

# FUS-driven zebrafish model of ALS identifies tribenzylamine as a candidate modulator of ALS-associated pathology

Yongbo Seo <sup>a,1</sup> , Jeonghyeon Lee <sup>a,1</sup> , Yunkyoung Lee <sup>b</sup> , Suhyun Kim <sup>a</sup>, Jeong-Soo Lee <sup>c</sup>, Hyunju Ro <sup>d</sup>, Sung-Hee Cho <sup>e</sup>, Jung Sang Park <sup>f</sup>, Myung Ji Kim <sup>g,\*</sup> , Hae-Chul Park <sup>a,\*</sup>

<sup>a</sup> Department of Convergence Medicine, Korea University College of Medicine, Seoul 04763, Republic of Korea

<sup>b</sup> Zebrafish Translational Medical Research Center, Korea University, Ansan, Gyeonggi-do 15588, Republic of Korea

<sup>c</sup> Microbiome Convergence Research Centre, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, Republic of Korea

<sup>d</sup> Department of Biological Science, College of Biosciences and Biotechnology, Chungnam National University, Daejeon, Republic of Korea

<sup>e</sup> Chemical Analysis Center, Korea Research Institute of Chemical Technology (KRICT), Daejeon 34114, Republic of Korea

<sup>f</sup> Orgasis Corporation, Suwon, Republic of Korea

<sup>g</sup> Department of Neurosurgery, Korea University College of Medicine, Ansan Hospital, Ansan, Republic of Korea

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## ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by motor neuron loss and declining motor function; however, effective therapies remain limited. To support unbiased therapeutic discovery, we aimed to develop a high-throughput phenotypic screening platform based on a transgenic zebrafish model expressing the human ALS-associated FUS-R521C mutant (mtFUS). This model was generated using a modified QF-based binary expression system and exhibited early-onset pathological features, including elevated oxidative stress, progressive neuronal degeneration, and impaired locomotor activity, thereby recapitulating the key aspects of FUS-associated ALS. Transcriptomic profiling revealed molecular signatures resembling those reported in patient-derived motor neurons, including dysregulated neuroactive ligand–receptor signaling, immune activation, and stress–response pathway alterations. Using this platform, we identified tribenzylamine (TBA) as a candidate compound that improves locomotor performance and significantly reduces reactive oxygen species levels. Integrated transcriptomic and biochemical analyses suggested that TBA induces coordinated molecular changes, including normalization of neuronal activity-related gene expression, modulation of immune and metabolic pathways, and restoration of hormone-related signaling. TBA reversed FUS-induced reductions in key neuronally active sex steroids, including estrogen and progesterone, and increased estrogen-responsive gene expression, suggesting a partial recovery of neuronally active sex steroid homeostasis. These findings support the mtFUS zebrafish model as a useful platform for ALS drug discovery and identify TBA as a candidate modulator of ALS-associated phenotypes, with effects linked to transcriptomic remodeling and neuronally active sex steroid signaling.

## 1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive degeneration of upper and lower motor neurons, leading to muscle weakness, paralysis, and respiratory failure [1,2]. Most patients with ALS have sporadic ALS, whereas approximately 10% have familial ALS associated with mutations in genes such as SOD1, TARDBP, C9ORF72, and FUS [3]. Despite substantial progress in understanding ALS pathology, currently approved

therapies, including riluzole, edaravone, and the SOD1-targeted antisense oligonucleotide tofersen, provide modest or subtype-specific benefits by slowing disease progression [4]. ALS exhibits significant genetic and pathological heterogeneity, broadly categorized into TDP–43-proteinopathies which account for most sporadic ALS cases and non-TDP–43 pathologies, such as those driven by FUS mutations. Given the complexity of ALS pathogenesis, conventional target-based drug discovery has had limited efficacy.

Phenotypic screening offers a complementary strategy by identifying

\* Corresponding authors.

E-mail addresses: [kjmj8686@korea.ac.kr](mailto:kjmj8686@korea.ac.kr) (M.J. Kim), [hcpark67@korea.ac.kr](mailto:hcpark67@korea.ac.kr) (H.-C. Park).

<sup>1</sup> These authors contributed equally to this study.

therapeutic candidates based on disease-relevant cellular and behavioral outcomes, rather than single molecular targets [5]. However, effective in vivo screening requires ALS models with early, quantifiable, and scalable disease-relevant phenotypes.

Among familial ALS genes, *FUS* mutations are strongly associated with early-onset ALS and rapid disease progression, with many pathogenic variants located in the nuclear localization signal (NLS) domain [6,7]. *FUS* is an RNA-binding protein involved in transcriptional regulation, RNA splicing, and mRNA transport [8–10]. ALS-linked *FUS* mutations impair nuclear import, resulting in cytoplasmic accumulation, stress granule formation and aggregation [11]. These changes suggest that *FUS* mutations contribute to the disease through loss-of-function and toxic gain-of-function mechanisms. Although *FUS* mutations account for a relatively small proportion of familial ALS, pathogenic *FUS* mislocalization due to impaired nuclear import is a central disease mechanism, and *FUS*-associated pathology is relevant across the ALS-FTD spectrum (14, 24, 34). Furthermore, RNA-processing abnormalities associated with *FUS* pathology overlap with molecular pathways implicated across broader ALS contexts, supporting the wider translational relevance of targeting *FUS*-associated disease mechanisms. [9,12–14].

Various in vivo and in vitro models, including *FUS* knockout, knock-in, and overexpression mouse models, have been developed to investigate *FUS*-mediated ALS pathogenesis [12,15–17]. However, many require extended periods to develop phenotypes and are limited by high maintenance costs and low scalability, which restrict their utility for early-stage drug screening. To address these limitations, we aimed to develop a neuron-specific zebrafish ALS model based on a Q-system-derived binary transactivation platform that enables stable overexpression of mutant human *FUS*-R521C (mtFUS), a pathogenic NLS-domain variant associated with *FUS*-linked ALS. This system, originally adapted from *Neurospora crassa*, provides improved expression efficiency and reduced leaky expression compared to conventional GAL4/UAS systems [18–20].

Using this platform, we performed a behavioral assay-based phenotypic screening and identified tribenzylamine (TBA) as a candidate compound that alleviates ALS-associated phenotypes. As TBA remains pharmacologically undercharacterized, we integrated comprehensive bulk and neuron-specific RNA-sequencing (RNA-seq) analyses to elucidate its molecular mechanisms. Together with biochemical evaluations, these transcriptomic datasets revealed that TBA treatment significantly attenuates oxidative stress, drives widespread transcriptomic remodeling, and modulates neuronally active sex steroid-related signaling pathways.

## 2. Materials and methods

### 2.1. Animals

All experimental procedures were approved by the Korea University Institutional Animal Care and Use Committee (KOREA–2023–0176) and were performed in accordance with the animal experiment guidelines of the Korea National Veterinary Research and Quarantine Service. Wild-type and Tg(NBT:DsRED) [21] zebrafish, along with transgenic lines carrying a QF-based binary reporter system, including Tg(HuC:QFDBD–2xAD-Crystallin:EGFP) and Tg(13QUAS:ZsGreen-Crystallin:mCherry) [20], were used in this study. The fish were maintained at 28.5 °C under a 14 h light/10 h dark cycle in egg water or E3 medium. The developmental stages were defined as days post-fertilization (dpf).

### 2.2. Generation of transgenic zebrafish

A transgenic zebrafish model with neuron-specific expression of mutant human *FUS*-R521C was generated using an IQ-switch effector system. The *FUS*-R521C mutation was introduced into the human *FUS* ORF (pENTR4\_FUS; Addgene #26366) using PCR with KOD-FX

polymerase (Toyobo, Osaka, Japan). The mutant *FUS* sequence was cloned into the Tol2 13QUAS:ZsGreen-p2a-terminator-Crystallin:mCherry vector using sequence- and ligation-independent cloning. The construct was linearized with *Bgl*II (New England Biolabs, Ipswich, MA, USA), and *FUS*-R521C was inserted downstream of the p2a sequence. The final plasmid, Tol2 13QUAS:ZsGreen-p2a-hFUS-R521C-terminator-Crystallin:mCherry, was verified using sequencing. For transgenesis, the construct was co-injected with transposase mRNA into one-cell-stage embryos. Founder (F0) fish were screened for integration, and stable F1 lines were established by selecting offspring with mCherry fluorescence in the eyes after outcrossing with wild-type fish.

### 2.3. Spinal cord cross section preparation, Immunohistochemistry (IHC), and Axon length quantification

Zebrafish larvae were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. For spinal cord cross-sectioning, the fixed specimens were embedded in a block containing 1.5% agarose and 5% sucrose, frozen, and sectioned at a thickness of 10 µm using a Leica CM1860 cryostat (Leica Biosystems). The samples were then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-*FUS* (1:1000, Invitrogen, Carlsbad, CA, USA), mouse anti-Hu (1:200, Invitrogen), mouse anti-Islet1/2 (1:200), and mouse anti-Mnx1 (1:200; Developmental Studies Hybridoma Bank, Iowa City, IA, USA). After thorough washing, the samples were incubated with Alexa Fluor 488-, 568-, and 647-conjugated secondary antibodies (1:1000; Invitrogen) were applied, and nuclei were counterstained with DAPI (1:1000). The sections were imaged using an A1 confocal microscope (Nikon, Tokyo, Japan) with a 40 × /1.25 objective. Z-stacks were acquired at 1 µm intervals and analyzed using the NIS-Elements software (Nikon). Whole-mount imaging was performed using an Eclipse Ti2 spinning disk confocal microscope (Nikon) with a 20 × objective. Images were collected at 1 µm intervals in the somite 2–4 region and processed using NIS-Elements. For motor axon length quantification, primary motor axons were measured from the whole-mount confocal z-stack images. The length of each axon was explicitly defined and traced from its exit point at the central nervous system (the ventral boundary of the spinal cord) extending distally to its first major branching or turning point within the myotome. Measurements were performed using the NIS-Elements software.

### 2.4. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

To detect apoptosis, 5 and 10 dpf zebrafish larvae were fixed in 4% paraformaldehyde at 4 °C overnight. The fixed larvae were treated with 0.25% trypsin for 5 min on ice, washed three times with phosphate-buffered saline with Tween 20 (PBST), and incubated with TUNEL solution from the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) at 37 °C for 1 h in the dark. After two washes with PBST, fluorescence microscopy was performed.

### 2.5. Oxidative stress detection

Live staining was performed to detect oxidative stress in zebrafish larvae using CellROX™ Orange Reagent (Invitrogen). At 5 dpf, the zebrafish larvae were treated with 5 µM CellROX™ reagent for 30 min in the dark. After three washes with PBST, the oxidative stress levels were measured using fluorescence microscopy.

### 2.6. Neuromuscular junction analysis

At 5 dpf, larvae were immunostained with mouse anti-SV2 (1:50; Developmental Studies Hybridoma Bank) and incubated with Alexa 647-conjugated  $\alpha$ -bungarotoxin (1:500; Developmental Studies Hybridoma Bank) for 15 min at room temperature (20–25 °C). Fluorescence images were obtained using an A1si laser scanning confocal microscope

(Nikon). Confocal images were captured at 1  $\mu$ m z-stack intervals and processed using NIS-Elements AR Analysis 4.30 software (Nikon) to calculate the area fraction of colocalized SV2 and  $\alpha$ -bungarotoxin signal.

## 2.7. RNA sequencing and transcriptomic analysis

Whole-larvae bulk RNA sequencing and neuron-specific RNA sequencing were performed as discovery-stage pooled transcriptomic analyses. For bulk RNA-seq, one pooled RNA sample per condition was generated using 20 whole larvae per group at 5 dpf larvae from four groups—sibling controls and Tg(HuC:mtFUS) larvae treated with vehicle or TBA—were pooled (~20 larvae per group) and homogenized in TRIzol™ reagent (Invitrogen) for RNA extraction. For neuron-specific RNA-seq, ZsGreen\* neurons were isolated via fluorescence-activated cell sorting (FACS) from Tg(HuC:QF×13QUAS:ZsGreen) and Tg(HuC:QF×13QUAS:ZsGreen:hFUS) larvae at 5 dpf (approximately 300 larvae pooled per condition), with modifications to previously described protocols [22,23]. This large-scale pooling strategy was explicitly adopted to overcome the insufficient total RNA yield from specific neuronal population. The larvae were anesthetized with tricaine (Sigma-Aldrich, St. Louis, MO, USA), deyolked, and dissociated using a Neural Tissue Dissociation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

The cells were washed, resuspended in a sorting buffer containing bovine serum albumin, and sorted using a BD FACS Melody system (BD Biosciences). To establish strict fluorescence baselines and completely exclude autofluorescent pigmented cells, non-transgenic stage-matched sibling larvae (Non-fluorescent, NF) were utilized as strict negative controls. Dead cells were simultaneously excluded using SYTOX Orange staining. The sequential gating strategy included scatter gating (FSC/SSC) for debris and doublet exclusion, live/dead discrimination, and specific isolation of ZsGreen-positive cells. Total RNA from bulk samples and sorted neurons was extracted and quality-checked before library preparation. All RNA samples used for subsequent library preparation demonstrated high integrity (RNA Integrity Number [RIN] > 9.5). Libraries were generated using the TruSeq Stranded mRNA Library Prep Kit (Illumina) and sequenced as 101 bp paired-end reads by Macrogen Inc. (Seoul, Korea) using an Illumina platform. Raw reads were assessed using FastQC (v0.11.7) and trimmed using Trimmomatic (v0.38). Reads were aligned to the zebrafish reference genome (GRCz11, NCBI\_106) using HISAT2 (v2.1.0), and transcript assembly and quantification as fragments per kilobase of transcript per million mapped reads (FPKM) and transcripts per million (TPM) were performed using StringTie (v2.1.3b). Differential expression analysis was conducted using edgeR with trimmed mean of M-values normalization, followed by an exact test. Genes with  $|FC| \geq 2$  and  $p < 0.05$  were considered differentially expressed. Functional enrichment analyses, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, were performed using g:Profiler. To rigorously validate these exploratory transcriptomic findings, the principal gene expression changes were independently verified by RT-qPCR using completely separate biological replicates ( $n = 3$  independent experiments).

## 2.8. Neuronally active sex steroid analysis

Neuronally active sex steroid levels were quantified using an ultra-performance liquid chromatography system (ACQUITY UPLC; Waters Corporation, Milford, MA, USA) coupled to a Xevo TQ-S triple quadrupole mass spectrometer, following established protocols [24,25]. For sample preparation, each experimental group included 3 independent biological replicates, with each replicate consisting of 30 pooled zebrafish larvae. The pooled samples were homogenized on ice in methanol/acetic acid (99:1, v/v) using a probe sonicator. Homogenates were centrifuged at 21,300  $\times g$  for 5 min at 4 °C, and the supernatants were collected. The pellets were re-extracted twice with the same solvent, resuspended in methanol/water (10:90, v/v), and subjected to solid-phase extraction using Oasis PRiME HLB cartridges (Waters). The

eluates were dried under nitrogen, reconstituted in the mobile phase, and analyzed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) for neuronally active sex steroid quantification.

## 2.9. Quantitative reverse transcription PCR analysis

ZsGreen\* neurons were isolated from Tg(HuC:QFDBD×13QUAS:ZsGreen) and Tg(HuC:QFDBD×13QUAS:ZsGreen:hFUS) zebrafish larvae at 5 dpf using FACS before RNA extraction. Total RNA was extracted using TRIzol™ reagent (Invitrogen), and cDNA was synthesized using the ImProm-II™ Reverse Transcriptase kit (Promega). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed using the FastStart Essential DNA Green Master Mix (Roche) on a LightCycler® 96 system (Roche). The amplification conditions were as follows: 95 °C for 10 min, followed by 40 cycles at 94 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s, with a final step at 85 °C for 15 s. The following primers were used for RT-qPCR:

**$\beta$ -actin forward:** 5'-AAGGCCAACAGGAAAAGAT-3'.  
 **$\beta$ -actin reverse:** 5'-GTGGTACGACCGGAGGCATAC-3'.  
**chrnb2l forward:** 5'-GCTTCAGGACTTTGGTTTGTGCG-3'.  
**chrnb2l reverse:** 5'-GATAAGCTGACCAAGGGACAC-3'.  
**ccl19b forward:** 5'-CGAATCCCTCAGAAAAGT-3'.  
**ccl19b reverse:** 5'-GCTCGGAAATGGTGC-3'.  
**pth2 forward:** 5'-CCACGCAACACACAGTCAAG-3'.  
**pth2 reverse:** 5'-GCAAGTTACTTTGCAGAGGTC-3'.  
**hmox1a forward:** 5'-TAAAAACGAAGTGGGGCGGT-3'.  
**hmox1a reverse:** 5'-TGTTTCAGACAGATCACTGCCA-3'.  
**prfl.2 forward:** 5'-AGTCATTGAAGAGTGTCCCTG-3'.  
**prfl.2 reverse:** 5'-CACTGCATTCTTACCAAGC-3'.  
**pdyn forward:** 5'-ATCTCCGATTGGACTCTGC-3'.  
**pdyn reverse:** 5'-CTTGGAGGTCCTCATCTGTGT-3'.  
**nmba forward:** 5'-GGAAATCTTTGGGCAACAGG-3'.  
**nmba reverse:** 5'-CTGAGCCTGAAGAGCAATTT-3'.  
**nmbb forward:** 5'-GTGGATTCTTCACATACCTAGTGC-3'.  
**nmbb reverse:** 5'-CTTGCTGTCCACGACACTTT-3'.  
**ptger4b forward:** 5'-CTTTCACGATATGGACCTCCAG-3'.  
**ptger4b reverse:** 5'-GCTCTTTCCGTGACTTTTCA-3'.  
**rhocb forward:** 5'-GATCAGTTCCTTGAGGTCTAC-3'.  
**rhocb reverse:** 5'-CTGGATAAGACAATGGCCTC-3'.  
**sorbs1 forward:** 5'-AGGGTGCACTTACTTTGAGG-3'.  
**sorbs1 reverse:** 5'-GTTTACACTTCTCTCAGCCAG-3'.

## 2.10. Larval behavioral analysis

Behavioral analysis was performed using the DanioVision system and EthoVision XT12 software (Noldus). Experiments were conducted between 10:00 and 16:00 using larvae from the same parental pair. The larvae were maintained at 28.5 °C under a 14 h light/10 h dark cycle and transferred to 48-well plates 1 h before testing. For spontaneous locomotion, 5 dpf larvae were acclimated to light for 10 min, followed by a 5 min recording. Light–dark transition responses were assessed after 10 min light adaptation, with locomotion recorded under alternating light and dark conditions. The total distance moved was measured in each phase. For the startle response assays, larvae were placed in the DanioVision observation chamber and acclimated in the dark for 30 min (Light OFF adaptation). Following the acclimation period, video tracking was initiated. To ensure absolute experimental consistency and eliminate any manual variability, an automated mechanical tapping stimulus was delivered exactly 5 min after tracking commenced using the DanioVision integrated tapping device set at a standardized intensity (Intensity level 4).

## 2.11. Bioactive compound library and drug treatment

A bioactive compound library (MedChemExpress, Monmouth Junction, NJ, USA) was used for the primary phenotypic screen. Compounds

were supplied in 96-well plates as 10 mM stock solutions in DMSO and stored at  $-80^{\circ}\text{C}$  until use. Non-toxic drug concentrations were determined by treating wild-type embryos with 10  $\mu\text{M}$  compounds from 6 hpf to 4 dpf, which were used for screening. For efficacy assessment, larvae were incubated in E3 medium containing 0.1% DMSO as the vehicle or with test compounds at the indicated concentrations. For the validation, rescue and mechanistic blockade experiments, TBA (MedChemExpress), hexestrol and fulvestrant (Selleck Chemicals) were used. The larvae were treated with 10  $\mu\text{M}$  edaravone, 0.5  $\mu\text{M}$  hexestrol, or 1–10  $\mu\text{M}$  TBA. The control group received E3 medium containing 0.1% DMSO. To evaluate drug efficacy, zebrafish larvae were incubated in E3 medium containing 0.1% DMSO as a vehicle control or with various concentrations of the test compounds. Assay quality was evaluated using the locomotion profiles of the sibling controls and mtFUS larvae, yielding a highly reliable Z-factor of 0.52. Furthermore, compounds were selected as hits only if they increased the average distance moved by at least 30% compared to the mtFUS and demonstrated statistical significance ( $p < 0.05$ ). Applying these objective criteria throughout the screening process, TBA was identified as the primary hit rate of approximately 0.33%. For the validation, rescue and mechanistic blockade experiments, tribenzylamine (TBA; Cat# HY-W027580, MedChemExpress), hexestrol (Cat# S2473, Selleck Chemicals, Houston, TX, USA) and fulvestrant (Cat# S1191) were used. Larvae were treated with 10  $\mu\text{M}$  edaravone, 0.5  $\mu\text{M}$  hexestrol or TBA at 1, 2, 4, and 10  $\mu\text{M}$ , respectively. For the pharmacological blockade, Tg(HuC:mtFUS) larvae were co-treated with 1  $\mu\text{M}$  fulvestrant and TBA. The control group received only E3 medium containing 0.1% DMSO.

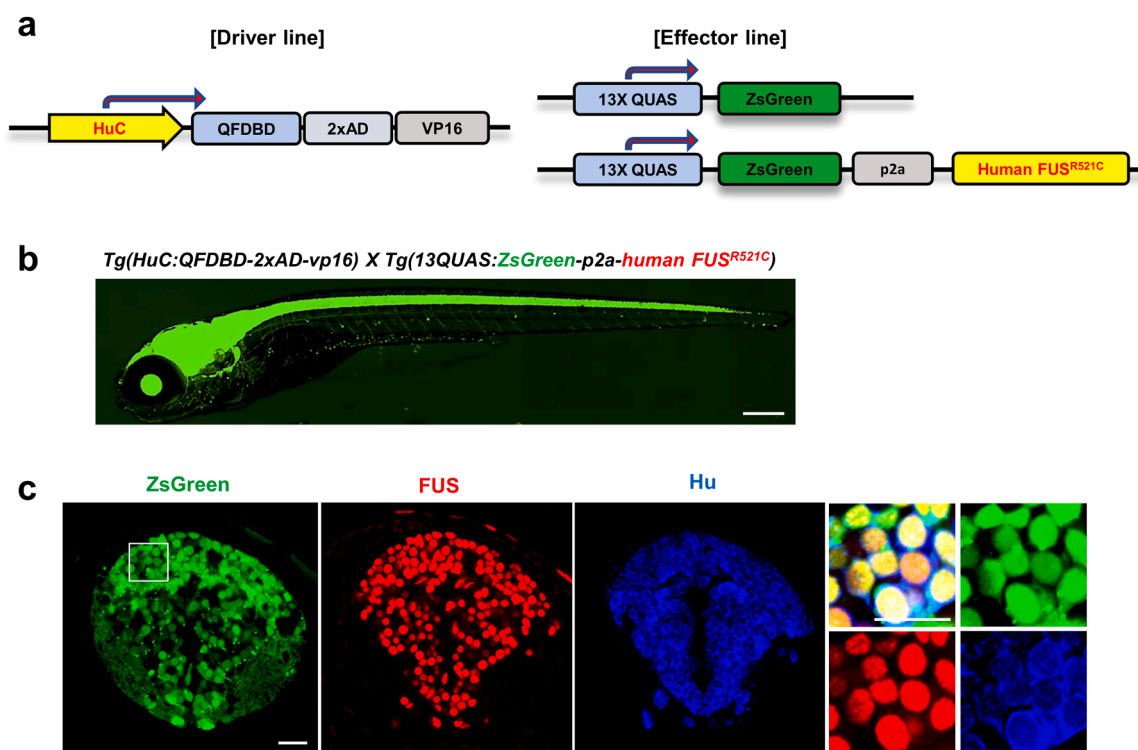
## 2.12. Statistical analysis

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). For two-group comparisons, an unpaired Student's *t*-test was used for normally distributed data, and the Mann–Whitney *U* test was used for non-normally distributed data. For comparisons among three or more groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for normally distributed data, and the Kruskal–Wallis test followed by an appropriate post hoc test was used for non-normally distributed data. Statistical significance was set at  $p < 0.05$ . Investigators were not blinded to group allocation or treatment conditions. However, all quantitative behavioral measurements were acquired and analyzed using the automated tracking system, thereby minimizing potential observer-related bias during behavioral assessment.

## 3. Results

### 3.1. Overexpression of mtFUS induced neuronal death and early mortality

To develop a larval zebrafish model of early-onset ALS for high-throughput phenotypic drug screening, we generated transgenic zebrafish that neuronally overexpress human mutant FUS-R521C, hereafter referred to as mtFUS. To induce robust transgene expression, we used a modified QF-based binary transactivation system that drives transgene expression at levels five times higher than the GAL4-UAS system [20]. We generated the driver line Tg(HuC:QFDBD 2xAD-VP16), in which the QFDBD transactivator is driven by the neuron-specific HuC promoter [26]. For the effector lines, we used Tg(13XQUAS:ZsGreen) and Tg



**Fig. 1.** Generation of transgenic zebrafish with neuron-specific mtFUS expression. **a.** Schematic of the transgenic constructs used for neuron-specific expression of mutant human FUS in zebrafish. The driver line uses the pan-neuronal HuC promoter to drive QFDBD–2xAD–VP16 expression, enabling neuron-specific activation (left panel). The effector lines contain 13 tandem QUAS repeats upstream of either the ZsGreen fluorescent reporter alone or ZsGreen linked to mutant human FUS-R521C, allowing visualization of transgene expression (right panel). **b.** Lateral views of Tg(HuC:QFDBD 2xAD X 13QUAS:ZsGreen-p2a-hFUS (R521C)) zebrafish at 3 days post-fertilization (dpf). Scale bar: 200  $\mu\text{m}$ . **c.** Transverse sections of the spinal cord of Tg(HuC:QFDBD 2xAD X 13QUAS:ZsGreen-p2a-hFUS-R521C) zebrafish at 3 dpf labeled for ZsGreen + neurons (green), FUS (red), and Hu (blue). Dorsal is at the top. Magnified views are shown on the right and demonstrate colocalization of ZsGreen with FUS and Hu, indicating neuron-specific expression of mutant human FUS. Scale bars: 10  $\mu\text{m}$ .

(13XQuas:ZsGreen-p2a-hFUS-R521C), which enables expression of either ZsGreen fluorescent protein alone (*HuC:ZsGreen*) or mtFUS together with ZsGreen (*HuC:mtFUS*), respectively (Fig. 1a). Immunohistochemistry of the spinal cord in *HuC:mtFUS* larvae at 3 dpf using a human FUS antibody and the neuronal marker anti-Hu revealed colocalization of ZsGreen fluorescence with anti-Hu and anti-FUS labeling, demonstrating that human mtFUS was specifically expressed in spinal cord neurons of *HuC:mtFUS* larvae (Fig. 1b, c).

We next assessed the survival of *HuC:mtFUS* zebrafish and observed that they died earlier than their non-transgenic siblings, with an average lifespan of approximately 13 dpf (Fig. 2a). To determine whether mtFUS overexpression induced neuronal degeneration, we performed TUNEL staining to detect apoptotic cell death in *HuC:ZsGreen* and *HuC:mtFUS* larvae. At 5 dpf, TUNEL-positive cells were first observed in the dorsal spinal cord of *HuC:mtFUS* larvae, where primary sensory neurons are located, but not in the ventral spinal cord, where motor neurons are located (Fig. 2b).

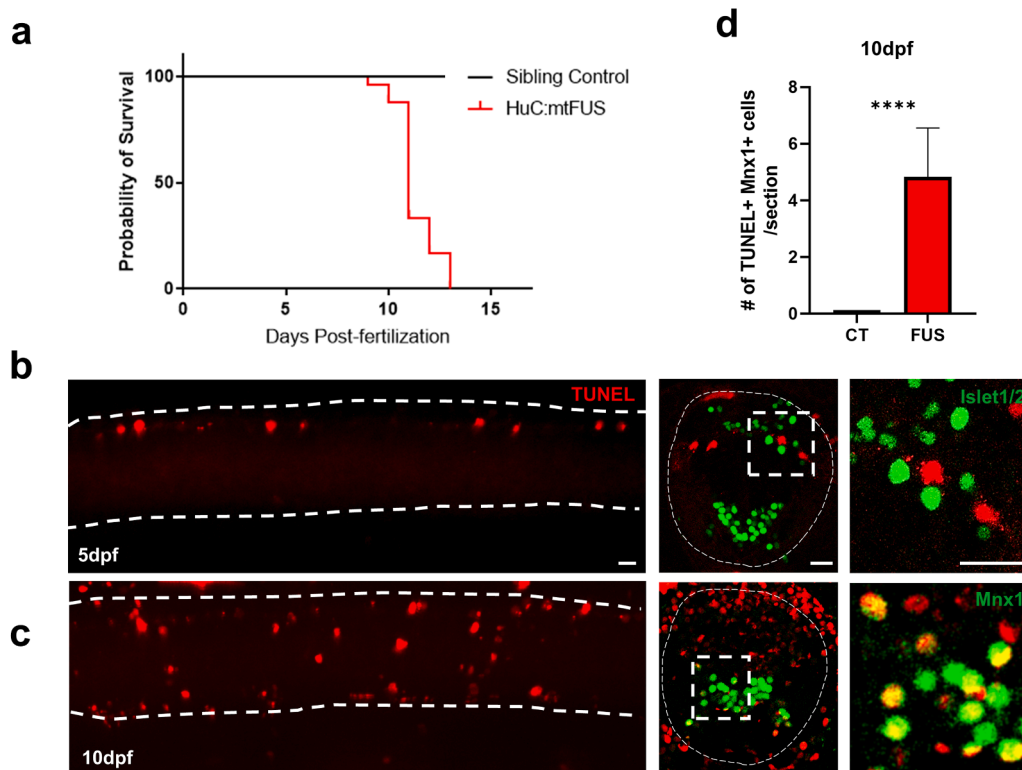
Immunohistochemistry using an anti-Islet1/2 antibody, which labels sensory and motor neurons, did not label TUNEL + cells in the dorsal spinal cords, suggesting that these apoptotic cells were interneurons (Fig. 2b). By 10 dpf, widespread TUNEL + apoptotic cell death was observed throughout the spinal cord of *HuC:mtFUS* larvae (Fig. 2c). Immunostaining with the Mnx1 antibody, a specific motor neuron marker, confirmed motor neuron cell death at 10 dpf (Fig. 2c, d). These results indicate that neuronal mtFUS overexpression induces early apoptotic cell death in putative interneurons, followed by motor neuron death.

### 3.2. Overexpression of mtFUS caused early locomotor defects

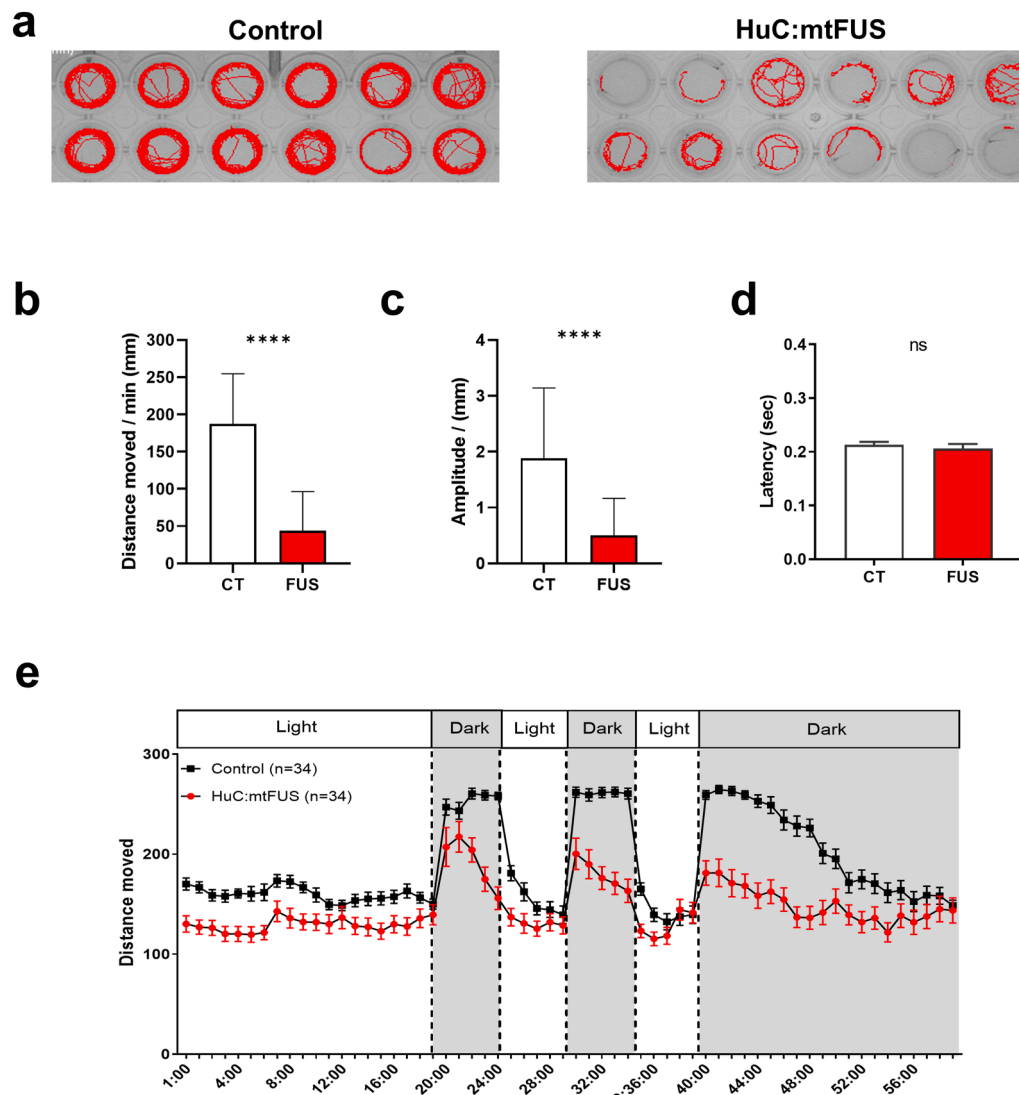
To determine whether mtFUS overexpression affected motor function, *HuC:mtFUS* larvae were placed in 48-well plates, and their locomotor activity was measured under light. *HuC:mtFUS* larvae showed impaired locomotor activity, with a shorter distance moved than that of control larvae (Fig. 3a, b). In the tapping-induced startle response assay, *HuC:mtFUS* larvae showed a marked reduction in total movement distance after stimulation compared with control larvae (Fig. 3c). Reaction latency was comparable between *HuC:mtFUS* and control larvae (Fig. 3d), suggesting preserved stimulus detection despite reduced movement.

Locomotor activity was further investigated using light–dark locomotion assay. The control larvae showed lower locomotor activity during the light cycle than during the dark cycle, producing the expected photomotor response pattern (Fig. 3e). In contrast, *HuC:mtFUS* larvae showed a reduced overall distance traveled. During the dark cycle, *HuC:mtFUS* larvae showed lower locomotor activity and reduced peak distance compared with control larvae. *HuC:mtFUS* larvae showed a gradual decrease in the peak distance moved, consistent with reduced locomotor endurance. However, *HuC:mtFUS* larvae did not exhibit delayed or absent responses to light transitions under the conditions tested. These findings suggest that neuron-specific mtFUS overexpression induces early motor dysfunction while maintaining responsiveness to visual stimuli in larval zebrafish.

As *HuC:mtFUS* larvae exhibited locomotor defects at 5 dpf without evidence of motor neuron degeneration at this stage, we next investigated whether motor axon length was altered. Measurement of axonal



**Fig. 2.** mtFUS expression led to early mortality, early dorsal spinal cell death, and later motor neuron death. **a.** Kaplan–Meier survival curve showing the survival of Tg(*HuC:mtFUS*) zebrafish and their non-transgenic siblings from 0 to 15 dpf. ( $n = 30$ . \*\*\*\* $p < 0.0001$ ) **b–c.** Representative whole-mount images of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining in Tg(*HuC:mtFUS*) zebrafish at 5 and 10 dpf. TUNEL-positive cells are shown in red along the spinal cord. Transverse sections show TUNEL staining combined with Islet1/2 immunolabeling at 5 dpf, which labels sensory and motor neurons (top row), and Mnx1 immunolabeling at 10 dpf, which labels motor neurons (bottom row). Magnified views highlight the dorsal region, where TUNEL-positive cells did not overlap with Islet1/2-positive neurons at 5 dpf but colocalized with Mnx1-positive motor neurons at 10 dpf; anterior is to the left and dorsal is at the top. **d.** Quantification of TUNEL-positive and Mnx1-positive cells in the spinal cord of Tg(*HuC:mtFUS*) zebrafish at 10 dpf. Data are reported as the mean  $\pm$  standard deviation (SD).  $n = 10$  independent experiments. Statistical significance was determined using an unpaired Student's *t*-test. Significance is indicated by \*\*\*\* $p < 0.0001$ . Scale bars: 10  $\mu$ m.



**Fig. 3.** mtFUS expression caused early locomotor defects with preserved sensory responsiveness. **a.** Representative tracking images of locomotor activity in sibling control and Tg(HuC:mtFUS) zebrafish at 5 dpf, recorded using DanioVision. **b.** Quantification of distance moved per minute in sibling control and Tg(HuC:mtFUS) zebrafish at 5 dpf. **c.** Startle response assay measuring movement distance after tapping stimulation in sibling control and Tg(HuC:mtFUS) zebrafish at 5 dpf. **d.** Startle response assay measuring reaction time to tapping stimulation in sibling control and Tg(HuC:mtFUS) zebrafish at 5 dpf. **e.** Light–dark locomotion assay showing locomotor activity in sibling control and Tg(HuC:mtFUS) zebrafish under alternating light and dark conditions. The experiment was independently repeated three times. Data are reported as the mean  $\pm$  SD;  $n = 24$  independent experiments. Statistical significance was determined using an unpaired Student's *t*-test. Significance is indicated by \*\*\*\* $p < 0.0001$ ; ns: not significant.

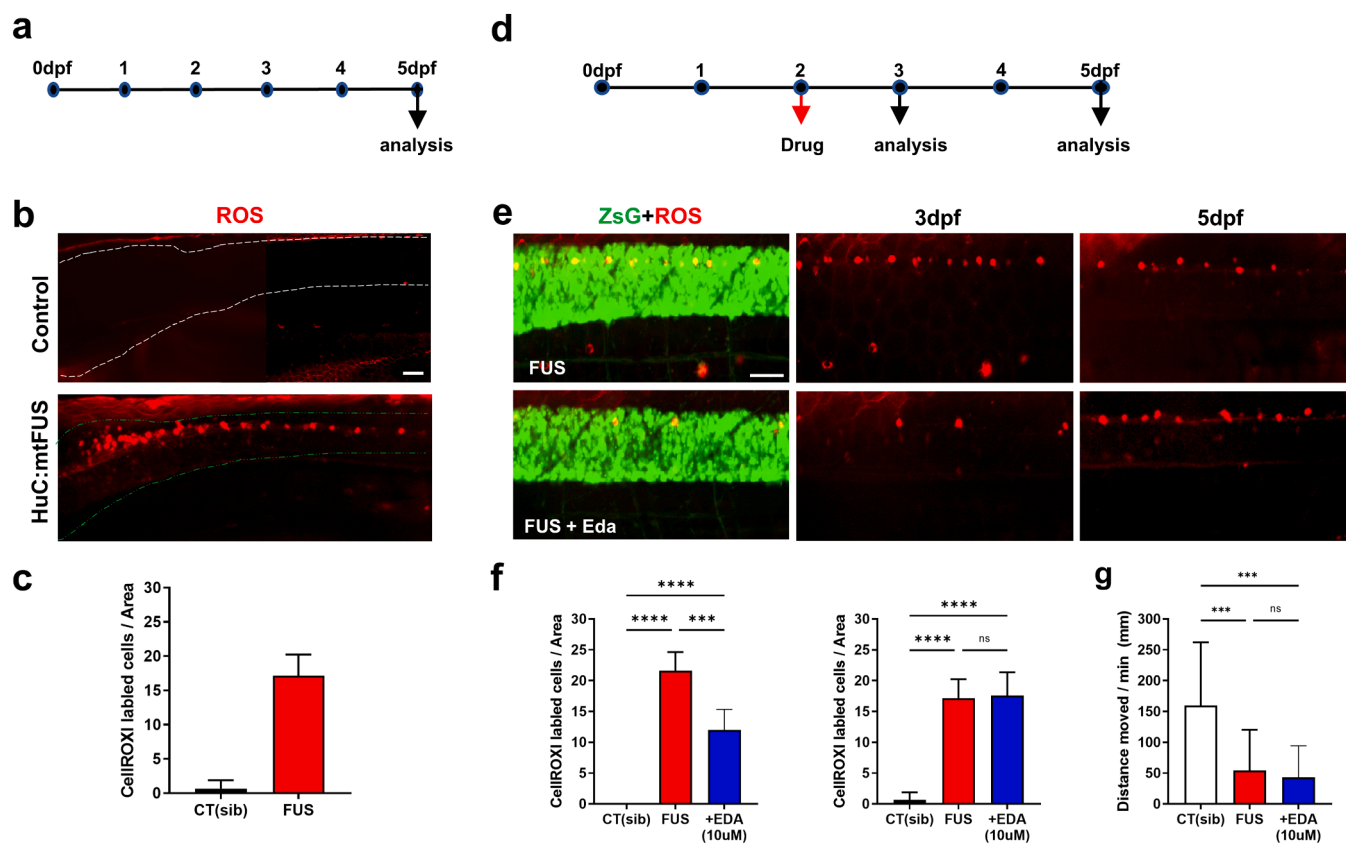
length revealed no significant difference between HuC:ZsGreen and HuC:mtFUS larvae (Supplementary Fig. 1a, b). In addition, neuromuscular junction (NMJ) analysis at 5 dpf using  $\alpha$ -bungarotoxin and anti-SV2 antibody, which label postsynaptic and presynaptic regions, respectively, showed no significant difference in the density or colocalization of presynaptic and postsynaptic markers between HuC:mtFUS and control larvae (Supplementary Fig. 2a, b).

### 3.3. Edaravone treatment transiently reduced oxidative stress in HuC:mtFUS larvae

As increased reactive oxygen species (ROS) levels are a hallmark of ALS pathology, we assessed ROS using CellROX™ reagent, a fluorescent reagent that becomes oxidized and retained within cells upon ROS exposure [27]. Control and HuC:mtFUS larvae were treated with CellROX™ reagent, and CellROX™ signals were measured at 5 dpf, when motor dysfunction appeared (Fig. 4a). CellROX™ fluorescence increased along the dorsal region of HuC:mtFUS larvae, from the spinal cord to the

hindbrain, similar to the dorsal cell-death pattern observed in the TUNEL assay (Figs. 2b, 4b, c).

To determine whether edaravone, an ROS-reducing drug approved for ALS treatment, reduced ROS levels or improved motor dysfunction in HuC:mtFUS larvae, we administered the drug on day 2 and subsequently analyzed ROS levels and locomotor activity (Fig. 4d). After 1 day of treatment, edaravone-treated HuC:mtFUS larvae showed fewer CellROX™ signals in the dorsal spinal cord than untreated larvae. However, 3 d after edaravone treatment, the number of CellROX™ signal-positive cells increased to a level similar to that in untreated larvae (Fig. 4e, f). In addition, the distance moved did not differ significantly between treated and untreated larvae at 5 dpf (Fig. 4g). These results indicate that edaravone transiently reduced cellular oxidative stress but did not restore locomotor behavior.



**Fig. 4.** Edaravone treatment transiently reduced oxidative stress but did not restore locomotion in Tg(HuC:mtFUS) larvae. **a.** Schematic diagram showing the workflow of the CellROX™ assay for detecting reactive oxygen species (ROS) levels in zebrafish larvae. **b.** Representative whole-mount images of CellROX™ staining in sibling control and Tg(HuC:mtFUS) zebrafish at 5 dpf. CellROX™-positive cells are shown in red along the dorsal spinal cord of Tg(HuC:mtFUS) zebrafish. **c.** Quantification of CellROX™-positive cells in the spinal cords of sibling control and Tg(HuC:mtFUS) zebrafish at 5 dpf. **d.** Schematic diagram of the edaravone treatment strategy. Tg(HuC:mtFUS) zebrafish were treated with edaravone (10  $\mu$ M) from 2 dpf, followed by ROS analysis at 3 and 5 dpf and locomotion analysis at 5 dpf. **e.** Representative whole-mount images of CellROX™ staining in untreated and edaravone-treated Tg(HuC:mtFUS) zebrafish. Top row: untreated Tg(HuC:mtFUS) zebrafish showing ZsGreen and CellROX™ signals at 3 and 5 dpf. Bottom row: edaravone-treated Tg(HuC:mtFUS) zebrafish showing merged ZsGreen + and CellROX™ + signals at 3 and 5 dpf. **f.** Quantification of CellROX™-positive cells in sibling control, untreated Tg(HuC:mtFUS), and edaravone-treated Tg(HuC:mtFUS) zebrafish at 3 dpf (left) and 5 dpf (right). **g.** Locomotion analysis of sibling control, untreated Tg(HuC:mtFUS), and edaravone-treated Tg(HuC:mtFUS) zebrafish at 5 dpf. Data are reported as the mean  $\pm$  SD;  $n \geq 10$  independent experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Significance is indicated by \*\*\*\* $p < 0.0001$  and \*\*\* $p < 0.001$ ; ns: not significant. Scale bars: 50  $\mu$ m.

### 3.4. Transcriptomic analysis of the HuC:mtFUS larvae revealed ALS-associated molecular signatures

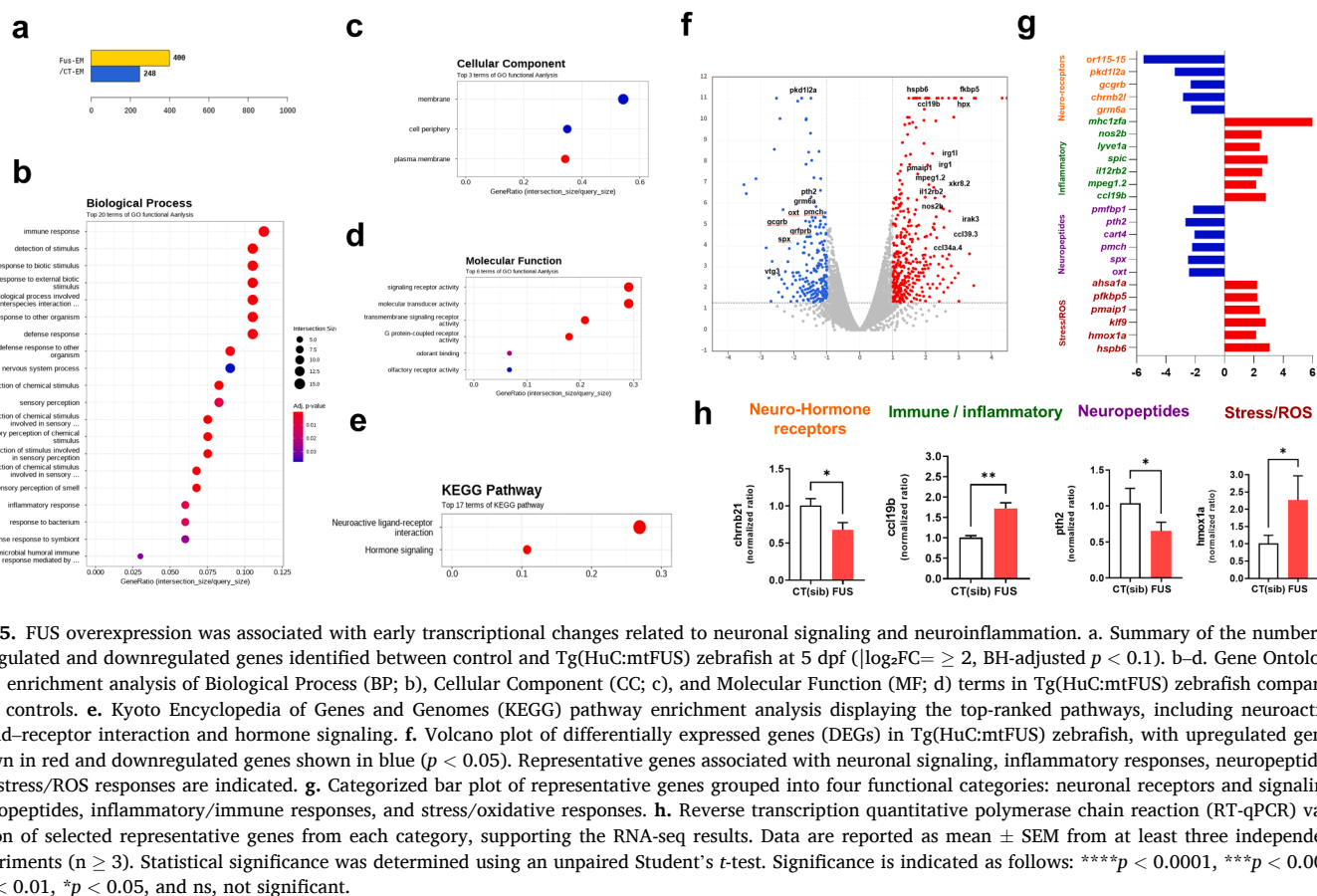
To characterize the molecular changes associated with the behavioral and cellular phenotypes observed in the HuC:mtFUS zebrafish model, we performed bulk RNA sequencing at 5 dpf. Differential expression analysis identified 400 upregulated and 248 downregulated genes in HuC:mtFUS larvae compared to controls ( $|\log_2FC| \geq 2$ , BH-adjusted  $p < 0.1$ ) (Fig. 5a). GO enrichment analysis showed that significantly enriched Biological Process terms were predominantly associated with immune activation and stimulus perception. The top-ranked processes included immune response, detection of stimulus, response to external biotic stimulus, defense response, sensory perception, and inflammatory response (Fig. 5b). These findings indicate that neuronal FUS overexpression is associated with widespread transcriptional changes linked to immune/inflammatory regulation and external stimulus processing.

In the Cellular Component and Molecular Function categories, enriched terms were primarily related to membrane-associated and receptor-mediated signaling, including plasma membrane, cell periphery, signaling receptor activity, transmembrane receptor activity, G protein-coupled receptor activity, and olfactory receptor activity (Fig. 5c–d). This suggests that membrane-associated receptor signaling and sensory transduction pathways are among the most prominently

affected processes, consistent with the impaired stimulus responsiveness observed in behavioral assays (Fig. 3c).

KEGG pathway analysis identified neuroactive ligand–receptor interaction and hormone signaling pathways among the most significantly enriched pathways (Fig. 5e), suggesting altered neuronal communication in the HuC:mtFUS model. Volcano plot analysis further showed downregulation of multiple neuronal receptor genes (or115–15, pkd112a, gcgrb, chrnb2l, and grm6a) and upregulation of genes associated with immune and stress responses (mhc1zfa, nos2b, lyve1a, il12rb2, ccl19b, hmox1a, and hspb6) (Fig. 5f). To characterize these transcriptional alterations, differentially expressed genes were categorized into four functional groups: neuronal receptor/signaling genes and neuropeptides, which were downregulated, and inflammatory/immune response genes and stress/oxidative response genes, which were upregulated (Fig. 5g). RT-qPCR validation of representative genes supported the RNA-seq results, showing downregulation of chrnb2l (neuronal receptor) and pmch (neuropeptide), and upregulation of ccl19b (immune response) and hmox1a (oxidative stress) (Fig. 5h).

To determine whether these transcriptomic changes originated specifically from neurons, we performed RNA sequencing on neurons isolated using FACS from HuC:mtFUS and HuC:ZsGreen larvae at 5 dpf (Supplementary Fig. 3a). Differential expression analysis identified 279 differentially expressed genes, including 154 upregulated and 125 downregulated genes (Supplementary Fig. 3b). KEGG pathway analysis



showed that neuroactive ligand–receptor interaction was enriched in bulk and neuron-specific datasets, suggesting shared alteration of neuronal communication pathways. Apoptosis and adherens junction pathways were significantly enriched, consistent with transcriptomic signatures reported in motor neurons from patients with ALS harboring FUS mutations [13] (Supplementary Fig. 3c). RT-qPCR validation further supported neuron-specific alterations, with *rhocb* (cell adhesion) and *prf1.2* (apoptosis) significantly upregulated and *nmba* (neurotransmitter receptor signaling) downregulated in *HuC:mtFUS* neurons compared with controls (Supplementary Fig. 3d). Collectively, these results indicate that neuronal overexpression of mutant FUS was associated with ALS-relevant transcriptomic features, including altered neuroactive ligand–receptor signaling and activation of immune, stress, and apoptotic pathways.

### 3.5. TBA improved ALS-associated phenotypes via multi-level transcriptomic profiles in the *HuC:mtFUS* model

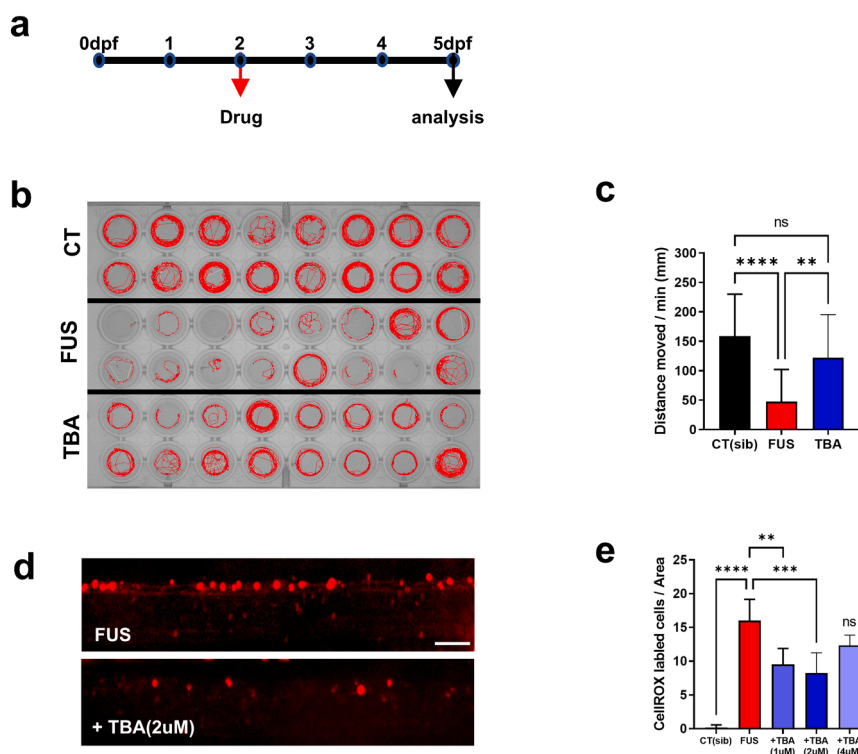
To identify compounds that improve mtFUS-induced behavioral deficits, we established phenotypic screening platform based on locomotor activity assay using the *HuC:mtFUS* zebrafish model (Fig. 6a). Screening of approximately 300 bioactive compounds (administered at 10  $\mu$ M from 6hpf to 4dpf) identified TBA as a hit compound that significantly improved motor impairment. TBA is a known natural constituent of the hops plant (*Humulus lupulus*) [28], and the antioxidant and estrogenic properties of hops-derived compounds [29] suggest that related pathways may be relevant to its biological effects. Following a dose-response assessment (1–10  $\mu$ M), treatment of *HuC:mtFUS* larvae with TBA from 2 dpf improved motor performance by 5 dpf, approaching control levels (Fig. 6b, c). Given the established link between ALS pathology and oxidative stress, we next examined whether TBA affected mtFUS-induced oxidative stress. TBA treatment

significantly reduced ROS levels at 5 dpf, coinciding with the observed improvement in motor function (Fig. 6d, e). These findings suggest that attenuation of oxidative stress may contribute, at least in part, to the functional improvement associated with TBA treatment.

To characterize molecular changes associated with TBA treatment, we performed RNA sequencing on *HuC:mtFUS* larvae with or without TBA treatment. Transcriptomic analysis showed broad transcriptional changes following TBA administration (Fig. 7a). GO enrichment analysis of Biological Processes showed strong enrichment of stimulus-response pathways, followed by metabolic processes, including carbohydrate, glucose, and hexose metabolism, as well as insulin-like growth factor receptor signaling (Fig. 7b). These results indicate that TBA treatment was associated with changes in stimulus-responsive and metabolic regulatory networks, consistent with the observed behavioral improvement.

To further characterize these changes, differentially expressed genes were grouped into functional categories (Fig. 7c). Cell cycle-related genes (*haus4*, *exo1*, *chk1*, *cdc6*) were downregulated, suggesting reduced expression of proliferative or stress-associated signaling genes. In contrast, genes associated with immune regulation (*socs3a*, *irf7*, *hspb6*, *alox5ap*), metabolic processes (*ucp3*, *igfbp1a/b*, *gcgrb*, *cyp24a1*, *arg2*), and neuronal activity (*npas4a/b*, *fosab*, *egr2*, *egr4*) were upregulated, indicating changes in immune, metabolic, and neuronal activity-related pathways.

We next assessed stimulus-evoked behaviors after TBA treatment. In the startle response assay, reaction time to tapping stimulation was comparable between *HuC:mtFUS* and wild-type larvae, suggesting preserved detection of mechanical stimuli (Fig. 3d). However, the reduced distance moved in response to mechanical stimulation suggests that the altered startle response in *HuC:mtFUS* larvae was primarily attributable to decreased motor output rather than defective stimulus detection. TBA-treated *HuC:mtFUS* larvae exhibited a significant increase in startle



**Fig. 6.** Phenotypic screening identified tribenzylamine (TBA) as a compound that improved locomotion and reduced ROS levels in Tg(HuC:mtFUS) larvae. **a.** Schematic of the TBA treatment strategy. Zebrafish larvae were treated with TBA from 2 dpf, followed by locomotor and oxidative stress analyses at 5 dpf. **b.** Locomotion tracking images of sibling control, Tg(HuC:mtFUS), and TBA-treated Tg(HuC:mtFUS) zebrafish at 5 dpf. **c.** Quantification of distance moved per minute among sibling control, Tg(HuC:mtFUS), and TBA-treated Tg(HuC:mtFUS) zebrafish at 5 dpf. **d.** Whole-mount images of ROS detection in the spinal cord of Tg(HuC:mtFUS) zebrafish at 5 dpf. ROS-positive cells in untreated and TBA-treated Tg(HuC:mtFUS) zebrafish were labeled using the CellROX™ assay. Scale bar: 50  $\mu$ m. **e.** Quantification of ROS-positive cells in untreated Tg(HuC:mtFUS) zebrafish and Tg(HuC:mtFUS) zebrafish treated with different concentrations of TBA. The data are reported as the mean  $\pm$  SD;  $n \geq 10$  independent experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Significance is indicated by \*\*\*\* $p < 0.0001$ , \*\*\* $p < 0.001$ , \*\* $p < 0.01$ ; ns: not significant.

response distance following mechanical stimulation (Fig. 7d). These findings indicate that TBA improved motor performance and stimulus-evoked movement in the HuC:mtFUS model.

To explore pathways related to the hypothesized estrogenic effects of TBA, we performed KEGG pathway analysis focused on hormone-related signaling. KEGG pathway analysis showed significant enrichment of neuroactive ligand–receptor interaction, hormone signaling, calcium signaling, and steroid biosynthesis pathways following TBA treatment (Fig. 7e), suggesting a potential association between neuroendocrine regulation and the observed phenotypic improvement. For estrogen-related signaling, several genes negatively regulated by estrogen (e.g., *amh*, *klf9*, *hpx*, *fkbp5*) were upregulated in HuC:mtFUS larvae compared with controls, whereas canonical estrogen-responsive genes (*egr2a*, *cyp24a1*, *vtg3*) were downregulated. TBA treatment shifted the expression of these estrogen-related genes toward control patterns, with *vtg3* showing the most robust induction (Fig. 7f), suggesting increased estrogen-related signaling.

### 3.6. TBA increased neuronally active sex steroid levels in the HuC:mtFUS model

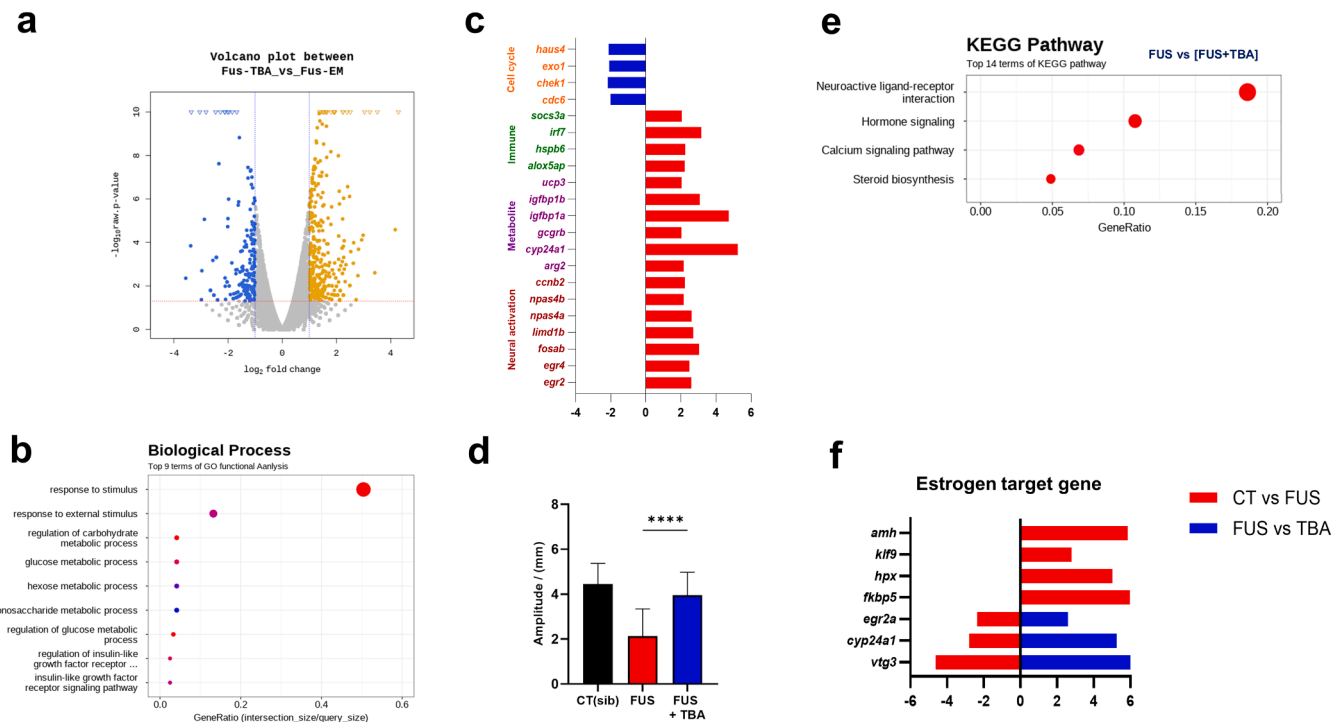
To determine whether the observed transcriptional changes were associated with alterations in steroid metabolism, we next examined neuronally active sex steroid levels. Based on the pathway-level findings, we hypothesized that TBA may modulate neuronally active sex steroid levels in the HuC:mtFUS model. To test this, we quantified key neuronally active sex steroids, including estrogen and progesterone, using LC–MS in control, HuC:mtFUS, and TBA-treated larvae (Fig. 8a). Compared with control larvae, HuC:mtFUS larvae showed significantly

reduced estrogen and progesterone levels. These findings indicate that FUS overexpression was associated with altered neuronally active sex steroid levels, suggesting a potential link between ALS-associated pathology and steroid metabolism.

We next assessed whether TBA treatment increased these reduced neuronally active sex steroid levels. Administration of TBA to HuC:mtFUS larvae significantly increased neuronally active sex steroid levels, with key estrogenic and progestogenic neuronally active sex steroids reaching levels comparable to those observed in controls (Fig. 8b–e). These results indicate that TBA increased neuronally active sex steroid levels reduced by FUS overexpression and suggest that neuronally active sex steroid-related pathways may be associated with its phenotypic effects.

To determine whether activation of estrogen signaling alone was sufficient to reproduce the observed functional improvement, we treated HuC:mtFUS larvae with hexestrol, a selective estrogen receptor agonist. Hexestrol treatment did not significantly improve overall locomotor activity but partially increased the startle response distance following mechanical stimulation (Fig. 8f). This selective improvement suggests that activation of the estrogen axis may enhance stimulus-evoked movement without fully improving motor capacity.

These findings suggest that estrogen signaling may contribute to stimulus-evoked behavioral improvement in the HuC:mtFUS model. To further determine whether estrogen receptor signaling is required for the therapeutic effect of TBA, we performed pharmacological inhibition using fulvestrant, a highly selective estrogen receptor antagonist. Co-treatment with fulvestrant significantly attenuated the TBA-mediated functional recovery in HuC larvae (Fig. 8g), demonstrating that the beneficial effects of TBA are dependent, at least in part, on active



**Fig. 7.** Tribenzylamine altered transcriptomic profiles and neuronally active sex steroid-related pathways in Tg(HuC:mtFUS) zebrafish. **a.** Volcano plot illustrating differentially expressed genes (DEGs). The plot displays DEGs between untreated Tg(HuC:mtFUS) and TBA-treated Tg(HuC:mtFUS) zebrafish. **b.** Gene Ontology (GO) enrichment analysis of DEGs. The graph displays significantly enriched Biological Process (BP) terms, including strong enrichment of the response-to-stimulus category following TBA treatment. **c.** Categorized bar plot of representative DEGs. The graph shows representative gene-expression changes grouped into four functional categories: cell cycle, immune regulation, metabolic processes, and neuronal activity. **d.** Quantification of startle response distance. The graph shows increased startle response distance after mechanical tapping stimulation in TBA-treated Tg(HuC:mtFUS) zebrafish compared with untreated Tg(HuC:mtFUS) zebrafish. Data are reported as the mean  $\pm$  standard error of the mean (SEM);  $n \geq 20$  per group. Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Significance is indicated by \*\*\*\* $p < 0.0001$ . **e.** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs. The analysis compares untreated Tg(HuC:mtFUS) and TBA-treated Tg(HuC:mtFUS) zebrafish and shows significant enrichment of neuroactive ligand-receptor interaction, steroid hormone biosynthesis, and hormone signaling pathways. **f.** Expression changes in selected estrogen-responsive genes. The horizontal bar graph displays relative expression changes of selected genes (*amh*, *klf9*, *hpx*, *fkb5*, *egr2a*, *cyp24a1*, and *vtg3*). Red bars represent FUS-associated dysregulation (CT vs FUS), whereas blue bars represent TBA-associated expression changes (FUS vs TBA).

estrogen receptor signaling. Collectively, these results suggest that TBA alleviated FUS-induced neuronal dysfunction in association with reduced oxidative stress and increased estrogen-related neuronally active sex steroid signaling. The decreased estrogen and progesterone levels in the HuC:mtFUS model suggest a possible link between FUS pathology and neuronally active sex steroid dysregulation. By increasing these neuronally active sex steroid levels, TBA may exert effects beyond its antioxidant activity. These findings identify TBA as a candidate compound for further ALS-related investigation, although additional studies are required to define its direct targets, mechanisms, and therapeutic potential.

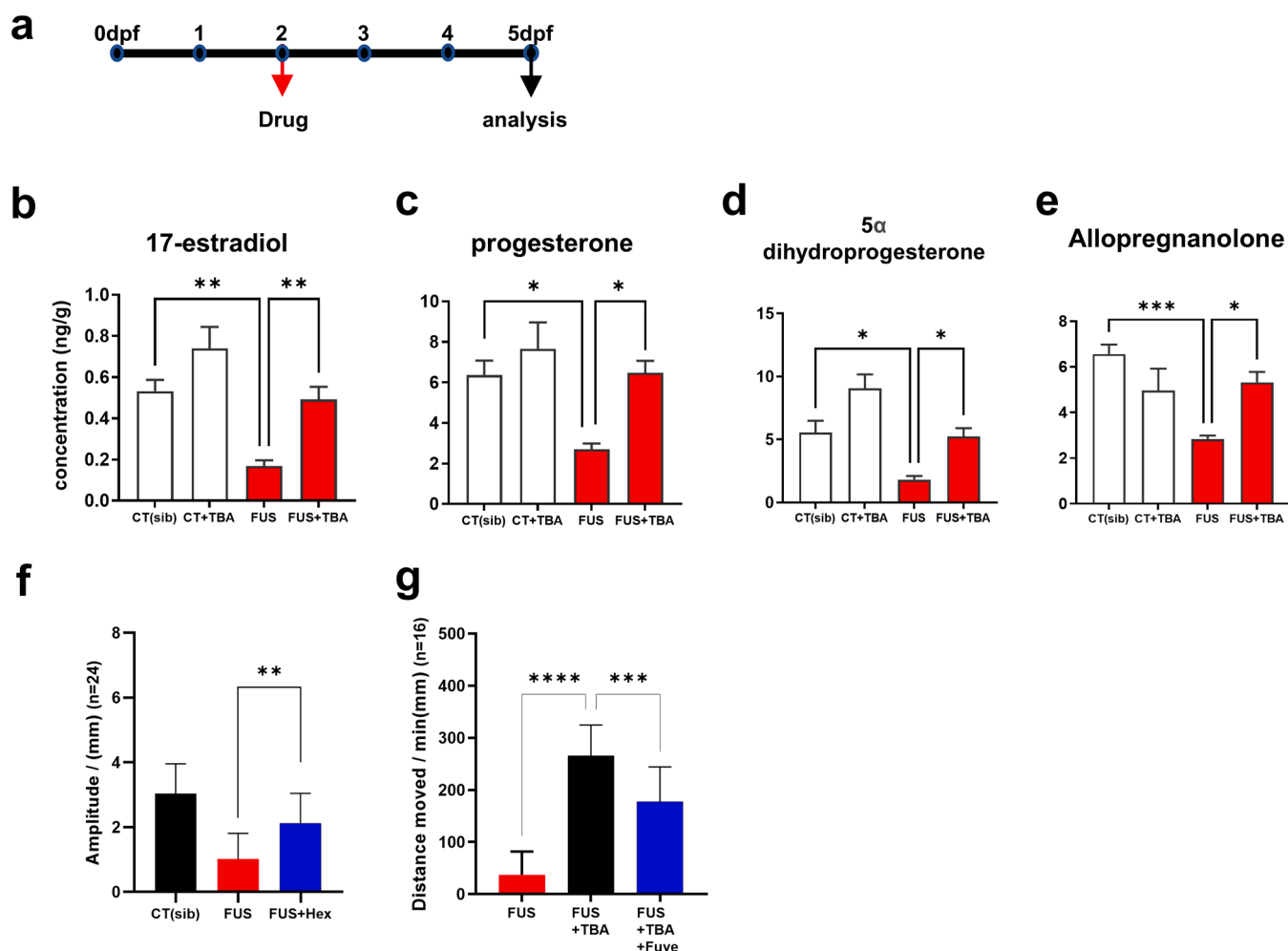
#### 4. Discussion

Despite extensive research, only a limited number of drugs, including riluzole, edaravone, and tofersen, are currently approved for the treatment of ALS. However, currently available therapies provide limited survival or functional benefit, highlighting the need for robust disease models that support high-throughput phenotypic screening of candidate therapeutics. Among familial ALS genes, FUS mutations are associated with early-onset and rapidly progressive ALS [30,31] making them important targets for disease modeling. Although various FUS-based models have been developed, FUS knockout models in mice and zebrafish exhibit only mild phenotypes, possibly because of compensatory mechanisms [15,32], whereas FUS knock-in mouse models and transgenic overexpression rat models more closely recapitulate ALS pathology but require several months to develop disease phenotypes and

incur high maintenance costs, limiting their utility for large-scale drug screening [16,33,34].

To address these limitations, we developed the HuC:mtFUS zebrafish model using a modified QF-based binary transactivation system that enables strong and neuron-specific overexpression of human mutant FUS(R521C) (mtFUS), with approximately fivefold higher efficiency than the conventional Gal4/UAS system [20]. This approach enabled rapid manifestation of ALS-like phenotypes at early larval stages. This model reproduced several ALS-relevant pathological and functional features, including early onset, clear locomotor deficits, pharmacological responsiveness, and scalability, supporting its use as a platform for early-phase phenotypic drug discovery. We acknowledge that the absence of a wild-type FUS (wtFUS) overexpression control is a limitation of the current study. Because FUS overexpression alone can induce cellular toxicity, the lack of a matched wtFUS comparator limits the ability to fully uncouple mutation-specific effects from general overexpression-related artifacts. However, the primary objective of this study was to establish a highly penetrant, early-onset phenotype for *in vivo* drug screening. Despite this constraint, the robust and reproducible phenotypes observed in the mtFUS model successfully enabled the identification of TBA as a disease-modifying candidate, highlighting the model's practical utility for early-phase phenotypic drug discovery.

Motor neuron degeneration is a defining feature of ALS and leads to progressive paralysis and eventual respiratory failure. In the HuC:mtFUS model, motor neuronal degeneration was first detected at 10 dpf. Early apoptotic cell death was initially observed in putative dorsal spinal interneurons at 5 dpf, before motor neuron loss. A similar phenomenon



**Fig. 8.** TBA treatment increased neuronally active sex steroid levels in Tg(HuC:mtFUS) zebrafish. **a.** Schematic diagram of TBA treatment and endogenous neuronally active sex steroid analysis. **b–e.** Quantification of endogenous neuronally active sex steroid concentrations (ng/g) of 17 $\beta$ -estradiol (**b**), progesterone (**c**), 5 $\alpha$ -dihydroprogesterone (**d**), and allopregnanolone (**e**) in zebrafish larvae at 5 dpf. Data are reported as the mean  $\pm$  SD;  $n = 50$  independent experiments. **f.** Startle response assay measuring movement distance after tapping stimulation in sibling control, Tg(HuC:mtFUS), and hexestrol-treated Tg(HuC:mtFUS) zebrafish (FUS+Hex) at 5 dpf. **g.** Quantification of the distance moved per minute in Tg(HuC:mtFUS) (FUS), TBA-treated Tg(HuC:mtFUS) (FUS+TBA), and TBA/fulvestrant co-treated Tg(HuC:mtFUS) (FUS+TBA+Fuve) zebrafish at 5 dpf. Data are reported as the mean  $\pm$  SD;  $n \geq 24$  independent experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Significance is indicated by \*\*\*\* $p < 0.0001$ , \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ .

has been reported in an SOD1 mouse model of ALS, in which inhibitory synapses between interneurons and fast motor neurons are lost early, with interneuron degeneration preceding motor neuron damage [35, 36]. Similar synaptic dysfunction in interneurons has been reported in TDP-43 and C9ORF72 mouse models of ALS [37], and early interneuron damage has been observed in patients with ALS [38]. The early increase in ROS and apoptotic cell death observed in the dorsal spinal cord of HuC:mtFUS larvae suggests that cells in this region may be vulnerable to metabolic stress. Together, these reports and our data suggest that early disruption of spinal interneuron-related circuits may contribute to ALS-associated neurodegeneration.

At 5 dpf, when dorsal spinal cell death was observed, the HuC:mtFUS model showed a marked reduction in locomotor activity, resembling early motor deficits associated with FUS-linked ALS. However, examination of NMJ morphology in the HuC:mtFUS model revealed no significant differences in NMJ density or synaptic integrity at 5 dpf, suggesting that early locomotor deficits in HuC:mtFUS larvae may primarily reflect central nervous system dysfunction rather than peripheral synaptic abnormalities. Elevated ROS levels contribute to protein misfolding, mitochondrial dysfunction, and neuronal apoptosis in ALS [39]. In the HuC:mtFUS model, we observed a significant increase in ROS levels, particularly in the dorsal spinal cord, where early cell death was

initiated. This increase was transiently reduced by edaravone, an ALS-approved drug with antioxidant properties [40]. Although edaravone reduced ROS levels, it did not restore locomotor activity, suggesting that oxidative stress reduction alone was insufficient to reverse the behavioral phenotype in this model.

Beyond phenotypic similarities, transcriptomic analysis of neurons in the HuC:mtFUS zebrafish model revealed gene-expression changes that overlapped with those reported in patient-derived motor neurons. Specifically, we identified upregulation of apoptosis-related genes, including prf1.2, which is involved in programmed cell death, consistent with findings in patient-derived iPSC motor neurons [14]. Similarly, genes associated with cell adhesion and neuroactive ligand–receptor interaction pathways were significantly altered, paralleling transcriptional changes observed in post-mortem ALS motor neurons [13]. These molecular similarities further support the relevance of the HuC:mtFUS model for studying selected ALS-associated disease mechanisms.

Using high-throughput phenotypic screening in the HuC:mtFUS model, we identified TBA as a candidate modulator of ALS-associated phenotypes. TBA was originally reported as a natural constituent of the hops plant *H. lupulus* [28], and the reported antioxidant and estrogenic properties of hops-derived compounds [29] suggest that hormone-related regulatory networks may be biologically relevant.

However, the pharmacology of TBA itself remains largely unexplored. TBA treatment improved locomotor deficits and reduced oxidative stress, indicating functional and cellular improvement in the HuC:mtFUS larvae. This broader phenotypic improvement contrasted with edaravone treatment in this model, which transiently attenuated oxidative stress without improving motor performance. These findings suggest that phenotypic improvement in this model may require modulation of multiple pathological processes rather than targeting oxidative stress alone. In this context, our findings are best interpreted not as confirmation of a defined TBA mechanism but as evidence that a hops-derived small molecule can modulate ALS-relevant phenotypes and pathways in a FUS-associated ALS model. This interpretation is supported by the known bioactivity of other hops constituents, which regulate estrogenic signaling, antioxidant responses, and neuroactive pathways [41–44].

A key pathway-level finding from this study is the association of neuroendocrine signaling, particularly neuronally active sex steroid regulation, with TBA-induced phenotypic improvement. Transcriptomic analysis revealed enrichment of hormone signaling and steroid biosynthesis pathways following TBA treatment, accompanied by shifts in estrogen-responsive gene expression toward control patterns. Consistently, biochemical analysis showed that FUS overexpression was associated with reduced endogenous neuronally active sex steroid levels, whereas TBA treatment increased them toward control levels. These findings suggest that neuronally active sex steroid dysregulation may be an underexplored component of FUS-associated ALS pathology and that neuronally active sex steroid-related pathways may contribute to functional improvement in this model. This interpretation is supported by convergent evidence from ALS models and human studies linking neuronally active sex steroids to disease modulation.

In SOD1(G93A) mice, 17 $\beta$ -estradiol enhances motor neuron survival and suppresses neuroinflammation [45], whereas ovariectomy shortens lifespan and estradiol treatment attenuates motor decline, supporting a role for gonadal hormones in disease progression [46]. Similarly, progesterone delays neurodegeneration, partly through activation of autophagy [47]. Consistent with these observations, human biomarker studies have reported reduced neuronally active sex steroid levels, including progesterone and allopregnanolone, in patients with ALS, inverse correlations with neuroaxonal injury markers, and overall decreases in estrogen and progesterone levels [48]. Moreover, the lower incidence and delayed onset of ALS in women may further support a protective role of endogenous estrogen [49–51]. Collectively, our findings support the use of this mtFUS zebrafish model as a scalable in vivo platform for ALS drug discovery and provide initial insight into molecular pathways associated with TBA-mediated phenotypic rescue.

Estrogen signaling alone was insufficient to fully reproduce TBA effects, as hexestrol improved stimulus responsiveness without restoring overall locomotion, suggesting that TBA may act through multiple pathways. Although our data suggest coordinated changes in oxidative stress, metabolism, and neuronally active sex steroid homeostasis, the direct molecular targets of TBA remain undefined, and causal links to functional recovery remain unclear. Future studies should identify the primary targets of TBA, define its interactions with endocrine and stress-response pathways, assess target engagement and pharmacokinetic properties, and validate efficacy across additional ALS models before its therapeutic potential can be determined. We also emphasize that our findings are currently confined to a FUS-based ALS model. Because FUS-ALS is mechanistically distinct from the TDP-43 pathology that characterizes most sporadic ALS and several hereditary ALS subtypes, the therapeutic relevance of TBA beyond FUS-associated disease remains to be established. Future studies should therefore evaluate its efficacy in complementary ALS models, including those driven by TDP-43 and C9orf72 pathology.

## 5. Conclusions

In this study, we established a HuC:mtFUS zebrafish model that recapitulates several pathological, functional, and transcriptomic features relevant to FUS-associated ALS while offering rapid disease onset, neuronal specificity, and scalability. This model enables high-throughput phenotypic screening and may provide a useful platform for early-phase drug discovery. Using this system, we identified TBA as a candidate modulator that improved motor function and reduced oxidative stress in HuC:mtFUS larvae. TBA treatment was associated with coordinated transcriptomic changes and increased neuronally active sex steroid levels, suggesting that neuroendocrine dysregulation may contribute to FUS-associated ALS phenotypes in this model. Together, these findings support phenotype-based screening as a useful discovery strategy and identify TBA as a candidate compound for further investigation, particularly through studies defining its direct targets, the causal role of neuronally active sex steroid-related pathways, the durability of phenotypic improvement, and efficacy in independent ALS models.

## Ethics

All animal experiments were approved by the Korea University Institutional Animal Care and Use Committee (KOREA-2023-0176) and performed in accordance with the animal experiment guidelines of the Korea National Veterinary Research and Quarantine Service.

## CRediT authorship contribution statement

**Suhyun Kim:** Formal analysis, Data curation. **Jeong-Soo Lee:** Methodology, Investigation. **Jeonghyeon Lee:** Writing – original draft, Formal analysis, Data curation. **Yunkyoung Lee:** Formal analysis, Data curation. **Yongbo Seo:** Writing – original draft, Formal analysis, Data curation. **Myung Ji Kim:** Writing – review & editing, Project administration, Funding acquisition. **Hae-Chul Park:** Writing – review & editing, Project administration, Funding acquisition. **Jung Sang Park:** Methodology, Investigation. **Hyunju Ro:** Methodology, Investigation. **Sung-Hee Cho:** Formal analysis, Data curation.

## Declaration of Competing Interest

The authors declare that they have no competing interests. This manuscript has not been published previously and is not under consideration for publication elsewhere. All authors have approved the manuscript and agree with its submission to Biomedicine & Pharmacotherapy.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2026.119761](https://doi.org/10.1016/j.biopha.2026.119761).

## Data availability

Data will be made available on request.

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