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An affective disorder in zebrafish with mutation of the glucocorticoid receptor

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Abstract

Upon binding of cortisol, the glucocorticoid receptor (GR) regulates the transcription of specific target genes, including those that encode the stress hormones corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH). Dysregulation of the stress axis is a hallmark of major depression in human patients. However, it is still unclear how glucocorticoid signaling is linked to affective disorders. We identified an adult-viable zebrafish mutant in which the negative feedback on the stress response is disrupted, due to abolition of all transcriptional activity of GR. As a consequence, cortisol is elevated, but unable to signal through GR. When placed into an unfamiliar aquarium (‘novel tank’), mutant fish become immobile (‘freeze’), show reduced exploratory behavior and do not habituate to this stressor upon repeated exposure. Addition of the antidepressant fluoxetine to the holding water and social interactions restore normal behavior, followed by a delayed correction of cortisol levels. Fluoxetine does not affect overall transcription of CRH, the mineralocorticoid receptor (MR), the serotonin transporter Serta or GR itself. Fluoxetine, however, suppresses the stress-induced upregulation of MR and Serta in both wildtype fish and mutants. Our studies show a conserved, protective function of glucocorticoid signaling in the regulation of emotional behavior and reveal novel molecular aspects of how chronic stress impacts vertebrate brain physiology and behavior. Importantly, the zebrafish model opens up the possibility of high-throughput drug screens in search of new classes of antidepressants.

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Conflict of Interest

The authors declare that they do not have competing financial interests.

Keywords

Stress; Depression; Anxiety; Glucocorticoid; Serotonin; Fish model

Perception of a threatening environmental stimulus elicits endocrine changes that in turn trigger a cascade of physiological and behavioral processes. This so-called 'stress response' is mediated by the hypothalamus-pituitary-adrenal (HPA) axis. Increased synthesis and release of CRH in the hypothalamus promote the release of ACTH (encoded by the *pomc* gene) from the pituitary gland into the circulation. ACTH stimulates the production of glucocorticoids from the adrenal gland, whose fish homolog is named interrenal organ. In teleost fish and humans, the major glucocorticoid hormone is cortisol (corticosterone in rodents). Cortisol levels not only increase in response to stress, but also exhibit a circadian rhythm, peaking during daytime in both zebrafish and humans[1,2].

In humans, hyperactivity of the HPA axis is the most consistent endocrine parameter associated with major depression[3, 4]. Moreover, even in non-diseased individuals, a high cortisol level in the circulation (hypercortisolemia) is considered a risk factor, predisposing to the development of the disease[5]. Correction, i. e. lowering, of cortisol is often used clinically to monitor the success of therapeutic intervention[4-6]. Both extreme short-term stress and mild chronic stress can precipitate affective disorders including depression and pathological anxiety, demonstrating a causal contribution of stress to long-term mood changes. However, it is unclear which component(s) of the HPA axis is/are responsible for the neural circuitry changes that result in depression. There is no obvious link between HPA-related hormones and the pharmacological treatments that have proven to be effective in many forms of depression, such as benzodiazepines (e. g., diazepam = Valium), which modulate GABA-A receptors, and selective serotonin reuptake inhibitors (SSRIs, e. g., fluoxetine = Prozac).

Understanding the molecular crosstalk between the HPA axis and depression is important, as it will inform the search for better therapies. In the brain, cortisol is known to signal through a ligand-dependent transcription factor, the glucocorticoid receptor (GR). Upon binding of cortisol, GR forms homodimers and translocates from the cytoplasm to the nucleus, where it binds specific DNA sequences called glucocorticoid response elements (GREs)[7], to regulate the expression of target genes in a tissue-specific manner[8, 9]. These GREs are often highly conserved and can serve as enhancers or repressors of gene transcription[10]. GR can also form heterodimers with other transcription factors, largely to repress transcription of target genes. GR is ubiquitously expressed and is only occupied by cortisol during the diurnal peaks or under stress. A related factor, the mineralocorticoid receptor (MR), is more sparsely expressed in the brain and has a tenfold higher ligand affinity than GR. Together, MR and GR act over a wide range of cortisol concentrations. Signaling through GR, within the physiological range, is thought to terminate the stress reaction and facilitate recovery and memory storage[11].

Both an excess and a shortage of GR signaling might be detrimental to brain function. Some evidence exists in rodents that glucocorticoids have negative effects on neurogenesis and synaptic plasticity in the hippocampus and that these effects are reversed by SSRI

administration over several weeks[12]. On the other hand, GR activity appears to be protective of the brain by dialing down the stress response. In the latter view, depression is characterized by 'glucocorticoid resistance', and the disease-causing culprit is excess of some other hormone, perhaps CRH[8,9,13]. In humans with functioning HPA axis, administration of Dexamethasone (Dex, a synthetic ligand of GR) suppresses cortisol. In depressed patients with hyperactivated HPA axis, however, this effect is blunted[11]. This is evidence in favor of glucocorticoid resistance in at least some forms of major depression.

We have identified an adult viable zebrafish mutant, *gr*^{s357} in which a single base-pair change completely disrupts GR transcriptional regulation of its target genes. We show that fish homozygous for the *gr*^{s357} mutation display a hyperactivated HPA axis, blunted suppression of cortisol by Dex and increased depression-like behavior in response to mild stress. Diazepam and Fluoxetine treatments, as well as social interactions, reverse the abnormal behavior. These results reveal a phylogenetically conserved link between the HPA axis and affective disorders in vertebrates and strongly support the view that glucocorticoid resistance, and not excessive GR signaling, contribute to the development of depression.

Materials and Methods

Positional cloning and genotyping of *gr*^{s357}

By linkage mapping using 1,230 meioses, the *s357* mutation was mapped to chromosome 14, between microsatellite markers *z9017* (0.08 cM) and *z22094* (0.16 cM). Four partly overlapping BAC clones, *zK10H23, zC221F10, zC143O2* and *zC119P14* covered this region (Zebrafish Genome Fingerprinting Project; http://www.sanger.ac.uk/). By blasting GenScan-predicted peptide sequences in this region to the NCBI protein database (http://www.ncbi.nlm.nih.gov/), nine genes were identified. The zebrafish *kohtalo/TRAP230* mutant complemented *gr*^{s357}, excluding this gene as a candidate locus. Coding regions from the other eight genes were RT-PCR-amplified and sequenced. One mutation was found in the GR cDNA, which was also confirmed in the genomic sequence. The WT cDNA sequence of GR matched the one submitted by Ozawa et al. (NCBI accession number AB218424).

All animals used for behavioral analysis were genotyped by using the markers *z9017* and *z22094*, which flank the *gr* locus, on genomic DNA template extracted from fin clips or by mismatch PCR restriction-fragment length polymorphism (RFLP) strategy on cDNA template. PCR primers [Forward 5'GGAAGAACTGACCTGCCTGT3'; reverse 5'TATCCGGCATAGAGGGTGTC3'] were designed to add a *DrdI* site to the WT allele adjacent to the mutation site. PCR products were then digested with *DrdI* and separated by 2% agarose gel electrophoresis to identify carriers.

Cell culture, transient transfection and luciferase assay

Transcriptional activity was measured in two cell lines, U2OS and COS-7. To transfect cells, WT (pCDNA3-GR^{WT}) and mutant (pCDNA3-GR^{R443C}) forms of Flag-tagged GR constructs were made. Anti-FLAG antibody (Sigma) was used to detect GR by fluorescent

immunocytochemistry. GRE-luciferase reporter gene was co-transfected, and luciferase activity was measured following standard procedures.

To transfect U2OS cells, the following reporter plasmids were used: *AP1-Luc* containing a single consensus AP-1 site upstream of the TK promoter, *pOS-344-Luc* containing the -344/+33 fragment of the osteocalcin gene, and *NFκB-Luc* containing a single NFκB-site. U2OS human osteosarcoma cells were maintained in DMEM (GIBCO) supplemented with 5% FBS. For reporter activity assays, cells were seeded into 24-well plates in DMEM/5% FBS at approximately 20,000 cells per well. The following day, the cells were transfected in FBS-free DMEM using 0.8 μl of Lipofectamine and 1.6 μl of PLUS reagent (Invitrogen) per well, with 20 ng reporter and 20 ng of *lacZ* plus 100 ng empty plasmid *p6R*. After transfection (3h), cells were re-fed with DMEM/5% FBS, allowed to recover for 3h, and either re-fed with DMEM/5% FBS containing 100 nM Dex or ethanol vehicle (AP1 and osteocalcin reporters) for twelve hours, or treated the next day with ethanol vehicle, TNFa (5ng/ml) or TNFa (5ng/ml) + 100 nM Dex for 6 hrs (NFkB reporter). After treatment, cells were lysed in 100 μl per well of 1x lysis buffer (PharMingen) and assayed for luciferase and β-galactosidase activity.

Behavioral testing and image analysis

Experimental fish were recorded in 4-7 day intervals, once or twice a week. Since the HPA axis is under circadian regulation all behavioral tasting were restricted to the early afternoon (5-8 hours after light onset; 13:00-16:00). Movies were recorded with a digital camera (HandyCam, Sony), transferred by Firewire to a computer, and saved on the hard disk. Offline analysis was carried with custom-written software in LabVIEW 8 (National Instruments) and Matlab 6.5 (The MathWorks Inc.).

Drug treatments

Long-term drug treatments with fluoxetine hydrochloride (Sigma, 0.8 μM), bupropion hydrochloride (Sigma, 3 μM) and RU-486 (mifepristone, Sigma, 2.5-10μM) were conducted on groups of fish (n=4 to 5) housed in 2 l tanks (1.5 l water volume) with solvent for the drug serving as the control. Drug-treated fish were fed once a day with live *Artemia salina*, followed by replacement of the water and drug one hour later. Short-term treatment with diazepam (Sigma, 5μM) was carried out by adding the drug to the fish tank 30 min before the assay. All drugs were also added to the water of the tank in which the behavioral test was carried out. Treatment with dexamethasone (Dex, Sigma, 25 μM) was carried out by adding the drug to the fish tank at 18:00 hours the day before the cortisol extraction. The next morning (9:00), fish were fed and Dex-containing water was replaced with system water.

cDNA isolation and quantitative RT-PCR

Adult fish were killed by immersion in Tricaine (Western Chemicals Inc, WA, USA). RNA, extracted from fresh brains, was dissolved immediately in Trizole reagent (Invitrogen) and kept on ice until processed (10-40 min). If brains were taken immediately following behavioral testing, heads were first frozen on dry ice and stored in -80°C before dissection and RNA extraction. cDNAs (Invitrogen) were prepared, and qRT-PCR (Applied

Biosystems Inc.) was performed according to the manufacturers' instructions. All RT-PCR expression data were normalized to zebrafish *ppia-1* (NM199957).

Whole-mount RNA *in situ* hybridization and vibratome sections

Adult fish were killed by immersion in Tricaine and fixed in 4% paraformaldehyde (Ted Pella, Inc.) in PBS overnight in 4°C or for 4h at room temperature. Whole brains were removed under a dissecting scope and stored in 100% methanol at -20°C. SP6 and T7 clones with fragments from the coding sequence of *pomca* (NM_181438), *crh* (NM_0001007379) and *serta* (NM_001039972) were used as templates to prepare digoxigenin (DIG)-labeled antisense riboprobes (Roche). Large probes were further digested to 200-300bp fragments. Whole mount *in situ* hybridizations were performed following a standard protocol. Following hybridization, brains were washed and mounted in 4% agarose (low melting temperature, Invitrogen) in PBS and sectioned at 100μm using a vibratome (Leica). BM purple (Roche) substrate was used for AP staining. Images were captured with a CCD camera, mounted on Leica stereoscope.

Cortisol measurement

Fish were anesthetized on ice, superficially dried with a paper towel, and the tail was removed. Blood was collected from the caudal vein with a heparinized capillary (Fisher Scientific), prefilled with sample buffer (1% BSA (Sigma), 2 TIU Aprotinin (Sigma) in PBS). Either blood was blown out into a tube with sample buffer to a final amount of 20μl, or capillaries were directly centrifuged in a 1.5 ml tube, and plasma was taken out of the capillary using a gel loading tip. Plasma was isolated by centrifugation at 4°C, 3000g for 10 min, and stored at -80°C. Cortisol plasma concentrations were determined using an enzyme immunoassay (EIA) kit (Cayman Chemicals Co, MI, USA, or Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany) or a Cortisol RIA kit (COAT-A-COUNT, Siemens Healthcare Diagnostics Ltd., Frimley, Camberley, UK). In order to reach the sensitive range of the standard curve, plasma was diluted 1:25-60 ($gr^{+/+}$ and $gr^{s357/+}$) or 1:500-1000 ($gr^{s357/s357}$) with EIA buffer or zero calibrator.

Statistical analysis

Statistical analysis was carried out with JMP8 software (SAS Institute Inc.). In all graphs, error bars represent SEM. For statistical comparisons, one-way or two-way ANOVA, followed by paired Student's *t*-tests, were used. The Shapiro–Wilk test was used to determine normal distributions.

Results

The gr^{s357} mutation generates an R to C substitution in the DNA binding domain of GR

The gr^{s357} mutant (originally named $utouto^{s357}$) was identified in a large-scale forward genetic screen for chemically-induced point mutations that disrupt larval behavior[14]. The mutant larvae move less actively than wildtype (WT) at 6 days after fertilization (6 dpf), but are morphologically indistinguishable from WT at all stages into adulthood. We employed standard genetic mapping techniques to identify the mutation in the zebrafish genome. Homozygous gr^{s357} mutant larvae are somewhat darker than their WT siblings, owing to

dispersed melanin pigment in their melanophores. This phenotype, which may be related to misregulation of alpha-melanocyte-stimulating hormone (α–MSH, a peptide hormone encoded by the *pomc* gene) was used to sort mutants from WT. A panel of polymorphic simple-sequence repeats were scanned to identify those that co-segregated with the mutant phenotype. About 600 individual homozygous mutants from a WIK/TL hybrid cross (see Materials and Methods) were then used to refine the chromosomal location.

Narrowing the locus to a small region on chromosome 14 and sequencing of positional candidates identified *gr*, the gene encoding GR, as the mutated locus (Fig. 1a). The zebrafish genome contains one copy of GR with high protein identity (47%) to the human GR protein, especially in the DNA-binding domain (97%) and the ligand-binding domain (73%)[15]. Sequencing of the mutant cDNA showed a missense mutation that replaces an arginine (R) at position 443 with a cysteine (C) in the DNA-binding domain. This domain is essential for transcriptional regulation by GR[16-20] (Fig. 1b-d). The protein structure model of GR (http://www.rcsb.org/pdb/) predicts that the positive charge of this arginine, which corresponds to R496 in rat, R484 in mouse and R477 in human GR, is critically important for binding of the second zinc finger knuckle to the negatively charged phosphate groups of the DNA backbone[21] (Fig. 1d). This suggested (and was verified below) that the gr^{s357} mutation generated a strong hypomorph or null allele of GR.

The mutation spares hormone binding and nuclear translocation, but eliminates transcriptional activity of GR

To determine which protein functions were disrupted by the R443C mutation, we expressed Flag-tagged GR, with and without the amino acid substitution, in COS-7 cells. In the absence of ligand, anti-Flag antibody detected GR protein in both nucleus and cytoplasm. In the presence of the synthetic GR ligand Dex, both WT and mutant GRs were now exclusively found in the nucleus (Fig. 1e), indicating that neither hormone binding nor subsequent nuclear translocation were disrupted by the mutation. This is consistent with previous structure-function studies showing that nuclear translocation does not depend on the protein domain carrying the substitution[16-20].

To assay the effect of the R443C mutation on transcriptional regulation by GR, WT or mutant GR were co-transfected with reporter plasmids that recapitulated GR-dependent transcriptional activation (GRE and GRE-tk) or repression (Osteocalcin, AP-1, NF-kB) in cell lines not expressing GR. Transcriptional activation through direct binding to a strong GRE was abrogated in two different cell lines (COS7 and U2OS), even at a Dex concentration that exceeded the maximal effect in WT tenfold (Fig. 1f; Suppl. Fig. S1). Similarly, transcriptional repression, which can either be mediated by direct DNA binding (Osteocalcin; negative GRE) or by interaction with other transcription factors, which tether GR to DNA (AP-1 and NF-kB), was also disrupted (Fig. 1g-i). Together our findings indicate that transcriptional activity of GR is largely eliminated by the *s357* mutation.

The stress axis is chronically elevated and dysregulated in the mutant

Disruption of GR is expected to abrogate cortisol-mediated negative feedback of physiological stress signals. Indeed we found substantially elevated cortisol levels in

homozygous *gr*^{s357} mutants (Fig. 2a). Cortisol varied by a factor of three, between individual animals, but was always several fold higher in mutants (1-3 μg/ml) than in WT sibling fish from the same tank (0.02-0.2 μg/ml). In WT, cortisol was slightly increased in response to acute confinement stress (keeping an adult fish in a narrow glass tube for 10 minutes) (Fig 2a). Homozygous mutants did not exhibit a further increase in cortisol following stress treatment, suggesting a ceiling effect. Heterozygous carriers had higher cortisol levels than WT in the non-stressed condition and responded more strongly to confinement stress, but never reached the levels seen in homozygous mutants (Fig. 2a, insert). Stress did not further raise cortisol levels in mutants, perhaps owing to a ceiling effect. To investigate if negative feedback by GR on the HPA axis is blunted by the mutation, we carried out a 'Dex suppression test'. Blood cortisol levels were measured in the early afternoon (13:00-16:00, matching the time of the day in which behavioral measurements were made, see below), following administration of Dex or vehicle in the evening (18:00) of the previous day (lights were on from 7:00 to 21:00). Whereas in WT and heterozygous carriers Dex treatment abolished the diurnal cortisol increase, cortisol levels remained high in mutants (Fig. 2b).

Quantitative real-time PCR on total RNA taken from the front part of the brain (including telencephalon and anterior hypothalamus) demonstrated chronic, two-fold increases of *crh* transcript levels in mutants compared to heterozygotes (Fig. 2c). Elevated levels of *crh* mRNA were seen in the preoptic area of the hypothalamus (the homologue of the mammalian paraventricular nucleus)[22] (Fig. 2d) and the lateral tuberal nucleus (Suppl. Fig. S2). *pomca* mRNA was likewise globally increased in mutants (data not shown), particularly in the lateral tuberal nucleus (Fig. 2e). Confinement stress resulted in an increase of *crh* and *pomca* transcripts by RT-PCR and in situ hybridization in heterozygotes, but had little, if any, effect in mutants (Fig. 2c-e). Together, these changes in peptide hormone expression indicate a lack of negative feedback on gene transcription in the HPA axis.

Mutants freeze when placed in a novel tank and fail to habituate to repeated stress treatments

To test if *gr*^{s357} mutants showed abnormal stress-related behavior, we observed the responses of WT, heterozygotes and mutants to a mildly anxiogenic environment[23, 24]. Single male fish were placed into a novel tank, which was well lit and had opaque, dark, non-reflective walls. The novel-tank test was repeated twice a week over three weeks. For each test, the fish's swimming trajectory was recorded with an overhead video camera, graphed over time and evaluated with respect to the animal's location and speed (Fig. 3a, b). After ten minutes, the fish were returned to their familiar community tanks. After being transferred into the novel tank, the fish often stopped swimming and sank to the bottom of the tank for 10 to 60 sec, before resuming normal swimming for the remainder of the observation period (Fig. 3c; top panel shows the velocity profile of a representative WT fish after five previous tests, in the third week after initial exposure to the novel tank). Most fish reacted most strongly in the first 60 s and then habituated. In some fish, however, 'freezing' bouts could occur through the entire observation period. Then fish could be immobile for up to 6 min out of the 10 min observation period and show only short swimming bouts across

the tank (Fig. 3c; bottom panel shows velocity profile of a homozygous mutant with prior exposure identical to the WT fish above).

While the behavior was variable between individuals, particularly the initial freezing response (minute 1 of the test), we observed strong population differences between the behavioral patterns of mutant and WT fish. After, >3 test, each separated by 4 to 7 days of recovery, mutants froze significantly more than WT and heterozygous carriers. They spent greater percentage of time in an immobile state during the entire 10 min (see Fig. 3c). We calculated the freezing index as the time spent without moving, divided by the total observation time. Plotting the freezing index showed significant difference between mutants and WT (Fig. 3d). Genotype differences were not detectable at the very first exposure to the novel tank, but gradually developed as a result of experience. WT fish froze less with each exposure, apparently habituating to repeated isolation (Fig. 3e; Suppl. Fig. S3a). Heterozygotes followed the WT trend, although their freezing index revealed a slower time course of habituation than WT (Fig. 3e). Similarly experienced mutants showed the opposite trend over the course of three weeks; they spent increasingly longer time freezing with each exposure, suggesting that they became sensitized to the novel tank (Fig. 3e; Suppl. Fig. S3b). Thus, zebrafish with one or two WT copies of GR appear to exhibit long-term (inter-trial) habituation to a novel tank, while homozygous mutants become sensitized to the aversive effects of this stressor. The conditioning regime chosen here thus amplified behavioral differences between the genotypes.

Mutants show reduced wall exploration in the novel tank

We evaluated the locations of fish in the novel tank over the 10 min test period, as a function of genotype and prior experience. ‘Heat maps’ were generated that allowed us to visualize the cumulative positions of the fish, with warmer colors representing preferred areas of the tank and cooler colors representing areas that the fish only rarely visit (Fig. 3f). Place preference was calculated by dividing the time spent in the central 50% of the tank volume (away from the walls; as indicated in Fig. 3g) by the total observation time. This metric was termed “wall avoidance index”. Wildtype and heterozygotes showed no preference for the walls during the first exposures. (Suppl. Fig. S3c). With repeated exposures, WT fish became increasingly likely to swim near the walls (index drops from >0.5 to around 0.2; Fig. 3h; Suppl. Fig. S3c, data collected from two different cohorts of fish). Mutants exhibited a greater degree of wall avoidance and did not habituate to weekly exposures, being equally likely to be found near the perimeter as in the inner area of the tank (index 0.5; Fig. 3h; Suppl. Fig. S3d). Thus, fish with presumably higher levels of anxiety tended to avoid the walls of the novel tank.

The behavioral differences between the genotypes, both in their freezing responses and in their location preferences, could not be attributed to a locomotor defect, as mutant fish swam with peak velocities very similar to WT, although their average velocities and their total distances traveled were suppressed (Suppl. Fig. S4a, b).

Social interactions reduce freezing responses of the mutant

Zebrafish are highly social animals[25]. According to the social buffering paradigm[26], human subjects are less prone to the anxiogenic effects of stressful environments when allowed interaction with other non-stressed group members and, inversely, show increased stress responses when isolated. We asked if freezing and place preference in the novel tank could be modified by exposure to a conspecific. For this experiment, we varied the configuration, leaving one wall of the tank unoccluded, enabling visual interaction with a WT fish in an adjacent tank (Fig. 4a). An empty tank served as a control. Analysis of swimming trajectories (Fig. 4b), cumulative 'heat maps' (Fig. 4c) and measurement of average positions (Fig. 4d) all revealed a clear tendency of zebrafish, regardless of genotype, to spend more time near the transparent wall. This tendency was stronger when the neighboring tank contained a conspecific. Intriguingly, when allowed visual interaction with other fish, mutants spent less time freezing, approaching the levels of the heterozygotes (Fig. 4e). Reduced freezing was not associated with measurably lower cortisol levels (Fig. 4f). We conclude that social interactions reversed freezing behavior in the mutant and did do so without correcting the HPA axis.

Acute diazepam and long-term fluoxetine treatments reverse depression-like behavior

To further investigate if freezing and reduced wall exploration behavior involves neural circuits associated with depression and/or anxiety, we treated fish with drugs known to have antidepressant or anxiolytic effects in mammals. Diazepam binds to the benzodiazepine binding site of the GABA-A receptor, where it acts like a positive allosteric modulator. Addition of diazepam (5 μM for 30 min) to the tank water lowered the freezing response of experienced mutants to levels seen in untreated heterozygotes (Fig. 5a; Suppl. Fig. S5a). Wall exploration was likewise increased to levels seen in untreated heterozygotes (Fig. 5c; Suppl. Fig. S5b). Heterozygotes treated with diazepam showed the lowest-possible freezing index (0; Fig. 5a) and the lowest-measured wall avoidance index (ca. 0.1; see Fig. 5c, top-right panel). Treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine (0.8μM) had a similar effect, when administered over four days, but not after shorter treatments (Fig. 5b; Suppl. Fig. S5c, d). Neither diazepam nor fluoxetine altered average swimming speeds in either mutants or heterozygotes (Suppl. Fig. S4c), indicating that these drugs did not exert sedative or other undesirable effects at the concentrations used. The selective norepinephrine and dopamine reuptake inhibitor bupropion (3 μM) had no effect on freezing behavior (Suppl. Fig. S6a). As expected, the potent GR antagonist RU486 (5 μM) had no behavioral effects on the mutants (Suppl. Fig. S6b-d). However, RU486 increased freezing responses in WT, without affecting swimming speed or place preference (Suppl. Fig. S6e-g), thus partially phenocopying the mutation.

We next asked if fluoxetine treatment lowered cortisol levels in stressed fish and/or corrected cortisol in behaviorally rescued mutants. After four days of treatment, i. e., immediately after the behavioral test, cortisol was unchanged (Fig. 5d). However, following two weeks of continuous fluoxetine treatment, cortisol was substantially reduced, albeit not to the comparatively low levels seen in heterozygotes and wildtype (Fig. 5e). Therefore, cortisol correction follows on the heels of, but is apparently not necessary for, behavioral improvement.

Fluoxetine and the *gr*^{s357} mutation reveal crosstalk between glucocorticoid signaling and the serotonergic system

To better understand how SSRIs impinge on the stress axis, we measured the expression levels of GR and MR by quantitative RT-PCR, following chronic mild stress (CMS) treatment. CMS was produced by isolating a fish for up to two weeks, with regular ad libitum feeding and daily handling. We found that *gr* mRNA itself was slightly but consistently increased (by ca. 25%) in the *gr*^{s357} mutant and was not detectably changed by CMS und the experimental conditions used here. Fluoxetine had subtle, if any, effects on *gr* expression (Fig. 5f). Transcript levels of *mr*, the gene encoding the mineralocorticoid receptor, were similar for all genotypes, suggesting that MR is not regulated by GR (Fig. 5g). Following chronic mild stress, *mr* mRNA was significantly increased (by ca. 100%); fluoxetine administration blunted this increase (Fig. 5g). It is possible that an attenuation of the MR-mediated stress response accounts for some of the beneficial effect of fluoxetine on behavior.

Chronic stress may affect expression of genes important for serotonergic signaling. We therefore analyzed the level and expression pattern of brain serotonin transporter (*serta*). Whole brain qRT-PCR revealed a subtle increase in *serta* expression between stressed and non-stressed states in wildtype, mutants and heterozygotes (Fig. 5h). This trend is detectable by 4 days of CMS and is significant by 2 weeks (Suppl. Fig. S7). Fluoxetine (4 days or 2 weeks) lowers *serta* levels to baseline levels in CMS-exposed fish, whereas diazepam does not, even when applied continuously for 4 days (Suppl. Fig. S7). Thus, regulation of *serta* transcription could be one mechanism, by which the therapeutic effects of fluoxetine and diazepam differ. Our measurements also suggested that serotonin regulation during stress and the effect of SSRIs are not disrupted by the *gr*^{s357} mutation. In situ hybridization, however, offered a more complex picture. Here, *serta* mRNA appeared to be absent from the superior raphe nucleus of the mutants (Fig. 5i). Pretectal nuclei expressed apparently normal levels of *serta* (Suppl. Fig. S8). Together, the observed fluoxetine effects on behavior, GR and MR expression, as well as apparent *serta* misregulation in the *gr*^{s357} mutant, are consistent with the notion that glucocorticoid signaling is cross-linked with the serotonergic transmitter system and that a serotonin imbalance predisposes depression-like behavior in zebrafish.

Discussion

We have presented evidence that disruption of GR causes a syndrome in adult zebrafish that resembles an affective disorder. The syndrome carries the molecular signature of chronic stress and the behavioral profile of depression, including decreased exploratory behavior and impaired habituation to repeated exposure to an anxiogenic environment. Molecularly, the mutation results in substitution of a highly conserved R to C in the second zinc finger motif of the GR protein, which is essential for DNA binding. This change abolishes both transcriptional repression and activation of target genes in a broad range of in vitro assays, including those requiring binding of GR homodimers to positive or negative GREs and heterodimerization with NF-kB or AP-1. We conclude from these data that the mutation has generated a null for most, if not all, genomic functions of GR. In agreement with our

findings on the molecular consequences of this mutation in zebrafish, a naturally occurring mutation of the equivalent residue (R477) in human GR[20, 27, 28] also disrupted transcriptional regulation by GR. Moreover, mutation of the corresponding amino acid residue in the androgen receptor (R615) causes androgen insensitivity syndrome in humans[29] suggesting that this conserved arginine is essential for the function of steroid hormone receptors in general.

We established that disruption of GR genomic activity results in a hyperactivated HPA axis. In particular CRH, ACTH and cortisol, are chronically elevated. Consistent with an exaggerated stress response, mutants overreact to an anxiogenic environment, a novel tank in which they are isolated from conspecifics. Wildtype and heterozygous fish habituate both in the short term (within the 10 minutes of the individual trial) and long term (between trials) to this stressful environment. Their behavior shifts from freezing and location indifference to strong exploratory swimming behavior near the walls. Homozygous mutants, on the other hand, largely remain in a freezing posture and do not show place preference between and during freezing bouts; they barely habituate within a trial and even become sensitized between trials. Treatments with diazepam and fluoxetine reduce freezing duration and wall avoidance behavior in the mutants and also augment and accelerate habituation in wildtype and heterozygotes.

In zebrafish, wall-hugging behavior, or thigmotaxis, in the novel tank appears to be an indicator of motivated, exploratory behavior. In agreement with previous studies[30-32], we observed that habituation to the novel tank results in a reduction of wall avoidance. It has been reported that intermediate doses of ethanol increased several indicators of anxiety, while thigmotaxis decreased[33]. On the other hand, d-Lysergic Acid Diethylamide (LSD) reduced freezing and other anxiety-related behaviors, and simultaneously increased thigmotaxis[34]. We show her that anxiolytic and antidepressant treatments similarly result in pronounced swimming near the walls. So, a consensus is emerging that, at least in the small, well-lit containers used by us and others, ‘thigmotaxis’ increases as stress levels drop, and vice versa. This may appear counterintuitive, since it is opposite to what has been reported in rat and mouse, but could be owed to ecological differences between diurnal telosts and nocturnal rodents. Zebrafish are visual specialists. For them, moving to the open field when threatened, where they cannot be ambushed by a hidden predator and can seek conspecifics to shoal with, may be an adaptive strategy. Further work is needed to resolve this important issue.

Is the freezing behavior observed here more closely related to anxiety or to depression? The pharmacology leaves this question unanswered, since freezing is reversed by both anxiolytic (diazepam) and antidepressant drugs (fluoxetine). So we have to consider behavioral parameters. Anxiety in zebrafish is characterized by darting across the tank and active exploration of escape routes[23,24,30]. We did not observe this behavior in our mutants. Also, behavioral differences between mutants and wildtypes became only detectable after repeated exposures, suggesting long-lasting, experience-dependent effects akin to learned helplessness. While we cannot know the emotions of a fish, we therefore interpret the freezing behavior and immobility of *gr*^{s357} mutants as manifestation of a syndrome more similar to depression than to anxiety. It cannot be excluded, however, that the mutant’s

behavior may reflect an extreme form of anxiety or that the boundaries of anxiety, panic, catatonia, despair and depression may be fluid for teleost fish.

Successful treatment with fluoxetine, as well as social interactions with other zebrafish leave the high cortisol levels of the mutant initially unchanged, apparently bypassing the HPA axis. Only after two weeks of fluoxetine treatment did we observe a reduction of cortisol. Thus, while disruption of GR-mediated transcriptional control is sufficient to cause an affective disorder in this zebrafish model, subsequent correction of cortisol levels is not necessary for normalization of the behavior. Instead, other stress-associated mechanisms, presumably in the emotional centers of the brain, are independently targeted by SSRIs and other successful treatments. Cortisol reduction occurs with a delay and appears to be consequence, rather than cause, of the successful therapy.

The neural mechanism by which HPA dysregulation conditions behavioral responses in zebrafish is currently unknown, but there is strong evidence that interactions between CRH and the serotonergic system may be involved. A subsensitivity of the serotonin system is strongly associated with depression in mammals[35,36]. In teleosts and amphibians, intracerebral injection of CRH modulates locomotor activity and place preference in a context-dependent manner. These CRH-induced behaviors are strongly potentiated or inhibited by co-administration of fluoxetine or a 5-HT_{1A} receptor antagonist, respectively[37,38]. In our zebrafish *gr*^{s357} mutant, brain serotonin transporter (Serta) expression appears to be decreased in the superior raphe nucleus. Serotonergic neurons from the raphe nucleus project broadly to many areas of the CNS and are important modulators of brain plasticity and neurogenesis, as well as locomotion and affective behaviors[40]. The therapeutic potency of SSRIs observed above could be related to restoration of normal serotonin function in the superior raphe nucleus with distributed effects on forebrain or brainstem motor circuits. In addition, as suggested by their slow time course of action, SSRIs might affect gene transcription, e. g., dampen the stress-induced expression of MR, as shown here in zebrafish.

Several mouse lines with conditionally altered GR expression or function have been generated[40,41]. (Constitutive knockout of GR is embryonic lethal in the mouse.) Depressive behaviors were observed in mice with heterozygotic GR knockout[42] or forebrain-restricted GR knockout[43]. However, total neuronal or glial GR knockouts[44], or antisense GR knockdown[45] either resulted in *reduced* anxiety or had no measurable effect on behavior. Paradoxically, a transgenic mouse with *over*expression of GR in the forebrain showed increased anxiety and/or depression-like behavior after exposure to chronic mild stress[46]. Thus, behavioral results in the mouse cannot be easily reconciled with data in human patients, maybe because tissue-restricted manipulations have incomplete or complex effects on the HPA axis and/or the limbic system.

Transcriptional regulation by GR is not the only mediator of cortisol signaling in the brain. Transgenic overexpression of MR in the forebrain was recently shown to dampen anxiety-related behavior in mouse[47]. In addition to the classical genomic activity of GR and MR, an alternative rapid, non-genomic signaling route for cortisol was shown, which may or may not involve GR or MR[48]. Apparently, this signaling is crucial for mediating glucocorticoid

effects on neuronal activity and plasticity, facilitating or inhibiting signaling of ion channels and neurotransmitter receptors[49-51]. Both cortisol's non-genomic effects and MR-dependent signaling are expected to be intact in the *gr*^{s357} mutant or may even be exaggerated due to increased cortisol levels. Since *gr*^{s357} mutants lack all GR genomic activity it may be a useful tool for disentangling the contributions of these other signaling pathways in the stress response.

Together, our findings demonstrate the striking degree of evolutionary conservation in the neuroendocrine circuits regulating emotion in vertebrates. Our data strongly support the hypothesis[8] that excessive activation of the HPA axis, by acquired or (in the case of this novel fish mutant) inherited glucocorticoid resistance, is an important contributor to the development of depression. As a diurnal species with cortisol rhythms similar to humans[1], zebrafish may provide a useful model system for neuropsychiatric research, complementary to the widely used rodent models. In a future application, our zebrafish *gr* mutant could potentially be used as a small-molecule screening tool[52] for the discovery of novel antidepressant pharmacologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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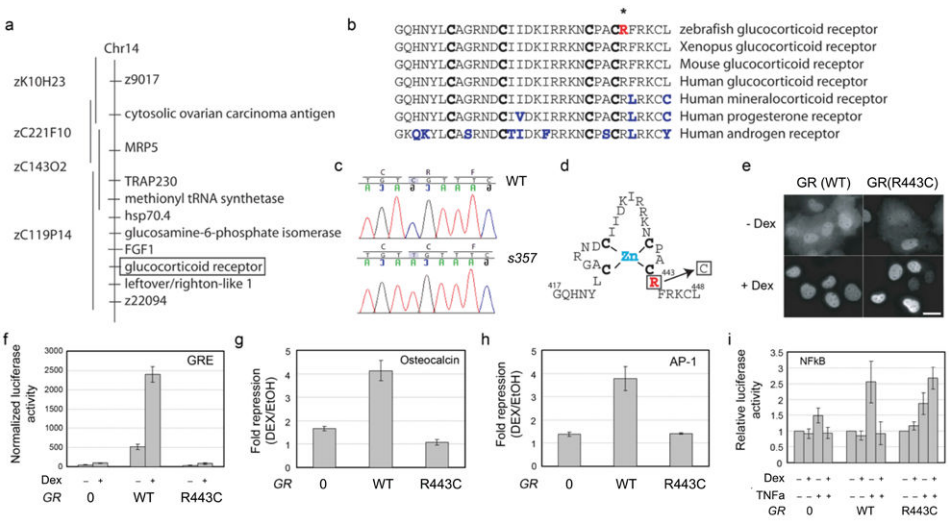

Figure 1. Forward genetic identification and biochemical characterization of a mutation that disrupts transcriptional activity of the zebrafish glucocorticoid receptor

a, BAC contig, spanning the interval between two microsatellite markers, *z9017* and *z22094*. The region contains nine predicted genes. **b**, Genomic sequence alteration identified in the *gr* gene. **c**, Sequence comparison of the zinc finger motif near the mutated amino acid residue (in red, marked with *). Four conserved cysteines in the zinc finger are in bold. Non-conserved amino acids are in blue. **d**, Schematic of the zinc-finger and the location of the R to C substitution. **e**, Subcellular localization of Flag-tagged WT and R443C GR protein, expressed in COS-7 cells and detected with anti-Flag antibody. Both showed nuclear translocation in the presence of 1 μM Dex. Scale bar is 10 μm. **f-i**, GR (R443C) is defective in transcriptional repression. U2OS cells lacking endogenous GR, were co-transfected with either empty vector ('0'), or expression constructs for either wildtype GR (WT) or mutant GR (R443C) and reporter constructs for PRE-tk (GRE) (n=2, p<0.0001) (f); Osteocalcin (nGRE) (n=5, p<0.0001) (g); AP1 (n=3, p=0.018) (h) or NFκB (n=4, p=0.0037) (i). Cells were treated overnight with 100 nM Dex (PRE-tk, AP1 and Osteocalcin) or for 6 h with either ethanol vehicle, TNFα or TNFα plus 100 nM Dex (NFκB). Reporter activity was expressed as fold repression of Dex treated/EtOH vehicle (g and h) or relative luciferase units (f and i).

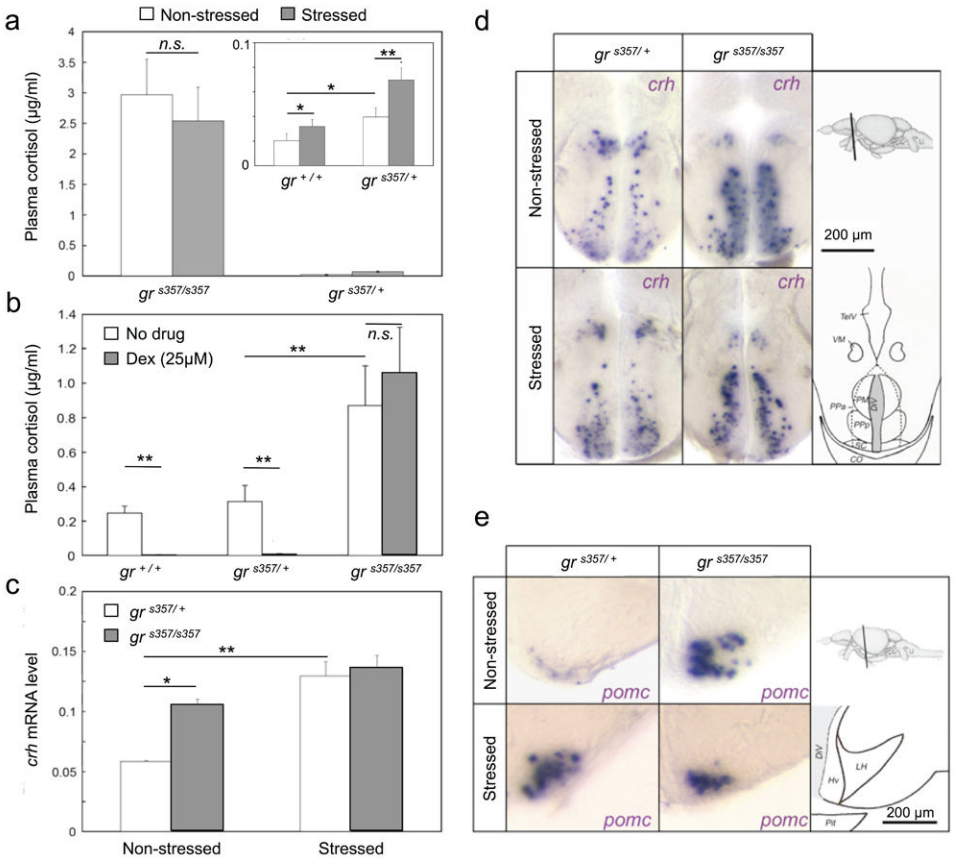

Figure 2. Hyperactivity of the HPA axis in *gr*^{s357} mutant zebrafish

a, Plasma cortisol levels determined by EIA before (non-stressed) and after confinement stress (stressed) in mutants (*gr*$^{s357/s357}$) compared to heterozygotes (*gr*$^{s357/+}$). Insert shows cortisol of heterozygous (*gr*$^{s357/+}$) and WT fish (*gr*$^{+/+}$) at different scale (*n*=4-10 each). Cortisol is highest in mutants. Cortisol is higher in heterozygotes than in WT. Stress increases cortisol in heterozygotes and WT (difference between *gr*$^{s357/s357}$ and *gr*$^{s357/+}$: $p<10^{-4}$, ANOVA F ratio = 160.24; difference between *gr*$^{s357/+}$ and *gr*$^{+/+}$: **p*=0.05; difference between non-stress and stress: ***p*=0.0051, ANOVA F ratio = 8.81; combined effect genotype × stress *p*=0.002, ANOVA F ratio=10.99; pooled data ANOVA: degrees of freedom = 3, F ratio = 60.82, $p<10^{-4}$). **b**, Dex suppression test. Dex was added at 18:00 the day before. Cortisol was measured at 13:00-16:00. Light on was at 7:00. In WT and heterozygotes, the diurnal cortisol spike is greatly suppressed (**$p<10^{-3}$). Mutants show no reduction ($p>0.05$;). **c**, RT-PCR expression of *crh*. RNA was extracted from the front part of the brain (including telencephalon and anterior hypothalamus) in non-stressed and stressed fish (*n*=3-9). *crh* mRNA is increased in mutants (**p*<0.05) and following confinement stress (**$p<10^{-3}$). **d**, Sagital sections showing *crh*-expressing cells in hypothalamic preoptic area. More signal is seen in mutants. Drawings on the right indicate position of section in a side view of the zebafish brain (top) and boundaries of brain nuclei and ventricle at the level of the stained section (bottom). **e**, Sagital sections showing *pomca*-expressing cells in the lateral tuberal nucleus (NLT) of the hypothalamus. Mutants and stressed fish show stronger signal. Drawings on the right, as in (d). Abbreviations: PPa and PPp, parvocellular preoptic

nucleus, anterior and posterior parts; PM, magnocellular preoptic nucleus; VM, ventromedial thalamic nucleus; TelV, telencephalic ventricle; DiV, Diencephalic ventricle; SC, suprachiasmatic nucleus; CO, optic chiasm; LH, lateral hypothalamic nucleus; Hv, ventral zone of periventricular hypothalamus; pit, pituitary.

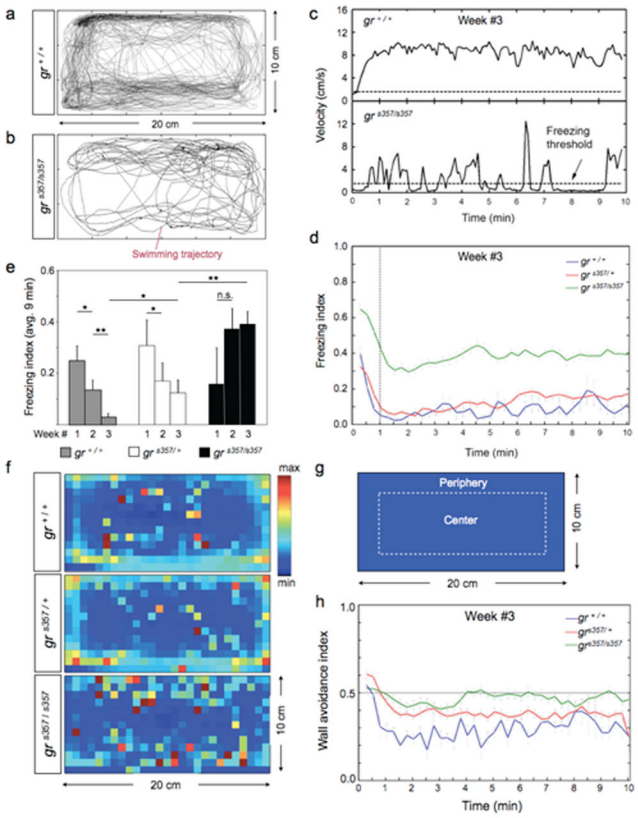

Figure 3. Exaggerated freezing responses and altered location preference in zebrafish *gr*^{s357} mutants after isolation in a novel tank

Naïve fish were tested in the novel tank on day 0 (week #1) and then tested again every 4-7 days during the next two weeks (*n*=4-8). Movements were recorded every 15 s over 10 min. **a**, Trajectory of a representative WT fish after 5 exposures to the novel tank. **b**, Trajectory of a representative mutant after 5 exposures. The mutant swims significantly less than WT. **c**, Plot of velocities over 10 min observation time. WT (top) shows short-term habituation within the first min and then swims at approx. constant speed for 9 min. Mutant (bottom) is immobile half of the time and swims in short spurts. Peak velocities are similar to WT. **d**, Freezing index (time spent immobile/observation time) of experienced fish in novel tank. Data were pooled and averaged ($gr^{+/+}$, *n*=47; $gr^{s357/+}$, *n*=115; $gr^{s357/s357}$ *n*=134). The index drops in the first min but remains high in mutants. WT freeze slightly less than heterozygotes. Freezing threshold (dotted line) is 1.6 cm/s. **e**, Freezing index, averaged over 9 min. The first minute of the observation period (short-term habituation) was not included. WT and heterozygotes show long-term habituation over three weeks; they freeze gradually less. Mutants become more sensitized or are unchanged (*p<0.05; **p<10^{-3}). **f**, Heat maps, showing the spatial preferences of animals in the novel tank after 5 exposures. The spatial bin size is 10×10 pixels. Warmer colors represent preferred locations (see scale on the right). Mutants (bottom) spend less time near the perimeter of the tank than WT (top) or heterozygotes (middle) (note the yellow and cyan pixels near the walls in the WT and heterozygotes). Orange and red pixels indicate places where the fish froze. A greater number of red pixels are seen in the mutants, and they tend to be at a distance from the walls. **g**,

Quantification of place preference. Video images of the tank were divided into two equally large compartments, as indicated. The wall-avoidance index was calculated as the time spent in the central compartment divided by total observation time. **h**, Wall avoidance in experienced fish. Mutants remain in the central compartment (index ca. 0.5). WT prefers the periphery, after short-term habituation index drops from 0.5 to 0.3. Heterozygotes show intermediate preferences. Error bars ± SEM.

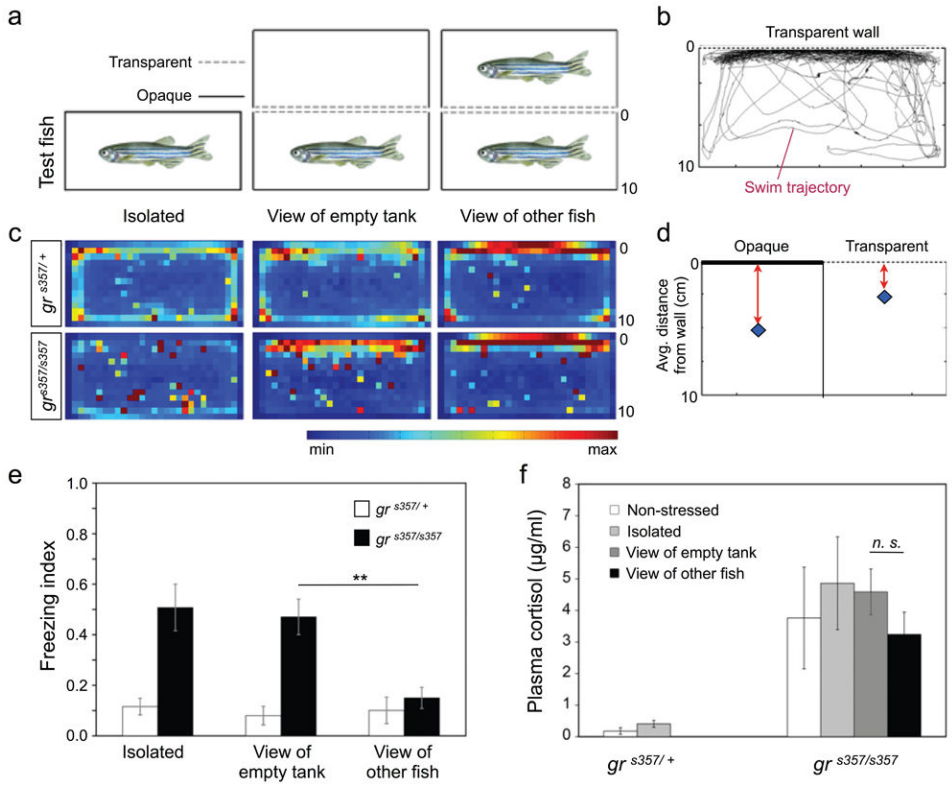

Figure 4. Social interactions reduce freezing and wall avoidance in zebrafish *gr*^{s357} mutants

a, Top view of the experimental setup. The tested fish are depicted in the bottom row of tanks. Behavior was tested in the tank with one transparent wall, allowing visual interaction with a non-mutant conspecific in a neighboring tank ('view of other fish', right panel). A standard tank with four opaque walls ('isolated', left panel) and a tank with one transparent wall with view of an empty tank ('view of empty tank', middle panel) were used as controls. **b**, Representative trajectory of a mutant fish with view of a conspecific. **c**, Heat maps, showing place preferences as in Fig. 4a. Heterozygotes (top panels) prefer the walls and corners of the tanks and particularly the wall that apposes the empty tank or the tank that contains the other fish. Isolated mutants show no preference for the walls. However, they prefer to swim close to the transparent wall, particularly when the neighboring tank contains another fish. **d**, Average fish distance from the opaque vs. transparent wall. SEMs are smaller than the size of the marker diamond. **e**, Freezing indices for two genotypes (n=9-19) as a function of their visual environment. Experienced mutants freeze much less when able to view a conspecific (**p=0.0013, ANOVA F ratio = 11.26; pooled data ANOVA: degrees of freedom = 5, F ratio = 4.64, p=0.001). Other differences were not statistically significant. **f**, Plasma cortisol, determined by EIA. Hormone levels do not change in the behaviorally rescued mutants (n=2-4).

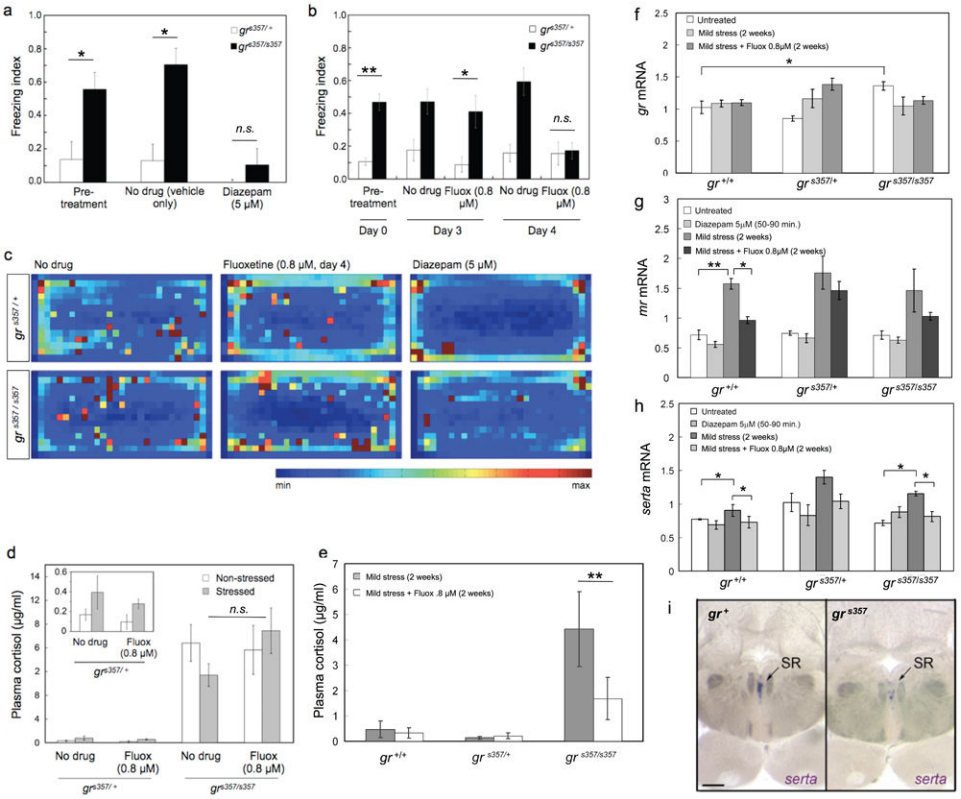

Figure 5. Effect of diazepam and fluoxetine on zebrafish behavior and gene expression

a, Freezing indices of experienced fish before ('pretreatment') and after diazepam (5μM, 30 min) or vehicle ('no drug', 30 min) treatments (n=5, significant difference between mutant and wildtype pre-treatment, *p=0.019, ANOVA F ratio = 6.17; no significant difference between treated mutants and wildtype, p=0.066, ANOVA F ratio = 2.97; no significant combined effect genotype × treatment, p=0.3464, F ratio = 1.09; pooled data ANOVA: degrees of freedom = 5, F ratio = 2.87, p<0.03). **b**, Freezing indices of experienced fish after fluoxetine treatment (0.8μM, 4 days). Same controls as in the diazepam experiment (n=10-36; significant difference between mutant and wildtype pre-treatment genotype difference, **p<0.0012, ANOVA F ratio = 11.0; no significant difference between treated mutants and wildtype, p=0.097, ANOVA F ratio = 2.38; no significant combined effect genotype × treatment, p=0.62, F ratio = 0.62; pooled data ANOVA: degrees of freedom = 5, F ratio = 6.91, p=10^{-4}). **c**, Heat maps of location preferences. The heterozygous fish shows a mild preference for the corners and walls, which is greatly increased following fluoxetine and diazepam treatments. Diazepam-treated fish (upper right panel) spent most of the time in the corners of the tank. The mutant is similarly affected, although its wall avoidance does not reach the levels of the diazepam-treated heterozygote (lower right panel). **d**, Plasma cortisol levels of experienced mutants and heterozygotes, determined by EIA before ('non-stressed') and after behavioral testing ('stressed') following 4 days of fluoxetine (0.8μM) or vehicle ('no drug') treatments (n=2-8). Insert shows heterozygote levels on a different Y-axis scale. Fluoxetine normalizes behavior without correcting cortisol levels (p>0.05). **e**, Plasma cortisol levels of naïve WT, mutants and heterozygotes, determined by RIA following 2 weeks of fluoxetine (0.8μM) or vehicle ('untreated 2 weeks') treatments

(n=3-7). Cortisol is partially corrected after two weeks of fluoxetine administration (**p<0.001). **f-h**, RT-PCR expression data of *gr*, *mr* and *serta* mRNA in total RNA extracted from whole brain of non-stressed fish ('Untreated'); following two weeks of chronic mild stress ('2 weeks CMS'); 50-90 min of 5μM diazepam treatment ('Diazepam 5μM'); or two weeks of CMS with 0.8μM fluoxetine treatment ('2 weeks CMS + Fluoxetine 0.8μM') (n=3-9 fish per group; *p<0.01, **p<0.0001). *gr* mRNA is largely unaltered by stress and drug treatments (f). *mr* mRNA is similar between genotypes, but upregulated in stressed fish of all genotypes and normalized by fluoxetine (g). At the whole-brain transcript level, there is no significant difference in *serta* expression between experimental groups (h), but see (i). **i**, In situ hybridizations on hindbrain sagittal sections reveal the absence or strong reduction of *serta*-expressing cells in the superior raphe nucleus (SR) of the gr^{s357} mutant.