The zebrafish histamine H3 receptor modulates aggression, neural activity and forebrain functional connectivity

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Abstract

Aim: Aggression is a behavioural trait characterized by the intention to harm others for offensive or defensive purposes. Neurotransmitters such as serotonin and dopamine are important mediators of aggression. However, the physiological role of the histaminergic system during this behaviour is currently unclear. Here, we aimed to better understand histaminergic signalling during aggression by characterizing the involvement of the histamine H3 receptor (Hrh3).

Methods: We have generated a novel zebrafish Hrh3 null mutant line using CRISPR-Cas9 genome engineering and investigated behavioural changes and alterations to neural activity using whole brain Ca2+ imaging in zebrafish larvae and ribosomal protein S6 (rpS6) immunohistochemistry in adults.

Results: We show that genetic inactivation of the histamine H3 receptor (Hrh3) reduces aggression in zebrafish, an effect that can be reproduced by pharmacological inhibition. In addition, hrh3−/− zebrafish show behavioural impairments consistent with heightened anxiety. Larval in vivo whole brain Ca2+ imaging reveals higher neuronal activity in the forebrain of mutants, but lower activity in specific hindbrain areas and changes in measures of functional connectivity between subregions. Adult hrh3−/− zebrafish display brain region-specific neural activity changes in response to aggression of both key regions of the social decision-making network, and the areas containing histaminergic neurons in the zebrafish brain.

Conclusion: These results highlight the importance of zebrafish Hrh3 signalling for aggression and anxiety and uncover the brain areas involved. Targeting this receptor might be a potential novel therapeutic route for human conditions characterized by heightened aggression.

KEYWORDS
aggression, anxiety, histamine H3 receptor, neural activity, whole brain calcium imaging, zebrafish

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1 | INTRODUCTION

Aggression is a behavioural trait characterized by the intention to harm others for offensive or defensive purposes. Aggression is highly conserved throughout evolution and is expressed by many species, including humans. Animals typically use aggression to acquire resources, establish dominance hierarchies, defend territories and protect their offspring, but in humans, exaggerated aggression is a frequent comorbidity of psychiatric disorders such as attention-deficit/hyperactive disorder (ADHD), conduct disorder (CD), dementia and schizophrenia. Aggressive patients not only harm themselves or others but are also frequently institutionalized creating a financial burden on society. In addition, violent crime in the mentally ill stigmatizes psychiatric diseases in general, which leads to the societal exclusion of individuals with noticeable symptoms. Violent patients also represent a therapeutic challenge, with few drugs available to treat this behaviour specifically. A better understanding of the neurobiological basis of aggression, leading to the development of novel treatment strategies for this behaviour is urgently needed.

Histamine is a biogenic amine that has an important role in numerous physiological and pathophysiological processes including allergy, inflammation, vasodilation, gastric acid secretion and neurotransmission. It is synthesized from the amino acid L-histidine and acts on four G protein-coupled receptors termed Histamine receptor H1-H4. In the mammalian brain histamine-synthesizing neurons are only located in one brain region, the tuberomamillary nucleus (TMN), which sends projections throughout the entire CNS. All histamine receptors, except the H4 receptor, are strongly expressed in the brain, enabling histamine to act as a neurotransmitter modulating important functions such as the sleep-wake cycle, cognition and energy metabolism. Dysregulated central histaminergic signalling has been associated with a number of psychiatric disorders including sleep disorders, ADHD, Alzheimer’s disease, Parkinson’s disease and schizophrenia.

The histamine H3 receptor (Hrh3) has recently gained attention in studies of behaviour and neuropsychiatric disease. Hrh3 is expressed predominantly in important brain areas such as the cortex, thalamus, hypothalamus, hippocampus, amygdala and basal ganglia. The receptor displays high constitutive activity, known to act as both an auto- and heteroreceptor to regulate neurotransmitter release, and is also expressed postsynaptically, for example to modulate dopamine signalling. Murine behavioural studies have revealed that genetic and pharmacological manipulation of Hrh3 alters sleep-wake cycles, anxiety, learning and memory and social behaviour, but, to date, a role for Hrh3 in aggression has not been reported.

The zebrafish (Danio rerio) has emerged as an important model for behavioural and neuroscience research and the study of complex neuropsychiatric disorders because of its conserved neurobiological features, rapid development and genetic manipulability. Robust tests have been established to characterize both larval and adult behaviour, and zebrafish models have been developed to study the neurobiological basis of ADHD, depression, autism spectrum disorder, schizophrenia and drug-induced disorders. The zebrafish is also a suitable species to study aggression, which can easily be measured by mirror-induced stimulation, a reproducible and non-invasive method that has provided essential insights in the neurobiological and genetic basis of this behaviour.

In the current study we used zebrafish to study the role of histamine receptor H3 (hrh3), the zebrafish homologue of the mammalian histamine H3 receptor, during aggression. Unlike other vertebrates including rodents, zebrafish do not express histamine in any cells outside the CNS, making this an ideal model to study central histaminergic neurotransmitter signalling without interference from non-neuronal histamine sources such as mast cells. Based on previous work in zebrafish implicating the other parts of the histaminergic system in aggression we hypothesized that genetic and/or pharmacological manipulation of hrh3 would alter zebrafish aggression and the neural circuits that underpin this behaviour.

2 | RESULTS

2.1 | histamine receptor H3 is expressed in discrete areas of the adult zebrafish telencephalon and hypothalamus

Mouse and human expression analyses have shown that the mammalian histamine H3 receptor is expressed in the cerebral cortex, thalamus, hypothalamus, basal ganglia, hippocampus and amygdala. In zebrafish larvae and juveniles, strong hhr3 expression has been observed in the telencephalon, with moderate expression levels in the hypothalamus, thalamus and optic tectum. To assess the potential role of the hhr3 receptor in aggression, we first mapped hhr3 distribution in the adult zebrafish brain (Figure 1). Similar to larvae and juveniles, hhr3 expression is limited to discrete brain areas and cell populations with the strongest expression in the telencephalon. Dorsal telencephalic subregions showed very strong signals, especially in the medial and lateral zone of the dorsal telencephalic area (Dm and Dl; Figure 1A-C), regions that are considered homologous to the mammalian amygdala and hippocampus respectively. We also noted strong expression in the posterior zone of the dorsal telencephalic area (Dp; Figure 1A-C) and moderate expression in the ventral, dorsal, supra- and post-commissural nuclei of the ventral telencephalic area (Vv, Vd, Vs and Vp; Figure 1A-C). Vd and Vv are particularly interesting as they have been proposed to be homologues of the mammalian striatum and septal formation respectively. We also detected strong hhr3 signals in hypothalamic brain areas including the anterior parvocellular preoptic nucleus (PPa; Figure 1B,C),...
ventral, caudal and dorsal zone of the periventricular hypothalamus (Hv, Hc and Hd), the periventricular nuclei of the inferior lobe of the hypothalamus (PVN) and the diffuse nucleus of the inferior lobe (DIL) (Figure 1D-F). Notably, we also found hrh3 expression in neurons around the posterior recess of the diencephalic ventricle (PR; Figure 1F), which is the area that is known to contain histaminergic neurons in the zebrafish brain.23 Outside the hypothalamus and telencephalon, we detected strong hrh3 expression in the periventricular grey zone of the optic tectum (PGZ; Figure 1D), moderate expression in the torus lateralis (TLa; Figure 1E), the posterior tuberal nucleus (PTN; Figure 1E) and the periventricular nucleus of posterior tuberculum (TPp; Figure 1D) and weak hrh3 expression in various thalamic nuclei (Figure 1D) and the griseum centrale (GC; Figure 1G).

2.2 | histamine receptor H3 deletion reduces aggression

Based on the expression pattern of hrh3 in key areas of the social decision-making network (SDMN) that controls social behaviours across species,27 we hypothesized that hrh3 could play an important role in aggression. We therefore generated a hrh3−/− knockout zebrafish line (hrh3 uol1) using CRISPR-Cas9 genome editing. These mutant fish are characterized by a 4 bp insertion within exon 1 of the gene resulting in an early stop codon (at amino acid 129 out of 473 amino acids for the full length protein) and a strongly truncated protein (Figure 2A,B; Figure S1). To evaluate whether these mutant fish show changes in aggression, we tested them in a mirror-induced aggression (MIA) test.28 Over a 30 min observation period, homozygous hrh3−/− knockout fish spent less time fighting the mirror compared to corresponding wild-type (WT) animals (F(1,19) = 5.509; P = .030; Figure 2C) suggesting reduced aggression. This behaviour was not the result of impaired locomotor activity, given that the total distance moved during the test was unaltered (Figure 2D). We also detected no difference in the time spent immobile (Figure 2E) or angular velocity (Figure 2F), indicating that the observed reductions in aggression are not caused by altered anxiety levels. We saw the same effect using pitolisant, an antagonist/inverse agonist of H3 histamine receptors. Specifically, we found a dose-dependent decrease in aggression that
achieved statistical significance at 3 µM (Tukey’s post-hoc test: \( P = .047 \); Figure 2G), again without affecting distance moved (Figure 2H), time spent immobile (Figure 2I) or angular velocity (Figure 2J). This shows that both genetic and pharmacological inhibition of \( hrh3 \) function decreases zebrafish aggression without impacting on locomotion or anxiety.

2.3 Lack of histamine receptor H3 alters aggression-induced neuronal activation and serotonergic signalling

The areas of the zebrafish brain that control aggression have not been fully characterized, but are likely to include some parts of the SDMN. To investigate the brain areas activated by MIA, we used the neuronal activation marker rpS6 to identify differences in activation between WT and \( hrh3^{-/-} \) fish (Figure 3B,D,F, Figure S2, Table S1). However, interaction with the mirror strongly increased the number of rpS6 + cells (Figure 3A,G, Figure S2) indicating that selected telencephalic and hypothalamic brain areas are activated during aggressive behaviour. In most investigated areas (Dl, Vd, Hc, Hv, PPa and LH) this activation was independent of the fish’s genotype (Figure 3B,D,F, Figure S2, Table S1). However, in the Dm, the number of aggression-induced rpS6 + cells was higher in \( hrh3^{-/-} \) mutant fish compared to WT (Tukey post-hoc test: \( P < .001 \); Figure 3A,C), while it was lower in \( hrh3^{-/-} \) mutant fish in the Vv (Tukey post-hoc test: \( P = .010 \); Figure 3A,E). In addition, we detected a higher number of aggression-induced rpS6 + cells in the region around the PR in mutants (\( P < .001 \); Figure 3A,G). Changes in neural activation in these three brain areas suggest that they may mediate the observed reduction of aggression after \( hrh3 \) deletion.

Next, we investigated whether \( hrh3^{-/-} \) mutant fish display correlated changes in neurotransmitter levels
in the forebrain and hypothalamus, given that Hrh3 is known to interact with serotonergic and dopaminergic signalling in mammals. Although serotonin (5-HT), dopamine (DA), homovanillic acid (HVA) and 3,4-Dihydroxyphenylacetic acid (DOPAC) levels were not significantly altered between genotypes, we found that 5-Hydroxyindoleacetic acid (5-HIAA) levels were lower in both telencephalon ($t_{(9)} = 3.939; P = .015$) and diencephalon ($t_{(12)} = 3.423; P = .025$; Figure 3H,I) indicating reduced serotonergic signalling. Gene expression analysis revealed that the expression of serotonin synthesizing enzymes, tryptophan hydroxylase 1a ($\text{tph1a}$) and tryptophan hydroxylase 2 ($\text{tph2}$), the degrading enzyme monoamine oxidase ($\text{mao}$) and serotonin transporters solute carrier family 6 member 4a ($\text{slc6a4a}$) and solute carrier family 6 member 4b ($\text{slc6a4b}$) did not differ between genotypes (Figure 3). This suggests that the reduced 5-HIAA levels might be the result of decreased synaptic release of serotonin.

FIGURE 3 Effects of histamine receptor H3 (hrh3) deletion on neuronal activation and neurotransmitter levels. A, Representative images depicting differences in rpS6 immunoreactivity between aggression-exposed wild-type (WT/MIA) and mutant fish (KO/MIA) in the telencephalon and hypothalamus. The selected counting areas are indicated in blue. Scale bar: 150 µm. B-G, Number of ribosomal protein S6 (rpS6)+ cells in (B) the lateral and (C) medial zone of the dorsal telencephalic area (Dl and Dm), (D) the dorsal and (E) ventral nuclei of the ventral telencephalic area (Vd and Vv), (F) the caudal zone of the periventricular hypothalamus (Hc) and (G) the area around the posterior recess of the diencephalic ventricle (PR) in hrh3−/− (KO) and corresponding wild-type (WT) fish under basal conditions (CO) and after 1 h aggression exposure (MIA). n = 3-4. Two-way ANOVA followed by Tukey’s post-hoc test in case of a significant interaction term. Otherwise significant main effects are indicated in the graph. $^*P < .001$, $^\dagger P < .01$, $^\ddagger P < .05$ (H and I) HPLC measurements of neurotransmitter and neurotransmitter metabolite levels in (H) the telencephalon and (I) diencephalon. n = 5-7. Bonferroni-corrected t tests.$^* P < .05$ vs 5-HIAA WT levels. Data are presented as mean ± SEM

2.4 Adult hrh3−/− fish display heightened anxiety-like behaviour, but do not show alterations in social behaviour

We next asked whether hrh3−/− zebrafish show a selective reduction in aggression or whether other behaviours are affected as well. In the open field test, hrh3−/− mutant fish displayed a hyperlocomotion phenotype moving further ($t_{(22)} = 3.480; P = .002$; Figure 4A) and faster ($t_{(22)} = 3.468;
In the novel tank diving test, hrh3\(^{-/-}\) fish spent less time in the top third of the test tank (\(U = 24.5; P = .005\); Figure 4C) and entered this area both later (\(U = 118.5; P = .006\); Figure 4D) and less frequently (\(U = 30.0; P = .014\); Figure 4E), suggesting heightened anxiety. In line with the findings from the open field test, mutants also moved faster and swam further during this test (Figure S4) suggesting that this behaviour is an expression of anxiety. In contrast, social behaviour was not affected by gene knockout, since none of the parameters investigated in the shoaling assay and visually mediated social preference test (nearest neighbour distance, inter-individual distance, time spent in the social interaction zone and time spent in the control zone) differed between WT and hrh3\(^{-/-}\) (Figure 4F-I). In summary these data indicate that hrh3\(^{-/-}\) fish show heightened anxiety levels in these behavioural tests, but no changes in social behaviour.

### 2.5 The anxiety phenotype of hrh3\(^{-/-}\) mutants has a developmental basis

The altered behavioural phenotype of adult hrh3\(^{-/-}\) fish and brain region-specific differences in hrh3 expression between larvae and adults\(^{25}\) raises the question whether larval hrh3 mutant fish show similar behavioural alterations. Larval zebrafish do not display aggressive-like behaviour before 30 dpf,\(^{31}\) however, assays to measure locomotion and anxiety-like behaviour in early life stages have been established.\(^{32}\) When tested in the larval unstimulated locomotion assay, we found that hrh3\(^{-/-}\) larvae move less than age-matched WTs (\(U = 45.0; P = .004\); Figure 5A). Detailed analysis revealed that this is the result of longer immobility periods in mutant larvae (\(U = 37.0; P < .001\); Figure 5B) supporting enhanced anxiety, similar to adult mutant fish. When analysing the swimming behaviour of moving individuals during the test (WT: n = 13, KO: n = 5) we found that hrh3\(^{-/-}\) larvae did not
suffer from a general locomotor deficit, given that hrh3−/− larvae did not exhibit changes to the structure of beat-glide swimming episodes (Figure 5C). Furthermore, both the bout duration and maximal velocity of larval beat-glide swimming episodes were similar between genotypes (Table S2). In the light/dark transition assay, mutant larvae displayed a similar locomotor phenotype as in the UL test (Table S2), also moving less than corresponding WT larvae (main effect genotype: \( P = .003 \); Figure 5D) and spending more time being immobile (main effect genotype: \( P = .012 \); Figure 5E), again supporting enhanced anxiety. However, the behavioural response to a dark stimulus (Figure 5D,E) and the bout duration during this test was unaffected by genotype (Figure 5F; Table S2). Finally, mutant fish showed altered kinetics in the visually evoked startle response. Specifically, mutants showed on average shorter c-bend (\( U = 314.5; P = .028 \); Figure 5G) and counter-bend durations (\( U = 282.5; P = .007 \); Figure 5H) as well as increased escape swimming (\( U = 284.0; P = .009 \); Figure 5I). These changes could be because of a bias in the type of escape response, short latency c-starts (SLC) or long latency c-starts (LLC). The SLC and LLC responses can be distinguished by latency, c-bend duration and angular velocity. Since orientation of looming stimuli to specimens can have an effect on escape latency,35 SLC and LLCs were distinguished based on the c-bend duration and angular velocity criteria.34,35 This revealed that mutants performed significantly more SLCs than LLCs (Tukey post-hoc test: \( P < .001 \); Figure 5J, Table S2), an effect that was absent in WT animals, suggesting a bias towards SLC responses that may also support heightened anxiety. Taken together these data suggest that larval hrh3−/− fish display locomotor alterations indicative of anxiety similar to adults.

### 2.6 Whole brain functional imaging reveals enhanced activation in telencephalic regions, and alterations in regional functional connectivity in mutant larvae

We investigated potential neural correlates of the larval behavioural phenotype by using light sheet microscopy to image whole brain neural activity in hrh3−/− compared with

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**FIGURE 5** Effects of histamine receptor H3 (hrh3) deletion on larval behaviour. A, Total distance travelled, (B) time spent immobile and (C) average velocity during beat-glide swimming of larval hrh3−/− (KO) and corresponding wild-type (WT) fish in the larval unstimulated locomotion (UL) assay. The arrow in (C) indicates the onset of the swimming bout. n = 15/group. Mann-Whitney U test. †\( P < .01 \) vs WT. D, Total distance travelled, (E) time spent immobile and (F) average swim bout duration in the larval light/dark transition (L/D) assay. n = 15/group. Two-way ANOVA. Significant main effects are indicated in the graph. †\( P < .001 \), †\( P < .01 \), *\( P < .05 \). G-I, Visual startle response (VSR) test. Larval KO fish displayed (G) shorter c-bend durations, (H) shorter counter-bend durations and (I) enhanced escape swimming compared to corresponding WT fish. n = 15/group. Mann-Whitney U test. †\( P < .01 \), *\( P < .05 \) vs WT (J) In addition, KO fish were more likely to perform short latency c-bends (SLC) compared to long latency c-bends (LLC) and not responding at all. n = 15/group. Two-way ANOVA followed by Tukey’s post-hoc test. †\( P < .001 \), †\( P < .01 \) vs SLC/KO. Data are presented as mean ± SEM.
WT 4dpf larvae. We crossed *hrh3−/−* with transgenic fish expressing the Ca^{2+} indicator GCaMP6s under control of the pan-neuronal promoter ELAV-like neuron-specific RNA-binding protein 3 (*elavl3*),\(^3\) to generate *hrh3−/−* and *hrh3+/+* fish that pan-neuronally express GCaMP6s.

Comparison of the peaks in fluorescence intensity from the temporal profiles of *hrh3−/−* vs *hrh3+/+* revealed differences in activity in a number of brain regions (Table S3). In particular, *hrh3−/−* larvae had higher activity in many forebrain areas including the telencephalon as a whole, subpallium, eminentia thalami, pineal gland, preoptic area and olfactory bulb (Figure 6a), which overlaps well with the larval *hrh3* expression pattern described previously\(^2\) and the adult *hrh3* expression pattern described in this study. There were also some significant changes in activity in some hindbrain regions including significant increases in activity in the inferior olive, and locus coeruleus, as well as significant decreases in activity in the area postrema and noradrenergic neurons of the interfascicular and vagal areas. These changes were in regions not showing strong *hrh3* expression in our study and previous work.\(^2\) In addition, changes were also observed with respect to measures of functional connectivity between brain regions when WT and mutant larvae were compared (Figure 6b). The correlation coefficients obtained between temporal patterns of neural activity for a number of pairs of brain regions were significantly altered when compared between the two experimental groups, which most frequently encompassed connections to the pretectum, interpenduncular...
nucleus, intermediate hypothalamus and dorsal thalamus. This suggests that hrh3 deletion alters regional connectivity in specific brain structures which could be contributing towards the observed behavioural phenotype.

3 | DISCUSSION

The histamine H3 receptor is an emerging, promising therapeutic target for a variety of psychiatric disorders. It is widely expressed in the brain, and has an important neurophysiological role in regulating the signalling of histaminergic neurons as well as other neurotransmitter systems in mammals. Here, we demonstrate that both genetic deletion of hrh3, the zebrafish homologue of the human H3 receptor, and treatment with the inverse histamine H3 receptor agonist pitolisant, reduces zebrafish aggression. The decreased aggression of null mutants correlates with changes to neuronal activation in the telencephalon (Dm and Vv) and hypothalamus (PR) suggesting that reduced hrh3 signalling in these brain areas affects the fish's response to its own mirror image.

Neurotransmitters such as serotonin and dopamine are important mediators of aggression. However, the role of the histaminergic neurotransmitter system in aggression is less well studied. Murine studies have shown that Hnmt KO mice display heightened aggressive behaviour in the resident-intruder and aggressive biting behaviour tests, an effect associated with elevated brain histamine concentrations. In contrast, germ-line Hrh1 KO mice do not show altered histamine levels, but are less aggressive in the resident-intruder task. This is in line with pharmacological experiments demonstrating that histamine H1 antagonists suppress isolation-induced fighting in mice, or muricide in rats, an effect potentially related to the known sedative actions of these compounds. Previous research in zebrafish showed that aggressive behaviour is also linked to histamine signalling in this species. Gene expression studies have revealed that dominant zebrafish display changes in the expression of hdc and hrh2 in the telencephalon and hypothalamus compared to subordinate fish. Furthermore, the behaviour of the aggressive fgfr1a mutant is caused by reduced histaminergic neurotransmitter signalling in the brain. However, the precise role of the histamine H3 receptor in aggressive behaviour has not been investigated.

In this study we show for the first time that genetic and pharmacological manipulation of the Hrh3 receptor reduces aggression in the MIA test. This assay makes use of the fact that zebrafish do not recognize their own mirror image and fight it. Similar to real fights, zebrafish attack and bite the mirror opponent, but there is no risk of harm which favours this test over dyadic fights from an ethical perspective. In addition, male and female zebrafish express similar aggression levels during this test permitting the use of mixed zebrafish groups, while dyadic fight experiments typically use male opponents only. Although the fish's interaction with the mirror also shows good correlation with other measures of aggression and is linked to altered serotonin levels, it should be noted that some postures seen in real fights cannot be reproduced in mirror fights such as antiparallel display or the fight resolution phase in general. However, such a lack
of fight resolution phase during MIA offered an opportunity for the current study, because the neuronal activation pattern observed would represent the aggression phase only, without the influence of potential winner and loser effects.

We next investigated whether the altered MIA behaviour can be explained at the behavioural level. Comprehensive behavioural testing of hrh3−/− mutants excluded a general locomotor deficit and social disturbances, but revealed enhanced anxiety levels of adult mutant animals in the novel tank diving test. Increased anxiety of histamine H3 knockout animals has also been reported in mice, but in a test-specific manner. Histamine H3 KO mice are more anxious in the acoustic startle test, but less anxious in the elevated plus and zero maze.47 Since hrh3−/− zebrafish did not show signs of anxiety during the MIA test such as enhanced freezing periods or erratic swimming, it seems likely that our mutant displays test-specific changes in anxiety as well. It would also indicate that the low aggression phenotype observed during MIA is independent from the enhanced anxiety phenotype shown in the novel tank diving test.

Despite the lack of hrh3 in our mutants, neither histamine levels nor hdc, hrh2a or hmnt expression was altered suggesting no major compensatory changes in this signalling pathway. However, deletion of hrh3 did result in reduced expression of hrh1. In mammals, the histamine H1 receptor is an important regulator of sleep-wake cycle, blockade of which often has sedative effects.48 However, in zebrafish hrh1 null mutants exhibit largely normal sleep-wake behaviours suggesting that the observed expression changes of hrh1 in hrh3 mutants (average reduction of 24.4%) have minor effects on zebrafish behaviour. In mammals Hrh3 also acts as a heteroreceptor regulating the release of other neurotransmitters including serotonin and dopamine.6 Our finding of reduced 5-HIAA levels in mutants advocates for a similar function in zebrafish and agrees with previous studies regarding the interaction of histamine and serotonin signalling.25 The unaltered expression of genes relevant for serotonin production, degradation and reuptake suggest that decreased serotonin release at the synapse might be responsible for the observed reduction in 5-HIAA levels.

rpS6 immunohistochemistry identified a number of brain areas activated by aggression in WT zebrafish and those that potentially mediate the reduced aggression phenotype of hrh3 mutants. The SDMN has been proposed as a key neuronal circuit controlling social behaviours including aggression across species.27,50 Essential nodes in this network include numerous areas of the basal forebrain such as the medial amygdala, medial bed nucleus of the stria terminalis, preoptic area (POA), anterior and ventromedial hypothalamus and lateral septum, as well as midbrain brain areas such as the central grey and the ventral tegmental area.50 In line with this idea, a wealth of mammalian studies have demonstrated that these brain areas are essential components of the mammalian aggression neurocircuitry.2,51-54 Nodes of the SDMN have also been identified in zebrafish, but many areas are currently still undefined or debated. Nevertheless, many of the brain areas investigated here have a putative mammalian homologue including Dm (pallial amygdala), Dl (medial pallium/hippocampus), Vd (striatum/basal ganglia/nucleus accumbens), Vv (lateral septum) and PPa (preoptic area).55 Here, we investigated for the first time whether MIA activates these components of the SDMN compared to controls not exposed to aggression. Strikingly, all of these selected brain areas showed enhanced rpS6 immunoreactivity after aggression suggesting an important role in mediating this phenotype. These results are also in line with previous studies linking the zebrafish preoptic area (POA) to aggression. Dominant WT zebrafish and fish exposed to dyadic fights show alterations in arginine vasotocin (AVT) neuron morphology in the POA and AVT concentrations respectively,56,57 reinforcing the importance of this area during fish aggression. Interestingly, MIA also activated hypothalamic brain areas (LH, PR, Hc, Hv), which are not currently considered to be a part of the SDMN suggesting that both the hypothalamus and SDMN act together to regulate aggression in this species.

Of note, three of the brain areas investigated here (PR, Dm and Vv) showed changes in aggression-induced neuronal activation in mutants, with higher numbers of rpS6+ cells in the PR and the Dm compared to WT fish. The area around the PR is the only part of the zebrafish brain that contains histaminergic neurons.23 Although we did not detect a difference in histamine levels between mutant and WT fish, our results suggest that enhanced histaminergic tone in mutants influences the function of many important brain areas such as the telencephalon. Alternatively, since other non-histaminergic neurons reside in this area as well, including dopamine- and serotonin-containing neurons, lack of hrh3 activity could activate these neurons during aggression. Further experiments would be necessary to dissect the precise effects of hrh3 knockout on different neurotransmitter signalling pathways. The histaminergic neurons send projections throughout the whole zebrafish brain (except cerebellum) including a major projection to the dorsal telencephalon.23 The enhanced activity of Dm neurons in mutants in response to aggression might thus be the result of enhanced input from PR histaminergic neurons. Dm has been suggested to be the functional homologue of the mammalian amygdala based on expression and lesion studies.26 The amygdala plays a role in a plethora of brain functions such as anxiety, fear and stress processing, emotional memory, as well as aggression.54 The activation of the Dm in mutants could also indicate altered emotional processing of the mirror image because of the loss of hrh3. In contrast, Vv, another important part of the zebrafish telencephalon that is homologous to the mammalian septal formation,26 showed lower mirror-induced activation in mutant fish. Unlike Dm, this area receives no major input from histaminergic neurons.23 It does, however, send extensive descending projections to the midline hypothalamus.59 Like the
amygdala, the septal formation has also been long recognized as an important component of the mammalian aggression neurocircuitry.\textsuperscript{24} Our data suggest that this is also the case in zebrafish and that hrh3\textsuperscript{−/−} deletion reduces output of these neurons to the hypothalamus during aggression.

When analysing larval fish, we noted that an anxiety phenotype is already present in hrh3\textsuperscript{−/−} zebrafish at 4 dpf. Specifically, hrh3\textsuperscript{−/−} larvae were less active than WT larvae in both the unstimulated locomotion assay and the L/D assay indicating heightened anxiety levels. In addition, the changes in kinetics of the visual startle response suggest that this type of escape response is altered because of gene knockout. hrh3\textsuperscript{−/−} larvae showed an increase in the probability of eliciting short latency responses when exposed to visual threatening stimuli. Interestingly, a similar finding has been made in socially isolated zebrafish larvae. When exposed to a social interaction, these larvae show enhanced social avoidance using high-acceleration escape swim bouts and a short latency C-start to escape from the situation,\textsuperscript{60} similar to our data. At larval stages, hrh3 expression is strongest in the telencephalon,\textsuperscript{25} indicating that this area may mediate the observed phenotypic differences. In line with this idea, we observed strong increases in brain activity in the larval telencephalon of mutants using whole brain functional imaging suggesting an activation of this brain area after hrh3 deletion. Furthermore, in agreement with the expression of hrh3 in larvae,\textsuperscript{25} we found activity changes in the eminentia thalami, pineal gland, preoptic area and olfactory bulb. Of note, increased activation of the eminentia thalami has previously been shown in response to sildenafil, a compound that reduces zebrafish aggression.\textsuperscript{44} In contrast, a number of brain regions not strongly associated with the expression of hrh3 exhibited significantly altered neural activity in mutant compared with WT animals. When considering the functional imaging data, it must be remembered that these indicate network-wide changes including connectional influences. For example, we observed activation of the predominantly noradrenergic locus coeruleus, which as well as showing H3 receptor agonist radioligand binding,\textsuperscript{61} is also known to have an extensive network of projections to multiple zebrafish forebrain regions including the olfactory tract and bulb, hypothalamus and preoptic area.\textsuperscript{62} We observed significantly altered measures of functional connectivity between a number of brain regions in the mutant compared to wild-type animals which most frequently involved the pretectum, interpenduncular nucleus, intermediate hypothalamus and dorsal thalamus. These data suggest that the deletion of hrh3 alters regional circuit connectivity, particularly in the case of hypothalamic and thalamic areas is entirely consistent with the reported distribution of hrh3 receptors in developing zebrafish\textsuperscript{25} and may offer an explanation for the altered behaviour observed in our mutants. Functional imaging of the response of these regions to specific behaviours would help to further delineate the circuitry that controls these behaviours in larval and embryonic zebrafish.

4 | CONCLUSION

This study demonstrates that the hrh3 receptor is important for zebrafish aggression. hrh3\textsuperscript{−/−} zebrafish are less aggressive and we have identified a number of brain areas that are activated by this behaviour, including the zebrafish SDMN. We have also uncovered brain areas that are modulated by hrh3 deletion. Larval behaviour and whole brain Ca\textsuperscript{2+} imaging experiments reveal a potential role for hrh3 in anxiety, and heightened neural activity in areas expressing hrh3 such as the telencephalon, subpallium, preoptic area and eminentia thalami as well as altered network dynamics because of gene knockout. Genetic and pharmacological studies in various animal models also point to the potential of histamine H3 modulation as a novel therapeutic treatment. Pitolisant, an inverse histamine H3 agonist, has recently been approved for the treatment of narcolepsy and a number of clinical trials for other neuropsychiatric conditions are currently underway.\textsuperscript{10} Here, we show for the first time that hrh3 deletion reduces aggressive behaviour, which might lead to a novel therapeutic treatment for human conditions characterized by excessive aggression.

5 | MATERIALS AND METHODS

5.1 | Animals strains, care and maintenance

Zebrafish were maintained at the Universities of Leicester and Exeter using standard husbandry protocols and in accordance with institutional guidelines for animal welfare. The fish were maintained on a 14 hour light/10 hour dark cycle and fed twice a day. Experiments were carried out using AB WT zebrafish, hrh3\textsuperscript{−/−} (allele name: hrh3\textsubscript{uol1}) and corresponding WT zebrafish on AB WT background or hrh3\textsubscript{uol1}:elavl3:GCaMP6s and hrh3\textsuperscript{3/+}:elavl3:GCaMP6s fish on a mixed casper/nacre/AB background from which pigmentless larvae were selected for imaging. Mixed sex groups were used for all experiments and animals were kept in groups of 10-20 fish until analysis. Animal experiments were approved by a local Animal Welfare and Ethical Review Body (AWERB) of the University of Leicester and conducted under UK Home Office project and personal licences.

5.2 | CRISPR-Cas9 genome editing

Hrh3\textsuperscript{3/+} zebrafish and corresponding WT fish were generated by CRISPR/Cas9 genome engineering following published protocols.\textsuperscript{63,64} Briefly, ChopChop software\textsuperscript{65} was used to identify a suitable target site for mutagenesis of the hrh3 gene. Cas9 nuclease expression plasmid pMLM3613
(Addgene plasmid #42251) and single-guide RNA (sgRNA) expression vector pDR274 (Addgene plasmid #42250) were used to generate Cas9 mRNA and single guide RNA respectively. One cell embryos from AB WT zebrafish were injected with in vitro transcribed (mMESSAGE T7 ULTRA kit (Life Technologies)) sgRNA and Cas9 mRNA. Somatic mutation efficiency was evaluated by T7 endonuclease I assay and embryos injected with efficient sgRNAs were raised to adulthood. Germline-transmitting founders were identified by screening the offspring of injected individuals by T7 endonuclease I assay and MiSeq Next Generation Sequencing. Heterozygous individuals carrying a 4 bp insertion at nucleotide position 178 of the coding region were identified by genotyping and Sanger Sequencing and incrossed to obtain homozygous mutant (hrh-3\textsuperscript{sal1}) and corresponding WT siblings.

5.3 | Mirror-induced aggression

To characterize aggression levels, the mirror-induced aggression (MIA) paradigm was used as previously described.\textsuperscript{19} Adult zebrafish were placed in a narrow tank with a mirror placed in front of the tank at an angle of 22.5° for 30 minutes. The fish’s interaction with its mirror image was recorded by a camera and FlyCap2 software. Distance moved, time spent immobile and angular velocity during the test was quantified using Ethovision XT12 video tracking software (Noldus, Wageningen, The Netherlands). A modified version of the MIA setup was used for the adult neuronal activation experiment described below. In this case, fish were placed in small tanks (10.5 × 5.5 × 10 cm; length × width × height) without an external mirror (Video S3). However, the walls of the tank acted as mirrors showing strong reflections of the fish in the tank on all four tank sides stimulating aggression levels for 60 minutes. The amount of time spent in agonistic behaviour in both experiments was scored manually by an experienced investigator blind to the experimental groups.

5.4 | Open field test

Locomotor activity was assessed with the open tank test as previously described.\textsuperscript{43} For this, zebrafish were individually placed in a large tank (37.5 × 21.5 × 15 cm; length × width × height) covered with foam rubber on the inside to avoid aggression-provoking reflections. Ethovision XT12 software was used to analyse the swimming trajectory of the fish to calculate total distance moved and velocity.

5.5 | Novel tank diving

Anxiety-like behaviour was measured with the Novel Tank Diving test, which is based on the animal’s natural instinct to seek protection by diving, freezing and reducing exploration. For this, fish were placed in a trapezoid tank (Length × Width × Height; 19 × 10 × 7 cm) and for analysis the tank was divided into three parts using Ethovision XT12 software.\textsuperscript{43} Fish which spent more time at the top, entered the top compartment more frequently or had a lower latency to enter this compartment were considered less anxious.\textsuperscript{57}

5.6 | Social behaviour

Shoaling behaviour and a social interaction test were used to evaluate social behaviour. For shoaling assays, a group of five familiar fish from the same hometank was placed into a large tank (37.5 × 21.5 × 15 cm; length × width × height) filled with aquarium water.\textsuperscript{43} After 5 minutes habituation, the fish were filmed from above for 20 minutes. Nearest neighbour distances (NND) and inter-individual distances (IID) were analysed using VpCore2 software (ViewPoint Life Sciences, Lyon, France). Social interaction was analysed in the visually mediated social preference test (VMSP) as previously described.\textsuperscript{68} A test fish was placed in the central compartment of the VMSP tank (Length × Width × Height; 19 × 13.2 × 9.3 cm), which is surrounded by four smaller compartments (Length × Width × Height; 9.2 × 6.5 × 9.3 cm). All walls of the tank are made of clear acrylic to enable fish to see each other and to facilitate video recording and tracking. In addition, small holes (1 mm diameter) in the dividing walls between the compartments permitted olfactory as well as visual stimulation of the test fish. One of the smaller compartments was then filled with three stimulus AB WT fish, while the other compartments remained empty. Time spent near the stimulus fish compartment (social interaction zone) was used as readout for social behaviour and compared to time spent near the opposite small compartment (control zone).

5.7 | Larval unstimulated locomotion and light/dark (L/D) transition assay

Individual 4 days post-fertilization (dpf) larval zebrafish were transferred to a darkened behavioural arena (30 mm diameter), within a behavioural chamber that contained an infrared light source and an overhead camera (Point Grey DragonFly 2, Point Grey, Richmond, Canada). The chamber also contained a fibre optic white light source to control the ambient lighting (300 lux) in the behavioural chamber. Fish were allowed to acclimatize for 10 minutes before behaviour was recorded. Digital video (audio video interleave (AVI) format) recordings were acquired at 15 frames per second (FPS) and captured using Flycap2 software. For the unstimulated locomotion (UL) assay, zebrafish locomotion was captured for
10 minutes. To investigate anxiety and exploration behaviour, zebrafish were exposed to a light/dark transition assay. For this, zebrafish locomotion was captured for 5 minutes in an arena illuminated by a fibre optic light white light source (300 lux). Subsequently at 5 minutes, the light source was switched off and behaviour recorded for a further 5 minutes in darkness (0 lux).

5.8 | Larval visually evoked startle response (VSR)

Larval zebrafish were placed in a small glass dish (height = 30 mm; diameter = 10 mm) within a larger dish (70 mm diameter) sat upon an infrared light source and were allowed to acclimatize for 10 minutes before any experiments were performed. The smaller dish contained sylgard 184 in the lower 20 mm of the glass dish to restrict the swimming space to the upper quadrant. The larger dish contained system water. A sheet of 60 g tracing paper was affixed to the outer surface of the larger arena wall to create a screen. Looming stimuli were generated using Psychtoolbox within MATLAB and projected onto the screen using a 100-lumen portable DLP pico projector, which was placed 15 cm away from the outer arena wall (iXunGo, Guangdong, China). MATLAB scripts used to generate the looming stimuli were provided by Prof M. MacIver (Northwestern University, Chicago, Illinois, USA), and experimental protocol was replicated from. The looming stimulus approach velocity was defined by the l/v ratio, which is an indicator of angular (θ) expansion over time. The l/v ratio is determined as the ratio between half the size of the approaching object (l) and the approach velocity (v). Zebrafish were presented looming stimuli (l/v = 1.25) three times with 2 minutes intervals. Bouts of visually evoked escape behaviours were captured at 500 FPS using an overhead camera.

5.9 | Larval behaviour analysis

Compressed digital video AVI files were uncompressed using VirtualDub (V1.10.4). The uncompressed AVI files were subsequently converted to micro fly movie format (ufmf: V1.0.0.0) using any2ufmf (http://ctrax.sourceforge.net/any2ufmf.html). The ufmf video files were subsequently imported into California Institute of Technology Fly Tracker (Ctrax; V0.5.16). Video files were processed in Ctrax. Tracking data such as co-ordinates and orientation were obtained for the duration of the recording and extracted in MATLAB format files. Tracking data were reviewed in MATLAB (VR2016a), and any errors were corrected using the ‘FixErrors’ MATLAB graphical user interface (GUI). To analyse swimming distance and velocity, reviewed, Ctrax-derived data were extracted and processed in R-studio. Swimming distance, velocity and swimming trajectories were analysed using R script from Jay et al70

To analyse specific behaviours exhibited by larva, reviewed Ctrax data were imported into Janelia Automatic Animal Behavior Annotator (JAABA; V0.5.0). Using JAABA, the reviewed Ctrax data were used to classify behaviours, for example, beat-glide swimming and startle response. For JAABA to identify episodes of a specific behaviour, the software required an initial training period. During this training period, example (s) of a given behaviour and non-behaviour were manually labelled. Once a behaviour was classified, the machine learning algorithm was executed to identify the behaviour across the video files. Raw data relating to specified data were exported as an excel file for statistical analysis. C-bend and counter-bend angles were measured manually using ImageJ. The user was blinded during all behavioural experiments and analysis.

5.10 | Drug treatment

Pitolisant was purchased from Tocris Biosciences (Bristol, UK) and dissolved in distilled water (VEH) to obtain a 50 mmol/L stock solution. To study the effects of pitolisant on aggressive behaviour, AB WT fish were pre-treated in small tanks containing different concentrations of drug (2 µmol/L and 3 µmol/L in system water) or VEH in system water for 1 hour. After the treatment period, fish were transferred to the MIA setup and their behaviour was recorded and analysed for 5 minutes.

5.11 | Ribosomal protein S6 (rpS6) immunohistochemistry

Neuronal activation in adult fish was visualized by rpS6 immunohistochemistry as previously described. Freshly collected brains were placed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS) for 24 hour, washed in PBS for 24 hour, transferred to 30% sucrose solution in PBS for 24 hour, frozen in Tissue-Tek OCT compound blocks (Sakura Finetek, Thatcham, UK) in 2-methylbutane (Sigma-Aldrich, St.Louis, US) and stored at −70°C until cryosectioning. Twenty-micrometre thick coronal sections of zebrafish brains were mounted onto Superfrost Plus slides (Thermo Scientific, Waltham, USA) and then processed for immunohistochemistry. To block non-specific binding,
sections were incubated with 5% normal horse serum, 0.2% bovine serum albumin and 0.3% triton-X in PBS for 2 hour at room temperature followed by incubation with rabbit anti-phospho-S6 Ribosomal Protein (rpS6) antibody (#2211, Cell Signaling, Leiden, Netherlands) in blocking solution at 4°C overnight. After washing in PBS, sections were incubated with biotinylated horse anti-rabbit secondary antibody (VECTASTAIN Elite ABC HRP Kit – PK6200, Vector laboratories, Peterborough, UK) for 2 hour at room temperature. Endogenous peroxidase activity was then quenched by hydrogen peroxide incubation (1.5% in methanol) and after washing, sections were incubated with Avidin Biotin Complex (ABC) solution (Vector laboratories) and developed with 3,3′-diaminobenzidine (DAB; Sigma-Aldrich).

5.12 | Cell counting

Images for cell counting were acquired with a GXM L3200B light microscope (GT Vision, Stansfield, UK) and ImageFocus 4 software (Euromex Microscopen BV, Netherlands). Slides were coded such that the investigator was blind to the treatment group under investigation. Selected brain regions of interest (ROIs) included the medial and lateral zone of the dorsal telencephalic area (Dm and Dl), the ventral and dorsal nuclei of the ventral telencephalic area (Vv and Vd), the anterior parvocellular preoptic nucleus (PPa), the ventral and caudal zone of the periventricular hypothalamus (Hv and Hc), the lateral hypothalamic (LH) nucleus and the area around the posterior recess of the diencephalic ventricle (PR). These ROIs were identified with the help of adjacent Nissl-stained sections and the Wullimann zebrafish brain atlas. Fiji software was used to obtain cell counts within these areas. In order to quantify the number of rpS6 positive cells within a ROI, an intensity-based background threshold was determined for each brain ROI. The background threshold was defined such that the maximum number of labelled cells was identified without inclusion of any background staining. A 150 × 150 µm counting frame was placed in ROIs without clearly visible anatomical borders (Dm, Dl, Vd, Vv), while for the remaining areas the number of rpS6 positive cells was quantified across the whole structure. Two to three consecutive sections were evaluated for each ROI per brain and the average values/brain region of each animal were used for statistical analysis.

5.13 | hrh3 in situ hybridization

Brain hrh3 expression was visualized by in situ hybridization as previously described. Sections were photographed using an optical microscope (GXM L3200B, GT Vision) and ImageFocus 4 software (Euromex Microscopen BV) and images were mounted in Adobe Photoshop version CS2 (Adobe systems).

5.14 | Real-time quantitative PCR

RNA from freshly frozen zebrafish brains was extracted using peqGold TriFast (Thermo Scientific), followed by DNase digestion (Ambion DNA-free™ DNA Removal Kit, Thermo Scientific) and reverse transcription using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). For relative quantification of mRNA levels, qPCR was performed on a CFX384 Touch™ Real-Time PCR Detection System (BioRad, Vienna, Austria) using the SYBR green detection protocol. Primers for genes of interest in the histaminergic and serotonergic neurotransmitter system (tph1a, tph2, mao, slc6a4a, slc6a4b, hdc, hnmt, hrh1 and hrh2a) were designed with Primer BLAST or taken from published articles (Table S3). Amplification of the correct amplicon was confirmed by Sanger sequencing. All samples were measured in triplicate. Actin beta1 (actb1) and ribosomal protein L13a (rpl13a) were used as reference genes for quantification of target gene expression. Quantitative measurements of target gene levels relative to controls were performed with the ΔΔ Cq method using the mean value of the control group as the calibrator. Group differences were expressed as fold changes.

5.15 | High performance liquid chromatography (HPLC)

HPLC was performed after micro-dissecting the diencephalon and hypothalamus of the adult brain in ice-cold PBS under a stereomicroscope as previously described. Samples were weighed, homogenized in 100 µL ice-cold 0.1 N perchloric acid, and centrifuged to collect the debris-free tissue supernatant. HPLC with electrochemical detection was used to measure dopamine (DA), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA). Separation was achieved on a 150 × 1.0 mm C18 column (LUNA(2), Phenomenex, UK) with a running buffer comprising 75 mmol/L NaH2PO4, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.6 mmol/L octane sulphonic acid, 10% methanol, pH 3.8., and solutes were detected electrochemically at a potential of 750 mV vs an Ag/AgCl reference electrode (ANTEC Intro, with VT-03 flow cell, ANTEC Inc, Netherlands). Data were collected and peak area for each compound was determined using a PC-based integrator (ChromPerfect, Justice Laboratories, USA). Samples were compared to standard solutions of known concentrations and the results were expressed as pmol/mg of tissue.
5.16 | Histamine EIA

Telencephalic and hypothalamic tissue samples were weighed and homogenized in 0.2 N perchloric acid. Tissue debris was pelleted by centrifugation (10,000 × g, 4°C, 5 minutes) and the supernatant was collected and neutralized with an equal volume of 1 mol/L potassium tetraborate (pH 9.1). Histamine EIA (IM2015, Beckman Coulter, Wycombe, UK) was performed according to the manufacturer’s instructions. Absorbance was read at 405 nm using a Tecan Infinite M 200 plate reader (Tecan, Maennedorf, Switzerland). Histamine concentrations were calculated by interpolation using a 4 PL curve fit, based on the optical densities of standardized concentrations vs those observed in the samples.

5.17 | Larval whole brain imaging

Whole brain functional imaging of the 4-day-old brain was performed according to. Briefly, hrh3+/−:elavl3:GCaMP6s and hrh3−/−:elavl3:GCaMP6s larvae were exposed to the anti-nicotinic neuromuscular blocker tubocurarine (4 mmol/L) until muscle tone was lost, transferred to a mixture of tubocurarine and 1.4% Low Melting Point agarose, and then drawn into a clear borosilicate glass capillary (940 μmol/L internal diameter). Each mounted fish was positioned vertically (head down), and optical sectioning was undertaken in the horizontal plane from the dorsal to ventral surface of the head. This was repeated for approximately 6 minutes generating 200 × 10 Z-plane image stacks, each spanning a total depth of 220 μm. At the end of each run, the larva was checked for a normal heart rate and blood flow by microscopy to ensure viability. The light sheet microscope used comprised a 488 nm Argon laser (Melles Griot, Didam, Netherlands) for illumination, a 20x/0.5 NA objective lens (Olympus, Southend-on-Sea, UK) with an intermediary magnification of 1x, followed by a 525/50 nm emission filter and 495 nm bandpass filter (Chroma, Olching, Germany) to collect emitted light, and a 5.5MP Zyla sCMOS camera (30FPS, 640 × 540 pixels, 4 × 4 binning, 40 ms exposure, Andor, Belfast, Northern Ireland) to capture images. The camera and rotational axis (Picard Instruments, Albion, USA) were controlled through μManager and the OpenSPIM plugin. Image processing and data extraction were performed as described previously using a custom Python image processing pipeline. From the temporal profile of fluorescence intensity generated for each region of interest (ROI), peaks in intensity were automatically identified and the signals for each ROI compared between WT and mutant animals using univariate statistics. In addition, functional connectivity between ROIs was analysed using a comparison of r-values generated for the temporal profile of fluorescence intensity obtained for each paired connection, within each treatment group.

5.18 | Statistics

Statistical analysis was performed using IBM SPSS Statistics 26 (IBM, Ammonk, USA) and GraphPad Prism 6 software packages (Graphpad Software Inc, San Diego, USA). Group differences were assessed by Student’s t test for normally distributed data or Mann-Whitney U test for non-normally distributed data. One-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test, one-way repeated measures ANOVA or two-way ANOVA was used to analyse data sets with more than two groups as appropriate. P-values < .05 were considered statistically significant. For the functional imaging data, the fluorescence intensity data from each ROI were time averaged and then the means for each treatment group compared using Mann-Whitney or t tests following testing for normality and homogeneity of variances (Minitab 16, Minitab Ltd, Coventry, UK). For functional connectivity assessment, a connectivity matrix was generated for each animal by calculating the Pearson’s correlation coefficient between the time series’ of all possible pairs of ROIs. To indicate where a significant increase or decrease in the strength of a connection between two ROIs occurred between the WT and mutant larvae, the r-values obtained for each connection were subsequently compared using Bonferroni-corrected Mann-Whitney tests (individual α = 0.0012, equating to 0.05/no. of connections per ROI).

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article or the supplementary information or are available from the corresponding author on reasonable request.
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