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Expression of dopamine and opioid receptors and its correlation with risk taking behavior in zebrafish

Sarah McCarrick

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Biology Education Centre and Department of neuroscience, Uppsala University
Supervisor: Svante Winberg

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List of abbreviations

cAMP – cyclic adenosine monophosphate
cDNA - complementary DNA
DA – dopamine
GABA - gamma-aminobutyric acid
PCA - principal component analysis
RNA - ribonucleic acid
2-ME – 2-mercaptoethanol
qPCR - quantitative polymerase chain reaction

Abstract

Background – Individuals cope with difficulties in life in different ways. Behavioural models have been developed to categorize animals with different risk taking behaviours. It is believed that willingness to take risks is associated with drug abuse. Drugs of abuse affect the brain´s natural reward system such as the dopaminergic, opioid and GABA system. In this study we wanted to investigate if there was a correlation between risk taking behaviour and the expression of the dopamine receptors D2, D3 and the opioid receptor delta in zebrafish. **Methods** - Risk taking behaviour was assessed by observing individual zebrafish in a novel tank. The fish were classified according to several parameters, but most importantly duration of stay at bottom of the tank, from which 12 bold and 12 shy fish were selected. The zebrafish brains were then analysed for the gene expression of the genes *oprd1a* and *oprd1b*, which codes for the opioid receptor delta, along with the genes *drd2a, drd2b* and *drd3* coding for dopamine receptor D2 and D3. **Results** – The statistical analysis of the gene expression showed a significant difference in expression of *drd2a* and *drd2b* in the groups of bold and shy fish. The bold fish had a higher expression of the dopamine receptor D2 compared to the shy fish. A significant linear regression between the expression of *drd2b* and the parameters duration at bottom and latency to top in the behavioural test was found. *Oprd1a, oprd1b* and *drd3* showed no significant difference or correlation with the outcome of the behavioural test. **Conclusions** – Dopamine receptor D2 exists in both pre- and postsynaptic form. The presynaptic ones are autoreceptors and mediate negative feedback to the presynaptic neuron, and therefor regulate the release of dopamine. D2 has been shown to have a very critical role in DA transmission. Lower expression of D2 receptors would lead to less negative feedback hence larger dopamine release and more pleasure. It is though that low levels of the presynaptic receptor leads to larger susceptibility to drug abuse.

Side project – The hole test

A behavioural test was developed to see if determination of risk taking behaviour could be performed faster and in a less stressful way than the novel tank diving test. Risk taking behaviour was assessed by placing a partition with a hole in the tank. Depending on when the fish swam through the hole to the other side, different risk taking groups were formed. The outcome of the hole test showed a correspondence of 67 % with the outcome of the novel tank diving test. The hole test can hopefully after further development and more testing become a reliable behavioural test.

Introduction

Variation between individuals is a well-known phenomenon in both humans and animals (Koolhaas et al, 2010). One type of variation occurs in the behavioral repertoire, since individuals can cope with challenges they are faced with in different ways. Animals can be categorized in distinct behavioral phenotypes, often called behavioral syndromes or coping styles (Coppens et al, 2010). This focuses on two distinct patterns of reactions to a stressful situation and can be distinguished by many different terms such as proactive versus reactive, hawk versus dove and shy versus bold (Coppens et al, 2010, Koolhaas et al, 2010).

The terms bold and shy refer mainly to the willingness of taking risks among individuals, especially in novel environments (Coleman and Wilson, 1998). Shy individuals are characterized by a passive reactivity to stress combined with very little behavioral initiative, whilst the bold individuals are the opposite meaning a strong active response to stress (Koolhaas et al, 2010). Boldness is a factor that potentially may influence many aspects of an organism's life, such as interactions with predators, prey but also other environmental factors (Coleman and Wilson, 1998). It is thought that behavioural expression of different coping styles should be related to individual variation in the underlying neurobiological mechanism (Coppens et al, 2010).

A willingness to take risks has been implicated as a factor affecting substance abuse vulnerability (Blanchard et al, 2009). The brains natural reward systems are evolved to respond to natural pleasure such as sex and food. This natural award system is also what the addictive drugs effect s and that is why we feel pleasure when taking these drugs (Kelley and Berridge, 2002). It is thought that the association between sensation seeking, including boldness, and susceptibility to substance abuse may reflect different reward sensitivities between individuals (Blanchard et al, 2009). Understanding why some individuals are more vulnerable to drug addiction than others remains as one of the main difficulties among researchers in the field (Volkow et al, 2009)

Zebrafish as a model organism

Zebrafish, *Danio rerio,* is a widely used model organism in biological and biomedical research due to its inexpensive and easy housing, along with its extensively described genome (Maximino et al. 2010). The fact that zebrafish breed all year around and females can spawn every 2-3 days, along with a short generation time makes it an excellent model organism in that aspect (Spence et al, 2008).

Zebrafish is a good organism in the study of molecular mechanisms of among others neurobehavioral functions and for screening pharmacological and toxicological effects (Bencan and Levin, 2008). It has been shown that neurotransmitter systems seen in fish resemble those of humans and other mammalian species, such as the dopaminergic system (Winberg et al, 1993). Zebrafish and mammals have similar types of neurotransmitter receptors (Bencan and Levin, 2008), which makes zebrafish a valid, reliable and efficacious model in the research of stress and brain functions (Egan et al. 2009).

Behavioral tests

The behavioral repertoire of the zebra fish is complex and has necessitated the development of several behavioral models (Gerlai, 2000). The novel tank diving test was developed to study risk-taking behavior for the potential use for testing the effects of drug abuse (Gerlai, 2000), and has

now shown to be a valid and efficient test of behavioral stress response (Levin, 2011). The novel tank diving test is now the most widely studied model of anxiety in zebrafish (Maximino et al. 2010).

When a zebra fish is placed in a novel tank the innate behavior is to seek protection and therefor they dive and remain at the bottom until they feel safe, as a response to stress (Levin et al. 2007). The novel tank is divided into 3 zones; bottom, middle and upper zone and this is used to measure the duration of time the experimental fish spends in each zone (Gerlai, 2000). Zebrafish have a natural tendency to spend the majority of time at the bottom when introduced to a novel environment and gradually expand their position of swimming, including the higher portions of the tank (Levin et al. 2007). The choice of position can therefore be considered as the index of anxiety, and can be compared with the position choice of closed vs. open arms in the elevated plus maze and thigmotaxis vs. center of an open field with rodents (Levin et al. 2007). Freezing, thigmotaxis, erratic swimming and bottom dwelling are also proposed as indices of anxiety (Maximino et al. 2010). By using video-tracking tools, registration of zebra fish anxiety in the novel tank diving test shows high reliability (Egan et al. 2009).

Natural reward systems

Drugs of abuse act on systems evolved to mediate natural pleasures (Kelley and Berridge,2002), where three major brain systems appear to be involved – dopamine, opioid and GABA (Koob, 1992). In this study the dopamine receptors D2 and D3 along with the opioid receptor delta is examined.

Dopamine

Dopamine has a lot of different functions in our brain and the dopamine receptors are widely expressed in the central nervous system since they are involved in the control of locomotion, cognition, emotion and affect as well as neuroendocrine secretion (Missale et al, 1998). Moreover, it is thought to play a major role in addiction because its presence in the central reward pathways is important for remaining responses to reinforce such as cocaine (Blanchard et al, 2009). Addictive drugs, among others cocaine, heroin and amphetamine, all increase dopamine concentration in forebrain structures by diverse mechanisms (Montague et al, 2004). There are five distinct, all G protein coupled, dopamine receptors which can be divided into two subfamilies, the D1- and D2-like receptors. The D1-subfamily consists of D1 and D5, whilst the D2-like includes the D2, D3 and D4 receptors (Vallone et al, 2000). The D2-like receptors have both a pre- and postsynaptic location and inhibit the activity of adenylyl cyclase, and D2 and D3 receptors inhibit the endogenous cAMP level in several cell types (Vallone et al, 2000). The presynaptic receptors functions as autoreceptors, and produce feedback inhibition of dopamine transmission, which leads to decreased neuron excitability and less DA release (Bello et al, 2011). Autoreceptors inhibits the presynaptic neuron so it stops firing, and therefor controls the dopamine release.

Opioids

Opoid peptides are involved in three major functions: modulation of nociceptive response to painful stimuli and stressors, reward and homeostatic adaptive functions such as food, water and temperature regulation (Koob, 1992). Peptides and their receptors are present in areas associated with reward, motivation, learning and stress (Charbogne et al, 2014). There are three different

kinds of opioid receptors named mu, delta and kappa, which are all G protein coupled receptors (Chung, 2013). Stimulation of the opioid receptors also inhibits adenylat cyclase. Delta receptor activity reduces levels of anxiety and depressive-like behaviors among other thing (Charbogne et al, 2014). Opioid receptor genes of the gamma and delta classes have been implicated as genetic candidates for alcoholism-associated phenotypes in animals and also risk for alcoholism in man (Town et al, 2000).

Hole test
A side project was also done – a pilot study to see if we could select fish on risk taking behavior in a faster and less stressful way compared to the novel tank diving test. A test we refer to as the hole test was developed and tried. An opaque partition was placed into a tank with a population of fish, confining the fish into a smaller compartment. After a few hours of acclimatization a hole in the partition was revealed along with that food was distributed on the other, empty side of the partition. Depending on the time elapsed before a fish swam through the hole to reach the food, individuals could be categorized into groups with different risk taking behavior.

Project focus and aim
The main purpose of this study was to analyze the possible differences in expression of the genes of dopamine receptors *drd2a*, *drd2b* and *drd3* along with the opioid receptors *oprd1a* and *oprd1b* between fish with different risk taking behavior. A side project was also done, where the significance of the behavioral hole test was determined.

Material and Methods

Zebrafish
Captivity bred adult AB-strain Zebrafish (*Danio rerio)* were housed at approximately 27 °C in the lab at Uppsala University Biomedical Center. The lab had a 14:10-h light/dark cycle, with lights on from 7.00 am until 9.00 pm. The water used in the fish tanks was Uppsala municipal tap water (pH 7.2-7.6) of which 15 % was exchanged daily. Fish were fed once daily with Tropical flake food (Sera San) and Artemia. The use of animals was approved by the Uppsala Ethical Committee and the guidelines of the Swedish Legislation on Animal Experimentation were followed.

Zebrafish were sorted by sex and a total of 131 males were put into 4 tanks of 9.4 l, with 24-37 fish in each. Fish were left between 5-9 days to acclimatize to their tank prior to testing.

Hole test
A black plastic partition was placed 11 cm from the backside of the housing tanks of 9.4 liters (24-37 fish in each), leaving a space of approximately 2,3 liters (~11 x 21 cm) for all the fish in the tank. The Fish were then left for 2.5-3.5 hours for acclimatization, before a hole (2 cm diameter) was revealed. Food was immediately supplied on the other side and the fish were observed for a total of 4 hours. The time that elapsed before the fish swam through the hole and reached the other side with food was used to estimate their risk taking behavior which resulted in 3 different groups. Fish that swam through in the first 30 minutes were considered fast, and the fish that never swam through during the 4 hours were considered slow. The intermediate fish

were those that swam through between 30-240 minutes after revealing the hole. The fish that were classified as fast can be considered somehow high risk takers, and slow as low risk takers.

Figure 1. Arena of the hole test. The fish were confined into the small area of approximately 2.3 liters and left to acclimatize for 2.5-3.5 hours before the hole was revealed and food was distributed on the other side of the partition. The fish were there after observed for a total of 4 hours and classified into 3 different risk taking groups based on when they swam through the hole.

The fish considered as fast and slow were transferred to individual tanks immediately after the hole test, whilst the intermediates were placed in a mutual tank. The fast and the slow fish were given 5-9 days of acclimatization in their individual tanks before the next step, the novel tank diving test.

Novel tank diving test

Zebrafish were each transferred to 250 ml beakers just before they were individually placed in a 1.99 l experimental tank (length; 24.2 x height; 19.6 x width; 4.2 cm), maximally filled with housing water. 4 tanks were placed next to each other and therefore 4 fish were tested at the same time. Every arena (tank) was divided into 3 zones; bottom, middle and top. The swimming behavior was recorded and analyzed by Ethovision XT 9 (Noldus, The Netherlands). The zebrafish were recorded for 15 minutes, starting after 45 seconds of acclimatization of the novel tank. The time spent in each zone, and especially time of duration in the bottom zone was considered the main index of anxiety. To avoid interaction and disturbance between the fish, duct tape was used to cover the side headed towards another tank. Between each test, the tanks were first rinsed with tap water and cleaned with 90 % ethanol and then rinsed with housing water. The fish were tested in a randomized order.

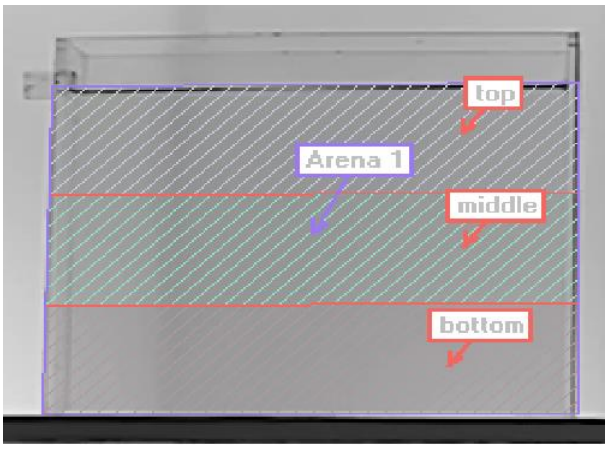


Figure 2. The arena of novel tank diving test. Each arena was formed after the water level in the tank, which was divided into 3 zones: top, middle and bottom. This was done using Ethovision 9.0 (Noldus, The Netherlands)

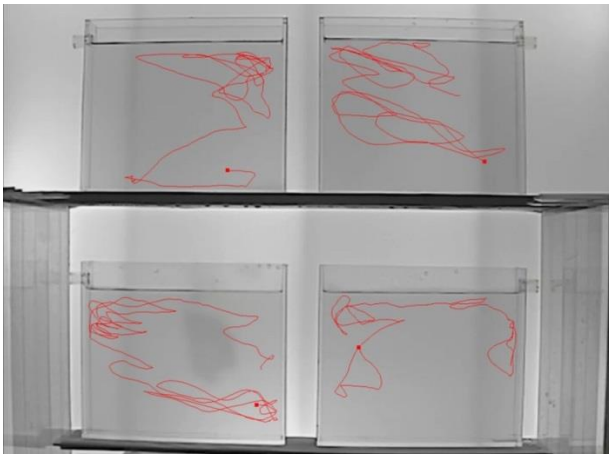


Figure 3. The novel tank diving test. Four tanks were recorded at the same time using Ethovision 9.0 (Noldus, The Netherlands). The red lines represent the registration of the movement of the fish.

Analysis of behavior

The fish were divided by a tertiary split according to their total time of duration spent in the bottom zone of the tank. The groups with the longest and shortest duration were selected and their data were used in a principal component analysis, using Simca 13.0 (Umetrics AB, Sweden). The variables that were used in the multivariate analysis were duration at bottom, duration at top, latency to top, total time moving, total time freezing, velocity and distance moved. From the PCA, 12 of the most extreme bold and 12 of the most extreme shy were selected. The statistical program R (R Development Core Team, 2008) was then used to confirm the differences in duration at bottom and latency to top between the groups of bold and shy fish by using a non-parametric Mann Whitney U-test.

Sampling

After at least 15 days of isolation following the Novel tank diving test, the fish were transferred to a beaker containing ice-cold housing water for a quick anaesthetization before decapitation. Whole brains were quickly removed and immediately put on dry ice and stored at -80 °C. During this step one of the shy test animals had to be removed from the trial due to failed sampling attempt.

RNA extraction
Total RNA was extracted from the zebra fish brains using Gene Elute Mammalian Total RNA miniprep kit (Sigma Aldrich). 2-mercaptoethanol (2-ME) was added to a volume of lysis solution, 10 µl of 2-ME to each ml of lysis solution. 350 µl of the lysis/2-ME solution was added to each eppendorf tube containing the frozen tissue which then was homogenized by hand using a pestel. The samples were centrifuged at 12500´ g for 3 minutes. The supernatant was collected and pipetted into a GenElute Filtration tube, there after centrifuged for 30 seconds at 8500´ g and the filtration column was discarded. 350 µl of 70 % ethanol was added to the filtrated lysate and mixed thoroughly using the pipette. Up to 700 µl of the mixture was transferred into a GenElute binding column subsequently centrifuged at 8500´ g for 30 seconds. 700 µl of Wash solution 1 was added into the column and centrifuged once again at 8500 ´ g for 30 seconds. The binding column was transferred to a new 2 ml collection tube, and the flow trough was discarded. Subsequently 500 µl of Wash solution 2, diluted with ethanol, was added twice, and centrifuged at 8500´ g for 30 seconds after the first adding, and 2 minutes after the second adding. The column was centrifuged for an additional 1 minute at 12500´ g to further dry the column. The binding column was transferred to a new 2 ml collection tube, and eluted by adding 30 µl of RNAse free water into the column and centrifuging at 8500´ g for 1 minute.

Turbo DNA-free Kit (ambion, by life technology) was used for DNAse treatment, following the manufacturers protocol. Nanodrop ND-1000 was used to quantify the total RNA from the extraction. After this step one individual from each group was removed due to too low concentration of RNA. The RNA was then immediately put on dry ice and stored at -80 °C.

cDNA synthesis
Total RNA was used to synthesize cDNA using Maxima SYBR green /ROX (Thermo scientific). The manufacturer´s protocol came with the kit and was followed with start from step 4. Sampling from one fish belonging to the group of shy was removed due to error in cDNA synthesis.

qPCR
When the final step was reached, 20 individuals remained in the experiment, 11 in the group of bold and 9 in the group of shy. Maxima SYBR Green /ROX (Thermo scientific) was used also in the qPCR. cDNA was used as the template for qPCR with specific primers for *oprd1a, oprd1b, drd3, drd2a* and *drd2b.* The reference genes used were elongation factor 2 (*Ef2*), actin (*act1a*), peptidylprolyl isomerase A (*ppia*) and Hypoxanthine-guanine phosphoribosyltransferase (hprt). Each sample was tested in duplicate in a 364-well plate. The reaction mix (10 µl in total) consisted of 5 µl 2x Maxima qPCR Master Mix, 0.5 µl of each primer and 4 µl of a 1/400 dilution of cDNA. The thermo cycling programme consisted of one hold at 95°C for 10 minutes, followed by 40 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C and 30 seconds at 72 °C. After completion of these cycles, melting curve data were collected to verify specificity.

The reference genes were used to create a normalization index using geNorm v3.5. The quantity values from the qPCR were normalized using the normalization index.

Analysis of correlation between gene expression and bold and shy behavior
A t-test was used to compare brain gene expression in shy and bold fish. Further, linear regression was used to evaluate relationships between gene expression and behavior measured as duration at bottom in the novel tank diving test. The genes that showed significantly different expression in bold and shy, were also further analyzed with linear aggression and other behavioral measurements (latency to top, time moving and distance moved).

Comparison between the hole test and the novel tank diving test
The correspondence between the outcome of the novel tank diving test and the hole test was used to determine the relevance and significance of the hole test. The data from the 24 most extreme bold and shy fish from the novel tank diving test was compared to the behavior displayed by these fish in the hole test.

Results

Novel tank diving test
The zebrafish were divided by a tertiary split based on the overall duration spent in the bottom zone of the tank, forming groups of long, intermediate and short duration. The fish with short and long duration were considered high- and low risk takers and were therefore used in the next step.

The data of the groups of short and long duration at bottom were analyzed in a principal component analysis (PCA) with the variables of cumulative duration at bottom, cumulative duration at top, latency to top, moving, not moving along with distance moved and velocity. Two principal components explained 82 % of the variance ($R^2X(cum)$ =0.823, $Q^2X(cum)$ =0.609), see figure 4 and 5.

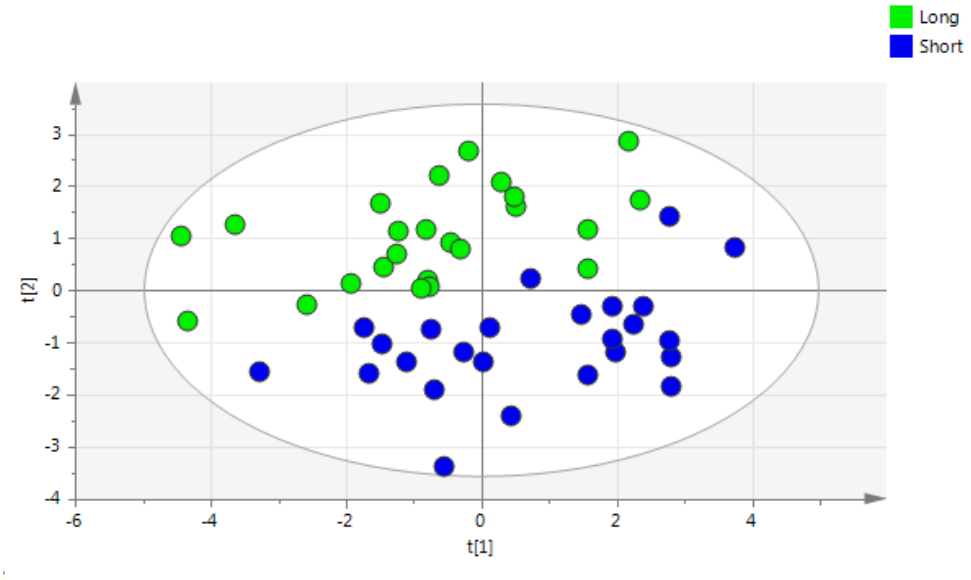


Figure 4. The principal component analysis (PCA) based on data from the novel tank diving test in male AB strain zebrafish. The plot shows the 25 individuals that spent the shortest respectively the longest duration in the bottom zone of the tank.

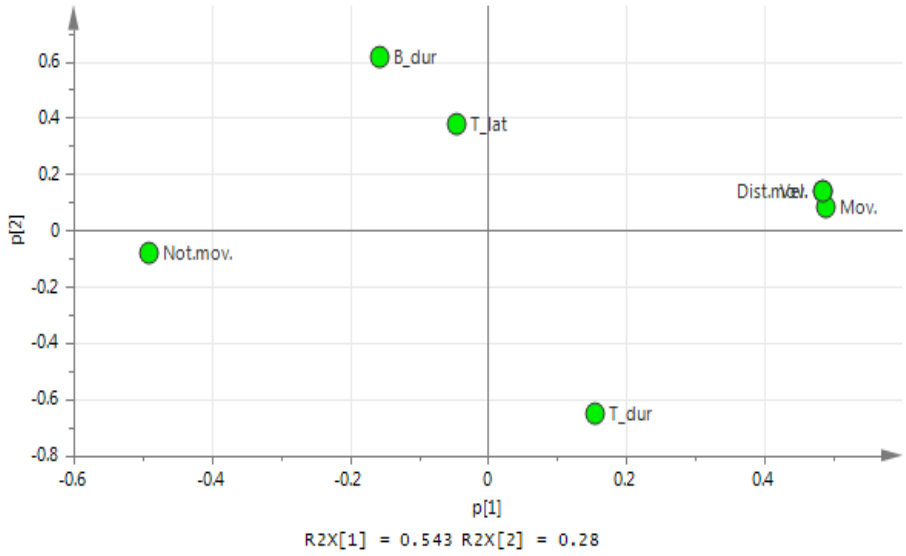


Figure 5. The loading plot of the principal component analysis, illustrates the test variables from the novel tank diving test that were included in the analysis. Variables that are located further away from the origin are the most important for differentiation between the groups. Not.mov= Total time not moving, B_dur; Duration at bottom, T_lat: Latency to top, Dist.mov: total distance moved, Vel.: Velocity, Mov: Total time moving, T_dur: Duration at top.

Using the principal component analysis (Figure 4 and 5), 12 of the most extreme bold and 12 of the most extreme shy were picked out, see figure 6. Due to that some fish were housed in different tanks before decapitation, this had to be taken in consideration during selection so that an equal distribution of large and small tanks between the groups of bold and shy was reached.

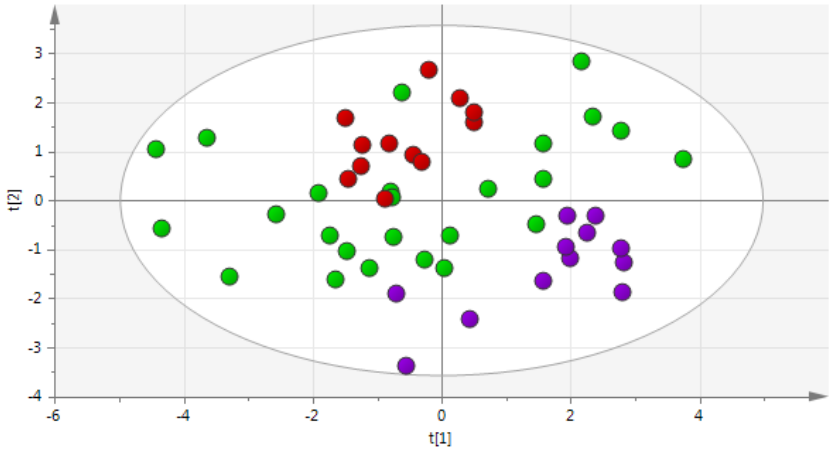


Figure 6. The Principal component analysis marked with the individuals that were selected as the most extreme ones. The red ones represent the 12 most extreme shy individuals, whilst the purple represent the 12 bold. The green ones are considered intermediates and were not further used in the experiment.

To confirm a correct dividing of the two groups, classical statistics was used in the statistical program R (R Development Core Team, 2008). A non-parametric one-sided Mann Whitney U-test showed that the bold fish have a significantly shorter cumulative duration at the bottom than the shy fish (p=3.698e-07), see Figure 7 below. It also showed that the bold fish have a significantly shorter latency to reach the top zone of the arena compared with the shy fish (p=0.01483).

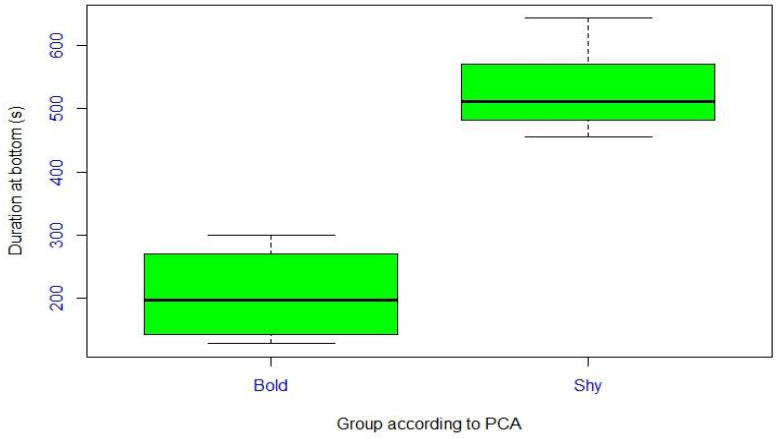


Figure 7. Shows the 2 groups formed from the PCA based on data from the novel tank diving test, and their difference in duration at bottom zone in the novel tank diving test. The Mann Whitney U-test confirmed that the groups are significantly different in duration at bottom.

Analysis of differences in gene expression between groups of bold and shy

Classical statistics in the statistical program R(R Development Core Team, 2008) was used. By using a t-test it could be determined if there was a difference in the gene expressions , using the quantitative data from the qPCR, between bold and shy zebrafish, see table 1.The results show that the expression of both *drd2a* (p=0.04038) and *drd2b* (p=0.0025) are significantly different between the groups of bold and shy fish.

Table 1.Shows the p-values determined from t-test, where gene expressions of oprd1a, oprd1b, drd3, drd2a and drd2b were compared between groups of bold and shy fish. The red color and * indicates that the results are significant.

Gene	**P-value (t-test)**
oprd1a	0.4134
oprd1b	0.09555
drd3	0.7538
drd2a	0.04038*
drd2b	0.002521**

The gene expression of *drd2a* and *drd2b* that differed significantly between the bold and shy Zebrafish are visualized in figure 8 and 9 below. The figures show that the bold fish have a higher gene expression of both *drd2a* and *drd2b*.

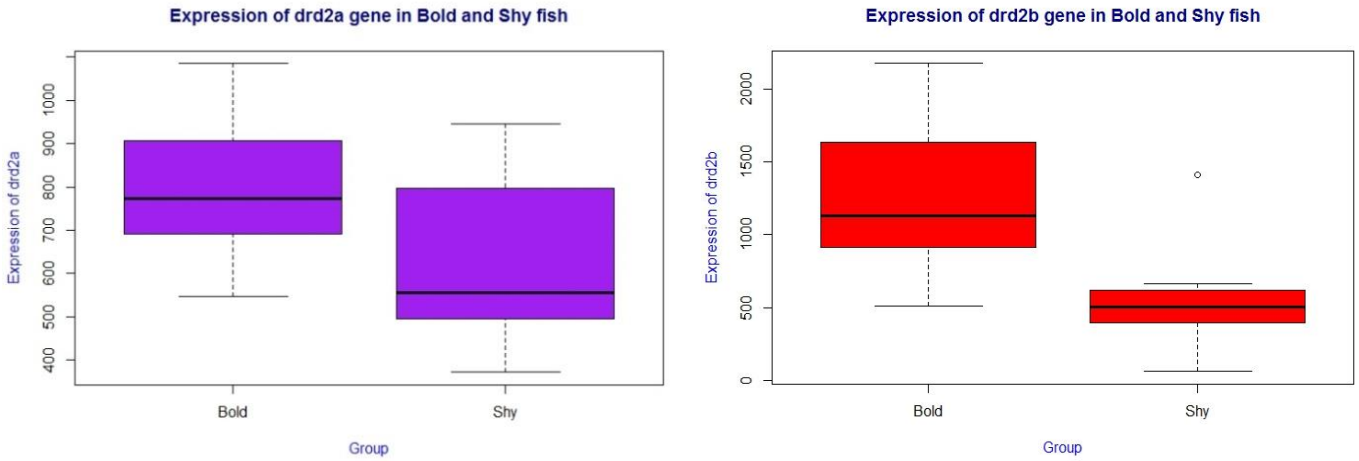


Figure 8 and 9. Shows the differences in gene expression of *drd2a* and *drd2b* between bold and shy zebrafish.

Correlation between gene expression and data from novel tank diving test

A linear regression analysis with the parameter duration at bottom was further done with all genes, see table 2. The result shows that expression of *drd2b* has a correlation with duration at bottom in the novel tank diving test with a R^2 value of 0.21, but with a p-value of 0.04 which indicates that it is statistically significant.

Table 2. Shows the results of linear regression between the genes displayed in the table and the duration at bottom in the novel tank diving test. The red color and * indicates that the result is significant.

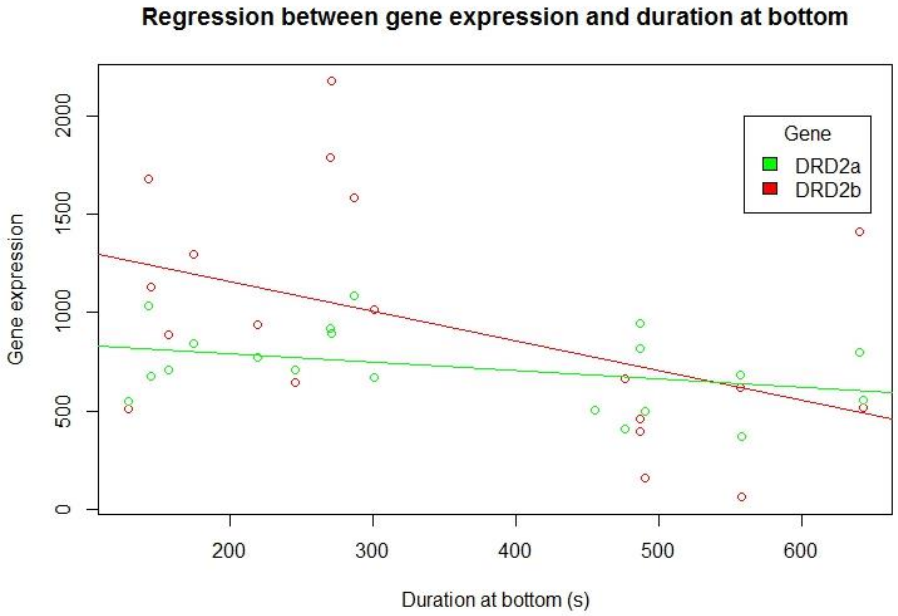
Gene	R^2-value	p-value
oprd1a	0.04089	0.3926
oprd1b	0.1243	0.1273
drd3a	0.008914	0.6922
drd2a	0.1386	0.106
drd2b	0.2133	0.04035*

A further regression analysis was done with *drd2a* and *drd2b* since they showed significance in the t-test. Regression analysis with the parameters latency to top, time moving and total distance moved was done and the results are shown in table 3 below.

Table 3. The linear regression between the genes *drd2a* and *drd2b*, and parameters from the novel tank diving test. The red color and * indicates that the result is significant.

Parameter	R^2value *drd2a*	p-value *drd2a*	R^2-value *drd2b*	p-value *drd2b*
Latency to top	0.03925	0.4024	0.277	0.03368*
Time moving	0.03462	0.4322	0.1715	0.06948
Distance moved	0.0107	0.6643	0.1494	0.09234

The correlation between the two genes *drd2a* and *drd2b* and the parameters duration at bottom and latency to top are visualized in figure 9a and 9b .



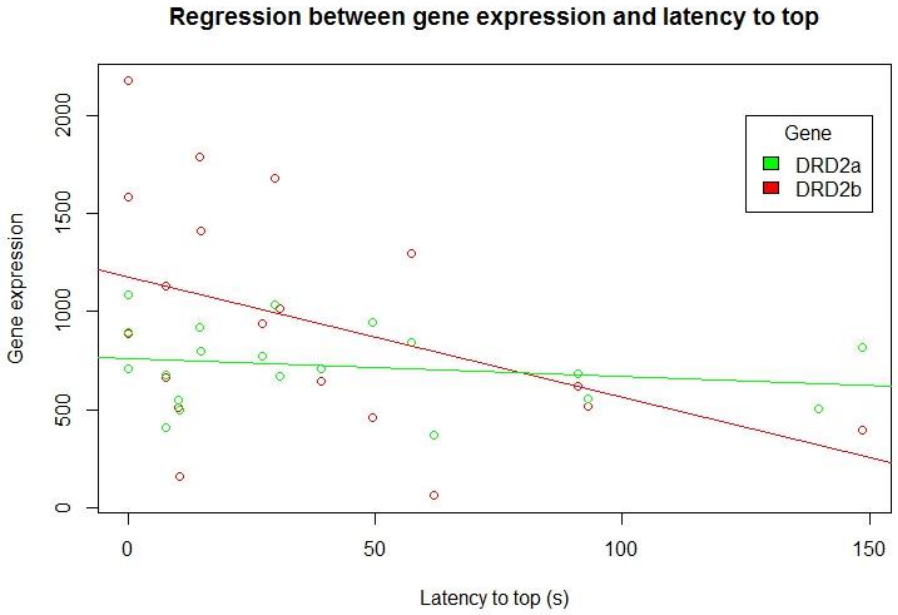


Figure 9a and 9b. Visualizes the linear regression of the genes *drd2a* and *drd2b* and the parameters duration at bottom and duration at top from the novel tank diving test.

Hole test

The hole test was a pilot project and an attempt to sort fish after risk taking behavior in an easier and less stressful way. The outcome of the hole test was therefore compared with the individuals of shy and bold picked out from the PCA, to see if the high risk takers according to the hole test correlated with the results of their risk taking in the novel tank diving test.

Of the 24 individuals that were picked out as bold and shy, 67 % showed the same extent on risk taking behavior in both the novel tank diving test and the hole test. In the group of bold, 6 were considered fast and 6 slow in the hole test. This gives a correlation of 50 %. In the group of shy 10 individuals had the same outcome of the two tests, which means that 2 individuals had not correlating results. This gives us a correlation of 83 % between the outcome of the hole test and novel tank diving test in the group of shy. The results are visualized in figure 11 below.

Figure 11. Shows the correlation between the outcome of the hole test and the novel tank diving test in the 24 individuals that were picked out as the most extreme ones in risk taking behavior based on data from the novel tank diving test.

Discussion

In this study the correlation between risk taking behavior and expression of the genes for the opioid receptors *oprd1a, oprd1b* and the dopamine receptors *drd3, drd2a* and *drd2b* was investigated. Zebrafish behavior was determined with the use of novel tank diving test, where duration at bottom was considered the most important factor.

The result shows that both *drd2a* and *drd2b* are expressed significantly different in the bold and shy fish, where the difference in the expression of drd2b was more pronounced than the difference in drd2a expression. Bold fish had a significantly higher expression of both *drd2a* and *drd2b* compared to the shy fish. There was also significant positive correlation between the expression of *drd2b* and the duration at bottom and latency to top. Thus, fish with high expression of *drd2b* spent less time at the bottom and were faster to reach the top zone of the arena than fish displaying a lower brain expression of *drd2b.*

As mentioned earlier, D2 is expressed partly as an autoreceptor which inhibits the presynaptic neurons release of dopamine when stimulated. This study suggests that the bold fish have a higher frequency of autoreceptors than shy fish, which would result in a stronger negative feedback on dopamine release in bold fish. The release of dopamine is hence more controlled. In the shy fish on the other hand, there are fewer autoreceptors which indirectly leads to less negative feedback and therefor a larger release of dopamine. It means a larger release of

dopamine per stimulation, since there are less D2 receptors present to perform negative inhibition (Bello et al, 2011). Possibly, the shy fish could therefor experience a stronger reward due to dopamine from dopaminergic stimulation, compared to the bold fish.

D2 and D3 receptors both belong to the same subfamily of dopamine receptors called D2-like receptors, and they both exist in the form of autoreceptor. In this study only D2 showed significant difference between bold and shy fish. This could be because D2 has a more important regulatory role. Bello et al (2011) claims that although there are five subtypes of DA receptors that orchestrate all DA postsynaptic responses, it is the D2 receptor that mainly convey the DA mediated auto inhibition of DA neuron activity, DA release and DA synthesis. D2 autoreceptors have the critical role in DA neurotransmission and therefor transcriptional regulation of drd2a in DA neurons may contribute to individual reactions towards natural rewards as well as drugs of abuse. Low levels of the presynaptic receptor can predict enhanced susceptibility to drug-seeking and drug abuse. (Bello et al, 2011).

Oprd1a and *oprd1b* expression did not show any significant difference between the groups of bold and shy fish, neither did it show correlation with the parameter duration at bottom from the novel tank diving test. The role of delta receptor in reward is much debated (Charbogne et al, 2014).

Hole test

The outcome of the hole test showed a 67 % correspondence with the results of the novel tank diving test in the 24 selected fish that were considered the most extreme bold and shy. Six out of the 12 that were assigned bold based on the novel tank diving test would have been assigned wrong if the hole test solely would have been used. The situation is less dramatic for the shy, were only 2 out of 12 would have been assigned wrong risk taking behavior. The fact that the hole test did not correspond a 100 % with the novel tank diving test does not say that the hole test cannot be used to determine risk taking behavior, since risk taking behavior can have many different indices. The novel tank diving test is based on a few chosen parameters, whilst the hole test could give indices of boldness and shyness in a different way. In the hole test the fish is tested in groups whereas as in the novel tank diving test the fish are tested individually. This may affect the outcome of the test. Zebrafish is a schooling species and isolation is likely to be stressful.

Rey et al (2013) performed something similar to the hole test. They used a hole of 3 cm in diameter, 10 minutes of habituation and then 35 minutes of total observation. All the sides of the tank were covered with black cover during the trial. They tested the same animals again after 10 months and only 8 % had a different outcome. They then continued to test the animals in other behavioral tests and the overall consistence was mostly significant and a behavioral syndrome was found. This clearly shows that the hole test can be used for scoring differences in risk taking behavior, although it needs to be further developed and validated as a model.

Conclusion

This study showed a significantly higher brain gene expression of drd2a and drd2b in bold compared to shy zebrafish males. This could be due to that dopamine receptor D2 has an important critical regulatory role in the brains dopamine system by acting as an autoreceptor.

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