Cell-selective labelling of proteomes in Drosophila melanogaster

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The specification and adaptability of cells rely on changes in protein composition. Nonetheless, uncovering proteome dynamics with cell-type-specific resolution remains challenging. Here we introduce a strategy for cell-specific analysis of newly synthesized proteomes by combining targeted expression of a mutated methionyl-tRNA synthetase (MetRS) with bioorthogonal or fluorescent non-canonical amino-acid-tagging techniques (BONCAT or FUNCAT). Substituting leucine by glycine within the MetRS-binding pocket (MetRSLtoG) enables incorporation of the non-canonical amino acid azidonorleucine (ANL) instead of methionine during translation. Newly synthesized proteins can thus be labelled by coupling the azide group of ANL to alkyne-bearing tags through ‘click chemistry’. To test these methods for applicability in vivo, we expressed MetRSLtoG cell specifically in Drosophila. FUNCAT and BONCAT reveal ANL incorporation into proteins selectively in cells expressing the mutated enzyme. Cell-type-specific FUNCAT and BONCAT, thus, constitute eligible techniques to study protein synthesis-dependent processes in complex and behaving organisms.
Cell type diversification and the execution of cell-type-specific functions is tightly coupled with the establishment of cell-specific proteomes and their proper modulation according to extrinsic and intrinsic signals. In fact, regulatory processes that act on protein synthesis, degradation and post-translational modification are permissive and sometimes instructive to higher-order organisational functions such as the adaptation of energy metabolism, immune defence or the acquisition and storage of memory. Thus, characterizing cell-specific proteome dynamics is key to understand physiological and pathological conditions at the level of single cells, organs and, ultimately, whole organisms. Facing ~10,000 different proteins in a mammalian cell\(^1\), in-depth identification of such a cell’s proteome, let alone the comparison with other cellular proteomes, is highly challenging. Proteome characterizations become even more demanding if temporal and spatial aspects, that is, proteome dynamics, are to be considered. Immense technological advances have allowed for increasingly efficient assessments of proteomes of eukaryotic cell lines, distinct tissues and readily cultivable cell types such as blood cells\(^2\)-\(^5\). Moreover, refined methods for protein fractionation and enrichment according to various criteria have led to successful characterizations of cellular subproteomes. For instance, post-translational modifications such as phosphorylation or ubiquitination provide a suitable handle for enrichment of the ‘phosphoproteome’\(^6\) or for proteins destined for degradation via ubiquitination\(^7\) or sumoylation\(^8\). In contrast, reducing sample complexity by selectively enriching for newly synthesized proteins is troublesome, since all proteins—old and new—share the same pool of 20 amino acids. Thus, until now, monitoring proteomes in multicellular organisms with cell-type-specific and temporal resolution has been impossible for cells that are tightly embedded in their respective tissues. As another complication, cellular and subcellular proteome turnover rates are usually low as recently demonstrated for the pool of synaptic proteins in mammalian primary cultures with a turnover rate of 0.7% of the synaptic protein content per hour\(^9\).

Bioorthogonal labelling strategies have been developed to globally label newly synthesized proteins or distinct post-translational modifications using the endogenous biosynthesis machinery (reviewed in ref. 10). Global metabolic labelling of newly synthesized proteins can be achieved using isotopic amino acids (as used in SILAC in combination with mass spectrometry\(^11\)), functionalized non-canonical amino acids\(^12\) or functionalized derivatives of the protein synthesis inhibitor puromycin\(^13\). The non-canonical amino acids azidohomoalanine (AHA) or homopropargylglycine (HPG) harbor an azide or an alkyne group, respectively, and these groups confer unique chemical functionality to their target molecules, which can subsequently be tagged with exogenously delivered probes for detection or isolation in a highly selective manner using copper-catalyzed [3+2] azide-alkyne-cycloaddition (CuAAC, ‘click chemistry’). AHA or HPG is used instead of methionine by the endogenous methionyl-transfer RNA (tRNA) synthetase (MetRS), that is, loaded onto respective tRNAs and incorporated into nascent polypeptide chains. The new chemical functionality of these proteins can be exploited for their identification (BONCAT, bioorthogonal non-canonical amino-acid tagging\(^14,15\)) and visualization (FUNCAT, fluorescent non-canonical amino-acid tagging\(^16-18\)). Both methods enable not only the analysis of activity-dependent global changes of cellular proteomes but also the identification of local synthesis hot spots in cellular subcompartments such as dendrites\(^17\). So far, AHA and HPG have been used to track new protein synthesis in a variety of model systems including bacterial\(^19\), mammalian cell culture models\(^16,17,20-21\) and larval zebrafish\(^22\). Recently, Tirrell and co-workers reported mutated variants of bacterial MetRS (EcMetRS), which charge the azide-harboursing amino acid azidonoleucine (ANL) onto methionine initiator tRNA, allowing its N-terminal incorporation into nascent proteins\(^23\). Importantly, in complex bacterial or mammalian cell mixtures, the residue-specific incorporation of ANL was restricted to cells expressing a mutant MetRS\(^25,26\).

The fruit fly Drosophila has recently been used in a number of studies that addressed subproteomes as diverse as the networks of proteins involved in the Hippo signalling pathway, innate immune reactions or the assembly of presynaptic active zones\(^27-29\). Moreover, high-coverage proteome analyses have been performed for Drosophila S2-cells and for different developmental stages of the organism, which resulted in valuable reference data sets, for example, for establishing comprehensive protein–protein interaction maps\(^30,31\). Here we present a method that adds cell selectivity as an important expansion for labelling proteomes in living Drosophila larvae and adult flies. A crucial step was to identify a leucine to glycine substitution within the binding pocket of murine or Drosophila MetRS (L1274G or L262G, respectively; collectively referred to as MetRS\(^1\) or MetRS\(^1\)) as a way to enable the enzymes to charge Drosophila methionyl-tRNA with ANL. On targeted expression of MetRS\(^1\) in flies, efficient cell-specific, time-dependent and concentration-dependent incorporation of food-supplied ANL into proteins is uncovered by FUNCAT. Moreover, BONCAT reveals a bulk number of labelled proteins in each of the tested cell types, implying that many proteins can be assessed simultaneously for cell-type-specific, temporally controlled and context-dependent synthesis of proteins.

**Results**

Drosophila proteomes are amenable to tagging by AHA. As previously shown, the non-canonical amino acid AHA (Supplementary Fig. 1a) can be used to detect newly synthesized proteins\(^11\). Therefore, to assess the principal amenable of Drosophila for metabolic protein labelling by FUNCAT and BONCAT, we grew larvae and adult flies on AHA-containing food and subjected them to either type of analysis as schematically depicted in Supplementary Fig. 1b. Using the red-fluorescent dye tetramethylrhodamine (TAMRA) in CuAAC reactions on larval body walls revealed efficient incorporation of AHA into muscles (Supplementary Fig. 2a) and any other tissue attached to this preparation such as motor neurons. Only low-background fluorescence levels were monitored when the reaction was applied to animals grown on AHA-free food (Supplementary Fig. 2b). Consistently, CuAAC-mediated coupling of a biotin-alkyne tag was efficient and specific for protein samples isolated from AHA-fed flies as revealed by western blot analysis (see accompanying paper by Niehues et al., Supplementary Fig. 10b).

Cell-specific in situ labelling of proteins by NCAT. Next, we replaced AHA in the food by the non-canonical amino acid ANL (Supplementary Fig. 1a). Neither FUNCAT nor BONCAT assays revealed incorporation of ANL into proteomes of wild-type flies (Supplementary Fig. 2c), implying that endogenous aminoacyl-tRNA synthetases are unable to use ANL as a substrate. Previous work, however, revealed that various mutations altering the methionine-binding pocket of the MetRS from Escherichia coli (EcMetRS) enabled the enzyme to couple ANL to tRNA and that the product is efficiently used in protein synthesis\(^23-26,32\). Among those mutant MetRS forms are the single amino acid mutant EcMetRS\(^1\) and the triple amino acid mutant EcMetRS\(^1\). The binding pocket of MetRS is evolutionary
well-conserved (Supplementary Fig. 3) and this led us to construct enhanced green fluorescent protein (EGFP)-tagged forms of wild type and mutated murine MetRS (mMetRS). As the mMetRS with the single amino-acid mutation L274G incorporated ANL most efficiently in cell culture assays (A.M. and D.C.D., unpublished observations), it was used to generate flies carrying the Gal4-inducible transgene UAS-mMetRSL274G-EGFP. Flies carrying the wild-type form, that is, UAS-mMetRSwt-EGFP, were generated as controls. Furthermore, UAS constructs encoding myc-epitope- or EGFP-tagged Drosophila MetRS variants mutated in the same manner at the respective position, that is, Leu$^{262}$ (Supplementary Fig. 3), were used to generate transgenic flies (UAS-dMetRSL262G,3xmyc, UAS-dMetRSL262G,EGFP). We then used FUNCAT to assess whether targeted expression of the mutated MetRS variants allows for cell-type-specific labelling of Drosophila proteomes. After testing various concentrations (Supplementary Fig. 5, also see Supplementary Note 1), ANL was food supplied at 4 mM throughout this study. As exemplified for dMetRSL262G-EGFP in Fig. 1, we found that at larval neuromuscular junctions only cells expressing any of the mutated MetRS variants show incorporation of ANL in either neurons (elavC155-Gal4; Fig. 1a), glia cells (repo-Gal4; Fig. 1b) or muscle cells (C57-Gal4; Fig. 1c). Likewise, ANL-labelled proteins were detectable only in appropriate subregions of wing disc epithelia when ptc-Gal4 was used to drive expression of the mutated enzyme (Fig. 1d). Expression of wild-type mMetRS-EGFP did not result in detectable labelling (Supplementary Fig. 4a), whereas C57-Gal4-driven mMetRS$^{L274G}$-EGFP expression leads to ample ANL incorporation into muscles of larval body walls (Supplementary Fig. 4b). On targeted expression of dMetRS$^{L262G}$-EGFP in muscle cells, TAMRA-tagged ANL-labelled proteins are found throughout the cell (Fig. 1c), that is, in the cytosol, abundantly in the nucleus and in the area of the bouton surrounding subsynaptic reticulum. This implies that ANL-harbouring proteins belong to different categories including soluble and membrane-associated proteins.

Cell-type-specific bulk labelling of proteins by ANL-BONCAT. Cell-specific labelling by FUNCAT could reflect incorporation of ANL into many different proteins or just into a few abundantly expressed proteins. To discriminate between these possibilities, we analysed cell-type-specific ANL-labelled proteomes by western blot (Fig. 2). Specifically, we expressed mMetRS$^{L274G}$-EGFP in larval muscles, adult neurons or adult glial cells and reared the animals on food with or without ANL. Protein lysates from larval body walls or adult heads (comprising neurons and glia) were subjected to CuAAC reaction with a biotin-alkyne affinity tag, subjected to affinity purification and then analysed by western blot using an anti-biotin antibody. Lysates from ANL-fed flies gave rise to strong signals virtually across the entire molecular weight range. Only a few distinct bands were detectable in control samples, most likely reflecting endogenously biotinylated proteins

Figure 1 | Cell-type-specific in situ labelling of proteins via FUNCAT. (a–c) ANL incorporation into larval proteins is monitored via FUNCAT on targeted expression of dMetRSL262G-EGFP in neurons (a, elavC155-Gal4; UAS-dMetRSL262G-EGFP), glia cells (b, repo-Gal4/UAS-dMetRSL262G-EGFP) and muscle cells (c, C57-Gal4/UAS-dMetRSL262G-EGFP) at larval neuromuscular junctions (muscles 6/7, segment A2). Co-staining with the neuron-specific marker anti-HRP (a–c) reveals that, wherever nerve terminal boutons (b), glial protrusions (g) and muscles (m) are in close contact, ANL-TAMRA signals are restricted to the dMetRSL262G-EGFP-expressing cell type (a–c). dMetRSL262G-EGFP is predominantly found in the cytosol, whereas TAMRA-harbouring proteins are detectable throughout cells including nuclei (n) and the bouton surrounding SSR area (c, dashed line). (d) Expression of dMetRSL262G-EGFP in a wing disc epithelium along the anterior–posterior border (ptc-Gal4;UAS-dMetRSL262G-EGFP) is accompanied by a respective confinement of ANL-Atto647N signals. The outline represents the shape of the entire disc. Scale bars, 10 μm (a,b); 5 μm (c); and 50 μm (d). SSR, subsynaptic reticulum.
(Fig. 2a–c; ‘anti-biotin’). This demonstrates that in all three cell types, ANL became efficiently incorporated into at least a broad variety of proteins. We next aimed to assess ANL incorporation into selected proteins. Western blot analysis on larval body wall extracts, which comprise both muscle and epithelial cells, typically reveals two bands when probed with antibodies against the scaffold protein discs large (Dlg), corresponding to isoforms DlgA and DlgS97. On muscle-specific expression of mMetRS<sup>1274G</sup>-EGFP both bands were detected at virtually the same ratio in BONCAT treated, affinity-purified extracts from ANL-fed larvae that expressed dMetRS<sup>262G</sup>-EGFP (Fig. 2b; ‘anti-candidate protein’). This demonstrates that both DlgA and DlgS97 are expressed in muscles as inferred previously from more indirect evidence<sup>33</sup>. We further detected ANL incorporation into the synaptic vesicle protein Synapsin in head lysates from flies that expressed dMetRS<sup>262G</sup>-EGFP ubiquitously (ubi-Gal4) (Fig. 2d; ‘anti-Synapsin’) and into the glial engulfment receptor Draper in head lysates from flies with repo-Gal4/UAS-mMetRSL274G+ANL–ANL (Fig. 2c; ‘anti-Draper8A1’). The latter also contained ANL-labelled Dlg (Fig. 2c; ‘anti-candidate protein’), pointing to hitherto largely unnoticed glial expression of Dlg in the adult brain.

Control samples from flies that received no ANL or expressed mMetRS<sup>1274G</sup> yielded no specific bands (Fig. 2). Very similar results were obtained when dMetRS<sup>262G</sup> variants were employed (Supplementary Fig. 5). Thus, cell-type-specific ANL-labelling can be tracked at both the level of bulk protein synthesis as well as at the level of individual proteins.

We performed two independent assays to substantiate that MetRS<sup>1274G</sup> replaces protein internal rather than just amino terminal methionine residues by ANL. First, we exploited that processing of the transmembrane protein Notch gives terminal methionine residues by ANL. First, we exploited that processing of the transmembrane protein Notch gives

| Figure 2 | Tagging of ANL-labelled proteins in Drosophila larvae and flies. | L3 stage larval body walls (a) or brains of L3 stage larvae (d) were dissected after chronic ANL feeding using 4 mM ANL concentration. Heads from adult Drosophila flies (b, c) were collected 0–3 days post eclosion after chronic ANL feeding using an ANL concentration of 4 mM. ANL is efficiently incorporated into muscle (a), neuronal (b) or glial proteins (c) when Drosophila larvae (a,d) or adult flies (b,c) express mMetRS<sup>1274G</sup>-EGFP or dMetRS<sup>262G</sup>-EGFP. ANL-labelled proteins were tagged by a biotin-alkyne affinity-tag, and purified via NeutrAvidin agarose. Tagged input (I, before NeutrAvidin purification), unbound (U, no ANL-containing proteins) and eluted (E, enriched ANL-labelled proteins after NeutrAvidin purification) fractions from ANL labelled and control samples were run in mirror-imaged order on SDS-PAA gels, blotted and probed with anti-biotin antibody. Effective ANL labelling and subsequent biotin tagging were verified for selected marker proteins (‘anti-candidate protein’), that is, Dlg (a) in muscles, Synapsin in neurons (b) as well as Draper I (c) and to a small amount Dlg (c) in glia cells. Furthermore, the intracellular domain (d) of the transmembrane protein Notch was found to be ANL labelled when dMetRS<sup>262G</sup>-EGFP is expressed ubiquitously. |
spectrometry and found internal peptides of the enzyme harbouring ANL instead of methionine (Supplementary Fig. 6).

**Limited side effects by chronic ANL incorporation.** Having documented MetRS\textsuperscript{LtoG}-mediated ANL incorporation into a wide range of proteins, we investigated putative side effects. To this end, we crossed heterozygous UAS-MetRS\textsuperscript{LtoG}-EGFP lines to various Gal4 strains and compared MetRS\textsuperscript{LtoG}-expressing offspring with their non-expressing siblings (Supplementary Fig. 7). In fact, we noticed that chronic mMetRS\textsuperscript{L274G}-EGFP-mediated incorporation of ANL into muscle proteins (C57-Gal4) caused an impairment in larval growth (Supplementary Fig. 7a), accompanied by reduced mobility in a larval crawling assay\textsuperscript{35} (Supplementary Fig. 8c) and followed by high lethality during pupal development. Notably, much less pronounced deficits in larval growth and no impairment in larval locomotion were observed when dMetRS\textsuperscript{L262G}-EGFP was expressed in the same manner (Supplementary Figs 7a and 8b), despite efficient ANL incorporation (Fig. 1c; Supplementary Fig. 5a–c). Also, depending on which MetRS\textsuperscript{LtoG} variant was used, chronic ANL incorporation into glia cells (repo-Gal4) had an effect at 2 mM ANL and at 8 mM ANL (Supplementary Fig. 7a) or no effect (Supplementary Fig. 7a) on the number of eclosed progeny. With one exception (repo-Gal4/UAS-dMetRS\textsuperscript{L262G}-EGFP; Supplementary Fig. 7a), we found a correlation between larval growth/eclosion rate and administered ANL concentration (Supplementary Fig. 7a). In case of pan-neuronally (elav\textsuperscript{C155}-Gal4) expressed MetRS\textsuperscript{LtoG}, however, we repeatedly found that at 2, 4 and 8 mM ANL, the number of eclosed flies was significantly reduced compared with the expected eclosion rate of 1.0 (Supplementary Fig. 7a). Notably, once eclosed survival of adults appeared largely unaffected for both elav\textsuperscript{C155}-Gal4- and repo-Gal4-driven dMetRS\textsuperscript{L262G}-EGFP (Supplementary Fig. 7b). As we did not observe extraordinary pupal lethality in elav\textsuperscript{C155}-Gal4-expressing MetRS\textsuperscript{LtoG} variants, we reasoned that MetRS\textsuperscript{LtoG}-expressing animals might be aggrieved during larval phases. An inspection of larval neuromuscular junctions by confocal microscopy using common markers did not uncover striking defects (Fig. 3) and, consistently, ANL-reared elav\textsuperscript{C155}-Gal4;UAS-MetRS\textsuperscript{LtoG}-EGFP larvae behaved normally in our crawling assay (Supplementary Fig. 8d,e). Reasoning that rather than a single major effect a variety of subtle side effects of ANL might collectively account for compromised vitality and behaviour, we assayed in addition to the above mentioned larval crawling assay the impact of ANL on adult behaviours and vitality. Focusing on neuronal ANL incorporation (elav\textsuperscript{C155};Gal4;UAS-MetRS\textsuperscript{LtoG}-EGFP), we used the rapid iterative negative geotaxis assay\textsuperscript{35,36}, the island assay\textsuperscript{37} and an ethanol sensitivity assay\textsuperscript{38}. In general, ANL per se did not have effects on any of the behaviours tested in wild-type flies (Supplementary Figs 8a,f, 9a,b, 10a,b and 11a,d). Moreover, in the majority of assays, chronic administration of ANL (that is, throughout the entire development) resulted in no discernible behavioural abnormalities (Supplementary Figs 9c–f and 11b,c). Deficits were only monitored in the negative geotaxis assay for both MetRS\textsuperscript{LtoG} forms (Supplementary Fig. 8g,h) and for mMetRS\textsuperscript{L274G}-EGFP-expressing flies in delayed platform clearance in the island assay, whereas the other performances were not affected (Supplementary Fig. 9c,d). Notably, no such deficits were observed when ANL was fed acutely (that is, 48 h; Supplementary Figs 8g,h, 10c–f and 11e,f).

**NCAT labelling correlates with duration of ANL exposure.** To validate the versatility of the method, we tested shorter feeding and labelling periods in larvae and adult flies. Using the OK371-Gal4 driver line to express dMetRS\textsuperscript{L262G}-EGFP in motor neurons, we evaluated NCAT signal intensity in the ventral nerve cord after 12, 24 and 48 h of ANL exposure in L3 larval body wall preparations (Fig. 4a,b). After feeding larvae for 12 h with 4 mM ANL, newly synthesized ANL-harbouring proteins were mainly detectable in the nuclei and to a lesser extent in the cytosol of cell bodies of motor neurons (Fig. 4a). After 24 h, bright staining was observable in the nuclei, the somatic cytosol and outgoing processes (Fig. 4a,b). Labelling intensities reached very high levels throughout the neurons after 48 h of exposure (Fig. 4a,b). We observed increasing variation of labelling intensities with increasing ANL incorporation times (Fig. 4b) possibly due to distinct inter-individual translation rates. Next, we performed the short-term labelling experiment in adult flies in conjunction with BONCAT analysis (Fig. 5a). Here we observed the same increase both in global protein synthesis as well as on single protein level for synapsin within adult head lysates from 24 to 48 h of feeding time (Fig. 5a).

During metamorphosis, the *Drosophila* nervous system undergoes a series of drastic changes and reorganization steps (reviewed in ref. 39). We wondered whether ANL labelling would outlast this phase and performed a metabolic pulse-labelling experiment in elav\textsuperscript{C155}-Gal4;UAS-mMetRS\textsuperscript{L274G}-EGFP animals. Larvae were allowed to feed ad libitum on ANL-containing food for 4 days. Half of wandering L3 stage elav\textsuperscript{C155}-Gal4; UAS-mMetRS\textsuperscript{L274G}-EGFP-expressing larvae were put onto food without ANL (chase group) until eclosion, whereas their siblings remained on ANL-supplemented food. Figure 5b shows the western blot profile for both the groups. Although there is a clear reduction in ANL-harbouring proteins both on the global level as well as for synapsin (Fig. 5b) in the chase group as compared with the long-term labelling group, a substantial amount of ANL-bearing proteins can be detected in the chase group. It remains elusive, however, whether the remaining signal is derived from ‘surviving’ larval proteins or whether ANL was reused, for example, following the apoptosis of neurons.

**Discussion**

Small chemical reporters have helped to shed light on the dynamics of a variety of biological molecules. This holds especially true for alkyne- and azide-bearing fatty acids, sugars and non-canonical amino acids, which can be covalently coupled in *CuAAC* reactions with fluorescent tags for visualization or with tags bearing an affinity moiety for separation and biochemical and mass spectrometry analyses. The non-canonical amino acids AHA and HPG have been used to explore protein und nucleosome turnover\textsuperscript{9,40,41}, to monitor local protein synthesis\textsuperscript{17,20} and to identify locally synthesized proteins in neuronal processes\textsuperscript{21,42} as well as to perform quantification of proteome dynamics\textsuperscript{43–46} in different cell culture models and in entire larval zebrafish\textsuperscript{22}. Importantly, these NCAT techniques enable no cell-type-specific resolution as all cells per se incorporate AHA and HPG. To overcome this limitation, the Tirrell and Schuman groups recently used heterologous expression of a mutant MetRS from *E. coli* carrying a triple amino-acid mutation in the methionine-binding pocket (EcMetRS\textsuperscript{NLI}) to permit incorporation of ANL into proteins made in mammalian (HEK293) cells\textsuperscript{26}. However, as pointed out by the authors, the EcMetRS\textsuperscript{NLI} mutant enables ANL incorporation only at the very N terminus of proteins as it charges ANL only onto the initiator methionyl-tRNA. As it is thought that about 80%\textsuperscript{7–9} of proteins undergo N-terminal methionine cleavage or excision this constitutes a severe limitation\textsuperscript{26}. Moreover, the same paper reports that attempts to generate transgenic worms expressing this mutated form under the control of an inducible cell-specific promoter were not successful. Using
the single amino-acid mutation leucine to glycine at position 234 in the murine MetRS or at position 262 in the Drosophila MetRS in the evolutionary well-conserved methionine-binding pocket, we were able to solve this problem. We report here the generation of transgenic flies, which allow targeted expression of MetRSL274G and the Drosophila dMetRSL262G enable incorporation of ANL into nascent proteins, which can be visualized using the previously described FUNCAT procedure or biochemically analysed on global proteome or single protein level with BONCAT. Importantly, FUNCAT and BONCAT confirm that only cells bearing the mutated enzyme incorporate ANL during translation. ANL-harbouring proteins cover the entire molecular weight range. Notably, identification of ANL-modified peptides in the tandem mass spectrometry analysis as well as the successful tagging of type I transmembrane proteins such as Draper, which undergo signal peptide cleavage, and the intracellular Notch fragment confirm the substitution of methionine by ANL at any position within polypeptide chains. ANL incorporation is detectable via FUNCAT as early as 12 h of exposing larvae to ANL-containing food with increasing signal intensities after 24 and 48 h. This finding is mirrored for 24 and 48 h of ANL exposure with BONCAT. Interestingly, metabolic pulse labelling of larval neuronal proteins with ANL using elav-Gal4;;UAS-dMetRSL262G-3xmyc expressing larvae revealed a substantial amount of ANL-harbouring proteins in flies 3 days post eclosion. Whether these proteins have been synthesized in larval stages and carried over into adult flies (many neurons will persist throughout metamorphosis) or whether these proteins reflect incorporation of recycled ANL from apoptotic cell material has to be clarified in future pulse-chase studies.

Proper protein function and localization are of critical importance for proteomic approaches that aim at characterizing cell-specific proteome dynamics with the ultimate goal to understand physiological and pathological conditions at the level of single cells. Despite the bulk incorporation of ANL at 4 mM, we observed only limited side effects in most of the here applied survival and behavioural assays, in particular when dMetRSL262G was employed. For instance, wings, which allow for a very sensitive readout of patterning defects, remained unaffected when ANL became incorporated into proteins of cells along the anterior–posterior compartment border (Fig. 1d). A reduced number of flies eclosed when ANL was chronically incorporated into neuronal and glial proteomes. In light of future applications, we consider it of pivotal importance that acute incorporation of ANL in adults showed no deficits in any of our assays. It is conceivable, however, that thorough controls for side effects are required according to the specific design of any given experiment. Moreover, our time course experiments showed that shorter times of ANL incorporation into proteins are compatible with detection by both FUNCAT and BONCAT, thus providing a means to minimize side effects even further. Indeed, the possibility to carry out acute pulse labelling by simply feeding ANL expands the range of potential applications tremendously, not at last in behavioural assays related to learning and memory.

With the here presented cell-selective non-canonical amino acid tagging, we addressed the limitations of the previously reported labelling methods with AHA or HPG. Especially, usage of the mutated MetRS (MetRSL262G) does not require a Met-free culture medium or Met-depleted nutrition to incorporate ANL.

Figure 3 | Neuronal ANL incorporation leaves NMJ morphology and prominent NMJ marker proteins unaffected. Motor nerve terminals at NMJs from elav-Gal4;;UAS-dMetRSL262G-3xmyc L3 larvae fed without (a,c) or with (b,d) 4 mM ANL were visualized by the surface marker HRP and assessed for either FUNCAT-mediated TAMRA signals in combination with immunofluorescent labelling of the homophilic cell adhesion molecule FasII (a,b) or co-stained for the active zone marker Brp and PMCA to visualize the postsynaptic SSR compartment (c,d). There are no obvious differences between ANL- and non-ANL-reared animals. Scale bar, 5 µm. NMJ, neuromuscular junction; SSR, subsynaptic reticulum.
into proteins. It, thus, enables both cell-selective labelling in living animals as well as physiological concentrations of methionine during the labelling procedure.

Using this technique, we investigated protein synthesis rates in a Drosophila model for Charcot–Marie–Tooth associated with mutations in glycine-tRNA synthetase (GARS) in an accompanying paper (Niehues et al., 2015). Transgenic flies carrying transgenes for GARS mutants or GARS wild type and dMetRSL262G-EGFP were analysed for differences in protein synthesis rates in motor and sensory neurons. ANL-FUNCAT and ANL-BONCAT experiments revealed a significant reduction of protein synthesis rate in larvae expressing GARS mutant isoforms, up to 40–68% of control. In contrast, using AHA was not sensitive enough to detect labelled proteins in genotypes in which translation is inhibited probably due to competition by methionine. Radioactive labelling, in turn, uncovered a similar reduction, which, however, required ubiquitous expression of the mutant GARS proteins to avoid dilution of the effect by non-expressing cells. The use of cell-type-specific metabolic labelling with ANL can resolve such a particular limitation.

We, thus, consider cell-type-specific ANL-FUNCAT and ANL-BONCAT as eligible techniques to study protein synthesis-dependent processes in a complex and behaving organism such as Drosophila. While this manuscript was under review, the non-canonical amino acid azidophenylalanine in conjunction with a mutated phenylalanyl-tRNA synthetase was introduced for cell-specific proteome labelling in transgenic worms50, further expanding the toolbox of metabolic labelling approaches. In the future, these types of in vivo labelling of de novo synthesized proteins will facilitate the correlation of molecular changes with processes occurring during synaptic plasticity, or during the course of neurodegenerative events. Furthermore, it will help to unravel the identity of complete secreted proteomes and could be combined with metabolic labelling approaches to tackle post-translational modifications and controlled degradation of proteins as well.

**Figure 4 | NCAT monitoring of protein synthesis over time.** Newly synthesized proteins were monitored by FUNCAT in motor neurons of OK371-Gal4; UAS-dMetRSL262G-EGFP third instar larvae that were exposed to ANL for 12, 24 or 48 h (a). Selective dMetRSL262G-EGFP expression in motor neurons was confirmed by EGFP fluorescence (a, upper panel). Fluorescence intensity of TAMRA tagged newly synthesized proteins increased with increasing ANL exposure times (a, lower panel). Identical confocal settings were used for acquisition of all images, and representative images are shown. Scale bar, 20 μm. (b) Quantification of fluorescence intensities confirmed the positive correlation between FUNCAT labelling intensity and the duration of ANL exposure. Averages ± s.d. relative to 24 h ANL exposure (100%) are shown. Mann–Whitney U-test, n = 10–11, **P ≤ 0.01, ***P ≤ 0.001 12 versus 24 h; **P = 0.001530673; 12 versus 48 h: ***P = 1.08E – 04; 24 versus 48 h: ***P = 9.34E – 05. s.d. (12 h ANL): 11.83; s.d. (24 h ANL): 59.28; s.d. (48 h ANL): 103.50.
Methods
Reagents and antibodies. All reagents were American Chemical Society (ACS) grade and purchased from Sigma unless noted otherwise. AHA was prepared as described\(^{42}\). ANL was prepared as described for AHA via copper-catalysed diazo transfer\(^{53}\). Biotinylated t-butyloxycarbonyl\(-\)lysine was used to protect ANL from NeutrAvidin purification. The signal intensities for both overall newly synthesized proteins and synapsin increases from 24 to 48 h of ANL incorporation periods. The assessment of protein-incorporated ANL into proteins and into synapsin, which is transmitted from larval neurons throughout the pupal phase into adult brain proteins, is incorporated ANL into proteins and into synapsin, which is transmitted from larval neurons throughout the pupal phase into adult brain proteins.

Flies. Unless stated otherwise stocks and crosses were grown at 25 °C in a 14 h/10 h dark-light cycle on Otto normal medium (ONM) containing Agar–Agar (0.83% w/v), mashed raisins (4% w/v), yeast (6% w/v), semolina (5% w/v), sugar beet syrup (2.6% w/v), honey (2.6% w/v) and Nipagin (0.13% v/v). Dominant markers associated with balancer chromosomes, CyO, Cy\(^{+}\)\(^{\text{egfp}}\), and TM6B, Tb Hu, were used to establish transgenic and recombinant lines and to identify genotypes of progeny at larval and adult stages in crosses of heterozygous flies. All Gal4 activator strains have been described as following: repo-Gal4 (pan-neuronal)\(^{52}\), repo-Gal4 (glia-specific)\(^{53}\), OK371-Gal4 (motorneuronal)\(^{54}\), C57-Gal4 (muscle specific)\(^{55}\), ptc\(^{59.1}\)-Gal4 (subregional in imaginal discs)\(^{56}\) and ubi-Gal4 (all cell types, obtained from Bloomington stock center).

Toxicity test of ANL to Drosophila larvae and flies. To test any putative toxicity of ANL to Drosophila larvae, the body weight of wandering L3 stage larvae was determined using an ultra fine scale (Sartorius). Body weights of either dMetr\(^{52,56}\)-EGFP- or mMetr\(^{52,56}\)-EGFP-expressing larvae were compared between larvae reared on ONM with 2, 4 and 8 mM ANL or without ANL. All Gal4 activator strains have been described as following: repo-Gal4 (pan-neuronal)\(^{52}\), repo-Gal4 (glia-specific)\(^{53}\), OK371-Gal4 (motorneuronal)\(^{54}\), C57-Gal4 (muscle specific)\(^{55}\), ptc\(^{59.1}\)-Gal4 (subregional in imaginal discs)\(^{56}\) and ubi-Gal4 (all cell types, obtained from Bloomington stock center).
ONM with 2, 4 and 8 mM ANL or without ANL. Parental flies were removed from the offspring before reaching the pupal stage. After hatching of the first offspring (3 days after the crosses started), the number of offspring was counted every second day and compared between the different groups expressing either dMetRSL262G-EGFP or mMetRSL274G-EGFP versus flies not expressing dMetRSL262G-EGFP or mMetRSL274G-EGFP. To determine the metamorphosis and eclosion rate of ANL-incorporating flies, we compared the group size relative with the segregation after Mendel’s law (expressing either dMetRSL262G-EGFP or mMetRSL274G-EGFP) reared either on ONM with 2, 4 and 8 mM ANL or without ANL. Statistical analyses were performed using one sample t-tests against a theoretical mean of 1.0. To investigate the correlation between ANL incorporation and eclosion rate, linear regression analysis with a 95% confidence interval was performed.

Investigation of the survival rate of MetRSL262G-EGFP-expressing flies was performed by crossing virgin female flies of different driver strains (elav-C176;Gal4 or repo-Gal4) to male flies of the UAS strain (either UAS-dMetRSL262G-EGFP, UAS-mMetRSL274G-EGFP), rearing them either on ONM with 2, 4 or 8 mM or on ONM without ANL. At 0–3 days post eclosion, 10 flies each (five female and five male) were transferred either to ONM with the same previous ANL concentration (for example, 2 mM ANL—2 mM ANL) or to ONM without ANL (for example, 2 mM ANL—no ANL). Over a total period of 14 days, the number of living flies was determined every day.

**Click chemistry (CuAAC) and detection of tagged proteins.** Visualization of ANL- or AHA-labelled proteins using FUNCAT. Larval body walls were dissected in haemolymph-like 3 (HL-3) solution (70 mM NaCl, 5 mM KCl, 20 mM MgCl2, 0.1 mM Ca2+, 115 mM sucrose, 5 mM trehalose, 5 mM HEPEs pH 7.2) with 0.1% Triton X-100, washed with two to three changes of HL-3 solution (in 1× PB: phosphate buffer, 0.1 M NaH2PO4, 0.1 M Na2HPO4 pH 7.2) for 1 min. After exchanging the solution to 4% paraformaldehyde in PB pH 7.2 body walls were incubated for 30 min at room temperature under gentle agitation. Following fixation, body walls were washed three times with PB (phosphate buffer red salin pH 7.2) and then mounted in Mowiol (4% v/v glycerol, 30% (v/v) Mowiol 4–88, 3% (v/v) PDB, 0.1% formic acid in water at flow rates of 0.15 ml/min for 1 h). After washing, the Mowiol was removed and the slides were incubated with 0.1% (w/v) SDS and 0.2% (v/v) Triton X-100. After an incubation with 8.8 mM iodoacetamide in 100 mM ammonium bicarbonate (in the dark, room temperature for 30 min) and digestion in 10% acetonitrile and 0.1% formic acid in water at flow rates of 0.15 ml/min for 1 h. 1 h later, the slides were incubated with 0.1% (v/v) TBTA, Sigma–Aldrich; 200 mM in dimethylsulfoxide; 1:1,000), biotin-PEO3-alkyne tag (Invitrogen; 1 mM; 1:5,000) or Atto647N tagged by mixing triazole ligand (TBTA, Sigma–Aldrich; 200 mM in dimethylsulfoxide; 1:1,000), TAMRA-alkyne tag (Invitrogen; 1 mM; 1:5,000) or Atto647N tagged by mixing triazole ligand (TBTA, Sigma–Aldrich; 200 mM in dimethylsulfoxide; 1:1,000) and another three times with 1% (v/v) Igepal (Sigma–Aldrich) before mounting.
25/C176
10
For larval behaviour analyses after acute ANL feeding, crosses were reared on ONM for 1–2 days and transferred onto fresh ONM for 4–6 h. After 72 h at 25 °C, the larvae were washed out of the food with warm tap water and rinsed into a mesh basket before being transferred onto ONM or 4 mM ANL-containing ONM with a brush. Another 24 h later, the larvae were tested in the larval crawling assay. We adopted our protocol for the larval crawling assay from ref. 35. Ten UAS-MetrZ-GFP-expressing third instar larvae of a certain group were centred on a 2% agarose (Biozym) containing Petri dish over a 0.2 cm2 grid graph paper and videotaped. The number of grid lines each larva crossed within 1 min was determined and a mean was taken from these measurements (counted as one replicate) for each group. The progeny of at least three independent crosses was analysed. Student’s t-tests were used to test for statistical differences in crawling behaviour between larvae raised on ONM and larvae raised on 4 mM ANL-containing ONM.

We adopted our protocol for rapid iterative negative geotaxis assay from refs 35,36. A climbing apparatus was generated by placing an empty polystyrene vial (25 × 95 mm, VWR) with a circle mark at 8 cm from the bottom on the fly-containing food vial. The two vials were fixed with tape. The flies were allowed to acclimatize to this setting for >5 min. The number of flies that passed the 8 cm mark within 10 s after being tapped to the bottom of the vial was recorded. This procedure was repeated 10 times with an inter-trial interval of 5 min and a mean was taken from these measurements (counted as one replicate) for each group. The progeny of at least three independent crosses was analysed. Student’s t-tests were used to test for statistical differences in climbing behaviour between flies raised on ONM and flies fed on 4 mM ANL-containing ONM. There were no differences between male and female performances. Therefore, the data were pooled.

We adopted our protocol for the island assay from ref. 37. Flies were videotaped while released on a 12.7 × 8.5 cm2 platform (island) in the middle of a wetaty soap bath (31.2 × 15 cm2). We analysed the percentage of flies jumping, running, sitting, or flying after being released on the platform (Student’s t-tests) and determined the time needed to clear the platform. Differences in the time to clear the platform between flies reared on ONM or on 4 mM ANL-containing ONM were analysed with repeated measurement ANOVAs. There were no differences between male and female performances. Therefore, the data was pooled.

We adopted our protocol for ethanol sensitivity from ref. 38. Vials (25 × 95 mm, VWR) containing 10 flies were sealed with a 1 mL 97% ethanol (Fisher, stained with commercially available blue food dye) moistened plugs. The flies were videotaped for 20 min. The number of mobile flies was determined every minute. We analysed resistance to ethanol intoxication by comparing the number of mobile flies over the time course of 20 min (repeated measurement ANOVAs) and by comparing half-maximal sedation (ST50, Student’s t-tests) and determined the median t-values needed to clear the platform. Differences in the time to clear the platform between flies reared on ONM and flies fed on 4 mM ANL-containing ONM. There were no differences between male and female performances. Therefore, the data was pooled.

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Author contributions

I.E., O.K., K.M., S.N., E.S., U.T. and D.C.D. designed research; I.E., O.K., K.M., S.N., J.A., A.M., J.B., T.Z., U.T. and D.C.D. analysed data; all authors commented on the paper; I.E., O.K., K.M., S.N., E.S., U.T. and D.C.D. performed research; I.E., O.K., K.M., S.N., T.Z. and D.C.D. wrote the paper.

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