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Long-Term Acetylcholinesterase Depletion Alters the Levels of Key Synaptic Proteins while Maintaining Neuronal Markers in the Aging Zebrafish (*Danio rerio*) Brain

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Acetylcholinesterase · Aging · Cholinergic system · Neuronal changes · Synapses

Abstract
Introduction: Interventions targeting cholinergic neurotransmission like acetylcholinesterase (AChE) inhibition distinguish potential mechanisms to delay age-related impairments and attenuate deficits related to neurodegenerative diseases. However, the chronic effects of these interventions are not well described. ***Methods:*** In the current study, global levels of cholinergic, cellular, synaptic, and inflammation-mediating proteins were assessed within the context of aging and chronic reduction of AChE activity. Long-term depletion of AChE activity was induced by using a mutant zebrafish line, and they were compared with the wildtype group at young and old ages. ***Results:*** Results demonstrated that AChE activity was lower in both young and old mutants, and this decrease coincided with a reduction in ACh content. Additionally, an overall age-related reduction in AChE activity and the AChE/ACh ratio was observed, and this decline was more prominent in wildtype groups. The levels of an immature neuronal marker were upregulated in mutants, while a glial marker showed an overall reduction. Mutants had preserved levels of inhibitory and presynaptic elements with aging, whereas glutamate receptor subunit levels declined. ***Conclusion:*** Long-term AChE activity depletion induces synaptic and cellular alterations. These data provide further insights into molecular targets and adaptive responses following the long-term reduction of AChE activity that was also targeted pharmacologically to treat neurodegenerative diseases in human subjects.

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Introduction

A subset of behavioral impairments during normal and pathological aging like Alzheimer's disease (AD) are associated with perturbations in the cholinergic system. Both humans and other models have shown changes in the biosynthesis and release of acetylcholine (ACh) in the aging brain [1, 2]. In conjunction, disruptions in choline uptake have been reported during old age [1–3]. Additionally, reductions in the binding affinities of the main cholinergic receptors are shown in both aging and AD [4]. This evidence has suggested that disturbances in cholinergic neurotransmission occur during both normal aging and age-related pathological conditions, which makes the components of the cholinergic system promising targets for interventions to ameliorate aging-related deficits.

One of the pharmacological interventions targeting the cholinergic system is treatment with acetylcholinesterase (AChE) inhibitors. AChE is the main enzyme responsible for the degradation of ACh, its inhibition is associated with increased cholinergic activity, and this activation has further impacts on anti-inflammatory pathways [5]. AChE inhibitors are widely used to treat cases of AD in human patients and improve behavioral abilities [6]. Additionally, in animal models, old subjects can benefit from treatment with AChE inhibitors with respect to behavioral performance as well as synaptic integrity [7]. Several studies examined the association between AChE inhibition and other cholinergic markers such as ACh content, and there is no distinct linear relationship between AChE inhibition and ACh content but rather mechanisms depend on the dose of the inhibition, as well as duration [8, 9]. The long-term effectiveness of treatment with AChE inhibitors and their molecular targets within the context of aging and neurodegenerative diseases are not well described.

Knockout AChE models can provide insights into the long-term effects of selective AChE activity depletion and its molecular targets. One of the AChE knockout models in mice that were homozygous mutants (AChE−/−) demonstrated that long-term loss of AChE activity in adults was associated with altered levels of other cholinergic components such as mAChRs and the high-affinity choline transporter [10]. However, in these AChE−/− mice, a liquid diet was implemented to have viable adult mutants due to underlying developmental and muscular abnormalities [11]. Additionally, in heterozygous mice (AChE+/−), the effects were not very robust since butyrylcholinesterase activity compensates for the loss of AChE. Assessing the long-term effects of reduced AChE activity and revealing its molecular effects at older ages, without any developmental abnormalities or confounded by the compensation from other cholinesterase activity, become challenging in these models.

Vertebrate knockout models like the zebrafish can be used to circumvent these limitations. Homozygous AChE mutants in zebrafish (*ache*$^{sb55/sb55}$) have completely abolished AChE activity and are not viable after 72 h post-fertilization [12], whereas heterozygous zebrafish mutants (*ache*$^{sb55/+}$, ache) have significantly reduced AChE activity without evident morphological and locomotor impairments, and they are viable and healthy at adult and older ages [13, 14]. Additionally, in zebrafish, AChE is the only enzyme hydrolyzing ACh since there is no typical butyrylcholinesterase [12]. More importantly, previous works indicated that heterozygous ache mutants had preserved cognitive and learning abilities at old age, while age-matched wildtype zebrafish experienced a significant cognitive decline as compared to young animals [14]. In the literature, molecular changes in the brains of ache mutants have been mostly analyzed at younger ages and only behavioral performances were assessed at old age [13–15]. Investigation of the neurobiological underpinnings of the preserved behavioral performance observed in ache mutants at old ages will demonstrate complementary evidence for the molecular targets of long-term selective depletion of AChE activity in the brain which will expand our knowledge in terms of potential AChE modulations for the treatment of age-related impairments as well as neurodegenerative diseases in humans. The present study aimed to assess the alterations in the brain levels of cholinergic, cellular, synaptic, and inflammatory markers in response to long-term and selective reduction in AChE activity at both young and old ages, which is expected to alter the deteriorating effects of aging in the group with chronically lower levels of AChE activity.

Materials and Methods

Animals

A total of 60 wildtype and *ache*$^{sb55/+}$ (ache) zebrafish were utilized. The ache line was obtained from the European Zebrafish Resource Center – Karlsruhe Institute of Technology and grown in our fish facility at Bilkent University. Both young (10–11 months old) and old (30–31 months old) male and female zebrafish were used, and the age ranges were determined based on the progression of age-related cognitive decline in zebrafish [14]. A subset of younger adult zebrafish (3.5–5 months old) was additionally included to assess time-specific alterations in the brain levels of AChE and ACh. All zebrafish were raised and maintained in a controlled and recirculating housing system, ZebTec (Techniplast,

Italy). For the dissections, procedures were followed as previously described [16]. All the tissues, including brains, gills, eyes, bodies, and tails, were placed into Eppendorf tubes and immediately snap-frozen in liquid nitrogen. Tissues were stored at −80°C for further protein and DNA extraction experiments. Tail samples were used for genomic DNA extraction and allele-specific q-PCR genotyping to distinguish ache mutants from their wildtype siblings. Genotyping experiments were carried out with respect to previously described protocols in this model [17, 18].

Protein Extraction

Frozen brain tissues were homogenized in 200 µL of 50 mM Tris-HCl (pH: 8) with a 1 mL syringe (26 gauge). Homogenized samples were divided into two equal amounts: the first part was homogenized further with a sonicator (UP 50H; Hielscher Ultrasonics GmbH, Teltow, Germany), and the second part was incorporated with 25 µL of 5X RIPA buffer (750 mM NaCl, 50 mM Tris-HCl pH: 8, 0.5% SDS, 5% NP-40) containing 12.5 µL of 10X protease inhibitor (05 892 970 001; Roche). Both homogenates were centrifuged (Himac CT15E, VWR Hitachi, Darmstadt, Germany) at 13,000 × rpm at 4°C for 20 min. Supernatants were collected, aliquoted, and stored at −80°C. The samples extracted with 50 mM Tris-HCl were used for the AChE and ACh assays, and the homogenates incorporated with RIPA buffer were used for Western blot experiments. Total protein amounts of both homogenates were determined with the Bradford assay using the Bradford Reagent (B6916; Sigma, St. Louis, MO, USA) with bovine serum albumin as a standard.

Assessments of Brain AChE and ACh Levels

Brain levels of AChE activity were determined using a commercially available colorimetric assay kit (Abcam, Cambridge, UK: ab138871), and the manufacturer's instructions were followed. Brain ACh levels were examined with the Amplex Red Acetylcholine Assay Kit (Thermo Fisher, Paisley, UK: A12217) by following the manufacturer's instructions.

Western Blot

The levels of the cholinergic, cellular, synaptic, and inflammatory proteins were assessed and compared between the groups. Western blot experiments were performed as previously described [16, 19, 20]. A minimum of two duplicates were performed for each sample. Loaded total protein amounts for detection and both the primary and secondary antibodies used in the current study are listed in online supplementary Table 1 (for all online suppl. material, see https://doi.org/10.1159/000534343). All antibodies, except the one directed against nicotinic ACh receptor alpha-7 (nAChR-a7), have been shown to work with zebrafish protein samples previously [16, 19, 20]. The antibody directed against nAChR-a7 was tested with zebrafish brain lysate and positive control (online suppl. Fig. 1). Protein band intensities were calculated using ImageJ software (NIH, Bethesda, MD, USA) by author MUT-S who was blind to the age and genotype of each subject. Band intensities were calculated as described previously [16, 19, 20].

Analyses of Brain Reactive Oxygen Species Levels

Frozen brain tissues from another cohort were placed in 150 µL of phosphate-buffered saline (L0615; Biowest, Nuaillé, France) and homogenized by passing through a 1 mL (26 gauge) syringe. 2′,7′-Dichlorodihydrofluorescein diacetate was used to detect the brain levels of free radicals. The protocol for this assay of reactive oxygen species (ROS) content in zebrafish brain tissue was followed as indicated previously [19]. The results are presented as relative fluorescence units.

Statistical Analyses

All statistical analyses were carried out with SPSS 19 software (IBM, Armonk, NY, USA). Assumptions of normality were checked with both Kolmogorov-Smirnov and Levene's tests. In all cases, these assumptions were fulfilled, and a two-way ANOVA was carried out with the factors of genotype with two levels (wildtype and ache) and age with two levels (young and old). Additionally, all dependent variables were investigated for possible sex-dependent differences with a three-way ANOVA. Significance levels in all cases were set to $p \leq 0.05$. Bonferroni multiple comparisons and simple effects analyses were further conducted in the cases of significant main effects or interactions. Graphs represented in the following figures were generated by GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

Results

Key Elements of the Cholinergic System Were Altered in the Brains of Ache Mutants

The induced mutation in this zebrafish line directly targeted the *ache* gene encoding for AChE [12]. Therefore, essential cholinergic neurotransmission markers, including AChE, ACh, and nAChR-a7, were examined. For the levels of AChE, a significant main effect of genotype was observed ($F(1, 20) = 87.649$, $p < 0.0005$), with ache mutants having reduced AChE enzymatic activity at both young ($p < 0.0005$) and old ($p < 0.0005$) ages as compared to the control groups. The main effect of age was also significant in AChE activity ($F(1, 20) = 4.388$, $p = 0.049$; Fig. 1a). There was an overall decline in old age, although post hoc analyses found no pairwise difference for this age effect; interaction between genotype and age was not significant ($F(1, 20) = 0.378$, $p = 0.546$).

The effect of genotype significantly altered ACh levels with ache mutants having lower ACh levels than wildtype controls ($F(1, 20) = 5.033$, $p = 0.036$; Fig. 1b). Pairwise comparisons indicated no significant difference between the genotype groups across different ages. No significant main effects of age or the interaction were observed on brain ACh levels (age: $F(1, 20) = 3.363$, $p = 0.082$, age by genotype: $F(1, 20) = 0.056$, $p = 0.815$).

Additionally, the AChE/ACh ratio was calculated and compared among the groups to understand the reciprocal dynamics of cholinergic signaling [21]. The genotype did not significantly alter the AChE/ACh ratio ($F(1, 20) = 0.865$, $p = 0.364$; Fig. 1c). Interestingly, aging significantly

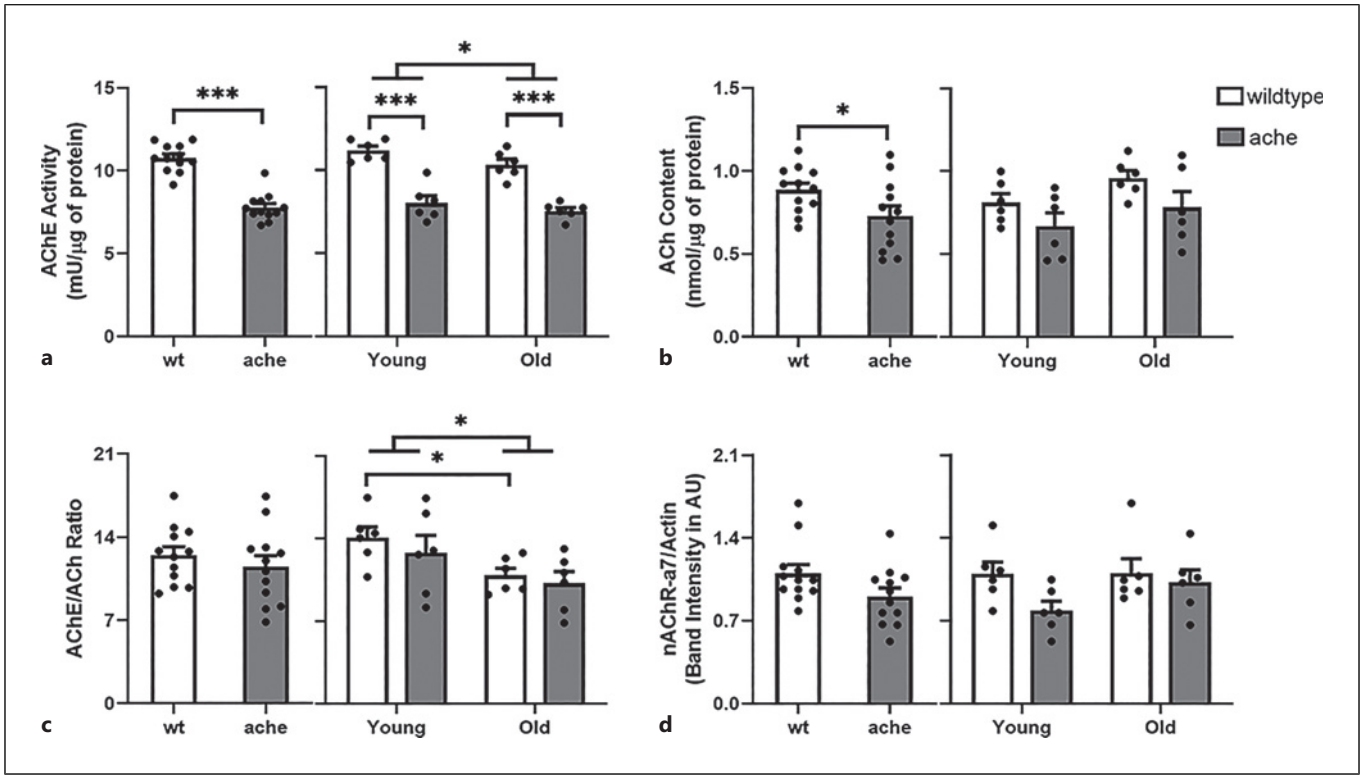

Fig. 1. A comparison in the brain levels of cholinergic transmission components between genotype and age groups. **a** AChE enzymatic activity was significantly reduced in both young (10–11 months old) and old (30–31 months old) ache mutants as compared to wildtype animals, and a significant overall age-related decline in AChE activity was observed. **b** A significant effect of genotype was revealed in brain ACh levels with ache mutants having reduced levels of ACh. **c** An age-dependent decline that was more robust in the wildtype group was revealed in the AChE/ACh ratio. **d** No significant change was observed in the protein expression levels of the cholinergic receptor subunit nAChR-a7. The group means + standard error (SE) are represented. *: p <0.05, ***: p <0.001.

reduced the AChE/ACh ratio ($F(1, 20) = 7.511$, $p = 0.013$), and pairwise comparisons showed that age-dependent decrease was significant in the wildtype zebrafish ($p = 0.044$), while it was not significant in the ache mutants ($p = 0.099$). Lastly, no significant interaction was found in the AChE/ACh ratio ($F(1, 20) = 0.088$, $p = 0.770$).

In order to understand the temporal dynamics of the exposure to depleted AChE activity, a younger subset of adult zebrafish (3.5–5 months old) was incorporated into the current work. Our results confirmed the significant main effect of genotype and age on AChE activity levels, as before. The 3.5–5-month-old ache zebrafish drove the age effect with significantly elevated AChE activity levels (online suppl. Fig. 2a), while its corresponding ACh levels were lower than those of the older groups. With regard to ACh levels, the effect of genotype was also significant, but only female old ache mutants had significantly lower ACh levels than the age-matched wildtype group (online suppl. Fig. 2b). The 3.5–5-month-old mutants had a significantly lower AChE/ACh ratio as compared to age-matched wildtype zebrafish, and this genotype effect was not significant in older groups pointing out possible differential adaptational responses at earlier stages of adulthood (online suppl. Fig. 2c). The last cholinergic marker was nAChR-a7, and no significant change was observed in nAChR-a7 protein levels (genotype: $F(1, 20) = 3.647$, $p = 0.070$; age: $F(1, 20) = 1.430$, $p = 0.246$; genotype by age: $F(1, 20) = 1.271$, $p = 0.273$; Fig. 1d, 2).

An Immature Neuronal Marker Was Upregulated in the Brains of Ache Mutants, while Glial Marker Levels Were Decreased

The levels of embryonic lethal abnormal vision (Drosophila)-like 3 (HuC), which was used as a marker of immature neuronal population, showed a significant genotype effect ($F(1, 20) = 10.377$, $p = 0.004$). Overall, ache mutants had significantly upregulated levels of HuC

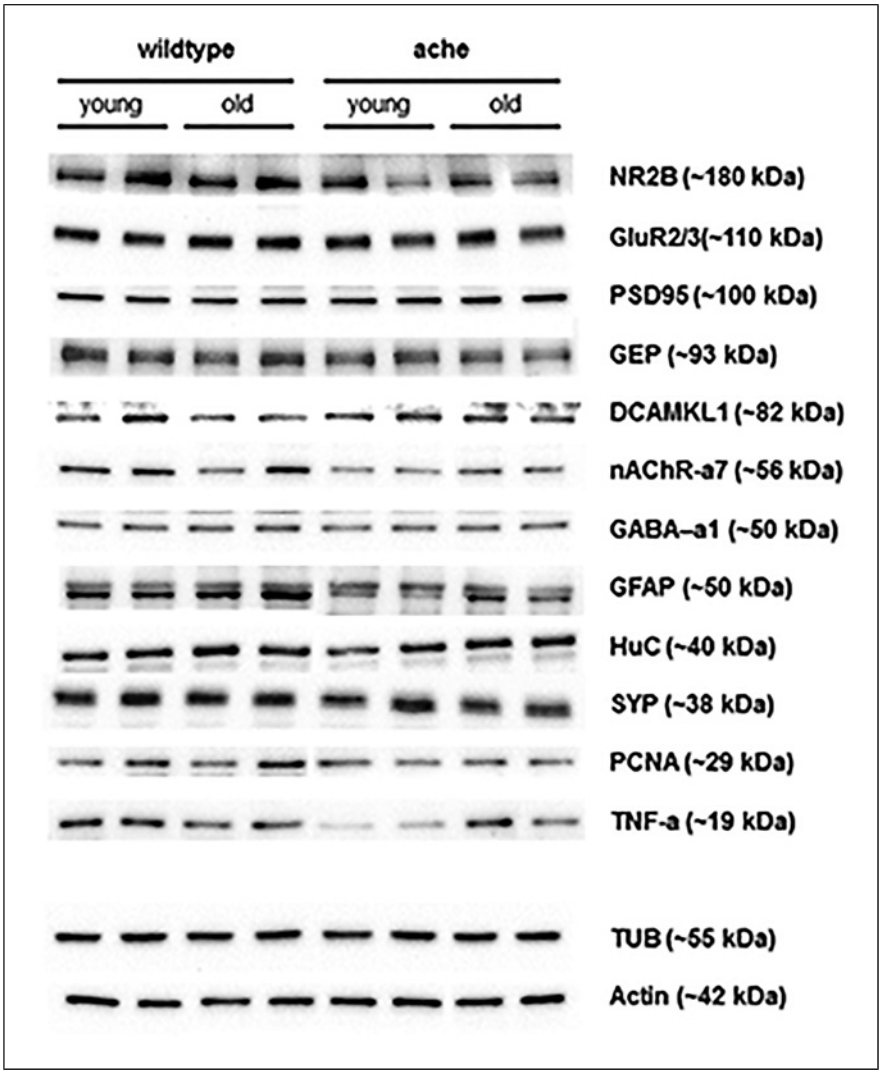

Fig. 2. Representative Western blot images for cellular, synaptic, and inflammatory proteins that were used in the current study to examine the effects of genotype and age. All antibodies gave bands at their expected molecular weights.

(Fig. 3a). Pairwise comparisons revealed that ache mutants had significantly higher levels of HuC in the young ($p = 0.022$) and old ($p = 0.050$) age groups relative to the wildtype control subjects. This differential segregation for genotype groups was also confirmed by using multivariate analyses (online suppl. Fig. 3a, b). Neither a main effect of age nor a genotype by age interaction significantly altered HuC levels (age: $F(1, 20) = 0.802$, $p = 0.381$; genotype by age: $F(1, 20) = 0.077$, $p = 0.785$). The other neuronal marker, doublecortin-like kinase 1 (DCAMKL1) expressed in post-mitotic migrating neurons, showed no significant main effect of genotype or age or interaction (genotype: $F(1, 20) = 2.687$, $p = 0.117$; age: $F(1, 20) = 0.407$, $p = 0.531$; genotype by age: $F(1, 20) = 2.479$, $p = 0.131$; Fig. 3b).

In terms of evaluating glial changes, the levels of the glial fibrillary acidic protein (GFAP) were investigated which can also change with respect to inflammatory status [22]. The genotype of the subjects coincided with differences in the GFAP levels, and this effect was marginally significant with ache mutants having down-regulated GFAP levels as an overall trend ($F(1, 20) = 4.111$, $p = 0.056$; Fig. 3c). Moreover, the effects of age ($F(1, 20) = 0.787$, $p = 0.385$) and genotype by age interaction ($F(1, 20) = 0.063$, $p = 0.805$) were not significant on GFAP. No significant main effects or interaction on proliferating cell nuclear antigen levels (PCNA), a global proliferation indicator, was revealed (genotype: $F(1, 20) = 0.435$, $p = 0.517$; age: $F(1, 20) = 0.065$, $p = 0.801$; genotype by age $F(1, 20) = 2.010$, $p = 0.172$; Fig. 3d).

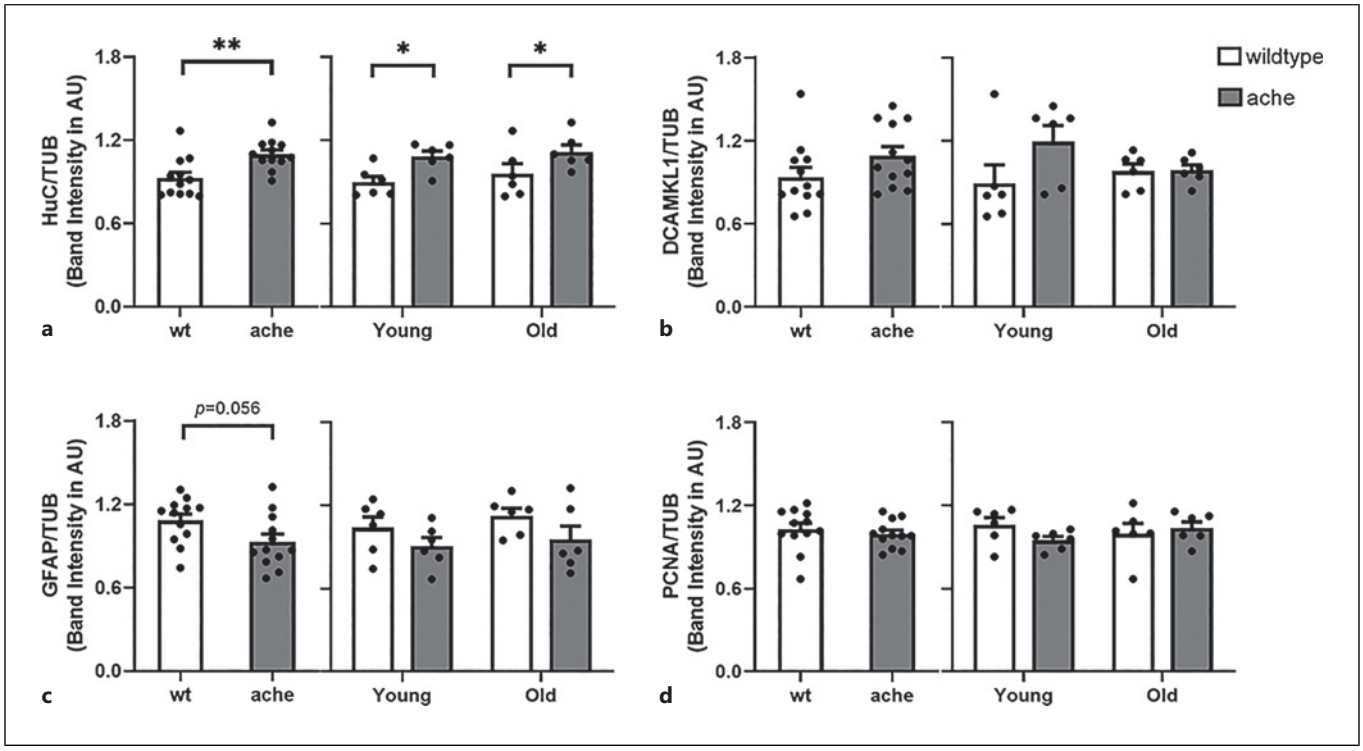

Fig. 3. Relative levels of neuronal, glial, and proliferation markers between genotype and age groups. **a** HuC protein expression levels were altered significantly depending on the genotype of the animal with ache mutants having higher levels of HuC at both young and old ages. **b** No significant changes were observed in the whole brain levels of the post-mitotic neuronal marker DCAMKL1. **c** Glial marker GFAP showed no significant changes. **d** Global proliferation marker PCNA was stable among age and genotype groups. The group means + SE are represented. *: p <0.05, **: p <0.01.

Excitatory Synaptic Proteins Decreased in Ache Mutants, while Age-Dependent Increases Were Observed in a Presynaptic Integrity Marker

Synaptic proteins representing excitatory, inhibitory, and presynaptic elements were investigated (Fig. 2). In post-synaptic density 95 (PSD95) levels, which is a clustering protein found at excitatory synapses, the main effects of genotype (F(1, 20) = 2.174, p = 0.156) and age (F(1, 20) = 1.584, p = 0.223), as well as a genotype by age interaction (F(1, 20) = 1.474, p = 0.239), were not significant (Fig. 4a).

The subunits of glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA), were examined as excitatory synaptic markers. The genotype of the animals significantly altered glutamate receptor subunits 2 and 3 (GluR2/3) levels which are AMPA receptor subunits (F(1, 20) = 5.265, p = 0.033; Fig. 4b). Overall, ache mutants had lower levels of GluR2/3 compared to wildtype controls. Pairwise comparisons revealed that old ache animals had significantly lower levels of GluR2/3 than old wildtype subjects (p = 0.017). The main effects of age and interaction were not significantly changing the GluR2/3 levels (age: F(1, 20) = 0.446, p = 0.512; genotype by age: F(1, 20) = 1.880, p = 0.186). Likewise, a significant genotype effect was shown in the levels of NMDA receptor subtype 2B (NR2B) (F(1, 20) = 13.816, p = 0.001; Fig. 4c). Mutants have lower NR2B levels, and post hoc analyses indicated this genotype-dependent reduction was significant at both young (p = 0.036) and old ages (p = 0.007); these results were also confirmed by multivariate approaches (online suppl. Fig. 3a, b). The effect of age (F(1, 20) = 0.132, p = 0.720) and genotype by age interaction (F(1, 20) = 0.288, p = 0.597) were not significant in NR2B levels.

Gephyrin (GEP) is a scaffolding protein that clusters gamma-aminobutyric acid type A receptors [23]. No significant main effect or interaction was found in the GEP protein levels (genotype: F(1, 20) = 0.001, p = 0.982; age: F(1, 20) = 3.936, p = 0.061; genotype by age: F(1, 20) =

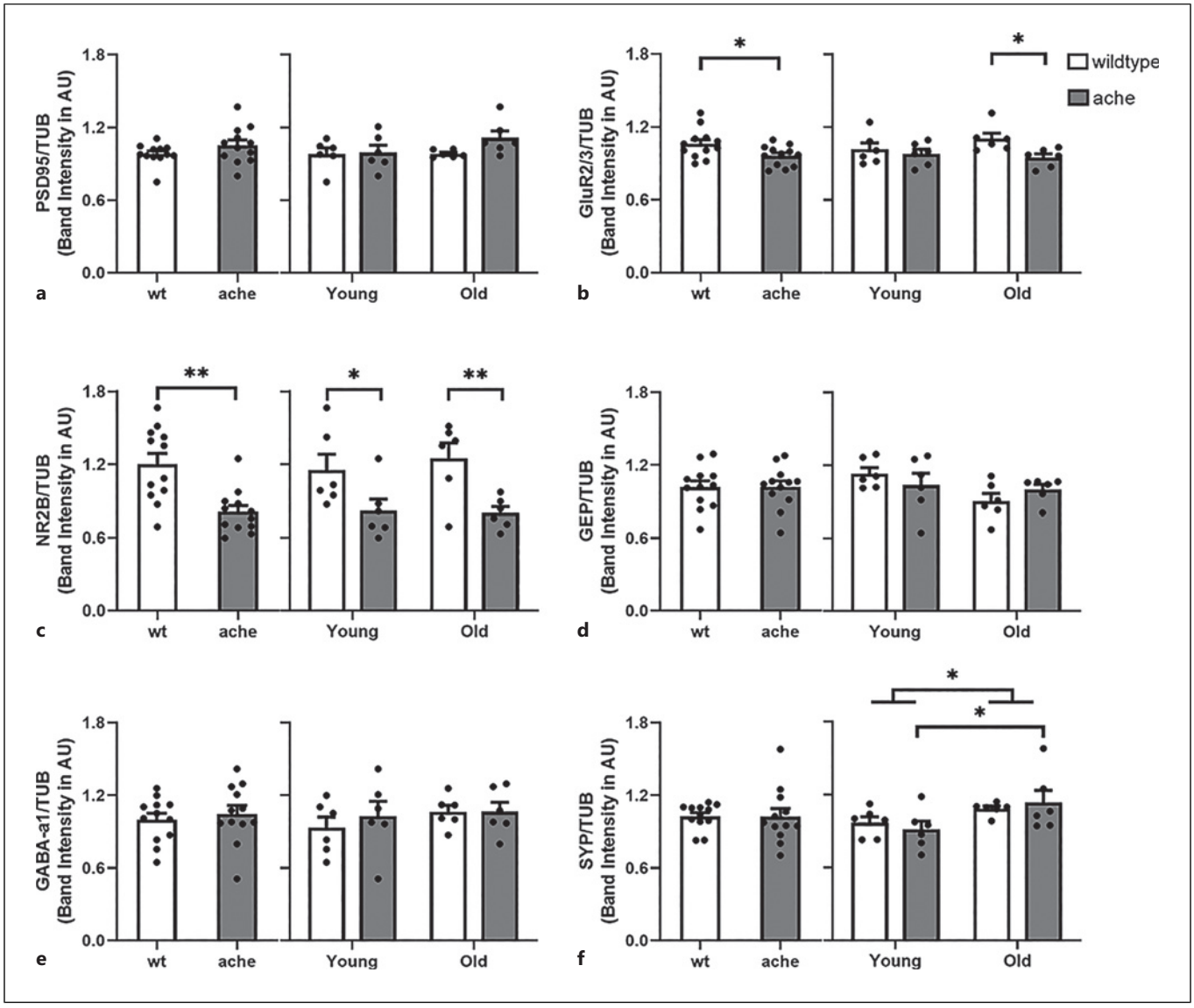

Fig. 4. Relative levels of synaptic protein between genotype and age groups. **a** No significant effect was observed in PSD95, a clustering protein in the excitatory neurotransmission system. **b** The levels of AMPA receptor subunits GluR2/3 were altered significantly depending on the genotype, and ache animals were characterized with lower levels of GluR2/3 at an old age. **c** Genotype effect was also significant on the levels of NR2B, an NMDA receptor subunit, and ache animals had significantly lower NR2B levels at both young and old ages. **d** The elements of the inhibitory neurotransmission system including GEP and GABA-a1 (**e**) were not significantly altered depending on the genotype and age of the animals. **f** The levels of SYP, a presynaptic integrity marker, showed an increase at older ages but this age effect was significantly driven by ache mutants, not the wildtype zebrafish. The group means + SE are represented. *: p <0.05, **: p <0.01.

2.058, $p = 0.167$; Fig. 4d). However, genotype and gender-driven changes were observed (genotype by gender: $F(1, 16) = 9.897$, $p = 0.006$; online suppl. Fig. 4a). These results demonstrated that wildtype female animals had significantly declining levels of GEP at old age ($p = 0.043$), while ache females had a lower baseline at a young age with stable expression levels of GEP throughout aging. GABA-a1 is the predominantly expressed subunit of the gamma-aminobutyric acid type A receptor, which is the clustering partner of GEP [23]. GABA-a1 levels did not fluctuate significantly with respect to the factors of genotype, age, or their interaction (genotype: $F(1, 20) = 0.289$, $p = 0.597$; age: $F(1, 20) = 0.889$, $p = 0.357$; genotype by age: $F(1, 20) = 0.278$, $p = 0.604$; Fig. 4e). Additionally, gender-specific

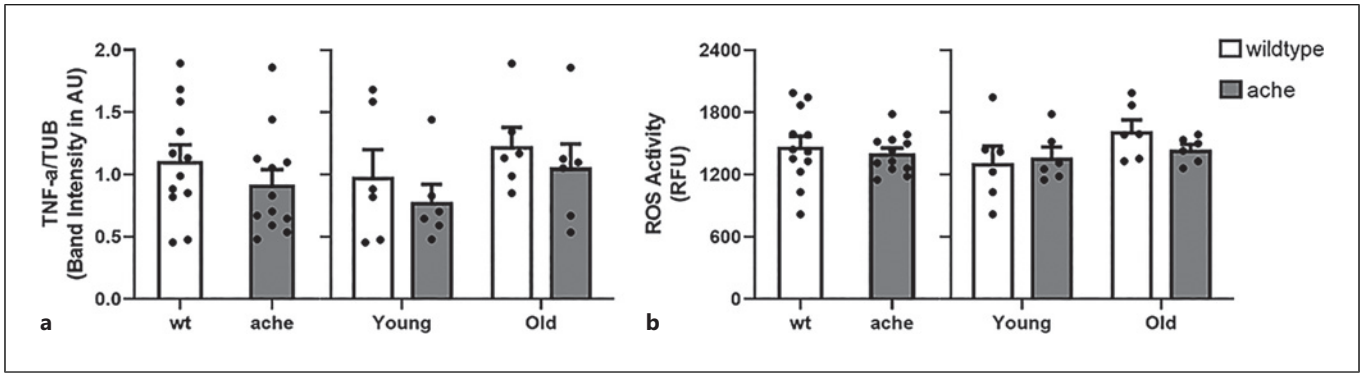

Fig. 5. Alterations in the levels of inflammatory and oxidative stress markers. **a** TNF-a, a pro-inflammatory protein marker, was not altered significantly by the factors of genotype or age. **b** No significant age- or genotype-dependent changes were observed in ROS activity levels, an indicator of free radical content. The group means + SE are represented.

modulations were found for GABA-a1. In young ache mutants, female subjects had lower levels of GABA-a1 as compared to young ache male zebrafish ($p = 0.015$; online suppl. Fig. 4b), which is consistent with the GEP measurements in young ache females that have a lower baseline.

Lastly, a protein marker of presynaptic integrity synaptophysin (SYP) was examined. No significant main effect of genotype was seen in SYP levels ($F(1, 20) = 0.001$, $p = 0.978$; Fig. 4f), while age-dependent increases were found ($F(1, 20) = 6.435$, $p = 0.020$). Pairwise analyses indicated an age-dependent increase in SYP was significant in the ache group ($p = 0.029$), whereas no changes were observed in wildtype subjects ($p = 0.223$). A genotype by age interaction was not statistically significant in SYP levels ($F(1, 20) = 0.639$, $p = 0.434$). Overall, this presynaptic marker demonstrates increasing protein levels at older ages but this age effect was significantly driven by ache mutants, not the wildtype siblings.

No Prominent Changes Were Observed in a Pro-Inflammatory Protein Marker, while the ROS Content Was Reduced in Ache Male Mutants

An altered brain microenvironment due to lower levels of AChE activity can modulate inflammation- and oxidative stress-related processes. The levels of a pro-inflammatory marker, tumor necrosis factor-alpha (TNF-a), and the activity of ROS were investigated to demonstrate these possible modulations. In TNF-a levels, no significant effects of genotype ($F(1, 20) = 1.120$, $p = 0.302$) or age ($F(1, 20) = 2.180$, $p = 0.155$) were revealed (Fig. 2, 5a). The genotype by age interaction was not statistically significant ($F(1, 20) = 0.007$, $p = 0.934$).

ROS activity was not affected significantly by genotype or age or the interaction between these factors (genotype: $F(1, 20) = 0.326$, $p = 0.575$; age: $F(1, 20) = 2.896$, $p = 0.104$; genotype by age: $F(1, 20) = 1.086$, $p = 0.310$; Fig. 5b). When ROS levels were analyzed by incorporating the factor of gender, a significant gender by genotype interaction was found ($F(1, 16) = 6.281$, $p = 0.023$). Genotype had a significant effect in male animals with ache males having a significantly lower ROS activity as compared to wildtype controls ($p = 0.040$; online suppl. Fig. 4c). However, in female animals of the wildtype groups, lower levels of ROS activity were observed as compared to wildtype male zebrafish ($p = 0.008$). In both TNF-a protein levels and ROS activity levels, numerical increases were seen at older ages but this pattern was not significant. Also, in the male groups, mutants have lower levels of ROS activity, which could have further implications on the decelerated aging profile previously reported in the literature [14].

Discussion

AChE inhibitors are widely used to treat age-related neurodegenerative diseases such as AD; however, their molecular impacts and temporal application patterns, such as long-term administration, remain largely unknown. Animal models with depleted AChE activity can provide powerful insights regarding these points. In this study, zebrafish ache mutants, in which previous studies measured neurobiological components at very young ages (3–6 months old) and behavioral attributes at older ages (12–24 months old) [13–15], were utilized across a wider

age scale. This helped us examine the temporal effects of sustained reduction of AChE activity itself and its molecular targets at young adulthood (10–11 months old) and old ages (30–31 months old). Our observations on reduced AChE activity in young and old mutants were in line with previous work conducted with very young ache mutants and ache embryos [12, 13, 15].

With respect to ACh, a previous study using the same mutant line had reported an increase in ACh levels in very young female animals (3–6 months old) [13]. Surprisingly, our data obtained from young adult (10–11 months old) and old (30–31 months old) ache mutants did not follow a similar pattern, such that we observed an overall decrease in ACh levels in the mutants. This difference likely results from the selected age range of the animals. For this reason, an additional subset of younger adult zebrafish (3.5–5 months old) was included in the current work for the assessment of AChE activity and ACh levels. A significant decrease in the AChE activity of the mutants was also evident at younger ages, and in the female group, numerical increases were observed in ACh levels. The AChE/ACh ratio was also compared between all age groups to understand the reciprocal dynamics between AChE and ACh levels [21]. Interestingly, mutants had a similar AChE/ACh ratio as compared to wildtype controls except for 3.5–5-month-old animals. At this age, ache mutants were characterized by a significantly lower AChE/ACh ratio indicating a status with low ACh hydrolysis and/or high ACh synthesis. This finding is in concordance with the previous literature [13, 15], but also it shows the importance of the duration of exposure to depleted levels of AChE activity. Mutants at younger ages have been exposed to low levels of AChE activity for 3.5–5 months which can be considered relatively short term when compared with the other groups. Therefore, the response of the cholinergic system might be changing with respect to this new microenvironment at earlier ages. However, after 10–11 months of exposure to low AChE activity, the system has adapted to these changes which is supported by the fact that the AChE/ACh ratio of mutants was indistinguishable from the wildtype controls. In this case, the homeostasis of the system is likely maintained by alternative pathways. Mice knockout models targeting the AChE activity indicated that declining AChE activity is accompanied by stable levels of choline acetyltransferase and vesicular ACh transporters regulating the synthesis and release of ACh [10]. However, the levels of $M_{1\text{-}2}$ and M_4 subunits of mAChRs declined significantly and their localization was changed in the striatum of mutant mice [10]. These results suggest that adaptive changes occur as a response to a sustained decrease in AChE activity and prominent alterations were seen in mAChRs. These observations imply that AChE may not be the only determinant. Previous literature suggested nonlinear mechanisms for the relationship between ACh and AChE and indicated that exposure to AChE inhibitors can enhance the levels of ACh in the synapse but this upregulation can lead to an autoinhibition, which is then followed by a reduction in ACh mediated by the muscarinic autoreceptors [9].

Additionally, while at young and old ages no genotype-driven changes were observed in the AChE/ACh ratio, an overall age-related decline was revealed. Previous studies have indicated a parallel age-related reduction in the elements of cholinergic indices such as AChE [24]. Interestingly, our results demonstrated that the AChE/ACh ratio which declined at old age was significantly driven by wildtype groups not the ache mutants. Mutants had relatively lower levels of AChE and ACh as compared to wildtype fish, but since both elements are lower, there is no clear difference in AChE/ACh ratio. The important point is that this ratio is maintained in the ache mutants across lifespan, while it is disrupted in the wildtype groups with increasing age. Therefore, long-term depletion of AChE activity can be associated with the stable state of cholinergic parameters that normally show age-related impairments as seen in wildtype groups.

Another cholinergic marker was the nAChR-a7 subunit of nAChR, which forms homomeric ion channels and is more permeable to calcium compared to other nAChRs [7]. Previous studies demonstrated that 14 days of administration of the AChE inhibitors increases the binding and number of nAChRs in the rat brain [7, 25]. Our data showed that in ache mutants with declined levels of AChE activity no change occurred in the levels of nAChR-a7. One reason for this observation can be related to the duration of the reduction of AChE activity. For example, 14 days of administration is a relatively short period compared to lifelong depletion of AChE activity for 10–31 months in the mutant animals and this difference in the time range may mediate adaptive responses in the organism. Taken together, our results demonstrated that persistent long-term reduction in AChE activity may not directly drive increases in the cholinergic components such as ACh and nAChR-a7 and this may be due to adaptation responses occurring over time. However, sustained reduction in AChE activity can exert stability in cholinergic components across lifespan.

One important point to consider is that long-term administration of AChE inhibitors and lifelong changes induced by mutation might have differential effects. In mice knockout models of AChE, inhibition of the AChE

enzymatic activity was around 50% and 100% in heterozygous and nullizygous mutants, respectively [11]. However, positron emission tomography studies indicated that administration of donepezil for 12 weeks resulted in 19–24% AChE inhibition in AD patients [26]. Parallel to that, administration of donepezil for 21 days was associated with a 39% reduction in cortical AChE activity in rats [27]. Although the assessment methods are different, in the current study AChE activity levels were inhibited by 27–29% in ache mutants compared to controls. Therefore, the extent of AChE inhibition was relatively subtle in ache mutants as compared to mice knockout models, which can be associated with no observation of robust changes in other cholinergic markers. However, the inhibition of the AChE profile in the current study shows similarities to the long-term administration of AChE inhibitors. Thus, it is important to investigate adaptative responses to sustained mild AChE activity reduction in potential cellular and synaptic targets.

Cellular alterations were assessed at protein levels in response to long-term reduction of AChE activity. Although previous studies did not directly measure the global levels of these cellular markers within a similar experimental paradigm, it was found that 4 weeks of treatment with the AChE inhibitor increased the number of 5′-bromo-2′-deoxyuridine-positive cells, indicating higher survival of newborn cells in healthy adult rats with no robust effects on neuronal differentiation [28]. Additionally, in this previous work, no changes were observed in proliferating cell nuclear antigen-positive cells after donepezil treatment, which was like our observations [28]. Our data in the mutant groups indicated elevated levels of HuC in both young and old mutants, and it likely reflects an overall increased survival in the neuronal cells independent of the age with long-term exposure to low levels of AChE activity. Similarly, another study indicated that after 3 weeks of donepezil treatment the number of 5′-bromo-2′-deoxyuridine-positive cells was elevated in a vascular dementia rat model, but donepezil treatment also led to a decrease in the intensity of GFAP-positive cells, indicating less glial activation [29]. Elevated levels of GFAP are known to be associated with functional disruptions in astrocytes, increases in pro-inflammatory cytokines, and will impair neurogenesis dynamics [22]. Our results indicated a marginally significant decrease in the global levels of GFAP in the ache mutants.

Another aim of the current study was to investigate the effects of long-term reduction of AChE activity on synaptic dynamics. Previous studies indicated that the pharmacological inhibition of AChE will lead to long-lasting alterations in NMDA receptors. It was shown that treatment with the AChE inhibitor in wildtype mice reduced the NMDA-mediated synaptic activity, and this suppressive effect was disrupted in the AD mice model [30]. NMDA receptors are characterized by having a high permeability to calcium in pathological conditions such as AD, the regions enriched in NR2A and NR2B receptor subunits are more affected, and it has been shown that NR2B-containing NMDA receptors are associated with more excitotoxicity in the cases of pathological aging [31]. On the other hand, in the case of normal aging, declining levels of NR2B at the older ages may be associated with behavioral impairments [32]. Our results showed a significant reduction in NR2B levels in both young and old mutants with low AChE activity. One important observation was that NR2B levels were not declining with aging in the mutant group, but rather NR2B levels were initially lower in the young mutants, and low NR2B levels were sustained at old ages. This overall NR2B reduction likely alters calcium dynamics enabling mutants to be more resistant to further excitotoxic damage, which warrants further studies. Additionally, previous studies utilizing dietary interventions such as caloric restriction demonstrate an induced initial reduction in excitatory synaptic markers that stabilizes their levels across the lifespan and protects against age-related synaptic impairments [32]. Parallel to NR2B, a decrease in the AMPA receptor subunits GluR2/3 was demonstrated in the mutants, with a more robust decrease observed in old mutants. AMPA-type glutamate receptors are permeable to sodium, potassium, as well as calcium, and the presence of the GluR2 subunit renders the receptor less permeable to calcium, as well as undergoing dynamic cycling in the synaptic region in response to plasticity-dependent changes, during long-term potentiation receptors lacking GluR2 are attached to the membrane [33]. A GluR2/3 reduction in the mutant group likely shows increased activity-dependent plasticity in old age. Alternatively, a decrease in the GluR2/3 subunit might permit more calcium influx and may occur due to homeostatic synaptic scaling as compensation for the NR2B-mediated changes in the calcium dynamics. Overall, changes in excitatory neurotransmission elements likely induce more resistance against excitotoxicity and augment plasticity after long-term exposure to the reduced AChE activity.

Additionally, inhibitory synaptic proteins including GEP and GABA-a1 were investigated with respect to aging and AChE activity. Previous studies indicated an age-related reduction in GEP levels which can lead to a vulnerability against excitotoxic events since the inhibitory elements are dysregulated [34]. A subtle decline in GEP was observed in wildtype control zebrafish during aging, while GEP levels were stable in mutants.

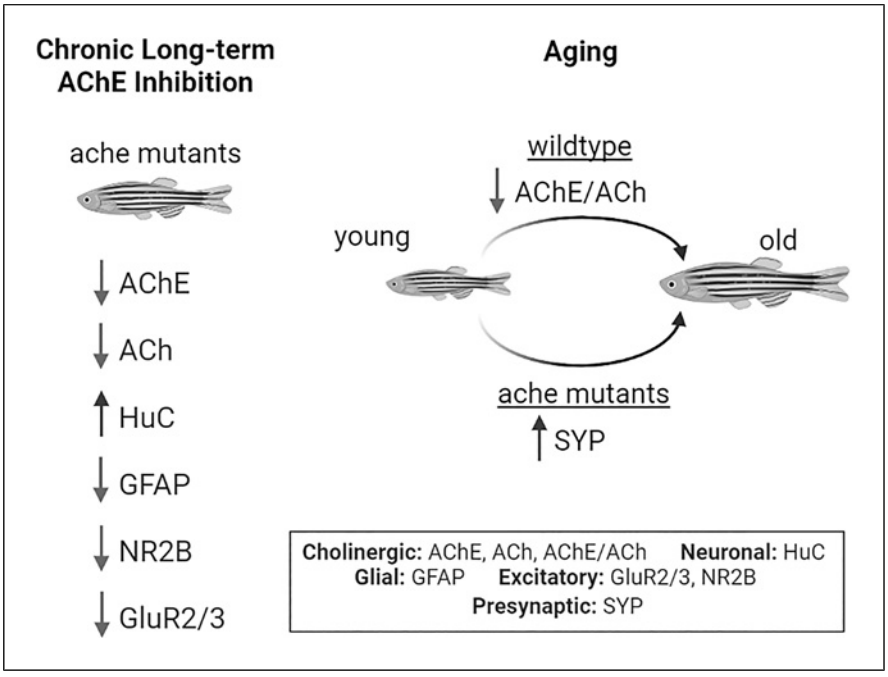

Fig. 6. Summary figure indicating alterations in response to long-term reduction of AChE activity and aging. Overall, brain levels of AChE activity and ACh content decrease significantly in the ache mutants. On the other hand, mutants as compared to wildtype controls are characterized by elevated levels of the immature neuronal marker, HuC, and reduced levels of the glial marker, GFAP, as well as excitatory synaptic proteins GluR2/3 and NR2B. In wildtype animals, the cholinergic ratio AChE/ACh declines with increasing age, while this index is stable in the mutants across the lifespan. Additionally, with aging, the levels of presynaptic protein SYP are elevated in the ache zebrafish. The figure was created with BioRender.

Additionally, stable levels of GABA-a1 were observed with aging in all groups, which was consistent with previous work [35]. SYP was used as an indicator of presynaptic integrity [36]. Previous studies reported a decline with aging in SYP levels using different animal models [19, 20, 37], and elevated SYP levels are correlated with better cognitive performance at old ages [36]. Our results demonstrated a different pattern such that SYP expression increased with aging. However, this increase was robustly driven by the mutant group, with old ache zebrafish having significantly elevated SYP as compared to the young ache group. Taken together, an age-related elevation of SYP in ache mutants can reflect maintained synapse number and presynaptic integrity at older ages.

Persistently low AChE activity can alter inflammatory mechanisms and oxidative stress-related processes. Previous studies suggested that treatments with AChE inhibitors trigger the cholinergic-mediated anti-inflammatory pathway, and this activation results in a reduction in the serum levels of pro-inflammatory cytokines [5]. It was shown that the administration of AChE inhibitors suppressed the systemic levels of pro-inflammatory cytokines such as TNF-a. Our results indicated no significant change in brain TNF-a levels of the ache mutants. One reason for this observation may be a differential regulation of pro-inflammatory cytokines between the brain and body. In previous work, systemic levels of the TNF-a were measured from the serum [5] whereas in our case brain levels of this marker were assessed. It is quite possible that there is less correspondence between brain levels and serum levels of TNF-a due to possible differences in the abundance of protein expression. This difference between plasma and brain expression levels has been observed in other studies examining growth hormone levels; for example, genetic and dietary interventions decreased the plasma levels of insulin-like growth factor-I, while no changes were detected in the brain levels of this hormone following these interventions [38]. For future studies, in addition to the measurements of brain levels of pro-inflammatory cytokines, serum samples from the peripheral blood will be investigated to identify brain-body interactions in the ache mutants regarding the cholinergic anti-inflammatory pathway. Additionally, ROS activity levels were analyzed. Previous work has shown that ROS activity is elevated with aging as well as in cases of brain insults [39]. Moreover, treatment with donepezil, an AChE inhibitor, decreases ROS activity that was induced by brain injury [40]. Our data indicated decreases in ROS activity might be driven by the depletion of AChE activity, and this pattern was observed in male mutants. Male wildtype animals had increased ROS activity compared to male ache zebrafish, as well as female wildtype zebrafish. This suggests that the depletion of AChE activity in males can reduce oxidative stress parameters, while in females gender-dependent susceptibilities may interact with these changes.

The current study was designed to reveal the expression changes in cellular, synaptic, and inflammatory targets of long-term depletion of AChE activity in young and old subjects (Fig. 6). Our results indicated that long-term reduction in AChE activity in the brains of mutants was accompanied by significant modulations in the levels of ACh to maintain the AChE/ACh ratio. Moreover, long-term depletion of AChE activity significantly upregulated the immature neuronal marker, HuC, independent of the age, whereas the glial marker, GFAP, was slightly downregulated in these mutants. With respect to synaptic proteins, the data demonstrated that reduced AChE activity was associated with significantly lower levels of glutamate receptors including GluR2/3 and NR2B especially at old age, whereas GABAergic inhibitory elements were relatively stable. Additionally, the presynaptic marker, SYP, demonstrated an age-related increase in the ache mutants. No significant change was observed in the levels of the pro-inflammatory marker TNF-a, but ROS content, an oxidative stress marker, was attenuated in male ache animals. Taken together, these data demonstrate that long-term depletion of AChE activity did not lead to an overall elevation in other cholinergic markers such as ACh and nAChR-a7, which may be an adaptive response of the cholinergic system against persistently low levels of AChE activity to maintain cholinergic homeostasis. However, long-term reduction in AChE activity exerts its effects on cellular and synaptic dynamics by balancing neuronal and glial markers, as well as inhibiting the key glutamatergic receptor subunits, which might induce more resistance in these mutants against age-related excitotoxicity.

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Statement of Ethics

The experimental procedures in the current study were approved by the local Bilkent University Laboratory Animals Ethics Committee with the approval dates: July 31, 2019, and June 15, 2016; no: 2019/23 and 2016/21, respectively.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

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Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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