The effect of human pharmaceuticals on dopaminergic and adrenergic gene expression in threespined sticklebacks

Zahra Hasson

Examinator, Johan Edqvist, IFM Biologi, Linköpings universitet
Tutor, Hanne Løvlie, IFM Biologi, Linköpings universitet
Denna rapport är ett examensarbete på kandidatnivå (16 hp) som har genomförts i samarbete med en studentkollega, Farhiya Hassan. Samarbetet har omfattat projektplanering samt insamling och bearbetning av data, medan studenterna var för sig har författat och strukturerat rapporten i alla dess delar.
Table of content

1  Abstract ......................................................................................................................... 4
2  Introduction ...................................................................................................................... 4
3  Material & methods ......................................................................................................... 6
  3.1  Previous behavioral experiment................................................................................... 6
  3.2  RNA extraction ............................................................................................................ 6
  3.3  cDNA synthesis .......................................................................................................... 7
  3.4  PCR .............................................................................................................................. 7
        3.4.1  Make primers for three genes (drd2, adrd2a and drd1b)...................................... 7
        3.4.2  PCR reaction scheme......................................................................................... 7
        3.4.3  PCR Master Mix ................................................................................................. 7
        3.4.4  Confirmation of cDNA and primers .................................................................... 7
  3.5  Statistical tests ............................................................................................................ 8
4  Results .............................................................................................................................. 8
  4.1  Gene expression in drd2 .............................................................................................. 8
  4.2  Gene expression in adrd2a ......................................................................................... 9
  4.3  Gene expression in drd1b ........................................................................................... 9
5  Discussion ......................................................................................................................... 12
  5.1  Conclusion .................................................................................................................. 13
  5.2  Societal & ethical aspect ........................................................................................... 13
6  Acknowledgment ............................................................................................................. 13
6  Referencess ...................................................................................................................... 14
1 Abstract

Many human pharmaceuticals have limited biodegradability and can end up in the aquatic environment. The effects of these pharmaceuticals on aquatic organisms is not fully understood. Ropinirole is a drug used to treat Parkinson’s disease. Ropinirole is a dopamine agonist that targets the dopaminergic system that many diverse organisms share. In this study, threespined sticklebacks (Gasterosteus aculeatus) were used to investigate the effect of ropinirole on wild animals. Wild captured sticklebacks were exposed to ropinirole, ropinirole and fluoxetine, or untreated control. The fish were sacrificed at two different time points to study the effects on gene expression after long and short-time exposure of the drug. Gene expression of two dopamine receptor genes (drd2 and drd1b) and one adrenergic receptor gene (adrd2a) is studied in this project. The fish brains were dissected, total mRNA isolated and translated to cDNA, and finally qPCR was done. The expression of drd2 and adrd2a genes did not differ across the treatment groups or time. Drd1b showed higher expression at long term of exposure relative to short time exposure to ropinirole, but no other differences were observed between treatment groups. Collectively, my results show that ropinirole, or ropinirole together with fluoxetine did not interact with the adrenergic receptor or the dopamine 2-receptor. Exposure to ropinirole longer time can upregulate genes, as seem for the gene drd1b. Overall, these results show that pharmaceuticals in the environment can affect gene expression on other animals than the targeted humans.

2 Introduction

Because many of the pharmaceuticals used by humans have limited biodegradability, these active substances can end up in aquatic environment and present a threat to aquatic organisms (Wienberger et al. 2014). Since many organisms have similar physiology as humans it has been suspected that they are also sensitive to drugs, originally designed to target humans (Wienberger et al. 2014). Substances from these drugs can bioaccumulate in fish and possibly affect a wide range of traits (Brooks et al. 2005; Schultz et al. 2010).

Both dopamine and serotonin are monoamine neurotransmitters and are released from neurons in the brain and peripheral nervous system. The function of these monoamines are still not fully understood, but it is suggested that some behaviour variation can be linked to the monoaminergic system (Winberg et al. 1993). Ropinirole is a drug used to relieve the symptoms of idiopathic Parkinson’s disease and treatment of restless legs syndrome (FASS 2017). Ropinirole is a dopamine agonist
with higher binding affinity to the D3 dopamine receptor than D2 or D4 receptor subtypes. The mechanism of action of ropinirole as a treatment for Parkinson’s disease is still not fully understood. It is suggested that the drug stimulates the postsynaptic dopamine D2-type receptor within the caudate-putamen in the brain (Cacabelos 2017). The D2 receptor in the brain plays important role in motor control as well as cognitive and emotional functions (Lammel et al. 2008). Stimulating dopamine receptors with the neurotransmitter dopamine influence most basic behaviour in both animals and humans, from searching for food to choosing partners (Messias et al. 2015). To understand such important traits the underlying genetic mechanisms should be studied.

It is important to understand how drugs such as ropinirole that ends up in the environment has consequences on behavioral responses of exposed individuals. The underlying genetic mechanisms should also be studied, to better understand how ropinirole interaction with different receptors in the monoaminergic system can affect gene expression for these receptors, and how this can affect the individuals.

To study the effect of these drugs on aquatic organisms, threespined stickleback (Gasterosteus aculeatus) was used. Sticklebacks are well known for their diverse and complex behaviours that have been widely characterized over the last century (Broad Institute 2007). The genome of sticklebacks is sequenced, and sticklebacks are in the forefront of research aimed to understand phenotypic diversity and adaption (Pechel et al. 2016; Broad Institute 2007). It has been predicted that monoamine levels and expression of genes related to the monoaminergic system will vary in the brain depending on the environmental exposure of psychoactive drugs. However, it is not very clear yet how pharmaceuticals affect gene expression, since only few studies have been done.

Both the dopaminergic and adrenergic receptors are G- protein coupled, but are different in terms of their pharmacology and signalling pathways (Cornil et al. 2010). However, some studies have shown that the pharmacological specificity among this subtype receptor family, is not perfect. Therefore, monoamines can cross-talk with receptors from other systems (Cornil at al. 2010). For this report, the dopaminergic and adrenergic system will be in focus, to study how ropinirole can interact with dopamine receptors but also adrenergic receptor and thereby effect their gene expression. Further, fluoxetine, which is a selective serotonin reuptake inhibitor (SSRI) (FASS 2015) was used to also manipulate the serotonergic system and investigate potential interaction with ropinirole exposure. Low serotonin levels in the brain leads to depression, anxiety
and aggression. Fluoxetine is therefore used in humans to increase the level of serotonin in the brain (Guzman 2013).

It is important to investigate gene expression to better inform the environmental risk assessment of pharmaceuticals. Therefore, the aim of this project is to measure gene expression in the stickleback’s brains, and test if some genes associated with dopaminergic (drd2 and drd1b) and adrenergic system (adrd2a) will be different in individuals exposed to chemical manipulations relative to control individuals.

3 Material & methods

3.1 Previous behavioral experiment

Young threespine sticklebacks were collected from Oxelösund, Sweden on Oct 26, 2017 and taken back to Linköping University (Fernandez 2018). Fish were exposed to one of 4 treatments: ropinirole only, fluoxetine only, both ropinirole and fluoxetine, or control (no drug). Some fish were sacrificed after 6 days of short time of drug exposure and others after 18 days of long term drug exposure. Whole brains were extracted from fish for these analyses.

3.2 RNA extraction

RNA extraction was done to isolate the RNA from the selected (brain) tissue. First, brain tissue was homogenized physically and chemically. Whole brains were placed in Lysing Matrix D tubes 1 ml TRizol and shook at 6.0 m/s for 40 s on the FastPrep-24 (MP biomedicals). The samples were then centrifuged at 12000 x g for 10 min at 4°C.

Next, 950 µl of the homogenate sample was transferred to a new tube with TRIzol and BCP (1-bromo-3-chloropropane). BCP has the task of separating the samples into a clear aqueous layer containing RNA and a lower pink organic layer containing DNA and proteins. The tubes were closed properly and shook vigorously by hand for 15 s and then incubated at room temperature for 5 min. The samples were centrifuged at 12000 x g for 15 min at 4°C.

The clear aqueous phase containing RNA was transferred to a new 1.5 ml tube containing isopropanol and RNA precipitation solution and vortexed at 1400 for 10 s. The samples were incubated at room temperature for 10 min and then centrifuged at 12000 x g for 10 min at 4°C. Now the RNA is in a pellet, so the supernatant was removed carefully. We then washed the pellet with 1000 µl 75% ethanol. The samples were then centrifuged at 7500 x g for 5 min at 4°C. To prevent ethanol from breaking down RNA
it was removed carefully and evaporated, and the pellet was dried. To
dissolve the RNA for further experiments 30 µl RNase- free water was
added to the pellet. We measured the final concentration of RNA using
1.5 µl of the RNA on the NanoDrop.

3.3 cDNA synthesis

The kit used for this procedure Thermo ScientificTM MaximaTM First
strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermofisher,
Sweden).

To remove contamination genomic DNA from RNA sample, 1 µl 10X
dsDNase Buffer, 1 µl dsDNase, 0.5 mg total template RNA and nuclease
free water to a total volume of 10 µl was added together to a sterile
RNase free tube on ice and mixed gently and centrifuged. The samples
were incubated for 2 min at 37°C in a water bath then chilled on ice,
briefly centrifuged and placed on ice. 4 µl 5X Reaction Mix, 2 µl Maxima
Enzyme Mix and 4 µl nuclease free water were mixed gently and
centrifuged. The tubes were incubated for 10 min at 25°C followed by 15
min at 50°C. The reaction was terminated by heating at 85°C for 5 min.

3.4 PCR

3.4.1 Make primers for three genes (drd2, adrd2a and drd1b)

A stock solution of primers #nmol made by adding to ×10 nuclease free
water. A working solution containing dilution 20 µl forward primer, 20 µl
reverse primer and 160 µl water (1:20 dilution) in aliquots for reactions
was also done.

3.4.2 PCR reaction scheme

The qPCR was run on an ABI Applied Biosystems 7900HT Fast Real-
time PCR system with following protocol: 5 min at 95°C, 45 cycles of
10 s at 95°C, 10 s at 60°C and 20 s at 72°C cooling to 40°C.

3.4.3 PCR Master Mix

The PCR mix contained 2 µl water, 1 µl forward and reverse primer
combined, 2 µl (1:32 dilution) cDNA and 5 µl SYBR green.

3.4.4 Confirmation of cDNA and primers

The cDNA product was confirmed by diluting all samples and NTC/RT-
to be 1:32. Two housekeeper were used ubs (Ubiquitin) and rp113a
(L12A ribosomal binding protein), and all samples were plated in
duplicated based on the mix and run as above. Melt curves and Cp values for each individual/sample were confirmed before analyses of genes of interest.

To test the primers 5 µl of cDNA was pooled for all individuals to make a general stock cDNA. 4 wells for each primer was run duplicate of normal and duplicate of NTC. Each well with primer duplicate contained 1 µl forward and reverse primer, 2 µl pooled cDNA, 5 µl SYBR Green and 2 µl water. Each well of NTC contained 1 µl forward and reverse primer, 5 µl SYBR Green and 4 µl water. Temperature/PCR reaction scheme was run as above. Melt curves and Cp values for each primer were checked and only primers with good values were used for final gene expression analyses.

All individuals produced good cDNA product and drd2, adrd2a and drd1b primers met criteria and were used to test for gene expression in all individuals. For relative quantification, the ΔCT method was used, comparing the target gene with the reference genes ubc and Rpl13a.

3.5 Statistical tests
All statistical analysis was performed with IBM SPSS statistic 25. To study the effect of the different treatments and time exposure on normalized gene expression of drd2b, adrd2a, and drd1b, a parametric one-way ANOVA was done with gene expression for each gene individually as a response variable. Because some of the gene expression data for some individual had invalid Cp values and melt curves we had to eliminate some data points. Therefore, not enough data points remained for the combined treatment (fluoxetine together with ropinirole) in short time of exposure.

4 Results
The three different genes in the dopaminergic and adrenergic system were expressed in stickleback’s brain in detectable levels.

4.1 Gene expression in drd2
Sticklebacks exposed to ropinirole do not seem to differ in gene expression of the drd2 receptor when compared to control group in short and long time of exposure (Table 1, Figure 1a, Figure 1b). No difference was observed between time points in gene expression when exposed to just ropinirole (Table 2, Figure 2a).


4.2 Gene expression in adrd2a

No significant difference in expression level in gene adrd2a across treatment groups and short and long time of exposure (Table 1, Figure 1c, Figure 1d). No difference in gene expression between time points when exposure to only ropinirole (Table 2, Figure 2b).

4.3 Gene expression in drd1b

No significant difference in gene expression of drd1b receptor was found within different treatments and the time of exposure (Table 1, Figure 1e, Figure 1f). A significantly higher expression of gene drd1b receptor was found in long time of exposure to ropinirole than short (Table 4, Figure 2c).

Table 1. Difference in gene expression in the brain of threespined sticklebacks after short and long-term exposure to monoamines manipulation. Statistic for gene expression of drd2, adrd2a and drd1b for exposure to ropinirole, ropinirole and fluoxetine and control. Short stands for short time of exposure and long for long term of exposure. Significant p-value<0.05.

<table>
<thead>
<tr>
<th>Gene</th>
<th>F-value</th>
<th>P-value</th>
<th>Total N</th>
</tr>
</thead>
<tbody>
<tr>
<td>drd2 (short)</td>
<td>0.880</td>
<td>0.380</td>
<td>8</td>
</tr>
<tr>
<td>drd2 (long)</td>
<td>0.611</td>
<td>0.548</td>
<td>39</td>
</tr>
<tr>
<td>adrd2a (short)</td>
<td>0.480</td>
<td>0.508</td>
<td>9</td>
</tr>
<tr>
<td>adrd2a (long)</td>
<td>0.012</td>
<td>0.988</td>
<td>39</td>
</tr>
<tr>
<td>drd1b (short)</td>
<td>0.480</td>
<td>0.508</td>
<td>9</td>
</tr>
<tr>
<td>drd1b (long)</td>
<td>0.493</td>
<td>0.615</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 2. Difference in brain gene expression for genes drd2, adrd2a and drd1b between short and long time exposure of ropinirole for threespined stickleback. Significant p-value<0.05 in bold.

<table>
<thead>
<tr>
<th>Gene</th>
<th>F-value</th>
<th>P-value</th>
<th>Total N</th>
</tr>
</thead>
<tbody>
<tr>
<td>drd2</td>
<td>2.731</td>
<td>0.121</td>
<td>15</td>
</tr>
<tr>
<td>adrd2a</td>
<td>0.093</td>
<td>0.765</td>
<td>16</td>
</tr>
<tr>
<td>drd1b</td>
<td>7.474</td>
<td>0.015</td>
<td>16</td>
</tr>
</tbody>
</table>
Figure 1. Gene expression levels in brains of threespined sticklebacks across treatment groups and time points. (a) drd2 short, (b) drd2 long, (c) adrd2a short, (d) adrd2a long, (e) drd1b short and (f) drd1b long exposure to the drugs. Control (white) = no exposure, ropinirole (grey), and combined ropinirole and fluoxetine (black). The expression was normalized according to housekeeping gene ubcHK and Rpl3a. Data are expressed as the mean ± SE.
Figure 2 Brain gene expression levels in threespined sticklebacks exposed to ropinirole. Gene investigated (a) drd2, (b) adrd1a, and (c) drd1b. Short- time exposure (white) and long-term exposure (black). The expression was normalized according to housekeeping gene ubcHK and Rpl3a. Data are expressed as the mean ± SE.
5 Discussion

I observed in this study no significant changes in brain gene expression for drd2 and adrd2a for fish exposed to ropinirole, ropinirole and fluoxetine, and untreated control, when short (6 days) and longer (18 days) exposure to the drugs. The gene expression of drd1b did not differ across treatment groups but was lower when sticklebacks were exposed to only shorter compared to longer time to ropinirole.

Comparing the treatment groups, we can see that gene expression for the combined experiment did not differ from the other groups. The reason behind it can be that fluoxetine dose not interact with the selected dopaminergic and adrenergic receptors because fluoxetine has good specificity of action to bind to target receptors in the serotonergic system (Perry et al. 1992).

The interaction of ropinirole in the dopaminergic system in sticklebacks is still not understood because only few studies have been done. Ropinirole is supposed to interact with the D2-receptor which is coded by the drd2 gene at least in various Parkinson’s models in mice, rats and Cynomolgus monkeys (Fukuzaki et al. 2000). In this study, no significant changes of the drd2 genes was observed, which suggests that ropinirole in threespined sticklebacks dose not interact with the D2-type receptor, and because of that did not affect gene expression. Therefore, the mechanism of action for ropinirole seem to differ between distinct species.

A pilot study was done to determine ropinirole dose response curve (how different doses affects individuals) because of the limited available data about ropinirole concentrations in water environment (Fernandez 2018). The concentration of ropinirole that was used in this study is 2500 ng/L, which might be too low of a concentration to induce a notable change in gene expression for the selected genes in sticklebacks. High concentrations of some psychoactive drugs have been shown in one study to increase gene expression profiles for genes associated with autism and Parkinson’s disease (Thomas et al. 2012). If the active substances of ropinirole continues to leak into the nature the concentration will eventually increase, so it would be relevant to also test the gene expression at higher concentrations of the drug.

Earlier studies have confirmed that exposure of low concentration of pharmaceuticals can affect a wide range of traits in organisms (Brooks et al. 2005, Schultz et al. 2010). This can be related to our results where the gene expression for drd1b increased after a longer term of exposure to ropinirole. This means that ropinirole can upregulate genes in the
dopaminergic system and possibly effect for instance stickleback’s behavior.

The sticklebacks that was used in this study is wild caught and it is unknow which drugs and concentrations they were exposed to before taking them in to the lab. In this study no difference in gene expression across different treatments of only ropinirole, ropinirole and fluoxetine and control were found, which could be explained that the gene expression was already affected before exposing them to drugs again (including the control). Future studies should therefore evaluate the here found results in fish raised under controlled condition and no prior exposure to drugs.

5.1 Conclusion
The effect of ecologically relevant doses of ropinirole found in our waste water on aquatic organisms as the threespined stickleback seems weak and therefore may have limited ecological consequences. However, longer exposure did alter gene expression suggesting that animals exposure to our residual medication via our wastewater are influenced by these. Investigating the effect of pharmaceuticals as ropinirole and fluoxetine on gene expression for more genes in the monoaminergic system, different organisms and concentrations are crucial for understanding of how these drugs that leak out into nature, influence the environment and other individuals.

5.2 Societal & ethical aspect
The amount of psychoactive drug that leaks to the environment has been rapidly increasing. Understanding the effects of these pharmaceuticals on organism’s genetic expression and behavior is important for ecosystem function. Therefore, we must raise awareness in the society about the effect of drugs.

6. Acknowledgment
I want to thank my supervisor Hanne Løvlie for her guidance and supervision during the whole project work. Many thanks to Robin N. Abbey-Lee, Sara Ryding and Anastasia Kreshchenko for their incredible patient support and directions during the lab work. I would also like to thank my co-lab worker Farhiya Hassan. Also, Xavier Fernández Sala and Shiori Kitano for helping with the statistics analyse and Madeleine Mickelsson and Hanna Ahlsén for evaluating my report.
6 References

Broad Institute. (2007), Stickleback genome project. (Read 2018-05-20)


FASS. (2017). Ropinirole Teva. (Read 2018-03-23)

FASS. (2015). Fluoxetine Accord 20 mg. (Read 2018-03-23)


