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Virtual reality reveals fine-scale alterations in behaviour following loss of the ADHD-linked gene *adgrl3.1* in zebrafish

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eLife Assessment

The study presents **valuable** findings on early behavioral phenotypes that arise in an ADHD-associated *adgrl3.1* mutant zebrafish, using a new behavioral approach with a closed-loop OMR assay. The evidence supporting the claims of the authors is **solid**; however, the validation of the method and analysis is **incomplete** and would benefit from more rigorous approaches and validation practices. This work will be of broad interest to developmental biologists and behavioral neuroscientists.

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Abstract

Zebrafish have been used a prominent model for high-throughput phenotypic screens of candidate risk gene mutations for several disorders. This also includes models for attention deficit/hyperactivity disorder (ADHD). Traditional behavioural tests, such as the forced light/dark assay, concentrate on basic locomotion measures. However, recently developed visually-driven locomotion assays, for example closed-loop systems using virtual reality, have allowed extraction of richer data on animal locomotion and decision-making under different sensory stimuli. Here, we have used such a system to assess the behaviour in *adgrl3.1* mutant fish, an established model for ADHD. Our results show that mutants exhibit a higher baseline excitability and a lower threshold for initiating motor events, demonstrating that collecting behavioural responses in an interactive environment enables a more precise characterisation of ADHD-relevant phenotypes associated with *adgrl3.1* disruption. More generally, we establish a scalable translational platform to screen gene-function relationships and possible therapeutic interventions, not only for ADHD but multiple neurodevelopmental disorders.

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is one of the most prevalent neurodevelopmental disorders in children and adolescents, impacting approximately 3-5% of the population worldwide (Castelpietra et al 2022 [↗](#), Erskine et al 2013 [↗](#), Geissler & Lesch 2011 [↗](#), Nigg et al 2020 [↗](#)). It is characterised by persistent and developmentally inappropriate patterns of inattention, hyperactivity, and impulsivity, and represents profound challenges to cognitive, social, and emotional functioning (Acosta et al 2004 [↗](#)). Beyond its core symptomology, individuals with ADHD frequently exhibit impairments in executive functioning and decision-making (Mowinckel et al 2015 [↗](#)), and face increased risk of comorbid psychiatric disorders (Katzman et al 2017 [↗](#)), including anxiety (Quenneville et al 2022 [↗](#)), bipolar disorder (Nurnberger et al 2014 [↗](#)), depression (Powell et al 2021 [↗](#)), and substance use disorders (Arcos-Burgos et al 2019 [↗](#)).

Substantial evidence supports the high heritability of ADHD, with genetic factors estimated to contribute up to 80% of the variance in risk (Faraone & Larsson 2019 [DOI](#), Yadav et al 2021 [DOI](#)). Genome-wide association studies (GWAS) and gene mutant analyses have identified candidate risk genes which modulate susceptibility to ADHD. Among these, genes encoding synaptic adhesion molecules, critical regulators of neural development, synaptic plasticity, and circuit specificity, have gathered increased attention (Burbach & Meijer 2019 [DOI](#), Sudhof 2018 [DOI](#)). One gene of particular interest is *ADGRL3* (formerly Latrophilin-3, *LPHN3*), which encodes an adhesion G protein-coupled receptor involved in synapse formation, cell adhesion, and plasticity, and is highly expressed in brain regions that govern attention, impulse control, and reward processing (Regan et al 2021 [DOI](#), Sando et al 2019 [DOI](#)). Variants of *ADGRL3* have been robustly associated with increased ADHD risk symptom persistence into adulthood and alterations in treatment responsiveness (Arcos-Burgos et al 2010 [DOI](#), Bruxel et al 2021 [DOI](#), Fallgatter et al 2013 [DOI](#), Kappel et al 2019 [DOI](#), Ribases et al 2011 [DOI](#), Vidal et al 2022 [DOI](#)). Interestingly, functional disruptions in *ADGRL3* not only impact synaptic organization but also appear to alter dopaminergic signalling, and are consequently crucial in treatment responsiveness to stimulant medications for ADHD (Fontana et al 2023 [DOI](#), Lange et al 2012 [DOI](#), Vidal et al 2022 [DOI](#)). Despite these strong associations, the mechanistic pathways by which *ADGRL3* dysfunction contributes to specific behavioural phenotypes remain poorly defined.

Zebrafish (*Danio rerio*) are a powerful model for investigating the neurogenetic underpinnings of ADHD, offering high fecundity, a well-characterized genome, and ethologically relevant quantifiable behavioural repertoires that complement rodent approaches (Fontana et al 2025 [DOI](#)). Consistent with this, zebrafish mutants lacking *adgrl3.1*, the orthologue of human *ADGRL3*, exhibit core ADHD-like phenotypes, including hyperactivity in larvae, and impulsivity and attention deficits in adults (Fontana et al 2023 [DOI](#), Lange et al 2012 [DOI](#), Regan et al 2021 [DOI](#), Sveinsdottir et al 2023 [DOI](#)). However, larval ADHD-related phenotyping has relied largely on the forced light/dark (FLD) locomotion test (Hillman et al 2025 [DOI](#)), which has commonly been used to measure anxiety-like behaviour (Burgess & Granato 2007 [DOI](#), Luchtenburg et al 2019 [DOI](#), Peng et al 2016 [DOI](#)). While useful for detecting overall activity differences, it cannot determine whether increased movement reflects hyperactivity, altered arousal, disrupted behavioural control, or anxiety-like responses (Blaser et al 2010 [DOI](#), Fontana et al 2022 [DOI](#)). This is a significant limitation given the substantial comorbidity between ADHD and anxiety disorders, estimated at 25–40%, in clinical populations (Koyuncu et al 2022 [DOI](#)). As a result, current larval assays therefore lack the behavioural resolution needed to connect *adgrl3.1* dysfunction to specific ADHD-relevant endophenotypes. This is particularly important as *ADGRL3* is implicated in synaptic organisation and dopaminergic signalling, suggesting a primary role in the mechanism of behavioural regulation rather than motor output alone.

To address this, we used a closed-loop virtual reality assay in freely swimming larval zebrafish (Bahl & Engert 2020 [DOI](#), Reynolds et al 2025 [DOI](#), Slangewal et al 2026 [DOI](#)). Closed-loop systems assess behaviour in an interactive context, in which the visual environment changes as a function of the animal's ongoing actions. This creates an interactive feedback loop based on the animal's real-time position and behavioural responses. Unlike static light/dark assays currently used within ADHD-related paradigms, this approach enables not only basic swim characteristics, such as general locomotion, but additionally allows precise measurements of cognitive traits including attentional, impulsive, and innate decision-making behaviours. This makes it possible to quantify how larvae adjust their behaviour in response to behaviour-contingent sensory feedback, beyond simple locomotor activity. These measures provide a more refined view of behavioural regulation than standard light/dark assays and capture phenotypes that are central to ADHD, but rarely assessed in larval models.

Here, we applied this paradigm to *adgrl3.1* loss-of-function larvae to test whether the effects of mutation extend beyond elevated locomotion to altered behavioural responses in an interactive environment. By separating overall activity from behaviour expressed under closed-loop feedback, this approach enables a more precise characterisation of ADHD-relevant phenotypes

associated with *adgrl3* disruption. Our findings provide new insight into the role of *Adgrl3* in neurodevelopment and establish a scalable, translational platform for the investigating gene-function relationships in disorders and therapeutic interventions in ADHD.

Material and Methods

Zebrafish

Zebrafish husbandry and experiments were approved by the Animal Welfare and Ethics Review Body at KCL and conducted under the Home Office project license PP7266180 to R.H. Embryos were placed into Danieau within a 90mm Petri dish, at a density of no more than 50 larvae per Petri dish. Zebrafish were maintained in an incubator at a temperature of 28.5°C and on a 14-hour ON/10-hour OFF light cycle. For experiments on larvae over 5 days, larvae were fed live rotifers as in line with the KCL Fish Facility feeding protocol. All experiments were performed on the AB wildtype line (ZIRC, 5th Generation) at 7 days post-fertilization (dpf), at which age sex cannot be determined.

Generation of *adgrl3.1* zebrafish knock out using the CRISPR method

CRISPR F0 mutants were generated following the protocol as described by Kroll et al. (2021) [but](#) using one-quarter of the recommended gRNA/Cas9 concentrations. Three crRNA oligonucleotides targeting *adgrl3.1* were designed with the IDT CRISPR design tool. For RNP assembly, each crRNA (50 µM) was individually annealed to tracrRNA (50 µM) by heating to 95 °C for 5 minutes, followed by immediate cooling on ice. The resulting gRNAs (15.25 µM) were then incubated with Cas9 protein (15.25 µM) at 37 °C for 10 minutes. Each RNP complex (7.125 µM) was subsequently combined in equal amounts, producing a final working concentration of 2.5 µM for each RNP. Control RNPs were prepared in the same way using the Alt-R® CRISPR-Cas9 Negative Control #1 crRNA (Integrated DNA Technologies). Microinjections were carried out by delivering 1nl of either the experimental or control RNP mixture into each AB wildtype embryo when at the 1-cell stage.

Genotyping of *adgrl3.1* zebrafish knock out

Six embryos from each injection batch were chosen at random and genotyped at 2 dpf. Genotyping was performed by PCR amplification of exons 6 and 8, with large indels detected by gel electrophoresis (Figure 1A [but](#)). To assess possible excision of the intervening genomic region, the exon 6 forward primer was paired with the exon 8 reverse primer, amplifying exons 6–8 plus flanking intronic sequences (Table 1 [but](#)). Larvae were classified as mutants if at least one mutation or excision was detected in any of the targeted regions.

Genomic DNA was extract from whole larvae using the HOTSHOT method (Meeker et al 2007 [but](#)) Larvae were lysed in 30 µl 50 mM NaOH at 95 °C for 20 min, cooled on ice for 5 min, and pH neutralized with 1:10 volume of 1 M Tris-HCl (pH 8). Samples were vortexed for 10 s and then centrifuged at 13,000 rpm for 1 min. PCR was carried out in 15 µl reactions using DreamTaq PCR Master Mix (Thermo Fisher Scientific), containing 7.5 µl 2× mix, 5.4 µl H₂O, 0.3 µl of each 10 µM primer, and 1.5 µl DNA template (supernatant from previous step). Cycling conditions were: 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s; followed by final 72 °C for 2 min and a 4 °C hold. PCR products were resolved on 1% agarose gels in 1× TAE buffer containing SYBR Safe DNA Gel Stain (1:10,000, Thermo Fisher Scientific). Gels were loaded with 5 µl DNA sample per well and run along 100 bp ladders (New England Biolabs) at 120 V for 30 min. DNA was visualized under UV illumination with a NuGenius Gel Documentation System. Across all experimental batches, 100% of CRISPR larvae were inferred to be mutants based on these genotyping results (Figure 1B [but](#)), aligning with (Kroll et al 2021 [but](#)) reporting that this method produces biallelic knockouts in over 90% of injected embryos.

Figure 1. Creation of *adgrl3.1* knockout mutant.

A) PCR approach with location and direction of gRNAs (red arrows), and location and direction of primers (large pink and green arrows), on each exon of *adgrl3.1*. **B)** Example of genotyping results showing indel mutations for all individual larvae in exon 6, likely indels in exon 8, and partial excisions between exons 6–8. For each sample a positive control was done using a primer pair in an unrelated genomic location (*tenm4* gene). H2O, negative water control; L, DNA ladder; WT, DNA from uninjected wild-type animals.

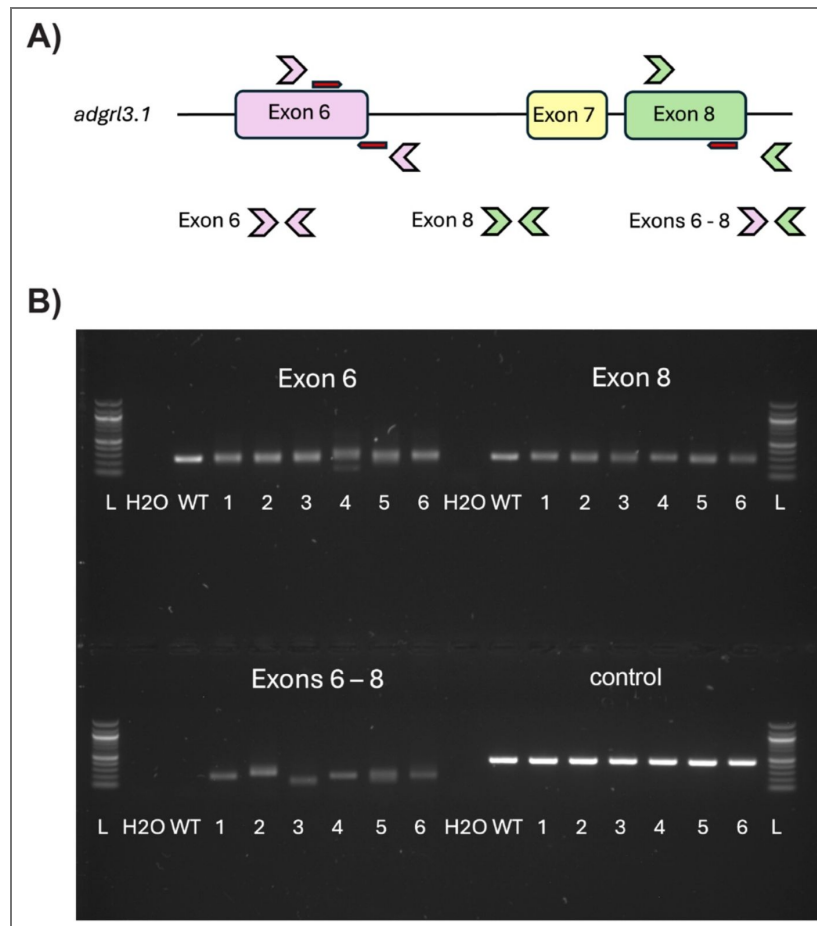


Table 1. Details of Exons across the CRISPRant mutants.

Exon 6 forward primer	CTCCCGTCACTGTTGACTATTG
Exon 6 reverse primer	CAGAGAGCAATGCACATACACA
Exon 8 forward primer	GCTGAACAGACAAAGGGAGAGT
Exon 8 reverse primer	TGAGCTATCTCATGCAAAAGGA
Exon 6 gRNA 1	CTGCTGAAATCTCCTGGCCA CGG
Exon 6 gRNA 2	TACTAACCTATGGTGCCAGG TGG
Exon 8 gRNA	GGCGGTGTCTTTTCCCCCAG GGG

Behaviour experiments in freely swimming larval zebrafish

The behavioural paradigm was performed using a custom closed-loop system, as previously described (Reynolds et al 2025 [DOI](#)). At 7 dpf, larvae were individually placed into custom-made circular acrylic dishes, measuring 120mm in diameter, 5mm in height, with a black rim and transparent base covered with diffusion paper. Dishes were filled with 50 ml of filtered fish water and left to acclimate for approximately 30 minutes in the system before experimentation. One computer was connected to 8 cameras and 4 projectors covering 8 dishes, therefore allowing tracking of up to 8 larvae simultaneously at a rate of 90 Hz. Combinations of stimuli were presented randomly. Swim bouts were considered turns rather than straight bouts if they had an absolute orientation change larger than 5°. Any trial where the animal does not move in the middle of the dish or could not be accurately tracked was removed automatically by the processing script.

Freely swimming paradigm

A custom visual stimulus system was developed using Panda3D and OpenGL Shader Language (GLSL) to render real-time dynamic grating patterns within the experimental arenas. Each larva was presented with a drifting sine wave grating at a constant speed of 0.9 cm/s, with a randomly assigned phase offset. Gratings moved either to the left or right relative to the larva's orientation. A total of 28 unique stimuli were generated by crossing stripe widths (wavelengths) of 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, and 0.32 arbitrary units (AU), corresponding to stripe widths ranging from approximately 0.64 mm to 41.14 mm (measured as one full cycle of black and white). Each of these was combined with one of two contrast levels (0.25 or 0.75) and one of two motion directions (leftward or rightward). Each stimulus was presented for 30 seconds, beginning with a 5-second rest period during which a black screen was displayed, followed by 25 seconds of drifting motion. Each fish experienced at least four trials of each stimulus, with the total experimental duration lasting one hour. Throughout the experiment, water temperature was maintained at approximately 26 °C.

Statistics and reproducibility

Descriptive statistics including normality were first completed to choose the appropriate test. All statistical analyses and tests were completed on Prism10 (GraphPad), and statistical significance was set at $p < 0.05$. Sample sizes were chosen to provide a high statistical power of > 0.8 and based on previous studies (Reynolds et al 2025 [DOI](#)). For all experiments, a minimum of $n = 62$ was run. Fish were chosen randomly within a Petri dish originating from between 2 - 6 parent batches per experiment, with batches collected and run on separate days.

Results

To investigate the role of *adgrl3.1* in ADHD-related behaviours using our VR approach, F0 CRISPRants were generated at the one-cell stage (see Methods) and tested at 7 days post-fertilization (dpf) in a closed-loop visual stimulation system (Figure 2A [DOI](#)).

At this developmental stage, the visual system is functionally mature, with retinotectal projections, lamination, and stable synaptic density established (Niell et al 2004 [DOI](#), Robles et al 2014 [DOI](#)). By 7 dpf, larvae also exhibit consistent beat-and-glide swimming behaviour (Budick & O'Malley 2000 [DOI](#), Fitzgerald et al 2019 [DOI](#)), making them well-suited for visual-motor behavioural assays while still representing a high-throughput model.

Larvae were placed into the closed-loop system (Figure 2B [DOI](#)), where their position and orientation were tracked in real time, and visual gratings were projected around them so that all visual stimuli were fixed to the animal's body axis, regardless of swimming trajectory. Crucially, this approach extends beyond classical locomotor assays conducted in static light/dark environments (Hillman et al 2024 [DOI](#)), where locomotor activity may conflate with anxiety-related responses (Blaser et al 2010 [DOI](#)). Instead, our paradigm used the optomotor response (OMR), feature-specific visual processing in which larvae align and swim with the moving gratings (Kist &

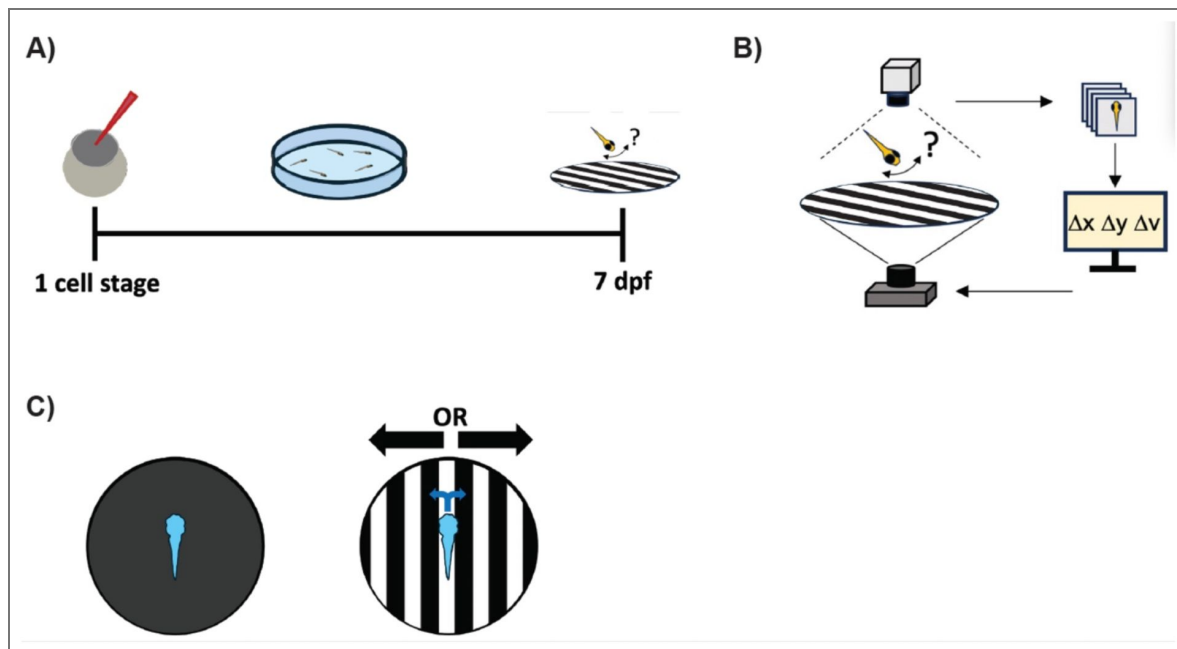


Figure 2. Experimental design of the *adgrl3.1* knockout mutant.

A) Schematic of the experimental workflow. F0 CRISPR-generated *adgrl3.1* mutants are created at the one cell stage and raised in a petri dish before being assayed at 7 dpf within the closed-loop virtual reality assay. **B)** Diagram of the closed-loop visual stimulation system. Individual larvae are tracked in real time within the arena (12cm diameter), with visual gratings updated and projected such that stimuli remained aligned to the animal's body axis. **C)** Optomotor response (OMR) stimulus paradigm. Each trial consisted of a 5 second baseline (black screen) followed by 25 seconds of drifting gratings moving either leftward or rightward. Gratings were presented at two contrast levels (0.25 and 0.75 AU) and across multiple spatial frequencies (stripe widths), allowing assessment of contrast- and frequency-dependent visuomotor responses.

Portugues 2019 [↗](#)). We designed a stimulus protocol comprising a 5 second baseline (black screen), followed by 25 seconds of gratings parallel to the zebrafish larvae moving either to the right or left (Figure 2C [↗](#)). Gratings were presented at two contrasts (0.25 and 0.75 AU) and at multiple stripe widths, as zebrafish responses follow a Gaussian-like dependence on spatial frequency, with a peak at the intermediate widths provided (Reynolds et al 2025 [↗](#)). This allowed us to assess whether loss of *adgrl3.1* alters not only overall locomotor drive as seen with previous paradigms assessing *adgrl3.1* mutants, but also specific visuomotor behavioural responses under different stimulus demands.

Increased locomotion in *adgrl3.1* mutants is independent from increased swim rate and bout duration

Our analysis first focused on the last 25 second stimulus period, during which moving gratings to either the left or right of the larvae's position were presented. This allowed us to separate behaviour from a possible general anxiety phenotype and instead identify any potential ADHD-associated behavioural ramifications from the *adgrl3.1* mutation. Quantification of bout speed (Figure 3A [↗](#)) showed that *adgrl3.1*^{-/-} larvae swam with significantly higher bout speeds than control-injected larvae (henceforth named "controls") (0.25 contrast: ****P < 0.0001; 0.75 contrast: **P = 0.039). This pattern of speed followed the expected Gaussian tuning to stripe width, with peak speeds occurring around 10.3 mm stripes, consistent with previous reports (Reynolds et al 2025 [↗](#)). Importantly, the increased speed phenotype is more pronounced under low contrast (contrast = 0.25) conditions than high contrast (contrast = 0.75) conditions. Here, *adgrl3.1*^{-/-} larvae swam noticeably faster than controls when at the optimal stripe width of 10.3 mm (0.25 contrast = 2.3412 cm/s ± 0.04716; 0.75 contrast = 2.3192 cm/s ± 0.06153), suggesting potential reduced sensitivity to weak visual cues. This effect was most pronounced under low-contrast conditions, particularly at 10.3 mm stripe width, indicating that genotype differences were larger when visual stimulation was weaker.

We next examined the duration of individual swim bouts (Figure 3B [↗](#)). Both control and mutant *adgrl3.1*^{-/-} larvae exhibited the expected Gaussian modulation of bout duration across stripe widths, with an increase from ~140 ms at 0.6 mm to ~170 ms at 10.3 mm before decreasing again. Here, *adgrl3.1*^{-/-} mutants showed a small rise in bout duration compared with control larvae, but this difference was only significant at 0.6 mm and 10.3 mm stripe widths under low contrast (0.25). Similarly, swim rate, measured as bouts per second, was ~1 bout/s across conditions (Figure 3C [↗](#)). Both control and *adgrl3.1*^{-/-} mutants displayed a Gaussian tuning to stripe width, peaking at 10.3 mm, with peaks of (control: 1.2785 Hz ± 0.03899; mutant: 1.3867 Hz ± 0.03147) at 0.25 contrast, and (control: 1.4091 Hz ± 0.03439; mutant: 1.4164 Hz ± 0.03712) at 0.75 contrast, similar to previous results for larvae in this assay (Reynolds et al., 2025 [↗](#)). However, overall, the swim rate was not dependent on *adgrl3.1*^{-/-} mutation, with a lack of significance in comparison to control (0.25 contrast: P = 0.1985; 0.75 contrast: P = 0.1531). This may suggest that *adgrl3.1* loss does not strongly alter bout duration or swim rate, and therefore the ADHD-related hyperactivity within this model is not expressed through extended bout execution or number of bouts, but rather through other locomotor parameters.

Locomotion phenotype of *adgrl3.1* mutants appears due to modifications in interbout interval and length of bouts

Next, we concentrated on the assessment of the interbout interval (IBI), where we observed the most pronounced difference between groups (Figure 3D [↗](#)). Control larvae exhibited variable IBIs, forming an inverse Gaussian curve with a minimum value between 5.6 - 10.3 mm stripe width (Contrast 0.25: 0.6936 secs ± 0.03086; Contrast 0.75: 0.6050 secs ± 0.02860). By contrast, *adgrl3.1*^{-/-} mutants had consistently shorter IBIs across all conditions (0.25 contrast: **P = 0.0012; 0.75 contrast: ***P = 0.007), with reduced variability. Frequent, stereotyped bouts may indicate impaired inhibitory gating and reduced behavioural flexibility, with a lack of transition in internal

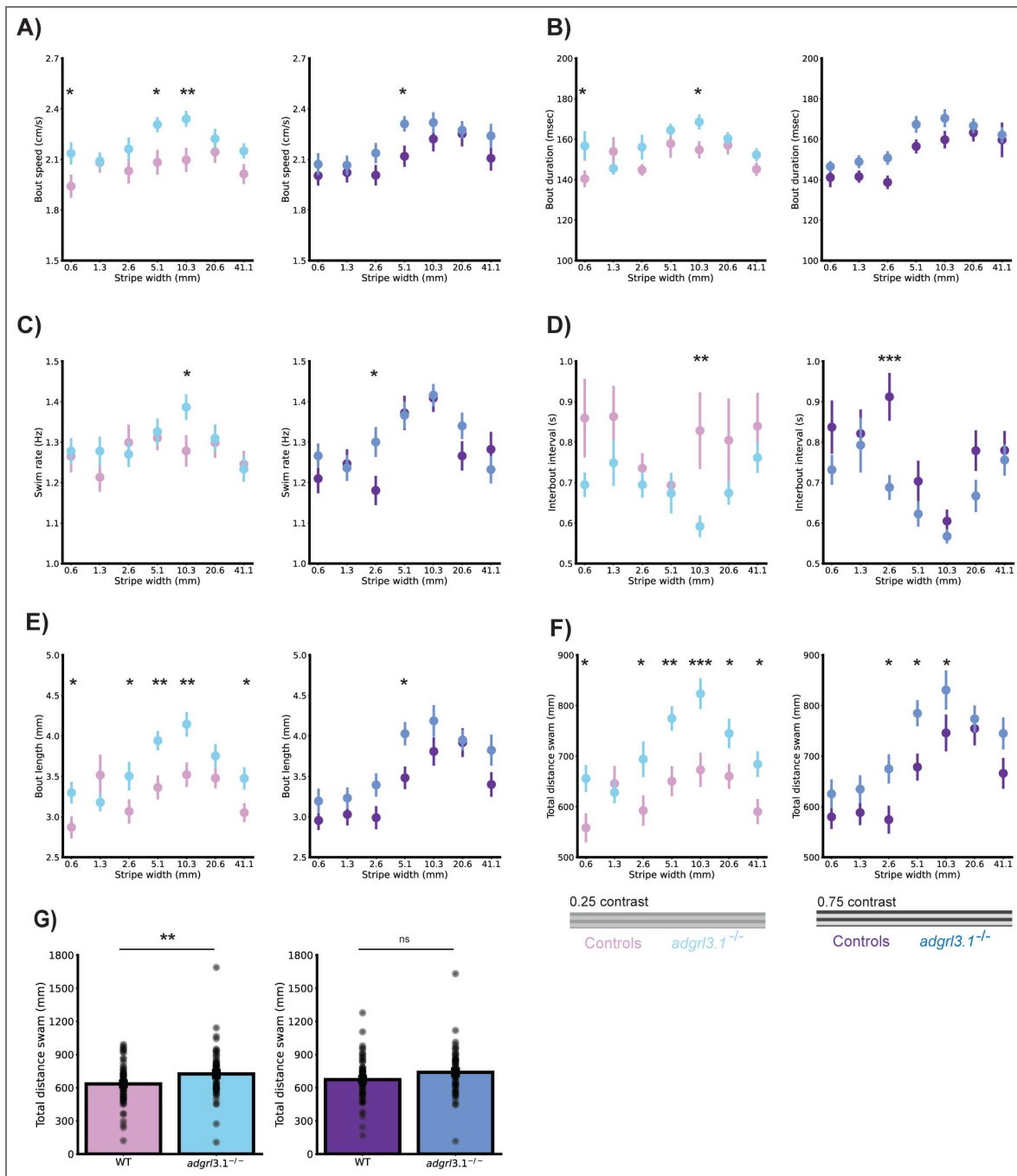


Figure 3. Increased locomotor output in *adgrl3.1* mutants is driven by altered bout structure.

A-F) Various swim behaviours measured across stripe widths and contrast conditions (Low contrast at 0.25 AU and high contrast at 0.75 AU) for WT (pink and purple colours) and *adgrl3.1* mutants (light and dark blue colours). **A)** Bout speed. **B)** Bout duration. **C)** Swim rate (bouts per second). **D)** Interbout interval (IBI). **E)** Bout length (distance travelled per bout). **F)** Total distance travelled. **G)** Total distance travelled at low (0.25) and high (0.75) contrast for *adgrl3.1* mutant and control larvae. A – G) $n = 62$ for *adgrl3.1* mutant larvae, $n = 70$ for control larvae. All error bars are mean $\pm$ s.e.m. over fish. Asterisks indicate significance * $P < 0.05$, *** $P < 0.001$, A-F) 2-way ANOVAs with multiple comparisons and corrected with Tukey test; G) two-tailed t-test with Welch's correction.

state (Johnson et al 2020 [↗](#)). This suggests that the loss of *adgrl3.1* elevates baseline arousal through shorter interbout intervals while preserving swim rate, producing a phenotype of heightened but less exploratory visuomotor activation.

Analysis of bout length also revealed that *adgrl3.1*^{-/-} mutants swam significantly longer distances per bout than control larvae (Figure 3E [↗](#)). This difference was most marked at low contrast (0.25: ****P < 0.0001), with peak bout lengths at 10.3 mm stripes (Control: 3.5221 mm ± 0.1518; Mutant: 4.1448 mm ± 0.1517). At high contrast (0.75: ***P = 0.0001), both groups followed a similar pattern, but group differences were smaller. These findings suggest that when visual stimuli are harder to detect, mutants respond with overextended motor output, possibly corresponding to over compensatory hyperactivity under ambiguous sensory conditions, consistent with attentional deficits.

Finally, we examined the total distance swum during each trial (Figure 3F-3G [↗](#)), facilitating clear comparisons with prior studies that measured baseline locomotor activity in *adgrl3.1* knockouts (Fontana et al 2023 [↗](#), Regan et al 2021 [↗](#), Sveinsdottir et al 2023 [↗](#)). Mutants consistently swam farther than control larvae, with peaks at 10.3 mm stripe widths both in the lower and higher contrasted stimuli (Contrast 0.25 ***P = 0.0003: Controls = 672.9481 mm ± 33.6654; Mutants = 823.5271 mm ± 30.0897.

Contrast 0.75 *P = 0.0437: Controls = 746.0387 mm ± 36.2865; Mutants = 830.8605 mm ± 38.8027). As with bout length, a significantly farther distance is travelled by the *adgrl3.1*^{-/-} mutants when at a lower contrast, reflecting sustained hyperlocomotion and high arousal, canonical features of ADHD-like behaviour. These findings of elevated locomotor output in *adgrl3.1*^{-/-} mutants replicates previous studies and validates the sensitivity of our closed-loop paradigm to ADHD-related phenotypes. In addition, it draws light to further granular analysis of ADHD-like behaviours, with *adgrl3.1* knockout larvae displaying hyperactive, stereotyped swimming patterns with reduced inhibitory control and increased motor drive.

Reduced behavioural variability in *adgrl3.1* mutants

To assess behavioural consistency across genotypes, we quantified the coefficient of variation (CV) of key locomotor parameters, namely IBI and average speed (Figure 4A [↗](#)). The CV of the IBI provides a measure of the variability in the timing between consecutive swim bouts, therefore reflecting how flexibly larvae initiate movement across repeated stimuli. Across all stimulus conditions, *adgrl3.1* mutant larvae exhibited markedly lower IBI variability compared to controls, with consistently smaller CV values and narrower distributions (0.25 contrast: ***P = 0.003; 0.75 contrast: **P = 0.0057). This pattern indicates that *adgrl3.1*^{-/-} mutants produce swim bouts with greater regularity, resulting in a more stereotyped behavioural profile. In contrast, control larvae displayed higher variability in IBI, reflecting a capacity for adaptive, spontaneous movement timing that supports flexible behavioural responses to changing sensory contexts. Importantly, this reduction in IBI CV in mutants was consistent across both stripe widths and contrast levels, pointing to a generalised decrease in motor control rather than a stimulus-dependent effect.

By comparison, the CV of average swimming speed remained stable across all stripe width and contrast conditions, although it was lower in *adgrl3.1*^{-/-} mutants than control larvae (0.25 contrast: ****P < 0.0001; 0.75 contrast: ****P < 0.0001). This indicates that while the magnitude of movements (i.e. how fast they swim) remains stable across stimuli, *adgrl3.1*^{-/-} mutants tend to produce these movements with less variability, reinforcing the view of a more rigid or stereotyped behavioural pattern. In sum, mutants showed reduced coefficient of variation for IBI and average speed across conditions, indicating more stereotyped locomotor structure.

Mutants show subtle contrast-dependent decline in vigilance within trials

We next quantified how orienting performance evolved over time within individual stimulus presentations. Larval orientation was segmented into discrete swim bouts, and movements aligning with the direction of visual motion (orientation change > 5°) were classified as successful

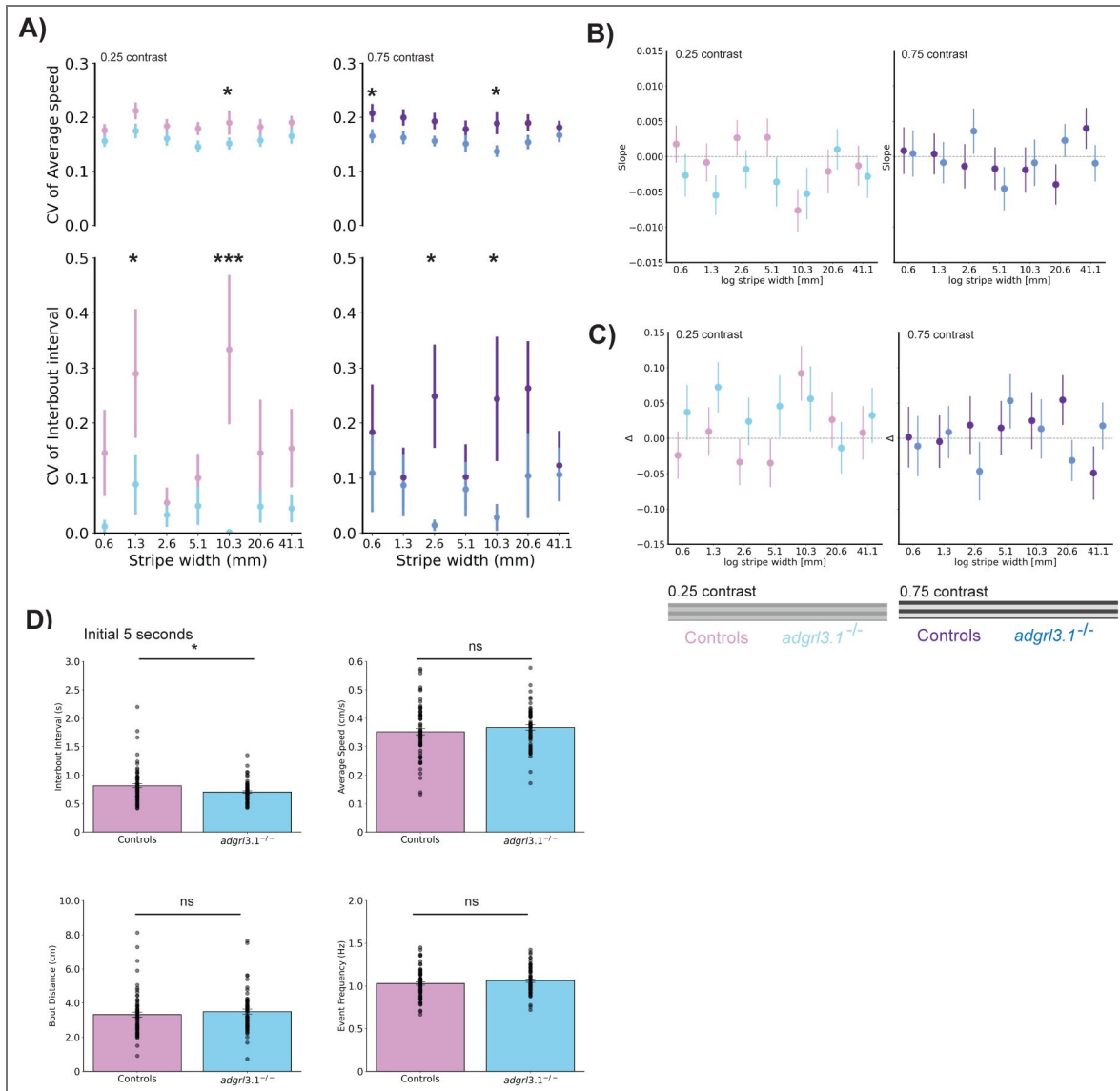


Figure 4. Reduced behavioural variability and subtle vigilance deficits are present in *adgrl3.1* mutants.

A) Coefficient of variation (CV) for interbout interval (IBI) and average speed across stimulus conditions to indicate flexibility in motor output. B) Within-trial slope of successful optomotor responses over stimulus conditions. Negative slopes indicate declining performance (vigilance decrement). C) Within-trial delta (Δ) in response success between early and late trial phases across stimulus conditions. Larger positive deltas indicate decline in performance over trials. C) Baseline locomotor behaviour during the pre-stimulus (black screen) period. A – D) $n = 62$ for *adgrl3.1* mutant larvae, $n = 70$ for control larvae. All error bars are mean $\pm$ s.e.m. over fish. Asterisks indicate significance * $P < 0.05$, *** $P < 0.001$, A-C) 2-way ANOVAs with multiple comparisons and corrected with Tukey test; D) two-tailed t-test with Welch's correction.

tracking responses. The proportion of successful responses was then analysed across sequential time bins within each trial. The *within-trial slope* represents the linear trend in successful responses over time: a negative slope indicates declining performance (vigilance decrement), whereas a positive slope denotes increasing engagement. Mean $\pm$ SEM slopes were calculated per genotype and stimulus contrast (Figure 4B [↗](#)).

Although genotype differences were not statistically significant (0.25 contrast: $P = 0.1484$; 0.75 contrast: $P = 0.8050$), qualitative trends emerged. At low contrast, mutant fish tended to exhibit more negative slopes than control larvae, suggesting a gradual decline in response success within a single trial. This trend implies that under more ambiguous visual conditions, mutants may lose vigilance or disengage more quickly. In contrast, at high contrast, slopes in both groups clustered around zero, indicating stable tracking performance throughout the trial. These subtle within-trial trends provide insight into short-timescale attention dynamics. Even in the absence of overt performance deficits, the directionality of the slopes suggests that *adgrl3.1* mutants may show diminished capacity to sustain visuomotor engagement when stimulus salience is low. This mirrors findings in ADHD populations, where attentional performance often deteriorates more rapidly under low-arousal or low-salience conditions (Strauss et al 2018 [↗](#), Tucha et al 2017 [↗](#)).

To complement the slope analysis, we also calculated the within-trial delta (Δ)—the difference in success rate between the first- and last-time bins of each trial (Figure 4C [↗](#)). Whereas slope captures the rate of change, delta quantifies the overall magnitude of performance change within a trial. Positive delta values indicate declining performance, while negative values reflect improvement over time. Consistent with the slope data, *adgrl3.1* mutants showed larger positive deltas under low contrast, indicating a greater overall drop in performance from trial start to end. At high contrast, deltas were near zero for both groups, reflecting stable performance. Across repeated trial blocks, no systematic genotype differences emerged (0.25 contrast: $P = 0.1353$; 0.75 contrast: $P = 0.6967$), suggesting that while overall learning or adaptation across trials was similar, subtle within-trial fluctuations in attention or vigilance were contrast dependent. Although genotype effects were not statistically significant at either contrast, mutants showed a tendency toward more negative slopes and larger deltas under low-contrast conditions. These analyses therefore suggest a possible contrast-dependent reduction in within-trial tracking stability in mutants, but this effect should be regarded as preliminary.

Baseline motor behaviour reveals altered temporal regulation in *adgrl3.1* mutants

Before the onset of each visual stimulus, all trials begin with a 5-second black screen during which larvae experienced no visual motion cues. This short pre-stimulus period provided a measure of spontaneous locomotor behaviour in the absence of visual motion cues. Analysing this phase allows assessment of baseline motor excitability, independent of visual feedback, providing a further test for hyperactive or impulsivity-like phenotypes.

To determine whether *adgrl3.1* mutants exhibit altered baseline motor control, we extracted behavioural data from this black phase for both control and *adgrl3.1*^{-/-} larvae (Figure 4D [↗](#)). For each fish, four parameters were computed – namely the IBI, average speed, bout distance, and bout frequency to uncover general activity rate. Among these metrics, only IBI showed a significant difference between genotypes, with *adgrl3.1* mutants exhibiting shorter IBI values compared to control larvae (control: 0.8163 secs, mutant: 0.7052 secs; $*P = 0.0109$). This reduction indicates altered temporal organisation of baseline swimming in the mutant larvae. By contrast, average speed, bout distance, and event frequency did not differ significantly between genotypes (all $p > 0.05$), suggesting that the overall magnitude and intensity of movement were comparable. This gives further evidence that the temporal structure of swimming, specifically the timing of movement initiation, is altered within *adgrl3.1*^{-/-} larvae. These findings highlight that loss of *adgrl3.1* increases the baseline excitability and a lower threshold for initiating motor events.

Together, our results show that the closed-loop virtual reality system allows for collection of richer data sets when assessing ADHD-relevant candidate risk genes and therefore improves the more detailed identification of phenotypes. This is important for the stratification of disorder phenotyping and the future translational use, such as in relation to drug discovery.

Discussion

We developed a closed-loop virtual reality paradigm to explore the behavioural consequences of *adgrl3.1* loss-of-function in larval zebrafish, providing the first high-resolution phenotyping of this ADHD-linked gene within a stimulus-driven context. Unlike conventional light/dark locomotion paradigms, our approach enabled context-dependent, visual-driven behavioural readouts, disentangling hyperactivity from anxiety-related locomotor responses (Fontana et al 2023 [↗](#), Regan et al 2021 [↗](#), Sveinsdottir et al 2023 [↗](#)). While confirming the previously reported hyperactivity within the *adgrl3.1*^{-/-} mutants, the assay revealed more granular mechanisms underlying this phenotype. Here, we found alterations in bout timing, reduced behavioural flexibility, and potential changes in sensory engagement.

A refined understanding of hyperactivity in *adgrl3.1* mutants

At a gross level, *adgrl3.1*^{-/-} mutants travelled greater distances than controls, consistent with prior studies (Fontana et al 2023 [↗](#), Regan et al 2021 [↗](#), Sveinsdottir et al 2023 [↗](#)). Crucially though, this increase does not arise from an elevated swim rate or average speed. Instead, hyperactivity was driven by a combination of shorter inter-bout intervals and longer bout lengths, resulting in higher frequency and extended bouts of swimming. This demonstrates that hyperlocomotion reflects a reorganisation of locomotor timing rather than a uniform increase in activity. Importantly, such fine-scale insights are not possible in standard light/dark paradigms.

IBI was the most sensitive metric, showing consistently reduced duration and variability across visual conditions. While control larvae exhibited flexible IBI patterns, mutants instead produced more rapid and stereotyped bout timings. Shorter and more consistent IBIs may reflect impaired inhibitory control, aligning with models of impulsivity in ADHD (Nigg 2001 [↗](#)), with more frequent bouts possibly indicating elevated baseline arousal, consistent with theories of dysregulated noradrenergic signalling in ADHD (Biederman & Spencer 1999 [↗](#), Sigurdardottir et al 2021 [↗](#)). However, reduced variability may also suggest diminished behavioural flexibility to changing environmental demands. Given that IBIs are linked to the integration of sensory input with internal state (Johnson et al 2020 [↗](#)), these findings suggest that *adgrl3.1* disruption alters this integration, promoting high-frequency, less variable responses.

Mutants also displayed longer swim bouts, particularly under low-contrast conditions, indicating altered responses to weak or ambiguous sensory input. This could reflect impaired discrimination of visual stimuli, driving compensatory motor output. In ADHD, such dysregulated effort allocation - producing either over- or under-engagement depending on task demands - is a recurrent theme in both behavioural and cognitive domains. By revealing these context-specific effects, the VR assay provides a translationally relevant model for studying how ADHD risk genes modulate sensory-motor decision-making.

Behavioural variability as an ADHD endophenotype

Further behavioural variability is seen through coefficient of variation. Control larvae displayed higher variability in IBI timing, whereas mutants showed significantly lower variability. Reduced trial-to-trial variability has been noted in some human ADHD studies, particularly in motor timing tasks, and has been proposed as a signature of dysregulated internal state modulation. The fact that speed variability remained similar across groups suggests that the *adgrl3.1* mutation primarily affects timing patterns rather than movement vigour, further underscoring the specificity of this phenotype.

Implications for ADHD models and future work

Together, these findings extend the characterisation of *adgrl3.1* mutants beyond gross hyperactivity to reveal distinct dimensions of impulsivity, reduced flexibility, and altered sensory–motor coupling. By resolving behaviour into quantifiable components, this approach provides a more precise link between gene function and behavioural phenotype than standard locomotor assays.

In standard larval assays, increased distance travelled is often treated as a unitary hyperactivity phenotype. Our data show that this is too coarse –the mutant phenotype can instead be decomposed into shorter interbout intervals, longer bout lengths, and reduced variability, indicating that elevated activity arises from specific changes in the temporal organisation of locomotion. This level of resolution is important because superficially similar hyperactive phenotypes may emerge from different underlying processes. Fine-grained behavioural analysis therefore provides a more informative bridge between gene disruption and behavioural expression than gross locomotor measures alone.

The present assay provides a controlled way to quantify how locomotor behaviour is organised under behaviour-contingent visual feedback. The observed genotype differences are interpreted as altered visuomotor behavioural expression in a dynamic environment. Future work combining this assay with pharmacological perturbation, circuit imaging, or explicitly validated behavioural tasks will be needed to determine how these changes map onto specific ADHD-relevant cognitive domains.

We demonstrate that hyperactivity emerges from shorter and more stereotyped IBIs combined with longer bouts, reflecting a shift in the temporal dynamics of motor output rather than indiscriminate increases in activity. These alterations mirror core ADHD phenotypes of impulsivity, dysregulated arousal, and reduced behavioural flexibility. By uniting genetic manipulation with fine-scale behavioural quantification, this work not only addresses critical gaps in our understanding of ADGRL3 function but also establishes a scalable platform for drug discovery and mechanistic dissection of neurodevelopmental disorders.

Data availability

All behavioural data and analysis code generated during this study have been uploaded to github.com/Hindges-Lab/Publications-Reynolds-2026-ADGRL and are publicly accessible.

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Additional information

Author Contributions

RH and PR conceived the overall study and designed the experiment. ER created the knockout, PR and CD-E performed all experiments, and PR analysed the data. PR wrote the first draft of the manuscript with input from all authors.

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Peer reviews

Reviewer #1 (Public review):

Summary:

Reynolds and colleagues provide a deep phenotypic analysis of behavior in *adgrl3.1* mutant zebrafish at larval stages using a closed-loop optomotor response (OMR) assay. The analyses conducted are interesting and extract new locomotor phenotypes with possible relevance to the role of *adgrl3.1* in ADHD. Reduced interbout interval (both in the OMR assay and in dark rest periods) and increased distance moved provide greater resolution on hyperactivity phenotypes already described in these mutants. Reduced variation in interbout interval and reduced variation in swim speeds throughout the assay provide new insights into how behavior is altered; the authors suggest that these findings reflect more stereotyped, less flexible behavior in *adgrl3.1* mutant animals. Analyses of task performance are interesting and could help understand how / whether animals maintain vigilance over time in the OMR assay and reveal trends in *adgrl3.1* mutants relative to siblings, but ultimately do not identify significant phenotypes for *adgrl3.1* mutants. While methods are extremely clear and analyses and phenotypic insights are solid, the authors do not provide sufficient support for assertions that their paradigm separates anxiety from locomotor activity or extracts phenotypes central to ADHD (impulsiveness, attention, etc). In some instances, interpretation of behavioral phenotypes in the context of disease is difficult to follow or not well supported with citations, etc.

Strengths:

- (1) Deeper phenotypic analysis of *adgrl3.1* locomotor phenotypes reveals changes to bout timing/initiation of locomotion as potentially causative for broader hyperactivity phenotypes previously reported.
- (2) Interesting dissection of OMR performance over time and variability in locomotor parameters, assessment of OMR performance in high- and low-contrast.
- (3) Methods are clearly described and considered to be rigorous.

Weaknesses:

- (1) The introduction does not clearly spell out why the closed-loop OMR assay is expected to capture phenotypes central to ADHD (impulsiveness, attention, etc). Similarly, it's stated in the discussion that hyperactivity is driven by shorter inter-bout intervals and longer bout lengths...reflecting a reorganization of locomotor timing," and that "such fine-scale insights are not possible in standard light/dark paradigms." But in fact, each of these parameters was

examined in the dark periods and could be assessed in a standard light/dark assay. As explained at the end of the discussion, this work provides a detailed analysis of locomotion and extracts new and interesting phenotypes, but the assertion that this is a function of the assay / that the assay is uniquely relevant to ADHD is not well-supported. The final statement of the introduction more accurately captures the advantages of the assay used: "this allowed us to assess whether loss of *adgrl3.1* alters not only overall locomotor drive...but also specific visuomotor behavioral responses under different stimulus demands."

(2) The statement early in the results that "this approach extends beyond classical locomotor assays conducted in static light / dark environments, where locomotor activity may conflate with anxiety-related responses" and later that the closed-loop OMR assay "disentangles hyperactivity from anxiety-related responses" are not well-supported. Anxiety states could influence performance on OMR (Braun et al., 2024, *Molec Psychiatry*).

(3) Some interpretations of the phenotypes are not well supported by citations and may be overstated. For example, "*adgrl3.1* elevates baseline arousal...producing a phenotype of heightened but less exploratory visuomotor activation." Since bout duration is increased alongside reduced interbout interval and increased total distance traveled, reduced exploration is not well-supported by the data. Later in the results, it's suggested that the increase in distance traveled reflects "over compensatory hyperactivity under ambiguous sensory conditions, consistent with attentional deficits." It's not clear what this means - references would be helpful to create links between hyperactivity and detection of ambiguous sensory conditions, and also between hyperactivity under these conditions and attentional deficits.

<https://doi.org/10.7554/eLife.111844.1.sa3>

Reviewer #2 (Public review):

In the study by Reynolds et al., the authors propose a new behavioral approach for ADHD evaluation using a genetically modified zebrafish model. The study is interesting and has potentially important implications for the field. However, there are several methodological, analytical, and validation-related issues that should be addressed before the study can be considered scientifically rigorous.

Comments:

(1) Introduction section

What is the epidemiological evidence supporting the prevalence of *ADGRL3* dysfunction in the human population? The authors should consider adding this information, as well as clarifying where *ADGRL3* mutations rank among other genetic variants associated with ADHD.

I suggest reconsidering the sentence "quantifiable behavioural repertoires that complement rodent approaches." Zebrafish studies do not simply complement rodent studies; they can serve as independent pharmacological and toxicological tools that may be used in parallel with rodent models.

The statement "forced light/dark (FLD) locomotion test" is too broad. Are the authors referring to the Visual Motor Response Test? If so, this is a robust assay that can evaluate not only anxiety-like responses but also locomotor state, arousal, decision-making, and potential cognitive impairment in larvae. A clearer description of the assay is necessary, especially to justify the statement that "while useful for detecting overall activity differences, it cannot determine whether increased movement reflects hyperactivity, altered arousal, disrupted behavioural control, or anxiety-like responses." In contrast, subtle behavioral changes across

light and dark phases can be highly informative when velocity, time moving, and anxiety-like responses are analyzed together.

(2) Methods section

The zebrafish husbandry section lacks several essential details. The authors should include fundamental information, such as the embryo medium used, how embryos were obtained, the age of the breeding adults, and how larval age was determined in hours post-fertilization. These details are necessary for proper interpretation and reproducibility of the data.

Why was the mutant DNA not sequenced? Although agarose gel electrophoresis can provide useful preliminary evidence of mutation, it cannot precisely determine the number or nature of base-pair changes. This information is essential because different mutations can have distinct impacts on gene function.

The sentence "All statistical analyses and tests were completed on Prism10 (GraphPad)" is insufficient. It should be specified which statistical tests were used, including assumptions tested, post hoc comparisons, correction methods, and how experimental replicates or batch effects were handled.

Overall, the methods section lacks sufficient information to support the scientific rigor of the study. It is unclear whether every individual evaluated behaviorally was injected and then only a subset was genetically confirmed, or whether stable breeding matrices were generated and all experimental individuals were derived from these parents. The manuscript mentions "2-6 parent batches per experiment, with batches collected and run on separate days," but the genetic origin and validation of these batches remain unclear.

If embryos were injected for each batch, how did the authors ensure that the mutation was homogeneous enough across individuals to consider them equivalent? How did the authors confirm that the mutation was homozygous or present across all relevant cells? Zebrafish embryos remain at the single-cell stage for only a short period before mitosis begins. Without detailed information regarding breeding timing, embryo collection, injection timing, and sequencing validation, it is difficult to determine whether the injected embryos developed homogeneous mutations or mosaic patterns.

Additionally, to claim a knockout model, protein-level validation, such as Western blotting or another protein expression assay, should be provided. At present, there appears to be some confusion between a knockout and a knockdown model.

To validate a new behavioral protocol, the authors should compare their assay with an established gold-standard behavioral paradigm using the same experimental batch. They should clarify why this comparison was not performed.

<https://doi.org/10.7554/eLife.111844.1.sa2>

Reviewer #3 (Public review):

Summary:

The study provides an in-depth phenotyping of a novel zebrafish larval model of ADHD. This topic is interesting, and the model and the approach are relevant and well-justified. While the paper has a massive amount of high-quality data, the general structure and presentation of this material lack focus and a clearly articulated rationale.

Strengths:

The paper is methodologically sound, well-presented, and well-illustrated. It has a clear logical rationale and reasonable experimental design.

Weaknesses:

The amount of high-quality data is impressive, yet the general structure and presentation of this material lack focus and a clearly articulated rationale.

- (1) First, it is unclear why VR is necessary here. It needs a better explanation in both the abstract and the intro section of the manuscript.
- (2) Second, data need to be better presented (most important things first, least important - shorter or move to the Supplementary materials). Currently, it is too much to be clear and easy to follow.
- (3) Discussion needs to better state the novelty and the significance of these findings. What does the study offer that is new? Why was it important to perform? What big questions does it address?
- (4) The authors should better discuss the study limitations and future directions of research.
- (5) There should be a stronger conclusion with a take-home message to emphasize what new information the study brings and why it is important.
- (6) The overall style of the paper should be improved. Currently, it reads like a dry bulleted CRO report, not a usual scholarly paper.
- (7) Optimize the text flow. Currently, the overall flow of the discussion needs to be smoother - it now reads as a selection of bulleted paragraphs, with few connections between them.

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Author response:

We would like to thank the editorial team and the reviewers for their thoughtful assessment of our manuscript. We are highly encouraged that the reviewers found our closed-loop OMR virtual reality assay to be a valuable new behavioural paradigm, and that our findings regarding the early behavioural phenotypes in *adgrl3.1* mutant zebrafish provide solid, high-quality data of broad interest to the community. We also appreciate your constructive feedback regarding the structural flow of our manuscript, the need for tighter conceptual framing, and the request for more rigorous methodological explanation and improved presentation. In our upcoming revision, we plan to directly address the suggestions raised to ensure clarity of our work.

For our revised manuscript, we will ensure to focus on fully contextualising our conceptual rationale and unique utility of our behavioural assay, ensuring a clear link to clinical relevance. This means we will provide further clarity on the rationale and relevance of the paradigm and interpretations of behaviours, while ensuring we are not overstating the absolute separation of anxiety-like states from hyperactivity and contextualising our findings within the broader literature. In addition, as suggested by the reviewers, we will improve the Abstract to explicitly articulate the unique advantages of the closed-loop OMR virtual reality paradigm over standard static assays and provide definitions. In addition, we will also revise our interpretations in the Discussion, such as around swim speed, exploration, and visual sensitivity, to ensure they are fully grounded and supported with clear flow.

Equally important to our revision is to clarify genetic validation, methods and statistical rigour as recommended by the reviewers. Therefore, we will update the Methods to explicitly

detail further husbandry details, such as the embryo medium used, breeding protocols, and the exact timings in hours post-fertilisation. To resolve any ambiguity surrounding our genetic model, we will provide further explanations and include details on genotype and sequencing protocols. Where needed, we will also update the statistical analysis and expand the Methods accordingly to detail all statistical reporting for clarity.

Finally, as suggested, we will also improve the overall structure, flow, and accessibility. In the Results, we will ensure the core behavioural phenotypes take primary focus throughout the writing. Furthermore, we will also improve the Discussion to ensure it is a unified, cohesive narrative that smoothly integrates our main behavioural findings with genetic model validation, limitations, and future directions. Additionally, we will update all visual presentations, figure formatting, and labelling to ensure accessibility and improved readability.

We are incredibly grateful to the reviewers for their insightful recommendations. We are confident that by integrating their revisions, we will substantially strengthen the clarity of the work and better demonstrate its impact.

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