INTERNAL DISEASES

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The influence of selective serotonin reuptake inhibitors on behavior of adult *Danio rerio* under depressive conditions

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Depression is one the most common form of mental diseases. Due to epidemiological studies, it is common in different age groups including young people. Depression pathogenesis from the point of system perspective view consists of morphofunctional and pathopsychological components. The former can be divided into neuroanatomical, neurophysiological, neurochemical parts. Laboratory animals as experimental models are an invaluable tool for investigating pathogenesis of depressive disorders and creating potential treatments. In our study *Danio rerio* was used as an experimental model of depression. In fish schools during separation in pairs, anxious and aggressive behavior towards each other was manifested. It contributes to formation of dominant-subordinate relationships. Before administration of fluoxetine and sertraline in all the groups studied, the lower level of fish swimming was observed. They were hyperactive. Aggressive behavior towards each other was not observed. On the third day of the experiment behavior of the fish in groups with fluoxetine and sertraline was different.

In group with sertraline 25 % of fishes come up to average level. In group with fluoxetine fishes remained at the bottom of the aquarium. On the fifth day of the experiment in the group with sertraline 25 % of fishes had an abnormal craniocaudal position of the body in space.
Keywords: selective serotonin reuptake inhibitors (SSRI), *Danio rerio*, depression.

Depression is one the most common form of mental diseases. Due to epidemiological studies it is common in different age groups including young people [1]. In terms of their socio-economic consequences, depression is far ahead of the other forms of mental diseases. It is characterized by "depressive triad", decreased mood and loss of ability to experience joy; impaired thinking and motor inhibition [2; 3]. According to the WHO, depression remains one of the leading causes of disability and maladaptation, loss of social and professional activity, divorce, interruption of interpersonal contacts or forced loneliness. The most severe outcome of depression is suicide. From 45 to 60 % of all suicides all over the world are committed by patients with depression [2]. Depression pathogenesis from system perspective consists of morphofunctional and pathopsychological components. The former can be divided into neuroanatomical, neurophysiological, neurochemical parts. Depression as a neuropsychiatric disorder related to underlying social defects or accompanied by social dysfunctions. These include autism, which is associated with deficits in processing social cues, and William's syndrome, which is characterized by an abnormally high enthusiasm for interacting with strangers. Other disorders that are not primarily social (e.g. schizophrenia and depression) may still interfere with normal social functioning. Therefore, developing and studying animal models with social deficits has far-reaching implications for many neuropsychiatric diseases, and studying these behavioral aspects requires developing specific behavioral assays [4; 5]. Rodents are traditionally used to model disorders associated with social deficits. Since its' high level of social interactions, rodents possess many complex social behavior traits that mimic human behaviors. Additionally, researchers have established sophisticated protocols for studying these behaviors in rodents [6]. These benefits recently make rodents current 'go-to' models for studying disorders associated with social deficits. However, rodent models possess certain drawbacks. They are expensive and labor consuming. Besides, they are predominantly nocturnal and highly sensitive to environmental disturbances, such as light, sound, temperature changes and odors. Furthermore, they are unable to scaling or high-throughput assays. These drawbacks pose a limit to the broader application of these models in disease research. Besides, period of pregnancy of animals in a few months lengthens preclinical studies. Development of new adequate models of depression in animals is an urgent task of modern neurobiology, neuropharmacology, neurology and psychiatry [7]. *Danio rerio* (Zebrafish) emerged as a promising new experimental model for studying various diseases of central nervous human system due to its high throughput, genetic and physiological similarities with humans, low cost and fast reproductive cycle. *Danio rerio* is a small tropical fish of cyprinid family that lives in nature in the coastal waters of the Indian Ocean. Groups of zebrafish naturally form compact aggregations, abehavior called shoaling, which emerges as early as 15 dpf (days post fertilization) [8]. Benefits of shoaling may include betterdetection of and defense against predators, enhanced foraging and increased mating choices [9]. While in most studies number of fish for testing in a shoaling assay was selected arbitrarily, a number that balances between minimizing animal usage

and reducing variability may be estimated using a method based on Shannon entropy [10]. Wild type zebrafish forms tight shoals. As will be discussed in later sections, experimental perturbations can lead to changes in shoal cohesion. A reduction in shoal cohesion is often interpreted as decreased social interactions among members of the group. However, simple measurements of aggregation cannot fully reveal the complex, interactive and inter-dependent forces between individuals [11] or collective dynamics of a group [12]. In addition to shoaling, a group of zebrafish can 'school'. While shoals are simple aggregations of individual fish, schools are shoals that exhibit polarized formations and synchronized motions. Density and group size affect shoal cohesion, but not polarization [13]. Acute treatment with alcohol strongly affects shoal polarization but only modestly inhibits cohesion, whereas nicotine significantly reduces cohesion but modestly affects polarization [14]. These differences indicate that schooling and shoaling are two differentially regulated behaviors and that assessing both behavioral endpoints together may more effectively characterize the effects of experimental treatments. An unsupervised machine learning approach to examine schooling of adult zebrafish was developed. Using this assay, the authors classified group behavior into distinct stereotypical states of polarization, and found out that genetic mutations may alter the proportion of time spent or the tendency to transition between these states. While this approach provides an innovative way to quantitatively evaluate the propensities of a group to adopt stereotypical states of schooling, it is limited to detecting static patterns of group formation as a whole and cannot reveal dynamic interactions among group members [15]. Compared to other model objects such as the fruit fly Drosophila melanogaster and the worm Caenorhabditis elegance a strong conservative relationship has been established between the human and Zebrafish genes. This makes this tropical fish an excellent model for studying complex biological processes such as development of the nervous, cardiovascular and hematopoietic systems as well as angiogenesis, apoptosis and toxic effects of various factors [7; 16]. Direct short-term (days) exposure to the herbicides glyphosate [17] and atrazine [18] reduced aggressive behavior and shoaling, respectively, whereas an 18-day exposure to intraperitoneally injected paraquat did not significantly affect social interaction [19]. Acute exposure to gold resulted in a temporary reduction in social preference behavior that may be related to elevated oxidative stress; the social inhibition effect was short lived and the treated fish recovered within several hours [20]. Chronic exposure to the EDC BPA reduced courtship behavior in females but increased their aggression towards mating competitors; females also preferred control males over BPA-treated males during courtship tests [21]. Nonylphenol, another EDC and xenoestrogen compound inhibited aggression and social preference behaviors by chronic exposure. 17α-ethinylestradiol, a synthetic estrogen and major component in oral contraceptive pills, is excreted from the human body in high amounts and accumulates in the environment. Its impact on zebrafish social behavior were examined in several studies to assess its influence on aquatic animals, revealing changes in social hierarchy and courtship in fish following exposure [22; 23]. Besides, adult male zebrafish fight to establish dominance and hierarchy, and to compete for important resources such as food and mates [24]. A simple way to assay aggressive behavior is to introduce target for the test subject to attack. A mirror is often used to allow the test subject to attack its own reflection [25]. Alternatively, a dummy fish or avideo recording of another fish can trigger aggression [26]. The number of times a test subject exhibits aggressive behavior, such as biting and charging, is counted to quantify its level of aggres-

siveness. Although this assay provides a simple means to quantify aggression, the lack of physical contact between aggressors and targets limits its ability to mimic natural fighting behaviors. Interestingly, live fish was not used as targets in this assay setup. Instead, when two fish interact through a transparent window, their behaviors were typically interpreted as social interaction (such as in a two-compartment social preference assay) rather than aggression. There are a number of practical advantages that allow researchers to maintain fish population effectively and reliably record changes in embryos and larvae. *Danio rerio* breeds easily. As a result of each pairing it is quite possible to get up to 200 eggs. Besides, the development of ex utero and the optical transparency of embryos during embryogenesis enable visual analysis of embryos at different stages of development and assessment of organogenesis [27]. By 24 hours after fertilization, a general plan of the body structure is already beginning to take shape and all precursor cells and brain tissue, eyes and the heart can already be easily detected using a routine light microscope. Embryogenesis ends by 72 hours after fertilization. The most important organs, including the cardiovascular system, gastrointestinal tract, liver and kidneys are already fully formed by 96 hours after fertilization. It is believed that such a rapid development in 96 hours of *Danio rerio* corresponds to the three-month development of the human embryo [28]. To our date hundreds of genetic mutants of *Danio rerio* have already been elaborated, the phenotype of which resembles and may be the clinical equivalent of diseases in humans. Several chimeric models with receptors and human signaling molecules have also been developed. Genes encoding specific receptors and signaling molecules are usually associated with development of cardiovascular pathology, diseases of hematopoietic, nervous systems, myopathies and myodystrophies, which allows us to analyze effectiveness of promising drugs [29; 30]. Modulating autism-related genes in zebra fish can induce autism related phenotypes. However, the endpoints assessed in these studies were primarily focused on developmental and physiological changes or other comorbid behavioral symptoms of autism such as anxiety, sleep disorders and seizures. For example, contactin associated protein-like 2 (cntnap2) knockout induced night-time hyperactivity [31] and chromodomain-helicase-DNA-binding protein 8 (chd8) morphants (Box 2) and mutants developed macrocephaly [32]. Researchers have started examining social behavior deficits in more recent studies. Knocking out autism gene postsynaptic protein, whose disruption at the genetic level is thought to be responsible for development of 22q13 deletion syndrome (Phelan-McDermid Syndrome) and other non-syndromic ASDs — shank3b [33] — induced deficits in shoaling, social preference and kin recognition [34]. Zebrafish with mutant S-adenosylmethionine synthase 1 (sam2), ortholog to the human FAM19A2 gene, were found to have shoaling [35] and social preference deficits [36]. The human FAM19A2 gene is located in the 12q14.1 locus, home to a copy-number variation (CNV) associated with intellectual disability and autism [37]. Zebrafish also demonstrated its rapid disease-modeling capability in recent study in which novel autism risk gene, CEP41, was identified by whole-exome sequencing. Zebrafish CEP41 morphant showed deficits in social preference behavior [38], providing experimental support for this new autism risk gene. A CRISPR-based targeted mutagenesis study systematically evaluated 35 autism and schizophrenia risk genes in an unsupervised machine learning assay for schooling. Significant behavioral changes were observed in the inner mitochondrial membrane peptidase 2 (immp2l) and sodium channel protein (scn1lab) mutants; immp2l knockout enhanced shoaling, whereas heterozygous mutation in scn1lab seemed to suppress all evi-

dent social interactions between individuals. Their human ortholog, IMMP2L, is associated with Tourette syndrome [39], and SCN1A is associated with autism [40] and Dravet syndrome [41]. Several other mutations also altered shoaling and schooling, but to a lesser degree. Zebrafish ortholog of schizophrenia risk gene DISC1 induced impaired shoaling response to stress when mutated [42]. Acute exposure to alarm substance (Box 2) or osmotic stress increased shoal cohesion in 5-dpf WT fish but not disc1 mutants, suggesting its role in the development of the hypothalamic-pituitary-interrenal (HPI) axis, the fish equivalent of the hypothalamic-pituitary-adrenal (HPA) axis. Knocking out adra1aa and adra1ab, the two zebrafish orthologs of human ADRA1A, causes fish to freeze in tight groups for prolonged periods of time [15]. Polymorphisms in the promoter region of the ADRA1A gene have been associated with schizophrenia, although not without controversies [43; 44]. A variety of pharmacological agents are used to treat depression. The selective serotonin [5-hydroxytryptamine (5-HT)] reuptake inhibitor (SSRI) family of antidepressants, especially fluoxetine (FLX), the active ingredient in well-known drugs such as Prozac, is generally the first line of pharmacological treatment [45]. The SSRIs exert their therapeutic actions by enhancing serotonergic neurotransmission through inhibition of 5-HT reuptake transporters on presynaptic neurons [46]. Critically, during brain development, 5-HT acts as a neurotrophic factor regulating neuronal proliferation, differentiation, migration, and synaptogenesis [47; 48] in addition to its prominent role in the programing stress axis [49; 50], also known as the hypothalamic-pituitary-adrenal (HPA) axis in mammals, which is highly plastic during development [51; 52]. In connection with this, the use of *Danio rerio* as an experimental model of depression seems to be particularly promising direction in psychopharmacology to test the effects of antidepressants.

Purpose

To elucidate behavioral differences in *Danio rerio* exposed to selective serotonin reuptake inhibitors (SSRI).

Methods and animals

The study was performed on *Danio rerio* males of natural color (n = 20). Before starting experiment fishes were kept in a spacious aquarium with 16 liter volume. Water temperature was +21°C. Soil components and aeration in aquarium were standard. pH 6.5, dH 11° and daily replacement of water of 15 % volume was strictly supported. Daylight was 9 hours. Adults were fed once a day with a standard diet. It is well established fact that in nature *Danio rerio* forms school. Being separated in pairs, they manifest anxious and aggressive behavior towards each other. It contributes to formation of dominant-subordinate relationships. To make experimental depression, two males were transplanted into separate vessels of 500 ml volume. The diet remained standard.

The animals were separated into equal three groups (see Fig. 1):

— control group;
— group administered to fluoxetine (F);
— group administered to sertraline (S).

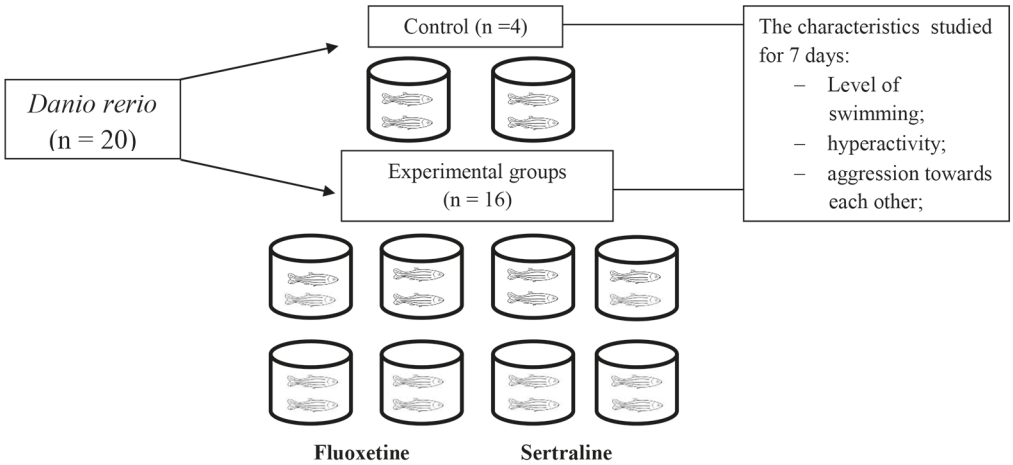

Fig. 1. The scheme of study

The males were held during the night. Their behavior was characterized as follows. The level of swimming in aquarium is lower, middle, upper, all levels; the hyperactivity is as "yes/no"; aggression towards each other is as "yes/no" (Fig. 1). It was considered that the lower level of fish swimming indicates depressive behavior of animals. Aggression is expressed in the fact that fishes attack each other [17].

Table 1. **The scheme of administration of selective serotonin reuptake inhibitors in reservoirwith *Danio rerio***

Group/ Substance	Fluoxetine	Sertraline
Concentration	500 μg/L (0,00005 %)	500 μg/L (0,00005 %)

Then antidepressants from the group of selective serotonin reuptake inhibitors were added to the vessels according to scheme (Table 1). Thereafter fishes behavior were monitored daily.

Results and discussion

On the day of the study before adding antidepressants in all the groups studied the lower level of fish swimming was observed. They were hyperactive and aggression against each other was not observed. An hour after the addition of substances, minor changes were detected in group C; 25 % of fishes swam at an average level (Table 2). On the third day of the study, hyperactivity was observed in all groups (Table 3). Other markers compared were different. In control group and group F all fishes swam at the lower level and showed aggression towards each other. In group C 25 % of fishes rose to an average level and aggression was 100 %. On the fifth day of the study in group C the craniocaudal body position in space abnormally changed in 25 % of fishes. Tail in relation to head was not less than 45 ° lower (Table 4). In this regard, it was decided to attach this characteristic to previously recorded markers. In control group all fishes showed lower level of swimming

and hyperactivity of 100 %. The difference between these groups was that in group F 50 % of fishes were aggressive towards each other. All fishes were hyperactive and 75 % of them swim at average level. In group C all fishes swam in upper level. Only 50 % were hyperactive and half of them showed aggression. On the seventh day after beginning the study in control group no significant changes were observed (Table 5). In group F 50 % of fishes swam at average level and 50 % of them showed aggression. In groups F and C 100 % fishes swam at all levels. *Danio rerio* from groups F and C did not demonstrate hyperactivity. They swam as before the experiment. Our observations are in accordance with the results obtained by other authors. It was showed that sertraline treatment improved depression-like behaviors by increasing locomotion and decreasing erratic movements and depressive phenotype. Reserpine-induced zebrafish model of depression demonstrated increased whole-body cortisol and 5-HT and decreased NA and reduced TH (tyrosine hydroxylase). Sertraline prevented increase in cortisol and NA and increased 5-HT and TH. Sertraline also prevented increase in cortisol, inhibited reuptake of 5-HT, and improved expression of TH [53]. In ordinary way hypolocomotor activity is used as a key symptom of depression in zebrafish. It was found that after removing fluoxetine, zebrafish larvae still showed hypolocomotor behavior, suggesting that there is a potential long-term effect of fluoxetine treatment [54]. The freezing as one of characters of depression phenotype was long-time in zebrafish mutant on glucocorticoid receptors. Plotting the freezing index showed significant difference between mutants and wild-type individuals. Genotype differences were not detectable at very first exposure to novel tank, but gradually developed as a result of experience. Wild-type fish froze less with each exposure, apparently habituating to repeated isolation. Fluoxetine lowered time of freezing in behavior of mutant zebrafish [55]. Another report concerning 6-day fluoxetine exposure during early zebrafish development to environmentally relevant level (0.54 μg·L–1 in water) showed that fluoxetine-treated pregnant women (54 μg·L–1 in water) reduces stress response (the arithmetic difference between the stress-induced and unstressed whole-body cortisol levels) in the adult females and males. The effect has been persisting for four generations [56]. The prolonged unpredictable strong chronic stress was studied using zebrafish model. 5-week prolonged unpredictable strong chronic stress induced overt anxiety-like and motor retardation-like behaviors in adult zebrafish, also elevating whole-body cortisol and proinflammatory cytokines — interleukins IL-1β and IL-6. Prolonged unpredictable strong chronic stress also elevated whole-body levels of anti-inflammatory cytokine IL-10 and increased the density of dendritic spines in zebrafish telencephalic neurons. Chronic treatment of fish with an antidepressant fluoxetine (0.1mg/L for 8 day) normalized their behavioral and endocrine phenotypes, as well as corrected stress-elevated IL-1β and IL-6 levels, similar to clinical and rodent data. CNS expression of bdnf gene, two genes of its receptors (trkB, p75), and gfap gene of glia biomarker, glial fibrillary acidic protein, was unaltered in all three groups. However, prolonged unpredictable strong chronic stress elevated whole-body BDNF levels and telencephalic dendritic spine density (which were corrected by fluoxetine), thereby somewhat differing from the effects of chronic stress in rodents [57]. To discuss our results further we found that in group C 50 % of fishes were hyperactive; in 25 % of fishes cranioaudal position of their body in space was still abnormal. By the eighth day of the experiment those individuals during the experiment with abnormality of craniocaudal body position died. The study showed that *Danio rerio* can be used as a model object to study the

Table 2. **The change of characteristics compared in an hour after administration of substances**

Groups	Characteristics		
	Level of swimming	Hyperactivity	Aggression towards each other
Control	Lower (100 %)	100 %	Absent (100 %)
F	Middle (100 %)		
S	Middle (25 %) Lower (75 %)		

Table 3. **The change of characteristics compared on third day of experiment**

Groups	Characteristics		
	Level of swimming	Hyperactivity	Aggression towards each other
Control	Lower (100 %)	100 %	100 %
F	Middle (100 %)		100 %
S	Middle (25 %) Lower (75 %)		100 %

Table 4. **The change of characteristics compared on fifth day of experiment**

Groups	Characteristics			
	Level of swimming	Hyperactivity	Aggression towards each other	Abnormal craniocaudal position
Control	Lower (100 %)	100 %	100 %	Absent
F	Middle (75 %) Lower (25 %)	100 %	50 %	
S	All levels (100 %)	50 %	50 %	25 %

Table 5. **The change of characteristics compared on seventh day of experiment**

Groups	Characteristics			
	Level of swimming	Hyperactivity	Aggression towards each other	Abnormal craniocaudal position
Control	Lower (100 %)	100 %	100 %	Absent
F	Middle (50 %) Lower (50 %)	100 %	50 %	
S	All levels 50 %	50 %	50 %	25 %

effect of antidepressants. *Danio rerio* model was not expensive and the experiment took only seven days. Fishes exposed to stress as separation from stock and restrictions in the swimming space but receiving sertraline earlier got out of depression. Perhaps the slower onset of fluoxetine is due to the fact that even in clinical practice antidepressant should be taken for a longer time to achieve therapeutic plasma concentrations of the substance. Of undoubted interest is the abnormality of craniocaudal position of fish's body exposed to sertraline followed by death of such individuals. This is of particular interest for further research.

Conclusion

The presented model may be promising to study other psychoactive substances as well as in preclinical trials of new substances.

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