The bullies are the leaders of the next generation: Inherited aminergic neurotransmitter system changes in socially dominant zebrafish, *Danio rerio*

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ABSTRACT

We studied the social hierarchy in zebrafish and assessed differences in neurotransmitters and behavior in the F1 generation offspring of dominant and subordinate zebrafish (*Danio rerio*). We used behavioral assays to study locomotion, ability to complete cognitive tasks, social interaction and aggression. To study the neurochemical changes, we applied quantitative polymerase chain reaction, high pressure liquid chromatography and immunohistochemistry. Social hierarchies were formed both by males and females when animals were kept in same sex pairs in the dyadic dominant-subordinate hierarchy test. The offspring of dominant animals were the leaders in social interactions, however aggression in the mirror-test was not altered in any group. Serotonin and noradrenaline levels were lower in the F1 generation subordinate animals when compared with dominant animals, but not compared with animals that were naïve to social hierarchy. The mRNA level of the rate-limiting enzyme in histamine synthesis, *histidine decarboxylase*, was significantly lower in dominant and subordinate larval zebrafish when compared with control animals. In the dominant adult zebrafish *tyrosine hydroxylase 1* mRNA level was lower compared with control animals, whereas *tyrosine hydroxylase 2* mRNA was not different. The result was verified with immunohistochemistry. There were gender specific differences between the dominant and subordinate animals, where the dominant females performed better in cognitive tasks such as the T-maze than subordinate females. This was not observed in males, as the behavior of the dominant and subordinate males did not differ. These results add to the understanding of the plastic nature of the central nervous system and show that neurochemical features in aminergic neurotransmitter systems are associated with social leadership and dominance.

1. Introduction

Social hierarchy exists within the majority of different animal populations including humans. This hierarchy affects both reproductive fitness and individual health resulting in aberrant functioning, which is a frequent symptom in neurologic and psychiatric disorders. The chronic psychosocial stress mediated by the subordinate status profoundly affects e.g. the immune system [1]. Dysregulation of the developing immune system can lead to neurodevelopmental disorders such as autism spectrum disorder (ASD) and schizophrenia [2]. A characteristic feature for ASD is the impairment of social skills especially in psychosocial stressful situations [3].

In fish, aggression might initially be used to form the social hierarchy, whereas when the hierarchy is established, there is no more need for aggressive attacks [4,5]. In humans, aggression has been studied by genome-wide association studies that identified associations with genes involved in dopaminergic and serotonergic neurotransmission and hormone regulation [6]. In rodents specific serotonergic modules affect aggression [7] whereas dopamine D2 receptor plays an important role in social hierarchy [8]. Social status, and especially the subordinate status, is associated with increased stress in fish [4]. The increased stress activates the hypothalamic-pituitary-adrenal axis in mammals and the equivalent hypothalamic-pituitary-interrenal axis in fish, affecting immune system functioning and hypothalamic functions [9], such as food intake [10] and reproductive success [11,12]. In the cichlid fish, *Astotilapia burtoni*, gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus are directly connected with social status, and these neurons grow in size and their connectivity changes within dominant males [13–15]. Other studies have linked the hypothalamic aminergic systems to social hierarchy. These neurotransmitter systems send widespread projections throughout the brain and communicate intensely with the PFC in mammals and dorsal telencephalon in fish [16]. Increased

dopamine signaling has been strongly linked to dominant behaviors in the male zebrafish [4] and male mice [17]. In zebrafish, boldness predicts social dominance in the dyadic contest and males are bolder than females [18], ensuring that there are sex-specific differences also in the zebrafish. The involvement of aminergic signaling in establishment of social hierarchy has been implicated, by significant impairment of the serotonergic system. Lack of serotonin is important in the etiology of depression; hence social defeat has been utilized as a way to study depression. Stimulation of the serotonergic system is associated with lower aggression in rats [19] and in *O. mykiss* [20,21]. Dopamine concentration in the forebrain of control, dominant and subordinate females is lower than in control, dominant and subordinate male fish [22]. Whereas the ratio of serotonin to its main metabolite 5-hydroxyindoleacetic acid is significantly lower in dominants compared with subordinates in both sexes, in the forebrain of males the ratio is higher when compared with females. Females tend to accept the social rank more easily than the males, a fact which may explain the discrepancy between the sexes in serotonin turnover [22].

We studied the effect of social hierarchy on the performance of zebrafish in two generations to identify the inherited properties of the hierarchy. We used naïve wild-type zebrafish that we challenged in the social hierarchy test and studied the behaviors of these dominant and subordinate male and female animals. The dominant and subordinate animals were then bred respectively, to acquire two different pools of fish, the dominant and the subordinate. The behavior and neurotransmitter levels in these animals were assessed to evaluate if the behavioral and neurochemical changes of social hierarchy are inherited.

2. Materials and methods

2.1. Animals

A wildtype Turku strain kept and raised in the laboratory [23] of both genders was used in our study. Fish were maintained according to standardized protocols [24] and the regulations of the European Convention. The animal experiment permits were acquired from the Regional Office of Southern Finland (ESAVI/3623/04.10.03/2012 and ESAVI/6100/04.10.07/2015). Adult zebrafish naïve to the dyadic social hierarchy test were used in the first behavioral experiments. To breed the dominant strain, the initially naïve animals which as a result of the dyadic social hierarchy test were defined as dominant males or females were later paired (at 10 months of age) and the offspring of these animals were collected and used as the dominant (DOM) group. The same procedure was done for the subordinate and control animals. The dominant, subordinate (SUB) and control (CTRL) fish were housed in separate groups until they reached adulthood.

2.2. Behavioral assays

Five different behavioral assays were performed according to the experimental setup presented in Table 1. In phase I-III we analyzed fish that had not been bred for social hierarchy (either dominance or subordinate behavior), whereas in phase IV-VII we analyzed fish that had been bred for either the dominant or subordinate trait. We refer to the fish in phase I-III as P generation and to the fish in phase IV-VII as F1 generation. All the fish were individually housed (tank size or V: 1,4 L, 5−6 cm wide and 22−27 cm long) which allowed follow up on personalities of fish and the correlation to dominant-subordinate outcomes. All fish were kept and bred under similar conditions to avoid that different housing conditions could account for differences observed in e. g. behavior. The walls of the holding tanks were transparent and the same water was circulated in all tanks, so the fish could see and smell each other, but not touch each other. In addition, the parents of the control fish were not subjected to dyadic social interaction.

Table 1
Experimental setup and approach.

	Fish status in regard to social hierarchy	Behavior and neuromodulator assays
Phase I	Animals naïve for the dominant-subordinate hierarchy: • 22 males, 18 females.	Locomotion (1) T-maze (2)
Phase II	Animals naïve for the dominant-subordinate hierarchy: • 22 males and 18 females.	Dominant-subordinate hierarchy (3)
Phase III	Social hierarchy established in initially naïve animals: • 11 male pairs resulted in 11 dominant males and 11 subordinate males, • 9 female pairs resulted in 9 dominant females and 9 subordinate females.	Locomotion (1) T-maze (2)
Phase IV	Breeding for dominant and/or subordinate animals: • Animals that are naïve to the dominant-subordinate hierarchy were used as controls.	
Phase V	Assessing the behaviors of offspring to dominant, subordinate and naïve animals.	Locomotion (1) T-maze (2)
Phase VI	Animals naïve for the hierarchy test, but that are offspring of either dominant, subordinate or control animals.	Dominant-subordinate hierarchy (3)
Phase VII	Dominant, subordinate and control animals.	Social leadership (4) Aggression (5) RT-qPCR HPLC

2.2.1. Locomotion (1)

General movement was quantitatively assessed in the individuals according to [23,25]. Briefly, six individual fish were simultaneously video-tracked in individual chambers made out of white plastic. The chambers had a diameter of 205 cm at bottom and 230 cm at top, contained 1 L of 25 °C fish water and the water column was 3 cm. Advanced and individual calibration of each arena was used. The chambers were divided into three digital arenas; inner (Ø = 5,5 cm), middle (Ø = 165 cm) and outer (Ø = 205 cm). The division of arenas into zones allowed assessment of the preference of fish for different areas in the chamber. The only difference between the zones was the proximity to the outer edge or to the central compartment of the chamber. Fish were habituated to the behavioral arenas for 1 min before tracking was started. Tracking was performed by EthoVision 3.1. software (Noldus) between 10:00−15:00 for a period of 10 min for each individual fish with a sample rate of 5 samples/second. The tracking was done in a brightly lit area (LUX 1108). The following parameters were analyzed: total distance moved (cm during 10 min), absolute angular velocity (degrees/second) and frequency entering the inner zone (times). Fish that had not been detected for more than 90 % of the time during the 10 min tracking period were excluded from the analysis. In total the basic locomotion pattern of 22 males and 18 females from the P generation and 30 males from the F1 generation were analyzed at the age of one year.

2.2.2. T-maze (2)

To assess reaction time in a cognitive task a T-maze was used according to Peitsaro [25]. Shortly, the T-maze is a simple labyrinth made of dark plastic where the fish perform individually. The size of the T-maze is as follows: starting chamber 8 cm wide and 15 cm long, start arm 45 cm long, the unpleasant shallow arm 8 cm wide and 30 cm long, and the pleasant deeper and larger basin 20cm × 20cm. The T-maze was filled with RT fish water. In the deep compartment the water depth was 9 cm and in the rest of the maze the water depth was 5 cm. The fish was moved from its home tank and placed into the starting point where it was habituated for 30 s, where after it was immediately allowed to explore the T-maze and the time the fish took to move to the pleasant area was measured. The fish could choose between 1) an arm that was

identical to the starting point with regard to depth and 2) an arm ending in a deeper and wider compartment containing plastic grass and stones to create a more safe and pleasant environment. The basic idea was that the fish would prefer the deep arm over the arm as shallow as the starting point, and hence the fish would not explore the maze after the initial 5 min habituation allowed to the entire maze, but rather swim directly to the deep basin from the starting point. In the initial trial fish were first allowed to habituate to and freely explore the system for 5 min, then moved to the starting point where they were kept for 30 s after which the time it took for the fish to reach the deep basin was measured, maximum time cut-off at 120 s. The trial was repeated three consecutive times for each individual each day the test was performed. The test was performed four times on the same set of fish, i.e. on day 1 and day 4 after assessing basic locomotion and before the social hierarchy had been established in the naïve P generation of adult zebrafish, and later on day 1 and day 4 after the end of the social hierarchy formation. In the F1 generation the behavior was assessed only after the social hierarchy formation. In total the time taken to reach the deep arm in the T-maze was assessed for 22 males and 18 females from the P generation and for 30 males from the F1 generation were analyzed at the age of one year.

2.2.3. Dominant-subordinate social hierarchy test (3)

Eleven male-pairs and nine female-pairs of the P generation zebrafish that had not previously been exposed to social hierarchy conditions were set up into individual aquaria (V: 3 L, 9−11 cm wide and 22−27 cm long or V: 1,4 L, 5−6 cm wide and 22−27 cm long) partially according to [4]. All the fish were weighed and fin clipped under anesthesia (MS-222, Sigma-Aldrich; Table 2) before the dyadic social hierarchy test, to be able to distinguish between the two individuals when housed together in the same tank and thereby assess the performance and behavior of the individual fish. The effect that MS-222 might have on behavior was not assessed. Video recordings of the individual pairs were performed with an iPad (Apple Inc.) each day for 1 min during the afternoons of the five day trial. The videos were later analyzed and the time each fish spent in the upper and/or lower compartment of the tank was scored, as well as how many times each fish attacked the other fish in the aquaria. After the five-day trial the fish were moved into individual tanks until basic locomotion and T-maze had been assessed, after which the fish were pooled as dominant or subordinate fish and kept in larger pools of fish to allow breeding. The F1 generation was assessed in a similar manner and in that case we only studied males. We chose 10 fish from the offspring of fish that in P generation had been dominant, 10 fish from the offspring that in P generation had been subordinate and 10 fish that were naïve to the social hierarchy test, and whose parents had not been exposed to the dyadic social hierarchy test. As with the fish in the P generation, the fish of the F1 generation were weighed and fin clipped under anesthesia and the protocol for assessing the formation of the dominance in the dyadic social hierarchy test was the same as described above for the P generation. In the case of these animals, they were kept in individual tanks until basic locomotion and T-maze had been assessed, after which the fish were pooled in groups as dominant or subordinate fish. The social hierarchy was repeated twice and in total 60 adult male zebrafish were used for this experiment from the F1 generation.

Table 2
Length and weight of both P and F1 generation zebrafish.

Sex	Hierarchy/genotype	Weight, g (mean ± stdev)	Length, cm (mean ± stdev)
P generation Male	Dominant	0.28 ± 0.03	2.58 ± 0.11
	Subordinate	0.28 ± 0.03	2.51 ± 0.14
P generation Female	Dominant	0.37 ± 0.07	2.62 ± 0.10
	Subordinate	0.35 ± 0.05	2.60 ± 0.11
F1 generation Male	Control	0.40 ± 0.10	2.67 ± 0.24
	Dominant	0.30 ± 0.05	2.49 ± 0.07
	Subordinate	0.28 ± 0.09	2.33 ± 0.15

2.2.4. Social leadership test (4)

Social leadership was assessed in the F1 generation of control (i.e. not offspring of fish that had been exposed to the dyadic social hierarchy paradigm and hence completely naïve to the social hierarchy), dominant and subordinate adult male zebrafish. Briefly, five adult individuals were transferred to a white circular tank with the diameter of 23 cm containing 1,5 L of 25 °C fish water and then video recorded for 10 min (14.31 frames/s) in a brightly lit sealed-off area (LUX2200). The videos were analyzed with idTracker [26] and further in MATLAB R2015a as previously described [26]. The behavioral assay was repeated three times with three groups of F1 generation fish (i.e. three groups of dominant, three groups of subordinate and three groups of control fish) collected from different breedings. In each analysis n = 5−6/group.

2.2.5. Aggression (5)

Aggression was assessed in the F1 generation male zebrafish as previously described [27] with minor modifications. Fish were placed individually into rectangular arenas (33 cm long and 14 cm wide) with fish water up to 5 cm. At either short end of each arena a mirror was attached at an angle of 25° allowing the fish to see a mirror image of itself. The arena was divided into two digital zones, a "mirror"-zone next to the mirror and a "no mirror"-zone in the end opposite to the mirror. The locomotion of adult male zebrafish was tracked with the EthoVision 3.1. software for 10 min with the rate of 5 frames/second after which the following parameters were analyzed: total distance moved within the arena (cm during 10 min), absolute meander (degrees/cm), time spent in the "mirror"-zone (seconds), and time spent in the "no-mirror"-zone (seconds). Fish that did not move more than 90 % of the time tracked were excluded from the analysis. The behavioral assay was repeated three times with three groups of adult male F1 generation fish, for dominant, subordinate and control fish, from different breedings. In each analysis n = 4/group.

2.3. RNA isolation and cDNA synthesis

From the dominant, subordinate and control groups of the F1 generation whole 15–20 7dpf larvae were collected in 1.5 mL microcentrifuge tubes and killed on ice-cold water for RT-qPCR. In total 6 replicates were made per group. RNA isolation was performed immediately using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Before cDNA synthesis, genomic DNA from the RNA samples was digested using the TURBO DNA-free Kit (Thermo Fisher Scientific/Ambion, Waltham, USA), followed by reaction purification using the RNeasy Mini Kit. cDNA was synthesized from 1−2 μg of RNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific/Invitrogen, Waltham, USA). Adult F1 male zebrafish from dominant, subordinate and control groups were sacrificed by decapitation and brains (n = 10/group) were dissected. RNA isolation was performed immediately from individual brains using the RNeasy Mini Kit, and the cDNA synthesis performed as above.

2.4. RT-qPCR analysis

All qPCR analyses were done using the LightCycler® 480 system with accompanying software (Roche, Basel, Switzerland). All samples were analyzed in duplicates using the LightCycler® 480 SYBR Green I Master (Roche). Primers for target genes were designed using the NCBI primer BLAST search tool, primer sequences and accession numbers are shown in Table 3. All primers were purchased from Oligomer (Immuno Diagnostic/Addlife, Stockholm, Sweden). The PCR reaction was set up according to the SYBR Green kit instructions. For all primers analyzed, the annealing temperature was set to 60 °C. The durations of the annealing and extension steps were set to 15 s and 20 s, respectively. Cp values were calculated using the Abs Quant/2nd Derivative Max analysis included in the software. Relative quantification of mRNA species was done according to Livak and Schmittgen [28], using *eef1a1l1* and *β-actin*

Table 3
Primers used for RT-qPCR.

Gene	Accession number	Forward primer	Reverse primer	Length
hdc	>NM_001102593.1	TTCATGCGTCCTCTCCTGC	CCCCAGGCATGATGATGTTC	94
hrh1	>NM_001042731.1	TCCTGATCCCGTCCGCACCA	CCCGACGGTATGCAGCGTCC	146
hrh2	>NM_001045338.2	GGCCACTAGGGGCGCACTTC	AGCGGAGCAGTGACCGCAAA	121
hrh3	>NM_001025518.1	CGCCACCGTCCTTGGGAACG	GGGGATGCAAAACCCGCCGA	130
th1	>NM_131149.1	GACGGAAGATGATCGGAGACA	CCGCCATGTTCCGATTTCT	95
th2	>NM_001001829.1	CTCCAGAAGAGAATGCCACATG	ACGTTCACTCTCCAGCTGAGTG	110
hcrt	>NM_001077392.2	TCTACGAGATGCTGTGCCGAG	CGTTTGCCAAGAGTGAGAATC	109
gfap	> NM_131373.2	GAAGCAGGAGGCCAATGACTATC	GGACTCATTAGACCCACGGAGAG	82
β-actin	>NM_131031.1	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	102
eef1a1l1	>NM_131263.1	CCAACTTCAACGCTCAGGTCA	CAAACTTGCAGGCGATGTGA	105

as internal control genes.

2.5. HPLC

The concentration of biogenic amines was assessed in brains of individual fish with high-performance liquid chromatography (HPLC) as previously described [29,30]. Briefly, brains were collected from dominant, subordinate and control groups of F1 generation adult fish (n = 5/group), flash frozen and processed for HPLC. Protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to manufacturer's instructions.

2.6. Immunohistochemistry

Adult F1 generation male zebrafish from each group (dominant, subordinate and control) were sacrificed by decapitation, the brains were dissected and fixed in 4% 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma-Aldrich, Saint Louis, USA) followed by 0.1 % paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer pH 7.0 o/n at 4 °C according to [31]. The next day the whole brains were rinsed several times with phosphate buffered saline containing 0.3 % Triton-x100 (PBSTx 0.3 %) followed by three washes in PBSTx 0.3 % lasting 1 h each. The whole brains were pre-incubated in a solution containing 4% normal goat serum (NGS), 1% dimethyl sulfoxide, and PBSTx 0.3 % o/n at 4 °C to increase permeability and decrease background staining. The next day the brains were moved to a solution containing 2% NGS, PBSTx 0.3 %, and the well characterized primary antibodies rabbit anti-histamine [32] and mouse anti-tyrosine hydroxylase purchased from Diasorin (Saluggia, Italy) [16]. The incubation in the primary antibody solution was done for 4 days at 4 °C. To remove the primary antibodies brains were washed once for 30 min and three times in PBSTx 0.3 % lasting for one hour each. After washes, whole brains were moved to the secondary antibody solution, containing highly cross purified goat anti-mouse conjugated with the 568 fluorophore (Alexa antibody, A11031, lot 822,389) and goat anti-rabbit conjugated with the 488 fluorophore (Alexa antibody, A11034, lot 1,751,340) at a dilution of 1:1000 in PBSTx 0.3 % o/n at 4 °C. The next day, the brains were washed again according to the same scheme: once for 30 min and three times in PBSTx 0.3 % lasting one hour each. This was followed by infiltration in 80 % glycerol o/n at 4 °C. All abovementioned washes were done at room temperature. To visualize the neurotransmitter network, the brains were mounted on glass slides in 80 % glycerol and spacers were built by attaching 4–5 small coverslips to each other with silicon grease, and analyzed by a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). To excite the 488 fluorophore, an argon-krypton laser was used and emission was collected at 500−550 nm. To excite the 568 fluorophore, a diode laser was used and the emission wavelength collected at 570−620 nm. Sequential scanning was used and no overlap was allowed between the wavelengths collected from the two different channels to avoid bleed through and false positive results. We used a HC PL APO CS 20x objective (DRY, NA 0.7) and 2 μm step size when acquiring the 3D image of the hypothalamus in the stained brains.

Results are presented as maximum projection images.

2.7. Statistical analysis

In the test for general locomotion a parametric test (One-way analysis of variance, followed by Tukey's multiple comparison test), or a nonparametric test (Kruskal-Wallis followed by Dunn's multiple comparison test) was used if the data was not normally distributed. The results from the dyadic social hierarchy test were analyzed by Two-way repeated measures analysis of variance. The results on reaction time in the T-maze were assessed with several statistical tests: Student's t-test, One-way analysis of variance followed by Tukey's multiple comparison test, Two-way repeated measures analysis of variance followed by Bonferroni's multiple comparison test or Tukey's multiple comparison test. Significant differences in the aggressive behavior were assessed by One-way analysis of variance, followed by Tukey's multiple comparison test. Quantitative RT-qPCR and HPLC results were analyzed with the parametric test (One-way analysis of variance, followed by Tukey's multiple comparison test) and/or nonparametric test (Kruskal-Wallis followed by Dunn's multiple comparison test) accordingly. Statistical significance was set to a minimum of $p < 0.05$ in all cases.

3. Results

3.1. Locomotor activity

Locomotion was assessed both before the social hierarchy test and immediately after the establishment of social hierarchy in both males and females of the P generation (Fig. 1). There were no significant differences in locomotion when the performance of male and female fish pre and post the social hierarchy test (Fig. 1a,b $p > 0.05$, One-way analysis of variance, n=9−11/group) was tested. However, the exploratory behavior of the subordinate females was significantly different from dominant females (Fig. 1c). The subordinate females showed a higher frequency of entering the inner zone after the social hierarchy test as compared with the situation before the social hierarchy test (H(8) = 17.48, $p < 0.05$, Kruskal-Wallis test, Dunn's multiple comparison test, $p < 0.05$) and in comparison with dominant females before social hierarchy test (H(8)=17.48, $p < 0.05$, Kruskal-Wallis test, Dunn's multiple comparison test, $p < 0.01$). In the F1 generation no significant differences were detected in the locomotion or exploratory behavior between the three groups; control, dominant and subordinate ($p > 0.05$, One-way analysis of variance, n=6/group, experiment was repeated three times with fish from different clutches, data not shown).

3.2. T-maze performance before social hierarchy testing

The males and females of the P generation performed differently in the T-maze before the social hierarchy test (Fig. 2). Females that later became dominant (PRE_DOM_F) took overall less time compared with females that later became subordinate (PRE_SUB_F) to enter the deep compartment (Student's t-test, t(10) = 2.490, $p < 0.05$). However, they
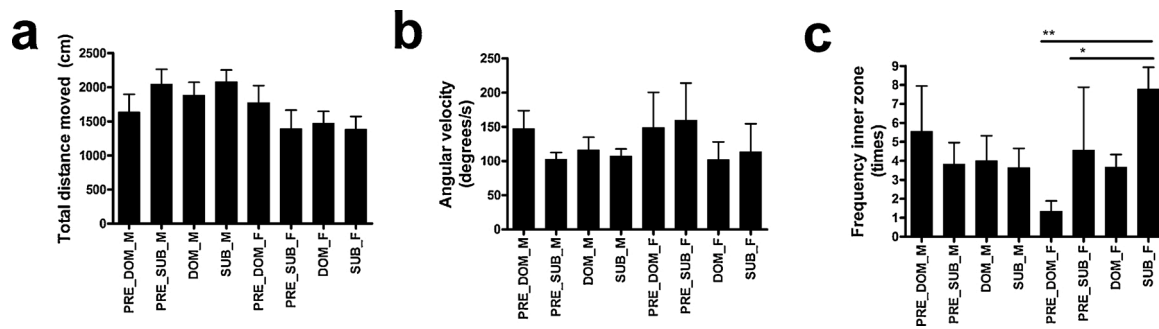
Fig. 1. Locomotion pre and post to the dominance hierarchy test; a) total distance moved (cm), b) angular velocity (degrees/s), and c) frequency of entering the inner zone (times). * = $p < 0.05$, ** = $p < 0.01$, Kruskal-Wallis test, Dunn's multiple comparison test. M=male, F=female, DOM=dominant, SUB=subordinate, PRE=before hierarchy test, POST=after hierarchy test, n=9-11 individuals/treatment. Graphs represent mean±SEM.
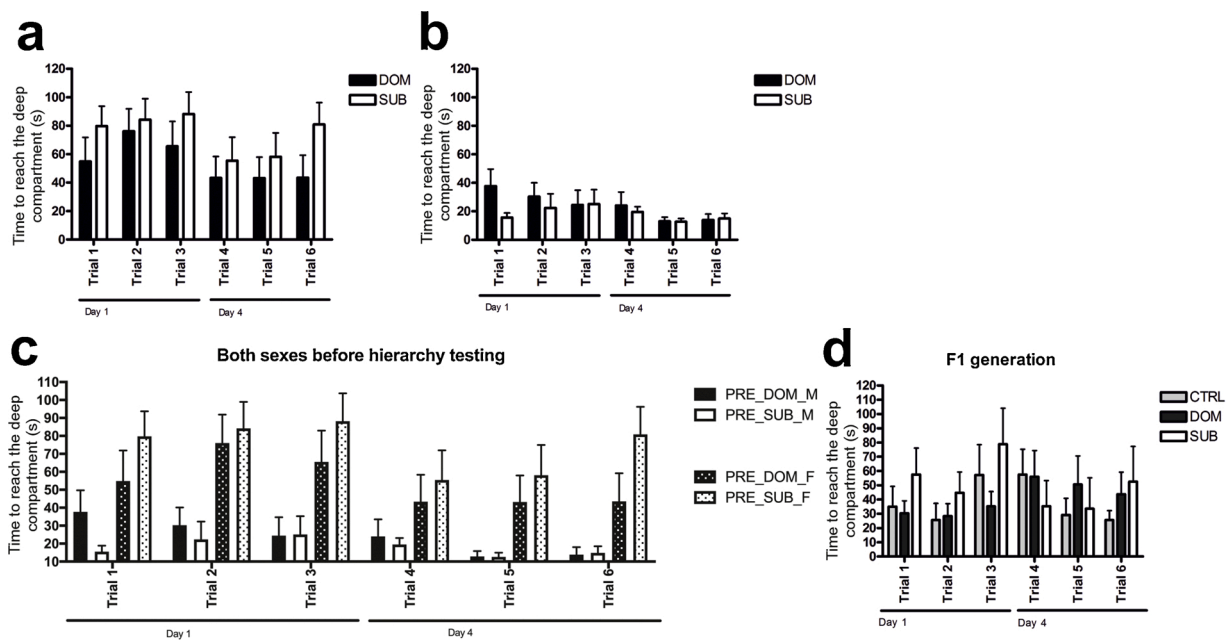

Fig. 2. Time spent to reach the deep basin was assessed by the T-maze test before the social hierarchy testing. a) Time spent by females before social hierarchy testing to reach the deep basin, n = 9. Student's t-test, $p < 0.05$. Two-way repeated measures analysis of variance, interaction of time and treatment $p > 0.05$, time $p < 0.01$, treatment $p > 0.05$. No significant differences between trials within groups, Tukey's multiple comparisons test. b) Time spent by males before social hierarchy testing to reach the deep basin, n = 11. Student's t-test, $p > 0.05$. Two-way repeated measures analysis of variance, interaction of time and treatment $p > 0.05$, time $p > 0.05$, treatment $p > 0.05$. No significant differences between trials within groups, Tukey's multiple comparisons test. c) The time spent by both sexes to reach the deep basin before the social hierarchy testing. $p > 0.05$ PRE_DOM_M vs PRE_SUB_M, $p < 0.001$ PRE_DOM_M vs PRE_DOM_F, $p < 0.001$ PRE_DOM_M vs PRE_SUB_F, $p < 0.001$ PRE_SUB_M vs PRE_DOM_F, $p < 0.001$ PRE_SUB_M vs PRE_SUB_F, $p < 0.05$ PRE_DOM_F vs PRE_SUB_F, One-way analysis of variance, followed by Tukey's multiple comparison test. Two-way repeated measures analysis of variance interaction of time and treatment $p > 0.05$, time $p < 0.001$, treatment $p < 0.001$, no significant differences between trials within groups, Bonferroni's multiple comparisons test, n = 9-11. d) Time spent by the F1 generation males to reach the deep basin before social hierarchy testing, n = 5. Two-way repeated measures analysis of variance, interaction of time and treatment $p > 0.05$, time $p > 0.05$, treatment $p > 0.05$, n = 5/group. M = male, F = female, DOM = dominant, SUB = subordinate, PRE = before hierarchy test. Graphs represent mean ± SEM.

did not improve their performance between trials, i.e. shorten the time they took to enter the deep compartment (Fig. 2a, Two-way repeated measures analysis of variance, interaction of time and treatment F(5,80) = 0.6305, $p > 0.05$, time F(5,80) = 3.748, $p < 0.01$, treatment F(1,16) = 1.152, $p > 0.05$, n = 9). Males that later became dominant (PRE-DOM_M) did not find the deep compartment faster than the males that became subordinate (PRE_SUB_M, Student's t-test, t(10) = 1.271, $p > 0.05$), nor did they show increased performance in the task to reach the deep basin when the different trials were compared (Fig. 2b, Two-way repeated measures analysis of variance, interaction of time and treatment F(5,60) = 0.6528, $p > 0.05$, time F(5,60) = 1.290, $p > 0.05$, treatment F(1,60) = 1.526, $p > 0.05$, n = 11). There was a gender difference as males were much faster in reaching the deep compartment of the maze than females (Fig. 2c, F(3,20) = 33.38, $p < 0.001$, One-way

analysis of variance followed by Tukey's multiple comparison test, $p > 0.05$ PRE_DOM_M vs PRE_SUB_M, $p < 0.001$ PRE_DOM_M vs PRE_DOM_F, $p < 0.001$ PRE_DOM_M vs PRE_SUB_F, $p < 0.001$ PRE_SUB_M vs PRE_DOM_F, $p < 0.001$ PRE_SUB_M vs PRE_SUB_F, $p < 0.05$ PRE_DOM_F vs PRE_SUB_F, and Two-way repeated measures analysis of variance, interaction of time and treatment F(15,180) = 1.096, $p > 0.05$, time F(5,180) = 5.268, $p < 0.001$, treatment F(3,36) = 7.8983, $p < 0.001$, n = 9−11). The adult F1 generation males did not show better and faster performance in the task to find the more pleasant deep basin (Fig. 2d, Two-way repeated measures analysis of variance, interaction of time and treatment F(10,60) = 1.450, $p > 0.05$, time F(5,60) = 1.612, $p > 0.05$, treatment F(2,12) = 0.2438, $p > 0.05$, n = 5/group) nor did the group alone (dominant, subordinate, control) have an effect on the time it took for the fish to complete the task of finding the deep basin ($p > $

0.05, One-way analysis of variance).

3.3. Establishing social hierarchy

In both male pairs and female pairs the social dominance-subordinate hierarchy was formed within five days (Fig. 3). During the course of the five days, one of the fish became dominant and the other one subordinate. The male fish developed a clear spatial hierarchy (Student's t-test, $t(8) = 4.247$, $p < 0.01$, n=11/group) with the dominant individual patrolling the top compartment of the tank (Fig. 3a, Two-way repeated measures analysis of variance, interaction of time and treatment $F(4,80) = 3.797$, $p < 0.01$, time $F(4,80) = 3.901$, $p < 0.01$, treatment $F(1,20) = 12.10$, $p < 0.01$, Bonferroni's multiple comparisons test, ** $= p < 0.01$, *** $= p < 0.001$) and the subordinate male spent most of the time freezing at the bottom of the tank. The male which at the end of the social hierarchy test was determined as the

dominant individual, had during the five-day trial attacked the subordinate male significantly more than vice versa (Student's t-test, $t(8)= 2.931$, $p < 0.05$). However, no significant differences were detected in the number of attacks performed by either individual on specific days during the five-day trial (Fig. 3b, Two-way repeated measures analysis of variance, interaction of time and treatment $F(4,80) = 1.150$, $p > 0.05$, time $F(4,80) = 2.512$, $p < 0.05$, and treatment $F(1,20) = 5.768$, $p < 0.05$).

In females the social hierarchy was also established within five days (Student's t-test, $t(8) = 6.634$, $p < 0.001$, n=9/group), but it was not spatially as clear as in the males (Fig. 3c, Two-way repeated measures analysis of variance, interaction of time and treatment $F(4,64) = 0.3077$, $p > 0.05$, time $F(4,64) = 0.2933$, $p > 0.05$, and treatment $F(1,64) = 3.463$, $p > 0.05$). The animal that at the end of the social hierarchy test was defined as the dominant female was generally chasing the animal that at the end of the social hierarchy test was defined as the subordinate
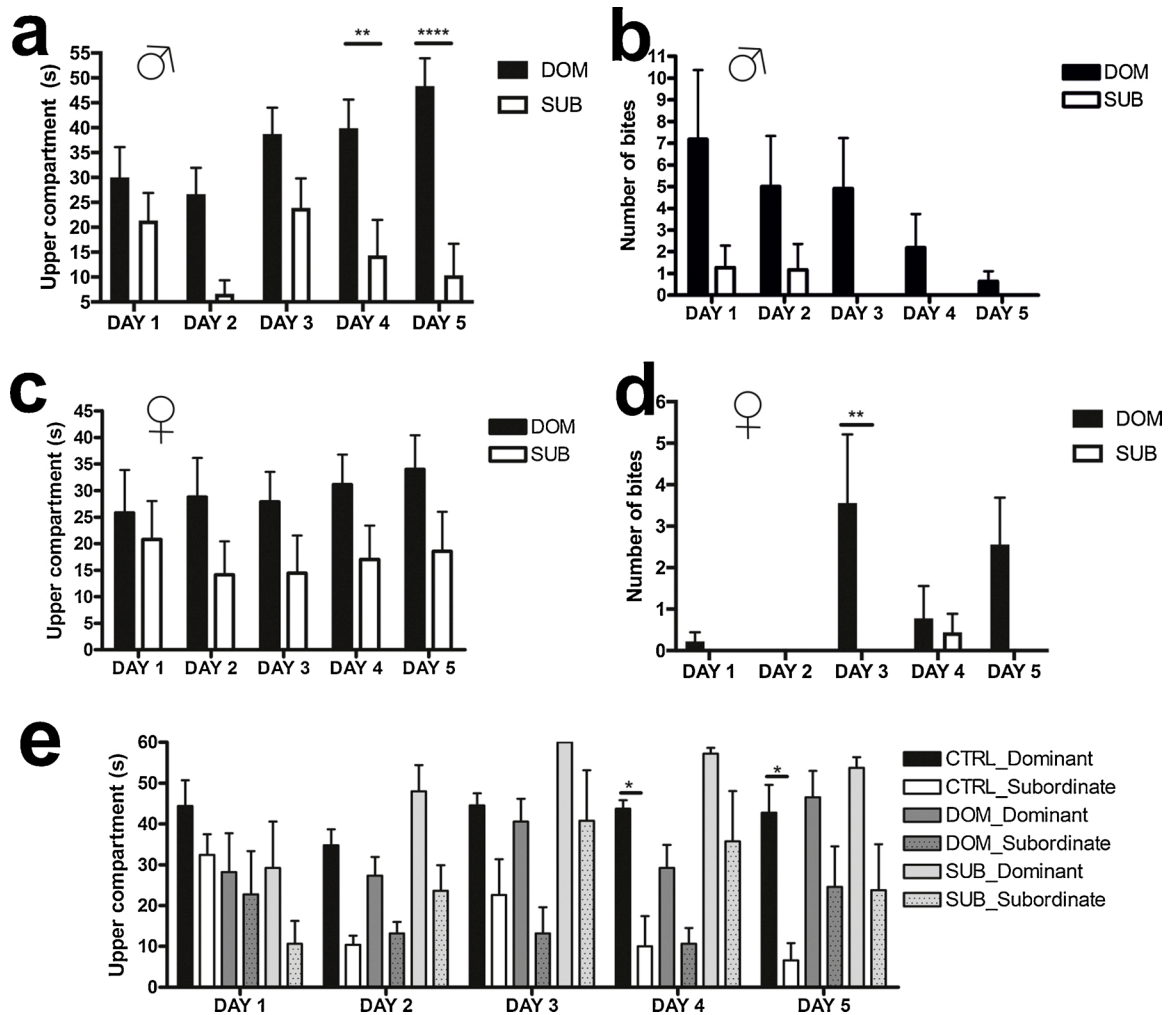

Fig. 3. Fish behavior during the social hierarchy test. Dominant fish spend more time in the upper compartment compared with the subordinate fish, and attack/bite the subordinate. a) Time spent by the P generation male fish in the upper compartment of the tank, n = 11. Two-way repeated measures analysis of variance, interaction of time and treatment $p < 0.01$, time $p < 0.01$, treatment $p < 0.01$, Bonferroni's multiple comparisons test, ** $= p < 0.01$, *** $= p < 0.001$. b) The number of times the males attacks/bites its test partner during a 1 min test time during 5 subsequent days. Two-way repeated measures analysis of variance, interaction of time and treatment $p > 0.05$, time $p < 0.05$, treatment $p < 0.05$, no significant differences between groups with the Bonferroni's multiple comparisons test. c) Time spent by the P generation female fish in the upper compartment of the tank during a 1 min test time during 5 subsequent days, n = 9. Two-way repeated measures analysis of variance, interaction of time and treatment $p > 0.05$, time $p > 0.05$, treatment $p > 0.05$, no significant differences between groups with the Bonferroni's multiple comparisons test. d) The number of times the female attack/bite the test partner during a 1 min test time during 5 subsequent days. Two-way repeated measures analysis of variance, interaction of time and treatment $p < 0.05$, time $p > 0.05$, treatment $p < 0.05$, Bonferroni's multiple comparisons test, ** $= p < 0.01$. e) The time spent by the F1 generation control (CTRL), dominant (DOM) and subordinate (SUB) males in the upper compartment of the tank during a 1 min observation period. Two-way repeated measures analysis of variance, interaction of time and treatment $p < 0.05$, time $p > 0.05$, treatment $p < 0.001$, Tukey's multiple comparisons test, * $= p < 0.05$. DOM = dominant, SUB = subordinate. Graphs represent mean ± SEM.

female (Fig. 3d, Two-way repeated measures analysis of variance, interaction of time and treatment $F(4,64) = 2.777$, $p < 0.05$, time $F(4,64) = 2.473$, $p > 0.05$, treatment $F(1,16) = 7.899$, $p < 0.05$, Bonferroni's multiple comparisons test, ** $= p < 0.01$). The subordinate females did not spend time freezing in the bottom part of the tank, rather the subordinate females avoided all contacts with the dominant females (Fig. 3c).

In the males of the F1 generation, only control fish (i.e. offspring of fish that had not been exposed to social hierarchy test) exhibited social hierarchy, whereas the offspring of the dominant or subordinate fish failed to establish social hierarchy within the group (Fig. 3e, Two-way repeated measures analysis of variance, interaction of time and treatment $F(20,96) = 1.890$, $p < 0.05$, time $F(4,96) = 2.358$, $p > 0.05$, and treatment $F(5,24) = 14.78$, $p < 0.001$). The social hierarchy in the control fish was observed at day 4 and 5 between the dominants and subordinates of the control strain (CTRL_Dominant vs. CTRL_Subordinate, Tukey's multiple comparison test, Day 4, $p < 0.05$, Day 5, $p < 0.05$).

3.4. T-maze performance after social hierarchy testing in the P generation

The performance in the T-maze (i.e. time to reach the deep compartment, Fig. 4) was reassessed in the dominant and subordinate animals of the P generation to test if the established social hierarchy

affected the performance in the T-maze. We found that the dominant females overall performed faster compared with subordinate females (Student's t-test, $t(10) = 4.048$, $p < 0.01$, n = 9). However, there was no difference in the performance when assessing the effect of time and treatment (Fig. 4a, Two-way repeated measures analysis of variance, interaction of time and treatment $F(5,80) = 0.8019$, $p > 0.05$, time $F(5,80) = 1.588$, $p > 0.05$, treatment $F(1,16) = 1.880$, $p > 0.05$). The dominant and subordinate males did not differ in time spent to find the deep compartment (Student's t-test, $t(10) = 0.8047$, $p > 0.05$, n = 11), nor did their performance in the T-maze differ (Fig. 4b, Two-way repeated measures analysis of variance, interaction of time and treatment $F(5,100) = 0.6676$, $p > 0.05$, time $F(5,100) = 2.100$, $p > 0.05$, treatment $F(1,20) = 0.2662$, $p > 0.05$). Also here we studied the gender difference and found that males were much faster in reaching the deep compartment of the maze compared with females (Fig. 4c, $F(3,20) = 34.47$, $p < 0.001$, One-way analysis of variance, followed by Tukey's multiple comparison test, $p > 0.05$ DOM_M vs SUB_M, $p < 0.01$ DOM_M vs DOM_F, $p < 0.001$ DOM_M vs SUB_F, $p < 0.01$ SUB_M vs DOM_F, $p < 0.001$ SUB_M vs SUB_F, $p < 0.001$ DOM_F vs SUB_F).

3.5. Aminergic system markers are altered in the offspring of dominant and subordinate fish

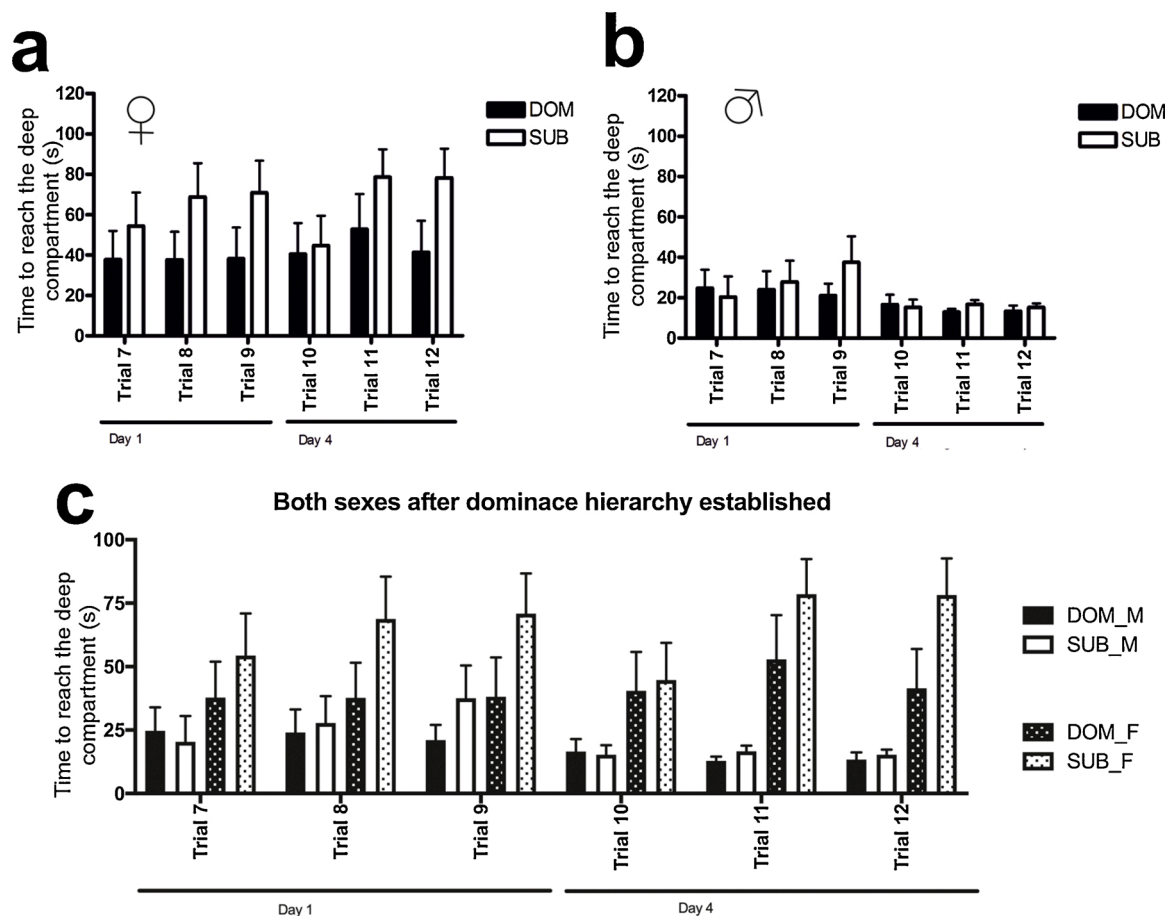To assess the role of aminergic systems in the dominant and

Fig. 4. Time needed to reach the deep basin in the T-maze assessed after the social hierarchy test to evaluate if the established social hierarchy affected the performance of fish in the T-maze. a) Time spent by P generation females to reach the deep basin, n = 9. Student's t-test, $p < 0.01$. Two-way repeated measures analysis of variance, interaction of time and treatment $p > 0.05$, time $p < 0.01$, treatment $p > 0.05$. No significant differences between trials within groups, Tukey's multiple comparisons test. b) Time spent by the P generation males to reach the deep basin, n = 11. Student's t-test, $p > 0.05$. Two-way repeated measures analysis of variance, interaction of time and treatment $p > 0.05$, time $p > 0.05$, treatment $p > 0.05$. No significant differences between trials within groups, Tukey's multiple comparisons test. c) The time spent by both sexes of the P generation to reach the deep basin after the social hierarchy testing. $p > 0.05$ DOM_M vs SUB_M, $p < 0.01$ DOM_M vs DOM_F, $p < 0.001$ DOM_M vs SUB_F, $p < 0.01$ SUB_M vs DOM_F, $p < 0.001$ SUB_M vs SUB_F, $p < 0.001$ DOM_F vs SUB_F), One-way analysis of variance, followed by Tukey's multiple comparison test. DOM=dominant, SUB=subordinate, M=male, F=female. Graphs represent mean±SEM.

subordinate zebrafish, we studied gene expression of key enzymes, receptors and proteins in the F1 generation of the animals at 7 dpf and in adulthood. The only transcript different in both dominant and subordinate 7 dpf zebrafish was *histidine decarboxylase (hdc)* as its mRNA expression was significantly lower in both groups than in control group (Fig. 5a, $F(2,15) = 9.944$, $p < 0.01$, One-way analysis of variance, followed by Tukey's multiple comparison test, $p < 0.01$, n=6). The rest of the transcripts assessed, *tyrosine hydroxylase 1 (th1), tyrosine hydroxylase 2 (th2), histamine receptor 1 (hrh1), histamine receptor 2 (hrh2),* and *histamine receptor 3 (hrh3)* were not different between the groups (Fig. 5b-f, Table 3). In adult F1 generation 2-year-old male zebrafish brains, we found a significant difference in the transcript levels of *th1* when the control group was compared with dominant animals (Fig. 5, h, $F(2,27) = 4.703$, $p < 0.05$, One-way analysis of variance, followed by Tukey's multiple comparison test, $p < 0.05$, n=10), but not for *th2, hdc, hypocretin (hcrt),* or *glial fibrillary acidic protein (gfap)* (Fig. 5g, i–k).

Significant differences were found in the amine levels in the F1 generation. The level of serotonin and noradrenaline was significantly lower in the offspring of subordinate fish when compared with the offspring of dominant fish (Fig. 6a $F(2,42) = 4.860$, $p < 0.05$, d $F(2,42) = 3.575$, $p < 0.05$, One-way analysis of variance, followed by Tukey's multiple comparison test, $p < 0.05$, n = 15) whereas histamine, dopamine and 3-methoxytyramine were not different (Fig. 6b,c,g). Additionally, the metabolites homovanillic acid, 3,4-dihydroxyphenylacetic acid and 5-hydroxyindoleacetic acid were significantly lower in dominant fish when compared with control or/and subordinate fish (Fig. 6e F $(2,42) = 4.295$, $p < 0.05$, f $F(2,42) = 7.732$, $p < 0.01$, h $F(2,42) = 9.878$, $p < 0.001$, One-way analysis of variance, followed by Tukey's multiple comparison test, $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively, n=15).

3.6. Social and aggressive behavior in the offspring of dominant and subordinate fish

Social leadership in the F1 generation, i.e. the offspring of dominant and subordinate fish in adulthood, was assessed initially as group behavior. We found that the offspring of the dominant fish of the P generation, i.e. the F1 generation dominant group, were social leaders, as they had the highest correlation for probability of being in front and leadership score when compared with the controls i.e. fish naïve to the social hierarchy test (Fig. 7a,b, n = 5/group). The offspring of subordinate fish of the P generation, i.e. the F1 generation subordinate group, showed also a positive correlation between being in front and leadership score as did the controls. However, this correlation was lower in the subordinate fish when compared with the controls (Fig. 7a,c, n = 5/group).

To further evaluate the social interaction on an individual level of the F1 generation, we assessed aggression in these fish. We did not observe any significant differences between the control, subordinate or dominant groups in time spent in the mirror zone, time spent in the zone farthest away from the mirror, locomotion or meander (Fig. 8a-d, $p > 0.05$, One-way analysis of variance, n=12).

3.7. Histamine and dopamine in the intact adult brain

Immunohistochemistry for histamine and tyrosine hydroxylase in the adult brain of F1 generation males was performed on brains from 2-year-old fish. Immunohistochemistry of tyrosine hydroxylase showed weaker tyrosine hydroxylase immunoreactivity in the hypothalamus of dominant males when compared with subordinate and control groups, in agreement with RT-qPCR of *tyrosine hydroxylase 1* mRNA (Fig. 9). Histamine-immunoreactive neurons were similarly distributed in controls, subordinates and dominant fish with a slight reduction observed in
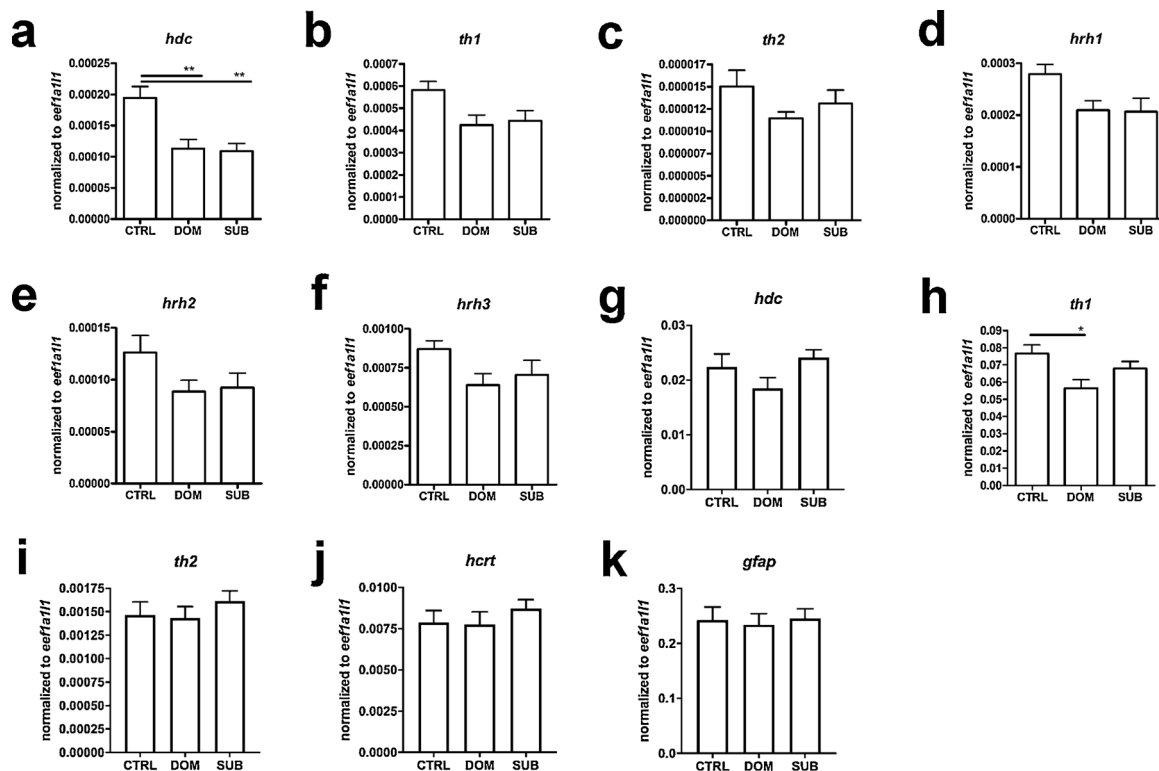

Fig. 5. Aminergic system markers (mRNA) assessed by RT-qPCR in whole larvae and brains of adult fish of the F1 generation. a) *histidine decarboxylase (hdc)* in larvae, b) *tyrosine hydroxylase 1 (th1)* in larvae, c) *tyrosine hydroxylase 2 (th2)* in larvae, d) *histamine receptor 1 (hrh1)* in larvae, e) *histamine receptor 2 (hrh2)* in larvae, f) *histamine receptor 3 (hrh3)* in larvae, g) *hdc* in adults, h) *th1* in adults, i) *th2* in adults, j) *hypocretin (hcrt)* in adults, k) *glial fibrillary acidic protein (gfap)* in adults. * = $p < 0.05$, ** = $p < 0.01$, One-way analysis of variance, followed by Tukey's multiple comparison test). CTRL = control, DOM = dominant, SUB = subordinate, larvae n = 6, adults n = 10. Graph represent mean ± SEM.
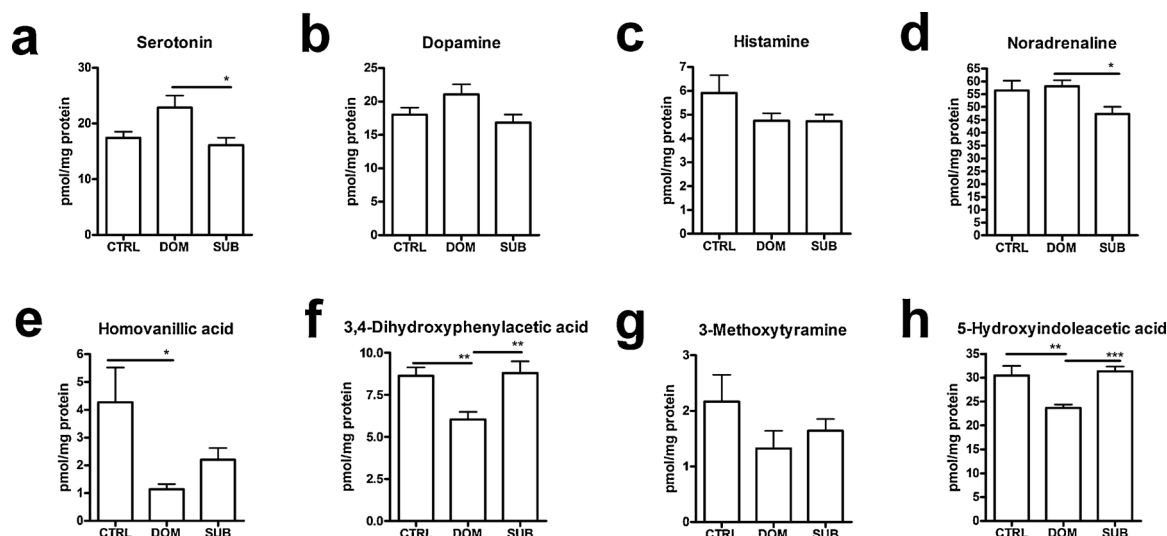
Fig. 6. Biogenic amine and metabolite levels in the brains of adult male zebrafish of the F1 generation assessed by HPLC. a) Serotonin, b) dopamine, c) histamine, d) noradrenaline, e) homovanillic acid, f) 3,4-dihydroxyphenylacetic acid, g) 3-methoxytyramine, h) 5-hydroxyindoleacetic acid. $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$, One-way analysis of variance, followed by Tukey's multiple comparison test. CTRL = control, DOM = dominant, SUB = subordinate, n = 15. Graph represent mean ± SEM.
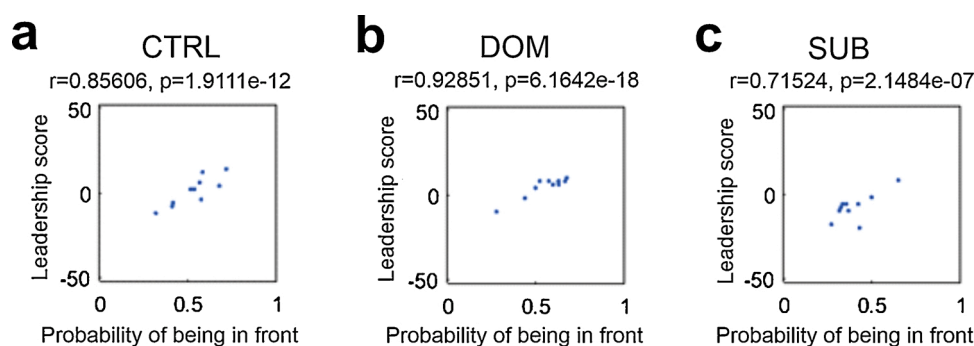

Fig. 7. Social leadership behavior of the males in the F1 generation. a) control, b) dominant, and c) subordinate adult zebrafish. CTRL = control, DOM = dominant, SUB = subordinate, n = 5, repeated three times.
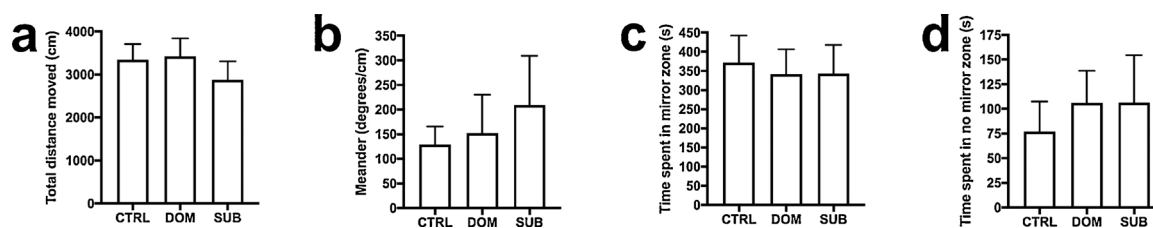

Fig. 8. Aggression of the males in the F1 generation. Fish were studied individually during 10 min to assess their aggressive behavior. a) Total distance (cm) moved during 10 min, b) meander (degrees/cm), c) time spent in the mirror zone (seconds), and d) time spent in the zone farthest away from the mirror (seconds). One-way analysis of variance, followed by Tukey's multiple comparison test. CTRL = control, DOM = dominant, SUB = subordinate, n = 12. Graph represent mean ± SEM.

histamine-immunoreactivity in the dominant and subordinate groups when compared to control (Fig. 9).

4. Discussion

Our results suggest that the offspring of dominant zebrafish are the leaders in a social context and that the levels of serotonin and noradrenaline are significantly lower in subordinate animals when compared with dominant animals. mRNA expression of the rate-limiting enzyme for histamine synthesis, *hdc*, as well as the rate-limiting enzyme for the dopamine synthesis, *th1*, are both significantly reduced when

dominant zebrafish are compared with the fish that are naïve to the social hierarchy test. Furthermore, we showed that both male and female zebrafish form social hierarchies and that the social status of the female animals correlated with their performance in a cognitive performance test, whereas the social status of the males did not correlate with better cognitive performance.

Histamine enhances learning in both non-stressed and stressed zebrafish [33] and earlier studies on social dominance hierarchy and aggression have strongly implicated the involvement of histamine [4,27, 34]. Only during the larval stage of the F1 generation were we able to detect a significantly lower level of the rate-limiting enzyme important
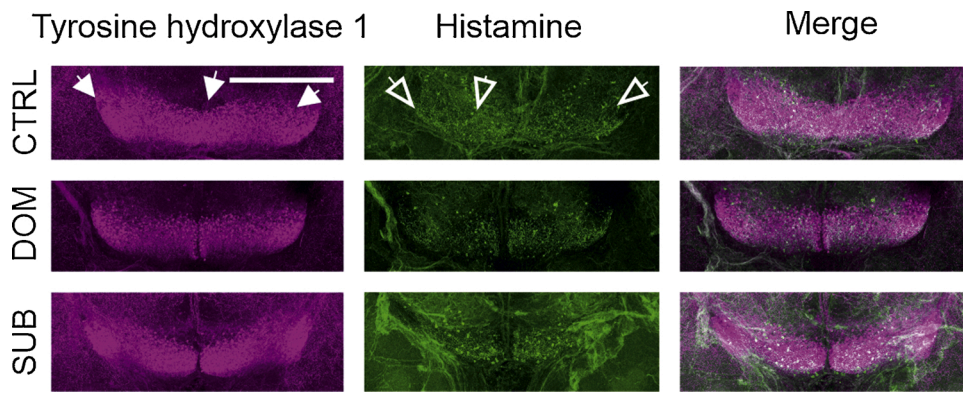
Fig. 9. Immunohistochemistry of histamine and tyrosine hydroxylase positive neurons in the adult male brains of the F1 generation. Throughout the images tyrosine hydroxylase immunoreactivity is depicted in magenta and histamine immunoreactivity is depicted in green. The brains are orientated in a similar manner in all sub-images, with the anterior to the top of the page, the posterior to the bottom of the page and the diencephalon and hypothalamus visualized from the ventral side of the brain. A horizontal view. CTRL = control, DOM = dominant, SUB = subordinate. n = 5/group. Scale bar =250 μm.

for the production of histamine, *hdc*, in both dominant and subordinate genotypes when compared with fish naïve to the social hierarchy test. Immunohistochemistry of histaminergic neurons suggested a slight reduction of histamine immunoreactivity in the adult brain of both dominant and subordinate fish when compared with the naïve fish, suggesting that the observed low level of *hdc* in the RT-qPCR analysis of F1 generation both dominant and subordinate larval fish can play a role on the development of the histamine levels in the adult intact brain. Previous studies on aggression, social hierarchy and dominance have implicated both serotonin and dopamine in the processes [4,7,8,18,22]. Our molecular RT-qPCR, cell biological immunohistochemical, and quantitative HPLC data support the findings by adding the aspect of inheritance just as the genome wide association studies performed in humans suggest [6].

In many species, e.g. the naked mole-rats (*Heterocephalus glaber*), social status is an essential feature in breeders and is associated with better breeding success than the actual sex of the animal itself. Additionally, the dominant and subordinate naked mole-rat individuals show great differences in hypothalamic brain volume; a difference that cannot be explained by sex [35]. *Astotilapia burtoni* exhibit differences in cell soma size that can explain their social hierarchy and reproductive success [15,36]. Also in zebrafish, dominance in males has been correlated with better reproductive success [37].

We did not observe any major changes in the basic locomotion of either P or F1 generation of fish bred for dominant or subordinate behavior. The subordinate females of the P generation showed higher exploratory activity compared with the dominant females. In general, the increased exploration activity is associated with increased anxiety and is suggested to be a behavior regulated by histamine [23,25]. Furthermore, the subordinate females had problems with the cognitive task in the T-maze; a feature also associated with histamine neurotransmission [33]. Expression of *hdc* has previously been shown to be unaltered in subordinate adult males [4]. However, to our knowledge this has not been assessed in subordinate female zebrafish. One factor possibly involved in this phenomenon is ependymin, previously shown to be involved in memory consolidation [38] and highly expressed in dominant rainbow trout [39]. In the case of rainbow trout the expression of ependymin was five times higher in dominants compared to subordinate animals [39]. This would allow the dominant animals to perform more quickly in the T-maze as compared with the subordinate animals. Whether there is a gender bias between the ependymin expression remains to be studied. Additionally, the hypothalamic estrogen receptor α has been associated with anxiety, social recognition and aggression [40] as well as learning in rodents [41]. In subordinate female zebrafish the estrogen receptor was reported to be three-fold lower when compared with dominant female fish [34] and might pose the answer to why the subordinate female fish in our study exhibited anxiety, subordinance and problems in performing quickly in the T-maze.

To date, several different forms of leadership are recognized; one of

them characterized by dominance and aggression [42,43]. Contradictory results have been obtained, because in different rat strains aggressiveness does not predict social dominance [44]. We analyzed the social leadership of the three different zebrafish groups in the F1 generation, and found that the dominant zebrafish exhibited the strongest social leadership behavior. Boldness, which could be seen as a form of social leadership, has been correlated with dominance in zebrafish, however it is not thought to be a direct consequence of social dominance [18]. In the dominant group of the F1 generation we observed significantly more brain serotonin than in the subordinate group of the same generation, and significantly more noradrenaline in the dominant group compared with the subordinate group in the F1 generation. The main metabolite 5-hydroxyindoleacetic acid of serotonin and 3,4-dihydroxyphenylacitic acid of dopamine were both lower in dominant animals when compared with both controls and subordinate animals, suggesting a reduced turnover of the amines. These changes in the two amines and their main metabolites could underlie and explain the social motivation, boldness and hence leadership behavior, as has been postulated earlier in humans [45]. However, when we assessed the aggressive behavior of the three groups in the F1 generation we did not observe a correlation between any group and aggression in the mirror test. The main aminergic system implicated in aggressive behaviors is serotonin [46]. Interestingly, serotonin was significantly lower in the subordinate group than in the dominant group, but the behavioral output was the same for both groups with no increase in aggression. Epigenetic changes may play a major role in regulating the altered gene expression we observed and reported on here.

Taken together, our data implies that some of the traits observed in the P generation of dominant and subordinate zebrafish are inherited by the next generation. We found that the F1 generation of dominant animals are strong leaders. These results add to our understanding of molecular mechanisms underlying social hierarchy, aggression, bullying and leadership.

Declaration of Competing Interest

The Authors declare no conflict of interest.

Acknowledgements

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