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Labeling and identifying cell-type-specific proteomes in the mouse brain

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Abstract

We develop an approach to tag proteomes synthesized by specific cell-types in dissociated cortex, brain slices, and the brain of live mice. By viral-mediated expression of an orthogonal pyrrolysyl-tRNA synthetase/tRNA$_{XXX}$ pair in a cell type of interest and providing a non-canonical amino acid with a chemical handle, we selectively label neuronal or glial proteomes. The method enables the identification of proteins from spatially and genetically defined regions of the brain.

Determining the proteomes of specific cells is a particular challenge in the mammalian central nervous system (CNS), where cell types form a network with dense chemical, electrical and physical connectivity1, 2. Efforts to elucidate proteomes of specific cell types in the CNS have used i) primary cell cultures or ii) cells that are acutely separated from tissue, by *ex vivo* manipulations, and purified on the basis of cell surface markers, for analysis3. However, the resulting cells are no longer in their physiological network, and may lose cellular structures (and their associated proteins), as well as chemical, electrical and physical connectivity3, 4. Thus, isolated and cultured cells are likely to have adapted proteomes; the proteomes of cultured cells and acutely isolated cells are clearly different from each other3, and it is unclear how these proteomes relate to the proteome *in vivo*.

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Data availability statement

The data sets generated or analyzed during the current study are available from the corresponding author upon reasonable request. The mass spectrometry data generated in this study has been deposited in the PRIDE database with accession number PXD008380. AAV vectors are available from the corresponding author upon reasonable request.

Author contributions

J.W.C. defined the direction of research. T.P.K. T. S.E. and R.J.E. designed all experiments. R.J.E. and V.B. designed the AAV vectors. V.B. performed the experiments in rat cortical preparations. T.P.K. performed the SCN slice experiments under the direction of M.H.H. L.C. performed experiments in live mice under the direction of M.G.S., E.C. performed experiments in live mice under the direction of M.T. T.S.E. provided ncAAs, analyzed lysates from live mice by fluorescent labeling and SDS-PAGE, performed the enrichments, and prepared samples for MS/MS. J.W.C., T.P.K. and R.J.E. wrote the paper with input from all authors.

Competing financial interests

The authors declare no competing financial interest.

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We previously reported stochastic orthogonal recoding of translation (SORT) for cell-type specific tagging of proteomes, to enable labeling and identification of proteins from targeted cells without dissection5, 6. In SORT an orthogonal pyrrolysyl-tRNA synthetase-tRNA$_{XXX}$ pair (where XXX indicates an anticodon sequence, and the pair is derived from *Methanosarcina* species, most commonly *M. Mazei* or *M. barkeri*) is expressed in the cell of interest. This pair recognizes an added noncanonical amino acid (ncAA) substrate containing a bioorthogonal cyclopropene group (CypK), and decodes a specific sense codon in competition with an endogenous aminoacyl-tRNA synthetase-tRNA$_{XXX}$ pair, leading to stochastic and low level tagging of the proteome with CypK. The tagged proteome can be labelled (SORT-M) or enriched and identified (SORT-E) (Supplementary Fig. 1). We have demonstrated SORT in *Escherichia coli*, cultured mammalian cells, and *Drosophila melanogaster* 5, 6, but not in any live vertebrate.

The CypK used for SORT in cultured cells and flies is prohibitively expensive for large animal experiments. We set out to develop SORT with Nε- (propargyloxycarbonyl)-L-lysine, AlkK (**1,** see Supplementary Fig. 2 for chemical structures), a substrate for PylRS7 that is 50 times cheaper than CypK to synthesize, metabolically stable in the mouse, and available in the mouse brain through drinking water8. We optimized selective labeling of an AlkK-tagged *E. coli* proteome with an Azide-AlexaFluor 647 conjugate (Az-AF647) (Supplementary Fig. 3).

We constructed AAV vectors in which a mCherry-P2A-*Mm*PylS genetic fusion is expressed from the neuron-specific, human synapsin 1 (hSyn1) promoter9, and $PylT_{CAU}$ (encoding Pyrrolysyl-tRNA$_{CAU}$) is expressed from the U6 promoter (Supplementary Fig. 4)8. We transduced primary dissociated rat neocortex, containing neurons and glia, with our hSyn1 mCherry-P2A-*Mm*PylS/PylT$_{CAU}$ (hSyn1-SORT$_{CAU}$) AAV in the presence of AlkK (Fig. 1A). Following transduction, mCherry was clearly visible in neurons (NeuN positive cells) but not in GFAP positive glia (Fig. 1B). We observed selective fluorescent labeling of this co-culture with Az-AF647 that was dependent on AlkK; labeling co-localized with both NeuN and mCherry, but not GFAP (Fig. 1B). Moreover, a lysate from the co-culture receiving AlkK was selectively labelled with Az-AF647. (Supplementary Figs. 5).

We transduced slices of the mouse suprachiasmatic nucleus (SCN)8 with the hSyn-SORT$_{CAU}$ AAV, added AlkK, and confirmed subsequent AAV-dependent mCherry expression (Fig. 1C). We selectively labelled tagged proteins with Az-AF647 (Fig. 1D. and Supplementary Fig. 6 for all full gels). We demonstrated that SORT can be extended to tagging proteomes in a set of glial cells (astrocytes and ependymal cells10, 11) by creating a GFAP mCherry-P2A-*Mm*PylS/$PylT_{CAU}$ (GFAP-SORT$_{CAU}$) AAV (Fig. 1C, D and Supplementary Fig. 4). GFAP-driven SORT led to the expected mCherry expression pattern in the SCN12 (Fig. 1C), and the GFAP positive proteome was labelled at a comparable level to the neuronal proteome (Fig. 1D). Circadian oscillations can be followed in SCN slices derived from Period2::LUC mice by bioluminescence, and these oscillations are known to be sensitive to cellular, electrical, or signalling perturbations of the SCN network8. We found that Per2::LUC organotypic SCN slices (slices that maintain their function in culture) that were transduced with hSyn I-SORT$_{CAU}$ or GFAP-SORT$_{CAU}$ AAVs and supplemented with AlkK retained robust circadian rhythms (Fig. 1E).

Unilateral stereotactic injections of the hSyn1-$SORT_{CAU}$ AAV into the corpus striatum of right hemisphere of adult mice were followed one week later by addition of AlkK to the drinking water. Coronal sections from the brains of the mice, after one week on AlkK, revealed asymmetric mCherry fluorescence, primarily localized to the right hemisphere (Fig. 2A, Supplementary Fig. 7A). mCherry fluorescence co-localized with NeuN but not with GFAP (Fig. 2B). We labelled protein extracts from the striatum of each hemisphere with Az-AF647 and observed selective labeling of the proteome from the right hemisphere (Fig. 2C). The efficiency of SORT tagging in mice was comparable to that in *E. coli* (Supplementary Fig. 8).

We compared the total striatal proteome of control mice, and mice subject to SORT tagging in both hemispheres (Supplementary Fig. 9). We found a strong correlation (Pearson correlation coefficient= 0.98) between the proteomes, comparable to the correlation between independent SORT proteomes (0.98) or the correlation between independent control proteomes (0.98). Similarly, we found an excellent correlation (0.98) between the proteomes of mice fed AlkK or injected with the hSyn1-$SORT_{CAU}$ AAV and the control proteome. The mice subject to SORT were healthy and maintained their body weight throughout the study (Supplementary Fig. 9).

We also carried out unilateral stereotactic injections of the hSyn1-$SORT_{CAU}$ or GFAP-$SORT_{CAU}$ AAV into the hippocampus in the right hemisphere of adult mice (Supplementary Fig. 7B, Supplementary Fig. 7C). We observed selective labeling of the proteome from the right hemisphere of the hippocampus via SORT-M, co-localizing either with neurons or GFAP positive cells (Supplementary Fig. 10), and demonstrating the approach can be used in different regions of the brain.

Numerous mouse strains have been created in which Cre recombinase is expressed in targeted cell types in the mouse brain13. We generated the hSyn1-FLEX-$SORT_{CAU}$ AAV (Supplementary Fig. 4), which facilitates the irreversible Cre-mediated flipping of an inverted mCherry-P2A-*Mm*PylS gene cassette, for expression from the upstream promoter14, 15. We observed SORT tagging when we injected the hSyn1-FLEX-$SORT_{CAU}$ AAV into the let striatum of Vgat-Cre mice (expressing Cre in inhibitory GABAergic neurons) (Fig. 2D), but not in mice lacking Cre recombinase. We also injected the hSyn1-FLEX-$SORT_{CAU}$ AAV into the hippocampus of the right hemisphere in Vgat-Cre mice and Vglut-Cre mice (expressing Cre subset of excitatory neurons) and observed proteome tagging in both cases (Fig. 2E-J and Supplementary Fig. 7D, E).

Next we tagged the proteome of neurons in the striatum, expecting to primarily tag proteins from medium spiny neurons (MSNs) involved in the post-synaptic side of dopaminergic synapses and glutamatergic synapses, and both the pre-synaptic and post-synaptic side of GABAergic synapses, as well as, potentially, interneurons16 (Supplementary Fig. 11). We developed a SORT-E protocol for AlkK tagged proteomes (Supplementary Fig. 12). We prepared lysates from the striatum of brains of mice bilaterally injected with the hSyn1-$SORT_{CAU}$ AAV and provided with AlkK, covalently labelled the resulting proteome with an azide-diazobenzene-biotin conjugate (**2**)17, 18 (Supplementary Fig. 2), captured the proteome on streptavidin beads, washed extensively, and then cleaved the diazobenzene

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linker to provide a SORT-E output (Supplementary Fig. 1). We observed clear enrichment of the SORT-E output with respect to non-tagged control outputs (Fig. 2K).

By tandem mass spectrometry (MS/MS) analysis we assessed the relative abundance of each protein in the SORT-E output to its abundance in the control output (Fig. 2L, Supplementary Data 1). For 96% of the 2119 proteins captured this ratio is >1, demonstrating the specificity of the enrichment protocol for SORT tagged proteins. We identified 1789 proteins, defined as SORT-E IDs, that are enriched in the SORT tagged proteome compared to the control (1604 proteins, S_0:1, false-discovery rate (FDR) < 0.05), or are found in three independent replicates of the SORT tagged proteome, but only zero or one time in six independent controls (185 proteins) (Supplementary Data 1). In contrast we found only 24 proteins that are enriched in the control proteome (7 proteins, S_0:1, FDR<0.05), or are only found in the control proteome (17 proteins). The SORT-E IDs define a set of proteins from the genetically targeted striatal neurons (Fig. 2L).

Established neuronal markers, are enriched in the SORT-E output, as are canonical markers of MSNs (Fig. 2L, Supplementary Table 1). Neuronal proteins were significantly enriched in SORT-E IDs (P(neuron part)= 2.8×10^{-7}), but not in SORT-E NS (P(neuron part)=0.17), with respect to the total striatal proteome (Fig. 2L). SORT-E IDs include several markers of GABAergic synapses, as well as established markers of the post-synaptic side of glutamatergic synapses and dopaminergic synapses (Supplementary Table 1, Supplementary Fig. 13)

The majority of glial markers are not enriched in the SORT-E output, consistent with the low expression of many glial markers in neurons (Fig. 2L), and most glial markers are de-enriched by SORT-E with respect to the striatal proteome (Fig. 2M). We found that proteins identified as glial-specific, in glia isolated from mouse brains, but not those identified as glial specific, from cultured glia3, are significantly de-enriched by SORT-E (P=3.38×10^{-5} and P=0.17, respectively) (Fig. 2N, O).

We have demonstrated a modular approach to co-translational proteome tagging in specific cells in primary culture, in mouse organotypic tissues slices and in the brains of live mice. Our approach complements a very recently described approach for cell specific proteome tagging19. Because our SORT approach uses viral injection in combination with specific promoters it provides independent special and genetic control of proteome tagging. Extensions of SORT may introduce additional chemistries into the mouse proteome20, including photo-crosslinkers to interrogate cell-type specific protein interactions, and may be extended to additional codons to provide greater proteome coverage5.

Online Methods

Additional information can be found in the "Life Sciences Reporting Summary" that accompanies this manuscript.

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Materials

AAV serotype 1 viral vectors were obtained commercially from the Penn Vector Core. All animal work was licensed under the UK Animals (Scientific Procedures) Act of 1986 with local ethical approval (MRC AWERB, Cambridge University). At least two mice were used for every condition tested, mice were not excluded, no randomization or blinding was used.

Chemical synthesis

All solvents and chemical reagents were purchased from Sigma-Aldrich or Fisher Scientific, and used without any further purification. The synthesis of Nε- (tert-butyloxycarbonyl)-L-lysine, "AlkK", (amino acid **1**) was previously reported6. The synthesis of Azide diazobenzene biotin, "ADB", (compound **2**) was as reported17.

Proteomic incorporation of AlkK via SORT into *E. coli* proteome

Chemically competent *E. coli* DH10B cells (50 μL) were doubly transformed with pBAD_wtT4L_MbPylT-AGA plasmid6 (2 μL, necessary for expression of PyltRNA-AGA) and pBK-MbPylS plasmid6 (2 μL necessary for expression of PylRS). Transformed cells were recovered in 1 ml S.O.B. (supplemented with 0.2% glucose) for 1 h at 37 °C. 100 μL of the recovery was used to inoculate 50 ml LB-KT (50 μg/ml kanamycin and 25 μg/ml tetracycline) and the cultures incubated overnight (37 °C, 250 r.p.m.). Overnight cultures were diluted to OD600 0.1 with LBKT½ (as LBKT, but with half the concentration of antibiotics) and divided into two 200 mL cultures. One culture was supplemented with AlkK (1 mM final concentration) and other with H_2O (200 μL). Cultures were then incubated (37 °C, 250 r.p.m.) for 6 h. Cells were harvested by centrifugation (4,000 r.p.m., 4 °C, 20 min) and then washed two times by resuspending in 25 mL of ice cold PBS, and collecting by centrifugation (4,000 r.p.m., 4 °C, 20 min) between each wash. The final cell pellets were immediately frozen for storage.

Optimised SORT-M labeling of *E. coli* protein lysates

Frozen cell pellets were thawed and resuspended in 8M urea (PBS, pH 7.8, 5 mL). Cells were lysed on ice using a microtip sonicator (amplitude 15, 11 m total sonication time, Soniprep 150 plus). Protein concentrations were determined by BCA assay (Pierce). For a typical SORT-M experiment 100 μL of protein lysate (~10 mg/mL) was first reduced with 3 mM DTT (3 μL of 100 mM stock solution, 1 hr at RT) and alkylated in 16.5 mM iodoacetamide (16.5 μL of 100 mM stock solution, 1 hr at RT in dark). The lysates were then ligated with the addition of 100 μM Azide-AlexaFluor647 dye (2 μL of 5 mM stock solution in DMSO, ThermoFisher), 5 mM aminoguanidine (5 μL of 100 mM stock solution) and freshly made 50X "click mix" (2 μL of 50 mM $CuSO_4$, 50 mM TBTA (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) and 100 mM sodium ascorbate made in 1:1 DMSO:H_2O, final concentration of Cu, TBTA and ascorbate in reaction should be 1, 1, and 2 mM respectively). The samples were then incubated at room temperature for 4 hrs or overnight with end-over-end rotation (Hula mixer). Reactions were quenched with the addition of 1 mM AlkK **1**. 2 μL aliquots (20 μg of protein) were used for 4-12% Bis-Tris SDS-Page gels and imaged on a Typhoon gel imager (excitation 633 nm/emission 670 nm).

The gels were then stained with Coomassie stain (Expedeon) and imaged on a BioRad Gel Doc XR System.

Plasmid construction

First, the hU6-$PylT_{CAU}$ gene was cloned by standard molecular biology protocols in a divergent orientation at the *XbaI* site upstream of the human Synapsin 1 promoter in pAAV-hSyn1-hChR2(H134R)-EYFP (Addgene #26973). Into this backbone, the mCherry::P2A::MmPylS, coding sequence was cloned between the *BamHI* and *EcoRI* sites. The GFAP mCherry-P2A-*Mm*PylS/$PylT_{CAU}$ was cloned by promoter replacement, the GFAP promoter refers to GfaABC1D promoter throughout. The hSyn1 FLEX mCherry-P2A-*Mm*PylS/$PylT_{CAU}$ (hSyn1-FLEX-$SORT_{CAU}$) was constructed by transferring *loxP* and *lox2272* sites from pAAV-hSyn-DIO-HA-hM3D(Gq)-IRES-mCitrine (a gift from Bryan Roth, Addgene plasmid #50454) into the hSyn1-$SORT_{CAU}$ plasmid via 4-piece Gibson assembly.

Primary culture of dissociated rat cortical neurons and glial cells

Dissociated rat cortical neurons (RCNs) were prepared as reported previously8. Brains of E17 fetuses from time-mated rats were dissected, trypsinised in presence of 1% (wt/vol) DNAse I (HT Biotechnology) and triturated with a 1 ml Gilson pipette. Dissociated cells were seeded at density of 20,000-50,000 cells/cm^2 onto poly-l-lysine coated coverslips or poly-l-lysine coated polystyrene plates. The cells were cultured in Neurobasal media supplemented with B27 and GlutaMAX (ThermoFisher) at 37°C under 5% CO_2 atmosphere. The cells were initially seeded in presence of 2.5% fetal bovine serum, switching to serum-free media after 24 hours. Subsequently, half of the media was exchanged every 5 days.

SORT in dissociated rat cortical neurons and glial cells

Dissociated cultures were transduced after culturing for 3 days by addition of 1-2 μL (~10^{12}-10^{13} transducing units) of pSynI-mCherry::P2A::MmPylS/$PylT_{CAU}$ AAV particles into a single well of 6-well plates or 8-well coverslips, and the media was supplemented with 1 mM AlkK **1** at this time. Half of the media was exchanged every 5 days with or without the corresponding amino acid. The cells were analyzed 14 days post transduction. For lysate analysis, cells were washed with HBSS and incubated for 30 mins on ice in RIPA buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.1% SDS; 1% Triton X-100; 1% Deoxycholate) supplemented with protease inhibitor cocktail (Roche). The lysates were centrifuged for 10 min at 20,000 x *g* (4°C), collecting the supernatant for analysis. For labeling and immunofluorescence, the cells were washed once with HBSS (ThermoFisher) and fixed with 4% (v/v) paraformaldehyde in HBSS.

Labeling of fixed cells, immunofluorescence and confocal imaging

Fixed cells were permeabilized by 0.5% Triton-X100 and blocked with 3% BSA for 1 hr at room temperature. For click labeling, 5 mM aminoguanidine (Sigma) and 5 uM Azide-AlexaFluor647 dye (ThermoFisher) solution was added to the cells, followed by addition of 1mM $CuSO_4$ (Sigma), 1mM sodium ascorbate (Sigma) and 1 mM TBTA (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine). The cells were incubated in the reaction

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mixture for 1 hr at room temperature and then washed with PBS. The cells were subsequently immunostained with anti-NeuN (Abcam, ab177487) and anti-GFAP (Abcam, ab4674) antibodies (1:300 dilution in 3% BSA) overnight at 4°C, washed again, and then stained for 1 hr at room temperature with corresponding secondary antibodies (A-31556 and A-11039, ThermoFisher) (1:300 dilution in 3% BSA). Confocal fluorescence images were acquired on a Leica SP8 inverted confocal microscope with appropriate filter settings. The presented images were created as maximum projections (in Z-axis) from Z-stacks stepping through the entire cell height.

SORT-M in SCN Slices

SCN organotypic slices were prepared from 7-9 day old C57/BL6 or Per2::LUC pups as reported previously3. Slices were transduced with 1 μL (~10^{12}-10^{13} transducing units) of hSyn1-mCherry::P2A::MmPylS/PylT$_{CAU}$ AAV particles. After two weeks slices were transferred to culture media with 15 mM AlkK **2**. The slices were imaged after one week of SORT labeling on a Leica DM IL LED inverted fluorescent microscope with QCapture Suite PLUS software. After imaging, the SCN slices were subsequently lysed by sonication in 8 M urea (PBS, pH 7.8) for subsequent fluorescent labeling of AlkK containing proteins. Protein concentrations were determined by BCA assay (Pierce).

Bioluminescence recordings of Per2::Luciferase SCN slices

Seven days after dissection of Per2::Luciferase SCN slices from the same litter, the slices were transduced with hSyn1-mCherry::P2A::MmPylS/PylT$_{CAU}$ or the corresponding GFAP AAV, transferred to a medium with 1 mM luciferin and placed into photo-multiplier tubes to record circadian oscillation as a bioluminescence signal as previously described8. After two weeks the medium was supplemented with 15 mM AlkK **1** and the bioluminescence signal was recorded for the successive 7 days during SORT protein labeling.

SORT in live mice

Adult male or female C57/BL6J, Vgat-Cre (JAX 016962) and Vglut-Cre (JAX 016963) mice aged 10 weeks, weighing between 18-26 g were housed on a 12 hour light/dark cycle in cages with *ad libitum* access to food and water. No randomization or blinding was uses. The only exclusion criteria for animals were health issues. For viral injections, animals were anesthetized with 2% isofluorane and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The mice received either unilateral or bilateral stereotactic injections of 2 μl of AAV1.hSyn1-mCherry::P2A::MmPylS/PylT$_{CAU}$, AAV5.GFAP-mCherry::P2A::MmPylS/PylT$_{CAU}$ or AAV1. hSyn1-FLEX-mCherry::P2A::MmPylS/PylT$_{CAU}$ particles (2.0 μL of ~5 x 10^{12} GC/mL; ~1 x 10^{10} viral particles) into the region of the striatum (-1.7 mm medio-lateral, +0.7 mm anterior, -3.6 mm dorsal relative to bregma) or the dorsal hippocampus (-2.0 mm medio-lateral, -2.55 mm anterior, -2.0 mm dorsal relative to bregma). Injections were performed using a 5 μl Hamilton syringe with a 33 gauge needle and a microsyringe pump controller (Micro4, World Precision Instruments) at flow rate of 0.5 μl/min. The needle was left in place at the injection site for 2 min before being slowly withdrawn. Two weeks after the surgery, the mice received AlkK, **1,** administered orally in the drinking water (15 % black currant-flavored water containing 30

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mg/mL AlkK **1**) or control drink with no AlkK **1** for one additional week. After the seven days of AlkK treatment, the mice were sacrificed, and the brains were either post-fixed in 4% paraformaldehyde and preserved in 30% sucrose for sectioning or dissected to remove the striatum or hippocampus, which was frozen on dry-ice for further analyses. To assess the effect of the different components of the SORT labeling system, we injected mice with hSyn1-SORT AVV and fed them AlkK for a week ”SORT-E” while in parallel creating three control groups: mice only injected with AAV ”AAV”, fed only AlkK “AlkK” or neither AAV nor AlkK “Control”. The striatal tissues from all four groups were lysed in 8M urea and separated on SDS-PAGE before subjecting the gel slices to LC-MS/MS,

Immunofluorescence microscopy and confocal imaging of brain sections

Fixed and cryopreserved brains (unilaterally injected) were cut into 40 μm coronal sections on a freezing microtome. For observation of mCherry fluorescence (PylS expression) throughout the brain, coronal sections from anterior to posterior were mounted with hardset VECTASHIELD H-1500 with DAPI (Vectorlabs) and images were acquired with a 4x objective on a Nikon Eclipse TE2000 microscope and processed in ImageJ (NIH). Immunostaining for co-localization was performed by overnight incubation with monoclonal rabbit anti-NeuN (Abcam, AB177487; 1:250) and polyclonal chicken anti-GFAP (MerckMillipore, AB5541; 1:500) and mounted with VECTASHIELD H-1500 with DAPI (Vectorlabs). Confocal images were acquired on a Zeiss LSM 710 confocal microscope and processed in ImageJ (NIH).

Fluorescent labeling of protein lysates

Cell, SCN and brain lysates (Striatal and Hippocampal) were first reduced in 3 mM DTT (1 hr at RT) and then alkylated in 16.5 mM iodoacetamide (30 min at RT in dark). For SORT-M using AlkK, the lysates were then supplemented with 100 μM Azide-AlexaFluor647 (ThermoFisher), 5 mM aminoguanidine, and “click mix” (1 mM $CuSO_4$, 1 mM TBTA (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) and 2 mM sodium ascorbate (final concentrations), made as 50X mix in 1:1 DMSO:H_2O). The samples were then incubated at room temperature for 4 hrs or overnight. All labeled samples were separated on 4-12% Bis-Tris SDS-Page gels and imaged on a Typhoon gel imager (excitation 633 nm/emission 670 nm). The gels were then stained with Coomassie stain (Expedeon) and imaged on a BioRad Gel Doc XR System.

Optimised SORT-E for *E. coli* lysate

Frozen cell pellets were thawed and resuspended in 8M urea (PBS, pH 7.8, 5 mL). Cells were lysed on ice using a microtip sonicator (amplitude 15, 11 m total sonication time, Soniprep 150 plus). Protein concentrations were determined by BCA assay (Pierce). For a typical SORT-E experiment 4 mL of protein lysate (~10 mg/mL) was first reduced with 3 mM DTT (24 μL of 500 mM stock solution, 1 hr at RT) and alkylated in 16.5 mM iodoacetamide (66 μL of 1 M stock solution, 1 hr at RT in dark). The lysates were then ligated with the addition of 100 μM azide-diazobenzene-biotin **2** (8 uL of 5 mM stock solution in DMSO), 5 mM aminoguanidine (20 μL of 1 M stock solution) and freshly made 50X “click mix” (80 μL of 50 mM $CuSO_4$, 50 mM TBTA (tris[(1-benzyl-1H-1,2,3-triazol-4-

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yl)methyl]amine) and 100 mM sodium ascorbate made in 1:1 DMSO:H_2O, final concentration of Cu, TBTA and ascorbate in reaction should be 1, 1, and 2 mM respectively). The samples were then incubated at room temperature overnight with end-over-end rotation (Hula mixer). Reactions were quenched with the addition of 1 mM AlkK **1**. 10 μL aliquots were taken at this stage as the input sample for subsequent SDS-PAGE analysis. The samples were precipitated by the addition of 10 volumes of ice-cold methanol and incubated at -20 °C overnight. Precipitated protein was collected by centrifugation (3200 rcf), and the supernatant decanted. The protein pellets washed with ice-cold methanol (2 x 25 mL), collected by centrifugation (3200 rcf), and re-dissolved in urea buffer (5 mL, 8 M urea, PBS, pH 7.8). 100 μL of high capacity streptavidin beads slurry (Thermo scientific) were added to the re-dissolved proteins and incubated with end-over-end rotation (Hula mixer) for 1.5 hours, then collected by filtration through a spin column (Mini Bio-spin® chromatography column Bio-Rad 731-1550). Unbound proteins were washed away by suspending beads in 1% SDS in PBS (500 μL) with 2 minute end-over-end rotation (Hula mixer), and then collecting the beads by a short centrifugation (1000 rpm, 5 second pulse). This washing process was repeated 5 times. An aliquot of the final wash was kept for analysis by SDS-PAGE. Specifically bound proteins were then eluted by resuspending the beads in 1% SDS in PBS supplemented with 50 mM $Na_2S_2O_4$ (100 μL) and incubating at room temperature for 30 minutes with end-over-end rotation (Hula mixer). The supernatant was collected by centrifugation (1000 rpm, 5 second pulse). The elution was repeated two more times (50 μL), and the fractions pooled. 10% of the elutant was used for analysis by SDS-PAGE.

SORT-E from the striatal tissue of live mice

Striatal tissue from five brains (bilaterally injected, n=3) were pooled and lysed in 1.5 mL of 8 M urea (PBS, pH 7.8) and homogenized with a disposable pestle. The tissue homogenates were sonicated in a water bath and subsequently cleared by centrifugation. Protein concentrations were determined by BCA assay (Pierce), and 1.3 mL of mouse striatal protein lysate (~8 mg/mL) was reduced with 3 mM DTT (1 hr at RT) and alkylated in 16.5 mM iodoacetamide (30 min at RT in dark). The lysates were then supplemented with 100 μM azide-diazobenzene-biotin **2**, 5 mM aminoguanidine and "click mix" (1 mM $CuSO_4$, 1 mM TBTA (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) and 2 mM sodium ascorbate (final concentrations), made as 50X mix in 1:1 DMSO:H_2O). The samples were then incubated at room temperature for 4 hrs with end-over-end rotation (Hula mixer). Reactions were quenched with the addition of 1 mM AlkK **1**. 10 μL aliquots were taken at this stage as the input sample for subsequent SDS-PAGE analysis. The samples were precipitated by the addition of 10 volumes of ice-cold methanol and incubated at -20 °C overnight. Precipitated protein was collected by centrifugation (3200 rcf), and the supernatant decanted. The protein pellet was wash with ice-cold methanol (5 mL), collected by centrifugation (3200 rcf), and re-dissolved in urea buffer (1.5 mL, 8 M urea, PBS, pH 7.8). 50 μL of high capacity streptavidin beads settled resin (Thermo scientific) were added to the re-dissolved proteins and incubated with end-over-end rotation (Hula mixer) for 1.5 hours, then collected by filtration through a spin column (Mini Bio-spin® chromatography column Bio-Rad 731-1550). Unbound proteins were washed away by suspending beads in 1% SDS in PBS (500 μL) with 2 minute end-over-end rotation (Hula mixer), and then collecting the beads by

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a short centrifugation (1000 rpm, 5 second pulse). This washing process was repeated 15 times. An aliquot of the final wash was kept for analysis by SDS-PAGE. Specifically bound proteins were then eluted by resuspending the beads in 1% SDS in PBS supplemented with 50 mM $Na_2S_2O_4$ (50 μL) and incubating at room temperature for 30 minutes with end-over-end rotation (Hula mixer). The supernatant was collected by centrifugation (1000 rpm, 5 second pulse). The elution was repeated three times, and the fractions pooled. 10% of the elutant was used for analysis by SDS-PAGE. The remaining elutant was concentrated to ~30 μL using a centrifugal filter unit (3000 kDa cut-off amicon), and loaded into a 10% SDS-PAGE gel (15 min run) before each lane was cut into two bands for LC-MS/MS analysis.

LC-MS/MS analysis (striatal SORT-E)

Each gel slice was subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd.) to minimise manual handling. The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system in line with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). In brief, peptides in 1% (vol/vol) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (vol/vol) acetonitrile 0.1% (vol/vol) formic acid, peptides were resolved on a 250 mm × 75 μm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1 min., 6-15% B over 58 min., 15-32% B over 58 min., 32-40% B over 5 min., 40-90% B over 1 min., held at 90% B for 6 min and then reduced to 1% B over 1 min.) with a flow rate of 300 nl min^{-1}. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization at 2.2 kV using a stainless steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary temperature of 250 °C.

All spectra were acquired using an Orbitrap Fusion Tribrid mass spectrometer controlled by Xcalibur 2.0 software (Thermo Scientific) and operated in data-dependent acquisition mode. FTMS1 spectra were collected at a resolution of 120,000 over a scan range (m/z) of 350-1550, with an automatic gain control target of 400,000 and a max injection time of 100 ms. Precursors were filtered with an intensity range from 5E3 to 1E20, according to charge state (to include charge states 2-7) and with monoisotopic precursor selection. The Data Dependent mode was set to TopSpeed and the most intense ions were selected for MS/MS. Precursors were filtered according to charge state (to include charge states 2-7) and with monoisotopic precursor selection. Previously interrogated precursors were excluded using a dynamic window (40 s +/-10 ppm). The MS2 precursors were isolated with a quadrupole mass filter set to a width of 1.6 m/z. ITMS2 spectra were collected with an AGC target of 5000, max injection time of 50 ms and HCD collision energy of 35%.

MS data analysis

MS raw files were processed using the MaxQuant software21 (version 1.5.0.0) and searched with the embedded Andromeda search engine22 against the reviewed mouse sequence database (Uniprot). Cysteine carbamidomethylation was set as a fixed modification and *N*-acetylation of protein and oxidation of methionine as variable modifications. Peptides with a minimum of seven amino acids were considered and the required FDR was set to 1% at

peptide and protein levels. The maximum allowed missed cleavages was set to two. Protein quantification was done by label free quantification (Fast LFQ) with default settings.

Downstream processing of mass spectrometry data was done with Perseus23 from the MaxQuant ProteinGroups output file, contaminations and reverse hits were removed by filtering. The remaining protein quantifications were log2 transformed.

Statistics and reproducibility

Figure 1b, the experiment was repeated 2 times, using independent cultures with similar results.

Figure 1c, the experiment was repeated 2 times, using independent slices, with similar results.

Figure 1d, the experiment was repeated 3 times, using independent slices, with similar results.

Figure 1e, the experiment was repeated 2 times, using independent slices, with similar results.

Figure 2a, the injection was repeated 3 independent times with similar results.

Figure 2b, the immunofluorescence and fluorescence was repeated on 3 sections from two independent mice with similar results.

Figure 2c, the gel and imaging was repeated 2 independent times with similar results.

Figure 2d, the gel and imaging was repeated 2 independent times with similar results.

Figure 2e, the injection was repeated 3 independent times with similar results.

Figure 2f, the immunofluorescence and fluorescence was repeated on 3 sections with similar results.

Figure 2g, the gel and imaging was repeated 2 independent times with similar results.

Figure 2h, the injection was repeated 3 independent times with similar results.

Figure 2i, the immunofluorescence and fluorescence was repeated on 3 sections with similar results.

Figure2j, the gel and imaging was repeated 2 independent times with similar results.

Figure 2k, each lane represents an independent SORT-E experiment derived from n=5 mice.

Figure 2l, Each Log2(SORT-E Output/Control Output) data point is calculated in Perseus using n=3 SORT-E output measurements and n=6 Control output measurements. The SORT-E IDs were defined from the Log2(SORT-E Output/Control Output) with a significance

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curve S_0=1 FDR<0.05, plus the proteins only identified in SORT-E output, but not in the control output (Supplementary Data 1).

The SORT-E ID proteome (1789 proteins) and the SORT-E NS (608 proteins) were submitted to DAVID bioinformatics24 with a background proteome of 3787 proteins (Supplementary Data 1) defined as proteins identified by LC-MS/MS across all our samples, including gel band fractionated (three bands) load of 15 μg total striatum protein lysate. From the GO:Cellular Compartment (CC_FAT) the Benjamini-Hockberg p-values for GO: 0097458 “Neuron Part” were reported for SORT-E ID and SORT-E NS.

Figure 2m, $Log_2(LFQ_{SORT\text{-}E\ Output} / LFQ_{Total\ Striatum})$, LFQ is the label free quantification value from LC-MS/MS analysis. The depletion of glial markers (Oligodendrocyte markers: Plp1, Mog, Mag, Cnp, Mbp, ,Enpp6, Bcas1; Astrocytes markers: Slc1a2, Scl1a3, GFAP, Aldh1l1, Vim, Glul, S100b) was calculated and plotted as Log_2 mean values with standard error of mean, n=3 independent experiments. Statistics were calculated with a one-sample t-test (H_0:μ=0) and the significance of the two tailed test were reported as *P-value<0.05 and ** P-value<0.005. P values were Plp1 (0.022), Mog (0.007), Mag (0.006), Cnp (0.037), Mbp (0.230), Enpp6 (0.049), Bcas1 (0.086), Slc1a2 (0.069), Scl1a3 (0.006), GFAP (0.0008), Aldh1l1 (0.165), Vim (0.0157), Glul (0.024), S100b (0.319).

Figure2n, Proteins that are >10 fold upregulated in cultured glia with respect to neurons in Sharma *et al*3 and are present in the SORT-E output were mapped onto proteins that are "SORT-E De-enriched " ($LFQ_{SORT\text{-}E\ Output} / LFQ_{Total\ Striatum} < 1$), and proteins that are "SORT-E Enriched" ($LFQ_{SORT\text{-}E\ Output} / LFQ_{Total\ Striatum} > 1$). The Euler diagram indicates the number of proteins found in each category. Hypergeometric distribution probability analyses were then used to calculate a P value for the significance of the proteins from cultured glia in "SORT-E De-enriched" and for the significance of the proteins from cultured glia in "SORT-E Enriched".

Figure2o, proteins that are >10 fold upregulated in acutely isolated glia with respect to neurons in Sharma *et al3* and are present in the SORT-E output were mapped and analyzed as described in panel **N**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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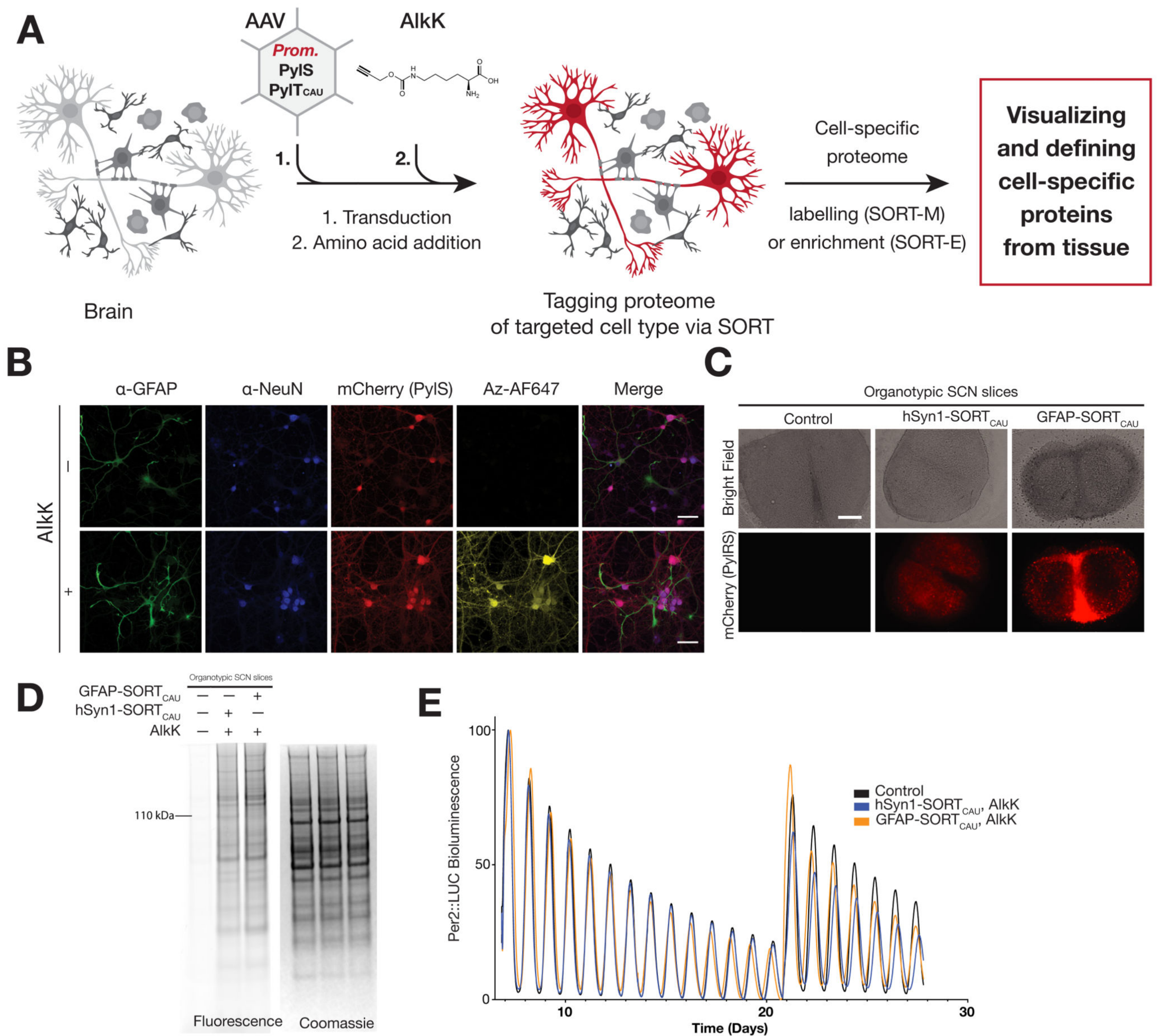

Figure 1. Cell-type specific labeling of proteomes.

A. Schematic of cell-type specific labeling in complex tissue. Promoter (Prom.) directs SORT tagging system to targeted cell type. **B.** SORT-M in the neurons of dissociated rat cortex. Selective labeling of neurons with Azide-AlexaFluor 647 (Az-AF647) in cells cultured with AlkK. Scale bar, 50 μm. **C**. mCherry::P2A::PylS expression in Organotypic SCN slices upon infection with the indicated hSyn or GFAP driven AAV SORT system. Scale bar, 300 μm. **D**. SORT-M labeling of proteomes from SCN slices infected with the indicated hSyn or GFAP driven AAV SORT system, provided with AlkK and labeled with Az-AF647. The Coomassie gel is a loading control for the fluorescent gel. **E**. Organotypic slices of the mouse SCN derived from Period2::LUC mice were transduced with the hSyn1-$SORT_{CAU}$ or GFAP- $SORT_{CAU}$ AAV and transferred to fresh media supplemented with

AlkK. Rhythms were followed by bioluminescence. Information on statistics and reproducibility for all panels are provided in the Online methods section.

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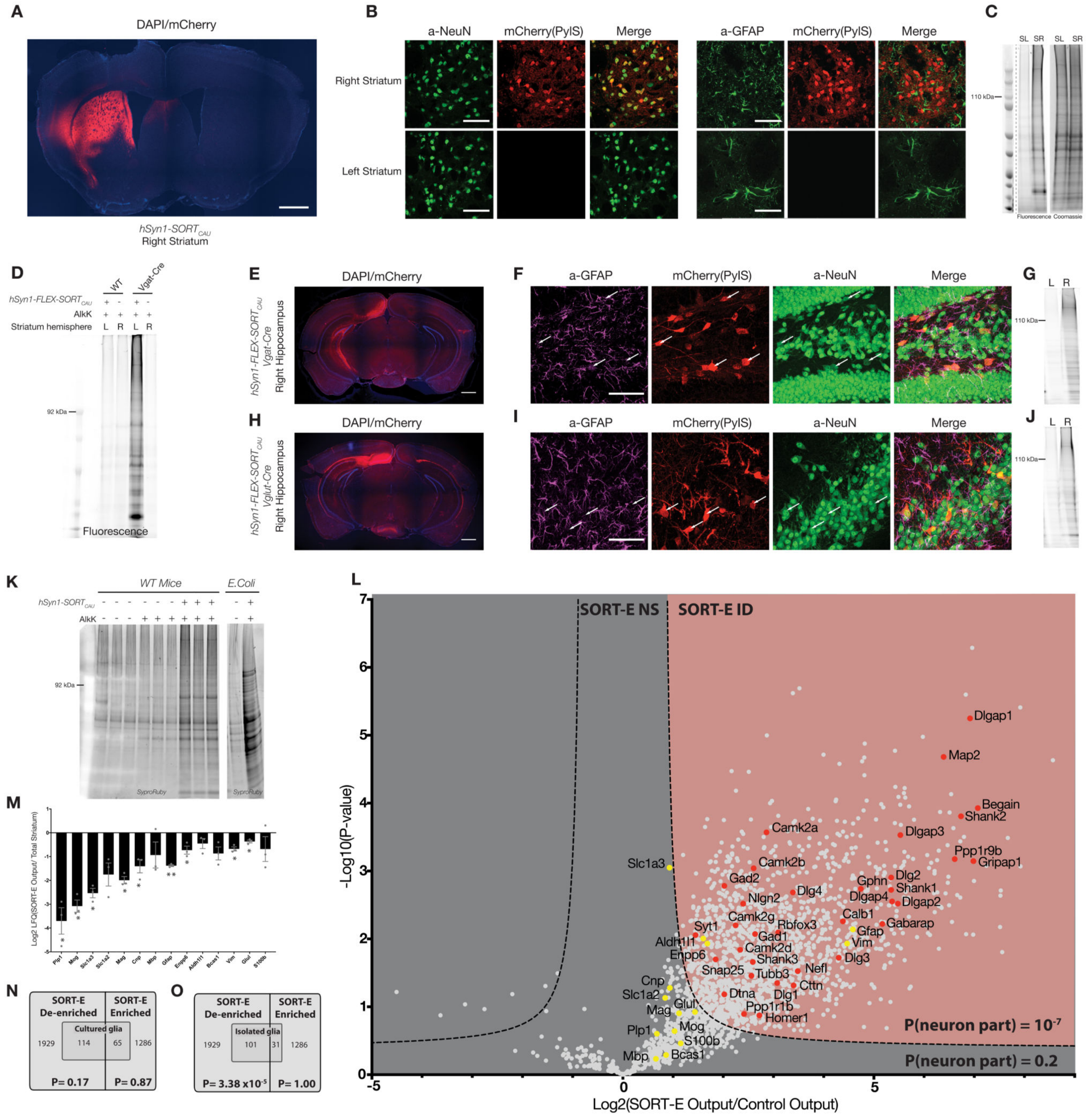

Figure 2. Spatially controlled cell-type specific labeling in live mice.

A. Section of a brain unilaterally injected with hSyn1-SORT$_{CAU}$ AAV into the right striatum and fed AlkK. Scale bar, 1000 μm. **B.** Fluorescence and immunostaining of brain sections from a mouse treated as in **A**. Scale bar, 50 μm. **C.** Az-AF647-labelled lysates from the right (SR) and left (SL) striatum of mice treated as in **A**. **D**. SDS-PAGE analysis of Az-AF647-labelled lysates from WT or Vgat-Cre mice injected with the hSyn1-FLEX-SORT$_{CAU}$ AAV in the left hemisphere, provided with AlkK. **E**. Section from a Vgat-Cre brain injected with hSyn1-FLEX-SORT$_{CAU}$ AAV in the hippocampal right hemisphere and fed AlkK. Scale bar,

1000 μm. **F**. Fluorescence and immunostaining for right hippocampus of a Vgat-Cre mouse, treated as described in **E**. Scale bar, 50 μm. **G**. SDS-PAGE analysis of Az-AF647-labeled protein extracts from the right (R) and left (L) hippocampus of Vgat-Cre mice, as described in **E**.. **H.-J.,** as for **E.-G.,** but using Vglut-Cre mice. **K.** SDS-PAGE of the elution from replicates of striatal SORT-E experiments. Each lane is eluted from five mice. **L.** (SORT-E output/control output) represents the ratios of the raw intensities. Dotted line represents the cut off curve for significance (S_0=1, FDR<0.05). Glial (yellow), neuronal (red). The *P* values for neuron part are from GO analysis. Each Log_2(SORT-E output/control output) data point is calculated in Perseus using *n*=3 SORT-E output measurements and *n*=6 Control output measurements. **M.** The y axis is the Log_2 ratio of the label free quantification value. The data show mean of independent three independent replicates, ± s.e.m. **P*<0.05, ***P*<0.005 for a ratio < 0. Statistics were calculated with a one-sample *t*-test (H_0:μ=0) and the significance of the two tailed test were reported **N.** Euler diagram indicates the partitioning of glial specific proteins in cultured glia between SORT-E enriched and de-enriched. **O.** As for **N**, but using glial specific proteins from acutely isolated glia. Information on statistics and reproducibility for all panels are provided in the Online Methods.

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