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Sight of conspecific images induces changes in neurochemistry in zebrafish

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Abstract

Zebrafish is gaining popularity in behavioural brain research as this species combines practical simplicity with system complexity. The dopaminergic system has been thoroughly investigated using mammals. Dopamine plays important roles in motor function and reward. Zebrafish have dopamine receptors homologous to mammalian counterparts, and dopamine receptor antagonists as well as alcohol have been shown to exert significant effects on this species as measured using HPLC or behavioural methods. The sight of conspecifics was previously shown to be rewarding in zebrafish but whether this stimulus affects the dopaminergic system has not been studied. Here, we present animated images of zebrafish to the experimental zebrafish subject for varying lengths of time and quantify the amount of dopamine, DOPAC, serotonin and 5HIAA extracted from the subject's brain immediately after the stimulus presentation using HPLC with electrochemical detection. We find conspecific images to induce a robust behavioural response (attraction) in experimental zebrafish. Importantly, dopamine and DOPAC levels significantly increased in response to the presentation of conspecific images but not to scrambled images. Last, serotonin and 5HIAA levels did not significantly change in response to the conspecific images. We conclude that our findings, together with previous studies, now conclusively demonstrate that the behavioural response induced by the appearance of conspecifics is mediated, at least partly, by the dopaminergic system in zebrafish.

Keywords

dopamine; serotonin; shoaling; social behaviour; zebrafish

INTRODUCTION

The zebrafish is becoming increasingly popular in behavioural brain research [1,2,3] as this species appears to strike an optimal compromise between system complexity and practical simplicity [4]. As such, it is promising to be a good translational tool with which mechanisms of complex phenotypes, including brain function and behaviour of mammals may be modelled and/or investigated [5]. The zebrafish is a highly social species, perhaps one of the most social among classical laboratory vertebrates [6]. In nature [7,8] and in the

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laboratory too [9,10,11] zebrafish form tight groups, shoals [12]. Shoaling is defined as aggregation of individuals characterized by distance among members of the group that is shorter than what would be expected in case of random spatial distribution of the individuals [9-12]. The shoaling response can be induced by the sight of conspecifics [13]. The conspecific individuals could be live stimulus fish [13,14,15] or animated images [16,17]. The latter has the advantage of being consistent and experimentally well controlled.

The presentation of conspecific stimulus fish has been shown to be rewarding in zebrafish [15]. This stimulus could be used effectively as the unconditioned stimulus (US) that reinforced acquisition of memory in maze learning tasks in zebrafish [18,19]. Alcohol, a drug of abuse known to engage reward mechanisms, has been shown to interfere with shoaling in zebrafish [14,20-23]. The mechanisms through which alcohol achieves this behavioural effect in zebrafish are not known. Furthermore, alcohol has been shown to engage numerous molecular targets and thus affects a large number of biochemical mechanisms, which manifests among other changes in altered neurotransmission in number of neurotransmitter systems [24]. The dopaminergic system is one of these neurotransmitters [25,26]. Given the important role of the dopaminergic system in reward [25, 27] and the recent demonstration of the sight of conspecifics having rewarding properties in zebrafish, it is possible that the dopaminergic system, at least partially, underlies the alcohol induced behavioural effects in zebrafish and that this neurotransmitter system is involved in shoaling.

Dopamine in particular and the dopaminergic system in general have been thoroughly investigated in mammals [27]. Among the many functions of this neurotransmitter system is its role in motor function and reward [27 - 29]. Importantly, zebrafish has been shown to have at least four dopamine receptors (D1-R, D2-R, D3-R and D4-R) that are homologous to mammalian counterparts both in terms of their amino-acid sequence and also in the nucleotide sequence of the genes corresponding to these receptors [30-33]. Indeed, a D1-R antagonist developed for mammals has been shown to have significant behavioural effects in zebrafish in a predicted manner: it significantly reduced shoaling responses in a dose dependent manner in zebrafish [34]. Thus given the rewarding properties of the sight of conspecifics, the known involvement of the dopaminergic system in reward, and this latter finding (the D1-R antagonist induced disruption of shoaling) it is likely that the dopaminergic system is involved in shoaling behaviour of zebrafish. However, there is a notable lack of detailed pharmacodynamic, pharmacokinetic and psychopharmacological data on how this particular D1-R antagonist works in zebrafish. As a result one cannot be completely certain about the specificity of this drug in zebrafish. Also, although alcohol has been shown to engage the dopaminergic system and also to disrupt shoaling, given alcohol's varied actions in the brain [35] and its effects on a range of behaviours other than shoaling in adult zebrafish [1; 14,21,23], one cannot draw a conclusion about causality. Briefly, however suggestive the above data may be, the case for the involvement of dopamine in shoaling is not yet closed.

In the current study we investigate this question by providing conspecific (or control, i.e. scrambled) animated images to zebrafish subjects and sampling neurochemical responses immediately after the presentation of these images. We focus our attention to the dopaminergic system and measure levels of dopamine and those of DOPAC (3,4-Dihydroxyphenylacetic acid), dopamine's metabolite, for the reasons explained above. We also measure levels of serotonin (5-hydroxytryptamine), and serotonin's metabolite 5HIAA (5-Hydroxyindoleacetic acid). Notably, all four of these neurochemicals have been shown to exhibit a developmental trajectory (increasing relative levels during ontogenesis) that correlated with the development of shoaling in zebrafish [36]. Furthermore, dysfunction of the serotonergic system has been associated with antisocial behaviour in humans [37] as

well as with fear (anxiety) [38] and aggression [39,40] and both aggression and fear may play roles in social behaviour, i.e. shoaling, in zebrafish [6,9].

METHODS

Animals and Housing

Experimental subjects were sexually mature, five month old, adult zebrafish (*Danio rerio*) of the AB strain. At this age, zebrafish reach 3.5 cm total length (from the tip of the nose to the end of the caudal fin) in our vivarium. The sex ratio of the experimental fish was approximately 50% males and females. The progenitors were obtained from ZIRC (Oregon, USA) but all fish used in this study were bred and raised in our facility (University of Toronto Mississauga Vivarium, Mississauga ON, Canada). Housing and maintenance were as described before [21]. Briefly, 1.2 litre nursery tanks housed the fry. The fry were fed *Artemia salina* (brine shrimp) until 3 weeks of age post-fertilization. Subsequently, fish were placed in a high-density multi-stage filtration fish rack system in 2.8 litre tanks (Aquaneering Inc., San Diego, CA, USA) with 15 fish per tank density. Fish were fed a mixture of Tetramin and spirulina flakes. Fish were randomly chosen for the studies described below.

Behavioural paradigm

To investigate neurochemical responses induced by the sight of conspecifics, we employed a slightly modified version of a behavioural paradigm described before [16,21]. Briefly, 24 hours before the behavioural tests began, experimental subjects were removed from their community tank and housed individually in 8 litre tanks in order to increase the effect of subsequent presentation of the social stimulus (the zebrafish images). Following this short isolation, subjects were placed singly in a 40 litre experimental tank (27 × 30 × 50 cm, width × height × length) that was flanked by a computer monitor (17 inch Samsung LCD monitor) on each side. In this tank zebrafish have been shown to be able to see and respond to animated images, including images of conspecifics [13, 16, 17]. Each monitor was connected to a PC that was running a custom software application, first described in [13], which allowed us to present five independently moving (animated) images. The images were either the picture of wild type zebrafish of a size identical to that of the experimental zebrafish, or rectangles of the same length and height as the zebrafish image. The rectangle contained the exact same pixels as the zebrafish image but the pixels were scrambled. The images were shown on a black background for varying lengths of time (see below) first on one side of the tank and subsequently on the other side with a starting side randomly chosen across experimental subjects. All images, when presented, moved horizontally in random directions with speeds varying between 1.5 and 4 cm/s, mimicking locomotory patterns seen in a natural zebrafish shoal. The image presentation served two purposes: One, stimulation that is expected to induce differential neurochemical responses depending on the particular stimulus presentation method employed (see below); Two, a paradigm that allowed us to quantify the behavioural responses of the experimental fish. Each experimental fish was monitored for 20 minutes and their behaviour was recorded using a hard disk drive video camera ((JVC Everio 32x HDD Camera, recording 30 frames per second). The digital recordings (MOD format) were transferred to an external hard drive and were later replayed on a desktop computer (Dell Dimension 8400) and analyzed using a custom designed video-tracking software developed in-house. Using this software, we quantified the distance of the experimental fish from the computer screen that presented the images. The distance values are expressed for 30 sec intervals of the 20 min recording session.

Fish were assigned randomly to one of the following five experimental groups. In the first three groups, the experimental fish was shown moving zebrafish images for either the last 5,

10 or 15 minutes of the recording session. In the forth group, the test subject was shown moving images of the scrambled image (the rectangle) for the last 10 min of the recording session. For all these four groups, the image presentation side was switched exactly in the middle of the presentation period (from left to right or from right to left side) to mimic a natural shoal leaving and moving back to the location of the experimental subject. Both in nature and in the laboratory zebrafish have been observed to leave and rejoin groups of zebrafish (a phenomenon termed “excursion”) and splitting of groups to subgroups has also been described [9-12]. Presenting the images on one side and subsequently on the opposite side of the test tank was aimed at mimicking this dynamic of zebrafish group forming. In the fifth group, no images were presented to the experimental zebrafish and these fish were shown the blank (black) computer screens only. The sample size (n) for each group was 10. The order of testing was randomized across the five zebrafish groups. The recording and quantification of behaviour were done blind.

Analysis of neurochemicals using HPLC

The experimental fish that received the above described stimulation were promptly decapitated after the behavioural session and their brains were dissected on ice and sonicated. The details of sample preparation methods and the HPLC (high precision liquid chromatography) procedures have been published elsewhere [41,42]. Briefly, the sonicates (each representing a single zebrafish) were centrifuged and the supernatant was analyzed with HPLC using a BAS 460 MICROBORE-HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN) together with a Uniget C-18 reverse phase microbore column as the stationary phase (BASi, Cat no. 8949). Standard neurochemicals (Sigma) were used to quantify and identify the peaks on the chromatographs. Levels of dopamine, DOPAC (3,4-dihydroxyphenylacetic acid, dopamine's metabolite), serotonin, 5HIAA (5-Hydroxyindoleacetic acid, serotonin's metabolite) were quantified. Results are expressed as nanogram (ng) of neurochemical per milligram (mg) of total brain protein.

Statistical analysis

The behavioural results were analyzed using repeated measure variance analysis (ANOVA) with interval (repeated measure factor with 40 levels) and stimulus treatment (with 5 levels) as the between subject factor. The neurochemical data were logarithm transformed to homogenize variances and subsequently the results were analyzed using ANOVA with stimulus treatment as the between subject factor (5 levels) followed by post hoc Tukey Honestly Significant Difference (HSD) test when appropriate.

RESULTS

The distance from the stimulus screen appeared to decrease upon the stimulus presentation and also appeared to be different among treatment groups according to what stimulus and how long was shown (figure 1). The apparently robust effects were confirmed by ANOVA, which detected a significant interval effect ($F(38, 1710) = 4.516, p < 0.001$), a significant stimulus treatment effect ($F(4, 45) = 8.622, p < 0.001$) and a significant interaction between these factors ($F(152, 1710) = 2.689, p < 0.001$). Given that post hoc multiple comparison tests, such as Tukey HSD, are inappropriate for the analysis of repeated measure designs, we performed the following calculations. We averaged the interval data for the period during which no stimulus was shown and for the period when the stimulus was shown and subtracted the value obtained for the no stimulus period from the stimulus period. In case of significant reduction of distance in response to stimulus presentation, the value obtained after this calculation is expected to be significantly below zero. For the no stimulus group, we performed a similar calculation but here we subtracted the average of the intervals of the first half of the session from the average of the intervals of the second half of the session.

The results are shown on figure 2. Comparison of the thus calculated values across the five groups is possible using Tukey HSD and comparison of the values obtained for each group with zero (random chance) using one sample t-test is also appropriate. These analyses confirmed that the stimulus treatment had a significant effect (ANOVA $F(4, 45) = 5.605$, $p < 0.001$) and that experimental subjects presented with zebrafish images all significantly decreased their distance to the computer screen as compared to the no stimulus group (Tukey HSD, $p < 0.05$). Tukey HSD also found that the difference between the scrambled image group and the no stimulus group was only bordering but not reaching significance ($p = 0.08$). In addition we also investigated whether the reduction of distance in response to the stimulus was significantly different from random chance, i.e. 0, and found that the scrambled images did not lead to a significant reduction ($t = -1.289$, $df = 9$, $p > 0.10$, one tailed t-test), but the zebrafish images did, irrespective of how long they were presented ($t > 1.953$, $df = 9$, $p < 0.05$).

Dopamine levels (relative to total brain protein) appeared different across the stimulus groups (figure 3), an observation that was supported by ANOVA, which found a significant stimulus treatment effect ($F(4, 45) = 21.203$, $p < 0.001$). Tukey HSD revealed that the fish presented with zebrafish images for 10 or 15 min had significantly ($p < 0.05$) higher dopamine levels as compared to the other three groups.

The pattern of results obtained for DOPAC was very similar to those we found for dopamine. Figure 4 shows a robust increase of DOPAC levels induced by the presentation of zebrafish images for 10 min while the shorter or longer presentation times, although still effective, do not appear to lead to such string changes. These observations were confirmed by ANOVA, which found a significant stimulus treatment effect ($F(4, 45) = 54.239$, $p < 0.001$), and also by the post hoc Tukey HSD test, which found all groups to significantly ($p < 0.05$) differ from each other except the no stimulus vs. scrambled image presented groups and also the 5 vs. the 15 min zebrafish image presented groups.

The results obtained for serotonin were markedly different from those of the above. The zebrafish image presentation appeared ineffective but the scrambled image seemed to reduce the level of this neurotransmitter (figure 5). ANOVA showed a significant stimulus treatment effect ($F(4, 45) = 7.088$, $p < 0.001$). Tukey HSD test found the group that received the scrambled image to significantly ($p < 0.05$) differ from all other groups whereas the other groups did not differ from each other.

Figure 6 shows the effects of different stimulus treatments on the levels of 5HIAA. ANOVA found a significant stimulus treatment effect ($F(4, 45) = 7.068$), $p < 0.001$) and Tukey HSD confirmed that the fish that received the scrambled image had significantly ($p < 0.05$) lower levels of 5HIAA than all other fish, while other groups did not differ from each other.

DISCUSSION

Our behavioural results showed that without the zebrafish images, the experimental subjects swam approximately 20-30 cm away from the stimulus screen placed adjacent to the side wall of the tank (considered random chance in a 50 cm long fish tank), which demonstrates that the fish had no preference towards any side of the tank. Upon presentation of the conspecific images, however, the distance from the stimulus screen significantly decreased, occasionally dipping below 10 cm, a value that is in accordance with what we observed in live shoals of zebrafish [12, 43]. These results confirm that presentation of conspecific images induces a robust behavioural response, a preference towards the images that manifests as moving towards and staying close to them. This response we previously interpreted as a sign of “social preference” or shoaling [16,17,21]. Notably, the scrambled

images, which otherwise contained the exact same pixels as the zebrafish images but not in the “right” pattern, did not induce this robust approach and image side preference. Interestingly, however, these images when switched from one side to the other did induce an apparent approach, which we may interpret as novelty induced exploratory activity, a speculation whose validity will need to be confirmed experimentally in the future. It is also notable that switching the side had the opposite effect when the images were conspecifics. Especially in the 10 min zebrafish stimulus group, but also to a lesser degree in the 15 and 5 min zebrafish stimulus groups, the response to the images was strongest when the images appeared the first time as compared to when the images reappeared the second time on the opposite side. We do not have a clear explanation for this but note that this finding is in line with what is known about the adaptive function of shoaling [6, 9-12 and references therein]. Zebrafish isolated singly in a novel tank is expected to be motivated to rejoin their shoal, an adaptive antipredatory response [12]. But this response is expected to habituate over time, that is, after the level of novelty induced fear is reduced [11], which may explain the somewhat reduced shoaling response as quantified by the less reduced distance from the stimulus screen upon the presentation of the shoaling image on the opposite side of the tank.

Examination of the neurochemical responses to these image presentations revealed some interesting, and somewhat unexpected, findings. Both dopamine and DOPAC levels increased in response to the presentation of conspecific images. Interestingly, however, unlike what we expected, the intermediate stimulus presentation length induced the most robust change. We speculate this may be due to the following reasons. One, it is likely that the effect of the sight of the conspecific stimulus does not manifest immediately at the neurochemical level. Processes associated with production of dopamine, with the release of dopamine and with its subsequent metabolism in the synaptic cleft may take time. This may explain the rising (left) side of the stimulus-response curve (see e.g. figures 3 and 4). What could explain the reduction of conspecific stimulus effect when the stimulus was shown longer than 10 min? A possible explanation for this is that the dopaminergic responses may not be induced by the presence but rather by the appearance of the conspecific stimuli. We speculate that the constant presence of the conspecific stimulus for a longer period of time is less effective because within a given period the stimulus appeared less frequently. If correct, this would explain the falling (right) side of the stimulus-response curve.

The fact that we found both dopamine and DOPAC levels to respond similarly is also worth discussing. Elevation of the level of the neurotransmitter is usually taken as evidence for increased production of the neurotransmitter while elevation of the level of its metabolites is often interpreted as due to increased neurotransmitter release and thus availability of the neurotransmitter for enzymatic degradation in and around the synaptic cleft [44]. Thus we suggest that the elevated dopamine and DOPAC levels induced by the sight of conspecific images are the result of increased dopaminergic function, which is due both to elevated dopamine release (synaptic transmission) and to increased dopamine synthesis.

Could this enhanced dopaminergic “tone” be non-specific? That is, could this be due to overall activation of the brain? This possibility must be considered as, for example, one could argue that shoaling may be associated with increased locomotor activity and also increased oxygen consumption as well as elevated metabolic activity. As a result, many processes associated with neuronal communication and synaptic transmission may be generally affected. This is a reasonable assumption, however, we have two pieces of evidence against it, at least in terms of whether the dopaminergic response reflects an overall activation of the brain or not. One, previously we found that the shoaling response induced by the presentation of conspecific images is associated with significantly reduced, and not increased, locomotor activity [17]. Second, and perhaps more compellingly, our current

results with serotonin and its metabolite 5HIAA also contradict the “general activation” hypothesis.

We found these neurochemicals to respond to the image conditions highly differently as compared to dopamine and DOPAC. While the level of the latter neurochemicals was significantly increased by the presentation of conspecific images, serotonin and 5HIAA levels did not change in response to these images. It is thus unlikely that overall activation of the brain is behind the conspecific images induced dopaminergic activation. Analysis of the serotonin and 5HIAA levels revealed another interesting finding. The scrambled images, unexpectedly, reduced the levels of these neurochemicals. At this point we have no explanation as to why. One, however, may speculate that perhaps the serotonergic system is involved in fear/anxiety in zebrafish similarly to mammalian organisms (including humans) [45-47]. If it is, one may further speculate that the appearance of novel moving objects (like the scrambled images) should induce fear which may be associated with serotonergic hypofunction [45-47]. Although this suggestion is highly speculative given the multiple and complex roles of the serotonergic system in a variety of different types and forms of anxiety, fear or panic related disorders, one may note that the observed apparent exploration of the scrambled images by zebrafish we saw here is not contradictory to the possibility of fear induced by these images. Predator inspection, a form of exploration associated with fear, is well documented in multiple fish species [48-51].

The last point we consider is the question of causality. Previously, using a D1-R antagonist, we have been able to disrupt shoaling responses without altering such performance factors as motor function or perception [34]. Here we induced shoaling responses using visual stimuli and found evidence for significant enhancement of the functioning of the dopaminergic system. We also found acute alcohol treatment to significantly interfere with both shoaling and the dopaminergic system [21]. It appears therefore, that together with our current results, the role of dopamine in shoaling in zebrafish is now well proven. What this role is, however, we do not yet know. One may suggest that dopamine mediates the rewarding aspect of seeing conspecifics [15], this suggestion is supported by the fact that D1-R antagonism did not affect motor function but did impair shoaling [34] and also by our current results suggesting that it is the appearance and not the mere presence of the social stimulus that engages the dopaminergic system. Nevertheless, one may also postulate that perhaps the motor responses associated with shoaling are also under the influence of this neurotransmitter system and it is these responses that led to the altered neurochemical levels. At this point, these possibilities cannot be unequivocally distinguished. Also, importantly, we do not yet know how other neurotransmitter systems may be involved in shoaling in zebrafish and also importantly what neural circuits, and in general which brain areas, may be involved. These important questions will be investigated in the future. Nevertheless, our current study together with prior work with zebrafish, now further demonstrates the utility of this species in the analysis of complex brain function and behaviour.

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Research Highlights

1. zebrafish were exposed to animated conspecific or scrambled images
2. conspecific images increased dopamine and DOPAC but not serotonin or 5HIAA levels.
3. intermediate length of exposure to conspecific images had the most robust effect
4. scrambled images reduced serotonin and 5HIAA levels
5. findings support the involvement of the dopaminergic system in shoaling in zebrafish

Figure 1.

The distance from the stimulus presentation screen significantly differs between stimulus groups. Mean $\pm$ S.E.M. are shown. $n = 10$ for each group. The grey symbols correspond to the 30 sec intervals during which no stimulus is shown. The black symbols correspond to the 30 sec intervals during which either the scrambled images (a rectangle containing the same pixels as in the zebrafish image but in a random, scrambled manner) or images of zebrafish were shown. The stimulus condition (i.e. which image and for how long was shown) is indicated above the graphs. The horizontal line shows random chance, i.e. 25 cm from the stimulus side. The vertical dashed line shows the interval during which the side of the stimulus presentation was switched. Note that fish in the no stimulus group had no significant side preference. Also note that fish in the scrambled stimulus group also did not swim significantly below chance level in response to the image presentation. Last, note the effect of zebrafish images (last three graphs).

Figure 2.

The change in the distance from the stimulus screen in response to the presentation of stimuli is significantly below random chance when the stimulus is the images of zebrafish but not when it is the scrambled image. Mean $\pm$ S.E.M. are shown. $n = 10$ for each group. Random chance is indicated by 0 on the Y axis. Note that a negative value represents reduction of distance in response to image presentation and a positive value an increase.

Figure 3.

Dopamine levels significantly increase in response to the presentation of zebrafish images but not in response to the scrambled images. Mean $\pm$ S.E.M. are shown. $n = 10$ for each group. Data are expressed as weight (ng) of neurochemical per weight (mg) of total brain protein. The colour coding of the stimulus conditions is indicated by the legend. Note that the strongest effect is seen after 10 min of zebrafish image presentation.

Figure 4.

DOPAC levels significantly increase in response to the presentation of zebrafish images but not in response to the scrambled images. Mean $\pm$ S.E.M. are shown. $n = 10$ for each group. Data are expressed as weight (ng) of neurochemical per weight (mg) of total brain protein. The colour coding of the stimulus conditions is indicated by the legend. Note that the strongest effect is seen after 10 min of zebrafish image presentation.

Figure 5. Serotonin levels significantly decrease in response to the presentation of the scrambled images but no change is seen in response to the zebrafish images. Mean $\pm$ S.E.M. are shown. $n = 10$ for each group. Data are expressed as weight (ng) of neurochemical per weight (mg) of total brain protein. The colour coding of the stimulus conditions is indicated by the legend.

Figure 6. 5HIAA levels significantly decrease in response to the presentation of the scrambled images but no change is seen in response to the zebrafish images. Mean $\pm$ S.E.M. are shown. $n = 10$ for each group. Data are expressed as weight (ng) of neurochemical per weight (mg) of total brain protein. The colour coding of the stimulus conditions is indicated by the legend.