Cell-type-specific metabolic labelling, detection and identification of nascent proteomes in vivo

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Summary.

A big challenge in proteomics is the identification of cell-type specific proteomes in vivo. This protocol describes how to label, purify and identify cell-type specific proteomes in living mice. To make this possible, we created a Cre-recombinase inducible mouse line (JAX stock number: 028071) expressing a mutant methionyl-tRNA synthetase (L274G), which enables the labelling of nascent proteins with the non-canonical amino acid Azidonorleucine (ANL). This amino acid can be conjugated to different affinity tags by click-chemistry. After affinity purification the labelled proteins can be identified by tandem mass spectrometry. With this method it is possible to identify cell-type specific proteomes derived from living animals, this was not possible to do with any previously published method. The reduction in sample complexity achieved by this protocol allows for the detection of subtle changes in cell-type specific protein content in response to environmental changes. This protocol can be completed in ≈10 days (plus the desired labelling period and MS analysis).

Key words: MetRS, MARS, cell-type specific proteins, newly synthesized proteins, ANL, Click chemistry, non-canonical amino acids, proteomics, metabolic labelling, BONCAT

INTRODUCTION:

Identification of cell-type specific proteomes and how these proteomes react to specific stimuli is a fundamental question for understanding function and dysfunction of living organisms. Identification of proteins by mass spectrometry (MS) has become a key technique for the study of cellular biology, complementing RNA and DNA sequencing. Unlike DNA or RNA sequencing, protein identification lacks the possibility of amplifying the original protein content, meaning that low abundant proteins might be difficult to detect. For this reason the ability to detect protein species using MS is much lower than the ability to detect RNA molecules using RNA-seq. This limits the identification of some protein species, especially in complex protein mixtures. To overcome this constraint and increase the dynamic range for protein detection, the sensitivity of the MS instruments has greatly improved as have the bioinformatics tools for protein identification and analysis. From the point of view of sample preparation, different strategies for decreasing sample complexity have been developed[1]. None of these strategies, however, preserves cellular integrity; consequently increasing sensitivity is often accompanied by a loss of information. This is especially notable in some cell-types such as neurons: they are morphologically complex with long axons and dendrites, which are lost during procedures aimed at sorting cells for analysis. One way to overcome this limitation is the use of bio-orthogonal strategies, based on the use of chemical reporters carrying alkynes, that can be used for protein purification. This approach has been widely used for the labelling of proteins, glycans and lipids[2], taking advantage of a certain degree of promiscuity of the relevant endogenous cellular machinery. Furthermore, the use of the cellular machinery for the incorporation of chemical reporters makes this strategy amenable to genetic manipulations[3-5]. For example, the expression of a methionyl-tRNA synthetase (MetRS) bearing a point mutation that alters the amino acid-binding site (MetRS L274G or MetRS*) allows for the incorporation of Azidonorleucine (ANL)[6-9] into proteins instead of methionine (Figure 1a). ANL has a slightly different structure from methionine and it is not recognized by the cell's endogenous MetRS. Therefore ANL cannot be incorporated into proteins in cells that do not express mutated MetRS. Thus, the expression of MetRS L274G under cell-type specific promoters limits ANL incorporation to proteins synthesized in the respective, specific cell-types. The approach described here allows for metabolic labelling of cell-type specific proteomes. ANL carries a reactive azide group, using a copper catalysed azide-alkyne ligation (BONCAT[4], BiOrthogonal Non-Canonical Amino acid Tagging), any alkyne can be covalently attached to ANL. Different molecules, like fluorophores or antigenic tags can be derivatized with alkynes. Here we used

biotin alkynes to covalently attach biotin to ANL in order to detect and pull-down cell-type specific proteins. We engineered a Cre-lox mouse line in which the expression of GFP-2A-MetRS L274G is controlled by Cre-recombinase expression (Figure 1b). Consequently, when MetRS L274G mice are crossed with animals expressing Cre under the control of a cell-type-specific promoter, metabolic labelling of proteins with ANL will occur only in the cell-type expressing Cre. This approach enables metabolic labelling of proteins in as many cell-types as specific Cre-driver lines exist. This method not only allows for the isolation of cell-type specific proteins: the enrichment of newly synthesized proteins decreases the complexity of the sample, increasing the sensitivity and enabling for the detection of small changes in proteomes in response to changes in the extracellular or intracellular environment. ANL, like natural amino acids, can be added to the animal's diet. We administered ANL in the drinking water of mice to label newly synthesized proteins. Although ANL contains an azide group, this compound is very stable and non-toxic, as has been shown for similar compounds[10].

Applications of the method:

The most compelling application of this technique is the possibility to label and identify proteins that were synthesized *in vivo*, in the natural environment of each cell-type (see Figure 2 for an overview of the method steps). One major advantage of the method is that the researcher can choose when to label the proteins to be identified, so it is possible to know when and where (which cell-type) the proteins were synthesized. Both timing and cell-type specificity make protein detection more sensitive to subtle changes in cellular protein content. Using this approach we purified and identified 3476 proteins from two different neuronal types in mice labelled with ANL for 21 days *in vivo*. To explore the sensitivity of our method we examined how the proteome of the excitatory neurons of the hippocampus changed after exposing the mice to an enriched environment (EE) during the labelling period. 225 proteins were identified that exhibited differences in expression levels between mice housed in standard cages and mice housed in EE cages. This proteome plasticity may underlie the changes in synaptic structures and neural circuits associated with the exposure of rodents to EE[11]. Additionally, it is possible to combine this method with other quantitative tags, such as isobaric or isotope tags for the comparison of several samples in one MS experiment. This method can also be used *in vitro* in primary cultures, or in tissue slices[11], simply by transfecting MetRS L274G controlled by the desired cell-type-specific promoter. The labelled and purified proteins can be analysed by Western blot for the study of candidate proteins, with the possibility of studying several proteins of interest with one affinity purification step. Click chemistry can be performed in fixed tissue[12], allowing for the visualization of newly synthesized proteins in situ. The use of fluorophore-click followed by imaging provides a general readout of global protein synthesis in tissues or cell cultures with spatial resolution (a.k.a. Fluorescence non-canonical amino acid tagging; FUNCAT). When click chemistry is combined with the in situ Proximity Ligation Assay (PLA[12]) it is even possible to visualize specific candidate proteins that were synthesized (*in vivo* or *in vitro*) during a defined period of time in a specific cell-type. The method described here can be also used for protein transfer experiments, which require knowledge about the source of proteins, for example in parabiosis studies[13,14]. This approach is very clean because ANL cannot be recycled in the transference organism provided it does not express MetRS L274G.

Comparison with other methods:

Classically, metabolic protein turnover has been measured using SILAC (Stable Isotope Labelling by Amino acids in Cell culture). The main advantage of SILAC is that it is quantitative. It is not, however, cell-type specific and the labelled proteins cannot be purified. Therefore, the resulting samples display a high protein complexity which limits

MS sensitivity. *In vivo* studies using SILAC are possible but they are limited by the high cost of the isotopic labelled amino acids. A newer technique; CTAP[15] (Cell-Type specific labelling using Amino acid Precursors) is also based on isotopic labelling, but in this case the isotopic labelling relies on amino acids precursors. For the application of CTAP, the necessary enzymes for the biosynthesis of essential amino acids have to be exogenously expressed, enabling cell-type specific labelling. Nevertheless, to date, this technique has not been implemented *in vivo*, and, similar to SILAC, labelled proteins cannot be isolated from the lysates. Labelling proteins with radioactive isotopes is another classical approach to study protein turnover. Radioactive labelling is very sensitive and can be used for the study of general protein turnover or, in combination with immunoprecipitation, for candidate-based studies. However, labelled proteins cannot be identified by MS and the labelling is not cell-type specific. In the last few years, puromycylation has become a widely used technique for the labelling of newly synthesized proteins[16]. The antibiotic Puromycin gets incorporated into peptide chains at the A site of the ribosome and later can be detected by antibodies. The advantage of puromycylation being easy to perform is counterbalanced by the fact that this technique generates many truncated or aberrant proteins. It is therefore not suitable for studies involving functional proteins. *In vivo* labelling with puromycin is limited to very short periods of time (minutes) due to its toxicity. Some attempts have been made to label in a cell-type specific manner with puromycin[17, 18] but so far there is no *in vivo* implementation. Finally, if we compare ANL labelling with AHA (L-Azidohomoalanine) the other widely used artificial amino acid, the main advantage of ANL over AHA is the cell-type specificity[19-21].

Method	**Suitable for Microscopy**	**Microscopy for specific proteins of interest**	**Protein Purification possible**	**Cell-type specific labelling**	**In vivo labelling**	***In vitro* labelling**
Radioactivity	yes	no	yes [a]	no	yes [b]	yes
SILAC	no	no	no	no	yes	yes
CTAP	no	no	no	yes	?	yes
Puromycin	yes	yes [c]	yes [c]	yes [c]	yes [c]	yes [c]
AHA	yes	yes	yes	no	yes	yes
MetRS*/ANL	yes	yes	yes	yes	yes	yes [e]

Table. 1: Comparison of metabolic protein labeling methods.

[a]only candidate-based, [b]potentially harmful and highly toxic, [c]generated proteins are often truncated: complicating the study of protein transport/half-life measurements**,** only short labelling times due to toxicity, puromycin can be incorporated at every amino acid position in the protein, so the labelled non truncated proteins can be substantially different from the natural ones. [e]MetRS* can be exogenously expressed in the cell-type of interest.

Experimental design:

The experimental design depends on the scientific question and the cell-type of interest. For *in vivo* experiments, ANL can be administered by the drinking water or by IP injections. Both administration systems work equally well,

but depending on the duration of the labelling it might be better to use one system or the other. For short periods of time (up to 1 week) IP injection is suitable, injecting the mice once or twice per day. It is important to take into account that a certain expertise is needed for IP injections, to avoid loss of the injected substance. For longer periods of labelling it is advised to add ANL to the drinking water, thus avoiding excessive physical manipulation of the mice. The number of replicates required per experiment varies depending on the scientific question; based on our studies we estimate that 3-6 biological replicates per MS condition will be necessary (replicates add accuracy to the results, this is a general premise for any MS based study). Animals that do not express the MetRS* gene are always needed as a negative control for the background e.g. due to nonspecific binding of proteins in the enrichment steps. The number and distribution of the cells to study can become a limitation of the method, if the cell-type of interest has a low cell number that is sparsely distributed in a huge tissue volume, this method may not be suitable for a proteomics study. There is, however, a good chance to study the proteome of a low abundance cell-type if it is grouped in a specific part of the tissue (e.g. a nucleus) suitable for dissection, increasing the signal-to-noise ratio. But, it is also important to take into account the rate of synthesis and the accessibility of ANL might vary between cell-types[22]. In our study the minimum number of neurons used per proteome was 130,000 to 200,000 for the Purkinje proteome[11, 23] and the entire cerebellum was used as starting material. As a rule of thumb, for neuronal samples if GFP expression from the cell-type specific X-Cre::MetRS* line is not detectable by Western immunoblot when 5-10 ∝g of the tissue lysate are loaded, it is very likely that the technique will not be sensitive enough for the identification of the specific cell-type proteins by MS.

General considerations for the experimental design:

a) ANL intake:

-Administration of ANL in the drinking water: for experiments aiming to label and identify neuronal populations of the brain, we recommend an average daily ANL intake of 0.9 ± 0.1 (mean ± S.D.) mg/day/g body weight for 21 days. Lower dosages or shorter times of labelling might be used for the labelling of other cell-types.

-Administration of ANL by IP injection: we recommended to test doses in the range of 4-400 mM of ANL. A 400 mM dosage administered by IP (IP volume: 10 ml/ kg) once per day for one week is recommended for labelling some neuronal populations (such as excitatory neurons of the hippocampus). Lower dosages (e.g. 4 mM) can be potentially used for labelling other cell-types or for longer labelling periods.

-Each investigator should determine the ideal concentration of ANL, depending on the cell-type to be labelled, the desired duration of the labelling and the specific experimental question.

b) Mouse diet:

Low methionine chow (0.1 %) can be administered to the mice; this step is optional (see attached recipe).

Note that the methionine content of non-defined mice chow is variable. Raising the mice with a defined diet will provide more reproducible results, avoiding potential methionine content variation between different batches of mouse chow.

c) Sample size:

-Animal numbers per proteome will largely depend on the abundance of the cell-type under study. In the case of specific neuronal types when labelling neurons with an approximate population of 130,000-200,000 neurons per brain or more, one brain is enough for protein purification.

-For the initial experiments to establish the administration route, the amount and the duration of ANL administration, the use of small groups of animals is possible as regular BONCAT (detection of labelled proteins using Western blot; e.g. using a biotin alkyne tag and then an anti-biotin antibody) is generally consistent among replicates (2 mice per condition). (see Figure 3 for an example of two different ANL administration protocols).

d) Replicates per proteome:

-Taking into account the intrinsic variability of in vivo experiments, we recommend that users obtain at least 3 to 4 replicates per proteome or condition. If the aim is to find subtle differences between conditions we consider a minimum of 6 replicates necessary. Due to many critical steps in the protocol we recommend to start with two to three times the number of animals as calculated for the desired number of biological replicates.

e) Controls:

-We recommend the following controls:

1_Positive control for click chemistry; for example any kind of cells labelled with AHA (for a basic *in vitro* labelling protocol see [4])

2_Negative control for background; label with ANL a control mouse line using exactly the same labelling conditions that are used for the X-Cre::MetRS* mice line under study. We recommend to use as control line the MetRS* without Cre, the use of wild type mice from the same strain is also possible. We did not observe any differences in the background levels between the above lines. At the end of the protocol, the obtained peptides in both samples will be compared and will be used as a background proteome.

General considerations for the analysis of MS samples:

a) Set precursor mass tolerance to 4.5 p.p.m., fragment ion tolerance to 20 p.p.m. (QExactive), max. 2 missed cleavages with fixed modification of Cys residues (carboxyamidomethylation +57.021 Da) and variable modifications of Met residues (Ox +15.995 Da), Lys residues (acetylation +42.011 Da), Asn and Gln residues (deamidation +0.984 Da) and of N termini (carbamylation +43.006 Da).

b) Calculate peptide identifications with FDR ≤ 0.01 for multiple comparisons, and proteins with one unique peptide per protein included for subsequent analyses.

c) Quantify proteins by measuring peptide ion intensity in ≥ 4 consecutive full scans using a label-free approach (LFQ).

d) Analyse identified proteins and log2 protein abundances using a bioinformatics platform (e.g. Perseus, R project).

e) Compare proteins purified in the X-Cre::MetRS* samples to the corresponding WT (or other control) samples, calculate the fold enrichment of each protein by subtracting the X-Cre::MetRS* intensity from the intensity measured in the paired WT (or other control) sample.

f) Consider only proteins detected exclusively in the ANL-treated X-Cre::MetRS* samples or found to be ≥ 3 times enriched in the ANL-treated X-Cre::MetRS* sample for further analysis (or significantly enriched using, for example, a

paired t-test). Proteins with no enrichment but quantified in both X-Cre::MetRS* and WT are counted as overlapping proteins.

g) Analyse differential protein abundance between conditions by a two-sided t-test (permutation-based FDR) for two sample groups or by ANOVA (followed by Post-hoc tests) for three or more sample groups. If the aim is to compare two proteomes or two conditions, the relative intensities and abundance changes between the two conditions should be analysed after all data are normalized (mean centering).

h) Visualize data using GO enrichment analyses (e.g. GOrilla, http://cbl-gorilla.cs.technion.ac.il/), Segmentation analyses, Principal Component Analyses, Heat maps and Volcano Plots using e.g. the Perseus software package and protein network analyses using String (e.g. www.string-db.org).

Expertise needed to implement the protocol:

For the implementation of the protocol it is necessary to have experience in the handling and managing of mice colonies. Basic knowledge in biochemistry is strongly recommended. MS expertise is required for the processing and analysis of the samples.

MATERIALS

REAGENTS

i) ANL synthesis

For ANL synthesis all the reagents found in[24] will be needed, use Boc-L-Lys-OH (N-alpha-t-Butylcarbonyl-L-Lysine) (BAA1107.0100 Iris Biotech) instead Boc-DAB

ii) Protein labelling and click chemistry

-Mouse crosses between a Cre- driver line and the MetRS*with the mutation (JAXnumber: 028071), we recommend the use of homozygote animals for MetRS* expression if possible.

CAUTION: All experiments with animals should be performed in accordance with relevant local guidelines and regulations, especially regarding animal welfare and Genetic Engineering.

-Low methionine diet (ssniff Custom, see attached recipe)

-Water, molecular biology grade (Sigma, ref. W4502)

-ANL**CRITICAL** (Synthesized as described previously[24]; alternatively, it can be obtained from Iris Biotech ref.HAA1625, other sources of ANL have not been tested)

-Maltose (Sigma, ref. M9171)

-SDS (Sigma, ref. 05030) **CAUTION** (toxic by inhalation, use lab coat, gloves and fume hood)

-Triton X-100 (Sigma, ref. T9284)

-Iodoacetamide (IAA) (Sigma, ref. I1149) **CAUTION** (toxic, use lab coat and gloves) **CRITICAL** store in the dark

-Triazole ligand (Sigma ref. 678937)

-Biotin-Alkyne (Thermo, ref. B10185)

-DST-Alkyne, synthesized as reported in[25] (Probe 20) **CRITICAL** do not use strong reducing agents when using this alkyne

-Copper (I) bromide, 99.999% (Sigma, ref. 254185), **CRITICAL** purity is very important; other brands might be used if they contain the same purity. Store desiccated at RT.
-DMSO (Sigma, ref. 276855)
-Complete EDTA-free protease inhibitor (Roche, ref. 04693132001) **CRITICAL** (has to be EDTA-free). **CAUTION** (toxic, use lab coat and gloves)
-Benzonase (Sigma, ref. E1014)
-PD-SpinTrap G-25 columns (GE healthcare) **CRITICAL** (other columns were tested and a considerable amount of protein was lost, other brands might be used but double check for protein loss)
-NeutrAvidin beads (Pierce, ref. 29200)
-ß-Mercaptoethanol (Sigma, ref. M6250) **CAUTION** (toxic, use lab coat, gloves and fume hood)
-SYPRO Ruby staining (Sigma, ref. S4942)
-BCA protein measurement kit (Thermo, ref. 23225) **CAUTION** (toxic for the aquatic life)
-NEM (Sigma, ref. 04259) **CAUTION** (toxic, use lab coat, gloves, and fume hood)
-HCl (VWR, ref. 30024.290) **CAUTION** (toxic, use lab coat, gloves, and fume hood)
-NaOH (Fluka, 35256) **CAUTION** (corrosive, use lab coat, gloves, and fume hood)
-Ammonium bicarbonate (Sigma, ref. 09830) **CAUTION** (toxic, use lab coat, gloves, and fume hood)
-Chicken antibody anti GFP (Aves, Cat 1020)
-Polyclonal rabbit biotin antibody (Cell Signaling, ref 5567)

iii) Mass Spectrometry
-Dulbecco's Phosphate-Buffered Saline (Thermo, ref. 14190-094)
-EDTA-free Protease Inhibitor Cocktail (Roche, ref. 04693132001) **CAUTION** (toxic, use lab coat and gloves)
-Microcon-10kDa Centrifugal Filter Unit with Ultracel-10 membrane (Merck, ref. MRCPRT010)
-Urea powder (Sigma, ref. U5378)
-Water, HiPerSolv CHROMANORM (VWR Chemicals, ref. 83645.290)
-Acetonitrile, LC-MS Grade (Carl Roth, ref. AE70.1) **CAUTION** (toxic, use lab coat, gloves, and fume hood)
-Tris(hydroxymethyl)-aminomethane, CELLPURE ≥99,9% (Carl Roth, ref. 3170) **CAUTION** (toxic, use lab coat, gloves, and fume hood)
-Ammonium bicarbonate (Honeywell Fluka, ref. 40867) **CAUTION** (toxic, toxic, use lab coat, gloves, and fume hood)
-NaCl (VWR Chemicals, ref. 27810.295)
-Formic Acid, Optima LC-MS Grade (Fisher Chemical, ref. A117-50) **CAUTION** (toxic, toxic, use lab coat, gloves, and fume hood)
-TCEP (Sigma, ref. 646547) **CAUTION** (toxic, use lab coat and gloves)
-Iodoacetamide (Thermo, ref. 90034) **CAUTION** (toxic, use lab coat and gloves) **CRITICAL** store in the dark
-Endoproteinase rLys-C (Promega #V1671).
-Trypsin Premium Grade, MS approved from porcine pancreas (Serva, ref. 37284.01)
-TFA ≥99,9%, for (peptide) synthesis (Carl Roth, ref. P088.1) **CAUTION** (toxic, use lab coat, gloves, and fume hood)
-Acetic Acid (Glacial) 100% (Merck Millipore, ref. 1.00063.1000) **CAUTION** (toxic, use lab coat, gloves, and fume hood)
-ZipTip C18 (Merck, ref. ZTC18S960)
-Dimethylsulfoxide, LC-MS Grade (Thermo Scientific, ref. 85190)

EQUIPMENT

i) If ANL is synthesized refer to the equipment in[24]

ii) For protein labelling and click chemistry

-Rotisserie-Rotator

-Lyophilizer

-Heating block/thermo mixer

-Manual vortex

-Centrifuges

iii) For protein Mass Spectrometry

-Speed-vac

-HPLC (e.g. Dionex RSCLnano or equivalent), coupled to a high-resolution tandem mass spectrometer (e.g. Thermo Orbitrap Elite, Q Exactive Plus / HF, FusionLumos or Bruker Impact-II, see[26] for review)

Software tools:

-Software package for analysing large-scale mass-spectrometric data sets; database search -MaxQuant (www.maxquant.org) or MS Amanda/Sequest as part of the Proteome Discoverer package (Thermo Fisher Scientific), data evaluation: Perseus (www.maxquant.org) or R project (www.r-project.org).

REAGENT SETUP

i) For protein labelling and click chemistry

-ANL Synthesized as described previously for AHA[24] with the next modification; use as starting material instead of Boc-DAB, Boc-L-Lys-OH (N-alpha-t-Butylcarbonyl-L-Lysine) in the same molar amounts.

CRITICAL: ANL purity is very important for its administration to the mice.

-All solutions for click chemistry must be free of EDTA, EGTA and other chelators to avoid inactivation of the copper (I) catalyst.

-**Protease inhibitors (PI): CRITICAL**: add PI to the solutions immediately before use, according to the manufacturer's instructions

-**1xPBS** (phosphate-buffered saline) 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM disodium hydrogen phosphate and 1.4 mM potassium dihydrogen phosphate. Adjust the pH to 7.4 or 7.8 with HCl or NaOH. Filter the solution and store at 4°C to avoid changes in concentration. Check and re-adjust the pH before each experiment. (**CRITICAL**: Preparing the PBS following this specific recipe is recommended, slightly less reactivity during click chemistry can occur with alternative compositions)

-**ANL administration by drinking water**: Prepare 1% (wt/vol) ANL + 0.7% (wt/vol) Maltose dissolved in the usual drinking water of the mice and sterilize by filtration. This solution is stable for several weeks at 4°C.

CRITICAL: Water containing ANL should be the only source of drinking water in the cage. Use sterilized water bottles for mice. The bottles should be replaced every 2-3 days including the ANL solution. Mouse body weight and water intake should be evaluated daily and ANL-containing bottles should be carefully scrutinized for contamination or clogging due to maltose precipitation.

- ANL administration by IP injections: ANL can be dissolved up to 400 mM in water, PBS or NaCl depending of the concentration of ANL used, to achieve an isotonic solution (OSM ≈300 and pH 6.5/8). This solution is stable for 5-6 weeks at 4°C.

-**PBS-PI** 1xPBS supplemented with Complete EDTA-free PI.

-**20% (wt/vol) SDS** diluted in molecular biology grade water. This solution is stable for several months at RT.

-**20% (vol/vol) Triton X-100** diluted in molecular biology grade water. This solution is stable for several months at RT.

-**Lysis buffer:** 1 % (wt/vol) SDS, 1% Triton X-100 (vol/vol), PI (diluted 1:4000 vol/vol), Benzonase (diluted 1:1000 vol/ vol) in PBS pH 7.4+PI. This solution is stable for several months at RT if the PI mixture and benzonase are added only before use.

-**Triazole ligand: 200 mM** stock solution in DMSO, **CRITICAL** Avoid exposure of the solution to air and water, use a fresh aliquot of DMSO to prepare the stock solutions. Aliquot in small volumes and store at -20°C. This solution is stable for 2-3 months, after melting always double-check that there is no precipitation. Triazole reactivity is variable among batches.

-**PD-SpinTrap G-25 columns exchange buffer** 0.04% (wt/vol) SDS, 0.06% (vol/vol) Triton X-100 in 1x PBS pH 7,8+PI. This solution is stable for several months at RT if the PI mixture is added only before use.

-**50mM Ammonium bicarbonate** +PI dissolved in molecular biology grade water. This solution is stable for 5-6 weeks at RT if the PI mixture is added only before use.

-**NeutrAvidin-binding buffer** 1% (vol/vol) Triton X-100, 0.15% (wt/vol) SDS in 1x PBS pH 7.4+PI. This solution is stable for several months at RT if the PI mixture is added only before use.

-**NeutrAvidin wash buffer 1:** 1% (vol/vol) Triton X-100, 0.2% (wt/vol) SDS in in 1x PBS pH 7.4+PI. This solution is stable for several months at RT if the PI mixture is added only before use.

-**NeutrAvidin wash buffer 2:** 50 mM ammonium bicarbonate+PI. This solution is stable for 5-6 weeks at RT if the PI mixture is added only before use.

-**NeutrAvidin Elution buffer:** 5% (vol/vol) β-mercaptoethanol, 0.03% SDS (wt/vol). Prepare fresh before use.

-**Iodoacetamide:** dissolve in molecular biology grade water up to 0.5 M, immediately before adding it to the samples.

ii) For protein Mass Spectrometry

Reagent set up:

-0.1M Tris/HCl, pH 8.5 diluted in LC-MS Grade water. This solution is stable for 5-6 weeks at RT. Double-check the pH value before use.

-0.1M Tris/HCl, pH 8.0 diluted in LC-MS Grade water. This solution is stable for 5-6 weeks at RT. Double-check the pH value before use.

-**UA buffer**: 8 M urea dissolved in 0.1M Tris/HCl, pH 8.5. Prepare fresh before use.

-**UB buffer:** 8 M urea dissolved in 0.1M Tris/HCl, pH 8.0. Prepare fresh before use.

-**Ammonium bicarbonate buffer:** 50mM ammonium bicarbonate dissolved in LC-MS Grade water. This solution is stable for 5-6 weeks at RT.

-**TCEP**: 10 mM TCEP dissolved in UA buffer. Prepare fresh before use.

-**Iodoacetamide:** Dissolve in UA buffer up to 0.5M. Prepare fresh before use.

It is **CRITICAL** that UA, UB, TCEP and Iodoacetamide are freshly prepared.

-0.5 M NaCl diluted in LC-MS Grade water. This solution is stable for 5-6 weeks at RT.

-**Wetting buffer:** 100% ACN. This solution is stable for 5-6 weeks at RT.

-**Equilibration buffer:** 0.1% (vol/vol) TFA in LC-MS Grade water. This solution is stable for 2-3 weeks at RT.

-**Elution buffer:** 90% (vol/vol) ACN, 0.1% (vol/vol) TFA in LC-MS Grade water. This solution is stable for 2-3 weeks at RT.

-**Loading buffer:** 5% (vol/vol) acetonitrile with 0.1% (vol/vol) formic acid in LC-MS Grade water. This solution is stable for 2-3 weeks at RT.

-**Buffer A:** 5% (vol/vol) dimethylsulfoxide and 0.1% (vol/vol) formic acid in LC-MS Grade water. This solution is stable for 2-3 weeks at RT.

-**Buffer B:** 5% (vol/vol) dimethylsulfoxide, 15% (vol/vol) water and 80% (vol/vol) acetonitrile and 0.08% (vol/vol) formic acid. This solution is stable for 2-3 weeks at RT.

-**rLys-C solution:** reconstitute the powder in the resuspension buffer provided with the kit (aliquots can be stored at - 20° C for up to 6 months).

-**Trypsin solution:** Reconstitute the powder in 50 mM acetic acid up to of 1 µg/µl (aliquots can be stored at -20°C for up to 6 months)

-**0.1% (vol/vol) TFA** diluted in LC-MS Grade water. Prepare fresh before use.

-**Elution buffer:** 90% (vol/vol) ACN, 0.1% (vol/vol) TFA in LC-MS Grade water. This solution is stable for 2-3 weeks at RT.

PROCEDURE

Protein labelling *in vivo*: TIMING days to weeks (the exact timing depends on the experimental purpose)

CAUTION: In many countries the application of this procedure in live mice will require special permission approved by the corresponding animal welfare authorities. Mice lose some weight during the first weeks of ANL administration; we recommend that the user requests permission for a maximum weight loss of 20% of body weight during the experiment. Other than that, we did not observe any major impact of ANL admiration on mouse heath. The protocol used for this paper was approved by the local government office (RP Darmstadt; protocols: V54-19c20/15-F122/14 and V54-19c20/15-F126/1012) and was compliant with the Max Planck Society rules.

Protein labelling:

1_Low methionine diet: start feeding the mice with this diet 1 week before starting the administration of ANL. This step is optional, but we recommend it for the labelling of neuronal proteins.

2 _X-Cre::MetRS* mice and control mice not expressing the mutant MetRS will receive ANL in the drinking water for the desired time period (e.g. 21 days) or by IP injection (up to 400mM ANL solutions) once or twice per day (morning and night).

-**CRITICAL**: Take into account that both room humidity and chow type will influence the water intake of the mice. It is important to keep all these parameters constant to get reproducible experiments or replicates. Check mouse health and water intake daily according to animal welfare regulations. Depending on the source and purity of the ANL, the animals may be reluctant to drink ANL-maltose water. ANL purity has less impact when administered by IP injection.

TROUBLESHOOTING

Tissue collection: TIMING depending on the tissue to collect (approximately 10 min per mouse)

3_Prepare tubes for the dissected tissue, weigh each of them and place them on dry ice.
4_Sacrifice the mice by an approved method according to regional animal welfare laws (e.g. by CO_2 or cervical dislocation), dissect the tissue pieces of interest, collect them and freeze them in the corresponding tubes. When necessary, pieces of tissue from several animals can be collected and stored in the same tube. Weigh tubes again after tissue collection to determine the wet weight of the tissue.

PAUSE POINT tissue pieces can be stored at -80°C for several months.

Preparation of samples for click chemistry: TIMING 4 h

5_Homogenize the tissue in 12 to 15 volumes of wet tissue weight (µl/mg) of lysis buffer with the help of a manual homogenizer until the tissue is completely lysed. Add more lysis buffer if the lysate does not appear clear. The suggested vol/wt ratio is recommended for brain tissue. Other tissues might need different buffer to tissue proportions.
6_Heat the homogenate to 75°C for 15min.
7_Centrifuge at 10°C for 15min at 16.000 xg.
8_Measure the protein amount of each sample using BCA and adjust all samples to the same protein concentration with lysis buffer. Protein concentration should range between 2-4 ∝g /∝l

PAUSE POINT tissue lysates can be stored at -80°C for several months.

9_Dilute the sample by the addition of 3 volumes PBS 7.4 + PI. Add 0.5M Iodoacetamide to a final concentration of 20 mM to alkylate the samples. Incubate the samples at RT in the dark for 1 to 3 hrs. Repeat one more time the addition of Iodoacetamide and incubation in the dark. (Depending of the tissue of interest a reduction step before the alkylation step[27] might be needed).

CRITICAL: Dissolve the Iodoacetamide just before adding it to the samples.

10_Equilibrate the PD-SpinTrap G-25 columns with PD-SpinTrap G-25 column exchange buffer and proceed with the buffer exchange of the samples, accordingly to the manufacturer recommendations. Prepare 80ul aliquots of the samples and freeze them at -80°C.

CRITICAL; if you use columns different from those described above, make sure that the proteins are not lost during the buffer exchange process.

PAUSE POINT lysates can be stored at -80°C for several months.

Click chemistry reaction: TIMING 18 h (overnight step)

Test a small fraction of the sample to evaluate the labelling success in the specific tissue and experiment. The aim of this first part of the protocol is to evaluate the labelling efficiency for proteins with the Cre-line of choice and identify/discard samples from animals with poor labelling.

11_Take 40 ∝l of the samples from step 10 and add 80 ∝l of PBS pH 7.8 + PI

CRITICAL; the ratio of lysate/PBS used here needs to be constant for all the samples.

12_Thaw aliquots of the biotin alkyne and triazole ligand. Set up reactions in sets of ≈6 samples. For each set of samples with a reaction volume of (120 ∝l) add in the following order and vortex for 20s after adding each of the reagents: 1.5 ∝l of Triazole (stock **40mM**), 1.5 ∝l of the alkyne (Stock **5mM**), and 1 ∝l of Cu(I) (stock **10mg/ml**). Dissolve the copper bromide in DMSO immediately before adding it to each set of samples.

CRITICAL; Fresh copper bromide suspension must be prepared immediately before adding it to the reaction by pipetting up and down several times (not by vortexing). The set up of the reactions has to be fast (less than 2 min per set of samples.)

13_Incubate samples overnight at 4°C with constant rotation on sample mixer 360°C rotator.

-Overnight incubation at 4°C.

14_Spin down at 16.000 x g for 5 min at 4°C. A small pellet should be visible.

15_Transfer the supernatant to a new tube. And keep at 4°C

PAUSE POINT Samples can be stored at -20°C until further analysis.

Western Blot analysis: TIMING 18 h (overnight step)

16_Mix 40 ∝l of the reaction with loading buffer according to the manufacturer's instructions, and boil the samples at 90° C for 10min.

17_Run SDS-PAGE gels and transfer them to PVDF membrane using a traditional wet transfer system, block the membrane and incubate with the recommended primary antibodies (both diluted 1:1000, **overnight incubation at 4C**) (see materials). We recommend using the Odyssey Li-Cor system and reagents if possible.

TROUBLESHOOTING

18_Choose animals with similar labelling efficiency to prepare the samples for proteomic analysis by MS.
For samples with very high ANL labelling (≈20x more signal in the X-Cre::MetRS* compared with the control) it is possible to perform the affinity purification (AP) with Neutravidin beads (Step 22 to 25) using a non-cleavable biotin alkyne followed by on bead digestion prior to MS. Also direct click as shown in [28, 29] could be tested. For samples with

lower labelling the use of a cleavable biotin alkyne (DST-alkyne) or an alkyne with a specific elution method is mandatory, this step favours the specific elution of labelled proteins versus background.

Alkyne dosage optimization: TIMING 2 days

19_Thaw an aliquot of the selected samples from step 10, to test several dosages (from 10 to 100 µ M) of the alkyne (DST or any other). See an example of alkyne dosage in Figure 4. For purification of neuronal proteins we recommend to use ≈20∝M of the DST alkyne (fig. 2B).
Perform steps 12 to 17 to determine the best signal to noise ratio by comparing the X-Cre::MetRS* samples to the negative control.

CRITICAL Do not use strong reducing agents (like DTT or β-Mercaptoethanol) to run the gels clicked with the DST-Alkyne. We recommend loading the samples with 5mM of NEM.

20_Click the remaining samples from the step 10 with the amount of alkyne that yields the best signal to noise ratio. Scale up the click reactions (Steps 12 to 17).

CRITICAL small changes in alkyne content can greatly change the result of the click chemistry. Be sure that identical volumes are pipetted after scaling up the reactions.

-ANL labelled protein purification using DST alkyne: TIMING 18 h (overnight step)

21_Take the clicked samples and perform a buffer exchange using the PD-SpinTrap G-25 columns with NeutrAvidin-binding buffer (see step 10). Take out a 40ul aliquot from each sample and keep the rest of the sample on ice.

22_Wash the neutravidin high capacity beads three times with NeutrAvidin-binding buffer. For the washes use 10 volumes of buffer per volume of dry beads. After each wash, resuspend the beads with a light vortex, and sediment the beads by centrifugation at 3000 x g for 5min at R.T.

23_After the last wash, calculate the volume of the dry beads and add the same volume of the NeutrAvidin-binding buffer (1:1 vol/vol), carefully resuspend the beads.

24_Mix 100∝g of clicked sample (in an approximate volume of 200 ∝l) with 4∝l of the beads obtained in the previous step. Incubate the mixture in a rotator overnight at 4°C.
CRITICAL: This basic reaction set-up might have to be up- or downscaled: the amount of beads used per ∝g of protein strongly depends on the amount of labelled proteins per ∝g of total protein.

Overnight incubation at 4°C

-Protein elution: TIMING 3h

25_Sediment the beads by centrifugation at 3000 x g for 5min at 4°C. Put the supernatant in a new tube, take out a 40ul aliquot from each sample and keep the rest of the sample at -80°C. Wash the beads three times fast and then three times for 10 min in a rotator with each of the following buffers (washes are performed as in step 22)

i) Binding buffer

ii) PBS+PI

iii) 50mM Amonium bicarbonate+PI

26_Elute the clicked proteins from the beads two times incubating for 30 min at RT in a rotator with one bead volume of elution buffer each time. Sediment the beads by centrifugation at 3000 x g for 5min at RT, combine the two elutions.

27_Take 1/3 (vol) of the combined eluate from the NeutrAvidin beads, run a SDS-PAGE gel and evaluate the samples by silver staining or SYPRO Ruby following the manufacturer instructions. Freeze the rest of the sample. See an example of eluted samples in Figure 2 and Supplementary Figure 1.

PAUSE POINT Eluted samples can be stored at -80°C.

TROUBLESHOOTING

28_Lyophilize the remaining sample to remove the β-Mercaptoethanol then resuspend in PBS with UA buffer. The amount of time needed for this step will depend of the volume to be lyophilized.

Mass spectrometry and protein identification

Protein Alkylation: TIMING 3.5h

The following steps have to be performed at RT (unless otherwise specified):

29_Load the samples onto the filter (Microcon-10) unit and centrifuge at 18,000xg for 15 min, discard the flow-through from the collection tube.

30_Add 100µL of UA buffer to the filter unit and centrifuge at 18,000 x g for 15 min and discard the flow-through from the collection tube.

31_Add 100µL 10mM TCEP and incubate at RT for 20min.

32_Centrifuge the samples at 18,000 x g for 15min, discard the flow-through from the collection tube.

33_Add 100 µL 0.05M Iodoacetamide solution and mix at 600 rpm in the thermo mixer for 1 min and incubate without mixing for 20min in the dark.

34_Centrifuge the filter units at 18,000 x g for 15 min. Discard the flow-through from the collection tube.

35_Add 100 µL of UB buffer to the filter unit and centrifuge at 18,000 x g for 15 min. Repeat this step twice. Discard the flow-through from the collection tube.

The proteins are now immobilized on the membrane. The amounts are typically invisible to the eye.

-Protein digestion: TIMING 20h (2x overnight steps)

36_Add 40 µL of UB buffer with Lys-C (enzyme to protein ratio 1:50 wt/wt) and mix at 600 rpm in thermo mixer for 1 min and incubate the filter units at 37°C for 20h without further agitation.

-Overnight digestion

37_Transfer the filter units to new collection tubes, and add 120 µL ammonium bicarbonate buffer with trypsin (enzyme to protein ratio 1:100 wt/wt) and mix at 600 rpm in thermo mixer for 1 min and incubate filter units at 37°C for 20h without further agitation.

-Overnight digestion

38_Centrifuge the filter units at 18,000 x g for 15 min at RT, add 50 µl 0.5M NaCl and centrifuge filter units at 18,000 x g for 10 min.
39_Add 150 µL 0.5M NaCl to the filter and centrifuge at 18,000 x g for 20 min.

Peptide desalting with ZipTips: TIMING 30 min per sample

40_Equilibrate C18 ZipTips by aspirating and dispensing 10 µL wetting buffer up and down three times slowly (3 s per pipetting step, i.e. 3 s up and 3 s down), followed by pipetting 3x 10 µL wetting buffer into waste.
41_Pipet 3x 10 µL equilibration buffer into waste (slowly, 3 s per pipetting step).
42_Acidify the sample by adding TFA up to 0.1% final v/v. Take 10 µl of the sample and pipet up and down ten times (slowly, 3 s per pipetting step).
43_Pipet 3x 10 µL of 0.1% TFA into waste (slowly, 2 s per pipetting step).
44_Elute desalted samples with 10 µL elution buffer by pipetting up and down 10x into a new tube (slowly, 3 s per pipetting step).
45_Evaporate the eluates in Speed-vac (RT).

PAUSE POINT samples can be stored at -20°C for several weeks

-LC-MS/MS analysis: TIMING 3.5h per sample

46_Reconstitute sample in loading buffer prior to MS measurement.

47_Dry the peptides using Speed-vac, reconstitute them in Loading buffer and load them on reversed phase columns (trapping column: particle size 3 µ m, C18, L = 20 mm, Acclaim PepMap 100, Thermo Fisher Scientific, ref. 164535; analytical column: particle size = 2 µ m, C18, L = 50 cm; Acclaim PepMap RSLC, Thermo Fisher Scientific, ref. 164540) using a nano-HPLC (e.g. Dionex U3000 RSLCnano).

48_Load the samples in the MS machine and elute Peptides in gradients of water based buffer A and acetonitrile based buffer B. Ramp gradients from 4% to 48% B in 178 min at a flow rate of 300 nl/min.

Ionize peptides eluting from the column using a Thermo nanoFlex ESI-source and analyze them using a Thermo Scientific Q Exactive Plus mass spectrometer (or equivalent high-resolution tandem mass spectrometer used for high-throughput proteomics). Acquire mass spectra over the mass range 350–1,400 m/z at a resolution of 70,000 (Q Exactive Plus) and acquire sequence information by data-dependent automated switching to MS/MS mode (scan range 200-2,000 m/z at a resolution of 35,000) using normalized collision energies based on mass and charge states (2-5) of the candidate ions (approx. equivalent to a collision energy of 30-35 eV for a reference ion of mass 500 and charge 1).

Bottom-up proteomics analysis: TIMING 2 days or more, (depending on the sample size)

49_Analyze raw MS data sets using MaxQuant (or other computational platforms suitable for processing high-resolution MS spectra, e.g. Proteome Discoverer) (see experimental design section for analysis details).

Table 2.TROUBLESHOOTING

Step	**Problem**	**Reason**	**Solution**
1-2	Water intake of the mice is too low (less than 1.5-2 ml per day)	Low purity ANL stock	-Decrease the percentage of ANL in the drinking water

17	Failed click chemistry	-Too much detergent due to incomplete buffer exchange	-Perform an additional buffer exchange step -precipitate the proteins
		-Poor quality of the reagents	-Prepare fresh Triazole ligand and check Copper (I) bromide purity
	Clicked proteins are not more abundant in the X-Cre::MetRS* mouse than in the wt mouse analysed by WB	-Too few cells for the labelling	-If possible dissect a region richer in your cell-type of interest
		-Inefficient click chemistry	-See previous problem
		-Labelling too low	-Revise the protocol of ANL administration into the mice
		-Alkylation step was not efficient	-Use fresh dissolved IAA, alkylate for longer periods of time (step 9), and implement a reduction step with TCEP previous to alkylation
27	Purified proteins are not more abundant in the X-Cre::MetRS* mouse than in the wt mouse	-Failed click chemistry	-Run the collected aliquots from steps 21 and 25 to verify proper click chemistry, if failed see previous problem
		-Too many beads used in the purification step	-Use the dosage of alkyne with best signal: noise labelling ratio (X-Cre::MetRS*/negative control) (see Figure 4)
		-Too much non-specific binding to the beads	-Use the minimum amount of beads possible. -Add a pre-clearing step with agarose beads
		-Incubation time with the beads is too long	-Add a final quick wash with 1% SDS

			-Reduce the incubation time of the sample with the beads
		-Too much detergent used in the elution step	-Decrease the SDS in the elution buffer
		-Insufficient resuspension after the lyophilization step	-Gently resuspend the proteins with a pipet

Anticipated results:

After delivering ANL in the drinking water of X-Cre::MetRS* mice for 21 days and using as starting material the entire hippocampus or cerebellum, we identified a total of 533 proteins differentially expressed between excitatory neurons of the hippocampus and inhibitory Purkinje neurons of the cerebellum (comprising unique proteins and common proteins expressed at different levels). We did not find any bias in our study towards preferential identification of longer or shorter proteins, or for proteins containing more or less methionine (Figure 5). The peptides containing ANL+/-Alkyne were not possible to identify consistently in the MS. This could be due to metabolic or chemical conversion of the ANL moiety leading to multiple products that are each only present in small quantities. But, we detected a significantly lower number of peptides containing methionine, consistent with the idea that the purified proteins indeed contain ANL (Figure 6).
One additional step for quality control comprises the evaluation of each biological replicate based on MS intensities. For example, the biochemical purification can suffer if the click chemistry is not optimal, if there is too much non-specific binding or elution in the purification step, or poor protein recovery after purification (see troubleshooting section). All of these potential problems will result in poor protein enrichment in the X-Cre::MetRS* sample with similar MS intensities observed in the experimental and negative control samples (see Supplementary Figure 2 and compare with Figure 2, c, d). In this case, when the enrichment between X-Cre::MetRS* and control samples is not significant (using t-test or a static cut-off of >3-fold), the replicate can be discarded. The exact fold-threshold of enrichment depends on the samples and data structure, but this is a general, important quality-control step.

Timing:

ANL labelling and protein purification:

-Protein labelling
Steps 1-2, variable, experiment dependent

-Tissue collection and sample preparation
Steps 3 to 4, 10min per mice
Pause point
Steps 5 to 8, 1-2h depending of sample number

Pause point
Steps 9 and 10, 3-4h depending of sample number
Pause point

-Click chemistry
Steps 11 to 15, 18h (Overnight step)
Pause point

-Western Immunoblot
Steps 16 to 18, 18h (Overnight step)
Pause point

-Alkyne dosage optimization
Steps 19 to 20, 2 days (2x Overnight steps)
Pause point

-ANL labelled protein purification
Steps 21 to 26, 21h (Overnight step)
Step 27 for Sypro Rubi 90min
Step 28 depending of the volume to be lyophilized
Pause point

Mass spectrometry and protein identification

-Alkylation
Steps 29 to 35, 3.5h

-Protein digestion
Steps 36 to 39, 2 days (2x Overnight steps)

-Peptide desalting
Steps 40 to 45, 30min per sample
Pause point

- C-MS/MS analysis
Steps 46 to 48, 3.5h per sample

-MS analysis
Step 49, 2 days or more depending of the sample size.

Figure legends:

Figure 1: ANL incorporation in the mutant MetRS and mouse design

a) Scheme showing the structure of the analogue of methionine Azidonorleucine (ANL), the tail of the artificial amino acid is larger than methionine. A point mutation in the methionyl-tRNA synthetase amino acid binding pocket (MetRS L247G or MetRS*), allows for the loading of ANL onto the methionyl-tRNA. Therefore, ANL can be incorporated to nascent proteins. **b)** Graphic representing wt and knock-in (KI) alleles in the R26-MetRS-L264G mouse line. In R26-MetRS-L264G animals the expression cassette is located in the *Rosa26* loci. Cre-dependent expression of GFP-2A-MetRS* is determined by a floxed polyadenylation signal (Figure and legend modified from [11]).

Figure 2: Workflow for cell-type specific protein labelling and purification

a) Workflow for the protein labelling in mice by adding ANL to the drinking water or by intraperitoneal injections. The tissue under study is collected and the proteins containing ANL are clicked (BONCAT), then the labelled proteins are isolated by affinity purification and eluted by cleaving the alkyne. Purified proteins are identified by MS. **b)** Western blot showing the BONCAT reactions of WT and GAD2-Cre::R26-MetRS* from mice supplied with drinking water containing 1% ANL for 21 days (left panel) and SYPRO Ruby staining (right panel) of the purified/eluted proteins comprising the Purkinje proteome (MetRS* lane). **c)** Plot showing the higher abundance in GAD2-Cre::R26-MetRS* mouse samples compared with unlabelled samples (peptide intensities) of proteins found in both groups. **d)** The Purkinje neuron proteome was obtained by the union of unique proteins to or enriched (>3-fold wt) in samples from GAD2-Cre::R26-MetRS* mice. The corresponding steps from the protocol are indicated in a), b), c) and d) (Figure and legend modified from [11]).

Figure 3: Dosage of ANL in drinking water

a) BONCAT signal from the hippocampus of CaMK2a-Cre::R26-MetRS* (MetRS*) mice. Mice were provided with ANL in the drinking water at/for the indicated dose and duration. There was a strong BONCAT signal obtained from the mice expressing the mutant MetRS* but not in WT or MetRS* animals provided with ANL or water, respectively. **b)** Quantification of the western blots shown in a) (Figure and legend modified from [11]).

Figure 4: Alkyne dosage testing

Western Immunoblot showing that the click chemistry efficiency depends on the amount of alkyne used. WT and Gad2-Cre-MetRS* (MetRS*) mice were provided with 1% ANL administered in the drinking water for 21 days, the cortex was dissected and clicked with different amounts of the biotin alkyne. Labelled proteins were detected by western blot with anti biotin antibody.

Figure 5: Analysis of eluted proteins

a) Distribution of frequencies and protein lengths identified in either the whole hippocampal proteome or the CaMK2a-Cre::R26-MetRS* proteome (ANL labelled and purified proteins). The hippocampus proteome is represented by red bars and light blue bars represent the CaMK2a-Cre::R26-MetRS* proteome. The overlap between both proteomes is represented by dark blue lines. **b)** Median methionine content of purified ANL labelled or unlabelled proteomes. Note that the methionine content of the CaMK2a-Cre::R26-MetRS* (CaMK2-HC) and the GAD2-Cre::R26-MetRS* (GAD2) proteomes was not significantly different from the other unlabelled proteomes from hippocampus, cerebellum or primary

cultures of glia. The upper and lower bounds of the box indicate the 75 and 25 percentiles of the data, respectively, and the whiskers indicate the 95th (upper) and 5th (lower) percentiles (Figure and legend modified from [11]).

Figure 6: Methionine content of the eluted peptides

Graph representing lower methionine content of the eluted peptides in the ANL labelled Camk2-Cre::R26-MetRS* (21 days) samples compared with peptides detected in no-ANL labeled hippocampus extracts. These data suggest that most of the purified proteins had ANL incorporated (that is not identified by MS). **a)** Percentage of methionine free identified peptides in non labelled crude hippocampus extracts and Camk2-Cre::R26-MetRS* proteome. **b)** Percentage of peptides with 1 and 2 methionines in non labelled crude hippocampus extracts and Camk2-Cre::R26-MetRS* proteome. In the graphs the bars indicate the 99 percentage confidence interval for biological variation

Supplementary Information:

Supplementary Figure 1

Supplementary Figure 2

0.1% Methionine mouse chow recipe

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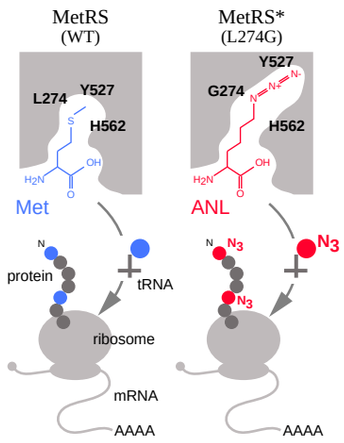
Figure 1

a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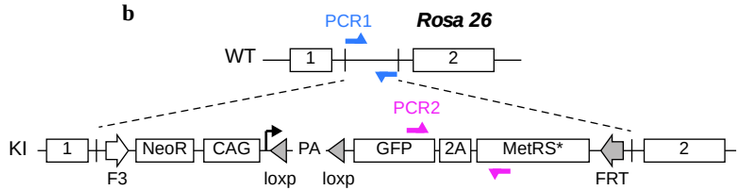

PCR1
Rosa 26
WT
1
2
PCR2
KI
1
NeoR
CAG
PA
GFP
2A
MetRS*
2
F3
loxp
loxp
FRT

Figure 2

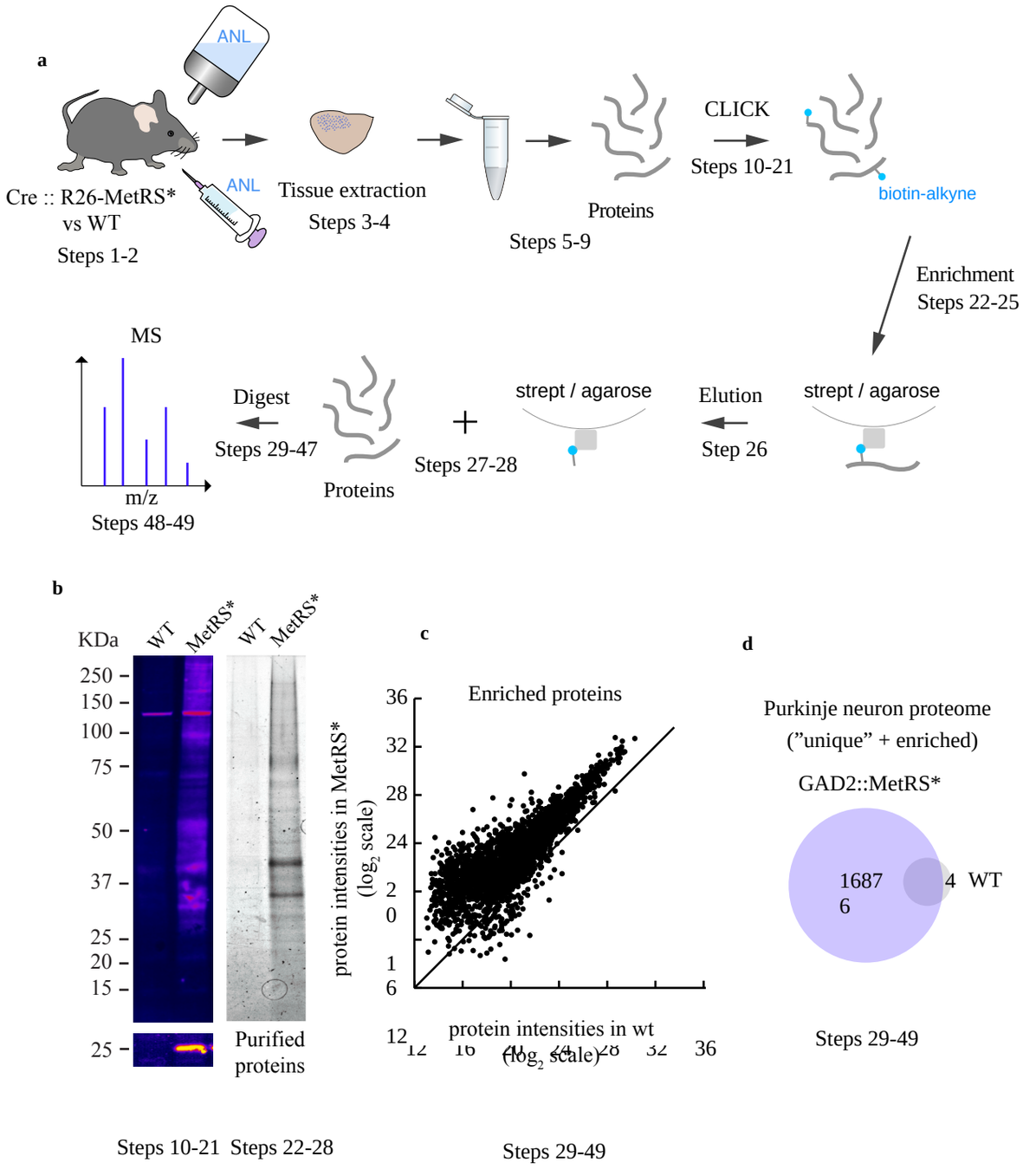

a
ANL
ANL
Cre :: R26-MetRS*
vs WT
Steps 1-2
Tissue extraction
Steps 3-4
Proteins
Steps 5-9
CLICK
Steps 10-21
biotin-alkyne
Enrichment
Steps 22-25
strept / agarose
Elution
Step 26
strept / agarose
Steps 27-28
Proteins
Digest
Steps 29-47
MS
m/z
Steps 48-49
b
KDa
WT
MetRS*
WT
MetRS*
250
150
100
75
50
37
25
20
15
25
Purified
proteins
Steps 10-21
Steps 22-28
c
Enriched proteins
protein intensities in MetRS* (log2 scale)
protein intensities in wt (log2 scale)
36
32
28
24
2
0
1
6
12
16
20
24
28
32
36
Steps 29-49
d
Purkinje neuron proteome
(”unique” + enriched)
GAD2::MetRS*
1687
6
4
WT
Steps 29-49

Figure 3

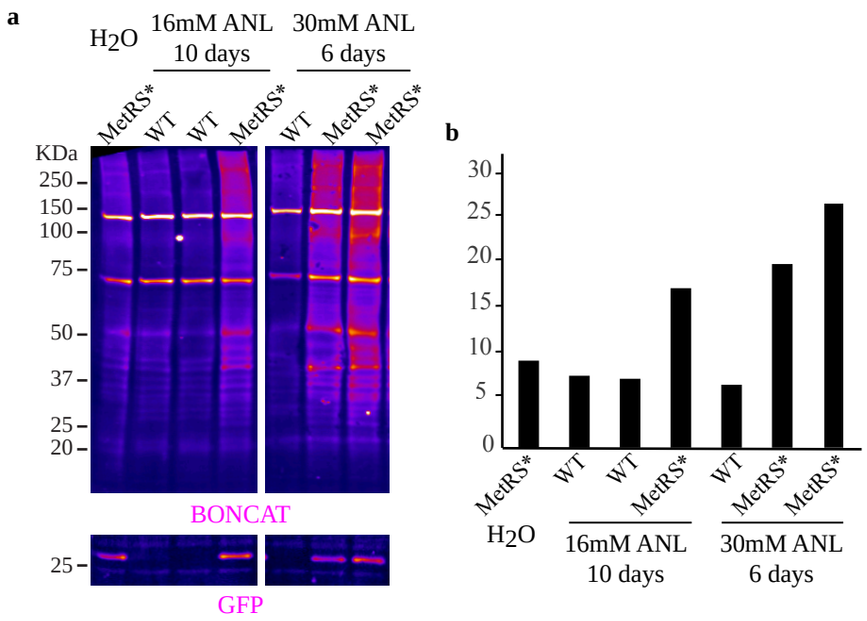

a
H2O
16mM ANL
10 days
30mM ANL
6 days
MetRS*
WT
WT
MetRS*
WT
MetRS*
MetRS*
KDa
250
150
100
75
50
37
25
20
BONCAT
25
GFP
b
30
25
20
15
10
5
0
MetRS*
WT
WT
MetRS*
WT
MetRS*
MetRS*
H2O
16mM ANL
10 days
30mM ANL
6 days

Figure 4

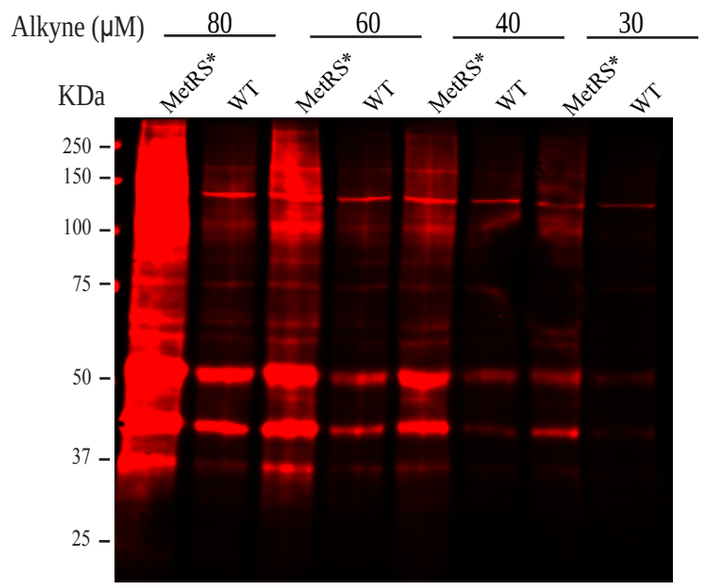
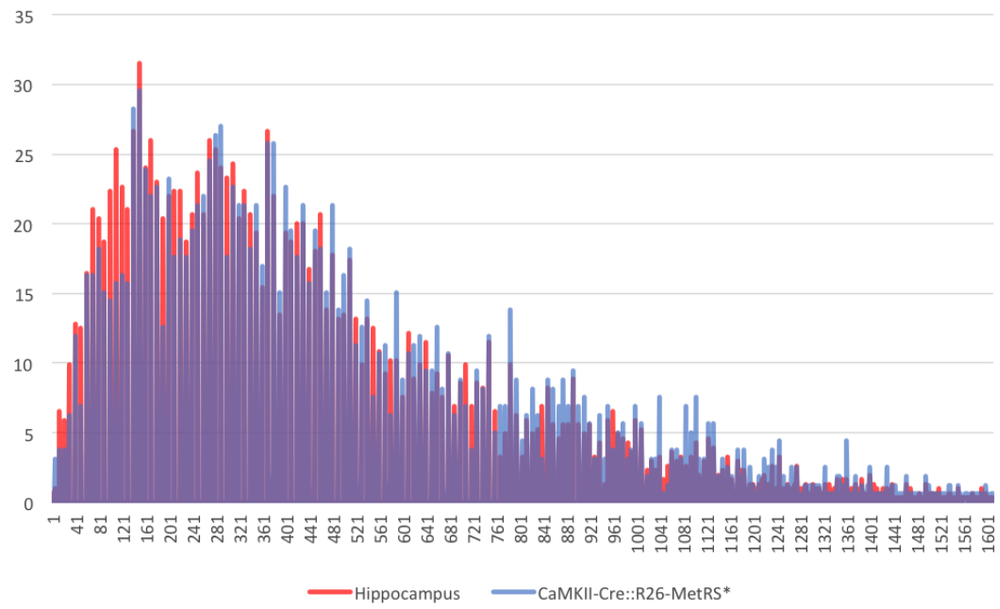

Alkyne (μM)
80
60
40
30
KDa
MetRS*
WT
MetRS*
WT
MetRS*
WT
MetRS*
WT
250
150
100
75
50
37
25
Anti biotin

Figure 5

a


35
30
25
20
15
10
5
0
1
41
81
121
161
201
241
281
321
361
401
441
481
521
561
601
641
681
721
761
801
841
881
921
961
1001
1041
1081
1121
1161
1201
1241
1281
1321
1361
1401
1441
1481
1521
1561
1601
Hippocampus
CaMKII-Cre::R26-MetRS*

b

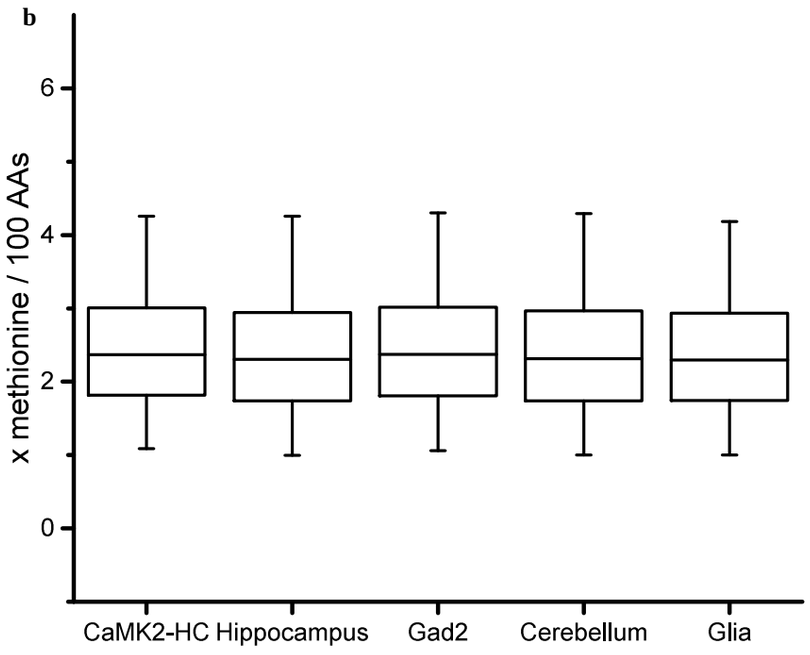
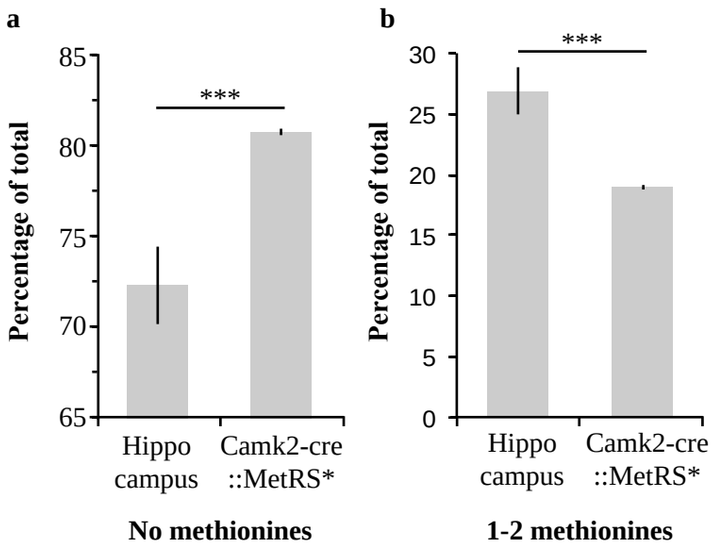
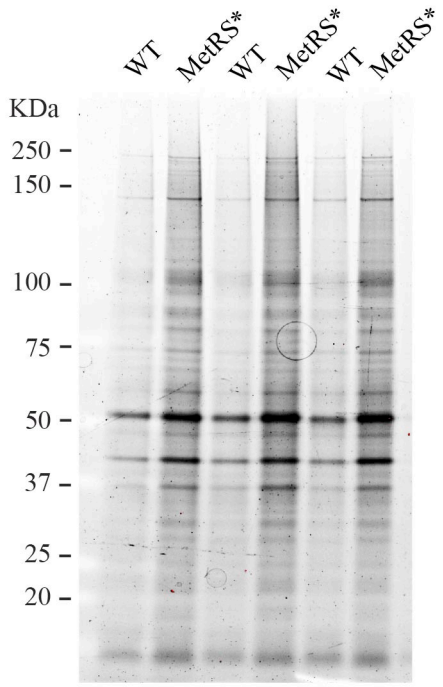

x methionine / 100 AAs
6
4
2
0
CaMK2-HC Hippocampus
Gad2
Cerebellum
Glia

Figure 6

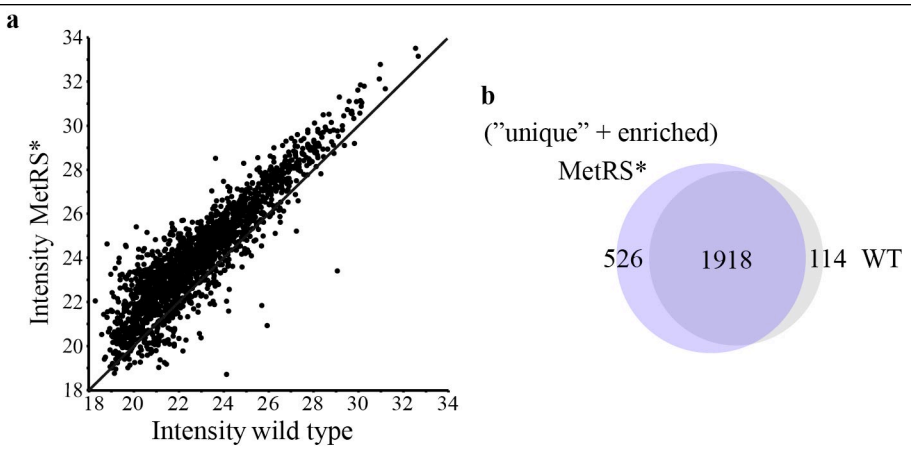

a
b
Percentage of total
85
80
75
70
65
***
Hippo campus
Camk2-cre ::MetRS*
No methionines
Percentage of total
30
25
20
15
10
5
0
***
Hippo campus
Camk2-cre ::MetRS*
1-2 methionines



Supplementary Figure 1

Sypro Ruby staining of eluted proteins

Gel stained with Sypro Ruby showing 3 biological replicates of cell-type specific eluted proteins derived from the negative control (wt mice) and Camk2-Cre::R26-MetRS* mice, labelled during 21 days with 1% of ANL administered in the drinking water. The hippocampus was dissected and used for the experiment (Figure and legend modified from[11]).



Supplementary Figure 2

example of a failed biological replicate

a) Plot showing similar abundance in Camk2-Cre::R26-MetRS* compared with WT mouse samples of proteins found in both groups (peptide intensities). **b)** Union of proteins unique to or markedly enriched (>3-fold WT) in Camk2-Cre::R26-MetRS* mice, showing a very low number of proteins (526).

NUTRITIONAL PROFILE [1]

Protein, %	***13.7***
Arginine, %	0.83
Histidine, %	0.49
Isoleucine, %	0.80
Leucine, %	1.20
Lysine, %	1.12
Methionine, %	0.10
Cystine, %	0.40
Phenylalanine, %	0.80
Tyrosine, %	0.40
Threonine, %	0.78
Tryptophan, %	0.20
Valine, %	0.80
Alanine, %	1.00
Aspartic Acid, %	1.00
Glutamic Acid, %	1.00
Glycine, %	0.99
Proline, %	1.00
Serine, %	1.00
Taurine, %	0.00
Fat, %	***5.1***
Cholesterol, ppm	0
Linoleic Acid, %	2.86
Linolenic Acid, %	0.05
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.05
Total Saturated Fatty A	0.64
Total Monounsaturated Fatty Acids, %	1.21
Polyunsaturated Fatty Acids, %	2.90
Fiber (max), %	***0.0***
Carbohydrates, %	***73.3***
Energy (kcal/g) [2]	***3.94***

From:	kcal	%
Protein	0.546	13.9
Fat (ether extract)	0.458	11.7
Carbohydrates	2.931	74.5

Minerals	
Calcium, %	1.21
Phosphorus, %	0.72
Potassium, %	0.41
Magnesium, %	0.01
Sodium, %	0.64
Chloride, %	1.15
Fluorine, ppm	0.0
Iron, ppm	87
Zinc, ppm	52
Manganese, ppm	211
Copper, ppm	5.0
Cobalt, ppm	0.3
Iodine, ppm	30.58
Chromium (added), ppm	0.0
Molybdenum, ppm	35.69
Selenium, ppm	0.46
Vitamins	
Vitamin A, IU/g	5.2
Vitamin D-3 (added), IU/g	0.9
Vitamin E, IU/kg	20.0
Vitamin K, ppm	2.00
Thiamin Hydrochloride, ppm	18.4
Riboflavin, ppm	10.0
Niacin, ppm	50
Pantothenic Acid, ppm	28
Folic Acid, ppm	4.0
Pyridoxine, ppm	4.9
Biotin, ppm	0.6
Vitamin B-12, mcg/kg	38
Choline Chloride, ppm	700
Ascorbic Acid, ppm	250.0

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.