

# Modulation of *Fgfr1a* Signaling in Zebrafish Reveals a Genetic Basis for the Aggression–Boldness Syndrome

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Behavioral syndromes are suites of two or more behaviors that correlate across environmental contexts. The aggression–boldness syndrome links aggression, boldness, and exploratory activity in a novel environment. Although aggression–boldness has been described in many animals, the mechanism linking its behavioral components is not known. Here we show that mutation of the gene encoding fibroblast growth factor receptor 1a (*fgfr1a*) simultaneously increases aggression, boldness, and exploration in adult zebrafish. We demonstrate that altered Fgf signaling also results in reduced brain histamine levels in mutants. Pharmacological increase of histamine signaling is sufficient to rescue the behavioral phenotype of *fgfr1a* mutants. Together, we show that a single genetic locus can underlie the aggression–boldness behavioral syndrome. We also identify one of the neurotransmitter pathways that may mediate clustering of these behaviors.

## Introduction

Many animal species exhibit behavioral syndromes (Sih et al., 2004a,b) [also called personalities (Boissy, 1995; Wolf et al., 2007), coping styles (Benus et al., 1991; Koolhaas et al., 1999), or

temperaments (Réale et al., 2007)], suites of two or more behaviors that correlate across environmental contexts. Behavioral syndromes constrain the direction of evolution, affect an animal's future fitness, maintain individual differences in behavior, and may explain maladaptive behaviors (Sih et al., 2004a; Wolf et al., 2007). From an adaptive evolution point of view, the existence of behavioral syndromes is puzzling, because greater flexibility in behavior would give animals a selective advantage (Sih et al., 2004a; Bell and Sih, 2007; Wolf et al., 2007). According to the aggression–boldness syndrome, animals that are more aggressive will also be bolder and more explorative in novel environments (Huntingford, 1976). Aggression–boldness has been described in many animals, including mammals (Benus et al., 1991), birds (Dingemanse et al., 2007), and fish (Huntingford, 1976; Brick and Jakobsson, 2001; Schjolden et al., 2005; Moretz et al., 2007; Bourne and Sammons, 2008). However, the mechanism underlying its formation is not well understood and has been suggested to be caused by either pleiotropic genetic (Deng et al., 1994; Dingemanse et al., 2007) or hormonal (Koolhaas et al., 1999; Veenema et al., 2003) constraint or selection driven by optimal adaptation to the environment (Bell, 2005; Bell and Sih, 2007), with each behavioral component having a separate genetic basis.

Similar to other vertebrates, there is a correlation between aggression and boldness in zebrafish, although the relationship varies depending on the strains analyzed (Moretz et al., 2007). Zebrafish display characteristic agonistic postures, including erection of the fins, biting, tail thrashing, and short bouts of fast swimming (Gerlai et al., 2000). Aggression is exhibited by both sexes (Moretz et al., 2007) and can be influenced by both habitat complexity and rearing conditions (Basquill and Grant, 1997; Marks et al., 2005). Furthermore, different wild-type strains show

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varying aggression levels suggesting a genetic component to its control (Moretz et al., 2007). Boldness, measured as the number of approaches to a novel object, has also been shown to differ between zebrafish strains, suggesting a genetic basis to this behavior as well (Wright et al., 2003).

In this study, we examine aggression, boldness, and exploration in a zebrafish mutant with reduced fibroblast growth factor receptor 1a (Fgfr1a) function, *spiegelanio* (*spd*) (Rohner et al., 2009). Our results provide evidence that modulation of a single genetic locus, *fgfr1a*, links the three components of the aggression–boldness behavioral syndrome, a phenotype that may be at least partly mediated through a reduction of histamine (HA) levels in the brain.

## Materials and Methods

**Animal strains, care, and maintenance.** Adult zebrafish were maintained using standard fish-keeping protocols and in accordance with institute guidelines for animal welfare. Two days before each behavioral assay, the fish were moved to an isolated room and kept under similar conditions to minimize environmental variance and interference from background noise during testing.

**Behavioral methods.** Behavioral analyses were performed on 3- to 6-month-old adult Tübingen wild-type, *spiegelanio*<sup>13R705H</sup> and *spiegelanio*<sup>hu3264</sup> fish of both sexes. Behavioral experiments were performed using Videotrack and LabWatcher from ViewPoint Life Sciences and a digital camera from Point Gray Research. All behavior experiments were completed between 1:00 P.M. and 4:00 P.M. to minimize circadian differences. One week before analysis, fish were habituated to the stress of being netted. Fish were daily subjected to two rounds of being caught in a fish net, held gently for 3 s, and released into a second tank. To reduce observer bias in the manual analysis of aggressive behavior, films were quantified by two independent observers who were unaware of the genotype being analyzed. Results presented in here show the mean of both observers' findings, and the correlation between the two results was estimated using the Pearson's product-moment correlation coefficient ( $r = 0.86$ ). In all experiments, the age, sex, and size was carefully matched between fish of different genotypes.

**Swimming behavior of fish in behavioral setups.** Photographs of behavioral setups and swimming traces (see Fig. 1) were captured using the reader function in Videotrack.

**Aggression.** The mirror-induced aggression protocol was modified from Gerlai et al. (2000). Adult fish were maintained in groups of 20 animals from birth until the day of testing. On the day before analysis, the fish were moved to a novel 10 L glass fish tank. During testing, fish were placed in a small glass aquarium measuring 15 × 10 × 30 cm. Both long sides and one short side of the aquarium were covered in a white opaque material. The fourth side of the tank was left clear, and a mirror was placed outside of the tank offset at an angle of 22.5° (Gerlai et al., 2000). The behavior of individual fish was recorded using a high-speed digital camera set to 100 frames/s and connected to the Videotrack program. Films were manually analyzed in LabWatcher (ViewPoint Life Sciences). The results were analyzed and processed in Excel (Microsoft).

**Boldness.** Boldness was measured using two protocols: the time spent close to a novel object and the time spent on the nonpreferred side of a condition place-preference tank. Novel object boldness gave more reproducible results and so was used when testing the effect of histaminergic pathway drugs on both wild-type and *spd* fish. "Novel object boldness" was measured as the amount of time spent close to a novel object (Wright et al., 2003). The novel object was made by filling a 15-cm-long clear plastic tube with dark modeling clay. The novel object was suspended at one end of the tank, midway in the water column. Boldness was measured as the total time spent within one body length of the object in a 10 min period. "Place-preference boldness" was measured as the amount of time spent on the nonpreferred side of a place-preference tank. Fish were placed into a 10 L tank, the preferred side of which was colored brown and the nonpreferred side of which was white with two black spots (Ninkovic and Bally-Cuif, 2006). Fish were allowed to habituate to this

setup for 2 d. Boldness was calculated as the percentage of time spent on the nonpreferred white side of the tank. Both behavioral protocols gave similar results and so were assumed to measure the same behavior in zebrafish: boldness.

**Exploration of a novel environment.** Exploration was quantified as the amount of time needed to explore the arms of a novel environment. For this experiment, a T-shaped tank with one long arm and two short arms (arm lengths, 30 × 30 × 45 cm) was used. The fish were not habituated to this setup. Fish were released into the long arm of tank, and the experiment started when they crossed a designated start line. Exploratory behavior was measured as the time taken to explore and swim within one body length of the end of each arm.

**Locomotion.** Locomotion was measured in a 10 L plastic fish tank with clear walls. Locomotion was defined as the distance swum in a 5 min period after a 10 min adjustment to the new tank.

Fish were tracked using a high-speed infrared camera with the Videotrack software set to a detection threshold of 11, inactive/small threshold of 5, and small/large threshold of 10. The mean total distance swam (small distance and large distance) was then calculated for each animal. Locomotion was used as a readout for general behavior of the fish. Because the aggression, boldness, and exploration tests all involved swimming, a reduction in locomotion (for example, after drug treatment) would skew the behavioral results.

**Olfaction.** The olfactory test was modified from Vitebsky et al. (2005). Adult fish were tested for their reaction to the aversive amino acid L-serine. A glass fish tank (15 × 10 × 30 cm) was covered with white opaque material. Two flexible plastic tubes were attached to opposing corners of the tank. The tubes were connected to a 15 ml Falcon tube filled with either saline (control) or 10<sup>-4</sup> M L-serine. The tank was divided into two areas, and the time spent in each area was measured using Videotrack. Olfaction was quantified as the time spent away from the source of aversive L-serine.

**Drug treatments.** Fluoxetine hydrochloride, tacrine hydrochloride, imetit dihydrobromide, and galantamine hydrochloride were purchased from Sigma-Aldrich. Drug doses were chosen in line with those used in other species (Morisset et al., 1996). In some experiments, fish were treated with drugs before their behavior was measured. Drugs were either administered intraperitoneally (1 mg/g tacrine hydrochloride, imetit dihydrobromide, or galantamine hydrochloride, once per day for 3 d; Sigma-Aldrich) or directly into the tank water (3 μg/ml fluoxetine hydrochloride for 3 h; Sigma-Aldrich) (Lynn et al., 2007). For control experiments, animals were treated with saline instead of drug.

**Histochemistry reagents.** *In situ* hybridization was performed according to Norton et al. (2008) and antibody staining according to Topp et al. (2008). The following *in situ* hybridization probes were used in this study: *dusp6*; *fgfr1a*; *mao*; *npvf*; *pomcb*; *slc6a4a*; and *slc6a4b*. For gene information, refer to www.zfin.org. Histamine *n*-methyl transferase (*hnmmt*) was amplified using the primers GGCTCAATGACTAAGATTCA AG and GCTCCATTCAAACACTTGT. *hnmmt* was cloned into the StrataClone PCR Cloning Vector pSC-A (Stratagene). The following antibodies were used: anti-phosphorylated extracellular signal-regulated kinase (pERK) [mitogen-activated protein kinase (MAPK); 1:1000; Sigma], anti-proliferating cell nuclear antigen (PCNA) (1:1000; Dako), anti-gonadotrophin releasing hormone (GnRH) (1:500; Sigma), and anti-histamine (1:2000; gift from Dr. P. Panula, University of Helsinki, Helsinki, Finland). TUNEL staining was performed using the Neurotacs 2 *in situ* apoptosis detection kit (Trevigen). Specimens were photographed with an Axioplan2 stereomicroscope for *in situ* staining (Carl Zeiss) or Axioplan2 ApoTome for antibody labeling (Carl Zeiss). Figures were mounted in Adobe Photoshop version CS2 (Adobe Systems). Nissl staining was performed on cryosections of the brain according to standard protocols.

**Radioimmunoassay.** Whole adult brains were snap frozen in liquid nitrogen and stored at -80°C until processing. Brains were homogenized by grinding up in 150 μl of 0.2N HClO<sub>4</sub>. After centrifugation at 10,000 × *g* for 5 min at 4°C, the supernatant was neutralized to pH 6–8 by addition of an equal volume of 1 M potassium borate and then passed through a 10 kDa filter (Nanosep 10 kDa; Pall Life Sciences, VWR). Two aliquots of 100 μl

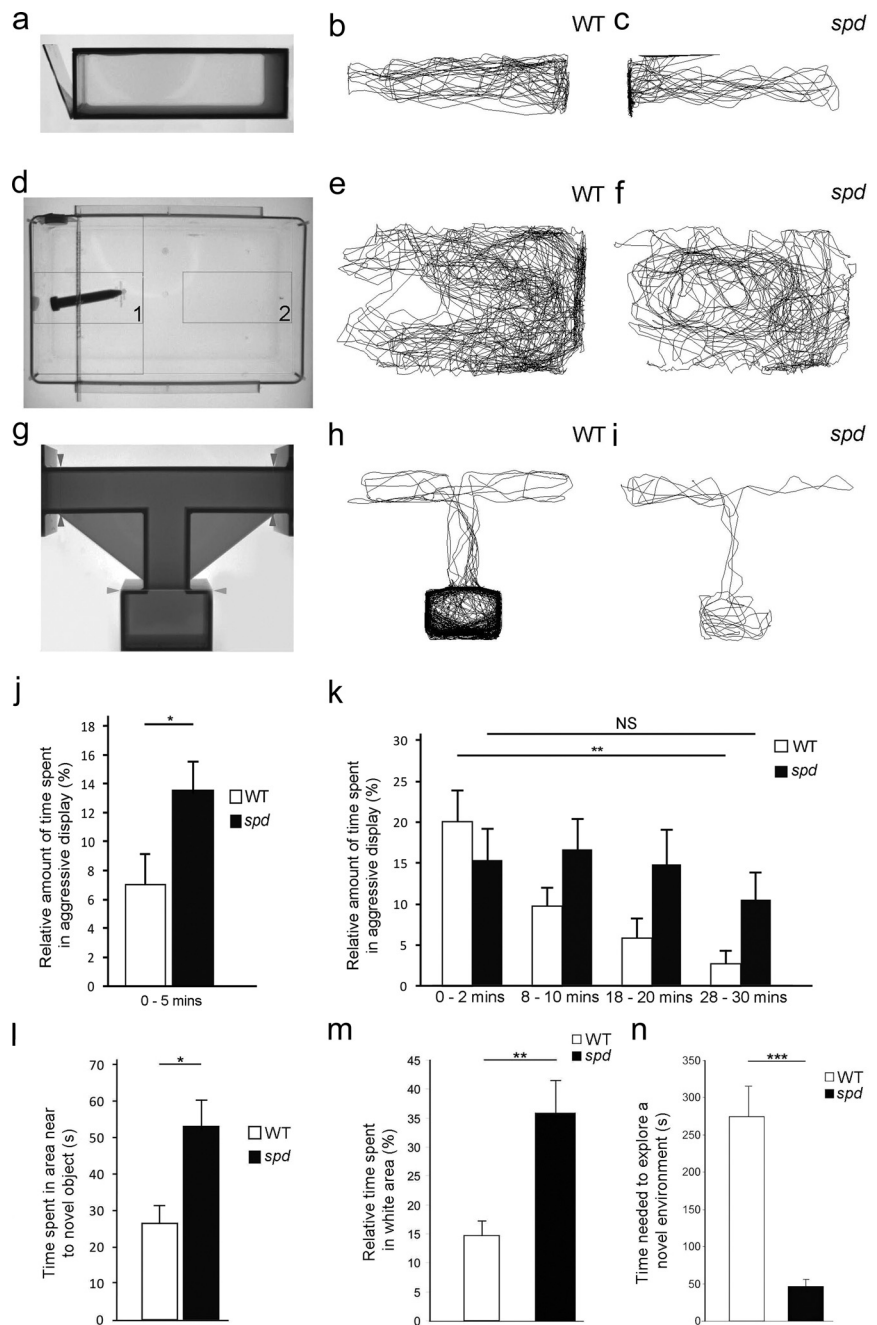
were analyzed for histamine levels by radioimmunoassay (RIA Histamine kit, Immunotech), following the instructions of the manufacturer.

**ELISA.** Blood plasma was collected from pools of three male and three female adult fish (each yielding between 1 and 5  $\mu$ l of blood, to make a total of 25  $\mu$ l). Fresh blood was mixed 1:10 with a citrate buffer and stored in heparinized hematocrit tubes. Blood samples were centrifuged at 4°C for 10 min, and the plasma was aspirated. ELISA was performed using either a testosterone immunoassay kit (DNOV002) or a 17 $\beta$ -estradiol immunoassay kit (DNOV001) from AbCys.

**Real-time PCR.** Quantitative PCR analysis (qPCR) was performed by Eurofins. Primers were designed and optimized by Eurofins (qPCR primer sequences available on request). PCRs were performed on an ABI 7900 SDS machine using a SYBR Green Universal Master Mix (Exiqon). cDNA was synthesized from 1  $\mu$ g of RNA extracted from whole brains. The PCR conditions were 56°C for 10 min, 94°C for 10 min, followed by 40 cycles of 60°C for 1 min and 94°C for 15 s. Analysis was performed in duplicate using 10 biological replicates for each genotype. All results were separately normalized to the expression level of four housekeeping genes: *gapdh*, *mapta*, *neurod*, and *tubb2b*. Because all housekeeping genes showed similar results, only data from *neurod* are presented in this study. qPCR results are shown as an expression ratio calculated relative to *neurod*. Relative expression levels were calculated using the arithmetic comparative method,  $(2^{-\Delta\Delta C_t})$ , which includes a correction between *E* (PCR amplification efficiency) for the target and housekeeping gene (Livak and Schmittgen, 2001). Results were expressed as relative expression ratios (REs) using *neurod* as a reference gene in the formula  $RE = (E_{ref})^{Ct_{ref}} / (E_{target})^{Ct_{target}}$ , where Ref is the housekeeping gene, target is the gene of interest, and Ct is the crossing point (Filby and Tyler, 2005). For statistical analysis, the Student's *t* test was performed with  $n = 10$  for both genotypes.

**qPCR primer sequences.** Primer sequences used for quantitative real-time PCR analysis of neurotransmitter encoding gene expression in *spd* and wild-type brains included the following: *hdc* forward, TCCAGCAGTAATCATCCA CTCGT; *hdc* reverse, TTAGAGGAGCGAGG CGTCTC; *hnm1* forward, GAAAGAGTTCAG TAAAAATGCTCCTGA; *hnm1* reverse, ATAT GATCCTGCCGTCCACC; *hrh1* forward, GA AACCTGCGTCCATCACG; *hrh1* reverse, AA GTTTTCGTTGCAGAGCGG; *hrh2* forward, CA CGCCCAATCTTCAAAGG; *hrh2* reverse, CC ATTTGTAGCGCTGTTATCGTC; *hrh3* forward, TTCTGGCTGCTGTGGATCAA; and *hrh3* reverse, TTGCTCGGACACAGTAATTTCG.

**Statistics.** All error bars denote SEM. For aggression, boldness, exploration, locomotion, cell counts, and qPCR analysis, we used Welch's two-sample *t* test allowing for unequal variances. For olfactory behavior, fluoxetine hydrochloride, tacrine hydrochloride, imetit dihydrobromide, and galantamine hydrochloride treatment data were analyzed by two-way ANOVA with genotype and



**Figure 1.** *spiegeldanio* mutants are more aggressive, bolder, and more explorative than wild-type fish. **a**, Photograph of tank used to measure aggressive behavior (top view). A mirror is placed on the left side of the setup, offset at an angle of 22.5°. **b**, Representative trace of wild-type swimming behavior in the aggression setup. **c**, Representative trace of *spd* swimming behavior in the aggression setup. **d**, Photograph of tank used to measure novel-object boldness (top view). The novel object is seen on the left side of the tank. Time spent in area 1 (near to a novel object) and area 2 (a control area away from the object) was compared. **e**, Representative trace of wild-type swimming behavior in the novel-object boldness setup. **f**, Representative trace of *spd* swimming behavior in the novel-object boldness setup. **g**, Photograph of tank used to measure exploration (top view). The time needed to swim within one body length of the two ends of the tank (shown by red lines and red arrowheads) after crossing an initial start line (green line and green arrowheads) was recorded. **h**, Representative trace of wild-type swimming behavior in the exploration setup. **i**, Representative trace of *spd* swimming behavior in the exploration setup. **j**, *spd* mutant fish (black bar) are more aggressive than wild-type siblings (white bar).  $p = 0.04$ ;  $n = 12$  wild-type and  $n = 10$  *spd*. *t* test. Interaction with a mirror image for 5 min. **k**, *spd* mutants maintain stable aggression levels (black bars), whereas wild-type fish (white bars) serially reduce aggression over time. Fish were exposed to a mirror image for 30 min, and behavior was recorded during 2 min time intervals. Wild-type 0–2 min versus wild-type 28–30 min:  $p = 0.02$ ;  $n = 7$  wild-type. *t* test. *spd* 0–2 min versus *spd* 28–30 min:  $p = 0.6$ ;  $n = 7$  *spd*. *t* test. **l**, *spd* mutants (black bar) are bolder than wild-type siblings (white bar), spending more time near to a novel object.  $p = 0.04$ ;  $n = 19$  wild-type and  $n = 17$  *spd*. *t* test. **m**, *spd* mutants (black bar) are bolder than wild-type siblings (white bar), spending more time on the nonpreferred side of a place-preference tank.  $p = 0.008$ ;  $n = 8$  wild-type and  $n = 8$  *spd*. *t* test. **n**, *spd* mutants (black bar) explore a novel aquarium faster than wild-type siblings (white bar).  $p = 0.0003$ ;  $n = 10$  wild-type and  $n = 10$  *spd*. *t* test.



drug. First, a model with interaction was fitted. If there was a significant interaction effect, the genotype difference was tested in the subgroups of drug treatment. If the interaction term was not significant, a two-way ANOVA without interaction was fitted. If appropriate, *post hoc* comparisons were performed by *t* tests with *p* value adjustment for multiple testing according to Holm (1979). When necessary, data were log-transformed to normalize the distribution. Statistical significance was depicted as follows: NS (nonsignificant),  $p > 0.05$ ; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . In all cases the number of animals tested is denoted by *n*.

## Results

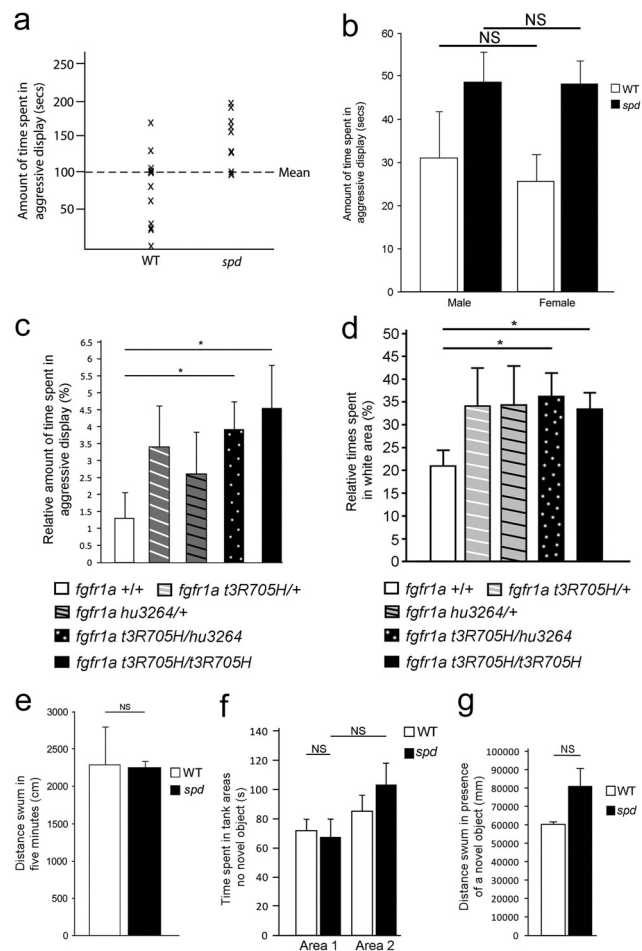
### Reduction of *Fgfr1a* increases aggression in zebrafish

During routine stock keeping, we noticed that *spiegeldanio* [*fgfr1a*<sup>t3R705H/t3R705H</sup>, also called *spd* (Rohner et al., 2009)] mutants showed an increased tendency to kill other fish and to bite conspecifics during pairing. To quantify these observations, we measured agonistic behavior by using mirror-image stimulation (Rowland, 1999; Gerlai et al., 2000; Clotfelter et al., 2007). Fish are unable to recognize their own mirror image and thus attack as if an intruder is present (Rowland, 1999). Homozygous *spd* mutants were clearly more aggressive than siblings during a 5 min period (Figs. 1*a–c,j*, 2*a*). Whereas wild-type fish serially reduced aggression levels after initiation, *spd* mutants maintained high levels of agonistic behavior throughout the experiment, failing to downregulate aggression over time (Fig. 1*k*). Agonistic behavior was not sex specific, in agreement with other studies of zebrafish aggression (Moretz et al., 2007) (Fig. 2*b*). We therefore used fish of both sexes in our subsequent behavioral analyses.

To confirm that alteration of *fgfr1a* activity underlies the observed behavioral defects, we measured aggression levels in fish mutant for a second *fgfr1a* allele, *fgfr1a*<sup>hu3264</sup>. Both *fgfr1a* alleles harbor strong hypomorphic mutations in the kinase domain of *fgfr1a* (Rohner et al., 2009) (Fig. 3*a*). However, *hu3264* shows variable penetrance and juvenile lethality as homozygotes, suggesting that it causes a more severe reduction of *fgfr1a* activity than the t3R705H allele (Rohner et al., 2009). The lethality of juvenile *fgfr1a*<sup>hu3264/hu3264</sup> fish precluded their use in adult behavioral experiments. We thus quantified aggression in transheterozygous fish (*fgfr1a*<sup>t3R705H/hu3264</sup>). Like homozygous *spd*<sup>t3R705H/t3R705H</sup>, transheterozygous *fgfr1a*<sup>t3R705H/hu3264</sup> fish showed a significant increase in aggression compared with siblings (Fig. 2*c*). Fish heterozygous for either allele showed a nonsignificant dominant trend increase in aggression compared with wild type, suggesting that a gene dosage effect may exist for this phenotype. We decided to use *fgfr1a*<sup>t3R705H/t3R705H</sup> in our subsequent experiments (hereafter referred to as homozygous *spd* mutants).

### *spiegeldanio* mutants exhibit hallmarks of the aggression–boldness syndrome

Because an aggression–boldness syndrome has been observed in some zebrafish strains (Moretz et al., 2007), we surmised that the increased aggression levels in homozygous *spd* might be paralleled by increases in boldness and exploratory activity. Boldness was quantified by using two behavioral assays. We first measured the amount of time spent close to a novel object [novel-object boldness (Wright et al., 2003)]. Neither wild-type nor homozygous *spd* fish showed a preference for either side of an empty boldness tank (Fig. 2*f*) or a difference in the distance swum in the presence of the novel object (Fig. 2*g*). However, homozygous *spd* mutants spent significantly more time within one body length of the novel object than wild-type fish (Fig. 1*d–f,l*), suggesting an increase in boldness. The second boldness paradigm recorded the amount of time spent on the nonpreferred side of a biased place-



**Figure 2.** Control behavioral experiments. *a*, Aggression levels overlap in both wild-type and *spd*. Each X represents one fish.  $n = 12$  wild-type and  $n = 11$  *spd*. Interaction with a mirror image for 10 min. *b*, Males and females of both genotypes show similar aggression levels. Males wild-type versus *spd*,  $p = 0.6$  and female wild-type versus *spd*,  $p = 0.2$ ;  $n = 10$  males and  $n = 10$  females per genotype. *t* test. *c*, Both transheterozygous (*fgfr1a*<sup>t3R705H/hu3264</sup>; black spotted bar,  $p = 0.03$ ) and homozygous (*fgfr1a*<sup>t3R705H/hu3264</sup>; black bar,  $p = 0.04$ ) *spiegeldanio* mutant fish are more aggressive than wild-type siblings (white bar). Heterozygous fish are not significantly more aggressive than wild-type (*fgfr1a*<sup>t3R705H/+</sup>; white hatches on gray bar,  $p = 0.35$  and *fgfr1a*<sup>hu3264/+</sup>; black hatches on gray bar,  $p = 0.38$ );  $n = 9$  wild-type,  $n = 14$  *fgfr1a*<sup>t3R705H/+</sup>,  $n = 9$  *fgfr1a*<sup>hu3264/+</sup>,  $n = 10$  *fgfr1a*<sup>t3R705H/hu3264</sup>, and  $n = 9$  *fgfr1a*<sup>t3R705H/hu3264</sup>. *t* test. *d*, Both transheterozygous (*fgfr1a*<sup>t3R705H/hu3264</sup>; black spotted bar,  $p = 0.03$ ) and homozygous (*fgfr1a*<sup>t3R705H/hu3264</sup>; black bar,  $p = 0.03$ ) *spiegeldanio* mutant fish are bolder than wild-type siblings (white bar). Heterozygous fish are not significantly bolder than wild-type (*fgfr1a*<sup>t3R705H/+</sup>; white hatches on gray bar, NS,  $p = 0.11$  and *fgfr1a*<sup>hu3264/+</sup>; black hatches on gray bar,  $p = 0.18$ );  $n = 9$  wild-type,  $n = 11$  *fgfr1a*<sup>t3R705H/+</sup>,  $n = 9$  *fgfr1a*<sup>hu3264/+</sup>,  $n = 9$  *fgfr1a*<sup>t3R705H/hu3264</sup>, and  $n = 13$  *fgfr1a*<sup>t3R705H/hu3264</sup>. *t* test. *e*, Both *spd* (black bar) and wild-type (white bar) show similar locomotion levels in a familiar environment.  $p = 0.93$ ;  $n = 10$  wild-type and  $n = 10$  *spd*. *t* test. *f*, Neither *spd* (black bars) nor wild-type fish (white bars) show a preference for either side of an empty novel object boldness tank. For depiction of tank areas, refer to Figure 1*d*. Area 1 versus area 2,  $F = 0.31$ ,  $p = 0.6$ ; wild-type versus *spd*,  $F = 3.41$ ,  $p = 0.07$ ;  $n = 7$  wild-type and  $n = 7$  *spd*. Two-way ANOVA without interaction. *g*, Both wild-type (white bar) and *spd* (black bar) fish swim a similar distance in the presence of a novel object.  $p = 0.07$ ;  $n = 12$  wild-type and  $n = 10$  *spd*. *t* test.

preference tank [place-preference boldness (Ninkovic and Bally-Cuif, 2006)]. Wild-type fish spent on average 14.7% of the time on the nonpreferred (frightening) side compared with 35.9% of time spent by homozygous *spd* mutants (Fig. 1*m*). The similar results of both assays (place preference and reaction to a novel

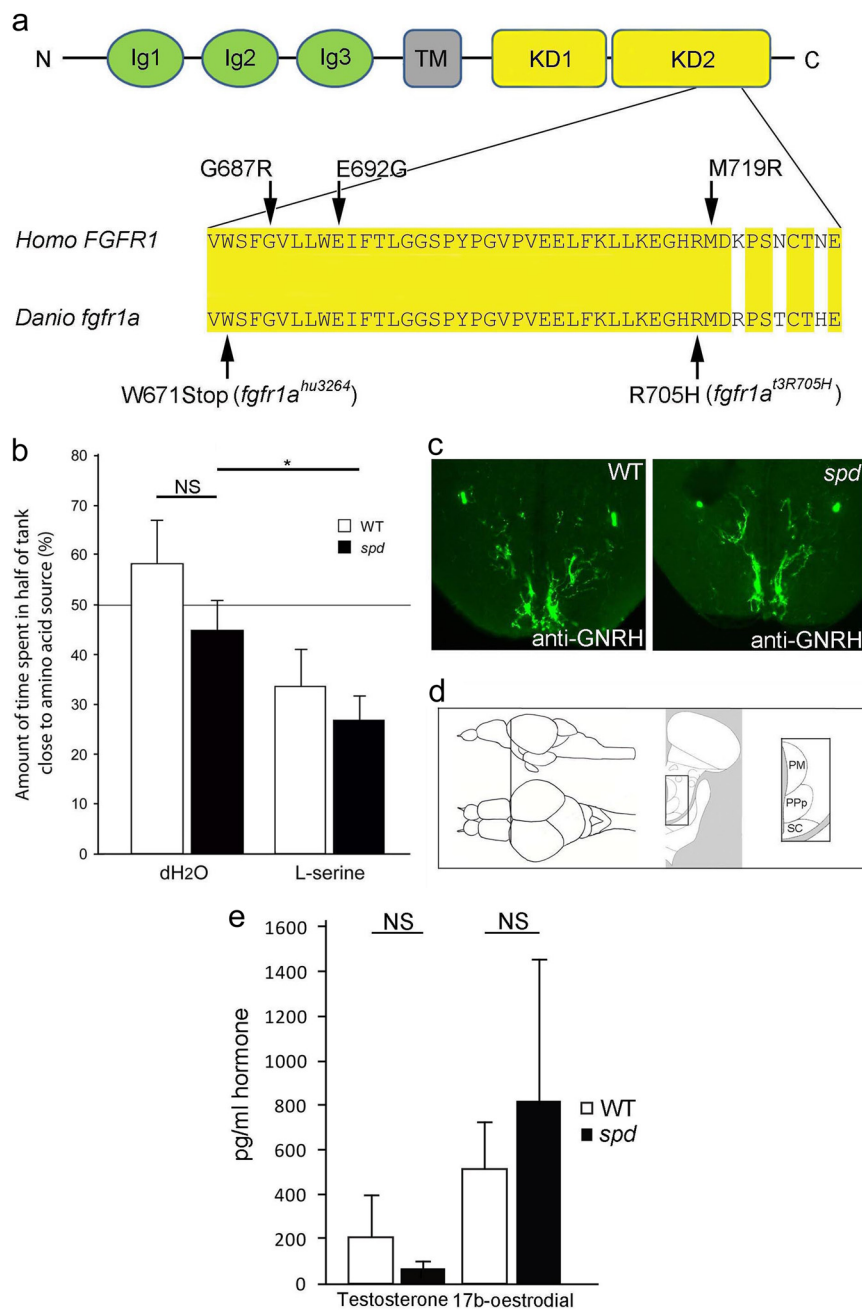
object) support the idea that they measure the same behavior in zebrafish. Together, the results of both these tests suggest that *spd* exhibits enhanced boldness compared with siblings. Similar to aggression levels, fish heterozygous for either allele show a nonsignificant trend increase in boldness, and both *spd* and transheterozygous fish show significantly increased boldness behavior (Fig. 2*d*).

We next analyzed exploratory activity in a novel environment, a T-shaped aquarium. We recorded the amount of time needed to swim within one body length of each end of the tank. Homozygous *spd* mutant fish explored this environment in significantly less time than wild-type siblings (Fig. 1*g–i,n*), thereby showing increased exploration. However, homozygous *spd* mutants did not exhibit changes in locomotion compared with wild type (Fig. 2*e*). Together, these results suggest that reduction of Fgf signaling in *spd* results in concomitant increases in aggression–boldness.

In humans, autosomal dominant loss-of-function mutations in *FGFR1/KAL2* cause Kallmann syndrome (KS), which comprises both anosmia/hyposmia and hypogonadotropic hypogonadism caused by a reduction of GnRH levels and hypoplasia of the olfactory bulb and tract (Dodé et al., 2003). KS is a developmental disease with highly variable penetrance; patients with the same mutation within *FGFR1* show a range of symptoms, from normosmia and fertility to complete KS. Because *spd* codes for one of the two zebrafish orthologs of *FGFR1/KAL2* (Fig. 3*a*), we asked whether homozygous *spd* mutants share phenotypic similarities with KS. We found that homozygous *spd* are able to detect the aversive amino acid L-serine (Vitebsky et al., 2005) (Fig. 3*b*) and so have at least some ability to detect odors. Homozygous *spd* also have no changes in the expression of GnRH in the preoptic area (Fig. 3*c*) and similar blood levels of testosterone and 17 $\beta$ -estradiol as wild-type fish (Fig. 3*e*). Therefore, homozygous *spd* mutants do not share obvious phenotypes with KS patients.

### *spd* mutants have a major reduction of Fgf signaling in the inferior lobe of the hypothalamus

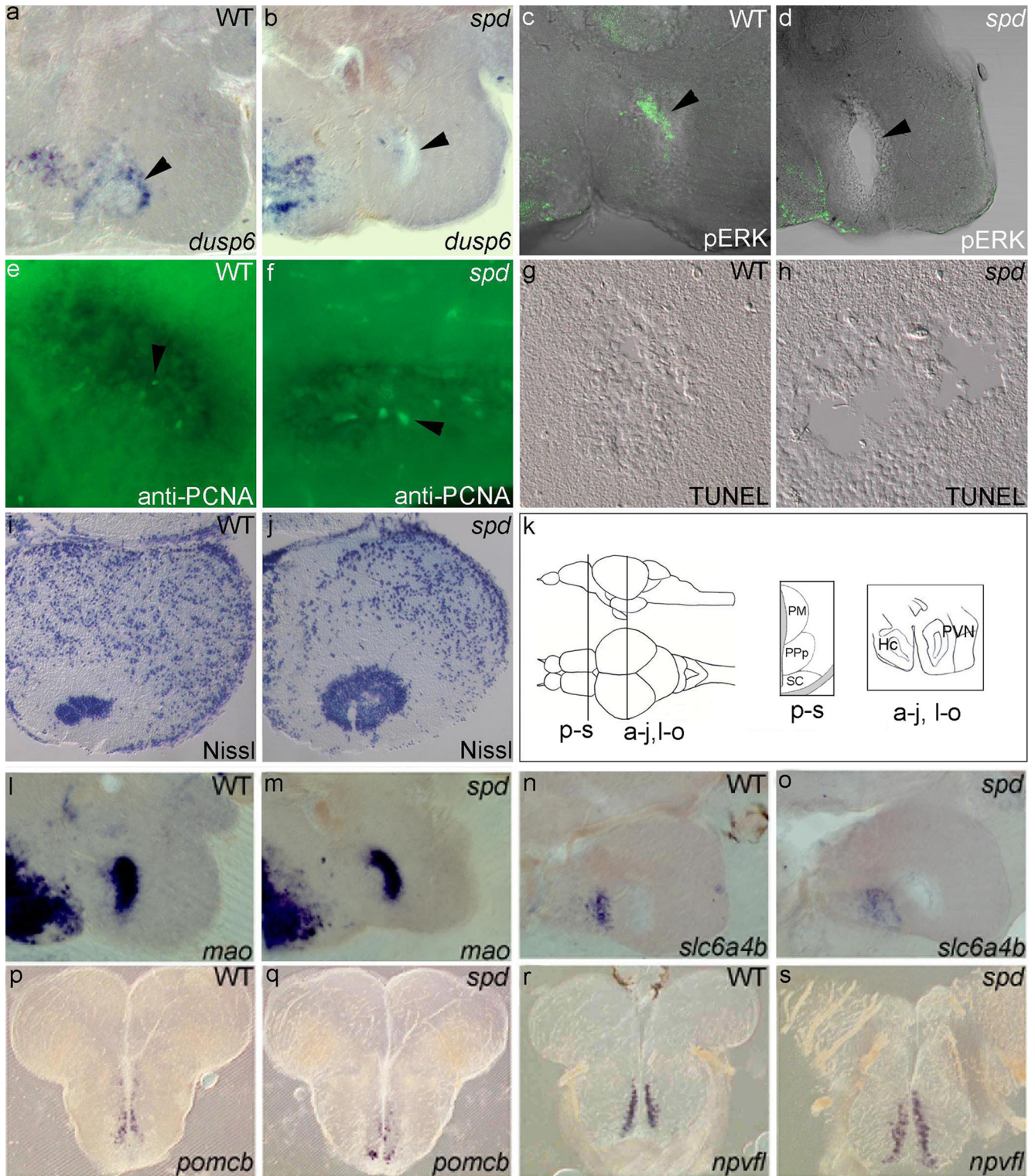
Compared with mice and medaka deficient in *fgfr1* activity (Deng et al., 1994; Yokoi et al., 2007), homozygous *spd* mutants have a surprising lack of early developmental phenotype, perhaps because of the presence of two paralogous *fgfr1* genes with similar expression patterns during development (Rohner et al., 2009). However, only *fgfr1a* is expressed in the adult zebrafish brain, and the expression of *fgfr1a* is globally similar in both wild-type and



**Figure 3.** *spd* mutants do not show a Kallmann syndrome-like phenotype. *a*, Diagram showing the position of mutations in the human FGFR1A (G687R, E692G, M719R) and zebrafish [hu3264 (W671Stop), *spd* (t3R705H)] *Fgfr1a* protein. Mutations are not conserved between species. *b*, Mutation of *fgfr1a* does not cause olfactory defects in zebrafish. Both wild-type (white bars) and *spd* (black bars) mutants are aversive to the smell of the amino acid L-serine but do not react to water. Wild-type versus *spd*,  $F = 1.615$ ,  $p = 0.208$ ; L-serine versus dH<sub>2</sub>O,  $F = 8.606$ ,  $p = 0.006$ ;  $n = 9$  wild-type and  $n = 8$  *spd*. Two-way ANOVA without interaction. *c*, Anti-gonadotrophin releasing hormone antibody labeling is similar in the anterior hypothalamus of both wild-type and *spd*. *d*, Diagram depicting the position of sections in *c* in the zebrafish brain. PM, Magnocellular preoptic nucleus; Ppp, posterior part of the parvocellular preoptic area; SC, supra-chiasmatic nucleus. *e*, There is no significant difference in levels of testosterone and 17 $\beta$ -estradiol in the blood of wild-type (white bars) and *spd* (black bars). Wild-type versus *spd* testosterone,  $p = 0.7$ ; wild-type versus *spd* 17 $\beta$ -estradiol,  $p = 0.67$ ;  $n = 18$  wild-type and  $n = 18$  *spd*. *t* test.

mutant fish. To examine the Fgf signaling defect in *spd*, we analyzed expression of the dual specificity phosphatase enzyme *dusp6* (Fig. 4*a,b*) and pERK (Fig. 4*c,d*), readouts of Fgf signaling in the adult brain (Topp et al., 2008). We observed a reduction of both *dusp6* and pERK in the periventricular nucleus of the inferior lobe of the hypothalamus (PVN; Fig. 4*a–d,k*). However, other brain areas, such as the central nucleus of the hypothalamus





**Figure 4.** *spd* have a reduction of Fgf signaling in the periventricular nucleus. **a–j**, High-magnification images of coronal sections through the adult zebrafish hypothalamus, showing *dusp6* *in situ* hybridization (**a, b**), pERK immunocytochemistry (**c, d**), PCNA immunocytochemistry (**e, f**), TUNEL labeling (**g, h**), and Nissl staining (**i, j**). *dusp6* and pERK are reduced in the PVN of *spd* (**b, d**) compared with siblings (**a, c**). PCNA and TUNEL labeling is similar between wild-type (**e, g**) and *spd* (**f, h**). The Nissl-stained PVN of *spd* (**j**) appears larger than wild-type (**i**). Arrowheads in **a–d** highlight the PVN. Arrowheads in **e** and **f** point to PCNA-positive cells. **k**, Diagram depicting the position of all sections in the zebrafish brain. **l–o**, The expression of *mao* (**l, m**) and *slc6a4b* (**n, o**) in the periventricular nucleus of both wild-type (**l, n**) and *spd* (**m, o**). Expression levels are similar in both genotypes. **p–s**, The expression of *pomcb* (**p, q**) and *npvfl* (**r, s**) in the preoptic area of both wild-type (**p, r**) and *spd* (**q, s**). Expression levels are similar in both genotypes. PM, magnocellular preoptic nucleus; Ppp, parvocellular preoptic nucleus posterior part; SC, suprachiasmatic nucleus.

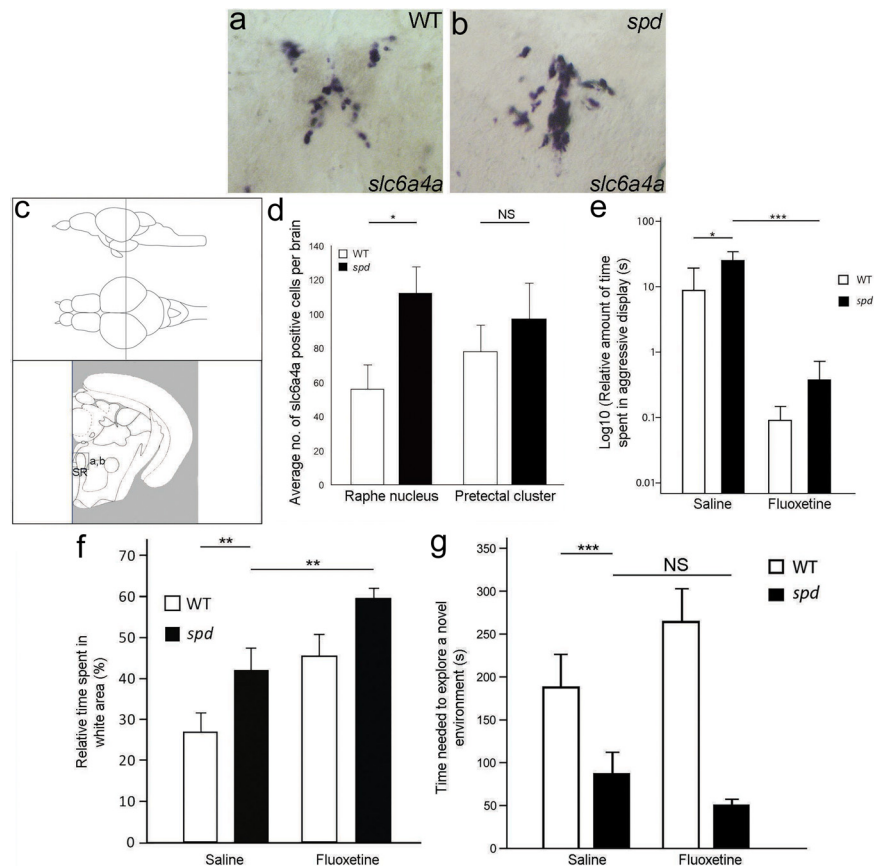
(Hc), were essentially unaffected (Fig. 4*a–d,k*). During closer inspection, the ventricle surrounded by the PVN appears larger in homozygous *spd* (Fig. 4*j*) than wild type (Fig. 4*i*), supporting our observation that Fgf function is altered in this region. However, analysis of anti-PCNA antibody (marking dividing cells; Fig. 4*e,f*) and TUNEL staining for apoptosis (Fig. 4*g,h*) did not reveal additional defects between wild-type and mutant hypothalami. We examined more genes expressed in the Hc and PVN (Fig. 4*l–o*). All of the genes that we analyzed were similarly expressed in the inferior hypothalamus of both genotypes, indicating that the PVN is not globally reorganized. Thus, homozygous *spd* mutants have a major reduction of Fgf signaling in the PVN.

We next expanded our characterization of the mutant brain to include other neural markers. The preoptic area of the anterior hypothalamus (POA) is another area of the zebrafish brain implicated in the control of aggression and dominance (Larson et al., 2006). In contrast to the PVN, the POA looked morphologically normal, and we found similar expression of POA marker genes in both wild-type and homozygous *spd* (Fig. 4*p–s*).

### Behavioral alterations in *spd* are caused by reduced histaminergic neurotransmitter signaling

We next aimed to identify the neurotransmitter pathways linking Fgf signaling and behavior. 5-HT acting via Htr1a (Gross et al., 2002; Clotfelter et al., 2007) and Htr1b receptors (Ramboz et al., 1996) has been identified as having a key role in the control of aggression and boldness/anxiety in fish and mouse. Treatment of fish with the selective 5-HT reuptake inhibitor fluoxetine hydrochloride increases 5-HT levels and has been shown to reduce aggression levels in fish (Perreault et al., 2003; Lynn et al., 2007). We first observed an increase in the number of neurons expressing the 5-HT transporter gene *slc6a4a* in the raphe nucleus of *spd* (Fig. 5*a,b,d*).

To assess the contribution of 5-HT signaling to the behavior of *spd*, we gave fish an acute nonsedative (see Fig. 8*a*) dose of fluoxetine and measured subsequent changes to aggression, boldness, and exploration. Surprisingly, although fluoxetine reduced aggression levels in both genotypes, it failed to blunt the increased aggression in *spd* (Fig. 5*e*). We next measured place-preference boldness and exploration. Modifying 5-HT signaling with fluoxetine caused a significant increase in the place-preference boldness levels of both wild-type and homozygous *spd* without rescuing the mutant behavior (Fig. 5*f*); thus, similar to the aggressive behavior, 5-HT neurotransmission does not underlie the increased boldness in *spd*. Conversely, fluoxetine treatment did not significantly alter exploratory activity in either homozygous *spd* or wild type (Fig. 5*g*), ruling out a role for 5-HT signaling in this behavior. We thus conclude that

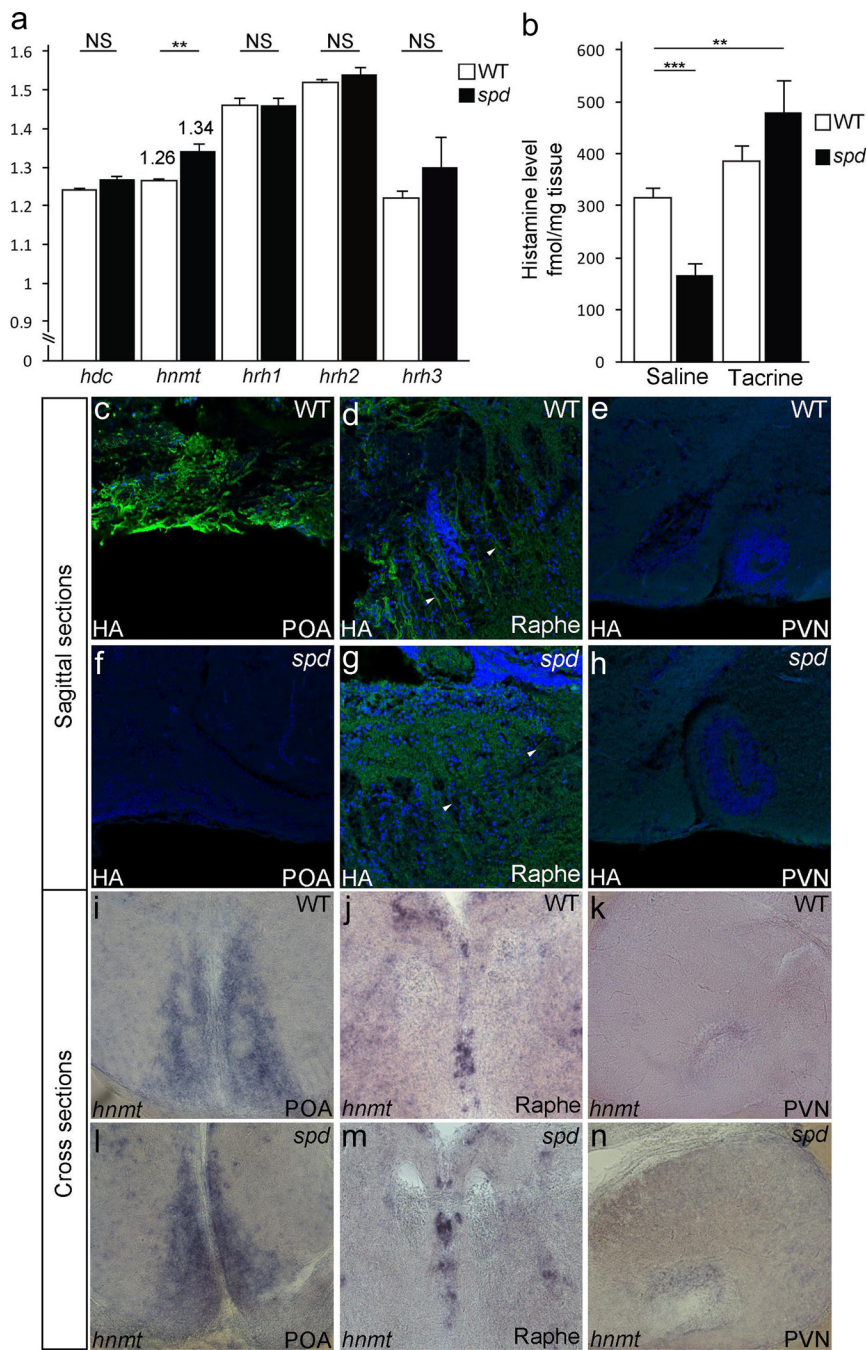


**Figure 5.** 5-HT signaling does not underlie the *spd* behavioral phenotype. *a, b*, High-magnification images of coronal sections through the adult brain showing the expression of *slc6a4a*. The number of *slc6a4a*-positive cells is increased in the dorsal raphe nucleus of *spd* (*b*) compared with siblings (*a*). *c*, Diagram depicting the position of sections *a* and *b* in the zebrafish brain. *d*, Quantification of the number of *slc6a4a*-positive cells in the dorsal raphe nucleus (SR) and pretectal cluster of wild-types (white bars) and mutants (black bars). Raphe nucleus,  $p = 0.02$ ; pretectal cluster,  $p = 0.5$ ;  $n = 6$  wild-type brains and  $n = 6$  *spd* brains. *t* test. *e*, Fluoxetine hydrochloride treatment does not rescue the *spd* aggression phenotype. Compare wild-type fish (white bars) with *spd* mutants (black bars) with or without fluoxetine treatment. wild-type versus *spd*,  $F = 2.134$ ,  $p = 0.04$ ; fluoxetine versus saline,  $F = 4.162$ ,  $p = 0.0002$ ;  $n = 10$  wild-type saline,  $n = 10$  *spd* saline,  $n = 10$  wild-type fluoxetine, and  $n = 10$  *spd* fluoxetine. Log10 transformation followed by two-way ANOVA without interaction. *f*, Fluoxetine hydrochloride treatment does not rescue boldness (measured in place-preference tank) in *spd*. Compare wild-type fish (white bars) with *spd* mutants (black bars) with or without fluoxetine treatment. Wild-type versus *spd*,  $F = 7.94$ ,  $p = 0.0074$ ; fluoxetine versus saline,  $F = 12.6$ ,  $p = 0.001$ ;  $n = 10$  wild-type saline,  $n = 13$  *spd* saline,  $n = 12$  wild-type fluoxetine, and  $n = 12$  *spd* fluoxetine. Two-way ANOVA without interaction. *g*, Fluoxetine treatment does not influence exploration in either wild-type or *spd*. Compare wild-type fish (white bars) with *spd* mutants (black bars) with or without fluoxetine treatment. Wild-type versus *spd*,  $F = 28.66$ ,  $p = 0.0001$ ; fluoxetine versus saline,  $F = 0.239$ ,  $p = 0.548$ ;  $n = 8$  wild-type saline,  $n = 8$  *spd* saline,  $n = 9$  wild-type fluoxetine, and  $n = 10$  *spd* fluoxetine. Two-way ANOVA without interaction.

5-HT signaling does not underlie the Fgf1a-dependent aggression–boldness syndrome. However, it is possible that the increase in *slc6a4a* expression seen in the dorsal raphe nucleus of mutants may be connected to the behavioral changes that we observe in a 5-HT-independent manner.

To broaden our analysis to include other neurotransmitter pathways, we used qPCR to examine the expression of 40 components of the dopaminergic, 5-HTergic, noradrenergic, and histaminergic neurotransmitter pathways, including synthesis enzymes, receptors, and transporters. The majority of these genes showed no change in expression, whereas the gene encoding *hnm1* was significantly upregulated in homozygous *spd* mutants compared with wild type (Fig. 6*a*), representing a 1.06-fold change in mRNA levels. *hnm1* curbs histaminergic signaling in the brain by converting HA to its metabolite *N*-methylhistamine (Haas et al., 2008). *hnm1* is widely expressed throughout the brain, with similar distribution in both wild type and *spd* (Fig.





**Figure 6.** *spd* mutants have reduced histaminergic signaling in the brain. **a**, Bar chart showing relative expression levels of histaminergic pathway genes in wild-type (white bars) and *spd* (black bars) brains. *hnmt* expression is significantly increased in *spd* compared with wild-type, whereas other genes are unaffected. *hdc*,  $p = 0.34$ ; *hnmt*,  $p = 0.007$ ; *hrh1*,  $p = 0.97$ ; *hrh2*,  $p = 0.48$ ; and *hrh3*,  $p = 0.63$ ;  $n = 10$  brains for each genotype. Expression ratio calculated relative to *neurod* followed by *t* test. The number above the bars depicting wild-type and *spd* *hnmt* show the exact expression level of this gene to aid comparison. **b**, Radioimmunoassay comparing levels of histamine in the brains of wild-type and *spd*, with and without tacrine treatment. Histamine is significantly reduced in *spd* compared with wild-type. Tacrine treatment increase histamine levels in *spd* but not wild-type. Wild-type versus *spd*,  $F = 25.13$ ,  $p = 0.0001$ ; tacrine versus salt,  $F = 9.83$ ,  $p = 0.0064$ ;  $n = 5$  brains for each genotype and treatment. Two-way ANOVA without interaction. **c–h**, Anti-histamine antibody labeling is reduced in the brain of *spd* compared with wild-type. Sagittal sections showing histamine antibody-positive fibers in green and DAPI in blue. *spd* mutants have a reduction of histamine in the POA (**c, f**) and raphe nucleus (**d, g**). No HA-positive fibers are seen in the PVN (**e, h**). White arrowheads show histamine-positive fibers in the raphe nucleus of both genotypes. The other green areas in the background of the image correspond to background in the antibody staining. **i–n**, *hnmt* is similarly expressed in the brain of wild-type and *spd*. Cross-sections showing expression in the POA (**i, l**), raphe nucleus (**j, m**), and PVN (**k, n**). Raphe, Raphe nucleus.

6i–n). This is in keeping with the widespread projection pattern of histaminergic neurons, whose cell-bodies are clustered in the Hc (Eriksson et al., 1998). Although the fold change of *hnmt* RNA was very small, it prompted us to investigate whether *spd* mutants have an imbalance in histaminergic neurotransmitter signaling. Analysis of neurotransmitter levels by radioimmunoassay (Fig. 6b) and with an HA-specific antibody (Fig. 6c–h) confirmed the reduction of HA in the mutant brain. The decrease in antibody labeling was seen in all brain areas, although a few HA-positive fibers were still seen in the raphe nucleus of *spd* (Fig. 6d,g). We reasoned that, if decreased HA was a relevant downstream component of impaired Fgf signaling, increasing HA levels should rescue some aspects of the *spd* phenotype. We treated homozygous *spd* mutants with a non-sedative dose (see Fig. 8b) of the Hnmt inhibitor tacrine hydrochloride (Horton et al., 2005) and observed an increase in HA levels in the brain (Fig. 6b). We then measured aggression, boldness, and exploration of tacrine-treated fish. Treatment with tacrine did not affect aggression or novel-object boldness levels in wild type, but reduced aggression and novel-object boldness in homozygous *spd* (Fig. 7a,b). Tacrine treatment had a weaker effect on place-preference boldness, only causing a nonsignificant trend reduction of boldness in *spd* (Fig. 8e). Thus, the novel-object boldness test appeared to be more sensitive to changes in HA level, and so we used it in our subsequent pharmacological experiments. Tacrine treatment increased the amount of time needed to explore the T-shaped tank in both wild-type and homozygous *spd* mutants, bringing both genotypes to the same exploration level (Fig. 7c). At low concentrations, tacrine hydrochloride is specific for *hnmt*, whereas at high concentrations it can also inhibit the acetylcholine breakdown enzyme acetylcholinesterase (Horton et al., 2005). To rule out a contribution of acetylcholine signaling to the *spd* behavioral phenotype, we treated homozygous *spd* mutants with a non-sedative dose (Fig. 8c) of the acetylcholinesterase inhibitor galantamine hydrochloride (Houghton and Howes, 2005) and measured aggression. Treatment with galantamine did not affect the aggression levels of either wild-type or mutant fish (Fig. 8f), ruling out a contribution of acetylcholine signaling to the aggressive behavior we observed.

In zebrafish, histaminergic neurotransmitter signaling is transduced by three receptors encoded by the genes *hrh1*, *hrh2*, and *hrh3* (Peitsaro et al., 2007). All three

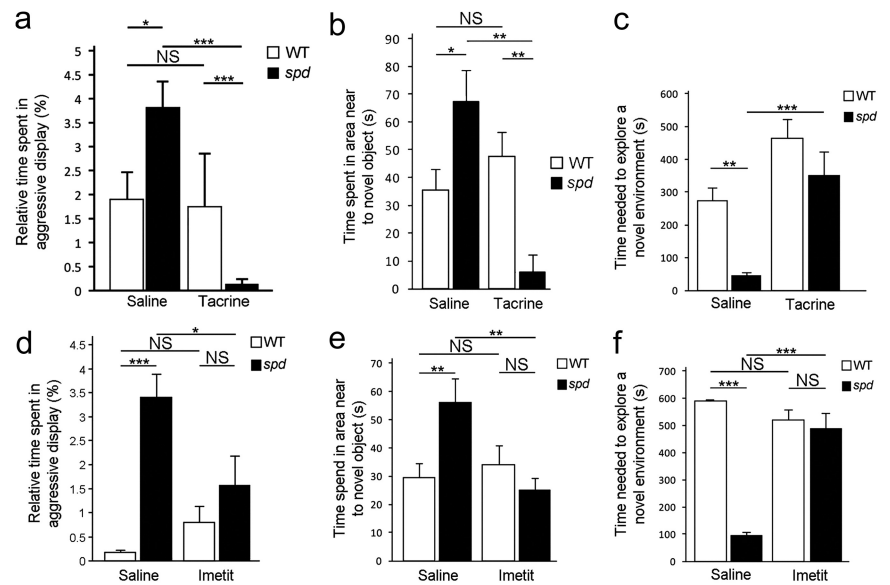


genes are expressed at similar levels throughout the brain of wild-type and homozygous *spd* mutant fish. In the PVN, only *hrh3* expression was easily detectable, whereas *hrh1* was absent and *hrh2* was only weakly expressed. To test whether the HA-linked behavior changes could be mediated by the histamine H3 receptor (Hrh3), we treated fish of both genotypes with a nonsedative dose (Fig. 8*d*) of the selective Hrh3 agonist imetit dihydrobromide (Garbarg et al., 1992). We measured aggression, novel-object boldness, and exploratory activity of imetit-treated fish. Treatment with imetit rescued the behavioral phenotype of homozygous *spd* mutants. Both aggression and novel-object boldness were reduced compared with wild-type siblings (Fig. 7*d,e*), whereas the amount of time needed to explore a T-shaped aquarium was increased to the level of wild-type fish (Fig. 7*f*). Therefore, the combined results from these experiments suggest that modulation of HAergic signaling can rescue the *Fgfr1a*-dependent aggression–boldness phenotype observed in homozygous *spd* mutants.

## Discussion

In this study, we have analyzed *spiegel-danio* mutant fish that have a reduction of *fgfr1a* activity in the brain. Homozygous *spd* mutants show increases in aggression, boldness, and exploration, three components of the aggression–boldness behavioral syndrome. These behaviors are related to a reduction in histamine neurotransmitter levels in *spd*, and pharmacological manipulation of HA signaling is sufficient to rescue the observed behavioral defects.

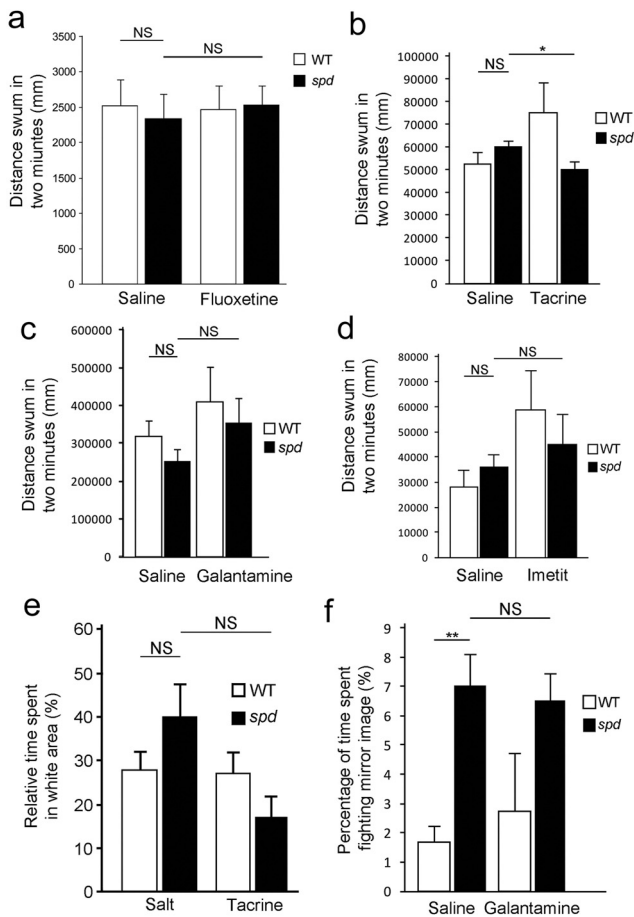
Although behavioral syndromes have been extensively studied, the mechanism underlying their formation is not well understood. Behavioral syndromes could be caused by either genetic or hormonal constraint (Koolhaas et al., 1999; Drent et al., 2003; Veenema et al., 2003; Dingemans et al., 2007) or by the selection of behaviors in response to environmental pressure (Bell, 2005; Bell and Sih, 2007). In this study, we have shown that polymorphism of a single gene, *fgfr1a*, is sufficient to induce parallel changes in three behaviors providing evidence that genetic constraint can influence the aggression–boldness behavioral syndrome. However, in another fish, the stickleback, the correlation between aggression and boldness varies depending on the testing environment (Bell, 2005; Bell and Sih, 2007). It thus seems likely that a combination of genetic or hormonal constraint and environmental conditions controls the expression of behavioral syndromes. Aggression is a malleable behavior, whereas boldness tends to be a more stable trait (Bell and Sih, 2007). Although our behavioral measurements were highly reproducible, we saw differences in aggression levels between zebrafish measured in different locations. Fish raised in Munich, Germany, were significantly more aggressive than those raised in



**Figure 7.** Changes in histaminergic signaling may underlie the aggression–boldness behavioral syndrome. *a*, Treatment of *spd* with the *hnm1* inhibitor tacrine hydrochloride decreases aggression in *spd* (black bars) but not wild-type (white bars) fish. Wild-type versus *spd* saline,  $p = 0.02$ ; wild-type versus *spd* tacrine,  $p = 0.0004$ ; wild-type versus wild-type tacrine,  $p = 0.9$ ; *spd* versus *spd* tacrine,  $p = 0.001$ ;  $n = 8$  wild-type saline,  $n = 8$  *spd* saline,  $n = 9$  wild-type tacrine, and  $n = 10$  *spd* tacrine. Log<sub>2</sub> transformation of data followed by ANOVA shows significant interaction, so both genotype effects were tested separately using a *t* test. *b*, Treatment of *spd* with the *hnm1* inhibitor tacrine hydrochloride decreases boldness in *spd* (black bars) but not wild-type (white bars) fish. Wild-type versus *spd* saline,  $p = 0.04$ ; wild-type versus *spd* tacrine,  $p = 0.006$ ; wild-type versus wild-type tacrine,  $p = 0.33$ ; *spd* versus *spd* tacrine,  $p = 0.006$ ;  $n = 8$  wild-type saline,  $n = 8$  *spd* saline,  $n = 6$  wild-type tacrine, and  $n = 6$  *spd* tacrine. ANOVA shows significant interaction, so both genotype effects were tested separately with a *t* test. *c*, Treatment with the *hnm1* inhibitor tacrine hydrochloride increases exploration time in both *spd* (black bars) and wild-type (white bars). Wild-type versus *spd*,  $F = 10.55$ ,  $p = 0.003$ ; saline versus tacrine,  $F = 22.29$ ,  $p = 0.001$ ;  $n = 10$  wild-type saline,  $n = 12$  *spd* saline,  $n = 10$  wild-type tacrine, and  $n = 10$  *spd* tacrine. Two-way ANOVA without interaction. *d*, Treatment of *spd* with the histamine H3 receptor agonist imetit dihydrobromide reduces aggression in *spd* (black bars) but not wild-type (white bars) fish. Wild-type versus *spd* saline,  $p = 0.00007$ ; wild-type versus *spd* imetit,  $p = 0.3$ ; wild-type versus wild-type imetit,  $p = 0.07$ ; *spd* versus *spd* imetit,  $p = 0.03$ ;  $n = 12$  wild-type saline,  $n = 10$  *spd* saline,  $n = 8$  wild-type imetit, and  $n = 8$  *spd* imetit. ANOVA shows significant interaction, so both genotype effects were tested separately with a *t* test. *e*, Treatment of *spd* with the histamine H3 receptor agonist imetit dihydrobromide decreases boldness in *spd* (black bars) compared with wild-type fish (white bars). Wild-type versus *spd* saline,  $p = 0.005$ ; wild-type versus *spd* imetit,  $p = 0.36$ ; wild-type versus wild-type imetit,  $p = 0.19$ ; *spd* versus *spd* imetit,  $p = 0.02$ ;  $n = 12$  wild-type saline,  $n = 12$  *spd* saline,  $n = 12$  wild-type imetit, and  $n = 12$  *spd* imetit. ANOVA shows significant interaction, so both genotype effects were tested separately with a *t* test. *f*, Treatment of *spd* with the histamine H3 receptor agonist imetit dihydrobromide reduces exploration in *spd* (black bars) but not wild-type (white bars) fish. Wild-type versus *spd* saline,  $p = 0.00001$ ; wild-type versus *spd* imetit,  $p = 0.64$ ; wild-type versus wild-type imetit,  $p = 0.1$ ; *spd* versus *spd* imetit,  $p = 0.001$ .  $n = 10$  wild-type saline,  $n = 10$  *spd* saline,  $n = 10$  wild-type imetit, and  $n = 10$  *spd* imetit. ANOVA shows significant interaction, so both genotype effects were tested separately with a *t* test.

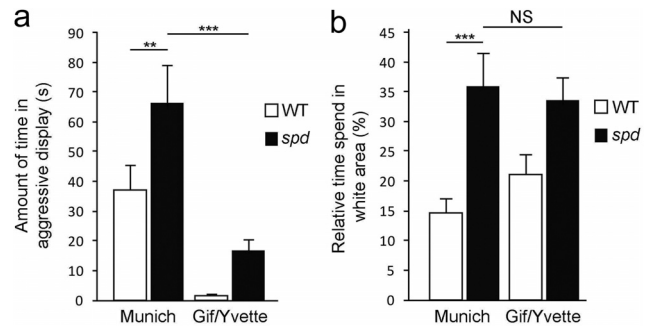
Gif-sur-Yvette, France. Nevertheless, *spd* were always more aggressive than wild-type regardless of testing location (Fig. 9*a*). Zebrafish aggression levels can be affected by the environmental conditions during development (Marks et al., 2005), and behavioral assays are sensitive to changes in feeding regimen and diet, lighting conditions, pH, time of day, and experimental observer (Wahlsten et al., 2006; Burgess and Granato, 2008). Although we made an effort to maintain similar conditions between laboratories, we cannot rule out the contribution of one (or all) of these variables to the alterations in aggression levels. Conversely, the boldness levels of both wild-type and *spd* were similar in both locations (Fig. 9*b*). Therefore, if the correlation between aggression and boldness varies in response to environmental factors, it may be the aggression component of the syndrome that is more likely to be modified.

Although *spd* mutant fish have a life-long reduction of Fgf signaling, it does not appear to adversely affect their lifespan, at least in the laboratory. Reduction of *fgfr1a* function therefore appears to occur within an acceptable limit of developmental plasticity (West-Eberhard, 2005): adult behavioral changes occur



**Figure 8.** Control behavior of drug treated fish. **a**, Fluoxetine hydrochloride does not affect locomotion levels of *spd* (black bars) or wild-type (white bars) fish in a familiar environment. Wild-type versus *spd*,  $F = 0.032$ ,  $p = 0.857$ ; fluoxetine versus saline,  $F = 0.056$ ,  $p = 0.832$ ;  $n = 6$  wild-type saline,  $n = 7$  wild-type fluoxetine,  $n = 8$  *spd* saline, and  $n = 6$  *spd* fluoxetine. Two-way ANOVA without interaction. **b**, Tacrine hydrochloride does not affect the locomotion levels of *spd* (black bars) or wild-type (white bars) fish in a familiar environment. Wild-type versus *spd*,  $F = 0.75$ ,  $p = 0.4$ ; tacrine versus saline,  $F = 4.71$ ,  $p = 0.04$ ;  $n = 6$  wild-type saline,  $n = 6$  wild-type tacrine,  $n = 6$  *spd* saline, and  $n = 6$  *spd* tacrine. Two-way ANOVA without interaction. **c**, Galantamine hydrochloride does not affect the locomotion levels of *spd* (black bars) or wild-type (white bars) fish in a familiar environment. Wild-type versus *spd*,  $F = 0.74$ ,  $p = 0.4$ ; galantamine versus saline,  $F = 1.23$ ,  $p = 0.28$ ;  $n = 6$  wild-type saline,  $n = 6$  wild-type galantamine,  $n = 6$  *spd* saline, and  $n = 6$  *spd* galantamine. Two-way ANOVA without interaction. **d**, Imetit dihydrobromide does not affect the locomotion levels of *spd* (black bars) or wild-type (white bars) fish in a familiar environment. Wild-type versus *spd*,  $F = 3.36$ ,  $p = 0.08$ ; imetit versus saline,  $F = 0.07$ ,  $p = 0.8$ ;  $n = 6$  wild-type saline,  $n = 6$  wild-type imetit,  $n = 6$  *spd* saline, and  $n = 6$  *spd* imetit. Two-way ANOVA without interaction. **e**, Treatment with the *hnm1* inhibitor tacrine hydrochloride causes a nonsignificant reduction in boldness in *spd* (black bars) but not wild-type (white bars). Wild-type versus *spd*,  $F = 0.56$ ,  $p = 0.45$ ; saline versus tacrine,  $F = 2.133$ ,  $p = 0.15$ ;  $n = 15$  wild-type saline,  $n = 13$  *spd* saline,  $n = 12$  wild-type tacrine, and  $n = 9$  *spd* tacrine. Two-way ANOVA without interaction. **f**, Treatment of *spd* (black bars) with the acetylcholinesterase inhibitor galantamine hydrochloride does not decrease aggression in *spd* (black bars) or wild-type (white bars) fish. Wild-type versus *spd*,  $F = 9.09$ ,  $p = 0.0046$ ; galantamine versus saline,  $F = 0.09$ ,  $p = 0.77$ ;  $n = 10$  wild-type saline,  $n = 12$  *spd* saline,  $n = 8$  wild-type galantamine, and  $n = 12$  *spd* galantamine. Two-way ANOVA without interaction.

in the absence of deleterious developmental phenotypes. In this study, we have shown that mutation of *fgfr1a* increases the number of zebrafish expressing aggression at the higher end of the natural spectrum (Fig. 2*a*). Although increased aggression is sometimes viewed as a negative trait, this result suggests that the *spd* behavioral phenotype may rather represent an extreme example of normal behavior (Garrod, 1902).



**Figure 9.** Comparison of the behavior of fish tested in two locations. **a**, Zebrafish raised and analyzed in Gif-sur-Yvette, France, were less aggressive than fish raised and analyzed in Munich, Germany, regardless of genotype. However, *spd* mutant fish were more aggressive than wild-types in both locations. Munich fish versus Gif-sur-Yvette fish,  $F = 5.62$ ,  $p = 0.02$ ; wild-type versus *spd*,  $F = 22.23$ ,  $p = 0.001$ ;  $n = 31$  Munich wild-type,  $n = 27$  Munich *spd*,  $n = 20$  Gif-sur-Yvette wild-type, and  $n = 23$  Gif-sur-Yvette *spd*. Two-way ANOVA without interaction. **b**, Zebrafish raised and analyzed in Gif-sur-Yvette, France, showed similar boldness levels as fish raised and analyzed in Munich, Germany. *spd* mutant fish were bolder than wild-types in both locations. Munich fish versus Gif-sur-Yvette fish,  $F = 0.29$ ,  $p = 0.59$ ; wild-type versus *spd*,  $F = 13.89$ ,  $p = 0.008$ ;  $n = 6$  Munich wild-type,  $n = 7$  Munich *spd*,  $n = 10$  Gif-sur-Yvette wild-type, and  $n = 11$  Gif-sur-Yvette *spd*. Two-way ANOVA without interaction.

*spiegeldanio* mutants harbor a mutation in *fgfr1a* that is a receptor for Fgfs, a large family of secreted signaling molecules with important roles during development (Thisse and Thisse, 2005). Zebrafish have multiple Fgf receptors that are coexpressed in the adult brain. For example, *fgfr1a*, *fgfr2*, and *fgfr3* are all expressed in the PVN, central nucleus, and diffuse nucleus of the hypothalamus (Topp et al., 2008). Both *fgfr2* and *fgfr3* are expressed normally throughout the brain of homozygous *spd* mutants. The most obvious reduction of Fgf signaling in *spd* is found in the PVN. The PVN has already been associated with the control of aggression in teleosts. Electrical stimulation of the nucleus of the lateral recess of the third ventricle (equivalent to the zebrafish PVN) causes aggressive interaction with a mirror in the Bluegill (Demski and Knigge, 1971). However, additional brain areas may also play an important role in linking aggression, boldness, and exploration. For example, a recent gene expression analysis of aggressive zebrafish identified gene expression changes in the telencephalon, optic tectum, hypothalamus, and hindbrain (Filby et al., 2010). It is clear that an in-depth analysis of the *spd* mutant brain is needed to resolve this issue.

Our analysis of *spd* mutants demonstrates that a reduction of Fgfr1a signaling results in increased expression of the HA breakdown enzyme-encoding gene *hnm1*, correlating with decreased levels of HA in the brain. Although *hnm1* expression was only marginally increased in *spd*, modulation of HA levels was sufficient to reverse the behavioral phenotype. However, such a slight change in the mRNA expression level of *hnm1* is unlikely to fully explain the changes observed. Thus, an additional (unidentified) mechanism may also disrupt HA levels in *spd*. Alternatively, the upregulation of *hnm1* may only occur in some discrete areas of the mutant brain. Our analysis of *hnm1* levels in whole zebrafish brain extracts precludes us from ruling out this possibility.

In zebrafish, similar to other animals, HA neurons are found exclusively in the caudal hypothalamus and send projections to many brain areas (Eriksson et al., 1998). We observed a strong reduction of HA-positive fibers throughout the brain of *spd* (Fig. 6*c–h*). Although the PVN (the area of the brain with the most striking reduction of Fgf signaling) does not produce HA, it is positive for both *hnm1* expression and the histamine receptor-



encoding genes *hrh2* and *hrh3* at weak and strong levels, respectively. Rescue of the behavioral phenotype of *spd* mutants with the *Hrh3* agonist imetit (Garbarg et al., 1992) provides evidence that *Hrh3* may underlie these behaviors, although we cannot explicitly rule out a contribution of *Hrh1* and *Hrh2* activity as well. *Hrh3* acts as both an autoreceptor and a heteroreceptor (Peitsaro et al., 2000) and has a complex role in the control of HA neurotransmission (Leurs et al., 2005; Jones and Kearns, 2011). *Hrh3* knock-out mice have a net decrease of HA levels in the cortex (Toyota et al., 2002) and show reduced anxiety in an elevated plus maze (Rizk et al., 2004) reminiscent of the *spd* phenotype. Histamine has been linked previously to several behaviors, including aggression, exploration, and boldness (Lecklin et al., 1998; Yanai et al., 1998; Peitsaro et al., 2003; Passani et al., 2004; Masaki and Yoshimatsu, 2006). Thus, HA may have a role as a general regulator of brain function rather than influencing specific behaviors (Wada et al., 1991).

To our knowledge, there are currently no studies linking Fgf and HA, making it difficult to understand how these signaling pathways might interact. However, our data suggest two possible mechanisms. Fgf signaling could act on *hnm1* expression independently of HA, reducing the activity of the enzyme. A reduction of *fgfr1a* in *spd* mutants would lead to a concomitant increase in *Hnm1* activity and HA turnover. Alternatively, *Fgfr1a* could interact with *Hrh3* signaling at the level of MAPK signaling. Stimulation of *Hrh3* receptors results in MAPK activation (Drutel et al., 2001), providing a possible substrate for interaction with Fgf that also uses MAPK as a target (Thisse and Thisse, 2005). Reduction of *fgfr1a* may decrease the sensitivity of *Hrh3*-expressing cells to *Hrh3* activation. Because we only observe a slight increase in *hnm1* expression levels in mutants, we tend to favor this second hypothesis. Together, our results suggest that Fgf signaling may sensitize the brain to changes in HAergic signaling levels, thus altering the expression of the aggression–boldness behavior syndrome. Nevertheless, it is important to remember that *spd* mutants have a life-long reduction of Fgf signaling, meaning that the behavioral phenotype we observe may stem from changes in brain development. It is therefore likely that other neurotransmitter pathways may also mediate aspects of the *spd* behavioral phenotype. Additional studies will be required to address this issue.

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