotinylation, Iba1, and βIII-tubulin in astrocytes and blood vessels in the hippocampus region from Rosa26 TurboID/wt/Aldh111 mice. f Clustering analysis of protein abundance data of region-enriched proteins with at least 4-fold enrichment over other regions in Rosa26 TurboID/wt/Aldh111 mice. STRING analysis identified networks of direct (protein–protein) and indirect (functional) interactions within core regional protein signatures in cortex/hippocampus and cerebellum. g Heatmap representation, based on enrichment Z-scores, of gene ontologies enriched in core regional proteins. h Volcano plot showing differentially enriched proteins comparing Rosa26 TurboID/wt/Aldh111 and Rosa26 TurboID/wt/Camk2a mice. For this analysis, all six brain regions were combined for both groups. Orange symbols (two-tailed T test unadjusted p ≤ 0.05 and ≥ 2-fold change) represent biotinylated proteins enriched in Camk2a neurons with neuron-specific proteins highlighted. Blue symbols (two-tailed T test unadjusted p ≤ 0.05 and ≥ 2-fold change) represent biotinylated proteins enriched in Aldh111 astrocytes and examples of astrocyte-specific proteins are highlighted in dark blue. i HCA of GSEA showing over-represented ontologies within neuronal-enriched proteins and astrocyte-enriched proteins. Representative gene ontology terms are highlighted. j Volcano plot showing enrichment (two-tailed T test unadjusted p ≤ 0.05) of MAPK and Akt/mTOR phospho-proteins in Rosa26 TurboID/wt/Camk2a compared to Rosa26 TurboID/wt/Aldh111 brains. For related MS data and additional analyses, see Supplementary Data 19-24. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. Source data are provided as a Source Data file. Source data are provided as a Source Data file.

These mice demonstrated biotinylation, as detected by Streptavidin-488, in Rosa26 TurboID/wt/Camk2a and Rosa26 TurboID/wt/Aldh111 brains while control brains lacked any biotinylation (Fig. 5c). Biotinylation in Rosa26 TurboID/wt/Aldh111 brains co-localized with Gfap- and Ndrg2-positive astrocytes with a preponderance of labeled blood vessels in the hippocampus (Fig. 5e) as well as other brain regions (Supplementary Fig. 9), with no evidence of gliosis (Fig. 5e). Furthermore, biotinylation was not observed in βIII-tubulin-positive neurons or Iba1-positive microglia, confirming astrocyte specificity in the Rosa26 TurboID/wt/Aldh111 brains (Fig. 5d). Given the prevalence of biotin labeling around and within blood vessels, we hypothesized that this signal may be due to astrocytic end-feet that are in close contact with blood vessels33. To confirm this, we immunolabeled the same brain sections for aquaporin 4 (AQP4), an aquaporin channel specifically located at the perivascular astrocyte processes, GFAP, and biotin. We observed global immunopositivity for AQP4 and GFAP in control and Rosa26 TurboID/wt/Aldh111 hippocampi, with biotin signal observed only in the Rosa26 TurboID/wt/Aldh111 brains (Supplementary Fig. 10a). At a higher magnification, we saw colocalization of biotin with AQP4 as well as GFAP (Supplementary Fig. 10b). Lastly, biotinylated proteins in Rosa26 TurboID/wt/Camk2a mice were similar to prior findings discussed above (Supplementary Fig. 9).

We enriched biotinylated proteins from Rosa26 TurboID/wt/Camk2a and Rosa26 TurboID/wt/Aldh111 brain regions and performed LFQ-MS, quantifying 3216 unique proteins. After enforcing a missing value threshold and imputing missing values, 2550 proteins remained in our analyses. First, we performed differential expression analysis, after normalizing for TurboID levels, from all brain regions comparing Rosa26 TurboID/wt/Aldh111 and controls. As a result, 1380 proteins with ≥ 2-fold enrichment in Rosa26 TurboID/wt/Aldh111 brains and 21 proteins with ≥ 2-fold enrichment in the control brains were identified (Supplementary Fig. 11a, Supplementary Data 19). GSEA of 1380 enriched proteins identified those involved in biological processes such as membrane organization and metabolic activity as well as proteins involved in molecular functions such as transporter activity, mitochondrial function, and calbindin binding (Supplementary Fig. 11b, Supplementary Data 20). Proteins within the cytoplasm, membrane, and other cellular components were significantly enriched in the Rosa26 TurboID/wt/Aldh111 proteome (Supplementary Fig. 10b, Supplementary Data 20).

Next, we analyzed the Rosa26 TurboID/wt/Aldh111 proteome for regional differences, complementary to the region-resolved neuronal proteomics described above and identified core groups of astrocyte-derived proteins that were highly abundant in a region-specific manner (≥ 4-fold change and p ≤ 0.05, Fig. 5f, Supplementary Data 21). We also identified networks of known direct (protein–protein) and indirect (functional) interactions (STRING) within core cortex/hippocampus and cerebellum protein signatures (Fig. 5g). GSEA of these core regional astrocyte proteomes revealed enrichment of metabolic processes in the cerebellum, glutamate receptor and ion channel activity in the cortex/hippocampus, microtubule and calmodulin binding in the pons/medulla, and protein heterodimerization and extracellular matrix in the spinal cord (Fig. 5g, Supplementary Data 22).

We next compared the proteomes from Rosa26 TurboID/wt/Camk2a and Rosa26 TurboID/wt/Aldh111 brain regions and identified 1061 proteins with cell type-specific differences (≥ 2-fold change and p ≤ 0.05, Fig. 5h), including 925 proteins more highly abundant in astrocytes and 136 proteins more highly abundant in neurons. We performed cell type enrichment analysis of these differentially expressed proteins using protein marker lists derived from existing reference proteomes of brain cell types3. We found astrocytic proteins such as Gfap, Fbxo2, and Hepacam enriched in Rosa26 TurboID/wt/Aldh111 proteome (Fig. 5h, dark red dots) while neuronal proteins such as Synap1, Camk2a, and Nptxr1 enriched in Rosa26 TurboID/wt/Camk2a proteome (Fig. 5h, dark blue). GSEA of the differentially expressed cell type-specific protein lists revealed enrichment of metabolic terms, including lipid metabolism, in astrocytes, while receptor (ionotropic and metabotropic), synaptic and signal transduction terms were enriched in neurons (Fig. 5i, Supplementary Data 23).

Finally, we used the adapted Lumixen approach described above to measure biotinylated signaling phosphoproteins from Rosa26 TurboID/wt/Camk2a and Rosa26 TurboID/wt/Aldh111 brain homogenates. In comparison to Rosa26 TurboID/wt/Camk2a brain, levels of astrocyte-derived signaling phospho-proteins in the Rosa26 TurboID/wt/Aldh111 brains were significantly lower even after accounting for differences in efficiency of TurboID labeling across cell types (Fig. 5j, Supplementary Data 24). This suggests that basal MAPK and Akt/mTOR signaling activity is higher in Camk2a neurons compared to astrocytes under homeostatic conditions in the mouse brain.

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To summarize, these findings demonstrate the ability of CIBOP to resolve proteomic differences between two brain cell types, neurons and astrocytes, and identify regional molecular differences in astrocytes at the proteomic level in their native state, without the need for isolation.

Discussion

We report a cell type-specific in vivo proteomic labeling approach in which the biotin ligase, TurboID, is expressed in a desired cell type using a Cre-lox genetic strategy. We have generated and validated the Rosa26TurboID mouse line that can now be bred with a plethora of well-validated existing non-inducible and inducible Cre mouse lines to achieve tissue as well as cell type-specific proteomic labeling. The broad cellular proteomic labeling allows us to capture a snapshot of the cellular proteome of a specific cell type while retaining the native state of the cell since the labeled proteins can be purified directly from brain lysates without cell type isolation. We term this approach cell type-specific in vivo biotinylation of proteins (CIBOP). To demonstrate the utility of CIBOP, we successfully performed proof-of-principle experiments to label the neuronal proteome using AAV (AAV-CIBOP) and genetic approaches (Tg-CIBOP), providing two complementary approaches for cell type-specific labeling in vivo. The AAV-CIBOP approach can be particularly useful when using complex genetic models involving multiple transgenes, thereby avoiding need for complicated genetic crosses. However, AAV delivery does introduce some undesired effects such as glial activation and some loss of specificity. On the other hand, transgenic-CIBOP provides an ideal approach without the artefacts of AAV delivery or glial activation. Using these approaches, we then used unbiased MS and targeted immunoassays of neuron-derived (Camk2a-CIBOP) and astrocyte-derived (Aldh1l1-CIBOP) biotinylated proteins from whole brain samples. LFQ-MS allowed us to identify over 2000 proteins and regional differences within Camk2a neurons and Aldh1l1 astrocytes. A major advantage of CIBOP is its depth of proteomic coverage, even with “single-shot” label-free MS approaches. We anticipate 3-4-fold greater proteomic coverage in future studies with tandem multiplexed approaches coupled with off-line fractionation. While we did not specifically perform neuron-specific phospho-proteomic profiling of neurons in our studies, we were also able to quantify over 140 neuron-derived phosphoproteins without enrichment. This depth of phosphoprotein coverage could be increased several-fold with phosphopeptide enrichment approaches which could reveal cell type-specific proteomic insights at the level of post-translational modifications that cannot otherwise be captured by other methods. Our successful application of CIBOP to contrast proteomic profiles of neurons and astrocytes in their native state, revealed proteomic markers for both cell types and identified activation of phospho-protein signaling involving the MAPK and Akt/mTOR pathways as being unique to neurons as compared to astrocytes. Our studies also demonstrate the validity of this approach for broad applications in neuroscience and outside the nervous system.

Since CIBOP involves biotinylation of lysine residues, it is important to consider potential implications of excessive biotinylation on protein and cellular function. Biotin is a water soluble, readily cell permeable, and a brain penetrant vitamin (vitamin H or B7) that is required as a co-factor for several enzymes involved in glucose, amino acid, and fatty acid metabolism. While biotin deficiency can cause growth retardation, skin and neurological diseases, high doses of systemically administered biotin are non-toxic. Since TurboID is highly efficient, biotin supplementation is necessary to prevent a relative biotin deficiency caused by TurboID shunting biotin away from biotin-dependent cellular processes. Appropriately, some concerns regarding toxicity of excessive biotinylation have been raised in the literature. In the present study, we did not observe any adverse effects of neuron-specific biotinylation in Rosa26TurboID mice, supported by electrophysiological studies. Proteomic biotinylation also did not alter astrocyte morphology or cause a glial response in the brain. Whether shortening the duration and/or degree of biotinylation can still achieve sufficient proteomic labeling needs to be determined. As CIBOP is extended to other cell types and tissues, investigators will need to optimize the duration of Cre-mediated recombination as well as the dose and duration of biotin supplementation to individual applications.

The biotinylated neuronal proteomes from AAV- and Camk2a-CIBOP approaches were highly enriched with proteins involved in neurotransmission and axon guidance and widespread neuronal cell body, axonal, and dendritic biotinylation with no glial labeling or activation. We captured synaptic proteins, ion channels, and membrane receptors including druggable targets. AKT1, a signaling protein that is a key member of the IGF1/PI3K/AKT1/mTOR axis that is relevant to synaptic functioning and memory, is a druggable target that was identified in the AAV and Tg proteomes, highlighting consistency across both CIBOP datasets and emphasizing important roles for this pathway in neuronal physiology. At the sub-cellular level, cytosolic and membrane associated proteins such as ion channels, as well as organelles such as mitochondria, ER and Golgi apparatus, and vesicles, were abundant in the enriched neuronal proteome. We attribute the broad proteomic labeling to a combination of the biotinylation radius (~10 nm) of biotin ligases, the promiscuous nature of protein biotinylation by TurboID, and the use of a nuclear export sequence (NES) in the transgene of Rosa26TurboID mice. The low coverage of nuclear proteins observed is attributed to the NES sequence, which was chosen in order to increase the chances of labeling the extra-nuclear proteins while limiting toxicity via nuclear proteomic biotinylation. The labeling of secreted and vesicular proteins also suggests that simultaneous profiling of secreted or released proteins in tissues and biofluids may be feasible. Indeed, secretome profiling using in vivo TurboID via AAV delivery was successful in a non-brain context. Our ongoing studies with Rosa26TurboID mice, in brain and non-brain disease contexts, will determine whether cellular origin of secreted proteins can indeed be measured in biofluids as biomarkers of underlying cellular mechanisms of disease.

The Camk2a-CIBOP transgenic strategy also successfully labeled neurons in the adult brain and, regardless of brain region, the proteome showed enrichment of neuronal synaptic proteins such as Dlg4 (PSD-95) and Gap43. With this approach, we had the opportunity to characterize regional proteomic differences within the Camk2a neurons and found core protein signatures unique to the cortex and hippocampus, striatum/thalamus, pons/medulla, and cerebellum while overcoming limitations associated with AAV-CIBOP. Glutamatergic synaptic transmission, calcium signaling, and synaptic plasticity encompassed the proteomic signature of cortical and hippocampal Camk2a neurons. Alternatively, cerebellar proteomic signatures point to mRNA processing, splicing, and calcium transporting ATPase activity. Unique proteomic signatures of pontine and medullary neurons with increased expression of metabolic proteins, inflammatory proteins, and pigmented granules agrees with the expected enrichment of axonal tracks and islands of neuronal cell bodies in the brain stem, particularly pigmented neurons. Collectively, these data emphasize the diverse functions of proteins within Camk2a neurons and how each brain region may be functionally distinct from the other. Furthermore, these functionally distinct characteristics reflect neuronal-specific mechanisms within brain regions that may be vulnerable to neurological diseases.

Indeed, several protein/gene-disease associations were enriched among core regional neuronal protein lists. In cortical and hippocampal Camk2a neurons, we identified signature proteins linked to epilepsy (e.g., Dlg4) and schizophrenia (e.g., Grin2a). Several studies
have shown that the GRIN2A gene, which encodes a glutamate (N-methyl-D-aspartic acid [NMDA]) receptor subunit protein, contains a polymorphism in the promoter that predisposes individuals to schizophrenia11–14. Grin2a is also known to interact directly and indirectly withDlg4 (PSD-95), which regulates glutamate NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking at the synapse15 with significant roles in Alzheimer’s disease (AD) biology16. Interestingly, regional differences in phospholipase C isoenzyme (Plcb1, Plcb4), druggable targets identified in the Camk2a proteome, expression in the brain are known to underlie several brain disorders including epilepsy, schizophrenia, and AD17. The neurotransytic tyrosine kinase receptor type 2 (NTRK2), a protein significantly enriched in our cortical and hippocampal Camk2a proteome associated with mood disorders, is a specific receptor for neurotrophin brain-derived neurotrophic factor (BDNF) which is implicated in the pathophysiology of bipolar disorder18.

In the cerebellum, core signature proteins were expectedly associated with ataxias, which are defined by Purkinje cell and cerebellar circuitry dysfunction19. One such protein we identified is Caca1a, a α1A-subunit of voltage-gated P/Q-type calcium channels (Cav2.1), which contains a mutation that causes spinocerebellar ataxia type 6 (SCA6) and episodic ataxia type 520,21. Lastly, proteins related to mitochondrial dysfunction across different brain regions. The biotinylated proteins appeared to be within astrocyte cell bodies and along blood vessels, which is expected given that astrocytic end-feet contact blood vessels to form the glia limitans of the blood brain barrier22. The astrocyte proteome showed enrichment of metabolic (e.g., Aldoc), glial (e.g., Gfap), and lipid binding (e.g., Lrp1) proteins. In addition, we identified synaptic terms in the astrocyte proteome. One explanation for this unexpected finding is the synaptic proteins were potentially phagocytosed by astrocytes since they actively contribute to synapse pruning and elimination23. Complementary to Camk2a-CIBOP, we characterized the regional proteomic differences within the astrocytes and found core protein signatures unique to the cortex and hippocampus, pons/medulla, cerebellum, and spinal cord. Sodium ion transport, biotin carboxylase activity, and pyruvate metabolic process encompassed the proteomic signature of cerebellar astrocytes while terms such as glutamate receptor activity, long term potentiation, and ion channel complex dominated the cortical and hippocampal astrocyte proteome. Alternatively, pontine and medullary proteomic signatures point to microtubule binding and regulation of neurotransmitter transport. Extracellular matrix, collagen, and protein heterodimerization were unique to the spinal cord. We were also able to distinguish cell type-specific proteomes by comparing astrocyte and neuronal proteomes. As expected, processes such as lipid metabolism, solute transport, mitochondrion, and metabolic activities were unique to the astrocyte proteome while synaptic transmission and long-term potentiation were relatively enriched in the neuronal proteome. Collectively, these data highlight the various roles of astrocytes within mouse brain regions under native states and how their proteome is distinct from neurons.

Using the adapted Luminex approach to directly measure biotinylated proteins of interest from brain homogenates, we found that several MAPK (e.g., pERK1/2 and pMEK1/2) and Akt/mTOR (e.g., pGSK3α and β) signaling phosphoproteins are highly abundant in neurons while majority of cytokines in the brain, with the exceptions of IL-10, IL-2, TNF-α, IL-1α, and RANTES were mostly of non-neuronal origin. Previous studies have shown that neurons may produce cytokines such as IL-2 and TNF-α under homeostatic conditions44–45. We also resolved differences in neuronal MAPK and Akt/mTOR signaling activation across brain regions, with cortical Camk2a neurons exhibiting the highest level of baseline activation Akt/mTOR signaling and striatal/thalamic neurons exhibiting highest level of MAPK. From a disease standpoint, recent studies have shown that increased MAPK protein expression and MAPK pathway activation (e.g., ERK signaling) are highly characteristic and potentially causal, in neurodegenerative diseases such as AD44–45, where they are associated with cognitive decline and pathological burden. MAPK signaling is also critical for synaptic mechanisms such as long-term potentiation and response to stress or injury46. Akt signaling activates the mTOR pathway that regulates mRNA translation, metabolism, and protein turnover, and is highly relevant to the pathogenesis of lysosomal storage disorders, genetic neurodevelopmental disorders, and neurodegenerative disorders46. The ability of CIBOP to label and quantify neuron-specific MAPK and Akt signals from whole brain can be highly relevant to understanding the spatio-temporal dynamics of these signaling pathways in disease pathogenesis. While our observed regional differences in neuron-derived cytokines are exploratory, the inverse correlation between MAPK and Akt signaling (high in the cortex but low in cerebellum) and cytokine levels (low in the cortex but high in the cerebellum), warrant further investigation47. Using the adapted Luminex approach to contrast neurons and astrocytes, we also found that neurons exhibit higher levels of basal signaling activity involving the MAPK and Akt pathways as compared to astrocytes, validating our mass spectrometry findings of higher levels of signaling proteins in neurons. The results presented in this manuscript also lay the foundation for future studies to determine whether neuroinflammatory stimuli, neuropathology and injury can alter signaling pathway activation and cytokine production in neurons and glia with spatial resolution.

In conclusion, we have generated and validated the Rosa26TurboID mouse line that enables CIBOP, a powerful experimental approach to investigate molecular changes occurring at the proteomic level while retaining the native state of the cell. Data obtained using this versatile in vivo system showcases breadth of proteomic labeling in neurons and astrocytes, regional Camk2a neuronal and astrocyte proteomic signatures, and neuron and astrocyte-specific phosphoprotein signaling (MAPK and Akt/mTOR) and cytokine signatures with relevance to several neurological disorders. These native state Camk2a neuronal and astrocyte proteomes represent key resources for the neuroscience community. This Rosa26TurboID mouse model and our validated experimental workflow also provide a framework to resolve cellular mechanisms underlying physiological or pathological conditions of complex tissues.

**Methods**

**Construct generation and cell culture studies.** The V5-TurboID-NE5_pCDNA3, a gift from Alice Ting48 (Addgene plasmid # 107169) was used to generate AsiS1-Kozak-V5-TurboID-NE5-stop-Mlu1 construct. This was then cloned into the pK26 CAG AsiS1/Mlu1 targeting vector, a gift from Ralf Kuehnl49 (Addgene plasmid # 74286), to generate the a Rosa26 (chromosome 6) targeting vector containing the CAG promoter, a floxed STOP site (loxP-STOP-loxp), and V5-TurboID with a nuclear export signal (TurboID-NE5). This Rosa26TurboID targeting vector was verified in- vitro for Cre-mediated TurboID expression and biotinylation in HEK293 cells (Supplementary Fig. 1a, b).
Human embryonic kidney 293 (HEK293 from ATCC CRC-1573) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo, 10090-145) and 1% penicillin-streptomycin (Lonza) in a 3% CO₂ atmosphere. For transient transfection, cells grown to 70–80% confluency in 6-well plates were transfected (Lipofectamine 3000, Thermo, L3000001) with 2.5 μg/well of Rosa26TurboID targeting vector and Cre plasmid (CMV-Cre, a gift from Dr. Xinqing Huang, Emory University), Rosa26TurboID targeting vector alone, or Cre plasmid alone according to manufacturer’s protocol. Untransfected cells also served as negative controls. Twenty-four hours post-transfection, cells were treated with 200 μM biotin for another 24 h. Subsequently, cells were rinsed with cold 1X phosphate buffered saline (PBS) and harvested in uraesis buffer (8 M urea, 10% triton X-100, 0.5 M NaH₂PO₄, pH 8.5) containing 1X HLTE protease inhibitor cocktail without EDTA (Thermo, R7786). The cells were sonicated for 3 rounds consisting of 5 s of active sonication at 25% amplitude with 10 s incubation periods on ice between sonication. Lyed cells were then centrifuged for 5 min at 15,000 × g and the supernatants were transferred to a new tube. Protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo, 23225). To confirm protein biotinylation, 10% of cell lysates were resolved on a 4–12% Bis-Tris gel, transferred onto a nitrocellulose membrane, and probed with streptavidin-

### Generation of the Rosa26TurboID mouse

The Rosa26TurboID targeting vector was electroporated into C57BL/6 embryonic stem (ES) cells (from Taconic, EC Cell Line # JMB012) at Texas A&M Institute for Genomic Medicine (TIGM). For confirmation of homologous recombination in ES clones, they were microinjected into albino germine (Ozgene) and implanted into pseudo pregnant (C57BL/6N female) mice, obtained from Taconic, using a mini-injector (Ismatec, 400R). Mice were given water supplemented with biotin (37.5 mg/L) for 2 weeks until euthanasia at 3 months of age. Consistent with previous publications, we have previously validated the Camk2a-CreERT2 line for neuron-specific labeling and non-leaky Cre activity.

### Animal studies

Approval from the Emory University Institutional Animal Care and Use Committee was obtained prior to all animal-related studies (IACUC protocols # PROTO201800252 and PROTO201710001). All mice used in the present study were housed in the Department of Animal Resources at Emory University under a 12 h light/12 h dark cycle with ad libitum access to food and water. Animals were housed in the vivarium under standard conditions for mice (temperature 72 °F, humidity range 40–50%). All procedures were approved by the Institutional Animal Care and Use Committee of Emory University and were in strict accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (NIH Publications 80–23, revised 1985).

AAV approach for Cre recombinase delivery to neurons in Rosa26TurboID mice: 2-month-old mice were anesthetized with isoflurane, given sustained-release buprenorphine subcutaneously (0.5 mg/kg), and immobilized on a stereotaxic apparatus. The mice were maintained on 1–buprenorphine subcutaneously (0.5 mg/kg) and 1% penicillin-streptomycin at 3% CO₂ atmosphere. For transient transfection, cells grown to 70–80% confluency in 6-well plates were transfected (Lipofectamine 3000, Thermo, L3000001) with 2.5 μg/well of Rosa26TurboID targeting vector and Cre plasmid (CMV-Cre, a gift from Dr. Xinqing Huang, Emory University), Rosa26TurboID targeting vector alone, or Cre plasmid alone according to manufacturer’s protocol. Untransfected cells also served as negative controls. Twenty-four hours post-transfection, cells were treated with 200 μM biotin for another 24 h. Subsequently, cells were rinsed with cold 1X phosphate buffered saline (PBS) and harvested in uraesis buffer (8 M urea, 10% triton X-100, 0.5 M NaH₂PO₄, pH 8.5) containing 1X HLTE protease inhibitor cocktail without EDTA (Thermo, R7786). The cells were sonicated for 3 rounds consisting of 5 s of active sonication at 25% amplitude with 10 s incubation periods on ice between sonication. Lyed cells were then centrifuged for 5 min at 15,000 × g and the supernatants were transferred to a new tube. Protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo, 23225). To confirm protein biotinylation, 10% of cell lysates were resolved on a 4–12% Bis-Tris gel, transferred onto a nitrocellulose membrane, and probed with streptavidin-

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regions (excluding hippocampus), which resulted in \( n = 4 \) region and \( n_{\text{total}} = 18 \), while the control Camk2a brain regions did not contain technical replicates (Fig. 3a, b). Only data from regions that contained at least 11 valid intensity values were filtered to contain at least 11 valid intensity values in each group, \( n_{\text{total}} = 22 \). The MaxQuant output data from the transgenic cohort 1 and 2 brain regions were first filtered to contain at least nine valid intensity values in the transgenic IP samples and then processed as described above with Perseus (Supplementary Data 9). Lastly, the MaxQuant output data from the transgenic cohort 2 brain regions were then filtered to contain at least 11 valid intensity values in each group, \( n_{\text{total}} = 22 \). The MaxQuant output data from the transgenic cohort 1 (Camk2a only) brain regions were first filtered to contain at least nine valid intensity values in the transgenic IP samples and then processed as described above with Perseus (Supplementary Data 9).

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streptavidin Alexa-flour 488 (1:500, Thermo, S12123) for 1 h at room temperature. After primary antibodies, the sections were rinsed 3x in TBS containing 0.5% BSA at room temperature for 10 min each. Then, the sections were incubated in the appropriate fluorophore-conjugated secondary antibody (1:500; donkey anti-mouse Alexa Fluor 594, Thermofisher, A-21203; donkey anti-rabbit Alexa Fluor 647, Thermofisher, A-32795) and streptavidin Alexa-flour 488 at room temperature for 10 min each. The sections were incubated with DAPI for 10 min, washed 3x in TBS for 10 min, dried, and cover slipped with ProLong Diamond Antifade Mountant (Thermofisher, P36965). Sections were imaged as z-stacks on a Nikon A1R HD5 inverted confocal microscope with a 40x objective (NA 1.3) using NIS-Elements Imaging software. Images were processed and maximum intensity projections were created using Image J.

Quantification of cytokines and signaling phospho-protein levels in brain homogenates. Luminesex multiplexed immunassays were used to quantify phospho-proteins within the MAPK (Millipore 48-660MAG) and PI3K/Akt/mTOR (Millipore 48-612MAG) pathways as well as 32 panel of cytokine/chemo-homogenates assayed using Image J software.

Confocal microscopy. 30 μm thick free-floating brain sections were washed, blocked and permeabilized by incubating in TBS containing 0.25% Triton X-100 and 5% horse serum for 1 h at room temperature. After primary antibodies, the sections were rinsed 3x in TBS containing 0.5% horse serum at room temperature for 10 min each. Then, the sections were incubated in the appropriate fluorophore-conjugated secondary antibody (1:500; donkey anti-mouse Alexa Fluor 594, Thermofisher, A-21203; donkey anti-rabbit Alexa Fluor 647, Thermofisher, A-32795) and streptavidin Alexa-flour 488 at room temperature for 10 min each. The sections were incubated with DAPI for 10 min, washed 3x in TBS for 10 min, dried, and cover slipped with ProLong Diamond Antifade Mountant (Thermofisher, P36965). Sections were imaged as z-stacks on a Nikon A1R HD5 inverted confocal microscope with a 40x objective (NA 1.3) using NIS-Elements Imaging software. Images were processed and maximum intensity projections were created using Image J.

Acute hippocampal slice preparation. Acute hippocampal slices were prepared from 3-month-old Rosa26TurboID/fl/ and Camk2a mouse or littermate control Rosa26TurboID/fl after receiving tamoxifen and bixin supplementation. Mice were first anesthetized and perfused with ice-cold cutting solution (in mM) 87 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 7.5 MgCl2, 0.5 CaCl2, 10 glucose, and 7 sucrose. Thereafter, the brain was immediately removed by dissection. Brain slices (300 μm) were sectioned in the coronal plane using a vibrating blade microtome (Leica Biosystems, VT1200S) in the same solution. Slices were transferred to an incubation chamber and maintained at 34 °C for 30 min and then at room temperature (23–25 °C). During whole-cell recordings, slices were continuously perfused with (in mM) 124 potassium gluconate, 2 KCl, 9 HEPS, 4 MgCl2, 4 NaATP, 3 L-Ascorbic Acid, and 0.5 NaGTP. Pipette capacitance was neutralized in all recordings and electrode series resistance compensated using bridge balance in current-clamp mode. Recordings with series resistance > 20 MΩ were discontinued. Constant current injection maintained the membrane potential of pyramidal neurons during whole cell recordings at −65 mV. Action potentials trains were initiated by somatic current injection (300 ms). To measure passive parameters in each recording, a −20 pA current step was utilized. For analysis of all passive and active parameters, Clampfit software (Molecular Devices) and custom python scripts were utilized.

Electrophysiology. Pyramidal neurons were targeted for somatic whole-cell recording in the CA3c region of hippocampus using gradient-contrast video-microscopy on custom-built or commercial (Bruker) upright microscopes. This region was selected based on high level of Camk2a expression in these neurons32. Electrophysiological recordings were obtained using Multiclamp 700B amplifiers (Molecular Devices). Signals were filtered at 10 kHz and sampled at 50 kHz using a Digidata 1440B (Molecular Devices) digitizer. For whole-cell recordings, borosilicate patch pipettes were filled with an intracellular solution containing (in mM) 124 potassium gluconate, 2 KCl, 9 HEPS, 4 MgCl2, 4 NaATP, 3 L-Ascorbic Acid, and 0.5 NaGTP. Pipette capacitance was neutralized in all recordings and electrode series resistance compensated using bridge balance in current-clamp mode. Recordings with series resistance > 20 MΩ were discontinued. Constant current injection maintained the membrane potential of pyramidal neurons during whole cell recordings at −65 mV. Action potentials trains were initiated by somatic current injection (300 ms). To measure passive parameters in each recording, a −20 pA current step was utilized. For analysis of all passive and active parameters, Clampfit software (Molecular Devices) and custom python scripts were utilized.

Data availability
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD027488 and PXD027489. The MassIVE database (downloaded from https://www.proteomecentral.uniprot.org/help/reference_proteome) was used for searches of mass spectrometry data. Source data are provided with this paper.

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The authors declare no competing interests.

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