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Non-canonical amino acid labeling *in vivo* to visualize and affinity purify newly synthesized proteins in larval zebrafish.

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Lay-summary:

Determining what neural circuits and proteins are involved in encoding memories is a central goal in neuroscience. Here we demonstrate that protein synthesis, known to underlie long-term memory formation, can be visualized in the intact larval zebrafish. Non-canonical amino acids bearing small bio-orthogonal azide groups are introduced into proteins *in vivo* using the cells' own translational machinery. Via a 'click reaction', the newly synthesized, azide bearing proteins are labeled with either a fluorescent or a biotin alkyne, which allows for the identification and visualization of newly synthesized proteins. We show that azidohomoalanine, the non-canonical amino acid, is metabolically incorporated into newly synthesized proteins, in a time- and concentration-dependent manner, but has no apparent toxic effect and does not influence simple behaviors. This enables fluorescent labeling of newly synthesized proteins in whole mount larval zebrafish. This is a powerful tool to investigate new global protein synthesis under a variety of conditions to link behavior to specific changes in the proteome.

Abstract

Protein expression in the nervous system undergoes regulated changes in response to changes in behavioral states, in particular long-term memory formation. Recently, methods have been developed (BONCAT and FUNCAT), which introduce non-canonical amino acids bearing small bio-orthogonal functional groups into proteins using the cells' own translational machinery. Using the selective 'click reaction', this allows for the identification and visualization of newly synthesized proteins *in vitro*. Here we demonstrate that non-canonical amino acid labeling can be achieved *in vivo* in an intact organism capable of simple learning behavior, the larval zebrafish. We show that azidohomoalanine is metabolically incorporated into newly synthesized proteins, in a time- and concentration-dependent manner, but has no apparent toxic effect and does not influence simple behaviors such as spontaneous swimming and escape responses. This enables fluorescent labeling of newly synthesized proteins in whole mount larval zebrafish. Furthermore, stimulation with a GABA antagonist that elicits seizures in the larval zebrafish causes an increase in protein synthesis throughout the proteome, which can also be visualized in intact larvae.

Introduction

Both chemical stimuli and changes in behavioral states alter protein expression in the nervous system. In particular, studies in many different model organisms have shown that protein synthesis, during or shortly after learning, is an essential step in the formation of long-term memory [1]. In 1964, Agranoff *et al*. showed that the protein synthesis inhibitor (PSI) puromycin injected intracranially into the goldfish produces impairment of memory for a shock avoidance task and that this impairment is time- and PSI concentration-dependent [2,3]. Since then, protein synthesis has been shown to be necessary for long-term memory formation in a variety of learning paradigms, including appetitively and shock-motivated discrimination learning, passive and active avoidance learning, shuttle box learning, and long-term habituation [reviewed in 1].

While it is now clear that long-term memory requires new protein synthesis, the identification of newly synthesized proteins has been sparse and limited to individually identified candidate proteins. Advances in mass spectrometry based approaches now permit the characterization and quantification of proteins, especially when paired with approaches such as stable isotope labeling with amino acids in cell culture (SILAC) [4], which allow for comparative quantification between proteomes of differentially stimulated cell populations. However, the proteome of the nervous system is complex and without a chemical handle to enable affinity purification of the newly synthesized proteins specifically, proteins of low abundance will likely be missed.

In addition, the identification of cells or neural circuits that show increased protein synthesis in response to memory formation would allow us to understand the components of memory circuits that undergo long-term modifications after learning. Genetically encoded fluorescent tags, such as GFP, have revolutionized cell biology by permitting visualization of fusion proteins of interest *in vivo* [5]. However, the size of GFP and the requirement for genetic manipulation of the target

protein may interfere with its endogenous function, while at the same time only permitting investigation of a small number of candidates at once.

Recently, new techniques for labeling a variety of molecules based on the principle of bio-orthogonal metabolic labeling have been developed [6]. Here, small functional groups that are commonly absent in the cellular environment, most prominently ketones and azides or alkynes, are introduced using the cells' own synthetic machinery. Using this approach sugars [7], lipids [8], virus particles [9], DNA and RNA [10] have been labeled and subsequently visualized using fluorescent dyes or enriched and identified using affinity reagents. Bertozzi and coworkers, in particular, have demonstrated *in viv*o labeling of glycans in living organisms ranging from rodents [11, 12], to larval zebrafish [13, 14, 15] and *C. elegans* [16].

Using a similar approach, bio-orthogonal non-canonical amino acid tagging (BONCAT)[17, 18] and fluorescent non-canonical amino acid tagging (FUNCAT) [19] have been used to tag and identify or visualize newly synthesized proteins. BONCAT and FUNCAT utilize non-canonical methionine derivatives, such as the azide-bearing azidohomoalanine (AHA), to bio-orthogonally label newly synthesized proteins. AHA can cross cell membranes and be charged onto methionine tRNAs by the endogenous methionyl-tRNA synthetase (MetRS). During protein synthesis, AHA is introduced in place of methionine resulting in the introduction of azide groups into the newly synthesized proteins. These azide groups can be used to tag proteins with either an alkyne affinity tag (BONCAT) or an alkyne fluorescent tag (FUNCAT) via selective Cu(I)-catalyzed or strain-promoted [3+2] azide-alkyne cycloaddition [20, 21, 22]. Affinity tagged proteins can be quantified using immunoblot analysis or separated from the preexisting proteome by affinity purification and identified by tandem mass spectrometry. Fluorescent tags can be used to visualize newly synthesized proteins, including those proteins of interest whose identities may not be known. Alternatively, the alkyne moiety may also be introduced into newly synthesized proteins by replacing methionine with the non-canonical amino acid homopropargylglycine (HPG) and subsequently labeled using azide bearing affinity or fluorescent tags. Azides and alkynes are small, so light labeling with AHA or HPG is likely to only cause modest, perhaps even insignificant, perturbations of protein

folding, localization [17] and therefore function of the labeled protein *in vivo*. Furthermore, azides and alkynes are stable under biological conditions and essentially absent from vertebrate cells, which makes the azide-alkyne ligation ('click chemistry') very selective.

BONCAT and FUNCAT techniques have already successfully been applied to study the proteome of HEK293 cells during a two-hour time window [17], as well as investigate local protein synthesis in dissociated hippocampal cultures [19]. Furthermore, metabolic AHA incorporation has been used to identify regions of the drosophila genome that show high levels of histone turnover [23], show that Chlamydia co-opt the functions of the lysosomes of their host cells to acquire essential amino acids [24], as well as demonstrate that treatment of primary sensory neurons with the cytokine interleukin-6 or the neurotrophin nerve growth factor (NGF) increases nascent protein synthesis in axons [25]. Recently, these techniques have also been used to indicate that the transmembrane receptor DCC may regulate protein synthesis in a localized manner within the cells as DCC was found to overlap with areas of new protein synthesis at the tips of filopodia in commissural neurons [26]. However, these studies have only used the techniques *in vitro*. Given the role of protein synthesis in learning and memory, developing BONCAT and FUNCAT for use in an intact organism in which simple forms of learning may be investigated is the essential next step.

In this study we describe the application of these techniques *in vivo*, in the 7-day-old larval zebrafish. The larval zebrafish is an excellent model organism as it is a genetically tractable, simple vertebrate, which is transparent and therefore ideal for imaging. Furthermore, zebrafish larvae have a well-defined behavioral repertoire [27], and the range of experimental paradigms to test this has recently been expanded to include associative conditioning [28]. Larval zebrafish can absorb small chemical compounds directly from their surrounding medium, all of which makes them not only amendable to chemical screens and an emerging human disease model, but also an excellent system to study the applicability of bio-orthogonal metabolic labeling of newly synthesized proteins *in vivo.*

Here we show that AHA is metabolically incorporated into newly synthesized proteins, in a time- and concentration-dependent manner, but has no apparent toxic effects and does not influence simple behaviors. This enables fluorescent labeling of newly synthesized proteins in whole mount larval zebrafish. Furthermore, we find that stimulation with the GABA antagonist, pentylenetetrazole (PTZ), causes an increase in protein synthesis throughout the proteome, which can also be visualized in intact larvae.

Results & Discussion

The BONCAT and FUNCAT protocols were adapted to larval zebrafish (Figure 1a). All larvae, unless otherwise noted, were analyzed at 7dpf. We incubated larvae in E3 embryo medium supplemented with the methionine surrogate AHA (Figure 1b) for a period of 0-72h immediately prior to harvesting, with the aim of incorporating the azide group into newly synthesized proteins throughout the zebrafish proteome. To quantify successful incorporation of AHA into protein, larvae were washed, anesthetized, homogenized and the resulting lysate was reacted with biotin-alkyne in the presence of CuBr and the triazole ligand (see methods). This allowed for detection and quantification of newly-synthesized biotin-labeled proteins using western blot analysis or for affinity purification of the newly synthesized proteins (BONCAT). To visualize newly synthesized proteins following AHA exposure, larvae were washed, anesthetized, fixed and permeabilize. Whole mounted larval zebrafish were reacted with AlexaFluor-488-alkyne in the presence of $CuSO_4$, the reducing agent *tris*(2-carboxyethyl)phosphine (TCEP) and the triazole ligand, before imaging using a confocal microscope (FUNCAT). This allowed for visualization of new protein synthesis, in the intact larval zebrafish.

Previously, Dieterich *et al*. showed that metabolic labeling of mammalian cell culture with AHA does not alter global protein synthesis rates or promote ubiquitin-mediated degradation, indicating that AHA incorporation does not cause severe protein misfolding or degradation [17]. To ensure that incubation and incorporation of AHA into newly synthesized proteins is not toxic for the living

animal, larvae were exposed to E3 embryo medium supplemented with 0 to 20mM AHA, or 10mM methionine, for 6 to 72h. Larvae were scored as healthy, if after incubation they were still responsive to light touch. No significant toxic effects were observed when larvae were incubated with 1-10mM AHA, even after 72h incubations (Figure 2a). Only incubations with extremely high (20mM) concentrations of AHA were toxic beginning around 24h after onset of incubation. This indicates that incubation with low to moderate concentrations of AHA, even over extended periods of time is not toxic to the living animal. In the remainder of the studies reported here concentrations $\leq$ 4mM AHA were used.

Next, we explored whether incorporation of AHA causes changes in simple behaviors. We conducted a series of behavioral tests after incubation in E3 medium supplemented with 4mM AHA, for 0-48h. First we investigated spontaneous swimming behavior. 7-day-old larval zebrafish were incubated in 4mM AHA for 0-48h prior to observation, and then placed individually into a 1 cm by 7.5 cm swimming chamber (supplementary Figure S1) and their spontaneous swimming bouts were recorded for a period of 15 min. Sample traces of spontaneous swimming behavior are depicted in supplementary figure S1. There was no significant difference in the number of individual spontaneous swimming bouts initiated between 48h AHA incubated, 24h AHA incubated and control larvae, although there was a small, not significant decrease in the 48h and 24h AHA groups as compared to the control group (Figure 2b). There was also no difference in the number of AHA incubated and control larvae that failed to exhibit spontaneous swimming bouts during the 15-minute trial period (Figure 2b).

To study whether AHA incubation causes deficits in visual tracking, 7-day-old larvae were tested for the optokinetic response [29] after incubation in 4mM AHA for 24-48h. Larvae were immobilized in 0.4% low-melting point agarose in a circular array of LEDs, which delivered a spot of white light that moved in a horizontal plane around the immobilized larvae. Similar to control larvae, AHA incubated larvae were able to track the light stimulus, producing smooth tracking eye movements and rapid saccades (Figure 2c, supplementary video), indicating

that neither visual acuity nor neural circuits underlying visual tracking behavior seem to be affected by prolonged incubation with 4mM AHA. To further test whether AHA incubation altered visual acuity and simple reflexive behaviors, we tested the animal's startle response to light flash and dark flash. Larvae were placed in a circular array of LEDs, which delivered either a light flash or a dark flash while the response of the larva was monitored. Figure 2d shows a representative startle response to a light flash in an animal following a 24h incubation with 4mM AHA. The larva is clearly exhibiting a stereotypical C-bend escape response [30] indicating that AHA has no effect on the motor function associated with escape behavior. Furthermore, incubation with 4mM AHA for 24-48h did not alter the percentage of larval zebrafish that responded to either light or dark flash (Figure 2e) nor did it affect the delay in response to either light or dark flash (Figure 2f). Therefore, we conclude that AHA incorporation is not toxic and has no effects on simple behaviors at low concentrations (4mM), even over prolonged incubation periods, making it suitable for labeling newly synthesized proteins *in vivo*.

To determine whether AHA is metabolically incorporated into newly synthesized proteins, we tagged lysates prepared from larval zebrafish incubated for 0-72h with 4mM AHA with biotin-alkyne in the presence of the Cu(I) catalyst. Subsequent dot blot analysis with a biotin antibody revealed successful incorporation of AHA into proteins in an incubation-time dependent manner. A sample dot blot is shown in Figure 3a, along with quantification of several experiments. After only a 6h incubation period with E3 embryo medium supplemented with 4mM AHA, statistically significant ($p<0.005$) AHA incorporation could be detected. After 24h, 48h and 72h incubations, approximately 140ng (±8ng), 375ng (±34ng) and 699ng (±72ng) of biotinylated protein were detected per homogenized larva, respectively. The total soluble protein per larva under the experimental conditions we used was 6.38μg (±0.53μg). From this we can estimate that 24h, 48h or 72h incubation with 4mM AHA leads to labeling and tagging of approximately 2.2%, 5.9% and 10.9%, respectively, of the total soluble protein per larval zebrafish. However, as different proteins may show different levels of AHA incorporation, and therefore different biotin signal strength, the analysis given here

should be regarded as semi-quantitative.

To verify the specificity of AHA incorporation into newly synthesized proteins, we incubated larval zebrafish in E3 embryo medium supplemented with AHA along with low concentrations of the protein synthesis inhibitor puromycin. These very low concentrations of PSI did not have a toxic effect on larval zebrafish (data not shown). Although abundant biotin signal was detected in lysates of larval zebrafish incubated with AHA only, no signal was detected when larval zebrafish were incubated without AHA, and a significantly lower signal was detected when larval zebrafish were incubated in AHA in the presence of puromycin (Figure 3b). Furthermore, when the concentration of PSI in the incubation medium was increased from 2.5μg/ml to 5μg/ml, a significant decrease in AHA labeled and biotinylated proteins was observed. However, no further decrease was observed when the PSI concentration was further increased to 10μg/ml.

The above results confirm that BONCAT labels newly synthesized proteins with high specificity in the larval zebrafish. In addition, we observed that AHA incorporation in larval zebrafish scales non-linearly with incubation time (Figure 3a) and we assume that an incorporation plateau would be reached after even longer incubation periods. Also, labeling was AHA concentration-dependent (Supplementary Figure S3). While no signal was detected when 4-day-old larval zebrafish were incubated with 0mM AHA, increasing the concentration of AHA in the incubation medium from 1mM to 4mM resulted in a detectable signal increase. Furthermore, AHA was incorporated not only into a few select proteins, but into a large variety of newly synthesized proteins throughout the proteome over time, as is shown by the abundance of protein bands on the western blot of affinity purified biotinylated proteins from whole larval zebrafish lysates reacted with the biotin-alkyne and probed against biotin (Supplementary Figure S2a). Biotin signal detected in the samples not incubated with AHA are likely a result of endogenous biotinylation.

To examine whether AHA is also incorporated into newly synthesized proteins in deeper structures such as the nervous system, we incubated 4-day-old transgenic HuC::GFP larval zebrafish with 4mM AHA for 48h. HuC encodes an RNA-

binding protein that serves as an excellent early marker for differentiating neurons and the HuC::GFP line is a stable zebrafish transgenic line in which GFP is expressed specifically in neurons [31]. With the exception of a few cells in the olfactory pit and the lateral line, the majority of these neurons are not surface structures. As before, whole zebrafish lysates were labeled with the biotin-alkyne, affinity purified, and then analyzed using western blot probed against GFP. Only in the sample that was incubated in 4mM AHA for 48h, were we able to affinity purify AHA-labeled, biotin-tagged GFP, indicating that AHA is not only incorporated into newly synthesized proteins in surface structures of the larval zebrafish, but also in the nervous system, the sole area of GFP expression in the HuC::GFP transgenic line (Supplementary Figure 2b).

We next optimized the labeling and reaction conditions to maximize specific visualization of newly synthesized proteins (FUNCAT) in the intact larval zebrafish. For this purpose we used the mutant zebrafish line *nacre*, which lacks melanophores throughout development [32] and thus is relatively transparent and ideal for imaging. Larval zebrafish were, as before, incubated in E3 medium supplemented with 4mM AHA for 0-72h. Larvae were anesthetized, fixed, and permeabilized, before whole mount samples were reacted with 5μM AlexaFluor-488-alkyne, in the presence of $CuSO_4$, TCEP and the triazole ligand, at room temperature overnight. After several washes in PBDTT buffer, samples were immobilized in 0.4% agarose and imaged using a confocal microscope.

Incubation of larval zebrafish with 4mM AHA followed by reaction with Alexa-488-alkyne resulted in an incubation-time dependent fluorescent labeling of newly synthesized proteins throughout the larval zebrafish (Figure 4a). Low fluorescent signals, especially in the muscles of the tail, could be detected after as little as 12h incubation with AHA. Other structures, including the brain, spinal cord, liver, intestines and heart could be readily visualized after 24h incubation with AHA. Specifically, sensory organs such as the neuromasts of the lateral line (indicated by arrow heads in Figure 4a, 72h incubation dorsal view panel) and the olfactory pit (Figure 4c) seem to be areas of especially high levels of fluorescence. Furthermore, deeper structures such as the optic tectum, cerebellum (Figure 4b), and the spinal

cord (Figure 4d), are not only readily labeled and tagged using the AlexaFluor-488 alkyne, but show differences in fluorescence intensity on the cellular level. In the case of the spinal cord, we believe this population of brightly labeled cells corresponds to Rohon-Beard neurons [33] (Figure 4d, as indicated by arrows). To verify that the fluorescent signal observed in the above experiments represents incorporation of AHA into newly synthesized proteins, larval zebrafish were incubated in E3 medium containing 4mM AHA in the presence of 5μg/ml puromycin (Figure 4e). In agreement with previously described results from lysates, abundant fluorescent signal was detected in whole mounts of larval zebrafish incubated with AHA only, while no signal was detected when larval zebrafish were incubated without AHA, and only background signal was detected when larval zebrafish were incubated in AHA in the presence of puromycin. These results suggest that FUNCAT may be used to identify regions of protein synthesis, specific cells or groups of cells that are metabolically active, during the AHA incubation window in intact larval zebrafish.

To further investigate whether BONCAT and FUNCAT can be used to identify changes in protein synthesis *in vivo,* larval zerbafish were exposed PTZ, a GABAergic receptor antagonist that induces epileptic-like neuronal discharges and seizure-like behaviors in rodents and zebrafish [34-36]. It has been shown that exposure to PTZ induces expression of immediate early genes in larval zebrafish [34], and leads to changes in postsynaptic GABA receptor expression [37] and hilar neurogenesis [38] in rodents.

Larval zebrafish were exposed to 15mM PTZ for two two-hour periods, 24h and 8h before anesthesia while being incubated in 4mM AHA for 30h. The amount of biotinylated protein per larva was detected using dot blot analysis, as previously described. We observed a significant increase in the amount of biotinylated protein in larval zebrafish exposed to PTZ during AHA incubation, as compared to larvae that were not exposed to PTZ (Figure 5a), indicating that PTZ induces an increase in protein synthesis. This increase in biotinylated protein signal is not specific to one or a few protein bands, but seems to be the result of a general increase of protein synthesis throughout the proteome as detected by western blot analysis of affinity

purified samples (Figure 5b). Furthermore, using the FUNCAT technique, we were able to visualize an increase in fluorescent signal in the brain and tail muscles in larval zebrafish that had been incubated in 4mM AHA for 48h and exposed to 15mM PTZ for two two-hour periods (Figure 5c). These results indicate that chemical stimulation with the GABAergic receptor antagonist PTZ induces an increase in protein synthesis, which can be quantified and localized using the BONCAT and FUNCAT techniques in larval zebrafish.

In this study we have shown that the BONCAT and FUNCAT techniques, which introduce bio-orthogonal chemical groups into newly synthesized proteins using the endogenous cellular translation machinery, can be applied to the live, 7-day-old larval zebrafish. This enables the enrichment and quantification of newly synthesized proteins, when using an affinity tag such as the biotin-alkyne, and the visualization of protein synthesis, when using fluorescent-alkyne tags, such as the AlexaFluor-488-alkyne. Furthermore, we have shown that chemical stimulation with the proconvulsant PTZ increases protein synthesis, which can be detected using the methods developed in this study.

BONCAT and FUNCAT techniques enable labeling of newly synthesized proteins only when methionine is substituted by non-canonical amino acids during translation. However, AHA competes with endogenous methionine for charging onto methionine tRNA by the somewhat promiscuous MetRS. Previous work by the Tirrell group has shown that the charging rate of AHA relative to that of methionine onto methionine tRNA in bacterial cells is 1/390, as indicated by the specificity constant k_{cat}/K_m [39], suggesting that not all newly synthesized proteins may incorporate AHA in the presence of endogenous methionine. Furthermore, only proteins that contain at least one methionine residue can be labeled. This, however, is not an important factor in zebrafish, as 97.97% of zebrafish proteins contain at least one non-terminal methionine. Only two of 27,014 currently annotated zebrafish proteins contain no methionine at all (NCBI *Danio rerio* protein database, 5.17.2011).

Recent work using bacterial cells has opened the door to increasing the specificity of these techniques. A different non-canonical amino acid,

azidonorleucine (ANL), can be used for the BONCAT / FUNCAT reaction [40]. ANLs' azide bearing side-chain is too bulky to fit into the binding pocket of wild-type MetRS and can therefore not be charged onto methionine tRNA in wild-type cells. However, introducing specific point mutations into the MetRS sequence enables charging of ANL. This permits genetic restriction of the tagging techniques by selective expression of the mutant MetRS in cell populations of interest. We are currently constructing transgenic fish in which the mutant MetRS is placed under the control of a specific promoter. Subsequent incubation with ANL will enable us to observe labeling and downstream identification of newly synthesized proteins in specific cell populations, as oppose to the whole organism.

Recently, the larval zebrafish has become a model organism for small molecule screens, permitting identification of small neuroactive molecules, which alter motor activity [41] or circadian rhythm [42]. In the future, the FUNCAT and BONCAT techniques can be paired with different chemical stimuli that cause behavioral changes to investigate underlying adjustments of the proteome in distinct regions of the nervous system. Even complex tasks known to be protein synthesis-dependent, such as long-term memory formation, can now be tackled with these techniques to elucidate which neurons and neuronal circuits are affected or involved.

Methods

Reagents

All chemical reagents were of analytical grade, obtained from Sigma unless otherwise noted, and used without further purification. We prepared AHA as described previously [43]. The AlexaFluor-488 alkyne was purchased from Invitrogen (catalog number A10267), while the biotin-alkyne tag was purchased from Jena Biosciences (catalog number TA105).

Zebrafish stocks and husbandry.

Adult fish strains AB, HuC::GFP and *nacre* were kept at 28°C on a 14h light/10h dark cycle. Embryos were obtained from natural spawnings and were maintained in E3 embryo medium (5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, 0.33mM $MgSO_4$; [44]).

Toxicity and behavioral tests

To test AHA toxicity larvae were placed five at a time in a 24-well Falcon culture dish well. Each well contained approximately 2ml of embryo medium. Medium was replaced with embryo medium supplemented with 0-20mM AHA or 10mM methionine at the appropriate time point. Larvae were checked for response to light touch at 7 dpf.

For other behavioral tests larvae were incubated in 10ml of embryo medium or embryo medium supplemented with 4mM AHA for 24-48h in a 6-cm petri dish. To monitor spontaneous swimming bouts larvae were placed individually in a 1cm by 7.5cm behavioral chamber and spontaneous swimming was recorded using a webcam for 15min. Subsequently, swimming bouts were scored. The optokinetic response was measured by immobilizing 7dpf larval zebrafish in a drop of 0.4% low melting point agarose (Promega) in embryo medium. Immobilized larvae were placed in a circular array of LEDs, which delivered a spot of white light that moved in a horizontal plane around the immobilized larvae. The optokinetic response was recorded using a high-speed camera (Redlake MotionScope M3) and eye movements were analyzed using Matlab. The startle response was measured by placing larval zebrafish in 5-cm petri dish in a circular array of LEDs. LEDs delivered 50ms light or dark flashes, while a high-speed camera mounted above the arena recorded responses. Response onset was scored.

Copper-catalyzed [3+2] azide-alkyne cycloaddition chemistry and detection of tagged proteins.

Zebrafish larvae were incubated in embryo medium supplemented with AHA after which larvae were washed three times in 25ml embryo medium. Larvae were moved into a 1ml Eppendorf tube in ~1ml of embryo medium and anesthetized on ice for one hour. Remaining medium was removed and anesthetized fish were washed once with 1ml of ice cold PBS + Protease Inhibitor (PI; Roche, complete ULTRA Tablets, Mini, EDTA-free Protease Inhibitor cocktail tablets). PBS+PI was removed and replaced with 100µL of fresh PBS+PI. Zebrafish larvae were homogenized using a Kontes Pellet Pestle Motor. 1% SDS and 1µL of Benzonase (≥500U) were added and the lysate vortexed and heated at 95°C for 10min. Lysate was allowed to cool to room temperature, before 400µl of PBS+PI and 0.2% triton X-100 were added. Then, lysates were centrifuged at 15,000g at 4°C for 10min. Supernatant was transferred to a new 1ml Eppendorf tube. For BONCAT, samples were reacted with 10µM biotin-alkyne in the presence of 200µM triazole ligand (*tris*[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine, 97%) and 5mg/ml CuBr suspension and incubated at 4°C with agitation overnight. Samples were then centrifuged at 4°C for 5min at 5,000g to pellet CuBr. Supernatant was moved into a new 1ml Eppendorf tube. To remove excess, unligated biotin-alkyne, samples were applied to a PD MiniTrap G-25 size exclusion column (GE Healthcare). Samples were then analyzed using "dot blots" and affinity purified as described in [18]. For western blot analysis of affinity purified samples, 25µL of washed NeutrAvidin beads (Thermo Scientific) previously incubated with sample were heated at 95°C for 5min in 50µl of LDS sample buffer (Invitrogen) containing reducing agent (Invitrogen). Proteins were separated on precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) and transferred to PVDF membranes and blocked in PBST (PBS+0.1% Tween-20) containing 5% milk. For detection, membranes were probed with goat anti-biotin (Biomol) and mouse anti-goat LI-COR-IR 800 secondary antibody and analyzed using the Odyssey Infrared Imaging System (LI-COR).

To image AHA labeled proteins, larval zebrafish were incubated in embryo medium supplemented with AHA, washed and anesthetized as described above. Remaining embryo medium was removed and replaced with ~1ml of fixation solution (4% PFA,

88mM sucrose in PBS). Larvae were fixed at room temperature for 3h, dehydrated in 100% methanol and stored at -20°C overnight. Larvae were rehydrated through successive 5min washes with 75% methanol in PBST, 50% methanol in PBST, 25% methanol in PBST and finally PBST. This was followed by two washes in PBDTT (PBST + 1% DMSO and 0.5% Triton X-100) and an hour permeabilization in Protease K (10µg/ml in PBST). After permeabilization, larvae were briefly washed with PBST and then immediately post-fixed for 20min. Larvae were washed twice for 5 minutes with PBST and three times for 5min with PBDTT, before blocking (5% BSA, 10% goat serum in PBDTT) for at least 3h at 4°C. Larvae were washed three times in PBST (pH 7.8), before being conjugated to the probe by addition of 200µM triazole ligand, 5µM AlexaFluor-488-alkyne, 200µM $CuSO_4$ and 400µM TCEP at room temperature overnight with gentle agitation. Samples were washed four times for 30min in PBDTT+0.5mM EDTA, and twice for 1h in PBDTT, before being rinsed in PBST and immobilized on Matek dishes using 0.4% low melting point agarose. Images were obtained using a Zeiss LSM780 laser scanning confocal microscope with 10X or 20X air lens. AlexaFluor-488 was excited with the 488nm line of an argon ion laser and the emitted light was detected between 510 and 550 nm. We performed all post-acquisition processing and analysis with ImageJ (NIH). Significance was tested for using the two-tailed T-test and error bars represent standard deviation.

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Figure Legends

Figure 1. Labeling of newly synthesized proteins for identification (BONCAT) and visualization (FUNCAT) in larval zebrafish. (a) Scheme depicting metabolic labeling of newly synthesized proteins in 7-day-old larval zebrafish using AHA incorporation and Cu(I)-catalyzed [3+2] azide-alkyne cycloaddition. TCEP, *tris*(2-carboxyethyl)phosphine. (b) Chemical structures of methionine and azidohomoalanine (AHA).

Figure 2. At low concentrations, AHA exposure is not toxic and does not significantly alter simple behaviors. (a) Survival rate of 7-day-old larval zebrafish when incubated with AHA (0 to 20mM, 6 to 72h) or Methionine (10mM, 6 to 72h), n=20. (b) Quantification of spontaneous swimming behavior of larval zebrafish after AHA incubation (4mM, 0h to 48h). Percentage of larvae that show no spontaneous swimming behavior per 15-minute interval. Mean swimming bursts per 15-minute interval, n = 10-12. Differences are not statistically significant. (c) Traces depicting the angle of eye rotation during a typical optokinetic response after AHA incubation (4mM, 0h to 48h). (d) Sample startle response upon light flash after AHA incubation (4mM, 24h). (e) Mean response percentage to light or dark flash after AHA incubation (4mM, 0 to 48h), n=5 larvae, flashed three times each. Error bars represent standard deviation of response percentage. Differences are not statistically significant. (f) Mean delay in response to light or dark flash after AHA incubation (4mM, 0 to 48h), n=5 larvae, flashed three times each. Error bars represent standard deviation of response time. Differences are not statistically significant.

Figure 3. AHA is metabolically incorporated into larval zebrafish proteins *in vivo*. Sample immunoblot and quantification of immunoblots of lysates from AHA-treated 7-day-old larval zebrafish reacted with biotin-alkyne (10μM) for 12h, probed with antibody against biotin. (a) Larval zebrafish were incubated with 4mM AHA for 0 to 72h, n=4 (b) Larval zebrafish were incubated with AHA (0 or 4mM) or 4mM AHA in the presence of puromycin (2.5μg/ml to 10μg/ml) for 48 h, n=3. ***$p<0.001$.

Figure 4. Imaging of newly synthesized proteins after *in vivo* labeling. (a) 7dpf larval zebrafish were metabolically labeled with 4mM AHA for 0 to 72h prior to fixation and reacted with 5μM AlexaFluor-488-alkyne tag for 12h. Left panel, lateral view; right panel, dorsal view. Arrow heads indicate neuromasts of the lateral line. (b), (d), (e) 7-day-old larval zebrafish labeled with 4mM AHA for 48h imaged at higher magnification. Dorsal views of (b) optic tectum and cerebellum, (c) olfactory pits, (d) horizontal cross-section of tail, showing tail muscles and spinal cord. Arrows indicate potential Rohon-Beard neurons. Scale bar in (a), 150μm; in (b), (d), (e), 20μm. (e) Larval zebrafish were metabolically labeled with 4mM AHA for 0h, 48h or 48h in the presence of 5μg/ml puromycin. Dorsal view, scale bar is 100μm, n=5.

Figure 5. The GABA antagonist PTZ induces increased protein synthesis in larval zebrafish. (a) Sample immunoblot and quantification of immunoblots of lysates from

7-day-old larval zebrafish reacted with biotin-alkyne tag (10μM) for 12h, probed with antibody against biotin. Zebrafish were incubated with 4mM AHA (0h or 30h) or with 4mM AHA for 30h as well as 15mM PTZ for two periods of 2h, 20h and 6h before harvesting, n=3. ***p<0.001. (b) Western blot of biotin affinity-purified lysates of zebrafish incubated with 4mM AHA for 30h with or without 4h 15mM PTZ exposure. (c) Imaging of 7-day-old larval zebrafish after 48h 4mM AHA incubation with or without 4h 15mM PTZ exposure, reacted with AlexaFluor-488-alkyne (5μM, 12h); dorsal view. Scale bar is 150μm; n=6.

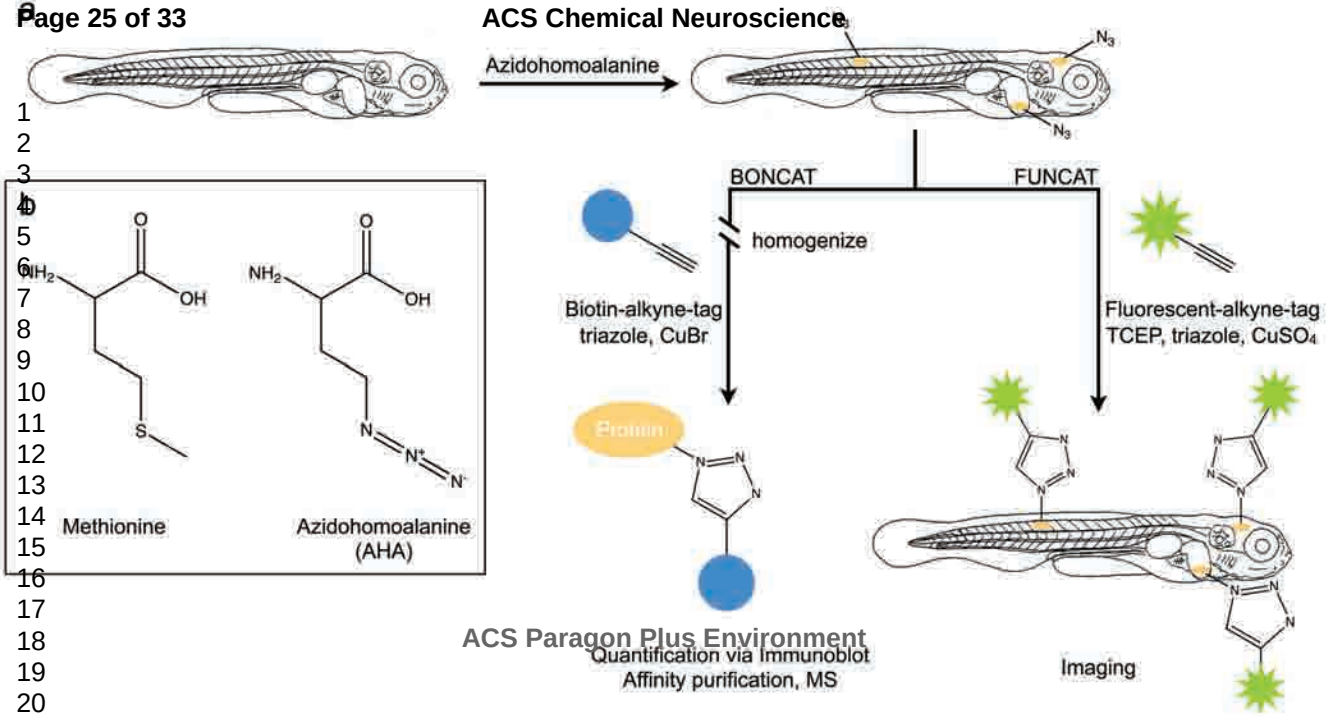
Supplementary Figures

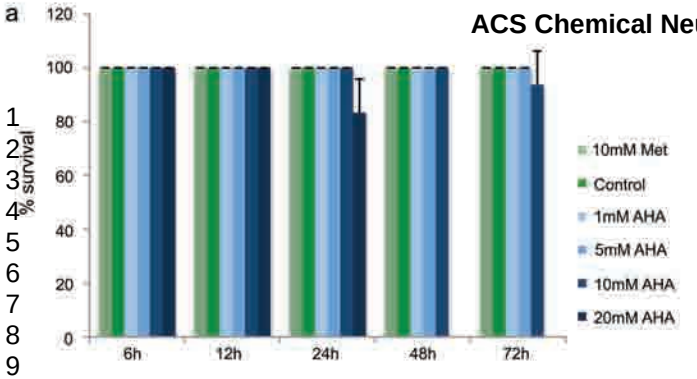
S1. Tracking spontaneous swimming behavior of 7-day-old larval zebrafish with AHA incubation (4mM, 0 to 48h). 15min interval; frame captured every 10s.

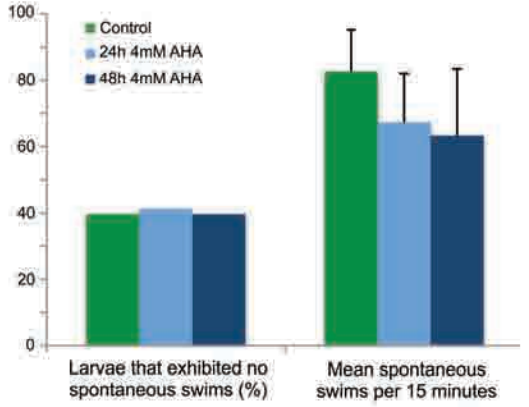
S2. AHA incorporation occurs throughout the proteome. (a), (b) Western blot analysis of biotin affinity purified lysates of zebrafish incubated with 4mM AHA for 0 to 72h. (a) Probed with an antibody against biotin. (b) HuC::GFP larval zebrafish lysates probed with an antibody against GFP.

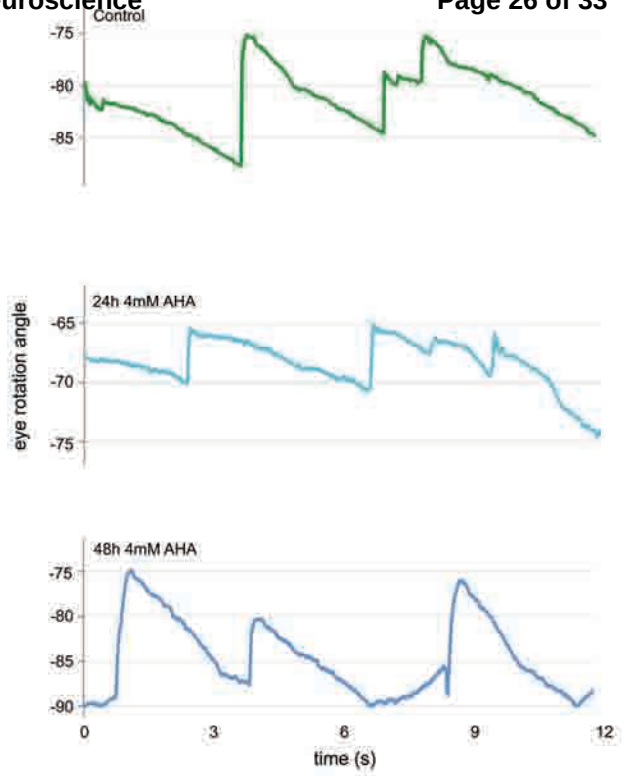
S3. AHA incorporation is AHA concentration-dependent. Immunoblot of lysates from 4-day-old larval zebrafish reacted with biotin-alkyne (10μM) for 12h, probed with an antibody against biotin. Larval zebrafish were incubated with 0 to 4mM AHA for 48h.

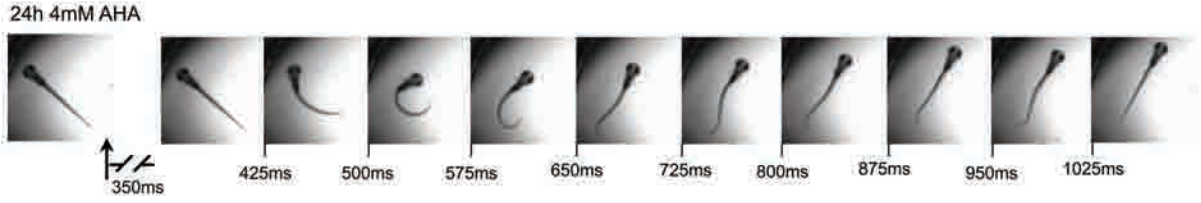
Supplementary video. Optokinetic response of 7-day-old larval zebrafish with 48h 4mM AHA incubation.

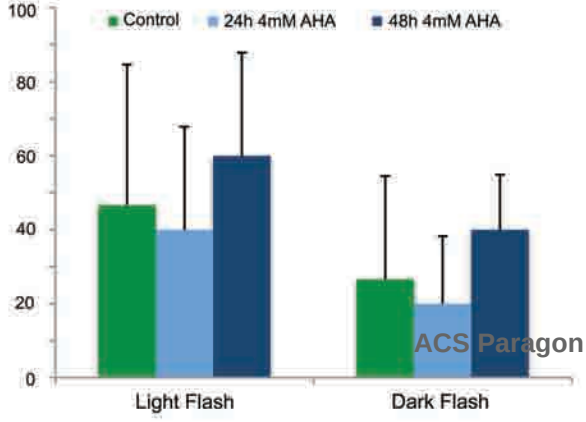


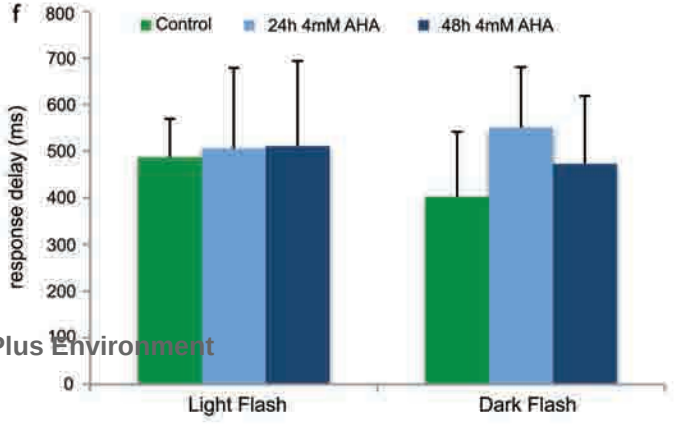


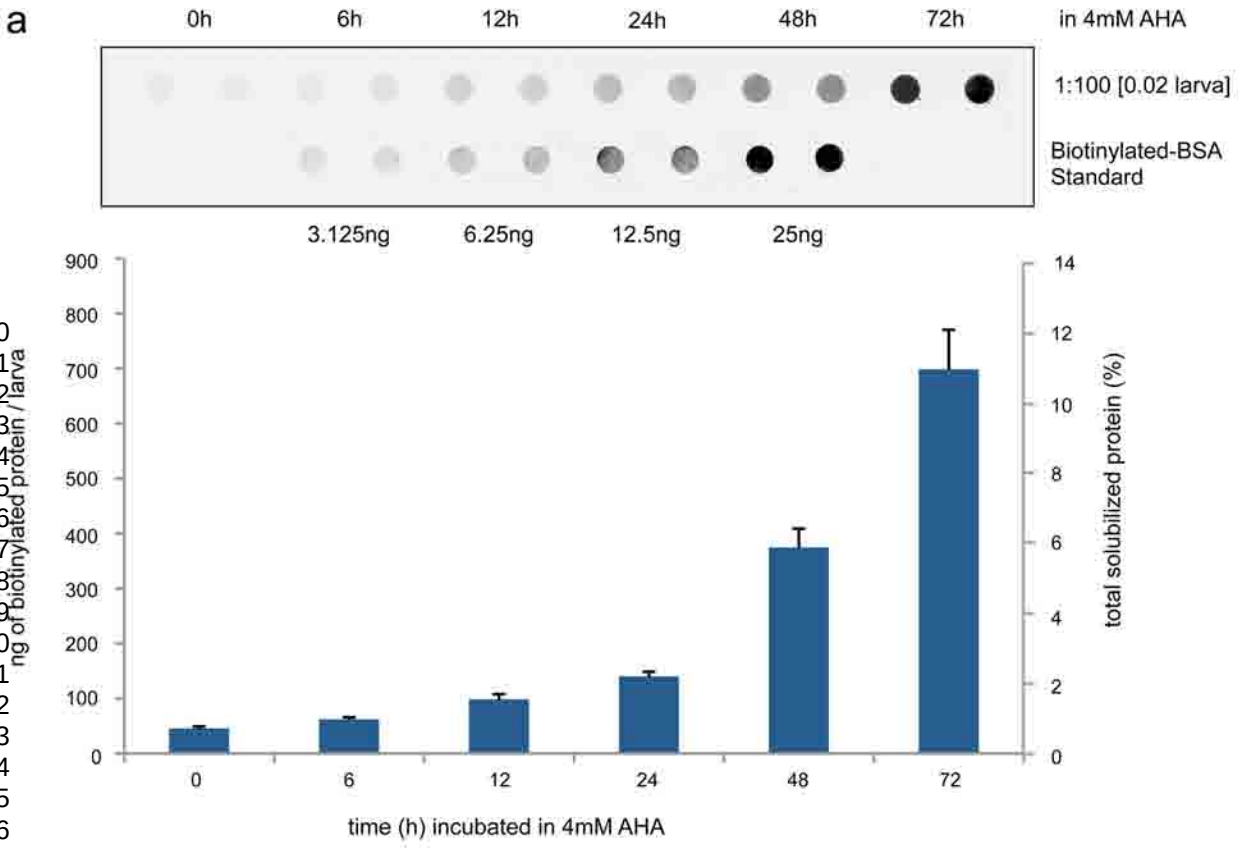


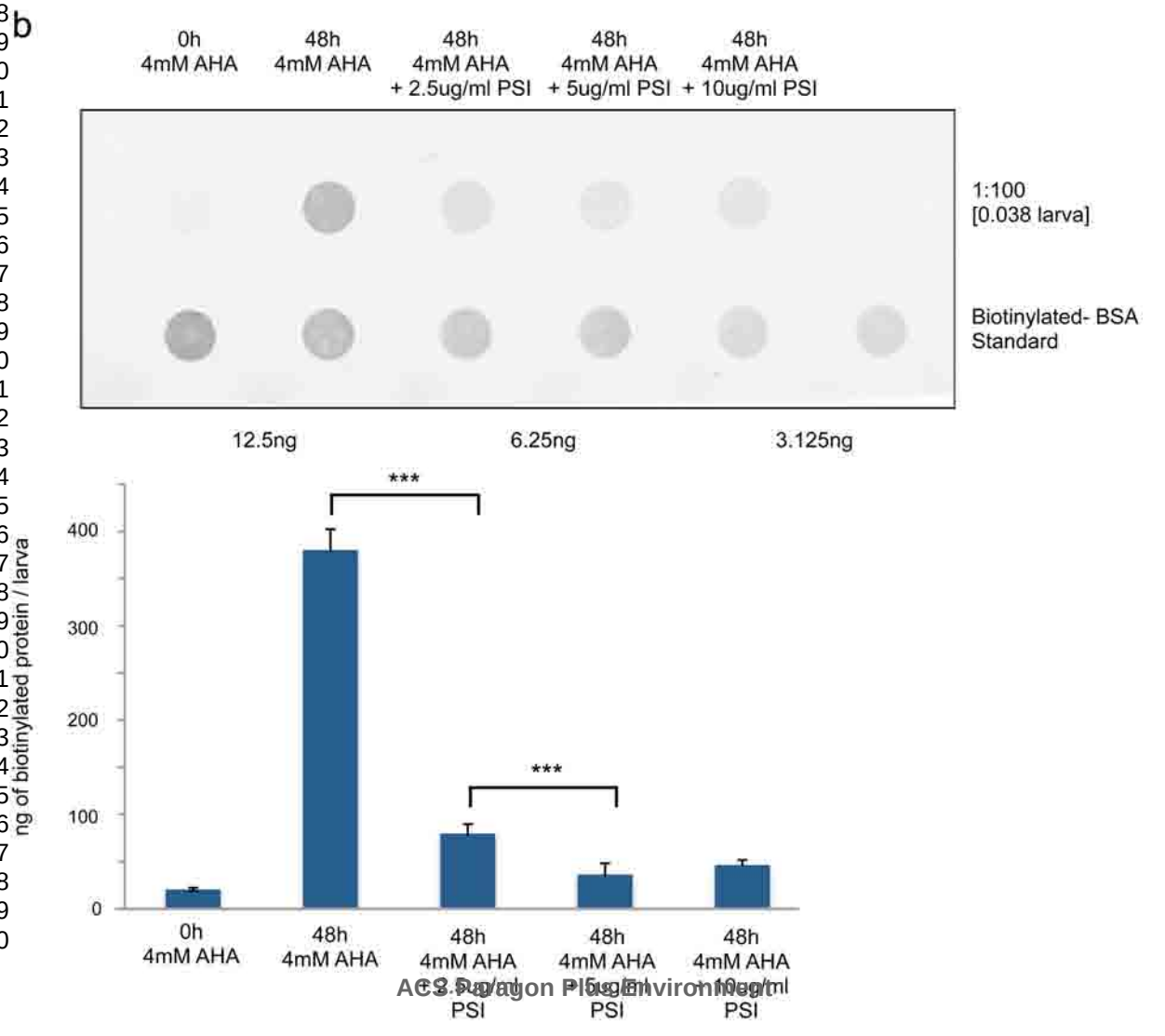


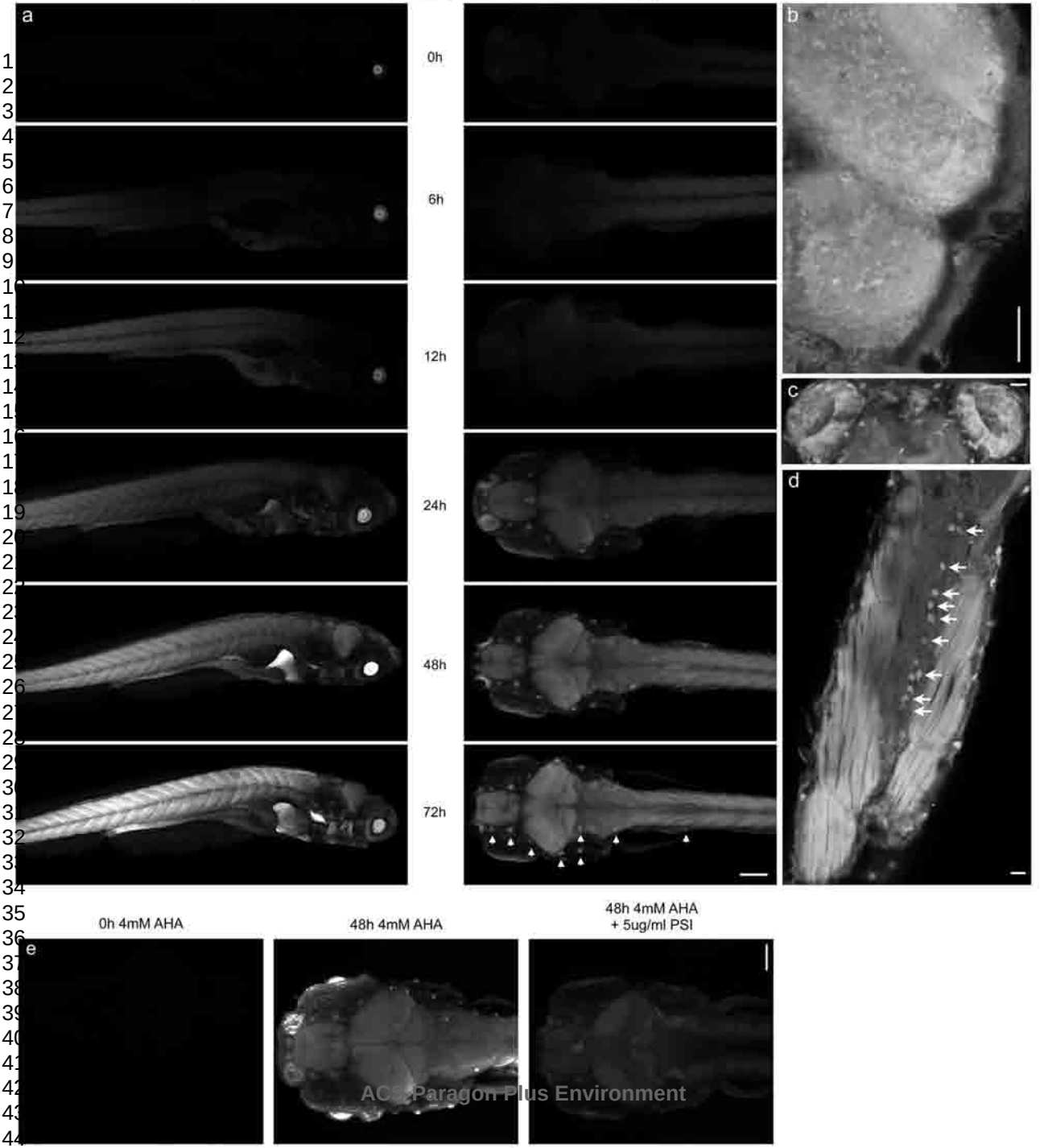


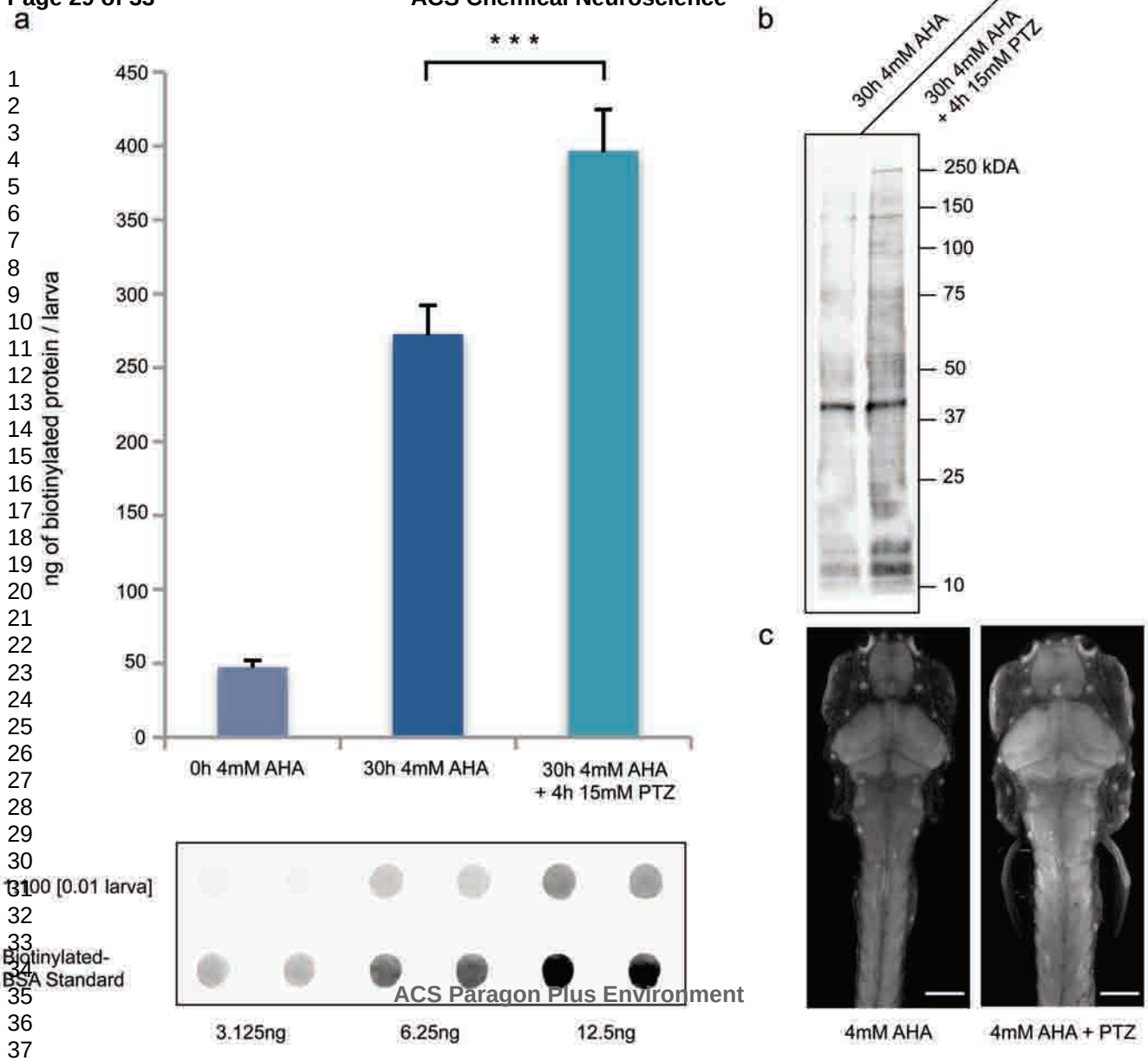






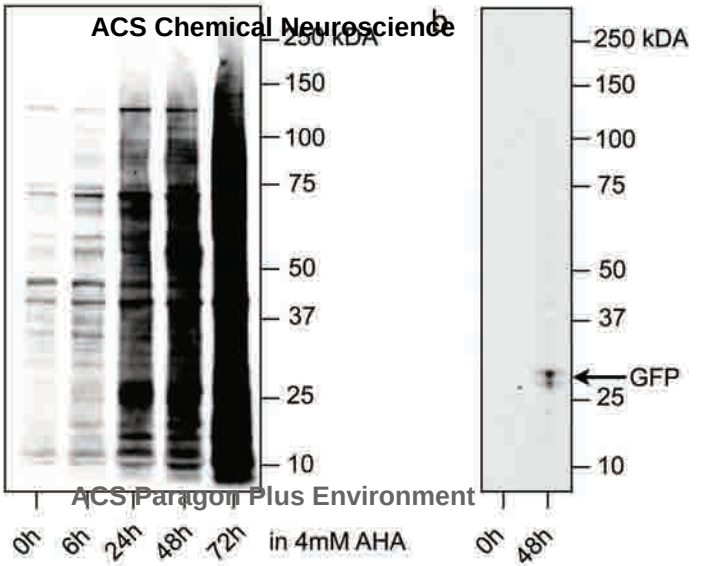






S1.

S2.



S3.