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Impact Of Psychopharmaceuticals On Visual System Of Non-Targeted Aquatic Organisms

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Impact Of Psychopharmaceuticals On Visual System Of Non-Targeted Aquatic Organisms

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Resumo

Os antidepressivos estão entre os fármacos mais identificados em sistemas aquosos e, como poluentes orgânicos emergentes, podem exercer efeitos negativos em organismos aquáticos não alvo. Em consequência do aumento continuado das concentrações no meio ambiente, os impactos biológicos destes compostos nos peixes têm sido discutidos. A exposição aos produtos farmacêuticos tem originado alterações no comportamento, reprodução e desenvolvimento. Além disso, estudos demonstraram que a exposição aos antidepressivos resulta num aumento substancial na mortalidade, atrasos no desenvolvimento, anomalias morfológicas e alterações patológicas no cérebro, coração e rim cranial e caudal. O desenvolvimento do cérebro e do sistema visual é particularmente suscetível aos efeitos da exposição pré-natal a substâncias neuroativas. A avaliação da resposta motora visual no peixe-zebra demonstrou que os antidepressivos modificam o perfil da locomoção espontânea com maior regularidade, apresentando variação significativa nos efeitos comportamentais. Em peixes, a análise de expressão génica global pode revelar efeitos de tóxicos em vias bioquímicas inesperadas, elucidar mecanismos de toxicidade e ser utilizada para avaliação de perfis de expressão génica para definir diferenças/semelhanças nas respostas de organismos aos tóxicos. O objetivo deste estudo é resumir o conhecimento atual sobre os impactos dos compostos neuroativos em animais não visados que vivem em águas superficiais, especialmente ao nível do cérebro e do sistema visual.

Palavras-chave: Antidepressivos; Peixe; Impacto Biológico; Expressão Génica; Sistema Visual

Abstract

Antidepressants are among the most identified pharmaceuticals in aqueous systems, and, as emerging organic pollutants, can exert negative effects on non-target aquatic organisms. As the concentrations in the environment are incessantly increasing the biological impacts of these compounds in fish have been under discussion. The exposure to these pharmaceuticals products has been producing alterations in behavior, reproduction, and development. Also, studies demonstrate that exposure to antidepressants result in a substantial rise in mortality, developmental retardation, morphological anomalies, and pathological changes in brain, heart, and cranial and caudal kidney. The development of the brain and visual system is particularly susceptible to the effects of prenatal exposure to neuroactive drugs. Assessing the visual motor response in zebrafish, demonstrated that antidepressants most regularly modify the profile of spontaneous locomotion having significant variation in behavioral effects. In fish, global gene expression analysis can reveal effects of toxicants on unexpected biochemical pathways, elucidate mechanisms of toxicity and be utilized for assessment of gene expression profiles to define differences/similarities in responses of organisms to toxicants. The aim of this study is to summarize current knowledge about the impacts of neuroactive compounds on non-target animals living in surface waters, especially at the level of the brain and visual system.

Keywords: Antidepressants; Fish; Biological Impact; Gene Expression; Visual System

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List of Abbreviations

5-HT	Serotonin
5-HTT	Serotonin transporter
actb1	Actin, beta 1
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APB	DL-2-amino-4-phosphonobutyric acid
atoh7	Atonal bHLH transcription factor 7
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DA	Dopamine
DC	Double single
DMSO	Dimethyl sulfoxide
dpf	Days post-fertilization
EDCs	Endocrine Disrupting Compounds
ef1	Elongation factor 1
EPs	Emerging Pollutants
FET	Acute toxicity test
GABA	Gamma-aminobutyric acid
GPCR	G protein-coupled receptor
hh	Hedgehog
HPA	Hypothalamic-pituitary-adrenal
hpf	Hours post-fertilizations
HPLC	High performance liquid chromatography
INL	Inner nuclear layer
IPL	Inner plexiform layer
LSC	Long single
lws	Long-wavelength sensitive
$MAO_{A/B}$	Monoamine oxidase A/B
MAOIs	Monoamine oxidase inhibitors
NDRIs	Norepinephrine-Dopamine reuptake inhibitors
NE	Norepinephrine
NMDA	N-methyl-D-aspartate
NSAIDs	Nonsteroidal anti-inflammatory drugs
OD	Ocular dominance
ONL	Outer nuclear layer
OPL	Outer plexiform layer
otx2	Orthodenticle homeobox 2b

PAHs	Polycyclic aromatic hydrocarbons
pax6	Paired box 6
pde6c	Phosphodiesterase6C
Phe	Phenanthrene
PPCPs	Personal care products
RGC	Retinal ganglion cell
rh2	Rhodopsin-like
rho	Rhodopsin
RNE	Retinal neuroepithelium
rx3	Retinal homeobox 3
shh	Sonic hedgehog
six3	Six homeobox 3
SNRIs	Serotonin and norepinephrine reuptake inhibitors
SSARIs	Serotonin antagonist reuptake inhibitors
SSC	Short single
SSRIs	Selective serotonin reuptake inhibitors
sws1	Short-wavelength sensitive 1
sws2	Short-wavelength sensitive 2
TCAs	Tricyclic antidepressants
VMR	Visual motor response
WWTPs	Wastewater treatment plants

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Previous Note

Within the scope of this thesis, it is intended to clarify that the Chapter 6 – Experimental Planning, is after the conclusion of the remaining chapters since it describes all the methodology that would be applied in the development of the work but with the global situation due to SARS-CoV-2 it was not possible to carry out the same practical work.

Chapter 1 . Emerging Pollutants

1. Overview Of Emerging Pollutants

Emerging pollutants (EPs) are considered a group of chemicals that constitute a series of risks inherent to the environment or toxicity to the human and for the vast majority of EPs, there are no adequate monitoring methods and the regulatory processes are not yet well understood (1). There is a vast diversity of EP classes that include: radioactive metals (2), endocrine disrupting compounds (EDCs) (3), human and veterinary pharmaceuticals, disinfection by-products, personal care products (PPCPs), polycyclic aromatic hydrocarbons, perfluorinated compounds, heavy metals, surfactants, pesticides, flame retardants, illicit drugs, hormones and algal toxins (4,5). Knowing that these types of compounds have become an indispensable part of today's society and are part of the modern life revolution, it is expected that the main origins of these components are agricultural, industrial or municipal sources (6). Table 1 summarizes the sources of the major categories of EPs in the aquatic environment.

Table 1. Sources of EPs in aquatic ecosystem (Adapted from: (7)).

Categories	Main Subclasses	Main Sources	
		Distinct	Nonexclusive
PPCPs	Fragrances, disinfectants, UV filters, and insect repellents	Domestic wastewater (bathing, shaving, spraying, swimming.)	Sources that are not exclusive to individual categories include: Industrial wastewater (product manufacturing discharges) Landfill leachate (improper disposal of used, defective or expired items)
Steroid hormones	Estrogens	Domestic wastewater (excretion) Run-off from concentrated animal feeding operations and aquaculture	
Pharmaceuticals	NSAIDs, lipid regulator, anticonvulsants, antibiotics, β-blockers, antidepressants, and stimulants	Domestic wastewater (excretion) Hospital effluents Run-off from concentrated animal feeding operations and aquaculture	
Surfactants	Non-ionic surfactants	Domestic wastewater (bathing, laundry, dishwashing) Industrial wastewater (industrial cleaning discharges)	
Pesticides	Insecticides, herbicides and fungicides	Domestic wastewater (improper cleaning, run-off from gardens, lawns and roadways.) Agricultural run-off	
Industrial Chemicals	Plasticizers, fire retardants	Domestic wastewater (leaching out of the material)	

Abbreviations: PPCPs-Personal Care Products; NSAIDS-Non-steroidal Anti-inflammatory Drugs

During the last few years, EPs have been detected in different aquatic sources, from drinking water, groundwater and surface water, wastewater treatment effluents and even in terms of precipitation, which triggers a great set of new and serious potential poisonings and an increase in environmental health concerns worldwide (7–9). Several studies have been carried out in order to monitor the existence, distribution, destination and ecotoxicology of several EPs in soils (10), marine sediments (11), wastewater, aquatic environments and human bodies and other living organisms (1,12). These compounds have a wide dispersion at a global level, being found traces of anthropogenic contamination that vary from the stratosphere to the deep oceans, pole to pole, in all modernized society, wildlife and consequently in the food chain and, considerably in the majority of the population, including newborns (13). In order to minimize the impact of EPs on the environmental level, analytical methods have been developed for the detection of these substances in the

environment and in living organisms, as well as different technologies that provide different remediation strategies (14,15). This chapter will provide a summary of the occurrence, transport, and distribution of EPs with a focus on aquatic environments.

1.1. EPs In Aquatic Environments

1.1.1. Municipal Wastewater

Municipal wastewater poses a serious risk to water systems as it is formed by complex mixtures of a large set of unknown compounds that could be dangerous for aquatic animals and humans, becoming a worldwide issue of environmental concern (16). Over time, it was verified that most wastewater treatment plants (WWTPs) were not built with the purpose of removing EPs from wastewater flows and such compounds end up passing through WWTPs due to their persistence, partial degradation, hydrophilicity and continuous introduction into drinking water chains of the hydrological cycle (7,17). Current physical-chemical analysis strategies that assess wastewater compliance can only detect a limited amount of some chemicals, making them ineffective in assessing the toxic and interactive effects of chemicals that coexist in these locations (18). As such, it is very important to proceed with the identification of these contaminants, particularly ill-informed or uninformed EPs, in order to develop new pre-treatment techniques or measures to optimize existing treatments (19).

The rate of use/consumption of substances considered as EPs are one of the main parameters that determine the quantity of these compounds that ends up reaching WWTPs. PPCPs have been found across 25 different countries and in Spain around 36 emerging chemical compounds derived from PPCPs, including 33 fragrances, have been detected in WWTPs (20,21). Pharmaceuticals are another class of EPs often found in wastewater due to the fact as they are mostly ingested orally and metabolized in the human body, they end up being excreted through urine and faeces and the rate of excretion plays an important role in introducing these compounds into water (22). The concentrations of micropollutants in conventional WWTPs in different countries range between 0.1 and 10 μg/L but for some pharmaceuticals (acetaminophen, caffeine, ibuprofen, naproxen and salicylic acid), one biocide (triclosan), one surfactant (nonylphenol) and one industrial chemical (DEHP) the values were relatively higher. The compounds with concentrations higher than 10μg/L in WWTP influents were ibuprofen, atenolol, caffeine and nonylphenol (7). The high levels of caffeine found can be explained by the high consumption of coffee, teas and soft drinks and ibuprofen by the easy access to this type of compound in pharmacies.

The average measured concentrations of neuroactive pharmaceuticals in WWTPs effluents have been reported at between 0.001 and 0.33μg/L for Selective Serotonin Reuptake Inhibitors (SSRIs) (e.g., fluoxetine, sertraline and citalopram), Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs) (venlafaxine), Tricyclic antidepressants (TCAs) (amitriptyline) and benzodiazepines (diazepam) (23,24). Antibiotics have been detected in concentrations ranging from 3.67 to 53.05 μg/L in wastewater effluents from hospitals (25). A study carried out in Greece showed that EDCs presented the highest risk of all emerging pollutants in wastewater with values of 17.40 μg/L (26).

1.1.2. Surface Water

The study and understanding of biogeochemical cycles are very important in the identification of environmental impacts caused by the introduction of potentially dangerous substances in the ecosystem, essentially by anthropogenic input (27). There are different constituents of a biogeochemical cycle, including the aquatic ecosystem, where different types of chemicals are introduced, and which can be converted into toxic or innoxious materials for these environments (28). The discharge of WWTPs effluents is one of the main causes that lead to the presence of EPs in surface waters compared to other types of sources (29) and, these compounds are exposed to the most diverse sources of natural attenuation since dilution in surface water, sorption onto suspended solids and sediments, direct/indirect photolysis and aerobic biodegradation (30).

In the case of pharmaceutical compounds, it is known that due to the dilution of river waters, they end up being found at lower levels, about an order of magnitude lower, compared to those found in WWTPs effluents (31). In general, the levels of EPs in surface waters change according to the seasons, with a steady increase in EP levels during dry weather and a notable reduction in wet weather. However, samples taken during the summer revealed that, in the case of pharmaceuticals, the levels were lower than those found in the winter, probably due to i) increased biodegradation of pharmaceuticals at higher temperature and ii) greater dilution when the summer is wetter. The rainy season, by itself, does not always cause a reduction in the levels of EPs released into the waters (32).

There is a large number of articles that focusing on the study of contamination of the waters of lakes and rivers have found that the levels of contamination of these waters by different EPs are in the order of ng/L, especially for pharmaceuticals and PPCPs (33). As previously mentioned, PPCPs are one of the main groups of EPs found in the aquatic environment, also being the most common in surface waters with an occurrence recorded in at least 30 countries with concentrations between 0.029-7.8x10^6ng/L, showing the anthropogenic contribution in introduction of these compounds into the waters (21). After analyzing surface water samples from different countries, it was found that the EPs most found are NSAIDs, carbamazepine, sulfamethoxazole and triclosan with the highest concentrations recorded in Costa Rica because of discharge of hospital effluents and other highly contaminated waters (34).

On a Chinese lake (Luoma Lake) were reported 6 EDCs with concentrations between 93.68 – 1857ng/L (35) and in France were reported 13 EDCs with a concentration of 286ng/L for bisphenol A (36). Basically, the pollution of EPs in the natural water bodies of the densely populated regions are more critical because of the substantial usage of these products by the large population and, population aging contributes to the high occurrence levels of pharmaceuticals.

1.1.3. Groundwater

The contamination of groundwater by EPs is lower than the surface water and is mainly due to various anthropogenic activities including the improper discharge of industrial and municipal wastewater, application of recycled wastewater for irrigation purposes, landfill leachate, interaction between groundwater and surface water, mobilization facilitated by subsurface geochemistry, and seasonal changes (37,38). The biggest source

of groundwater contamination is soil through the introduction of pesticides and similar compounds, but emerging pollutants can also be introduced via artificial recharge using reclaimed water and bank filtration (39).

The anthropogenic activities previously mentioned have an important role in relation to the governance of the flow and underground transport of EPs, eventually causing a decrease in the amount of these pollutants from the sources to the groundwater. The physical and chemical properties of these compounds are important in the processes of transferring these compounds to water (40). For different European countries and the United States of America, it was found that most of the detected EPs (mostly pharmaceuticals) were found in concentrations below 100ng/L in groundwater, establishing a good relationship between the levels of these compounds in these waters with the occurrence of other synthetic contaminants and land application (41).

In karst waters, and due to the dilution caused by precipitation, PPCPs were detected in the order of 4.6ng/L, with the antimicrobial triclocarban and cardiovascular drug GF being the most frequently detected (42). Interestingly, it was found that the levels of PPCPs in riverside groundwater in some places in China are higher during the dry season, with bezafibrate having an average concentration of 125ng/L (43). Regarding the class of pesticides, several studies have found that the average levels of these substances in groundwater can reach 478000ng/L in the European continent. This may be due to the fact that the climate and fertile soils in Europe originate high quality products, making the European Union one of the main producers and exporters of agricultural products in the world (44).

1.1.4. Drinking Water

The quality and safety of drinking water plays one of the most crucial roles in establishing the quality of life in today's society. As such, problems with the existence of pollutants in the production and distribution of drinking water constitute an enormous impact on public health (45). The existing literature has revealed that the existence of EPs in the final waters of drinking water treatments occurs at levels below the limits of detection or quantification of the methods used (32). Water sources and seasons are one of the main parameters that influence the presence of EPs in drinking water, in which samples collected in winter showed higher levels than in summer. Even today, there are several countries that continue to face serious problems with the provision of high quality drinking water to their inhabitants, with the treatment of drinking water playing a crucial role in the removal of EPs from drinking water, requiring an exhaustive analysis (46,47).

In Brazil, about 31 EPs and pesticides were detected during the rainy season and 27 during the dry season, in a water reservoir in concentrations between 0.6–4700ng/L. After an analysis of these waters, it was found that these compounds at the levels presented did not pose a risk after the treatment of raw water in drinking water, but some of the EPs were dangerous for the biota that was present in the reservoir (5). As mentioned for groundwater, some countries in Europe and the United States reported EP levels in drinking water below 100ng/L with the exception of caffeine and carbamazepine (> 600ng/L), in which the high concentrations of this anticonvulsant can be explained for his great persistence (48). In 2011, 25 PPCPs were found in drinking water with salicylic acid being the most detected, while carbamazepine and atenolol were detected in more than 30% of contaminated water sources but in concentrations less than or equal to 2ng/L (37).

In general, the levels of EPs in drinking water are not as high as in other waters, allowing countries to mitigate the adverse impacts of pollutants in these systems. However, it is necessary to take into account that there are other EP transformation compounds and by-products that can cause risks and adverse effects to public health and ecosystems, needing to be monitored, and drinking water has to be extremely examined in order to be secure your consumption.

1.2. Transport, Transformation And Bioaccumulation Of EPs On The Environment

Soils are a very suitable place for the retention of certain emerging pollutants in high concentrations. Regarding sediments, these are a crucial source of contaminants that are discharged into lakes and rivers, groundwater, and estuaries. Several studies have been using the retention capacity of EPs in sediments and soils to assess historical discharges that were sources of contamination, as well as breaks/transformation and other types of remobilization processes (49). Over the years, researchers have been focusing on the occurrence and detection of EPs in the environment, nevertheless it is known that these compounds have a great capacity for migration to water courses through different direct or indirect routes. Consequently, because of these migrations, there is a bioaccumulation of EPs along the food chain, causing health risks for the different living beings that constitute ecosystems (50). As such, the study of the transport and bioaccumulation of these contaminants has been the focus of major studies.

Some researchers have found that when sediments have low hydraulic conductivity, they create perched aquifer conditions that end up creating temporary effluent storage with spills. As such, they conjectured that processes such as tautomerization and methylation have significant roles in the transformation of EPs. The lack of guidelines and the leakage of pipelines (in the case of benzotriazoles) in wastewater, are pointed out as the most probable causes of the entry of pollutants into aquifers, creating a greater risk of contamination for groundwater that is later used as a source of potable water (51). Increasingly, attention is being paid to plastic debris on the coastlines as they can be considered passive samplers because they concentrate organic airborne contaminants through sorption mechanisms or specific interactions that can cause them to be transported to other systems such as different aquatic environments (from rivers to oceans). EPs are absorbed and moved from the coast to the oceans due to adsorption and interaction with microplastics affecting a large part of the marine habitat (52). Due to the continuous destruction of marine biodiversity by different causes, including the occurrence of EPs in the waters, a decrease in the productivity of aquatic organisms is expected.

The most common transformation methods in environmental and biological systems include chemical transformation (dissolution, exchange of surfactants, redox reactions, influence of other inorganic chemicals), physical transformation (aggregation and/or agglomeration, adsorption, deposition), biologically mediated transformation (distribution among organisms, bioturbation, ingestion-egestion dynamics related transformations) and interaction with macromolecules (hydrophobic/electrostatic interactions, ligand exchange, flocculation and hydrogen bonding) (53).

A mass flow assessment, in 2018, revealed that the Ciliwung River in Jacarta carries between 5 to 17 tons of pollutants, including EPs, to the Java Sea (54). In the case of pharmaceuticals, even if they belong to the same therapeutic group, their biodegradation along water courses shows great variability. In the case of NSAIDs, it

was found that diclofenac shows low biodegradation while ibuprofen and ketoprofen are biodegraded to a much greater extent (55). It is very important to understand the behavior of the different contaminants at the environmental level and, as such, it becomes crucial to underline the influence of chemical processes in these situations. One of the strategies currently used to replace the ozonation technique is the use of hydrogen peroxide and light-assisted systems to improve the action of ozone on pollutants (56). In the case of some classes of endocrine disruptors, treatment by photocatalytic oxidation has shown very effective results (57). Regarding the physical processes, the chitosan microspheres have been showing an enormous capacity in the elimination of EPs from the waters with a maximum capacity between 0.99 – 1.27 mmol per gram of chitosan spheres (58). The methods of ultrafiltration, nanofiltration and granular activated carbon are able to remove contaminants from water under low DOC conditions (59). In addition to the physical and chemical processes, there is also biological transformation that involves, for example, the use of *Trametes versicolor* and *Myceliophthora thermophila* that effectively eliminate mixtures of EDCs in water and also in wastewater (60). In addition to these, it was found that the enzyme bisphenol dioxygenase from organism *Paraburkholderia xenovorans* LB400 can metabolize the drug carbamazepine about 40 times more than the methods previously used for this purpose (61). It is accepted by the scientific community that complex natural processes can be used to remove EPs in the wastewater located, since acesulfame can be removed by about 90% through intensified wetland systems using a tertiary treatment sand filter (62).

After having exposed the zebrafish to different mixtures of EPs that are detected more frequently (caffeine, imidacloprid, 2-hydroxy atrazine, tebuthiuron, atrazine, and bisphenol A) with three different levels of exposure, it has been found that there were changes in the expression of target genes, mainly the induction of the expression of an estrogen exposure marker *(cyp19a1b)*. In contrast, studies carried out in rivers found that there were no indications of harmful effects on zebrafish due to low levels of EPs, but intensive and frequent agricultural activities near water courses can lead to unexpected peaks of EPs pollution and affect negatively the habitat of organisms (63). On the other hand, emerging pollutants are often detected in aquaculture products, with pesticides found in fish samples in China. In addition, in Liaoning and Mongolia, atrazine and linuron were found to be in higher concentrations than other pesticides in fish samples. This evidence draws attention to the consumption of aquatic products that should receive more attention due to the potential risk they entail (64). It has been verified over the years that EPs accumulate in organisms and, as such, carry ecological risks that can affect human health through the food chain. On the other hand, very little is known about the transformation mechanisms and respective metabolites of EPs in organisms, and it is expected that more research will be done in this area in the future.

1.3. Antidepressants As Emerging Pollutants

Antidepressants are pharmaceutical active compounds and are considered emerging pollutants due to their omnipresence at trace levels in the environment. Their problem is related to lack of knowledge of their impact on environment, including aquatic environment and human health. Several studies have demonstrated the existence of human drugs in untreated and treated drinking water, groundwater, sewage from hospitals and surface water (12). The presence of these residues in surface water comes mainly from the excretion of

pharmaceutical products by patients after therapy, incomplete disposal at WWTPs and direct disposal of unused and expired medicines. Antidepressants are excreted mostly in the form of metabolites (biologically active or not) but part of the administered dose remains unchanged. Thus, the percentage of parent compound present in the urine varies according to the pharmaceutical, e.g. citalopram is excreted unchanged in approximately 12%–20%, whereas fluoxetine in 10%, paroxetine 3%, venlafaxine 1–10% and sertraline 0.2% (65).

Another important aspect is due to a deconjugation phenomenon which is very likely to occur in WWTPs. The most relevant consequence of this deconjugation process is the increase of biologically active compounds in influents and, consequently, in effluents and sludge. The hypothesis on the deconjugation of pharmaceutical conjugates is supported by investigations which concluded that the concentration of parent compound found in WWTPs effluents is considerable higher than the concentration of the conjugated form, contradicting the excretion patterns and underlining the possibility of, at least, partial cleavage of the conjugates (66). Even in low concentrations, antidepressants may cause several effects on aquatic environment as a result of disturbing homeostasis throughout the central and peripheral nervous system, both in vertebrates and invertebrates, and by modifying the regulation of neurotransmitters, such as serotonin, norepinephrine and dopamine. Psychiatric drugs are among the most toxic compounds to aquatic organisms, and there are several studies focused on the biological activity of SSRIs, particularly fluoxetine (67).

Wastewater treatment is important to remove potential toxic compounds but was not originally designed to eliminate xenobiotics, allowing the entrance of pharmaceuticals and their metabolites in the environment. Thus, several studies have shown that most of the methods commonly used in WWTPs to remove antidepressants are ineffective, leading to the gradual increase in the concentration of these substances in water. As such, WWTPs have been described as one of the main routes of entrance of pharmaceuticals in the aquatic environment (68). The concentration of these compounds in the aquatic environment can be affected by i) the partitioning to the sediments and the particulate matter; ii) flow rate of the receiving surface water, which will determine their dilution factor; iii) biodegradation; iv) photodegradation; v) other transformation reactions (e.g. abiotic mechanisms); vi) and uptake by biota. There has been a problem of increased concern due to the increased detection of pharmacological agents in the environment, as they may pose a threat not only to human health but also to wildlife via, for instance, drinking water or food chain (69). Due to these problems, European strategy for the water policy, implemented by the Directive 2013/39/EU, concludes that monitoring of pharmaceuticals and other emerging pollutants in surface water is crucial for obtaining a larger set of data on the most affected surface waters, to address the risk posed by those compounds, allowing the implementation of future measures that will help to prevent and control the risks posed by those substances (70).

The presence of pharmaceutical products in aquatic environments is well known in several European countries, however in Portugal there is still a lack of knowledge about its occurrence, destination and environmental risk. In fact, only a few studies have been published, focusing mainly on the rivers of northern Portugal and the contribution of WWTPs and hospital effluents for the release of pharmaceuticals into the Portuguese environment has been also described (71).

A study carried out in the WWTPs of Olhalvas and Coimbrão, shows that removal efficiency of pharmaceuticals varied from not eliminated for psychiatric drugs to around 98% for NSAIDs/analgesics. The most frequently detected pharmaceuticals are the NSAIDs/analgesics ibuprofen, acetaminophen, ketoprofen, and the metabolite salicylic acid together with the psychiatric drugs fluoxetine and carbamazepine. The presence of antidepressants fluoxetine, sertraline and venlafaxine were detected along Lis river but only fluoxetine was quantified (2.01–10.0ng/L). In two German rivers were reported levels of venlafaxine up to 180ng/L (68). In Beijing municipal WWTP influent were detected antidepressants with a concentration range of 89ng/L for chlorimipramine, 67–261ng/L for citalopram, 0–17ng/L for fluoxetine, 29–106ng/L for sertraline, and 435ng/L for fluvoxamine. On the other hand, the concentrations of paroxetine, citalopram, fluoxetine, and sertraline ranged from <MQL to 53ng/L in a Montreal WWTP influent in Canada and a fluoxetine concentration of 177ng/L was measured in a Las Vegas WWTP influent (72).

Chapter 2 . Antidepressant Drugs

2. Background

Depression is considered a chronic, severe, and often life-threatening illness with significant impairment in psychological, occupational, and social functioning. This condition is characterized by a variety of symptoms that include changes in mood, sleep, appetite, cognition, motivation as well as a reduced ability to experience pleasure or thoughts of suicide. Major depressive disorder is operationally, but not biologically, characterized as having a sufficient number of these symptoms, and substantial distress or impairment in functioning, for at least two weeks (73). The prevalence rates for depression are estimated to be around 3.2% in patients without comorbid physical illnesses and 9.3% – 23.0% in patients with chronic conditions. It is considered the 4th cause of disability around the world and is estimated to be the 2nd leading cause of disability by this year, affecting about 300 million people regardless of gender, ethnicity, geographical location, and socioeconomic status (74).

In order to treat this type of condition, psychopharmaceuticals are known to be one of the most prescribed drugs worldwide, making the problem of over-prescription one of the main topics discussed in the field of medicine and psychiatry. The problem of over-prescription is related to the bad diagnosis of the disease, that is, several professionals prescribe this type of treatment without referring patients to a diagnosis by a specialist (psychiatrist or psychologist). Besides that, antidepressants are used in people with some depressive symptoms and not only in those with at least 5 symptoms or more severe symptoms (75). In addition, there has been an increase in the use and misuse of this type of drugs in practically all industrialized nations worldwide. For example, France is one of the countries that has one of the highest consumption rates globally, in which about a quarter of the population uses at least one psychotropic agent, revealing one of the international problems that could lead to serious ramifications regarding costs medical systems worldwide, as well as patient safety (76). As such, this problem should be of interest not only to health professionals but also to the government, health authorities and lay people.

One of the greatest examples of overprescribing psychiatric drugs is antidepressants due to their widespread use. This class of drugs is dispensed more quickly than the other classes of existing psychotropic drugs, presenting an almost two-fold increase since the beginning of this century (77) and according to OECD indicators, the consumption of these pharmaceutical substances doubled between 2000 and 2017, which may be an indicator of the existence of more effective diagnostic methods for the recognition of depression, as well as the availability of different therapies, guidelines and changes in provider and patient behavior (78,79). The consumption of these substances depends on how depression is diagnosed, treated and in its prevalence in each country with the highest consumption of antidepressants recorded, in 2018, in Iceland, more than twice the OECD average, followed by Canada, Australia and the United Kingdom (79). Several studies have revealed that antidepressants may have minimal or no efficacy in patients with mild to moderate levels of the disease, which is the one that most patients have (80–82). Interestingly, over the last decade the variety of antidepressant drugs has not undergone major changes, so it is thought that in the near future they will not undergo a major revolution (77). Unexpectedly, it was found that there is a poor alignment between the prescription of antidepressant drugs and the prevalence of psychological disorders. Thus, it was observed that the huge increase in the use of antidepressants is not closely related to an increase in the diagnosis of depression or other type of psychological disorders for which they are used (83). In view of this situation, some

researchers suggest that there are different factors behind the over-prescription, in addition to the patient's health, and it is expected that the demographic group most affected by the overuse of psychotropic drugs is the elderly, particularly those over 80 years of age, presenting a greater vulnerability to adverse reactions to drugs (84).

Among their characteristics, antidepressants can pass through cell membranes and are relatively persistent so as not to be inactivated before having the desired therapeutic effect. Regarding the particular case of drugs related to the nervous system, and in addition to the mentioned intrinsic properties, they have great relevance in the regulation of behavior, having the ability to directly affect the central nervous system (CNS) and interfere with neuroendocrine signaling (85,86). This chapter will discuss the different treatment approaches at the level of antidepressants, neurochemical circuits involved and the effects of waterborne antidepressants on non-target animals living in surface waters.

2.1. Monoamine Hypothesis And Monoamine Based Pharmocological Treatment Aproaches

2.1.1. Monoamine Hypothesis

The amine hypothesis of depression was formulated in the 1950s and suggest that depression is correlated with a deficiency in the transmission within the monoamine systems, i.e., serotonin (5-HT), noradrenaline (NE), and dopamine (DA) (87). Two primary lines of evidence led to the development of this theory: 1) the effects of reserpine on serotonin and catecholamines and 2) the pharmacological mechanisms of action of antidepressant drugs. In the mid-50s it was found that reserpine precipitated depression in some of the patients and the depression produced by this pharmaceutical was reversed after the treatment was completed and following either rest or electric shock therapy (88). Reserpine was found to inhibit vesicular monoamine transporter, and consequently, depletes brain monoamines (serotonin and catecholamines), which provided evidence for the role of 5-HT, NE, and DA in depression (89,90). In addition, the second line of evidence was based on the pharmacological mechanisms underlying the action of monoamine oxidase inhibitors (MAOIs) and TCAs. For example, antidepressant drugs primarily target the monoamine neurotransmitters, such 5-HT, DA and NE, in an attempt to increase the concentrations of these at the synaptic level in order to active postsynaptic receptors. A few decades later, the SSRIs offered extra support for the monoamine hypothesis with some recent studies providing evidence that the monoamine hypothesis for major depression needs to be reviewed because the causes of depression are not so simple as depleted concentrations of serotonin, norepinephrine, and dopamine. Corroboration what was mentioned above is the fact that only a deficit in monoamine levels in healthy subjects does not lead to depressive symptoms (91). Therefore, the monoaminergic hypothesis already reviewed suggests that the decrease in monoamines may have a more modulating role that ends up influencing other types of neurobiological systems, such as other systems of neurotransmitters and neuropeptides and intracellular signaling, or should be present in the context of stressors (92).

2.1.2. Monoamine Oxidase Inhibitors

The antidepressant action of MAOIs may be related to alterations in the characteristics of neuroreceptors, changes that occur both in the amount and sensitivity of these, rather than with the blockade of the synaptic reuptake of the neurotransmitters itself. Drugs in this class inhibit the release of the enzyme monoamine oxidase (93). If the enzyme is not released, it is unable to breakdown biogenic amines (5-HT, DA, NE and epinephrine) and sympathomimetic amines (e.g. tyramine, benzylamine, etc) within the presynaptic cleft, thereby allowing more of those transmitters to be available in the presynaptic terminal and are easily available for release when action potentials reach the nerve terminal, which improves mood. Also affect both the histaminergic and adrenergic systems (94). There are two isoenzymes, MAO_A and MAO_B, and throughout the body its distribution varies. MAO_A is mainly responsible for the enzyme activity for the deaminate of 5-HT, NE, epinephrine, and melatonin. The breakdown of benzylamine and phenethylamine is responsibility of MAO_B (93,95). Currently the use of MAOIs is very rare, or else they are the last antidepressants to be used, owing to serious and potentially fatal food and drug interactions that can lead to hypertensive crisis (96). These substances cause side effects like orthostatic hypotension, dizziness, drowsiness, insomnia, and nausea (97).

2.1.3. Tricyclic Antidepressants

TCAs were classified based on the three-benzene ring molecular core, essentially, because the mechanism of action was unknown at the time of discovery. Consequently, the classification of TCAs diverges from other classes of antidepressant drugs, which are classified based on their mechanism of action. These class of antidepressants have a diverse pharmacological profile with remarkable pharmacological action at two reuptake transporters and three receptor proteins: inhibiting presynaptic norepinephrine reuptake transporters; inhibiting presynaptic serotonin reuptake transporters; blocking postsynaptic adrenergic α_1 and α_2 receptors; blocking postsynaptic muscarinic receptors and blocking postsynaptic histamine H_1 receptors (98,99).

The common mechanism of action of TCAs results from the inhibitions of norepinephrine and serotonin reuptake at the transport proteins causing an increase in concentrations of NE and 5-HT in the synaptic cleft. This allows for more of the neurotransmitters to be available at the receptor sites before reentering the neuron causing changes in physiological behavior of neuroreceptors having a positive effect on mood. The selectivity for NE or 5-HT transporters varies with compounds, but, as most TCAs are more selective for the NE transporter over 5-HT transporter (98,100). Like MAOIs, TCAs are not being used as options for the first line of treatment. This class of antidepressants, among several problems, can have a negative impact on the cardiac level and even cause death if taken in high doses. Furthermore, patients should be evaluated for suicidal tendencies during treatment (101). However, these drugs can be used in other types of therapies such as pain associated with fibromyalgia and diabetic neuropathy (102). The antagonism of adrenergic, muscarinic, and histaminergic receptors causes mainly the side effects of dizziness, memory impairments, and drowsiness, respectively (103).

2.1.4. Selective Serotonin Reuptake Inhibitors

SSRIs have been developed from TCAs to decrease affinity for adrenergic α_1, α_2, and β, histamine H_1, muscarinic, and dopamine D_2 postsynaptic receptors, increasing the affinity for inhibiting 5-HT over NE at their transporter proteins. Some SSRIs do not stimulate the release of 5-HT or NE presynaptically and may not have a direct action at postsynaptic 5-HT receptors, as such 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2c} (104–106). As a result of the increased activity of postsynaptic serotonin receptors by SSRIs, there is an increase in serotonin concentrations in the synaptic cleft due to inhibition of reuptake, rather than direct binding to post-synaptic receptors (107). In order to treat depression, SSRIs are among the most widely used drugs and are considered first-line treatment. They are generally well tolerated but are not devoid of adverse effects and people experiencing these effects from one SSRI may benefit by switching to another drug in this class (101). Overall, this class of antidepressants is safer than MAOIs and TCAs, has fewer adverse effects and is less likely to end in overdose death. These and the other reuptake inhibitors have the advantage of selectively targeting the neurotransmitters commonly associated with depression (5-HT, NE, and DA) (108). Among the most common side effects associated with SSRIs consume are nausea, insomnia, sexual dysfunction (94), anxiety, panic attacks, irritability, aggressiveness, psychomotor restlessness, hypomania and mania (109,110).

2.1.5. Serotonin And Noradrenaline Reuptake Inhibitors, Norepinephrine–Dopamine Reuptake Inhibitors, Serotonin Antagonist And Reuptake Inhibitors

SNRIs includes Venlafaxine, Duloxetine and Milnacipran and, the mechanism of action is based on the inhibition of the NE and 5-HT reuptake pump and, to a lesser extent, DA (111). Milnacipran has a preference for inhibition of NE reuptake, but also inhibits the serotonergic and noradrenergic reuptake, in addition to blocking the NMDA receptor (112). On the other hand, venlafaxine acts at the level of inhibition of 5-HT reuptake (at low dose) and NE reuptake blockade increases with increasing drug doses (113). Unlike TCAs, SNRIs have minimal or no pharmacological action at adrenergic (α_1, α_2, and β), histamine (H_1), muscarinic, dopamine, or postsynaptic serotonin receptors (114). Some researchers suggest that SNRIs could be more effective for the treatment of depression as compared to SSRIs; however, these differences are relatively small (115).

NDRIs act as a reuptake inhibitor for NE and DA by blocking the action of the DA and NE transporters. As a result, there is an extracellular increase in the concentrations of these two neurotransmitters and, consequently, an increase in dopaminergic and adrenergic transmission (116). SSARIs act by antagonizing serotonin receptors such as 5-HT_{2A} and inhibiting the reuptake of serotonin, norepinephrine, and/or dopamine. Additionally, most also antagonize α_1-adrenergic receptors (117).

It was quickly realized that there were quite different time scales between the beginning of the neurochemical and therapeutic effects of antidepressants, with potentiation of monoaminergic function occurring a few hours after administration and clinical improvement only a few days or weeks later (118). The most recent approaches in the area of antidepressants focus on the neurobiological processes that can be responsible for this delay, so that drugs can be found with a faster action. Current research has concentrated on

finding non-monoaminergic based receptor targets for treatment-resistant depression, particularly, the glutamatergic system has become a focal point for drug development research.

2.2. Antidepressants And Brain Neurochemistry

2.2.1. Immediate Presynaptic Effects of Antidepressants

Most of neurotransmitters are stored in vesicles at the nerve ending and are released during neurotransmission – Figure 1. An influx of calcium ions is caused by the propagation of electrical impulses along the nerve to the nerve ending, and this influx ends in the liberation of the neurotransmitter and the initiation of chemical transmission along the synapse to the next nerve cell. After diffusing across the synapse neurotransmitters interact with a very specialized protein, known as receptor, on the outside surface of the postsynaptic cell. As a result of a neurotransmitter-receptor-effector complex a biologic change occurs within the cell (119). Feedback mechanisms are the processes by which neurons regulate their own activity, and this requires the involvement of existing receptors at the nerve endings (autoreceptors). For example, α_2-adrenergic receptor is an autoreceptor located on noradrenergic nerve endings and when is stimulated inhibits the release of NE, allowing the regulation of the amount of neurotransmitter in the synapse. NE, DA and 5-HT are inactivated at the synapse, in part because, they are taken back into the nerve endings from which they were released (120).

The blockade of NE and 5-HT in the presynaptic nerve ending by antidepressants potentiates neurotransmission involving these compounds by increasing the quantity of free neurotransmitter in the synapse. Also, the inhibition of MAO in the nerve ending potentiates neurotransmission at certain synapses by avoiding degradation of catecholamines and 5-HT by this enzyme. Besides that, antidepressants can cause one important effect after a treatment called antagonism of many different receptors. This effect can cause a reduction in transmission for some neurotransmitter systems (121–123).

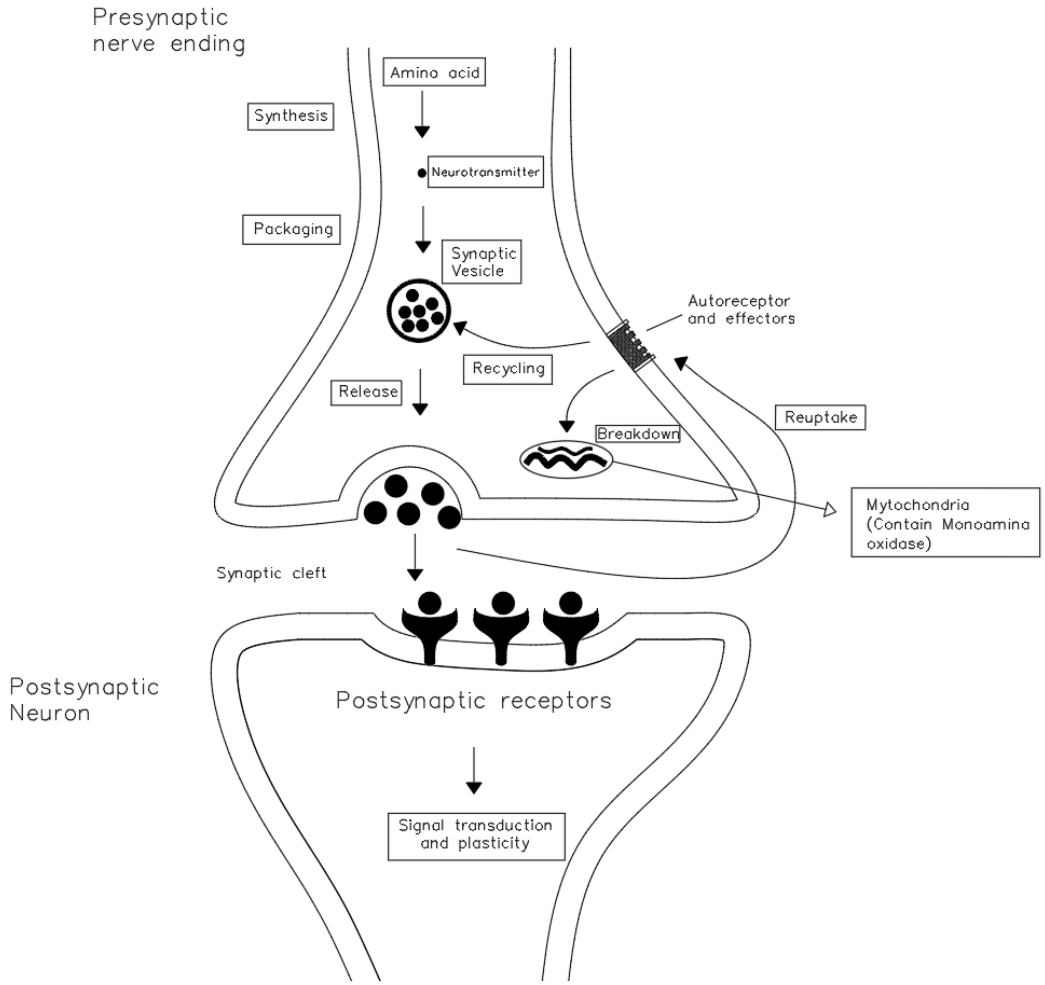

Figure 1. Diagram showing the general process of synaptic transmission. The arrival of the nerve impulse at the presynaptic terminal stimulates the release of neurotransmitter into the synaptic cleft. The binding of the neurotransmitter to receptors on the postsynaptic membrane stimulates the regeneration of the action potential in the postsynaptic neuron.

2.2.2. Postsynaptic Effects Of Antidepressants With Long-Term Treatment

A rodents clinical study showed that a long-term treatment with antidepressants caused loss of sensitivity of NE stimulated synthesis of cyclic adenosine monophosphate in slices from limbic forebrain of treated animals. This indicated that this down-regulation is selective for β_1-adrenoreceptor because β-adrenoreceptor down-regulation accompanies this desensitization for most antidepressants (124,125). Posteriorly, it was found that the postsynaptic effect of antidepressants was the result of actions at the presynaptic nerve ending and eliminating the presynaptic nerve endings researchers showed thar these drugs lost their postsynaptic effects (126). Antidepressants from many different classes can increase the level of catecholamines at postsynaptic receptor sites by inhibiting reuptake or preventing degradation of these biogenic amines by MAO and, as a result, this could lead to loss of sensitivity of the presynaptic autoreceptors. Consequently, it may increase de release of NE. The mechanism used to desensitize and down-regulate receptors is to increase the level of an agonist at its receptor site for prolonged periods (126,127). The loss of sensitivity hypothesis assumes that some catecholamine receptors are supersensitive in patients with depression and with antidepressant treatment the level of sensitivity would return to normal (128).

2.2.3. Neurochemical Theories

After administration of antidepressants, there is a time difference between the increase in monoamines and the observation of clinical changes in patients. This has led some researchers to assume that there are neurobiological adaptive changes that correlate over time with the start of the therapeutic response and that they may represent a more direct target for antidepressants than the initial action of these drugs for the reuptake of NE and 5-HT blockade. The development of some techniques, such as the ligand-receptor binding, allowed more targeted studies to be carried out on the effects of antidepressants at the level of monoamine receptor populations. In an initial phase, these investigations focused only on postsynaptic β-adrenoreceptors that were down-regulated through treatments with both TCAs and MAOIs (129).

However, it turned out that the theory of decreasing the activity of the β-adrenoreceptor, with the use of antagonists, which seemed to be a useful strategy was implausible, warning that neuroadaptive changes may represent homoeostatic mechanisms by which the brain of a healthy animal attempts to regulate monoamine neurotransmission in the presence of a monoamine enhancing drug (130). With the development of SSRIs, attention has turned to the role of $5\text{-}HT_{1A}$ autoreceptors that act at the level of inhibition of serotonin release from terminal nerves and, the continuous treatment with this class of drugs decreases the sensitivity of $5\text{-}HT_{1A}$ autoreceptors in animals and human beings. With this information it was suggested that the delay that occurred in the beginning of the therapeutic action of the SSRIs could be representative of the time necessary for the desensitization of the autoreceptors, ending up in a greater availability of neurotransmitter in the synapse (131).

As such, through the combination of SSRIs with other drugs that selectively block $5\text{-}HT_{1A}$ autoreceptors, mechanistically it would be possible to accelerate the initiation of the therapeutic action of SSRIs, however, from a clinical point of view this approach has not yet proved very useful (132).

2.2.4. Neuroplasticity Theories

As the molecular and cellular pathways that regulate neuronal function are increasingly clarified, the studies behind the neurotransmitter monoamine have been focusing on gene expression, intracellular signaling cascades and protein translation as central points for antidepressants action. There are several complex mechanisms that mediate neuroplasticity, for example postsynaptic Ca^{2+} signaling, trafficking of glutamate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) receptor subunits, regulation of presynaptic mechanisms of neurotransmitter and increased number and function of synapses. Studies suggests that synaptic plasticity mechanisms are affected by chronic stress and that antidepressant treatments reverse or oppose these effects (133).

2.2.5. Chronic Administration Of Antidepressants

Chronic administration of SSRIs and NRIs can increase synaptic plasticity and block synaptic deficits caused by stress (134,135). The actions of these two classes of drugs on synapse number are subtle and delayed possible because of the modulatory actions of NE and 5-HT neurotransmitter systems. Tests in rodent models showed that chronic administration of fluoxetine reinstates ocular dominance neuroplasticity even in adult

rodents and reinforce fear extinction training by causing fear circuitry to convert to a more immature and plastic state (136,137).

2.2.6. Intracelullar And Morphological Changes: Stress and Depression

It is known that chronic stress significantly alters neuronal circuits in the brain including disruption of intracellular signaling and the quantity and function of synapses. Rodent studies indicate synaptic loss in cortical and limbic areas associated with depression, especially the prefrontal cortex and hippocampus that are regions that control emotion, mood, and cognition in response to chronic psychological or physical stress (138,139). Besides that, a decrease in the formation of new neurons in the adult hippocampus was observed in response to stress (140). In prefrontal cortex and hippocampus chronic stress produces hypertrophy of neurons in the nucleus accumbens and amygdala. These effects could lead to disruption of behaviors that are regulated by these regions, including motivation, reward, and emotion (141,142).

At the molecular level, it is known that chronic stress induces changes in glutamate, transcription factors, intracellular signaling and gene expression, suggesting that stress increases extracellular glutamate and, consequently lead do excitotoxic damage (143). Brain-derived neurotrophic factor (BDNF) plays a crucial role in formation, guidance, and survival of neurons during development and in synaptic plasticity and survival in the adult brain - Figure 2. Chronic stress causes a decrease in the levels of this factor and in signaling pathways in rodents and post-mortem brains of individuals with depression (144). Studies indicates that mice with a single nucleotide polymorphism of BDNF (Val66Met) showed a decreased in the amount of synapses in the hippocampus and medial prefrontal cortex (145). Thus, an interruption in BDNF signaling contributes to the synaptic and behavioral deficits related to stress, verifying how exposure to stress and even several genetic factors alter the risk of suffering from depression. Behavioral actions of typical antidepressants in animal models are blocked by the deletion of BDNF but the administration of an infusion of BDNF in the prefrontal cortex or even in the hippocampus is sufficient to cause antidepressant effects (146). Moreover, fluoxetine induces synaptic plasticity in the ocular dominance dependent on BDNF and BDNF administrations are enough to create these effects (136). Antidepressant treatment also rises downstream signaling, as well as the cAMP and Ca^{2+} that increase the expression of BDNF (147).

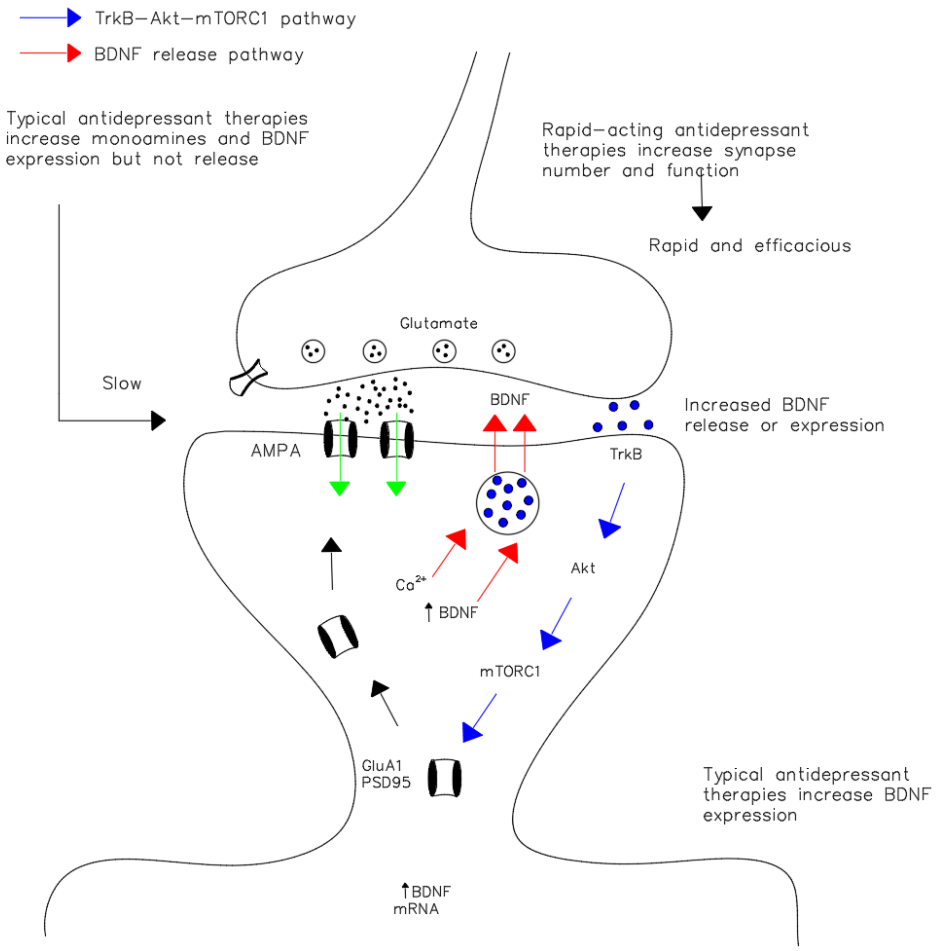

Figure 2. Simplified neurotrophic theory of antidepressant drug action. Stress and HPA axis: decreased BDNF signaling, decreased synaptic proteins, and decreased number and function of synapses. This leads to depression and relapse. HPA=hypothalamic–pituitary–adrenal; TrkB= Tropomyosin receptor kinase B; Akt=Protein Kinase B; mTORC1= mammalian target of rapamycin complex 1; BDNF= Brain-derived neurotrophic factor; AMPA= α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluA1=Ionotropic glutamate receptor; PSD95= postsynaptic density protein 95; mRNA=Messenger RNA.

2.3. Occurrence In Aquatic Environment And Bioaccumulation In Organisms

In order to determine the amount of these compounds in the environmental waters analytical methods have been validated, being the most used techniques based on chromatographic mechanisms (HPLC and Gas Chromatography) coupled to different types of detectors, such as mass spectrometric and spectrophotometric detectors (148). Antidepressants such fluoxetine and sertraline were detected in surface water and wastewater effluent, at levels up to 0.54µg/L and 0.929µg/L, and fluoxetine and its metabolite norfluoxetine were found in fish tissues in concentrations of 10µg/kg, signifying that these compounds have the capacity to bioaccumulate (149,150). Sertraline and norsertraline were also found in plasma, liver, and brain of fish living in an effluent-dominated stream in Matsuyama, Japan (151).

Regarding venlafaxine, it was found that its concentrations vary according to the regions. Measured concentrations of this drug were found at levels up 0.176–0.214µg/L in effluent, and 0.013–0.045µg/L in receiving streams at Canadian treatment plants (152). In Minnesota and Texas venlafaxine was measured at concentrations of 2.1 µg/L and 1.31µg/L, respectively (153). Venlafaxine has been described to be the most common antidepressant found in North American and European wastewater treatment plants and receiving

streams (149,154). A study of Melissa Schultz and colleagues, in 2010, shows that Venlafaxine was not detected in the brain tissue of the majority of wild whit shuckers *(Catostomus commersoni)*, but in animals that had detectable levels, the concentrations in brain tissue ranged from 0.1 to 1ng/g (154). Both venlafaxine and its metabolite (O-desmethylvenlafaxine), which is equally powerful to the parent compound in inhibiting 5-HT and NE reuptake, are consistently detected in municipal wastewater effluent (155). Therefore, these compounds are considered to be pseudo-persistent in fish, and it has been shown that venlafaxine is able to accumulate in the tissues of fish, including the brain, liver and muscles (23). Despite this information, the deposition of venlafaxine in embryos or its impact on the early development of fish has not yet been extensively studied. In turn, their deposition in the zygote can be a result of maternal transfer or absorption from the external environment in fishes. Nonetheless, venlafaxine deposition in the embryos may be an important route in affecting early developmental phenotypes, but this has not been tested before. A recent study has shown that although zebrafish embryos are able to degrade venlafaxine, trace amounts of this drug during embryogenesis are sufficient to affect brain development and behavior (156).

For citalopram, reported concentrations in wastewater effluent were 0.057μg/L, and 0.011μg/L in receiving water in Canada (152); in the USA concentrations were up to 0.07μg/L (154). Katerina Grabicova marked citalopram in *Hydropsyche sp.* and *E. octoculata* at a concentration of 3.6–4.2ng/g in a leech *(E. octoculata)* (23), but was not detected in the plasma of rainbow trout and guppies (157). These results show the need to introduce the monitoring of neuroactive drugs consumed by humans in organisms.

2.4. Impact Of Antidepressants On Aquatic Animals

Neuroactive drugs are considered one of the most ecotoxic drugs. Several studies developed have shown that these compounds, even at low levels, have the capacity to induce biological effects in aquatic organisms (fish and shellfish) as stress responses, inhibition of reproduction and physiological development and locomotion of fish and invertebrates (157–159) – see Table 2. Habitats under persistent exposure to pollutants often suffer a reduction in species richness and loss of community integrity (160). Fish are extremely sensitive to anthropogenic impacts and, because of that, they are becoming an useful toll as biomonitors in assessing the ecological status of aquatic environments (161,162). Consequently, fish in contaminated fields are regularly exposed to much higher concentrations or to different chemical forms than those typically found in the environment. In response to these stressors, these organisms undergo a series of biochemical and physiological changes, in an attempt to compensate for the challenges imposed and thus deal with stress (163,164). Thus, as fish are in the top of the food chain in aquatic environments is very important understand the effect of antidepressants in these animals (165).

As such, an increasing number of studies are reporting biological effects at lower concentrations but not at higher concentrations. It was observed that amphipod *E. marinus* had stronger phototaxis responses at concentrations of fluoxetine in the range of 10–100ng/L whereas no significant differences from the controls at higher concentrations of 1000ng/L (166). Studies on zebra mussels *(Dreissena polymorpha)* have shown that fluoxetine induces reproduction in males. This compound also has toxic effects on other tested species, causing changes comparable to those induced by other similar compounds, such as fluvoxamine and sertraline

(167). Fluoxetine was discovered to substantially impact mating behavior, such as nest building and defensive behavior, in male fathead minnow *(Pimephales promelas)* at lower concentrations as 1 μg/L. Males presented aggression, isolation, and repetitive behaviors at higher concentrations (168). Gulf toadfish *(Opsanus beta)* produced an increase in the number of aggressive behaviors in dominant individuals when treated with this drug (169). In the mosquito fish *(Gambusia affnis)*, exposure to fluoxetine induces changes in the reproductive behavior of males, with the exposed individuals less competitive in the search of the partner (170).

Fathead minnows were exposed over their complete life cycle to environmentally relevant concentrations (0.88 and 8.8μg/L) of the venlafaxine to study the influence on survival, development, and reproduction and, according to the results, this exposure at that levels produced no adverse effects in these organisms (171). A study of zebrafish embryos exposed to venlafaxine concentrations showed that there is a significant increase in embryo mortality and also revealed the appearance of multiple abnormalities, such as scoliosis and yolk sac edema in a percentage between 1.1% and 3.1% in embryos exposed to drug mixtures (including venlafaxine) (172). William Thompson, in 2017, reported that the exposure of embryos to this drug also induced an acceleration in early development as well as increased the hatching to 48 hours post fertilization (156). Furthermore, behavior was found to be affected by venlafaxine. Escape responses were delay in larval fathead minnows exposed to 5 μg/L venlafaxine over a period of 12 days (173). Some studies have shown that juvenile trout fish *(Oncorhynchus mykiss),* when exposed to venlafaxine, present changes in the response to stress and feeding behavior. This may include changes in brain function, as this antidepressant modulates the concentrations of NE, DA and 5-HT in the trout mesencephalon (174). Additionally, exposure to venlafaxine in *Pimephales promelas* resulted in changes in the expression of genes associated with neuronal action potentials, formation and growth of neuron sheaths, suggesting changes in brain function as a possible cause of altered stress, feeding and agonistic behaviors (175).

Another study conducted in zebrafish, but for the antidepressant amitriptyline, recorded a hatching rate of 50% at a concentration of 10μg/L. The results suggested that amitriptyline did not affect embryo hatchability, but significantly reduced incubation time. In zebrafish embryos exposed do amitriptyline occurred a clear increase of lipid peroxidation and oxidative stress (176). Also, an increase in lipid peroxidation as well changes in antioxidant enzyme activity were observed on early life stages of common carp *(Cyprinus carpio)* for TCAs at concentrations of 10, 100 and 500μg/L (177). Thus, it shows that antidepressants affect fish in different ways.

Table 2. Overview of antidepressants and main possible outcomes in organisms.

Classes [a]	Common Medications [a,b]	Mode of Action [a]	Main Outcomes [c]
Monoamine Oxidase Inhibitors		Inhibition the release of MAO	
Irreversible	Iproniazid, Tranylcypromine, Phenelzine, Isocarboxazid,	Both MAO-A and MAO-B	Hypolocomotion; Top dwelling; Decreased thigmotaxis and Increased heart rate
Reversible (Type A)	Moclobemide, Brofaromine	Specific for MAO-A	
Tricyclic Antidepressants	Amitriptyline, Clomipramine, Desipramine, Imipramine, Nortriptyline	Blockade of NA and 5-HT reuptake	Developmental Retardation; Morphological abnormalities; Pathological changes in brain, heart, and kidney; Body length; Decreased geotaxis
Selective Serotonin Reuptake Inhibitors	Citalopram, Escitalopram, Fluoxetine, Fluvoxamine, Paroxetine, Sertraline	Selective inhibition of 5-HT reuptake	Bioaccumulation in tissues; Alterations in swimming behavior; Alteration of camouflage; Impair of cryptic performance; Alterations in reproduction and growth; Decrease in oocyte and spermatozoan density
Noradrenaline Reuptake Inhibitors	Atomoxetine, Reboxetine	Selective inhibition of NA reuptake	Increase in climbing and diving; Decreased immobility
Serotonin-Noradrenaline Reuptake Inhibitors	Duloxetine, Desvenlafaxine, Venlafaxine	Inhibition of 5-HT and NA reuptake	Bioaccumulation in tissues (brain, liver, and muscles); Survival instincts affected; Embryo production reduced; Alterations in behavior; Alterations in neurotransmitters levels
Noradrenaline-Dopamine Reuptake Inhibitors	Bupropion	Inhibition of both NA and DA reuptake	Decrease food consumption; Improved attention; Delayed latency periods; Decrease escape velocity; Alterations in body length
Serotonin-2 Antagonist and reuptake inhibitors	Nefazodone, Trazodone	Blockade of 5-HT_2 receptors and inhibition of 5-HT reuptake	Activates a preference for light that trumps preference for color; Disturbed behavior
Noradrenaline and specific serotonergic antidepressant	Mirtazapine	Blockade of 5-HT_2 receptors and antagonist for 5-HT_3 and NA α2 receptors	Improve copulatory behavior; Bioaccumulation in tissues (liver, kidney, and brain)
Modern Antidepressants	Agomelatine	Stimulation of MT_1 and MT_2 receptors and blockade of 5-HT_{2C} receptors	Enhanced neurogenesis in ventral hippocampus; Significant decrease in duration of immobility, and an increase in the swimming time; Significant decreases in sperm count and motility.

[a] (178); [b] (179); [c] (180)

Abbreviations: MAO=Monoamine Oxidase Inhibitors; 5-HT=Serotonin; NA=Noradrenaline; DA=Dopamine; 5-HT_2= 5-hydroxytryptamine receptors (Gq/G11-protein coupled); 5-HT_3= 5-hydroxytryptamine receptors (Ligand-gated Na^+ and K^+ cation channel); MT=Melatonin Receptors

Chapter 3 . Visual System And Genetics

3. Background

The anatomy, histology, circuitry, and biochemistry of the eye are remarkably conserved among most classes of vertebrates. The observations that development of the eye also proceeds in very similar manners were not surprising (181). The zebrafish has become a crucial vertebrate model in developmental neuroscience because it is a valuable model for embryology, developmental biology, and genetic analysis. The similarities of its visual system to that of other vertebrates also make this animal an important model in the field of vision. The anatomical, physiological, and behavioral elements of zebrafish visual processing were explored in adult and in developing zebrafish. Its retinal anatomy continues to develop following hatching, giving an opportunity to connect the development of retinal structure with visual physiology and behavior. In addition, several genetic mutations have been developed which are used to examine the contributions of genetics to visual development and function (182).

Zebrafish have been proven a powerful forward genetic model for studying retinal development and associated diseases (183). The most of existing mutations were the product of numerous forward genetic screens to identify several genes essential to embryonic development, photoreceptor function, and survival (184,185). Those same features of high fecundity, external fertilization, relatively short generation time, and ease of the microinjection of one-cell stage embryos have facilitated the rapid adoption of gene-targeting technologies to test certain hypotheses of gene function in eye development (186,187), to generate reporter knock-ins, to generate accurate modifications through homology-directed repair, and to model human disease (188). Several genes required for the establishment of the retina anlage and its subsequent transition to optic vesicles have been identified. This chapter will provide an overview of the zebrafish's visual system, the genes most commonly addressed at the visual level and how emerging pollutants can induce changes in their expression.

3.1. Zebrafish Visual System And Development

3.1.1. Eye Anatomy

The zebrafish eye is very similar to that of fish species and appears to be emmetropic by 72 hours post-fertilization (hpf), which is the same time that extraocular muscles begin to be adult-like, and the optokinetic response is apparent (189). This organ must transmit both visible and ultraviolet wavelengths since the adult is responsive to ultraviolet wavelengths (190). The eye develops from no less than three different embryological tissues: i) neuroectoderm which provides rise to the neural retina, pigmented epithelium, optic stalk and ciliary margin, ii) skin ectoderm, which is induced to form the lens and subsequently the cornea and iii) head mesenchyme of neural crest cell origin that slightly forms connective tissue of the cornea and sclera (191). Thus, the eye begins with the optic primordia that emerges around 12 hpf (192). By 24 hpf the eyecups are properly developed (192), and around 30 hpf ganglion cells are found in a little area of the ventronasal retina (193–195). The retinal layers are visible across sections of the retina at 50 hpf (194). The highly conserved mechanisms of

development and function have provided significantly to our complete comprehension of visual processes and led to fast advances in understanding the basic mechanisms of diseases (191).

3.1.2. Retinal Anatomy

The zebrafish retina is constituted by seven main cell types derived from the neural ectoderm, six neurons and a single glial cell, the Müller cell – Figure 3 . The main classes of interneurons - the horizontal, bipolar and amacrine cells - can be subdivided into several subpopulations based on morphological, immunohistochemical and physiological profiles (196,197). The vertebrate retina consists of three nuclear layers and two plexiform layers. The outer nuclear layer (ONL) contains the cell bodies of the photoreceptors (rods and cones). The inner nuclear layer (INL) contains the cell bodies of the horizontal, bipolar and amacrine cells, and the ganglion cell layer contains the ganglion cell bodies. The plexiform layers are found between the nuclear layers and are where the synaptic connections between the retinal neurons take place. The outer plexiform layer (OPL) consists of the connections among photoreceptors, bipolar and horizontal cells, and the inner plexiform layer (IPL) consists of the connections among bipolar, amacrine and ganglion cells (182). In contrast to rodent models, the zebrafish is diurnal and its retina contains a large number of diverse cone subtypes in addition to rods (198,199). The cones are divided into four classes based on spectral sensitivity and morphology (198). The rod cell bodies are located vitread to the cone nuclei, and in the light-adapted retina, the thin rod inner and outer segments project beyond the cones to interdigitate with the apical microvilli of the pigment epithelium (200).

Anatomical researches revealed that the adult zebrafish have short single (SSC), long single (LSC) and double (DC) cone types (198) organized in a mosaic pattern (199). It is known that the adult zebrafish possesses at least four cone photopigments, each contained in an anatomically distinct cone outer segment and the rods seem to possess rhodopsin (201) under normal conditions (202). Some studies demonstrated that rod and cone opsin expression emerges as soon as 40–50 hpf in the ventral patch of the retina (194,199), which is sooner than the morphological differentiation of the photoreceptor types (203). It seems that photoreceptor growth is started by the expression of hedgehog genes, such as sonic hedgehog and tiggy-winkle hedgehog (204), which proliferated through the retina creating a wave of retinal neuron differentiation (204,205). Then, the cone types grow sequentially. All cone types and rods first appear in a small ventral patch on the retina and propagate over the retina, however the rods spread out in a different pattern than cones (199). The SSC appear first at about 4–5 dpf, the LSC appear at about 7 dpf and the DC finally appear beginning at about 10 dpf. Contrary, rods do not appear in the retina until 15–40 dpf (203).

There are several types of bipolar cells identified in the adult zebrafish retina. Studies showed that bipolar cells that ended in sublamina b had at least one of two different types of glutamate receptors, one that is sensitive to the glutamate analog APB (DL-2-amino-4-phosphonobutyric acid) and an APB-insensitive receptor and this last one resembled a glutamate-gated chloride mechanism (206). Some these bipolar cells possessed both types of receptor mechanisms. On the other hand, bipolar cells that terminated in sublamina a responded with an AMPA kainate-like conductance mechanism in response to glutamate and all were insensitive to APB. Other bipolar cell types ended in both a and b sublaminae and had both response types

(207,208). In terms of development, bipolar cells appear at about 60 hpf, which is especially late in development comparative to the other retinal neurons (194).

Relatively to horizontal cells, zebrafish adults appear to have two types: type A has a little round cell body with various processes and type B that has a longer cell body with fewer dendritic processes (209). These cells have low electrical conductance between cells compared to other fish (210). The neurotransmitters observed in zebrafish horizontal and amacrine cells are analogous to those found in other vertebrates. These cells seem to generate inhibitory inputs to the direct retinal pathway (i.e. photoreceptors to bipolar cells to ganglion cells) by the use of GABA in both horizontal and amacrine cells, as well as glycine in amacrine cells (98). In the developing larvae, horizontal cells start to disseminate laterally through the retina by 50 hpf and by about 3 dpf create the synaptic triads at photoreceptor synapses. Amacrine cells also appear at about 50 hpf (194).

Adult ganglion cells are categorized considering their response properties as phasic or tonic. These cells are the first retinal neurons to grow at about 28 hpf, and initially appear in the ventronasal retina (195). Their axons achieve the tectum by 2 dpf and by 3 dpf are dispersed through ten distinct areas of the tectum (211). Overall, the axons go in a focused direction with only some transient projections (193). Studies showed that the retinotectal projection is independent of neural activity and ganglion cell activity is required for the refinement of the retinotectal projection (212).

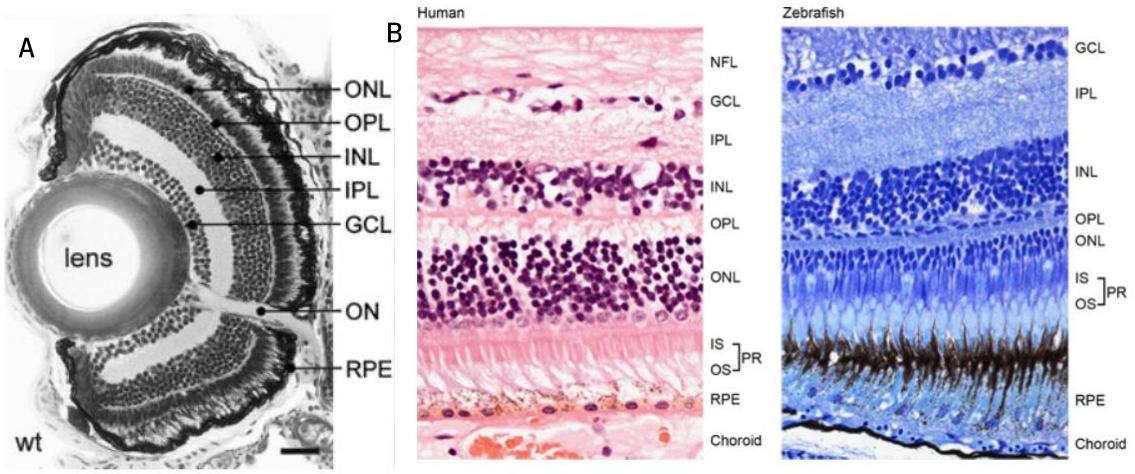

Figure 3. Retinal histology of 5 days old wild-type zebrafish (A) and cross-sectional histology of the human and zebrafish retina demonstrating similarities in the arrangement of cells and structural features that define the distinct retinal layers (B). RPE, pigmented epithelium; IS, inner segment; OS, outer segment; PR, photoreceptors layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; ON, optic nerve and NFL, nerve fibre layer. (From: (213,214)).

3.1.3. Eye Development

During the development of zebrafish, eye and lens morphogenesis, retinal histology and the expression of transcription factors show a high deal of solidity with other vertebrates – Figure 4. Through neurulation, expression of the transcription factors *six3a* and *pax6* in the anterior neural plate define the ocular tissues (215–217). During successive morphogenetic movements and inductive interactions, the eyes grow from bilateral paddle-shaped masses of cells that evaginate from the forebrain (192). Disruption of the *chokh/rx3* genes ends in a breakdown of the retinal progenitor cells to evaginate leading to an eyeless phenotype (216). Invagination of the central sections of this eye-mass and development of the optic lumen by 24 hpf contribute to the

formation of an optic cup – see Table 3 (192,194). The inner layer stays to proliferate and generates the neural retina, while the outer layer provides growth to the retinal pigment epithelium (RPE), possible across the action of *mitf* expression (218). The placing of the optic stalk is controlled by the expression of the *pax2* and *pax6* genes (219).

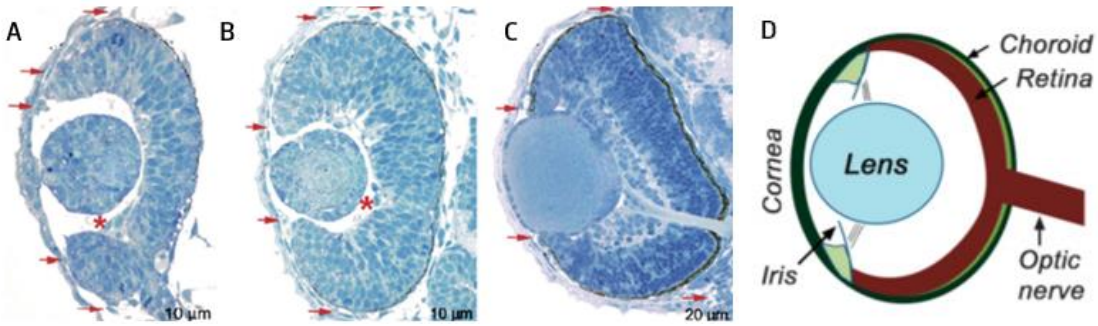

Figure 4. The development of the zebrafish eye, cornea and lens through micrographs. Histological sections at 24 hpf (A), 36 hpf (B), and 48 hpf (C). Between 36 and 38 hpf the neurogenesis and lamination of the retina develops quickly. The lens vesicle separates from the overlying ectoderm by 24 hpf and between 36 and 48 hpf the differentiation of the fiber cells continues rapidly. The periocular mesenchyme is present at 24 hpf, but more prominent at 36 and 48 hpf (arrows). Hyaloid vasculature is signaled with asterisk. (D) Diagram of the embryonic zebrafish eye. (Adapted from: (182,220)).

Table 3. Overview of zebrafish system [a].

	Structure	Function
Optics	Optic structures normal and eye forms at 12 hpf and adult-like by 72 hpf	Initially hyperopic and emmetropic by 72 hpf
Photoreceptors	Four cone outer segment types, rods SSC, LSC, DC, rods (4, 7, 10, >15 dpf)	At least four cone photopigments, rhodopsin
Horizontal cells	Types A and B, analogous to adults by 7–10 dpf	–
Bipolar cells	At least 13 cell types and very few cells at 7–10 dpf	Color opponent mechanisms, rods Cone follows anatomical development of cones and rods
Amacrine cells	Present by 50 hpf	–
Ganglion Cells	First retinal cells to develop (28 hpf)	Single unit: tonic or phasic response types
Tectum	Retinotopic organization and is attached to retina by 3dpf	ON and OFF phasic responses
Behavior	–	Optokinetic response developed by 73 hpf, biphasic dark adaptation function, circadian rhythm Acuity improves with age

[a]Adapted from (191)

Abbreviations: hpf=hours post-fertilization; dpf=days post-fertilization; SSC=Short Single; LSC=Long single; DC=Double single

3.1.4. Neurogenesis

Coming back to the retina, studies reported that retinal neurogenesis is a disciplined process. Like noticed in other species, the initial cells to leave the cell cycle differentiate into ganglion cells and then the neurogenesis process follows an approximate retinal order from internal to external (195). The first postmitotic cells and differentiation of ganglion cells are recognizable between 28 and 32 hpf, in the ventral patch, a section of mature neural development in the ventral nasal retina (192,193,195). Subsequently, differentiation extends dorsally across to the ventral temporal retina in a wave-like manner (221) associated with a wave of *sonic hedgehog* expression by the differentiating cells (205,222). The specification of ganglion cells needs the expression of the basic helix–loop–helix transcription factor *atoh7* required for the specification of the R8 photoreceptor. *Atoh7* is necessary for ganglion cell specification in several vertebrate species (223,224). In the absence of *atoh7* neuroblasts fail to be specified as ganglion cells and remain in the cell cycle giving rise to later born cell types (223). However, expression of *sonic hedgehog* by amacrine cells appears to mediate specification of the other retinal neurons (222). Therefore, the differentiation of the ganglion cells is followed by the differentiation of amacrine cells, interneurons, and retinal lamination. Lamination extends through most of the retina by 48 hpf (194). Müller glia are between the last to express the mature phenotype and their differentiation is promoted by signaling via the notch pathway (225,226).

3.2. Expression Of Genes Involved In Visual System

In fish, global gene expression analysis can be used to investigate the effects of toxicants on biochemical pathways, elucidate mechanisms of toxicity and be used for comparison of gene expression profiles to determine differences/similarities in responses of organisms to toxicants (227,228). Most studies approaching the impact of antidepressants on aquatic animals focus mainly on behavioral effects, reproduction, and growth. Little information exists about the impact of these drugs exclusively on the visual system in fish. Table 4 shows a summary of the most common genes in the visual system and their functions and temporal expression in zebrafish.

3.2.1. Rhoposin and Orthodenticle homeobox 2b Genes

Opsins are members of the G protein-coupled receptor (GPCR) superfamily that is characterized by a Schiff base linkage between a preserved lysine residue and a retinaldehyde chromophore (229) and are mainly responsible for starting the visual transduction cascade (230) but also the nonvisual processes (231). These are light-sensitive proteins produced by photoreceptors in the outer nuclear layer of the retina, being essential components of the visual pigments of vertebrates in the photoreceptors. Gene duplication events through vertebrate evolution provided rise to several opsin families. There are five groups of visual opsins, including *rhodopsin (rho)* and four cone opsin groups: rhodopsin-like *(rh2)*, short-wavelength sensitive 1 *(sws1)*, short wavelength sensitive 2 *(sws2)*, and long-wavelength sensitive *(lws)*. The rhodopsin gene *(rho)* is a photoreceptor and signal for phototransduction and is implicated in behavior regulation in invertebrates and vertebrates (150). Besides that is highly characterized GPCR and is the visual opsin that starts dim-light vision

in vertebrates (232). Because of its stability and abundance in rod photoreceptor outer segments, rhodopsin is responsive to detailed studies of structure and function, often through site-directed mutagenesis and in vitro expression (233). So far, nine visual opsins have been identified in zebrafish, including *rho*, four *rh2, sws2, sws1,* and two *lws*, a large complement even among teleosts (234).

In vertebrates, rhodopsin absorbs a photon that causes a change in the structure or electronic state of the 11-cis retinal chromophore. Consequently, a chain of events is initiated which leads to conversion of the energy of light to a neural signal—i.e., visual transduction (235). A study carried out on amphipods showed that when animals are exposed to low concentrations of fluoxetine there is a significant down-regulation of the *rho*, whereas for low concentrations of sertraline it becomes up-regulated, what can be explained by the different modes of action of these antidepressants. It can be speculated that these changes may be responsible for changes in the stimulation of light transduction and consequent modification in the behavior of amphipods to light (236). The advantage of studying opsin genes in the zebrafish is not only due to the availability of a genome sequence, but also to the considerable body of work assembled on the zebrafish visual system (182,237), and to the variety of existing and emerging tools available to study genes of interest in zebrafish (238). When this animal was exposed to triphenyl phosphate, down-regulation occurred in the expression levels of nine genes that code for opsins, which causes a reduction in the accumulation of these in the photoreceptors. This may result in an insensitivity of the larval visual response to light stimulation and a disruption in the development of the outer nuclear layer (239). In addition, the expression of genes relative to phototransduction *(opn1mw1, opn1sw1, cry5)* and eye photoreceptor cell development and maintenance *(atoh8, cyp1b1, per2)* in zebrafish embryos was modulated by benzo(a)pyrene and can cause a disturbance in ocular development (240).

Orthodenticle homeobox 2b *(otx2)* is involved in brain and eye development and acts in many first steps of ocular embryogenesis (241). After exposure to venlafaxine, the relative expression of *otx2* mRNA was significantly down-regulated in zebrafish, but when exposed to low concentration of sertraline this gene became up-regulated (242). It is known that misexpression of these genes leads to the development of anteriorized embryos with gastrulation defects (243).

3.2.2. Six homeobox 3 And Paired box 6 Genes

The homeodomain-containing transcription factors *six3* and *pax6* are crucial for the determination of retinal fate in the neuroectoderm (244) and also function in lens development (245). The four *six3/6* gene family members found in zebrafish might be a result of an extra genome duplication through early radiation of vertebrates (246). During early stages of eye development expression of zebrafish *six3* is analogous to that of the orthologous genes in mouse and chicken. *Six 3* transcripts could be found in the differentiating retina from 32hpf. At this stage a thin layer of *six3* staining is located in a part of the retinal neuroepithelium (RNE) near the ventral lens (217). Differential regulation of *six3* expression is revealed by the observation that just a short upstream sequence is needed for initial expression in the forebrain and eye primordia, while the entire upstream fragment of 3.2 kb is required for late expression in the eye (247).

Pax genes family is very important in embryonic development and organogenesis in many species. In vertebrate development, the *pax6* gene is expressed in the eye, the CNS, some of the sensory placodes, and the

pancreas. *Pax6* plays critical roles in lens development, and its expression level has critical effects on this process (248) but also looks directly implicated in the development of the retina and cornea while it is not needed for the initial formation of the optic ridge and vesicle (249). Loss of both alleles leads to lack of eye structures, nasal cavities and serious abnormalities in brain development producing postnatal lethality (250). The *pax6* dosage sensitivity regarding correct development of the eyes is exhibited upon overexpression of the gene in transgenic mice (251). In addition, the identification of eyeless as a *pax6* homolog in *Drosophila*, and the surprising demonstration that ectopic expression of eyeless or mouse *pax6* in different imaginal discs of *Drosophila* induced supernumerary eye structures on the wings, legs or antennae of the flies reinforced the idea of *pax6* as a principal control gene for eye development acting high up in the regulatory hierarch (252).

After a study carried out it was found that mouse with altered levels in *pax6* expression had defects in lens development (253). A down-regulation of *pax6* mRNA expression in *D. rerio* larvae exposed to amitriptyline has been verified, which may be an indication that waterborne antidepressants may have harmful effects on brain and eye development in *D. rerio*. Contrarily, the organism *X. tropicalis* did not reveal any changes in the expression of *pax6* mRNA when exposed to the antidepressants amitriptyline, venlafaxine, sertraline (242). In addition to antidepressants, there are other types of contaminants capable of causing ocular abnormalities. In the case of polycyclic aromatic hydrocarbons (PAHs), they are also present in the atmosphere and aquatic environment as a result of petrogenic and pyrogenic sources (254). Phenanthrene (Phe) is one of the examples of PAHs present mainly in the environment and, when the zebrafish was exposed to this compound, it was found that there was an induction of anomalies in the eye, a phototaxis response was impaired and there was an induction of apoptosis and reduced cell proliferation in the retina. Regarding gene expression, the study demonstrated a slight down-regulation of the *pax6* gene together with a slight up-regulation of *mitf*, which could be an indication that these two genes may be involved in ocular toxicity by Phe, and may explain the malformations observed , increased apoptosis and reduced cell proliferation (255).

Furthermore, the identification of *pax6* and the Brn-3 binding motifs in the *six3* upstream region required for late retinal expression also indicates regulatory functions for these two proteins. A direct involvement of *pax6* in regulating *six3* transcription was identified, detecting a high-affinity binding site for the *pax6* PD in the *six3* upstream region that is required for late retinal expression (217).

3.2.3. Retinal homeobox gene 3

It was concluded that mutations in the zebrafish retinal homeobox gene 3 *(rx3)* gene origin the eyeless phenotype and the role of *rx* genes in vertebrate eye development is important due to previous loss-of-function studies in mouse, medaka and zebrafish (216). Zebrafish and medaka *rx3* is the first paralogue to be expressed in eye precursors followed by *rx2/rx1*. Later in gastrulation, *rx3* becomes abundant inside the presumptive hypothalamus but repressed in the splitting optic primordia—in which *rx1/rx2* remains abundant. Remarkably, it is mutations/alterations in *rx3*—the paralogue with prevalent hypothalamic expression—that are linked with anophthalmia in medaka and zebrafish (256).

Assessment of gene expression in the developing eye field and brain determined that the phenotype of the zebrafish and medaka *rx3* mutants is especially specific to eye morphogenesis. Both the global morphological

organization of the brain as well as the early patterning of the eye field seems normal (257). It was shown that *rx3* is necessary for expression of *mab21l2* that plays a crucial role in early eye development by preserving the viability of undifferentiated progenitor cells in the immature retina, lens, and circumferential germinal zone. It was also shown that *mab21l2* expression in differentiated amacrine and ganglion cells indicate a second role later in eye development (258).

3.2.4. Visual System Homeobox 1 and 2 Genes

In the adult goldfish, expression of *vsx1* and *vsx2* is confined to the retina. The spatial expression of *vsx1* and *vsx2* mRNA in the adult goldfish retina was determined by *in situ* hybridization, and, in the mature portion of the retina, both mRNAs were confined to the INL in a pattern consistent with expression in bipolar cells (259). *Vsx1* is very expressed in a subset of postmitotic cells of the growth zone, but little expression is detected in cells of the retinal germinal zone or the iris epithelium (260). In contrast, *vsx2* is firmly expressed all over the germinal zone and the iris epithelium (259). The timing of onset of *vsx1* expression and its limitation to cells of the INL indicate that it may be vital for the development of this layer. *Vsx1* is initially expressed in a ventral patch of cells that seem intended to become INL cells. The expression of *vsx1* appears after *vsx2* and is down-regulated in this region, and, at these early stages of INL differentiation, the expression patterns of *vsx1* and *vsx2* are almost complimentary (261).

As development continues, *vsx1* expression expands along the middle of the RNE to eventually span the entire dorsal/ventral extent of the nascent INL. Thus, in the initial steps of retinal differentiation, *vsx1* may be directing postmitotic progenitor cells to adopt an INL phenotype. There are numerous ways in which *vsx1* can function to promote an INL cell fate. To render an undifferentiated cell capable of responding to environmental cues, *vsx1* may be needed for the transcriptional regulation of certain membrane receptors, which would let the cell to remain selectively "blind" to one set of extrinsic factors but responsive to other sets (262). Consequently, the expression patterns seen at the margin of the adult goldfish retina support the idea that *vsx1* may be necessary to sustain cellular differentiation, while *vsx2* may be implicated in regulating cellular proliferation and/or differentiation in both neural retina and iris.

Following the analysis of a transcriptome of six genes involved in the stimulation of light and photoperiodism *(rpe65a,clocka, per2, tefa, opn3 and vsx1)* it was found that these genes were enriched in the zebrafish brain after chronic exposure to dydrogesterone, which is a sign that this compound may affect ocular function in this vertebrate (263). We must be aware that the observed effects may depend on a large number of factors – i.e. type of compound, duration of exposure, concentration, stage of development, species under study – which makes its detection difficult and also the observation of several trends.

3.2.5. Phosphodiesterase 6C Gene

In wild-type zebrafish retina, photoreceptor progenitors withdraw from the cell cycle at 48 hpf to create the photoreceptor precursors in the ONL (195). These precursors will start to differentiate into rods and cones at 50 hpf in a ventral part of the retina (264). Then, cones progressively spread equally all over the retina; while rods spread occasionally in the retina, with higher density in the ventral region. By 3 dpf, these photoreceptors

are sufficient mature that the fish larvae will exhibit the first visually-evoked startle response (237). In *pde6c*, cones appear to grow following the normal course. They form outer segment by 3 dpf, but they degenerate starting at 4 dpf. *Pde6c* rods die subsequently as bystanders (185), even though they do not express the *pde6c* gene. They also develop initially in the retina, but look abnormal with more evident outer segment at 4 dpf (185), when cones first degenerate. However, rod amount is comparable to wild-type at least up to 6 dpf (265), and just starts to decrease in the central retina by 8 dpf (185). The degeneration of these photoreceptors will cause reactive gliosis as early as 7 dpf (266), and abnormal morphology in bipolar cells with dislocated nuclei and axonal processes at 8 dpf (185). These tissue-level effects begun by a cone-specific *pde6c* mutation. The molecular basis of these retinal defects can often be analyzed by profiling gene expression of the whole retina (267) which can identify expression change in a few cells (268).

This photoreceptor-specific defect will origin a succession of cellular changes, which in the end will change the well-being of the whole retina. For example, in a zebrafish mutant *pde6c*^{w59} (*pde6c*), an A>G point mutation was identified in the *pde6c* gene. This mutation was expected to produce a frameshift in the coding sequence and cause either in a truncated PDE6C or degradation of *pde6c* mRNA through nonsense-mediated decay. This mutation ultimately affects both cone and rod photoreceptors (185).

3.2.6. Sonic Hedgehog Gene

The differentiation of all cell types in the zebrafish INL, including amacrine, bipolar and horizontal cells and Müller glia, depends on sonic hedgehog *(shh)* secreted by amacrine cells. Mosaic experiments, in which wild-type cells were transplanted into *shh* mutants, reveal that *sonic hedgehog* functions as a short-range signal to direct the differentiation of these cell types (222). Additionally, development of the IPLs and OPLs also depends on *shh* activity, and *shh* plays as a short-range signal to direct IPL formation. Hedgehog (*hh)* signaling in zebrafish retinal neurogenesis is reminiscent of the role of *hh* in *Drosophila melanogaster* in directing the neurogenic wave of the eye imaginal disc (269). Coherent with this examination is the remarkably analogous function of the atonal gene in *Drosophila* and its zebrafish homolog, *atoh7* , in directing the differentiation of the first born neurons of the retina (270). As is the case for *Drosophila* atonal, *atoh7* expression in the zebrafish seems to be dependent on *hh* signaling (271). However, in compare to the decreased retinal differentiation observed in zebrafish *sonic hedgehog* mutants, blockage of *shh* activity in the chicken retina ends in augmented differentiation, i.e. increased production of ganglion cells (272), indicating that there are vertebrate-specific differences in the roles of *hh* signaling in the retina. In *Danio rerio*, a third wave of sonic and tiggy-winkle hedgehog expression continues in the RPE (273). *Hedgehog* signaling is crucial for the differentiation of all retinal cell types in the zebrafish, including not only neurons, but also glial cells, and therefore does not seem to confer any specificity concerning which cell fate is chosen (222).

3.2.7. Atonal bHLH transcription factor 7

Atoh7 is expressed in the developing retina in all vertebrates studied and shows a vital role in regulating retinal neurogenesis (274). In *Xenopus*, *xath5* is expressed in a closely limited set of cells in the developing neural retina, the olfactory placodes and the pineal gland. In the retina, *xath5* expression begins in retinal

progenitors just prior to cell cycle exit and onset of differentiation, but expression is downregulated before cells become fully mature retinal neurons (275). In mouse, zebrafish and chick, *atoh7* is additionally expressed in a similar restricted manner inside the retina immediately preceding the onset of retinal ganglion cell differentiation (276). *Atoh7* is specifically necessary for the differentiation of the retinal ganglion cell type as in both mouse and zebrafish *atoh7* loss-of-function mutants, RGCs are either drastically reduced or missing altogether (277).

Reductions in *atoh7* expression levels are associated with reductions in retinal neurogenesis, as shown by analysis of mouse *pax6* mutants and zebrafish midline signaling mutants, in which *atoh7* expression is changed (278). As *atoh7* plays such an important role in retinal development, it is important to understand how its expression is regulated.

Table 4. Overview about common genes involved in visual system of zebrafish. Information retired of ZFIN The Zebrafish Database Information Network and Uniprot Database.

Name	Acronym	Gene ID	Accession Number	Molecular Functions and Biological Processes	Temporal Expression
six homeobox 3a	*six3a*	30635	NM_131362.1	▪ Anatomical structure development ▪ Brain development ▪ Eye development ▪ Negative regulation of transcription, DNA-templated ▪ Optic nerve development and morphogenesis ▪ Regulation of transcription, DNA-templated	Eye and Retina: ▪ Pharyngula (24 – 48hpf) ▪ Hatching (48-72hpf) CNS and Brain: ▪ Segmentation (10-24hpf) ▪ Pharyngula (24-48hpf) ▪ Larval (72hpf – 30dpf)
six homeobox 3b	*six3b*	30636	NM_131363.1	▪ Anatomical structure development ▪ Brain development ▪ Embryonic camera-type eye morphogenesis ▪ Eye development ▪ Negative regulation of transcription, DNA-templated ▪ Optic nerve development and morphogenesis ▪ Regulation of transcription, DNA-templated ▪ Sensory epithelium regeneration	Eye and Retina: ▪ Pharyngula (24 – 48hpf) ▪ Hatching (48-72hpf) CNS and Brain: ▪ Segmentation (10-24hpf) ▪ Pharyngula (24-48hpf) ▪ Hatching (48-72hpf)
paired box 6a	*pax6a*	30567	NM_131304.1	▪ Anterior/posterior pattern specification ▪ Epithalamus development ▪ Forebrain development ▪ Habenula development ▪ Hindbrain development ▪ Retinal cone cell differentiation ▪ Retinal rod cell differentiation	Eye and Retina: ▪ Pharyngula (24 - 48hpf) ▪ Hatching (48 - 72 hpf) ▪ Larval (72 hpf - 30 dpf) CNS and Brain: ▪ Segmentation (10 - 24 hpf) ▪ Pharyngula (24 - 48hpf) ▪ Hatching (48 - 72 hpf) ▪ Larval (72 hpf – 30 dpf)
paired box 6b	*pax6b*	60639	NM_131641.1	▪ Anterior/posterior pattern specification ▪ Cornea development in camera-type eye ▪ Epithalamus development ▪ Eye development ▪ Hindbrain development ▪ Lens development and morphogenesis in camera-type eye	
retinal homeobox gene 3	*rx3*	30474	NM_131227.1	▪ Camera-type eye morphogenesis ▪ Embryonic camera-type eye morphogenesis ▪ Eye development ▪ Eye field cell fate commitment involved in camera-type eye formation ▪ Forebrain development ▪ Retina development in camera-type eye	

				▪ Neural crest cell migration ▪ Regulation of transcription, DNA-templated	
rhodopsin	*rho*	30295	NM_131084.1	▪ Retinal binding ▪ Photoreceptor activity ▪ Absorption of visible light ▪ Cellular response to light stimulus ▪ Detection of light stimulus ▪ Phototransduction ▪ Rhodopsin mediated signaling pathway ▪ Visual perception	Eye and Retina: ▪ Pharyngula (24 - 48hpf) ▪ Hatching (48- 72 hpf) ▪ Larval (72 hpf - 30 dpf) CNS and Brain: ▪ Pharyngula (24 - 48hpf) ▪ Hatching (48 - 72 hpf) ▪ Larval (72 hpf – 30 dpf)
phosphodiesterase 6C	*pde6c*	393845	NM_200871.1	▪ Embryonic retina morphogenesis in camera-type eye ▪ Photoreceptor cell maintenance ▪ Signal transduction ▪ Visual perception	Eye and Retina: ▪ Larval (72 hpf – 30 dpf) CNS and Brain: ▪ Hatching (48 - 72 hpf)
sonic hedgehog signaling molecule a	*shha*	30269	NM_131063.3	▪ Anterior/posterior pattern specification ▪ Brain development ▪ Camera-type eye development ▪ Central nervous system development ▪ Determination of left/right symmetry ▪ Diencephalon development ▪ Embryonic camera-type eye development ▪ Embryonic neurocranium and viscerocranium morphogenesis	Eye and Retina: ▪ Hatching (48- 72 hpf) ▪ Larval (72 hpf - 30 dpf) CNS and Brain: ▪ Segmentation (10 - 24 hpf) ▪ Pharyngula (24 - 48 hpf) ▪ Hatching (48 - 72 hpf) ▪ Larval (72 hpf - 30 dpf)
sonic hedgehog signaling molecule b	*shhb*	30444	NM_131199.2	▪ Embryonic neurocranium and viscerocranium morphogenesis ▪ Forebrain development ▪ Gliogenesis ▪ Neuron differentiation ▪ Neuron fate commitment ▪ Oligodendrocyte differentiation ▪ Proteolysis ▪ Regulation of gene expression	Eye and Retina: ▪ Adult (90-730dpf) CNS and Brain: ▪ Segmentation (10 - 24 hpf) ▪ Pharyngula (24 - 48 hpf) ▪ Hatching (48 - 72 hpf) ▪ Larval (72 hpf - 30 dpf)
visual system homeobox 1	*vsx1*	30598	NM_131333.1	▪ Axial mesoderm development ▪ Forebrain development ▪ Negative regulation of axial mesodermal cell fate specification ▪ Negative regulation of transcription, DNA-templated ▪ Paraxial mesoderm development ▪ Paraxial mesodermal cell fate specification	Eye and Retina: ▪ Pharyngula (24 - 48 hpf) ▪ Hatching (48 - 72 hpf) ▪ Larval (72 hpf - 30 dpf) CNS and Brain: ▪ Segmentation (10- 24 hpf)

				▪ Prechordal plate formation ▪ Regulation of transcription, DNA-templated ▪ Response to stimulus ▪ Visual perception	▪ Pharyngula (24- 48 hpf) ▪ Hatching (48- 72 hpf)
visual system homeobox 2	*vsx2*	796163	NM_131462.2	▪ Negative regulation of transcription by RNA polymerase II ▪ Regulation of neural retina development ▪ Regulation of transcription, DNA-templated ▪ Response to stimulus ▪ Visual perception	Eye and Retina: ▪ Pharyngula (24 - 48 hpf) ▪ Hatching (48 - 72 hpf) ▪ Larval (72 hpf - 30 dpf) CNS and Brain: ▪ Segmentation (10 - 24 hpf) ▪ Pharyngula (24 - 48 hpf) ▪ Hatching (48 - 72 hpf) ▪ Larval (72 hpf - 30 dpf)
atonal bhlh transcription factor 7	*atoh7*	58216	NM_131632.1	▪ Camera-type eye development ▪ Eye development ▪ Nervous system development ▪ Neural retina development ▪ Pigmentation ▪ Positive regulation of neurogenesis ▪ Regulation of cell cycle ▪ Regulation of mitotic cell cycle ▪ Regulation of transcription, DNA-templated ▪ Retina development in camera-type eye ▪ Retina layer formation ▪ Swimming behavior	Eye and Retina: ▪ Pharyngula (24 - 48 hpf) ▪ Hatching (48 - 72 hpf) ▪ Larval (72 hpf - 30 dpf)

3.3. Influence On Visual System

Since the retinofugal system is an extension of the CNS and consists in retinal ganglion cells that send their axons along the optic nerve to the visual brain areas, it has an important contribution to the current knowledge on mechanisms underlying the action of neuroactive drugs (279). Indeed, the retina and the optic nerve are the most accessible parts of the CNS and have a well-characterized and conserved morphology and function (280). The developing visual system is extremely vulnerable to the effects of prenatal exposure to neuroactive drugs. During the development of the CNS, serotonin has been described as a trophic factor influencing a wide range of developmental processes, such as cell division and differentiation, neuronal migration, synaptogenesis, and plasticity (281). One of the most important aspects regarding 5-HT homeostasis is related to the extracellular clearance of 5-HT through the serotonin transporter (5-HTT) which interrupts its postsynaptic actions through neuronal reuptake (282). As 5-HTT represents the primary target of several prescribed modern antidepressants has been receiving special attention since its discovery (283).

Studies have demonstrated that chronic treatment with fluoxetine during early postnatal development of the rat visual system produces abnormal patterns of topographical mapping and abnormally increased plasticity in subcortical visual pathways (284). Studies that used 5-HTT or MAO_A knockout models, described similar results which resulted in abnormal cortical cytoarchitecture and topographical patterning disruption of the somatosensory barrel and visual cortex (285,286). Indeed, fluoxetine administration disrupts the retinotectal topography leading to a topographical expansion of the retinotectal terminal fields both in the developing and in the mature brain and amplifies the lesion-induced plasticity of the intact pathways (287).

Assessing the visual motor response (VMR) in zebrafish, a simple but well characterized sequence of locomotory changes in response to a change in light conditions, showed that antidepressants most consistently altered spontaneous locomotion the common antidepressant classes having considerable variation in behavioral effects, such as whether effects were readily observed without an environmental stimulus or if effects were only noticeable or more pronounced (or abolished) when coupled with the light change. In this same study SSRI compounds show different behavioral effects despite supposedly having the same physiological mechanism. In fact, fluoxetine and paroxetine showed a decreasing activity with increasing dose, particularly after the light change where fish exposed to even the lowest dose treatment showed remarkable decreases in activity comparably to control fish. On the other hand, only fish exposed to the highest concentration of sertraline showed hypoactivity after the light change (288).

Conversely, other studies have demonstrated that chronic fluoxetine administration reinstates a juvenile-like form of ocular dominance (OD) plasticity in adulthood, which is indicated by a decrease in the response to stimulation of the deprived eye and promotes a complete recovery of visual functions in adult amblyopic rats (136). However, the effects induced by fluoxetine in adult visual cortical plasticity are surprisingly similar to those caused by environmental enrichment, a condition characterized by increased exploratory behavior and sensory-motor stimulation, which promote amblyopia recovery in adulthood through a reduction of intracortical inhibition (289).

Still in the field of neuroactive drugs, eye defects, including anophthalmia and folded retina, have been reported to occur in rodent offspring after early and late prenatal exposure to Methamphetamine, respectively (290,291).

Oil-exposed *sheepshead* larvae had decreased development of the retinal layers, culminating in a reduced optomotor response. The size of the pigmented epithelial layers in *sheepshead* larvae exposed to the highest PAH concentration were reduced. These layers perform a crucial role in absorbing light, storing and modifying vitamin A precursors for photoreceptors, and providing nutrients to photoreceptors (292). The mean diameter of the photoreceptor layer, essential for correct rod and cone function, was reduced in oil-exposed *sheepshead* larvae. Also, embryonic zebrafish exposed to growing concentrations of phenanthrene showed reduced retinal, pigmented epithelial, ganglion, and lens diameters (255). This indicate that phenanthrene could be damaging the photoreceptors themselves. It has further been shown that oil-exposed larvae had a downregulation in genes important in regulating photoreceptor function (293). Genes important in phototransduction and eye development *(arr3b, crx, gnat2, opn1mw, pde6c, pde6h, rgr, rho, rpe65a, and sws1)* were substantially downregulated in oil-exposed larvae. Also, vision-related, biological processes were between the most impacted pathways related with the exposure of crude oil in mahi-mahi and red drum larvae. Common genes found to be dysregulated after oil exposure in mahi-mahi were *crx, pde6c, rho, and rgr, and gnat2* and *rpe5a* in larval red drum. A reduction in *rpe65* can further inhibit correct rod function in zebrafish, while a downregulation of genes important in eye development occurred in larval zebrafish following individual PAH exposures, including *opn1mw, gnat2,* and *pde6h* (294).

It should be emphasized that drugs which allegedly have similar chemical structure, that interact with the same cell membrane components, and are used to treat the same or similar medical conditions, cannot be assumed to have the same biological effect on the nontarget organisms. As such, this leads to a potential complication for the environmental risk assessment of this type of compounds.

Chapter 4 . Zebrafish As A Model To Assess Sublethal Effects And Health Risks Of EPs

4. Zebrafish Model In Environmental Risk Assessment

Zebrafish is quickly becoming the preferred vertebrate model organism for developmental biology, drug discovery, evaluation of toxicological side effects of the potential drugs, and ecotoxicology (295). Small and low-cost to sustain, only a pair of adults breeds once a week, generating 100–200 offspring per brood, and their husbandry costs are 100 and 1,000 times lower than those of mice or other mammals (296). As its development is ex utero and they have optical transparency during embryogenesis and early larval stages simplify visual in vivo observation of early developmental processes and organogenesis. The functional and morphological changes can be observed in vivo or in whole mount fixed specimens with the use of vital dyes, fluorescent tracers, antibodies and riboprobes, and fluorescent markers (297). *Danio rerio* embryos grow up quickly, with the simple vertebrate body plan laid out within 24hpf. At this phase, embryo length is approximately 1.9mm, so many embryos fit easily within a single well of a 384 well plate (298). Most organs, including the nervous system, cardiovascular system, intestines, liver, and kidneys, can be examined at 5dpf, when the larva has just 3–4mm in length (296). These organs and tissues have shown analogous to mammalian counterparts on the anatomical, physiological, and molecular levels (297). As larvae can live in 50mL water, just micrograms of compounds are necessary for assays, demonstrating a major cost reduction in screening entire molecule libraries (296). As a result, zebrafish represents a distinctive vertebrate model for high-throughput chemical screening, making them helpful for toxicological evaluation (298). The principal emphasis of zebrafish study has been on developmental biology, observations from large-scale genetic screening permitted the identification of mutants phenocopying diseases and developmental pathologies comparable to humans. These showed the appropriateness of employing zebrafish as a model of human disease, drug discovery, and drug toxicity analyses (299).

Zebrafish tests contribute to the prioritization of drug candidates (300) and can be utilized for the choice of the chemical concentrations to be applied in further *in vivo* tests, thus decreasing the amount and cost of mammalian studies. The same principles that led to the implementation of zebrafish as a model for discovery and drug toxicology analyses also apply to the analysis of the toxic mechanisms and effects of emerging pollutants. The assessment of toxic effects in zebrafish embryos offers a holistic method because involves several aspects of the physiology, development, and functionality of complex organic systems. A range of assays are accessible for evaluating toxicity on the cardiovascular, gastrointestinal, renal, nervous, thyroid, digestive or skeletal systems (296,299).

4.1. Neurotoxicity Assessment

Danio rerio embryos and larvae are extraordinarily well suitable for developmental neurotoxicity experiments that merge cellular, molecular, and genetic approaches. Zebrafish embryos and early larvae are transparent and because of that particularity specific neurons and axon regions can be observed *in vivo* using transgenic lines or by injecting reporter dyes (297). Working at later developmental stages is achievable by using transparent zebrafish, as *casper* mutants. Particular types of neurons can be visualized in fixed intact zebrafish by whole mount immunohistochemistry or *in situ* hybridization (301,302). Besides that, the tiny size

of early-stage zebrafish allows performance of quantitative whole-animal assays in a 96-well microplate format for neurotoxicity screening. The zebrafish model has been used for assessing the toxic effect of different xenobiotics on specific cell types in the nervous systems, as dopaminergic neurons or the mechanosensory system (301,302).

4.2. Zebrasfish Visual System As A Model To Study Neuroscience

Analyzing the visual system offers understanding into neural processing. The use of the retina enables researchers to explore a separated neural network that is isolated from the rest of the CNS. Therefore, examining the development of the retinotectal pathway presents a chance to assess the mechanisms responsible for neural connectivity such as neural growth cones, guidance molecules, and synapse formation (303). Retinal growth in zebrafish (194) pursues a comparable progression as in other vertebrates, such as mice (304). In addition, zebrafish have a duplex retina (203) and exhibit visual behavior very early (189). Their retinal anatomy still developing after hatching, which is 3dpf (203). This gives a chance to evaluate the development of retinal structure with that of visual physiology and behavior. The number of eye (182), retinal (305) and retinotectal (303) genetic mutations were established and these attributes make the zebrafish a useful model studying visual development and neural development as well.

Chapter 5 . Brief Comment

5. Brief Comment: Impact of SARS-CoV-2 Pandemic On Mental Health

COVID-19 is a new strain of coronaviruses known to cause diseases ranging from cold to more severe illnesses such as SARS and MERS and was declared a pandemic by the World Health Organization (WHO) on March 11, 2020 (306,307). This disease, and the public health procedures employed to slow it, have deeply changed people's lifestyle, and is believed to be a threaten for physical and mental health. The unpredictable nature of the disease, the loss of control and personal freedoms, the contradictory communications from authorities, unexpected changes in plans for the immediate future, or concern for one's own health and well-being and that of one's relatives are examples of sources of stress associated with these outbreaks and pandemics (308). Nervousness and anxiety in a society affect everyone in a huge extent. Current data indicates that people who are kept in isolation and quarantine suffer substantial levels of anxiety, anger, confusion, and stress (309). Besides that, the studies that examined the psychological disorders during the COVID-19 pandemic have described that the affected individuals demonstrate numerous symptoms of mental trauma, such as emotional distress, depression, stress, mood swings, irritability, insomnia, attention deficit hyperactivity disorder, post-traumatic stress, and anger (309–311).

Some studies showed that prevalence of stress, anxiety, and depression, as a result of the pandemic in the general population, are 29.6, 31.9 and 33.7% respectively (312). The most recent global estimate d prevalence of depression is from 2017 and shows a proportion of 3.44% (ranging between 2 and 6%) (313). Results indicate that rates of depression in the general population might be 7 times higher during the COVID-19 outbreak. However, careful is necessary when interpreting these results, since the type of instruments and criteria used to ascertain depression might generally differ as well as the number of studies and countries included in the estimates (314). Several studies found higher rates of depression particularly between students (315). Young population might be more susceptible to uncertainty about the future of jobs, careers and economic crisis and they are also more exposed to social media (316).

Despite scarce information on the use of antidepressants during the COVID-19 pandemic, some reports showed that in United States of America prescriptions for anti-anxiety medications during rose 34.1%; for antidepressants, 18.6%; and for anti-insomnia drugs 14.8% (317,318). Besides that, according to aggregated Outcome Health data Australian general practices anti-anxiety drugs have soared by up to 31%, while antidepressant prescriptions have also risen steadily, up by double-figure percentages (13–22%) over last year for each of the eight weeks ending 6 June. Figure 5 illustrates the general growth in mental health prescriptions with the week 16 showing the largest increases in both new mental health conditions and an increase in medications prescriptions which have all continued to grow significantly, in particular antipsychotics (319). In this way, it can be expected that over the coming times there will also be an increase in the levels of psychotropic drugs found in water courses. Consequently, aquatic organisms may be exposed to a significant higher concentration of these drugs, which in turn will have an impact in these animals not yet addressed.

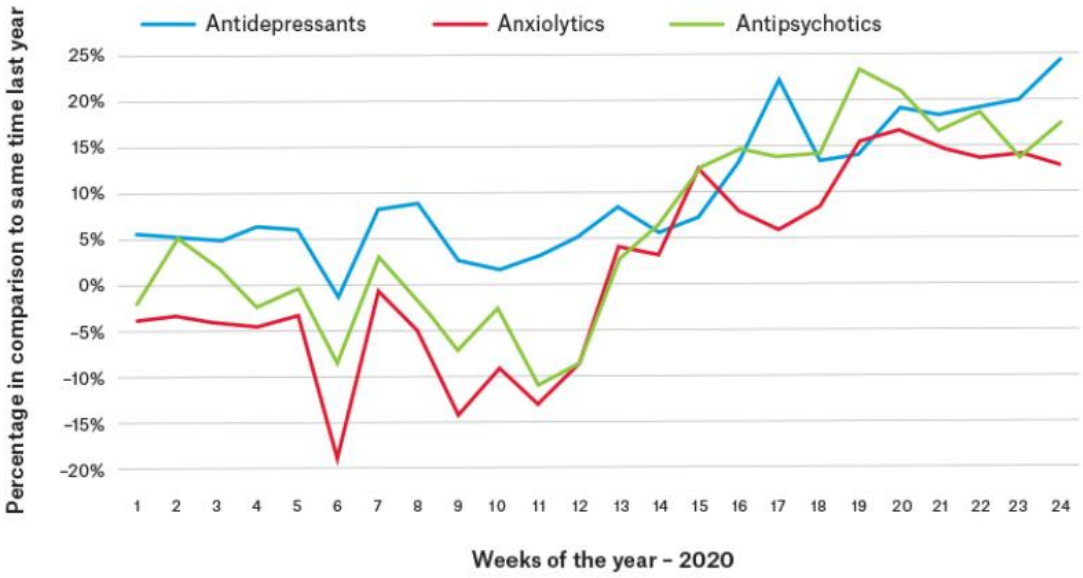

Figure 5. Rates of antidepressant, anxiolytic and antipsychotic use in 2020. (From: (319))

As mentioned at the beginning of this dissertation, next chapter 6 will be presented, which describes the experimental plan for carrying out the practical work before the pandemic, and that for public health reasons it was not possible to complete it. Thus, there is a summary description of what would be done at the laboratory level.

Chapter 6 . Experimental Planning

6. Experimental Planning – Exposure Of Zebrafish Embryos To Different Concentrations Of Venlafaxine

This chapter describes the selection of treatments, tests and experimental layouts that would be used to collect observations and data to analyze the effects of zebra fish exposure to venlafaxine, both at the morphological level and at the level of genetic analysis in the visual system. The adopted Planning follows the OECD guidelines for testing chemical compounds in fish embryos (320,321).

Hypothesis:

Changes of gene expression in zebrafish eye exposed to environmental relevant concentrations of antidepressants could reflect disruption in eye development with significant effects in visual system and visual system related behaviors.

Objectives:

Investigate the interactions between antidepressants and the development of the neurochemical machinery of the zebrafish's retina and visual pathways.

- Discover changes in the expression of genes involved in the development of the visual system.
- Understand the implications of these changes in the development of the eye and visual acuity of animals.
- Understand how these changes influence the performance related to vision, namely in the escape from predators and / or prey capture.

Acute toxicity test on fish embryos (FET)

6.1. Introduction

Guideline 236 describes a FET test with zebrafish. This test was created to determine the acute toxicity of chemicals at several embryonic stages of fish. The FET test is centered on studies and validation activities carried out on zebrafish. The FET test has been effectively applied to a wide variety of substances that show different modes of action, solubilities, volatilities and hydrophobicity.

6.2. Test Principle

Freshly fertilized zebrafish eggs will be exposed to the study antidepressant (venlafaxine) for a period of 144 hours. Observations will be made at 8, 24, 48, 72, 96 and 144hpf and some indicators will be recorded, such as mortality, delay on development, pigment, somites, tail detachment, yolk sac, edema, otolites, eyes, hatching, blood circulation, scoliosis and deformations and morphometric analysis.

6.3. Test Validity

For the test results to be valid, the following criteria apply:

a) The overall fertilization rate for all eggs collected must be ≥70%.
b) The water temperature must be maintained at 26±1°C on the test plates at any time during the test.
c) The overall survival of embryos in the negative control (dilution water) and, when relevant, in the solvent control must be ≥90% until the end of the 144-hour exposure.
d) Exposure to positive control (4.0mg/L 3,4-dichloroaniline for zebrafish) should result in a minimum mortality of 30% at the end of the 144-hour exposure.
e) The hatch rate in the negative control (and solvent control, if appropriate) must be ≥80% at the end of the exposure.

6.4. Equipment

It is necessary:

a) Aquariums of chemically inert material (glass) and with sufficient capacity in relation to the recommended number of animals.
b) Binocular loupe with a magnification capacity of at least 80 times for embryo selection.
c) Inverted microscope for morphometric analysis and visualization of malformations. If the room used to record observations cannot be adjusted to 26±1°C, methods are required to maintain the temperature.
d) Test chambers; for example, 6-well plates with a depth of approx. 20mm and 1L beaker.
e) Self-adhesive sheet or cover to cover 6-well plates and beakers.
f) Incubator with controlled temperature and photoperiod, allowing to maintain 26±1°C in the wells.
g) Pipettes with enlarged openings for collecting eggs and changing media.
h) Glass containers for preparing different test concentrations and dilution water (beakers, volumetric flasks, graduated beakers, and graduated pipettes).

6.5. Experimental design

6.5.1. Test Compound

The test solutions of the selected concentrations are prepared by diluting a stock solution. Stock solutions should preferably be prepared by mixing or stirring the test compound in the dilution water by mechanical means (for example, stirring and/or ultrasonification). In the present case, venlafaxine-HCl will be dissolved in de-chlorinated heated fresh water and the final nominal concentration in the exposure well plates will be 1 (low), and 10 (high) μg/L. The concentrations were chosen based on the levels reported in the surface waters. The highest concentration is close to the highest reported concentrations (172). The assay includes a negative control prepared only with the embryo culture medium and a positive control prepared with 4.0 mg/L 3,4-dichloroaniline. As the antidepressant in question dissolves easily, it is not necessary to use the

dimethylsulfoxide solvent (DMSO). If DMSO is used, a third DMSO control would be required and all exposure wells, except for positive and negative controls, would have a final solvent concentration of 0.01%. This DMSO control may be included due to the possibility that the solvent itself is capable of causing adverse effects on embryos, which, in the present case, is due to the ability of this compound to pass through cell membranes. (322). The culture medium used will be de-chlorinated fresh water.

6.5.2. Reproduction

a) Zebrafish eggs will be produced by spawning groups (in individual spawning tanks). In the case of spawning groups, males, and females (2: 1 ratio) are placed in breeding tanks a few hours before the onset of darkness the day before the test. As spawning groups of zebrafish can occasionally fail it is recommended to use at least three spawning tanks in parallel. To avoid genetic bias, eggs must be collected from a minimum of three breeding groups, mixed and selected at random.

b) For the collection of eggs, the spawning traps are placed in the spawning tanks before the beginning of darkness on the day before the test or before the beginning of light on the day of the test. To avoid predation of eggs by the adult zebrafish, the breeding traps are covered with an inert wire mesh of an appropriate mesh size (approx. 2±0.5 mm). If considered necessary, artificial plants made of inert material (for example, plastic or glass) can be attached to the mesh as a spawning stimulus. Worn out plastic materials that do not leach (eg. phthalates) should be used. Mating, spawning and fertilization take place within 30 minutes after the start of light and spawning traps with collected eggs can be carefully removed. It is recommended to rinse the eggs with dechlorinated water after collection.

6.5.3. Number Of Larvae Needed For Genetic Testing

Before carrying out the exposure tests to the different compounds, it was necessary to perform a small optimization test to find out the number of larvae necessary to obtain enough ocular material. For this purpose, embryos were placed in 24-well plates only in dechlorinated water and allowed to grow for 144 hours. Then, with the binocular loupe, the eyes were removed to 60, 90 and 120 larvae respectively- this procedure is demonstrated in Figure 10. The eyes were placed in eppendorfs, placed in RNAlater and stored in the chest at -80ºC for further analysis. Three replicates were performed. This step was carried out to find out how much RNA could be taken from each of the different eppendorfs in order not to use animals unnecessarily. However, only the RNA storage step was carried out later. It was not possible, in terms of time, to finish this stage.

6.5.4. Start Of Exposure And Test Duration

The test should start as soon as possible after egg fertilization and be completed after 144 hours of exposure. The embryos must be immersed in the test solutions before cleavage of the blastocyst begins, or at the latest, in the 16-cell phase. To start exposure with minimal delay, the eggs required per treatment group must be selected at random and transferred to their respective concentrations and controls no later than 90 minutes after fertilization. Thus, fertilized embryos from unexposed parents will be collected within 30 minutes

after the first light of the holding tank, selected with a magnifying glass and randomly placed in groups of ten in two 24-well plates filled with 2 mL of solutions and control prepared in hour, Figure 6 and Figure 7.

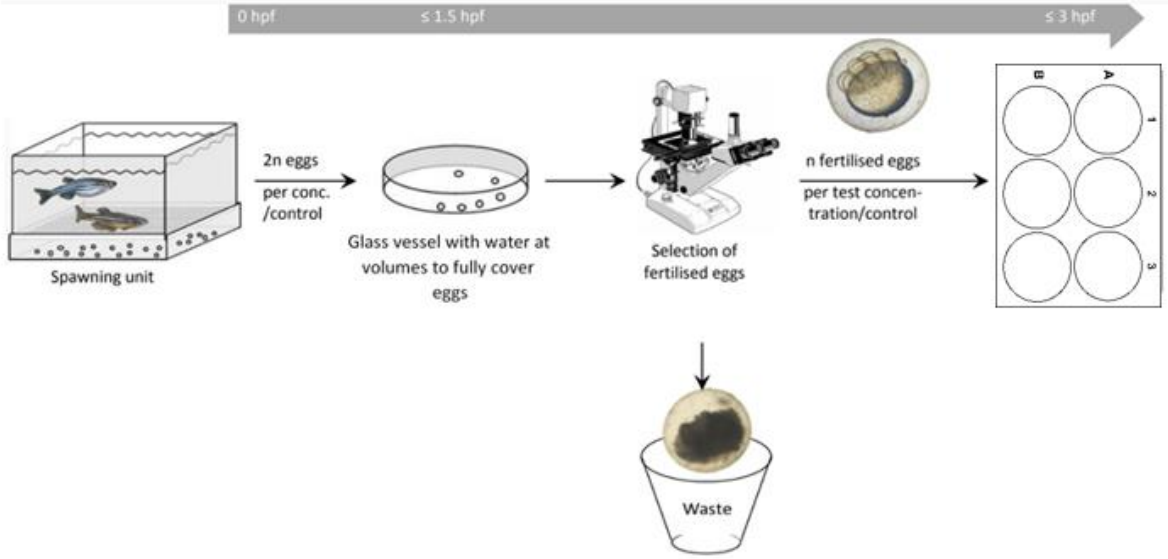

Figure 6. Outline of the zebrafish embryo's acute toxicity test procedure (from left to right): Laying, egg collection, selection of fertilized eggs with binocular magnifying glass and distribution of eggs in 6-well plates prepared with the respective concentrations/controls. (Adapted from: (321)).

Embryos that are alive and actively dividing are considered viable embryos. Zebrafish embryos will be exposed with venlafaxine in concentrations of 0 (controls), 1 (low) and 10 (high) μg/L. The embryos will be incubated at 26°C and 14 hours of light, being observed during 144hpf of incubation. The solutions for each well will be changed daily from the stock solutions made on the first day of exposure, to keep the nominal concentrations of oxygen and antidepressant constant during the test and to remove fungi or other organisms that could develop in the well. When the solution is changed, embryos from all plates will be viewed under an inverted optical microscope, photographed, mortality counted, and dead embryos removed. Controls will be done for each exposure plate to determine background mortality in the embryo set. Mortality can vary between groups of embryos and embryos of unexposed parents should generally exhibit less than 10% mortality. When mortality is greater than 10% in the control wells, embryos should be excluded from the data. For each exposure period there will be 4 plates of 6 wells, 3 beakers of 1L and 12 petri dishes. Each well of 6 well plates contain 10mL of medium with 25 embryos, each beaker contains 80mL of medium with 200 embryos and the petri dishes contain 20mL of medium with 50 embryos. This make a rate of 2,5 embryos/mL which is approximately the recommended (323).

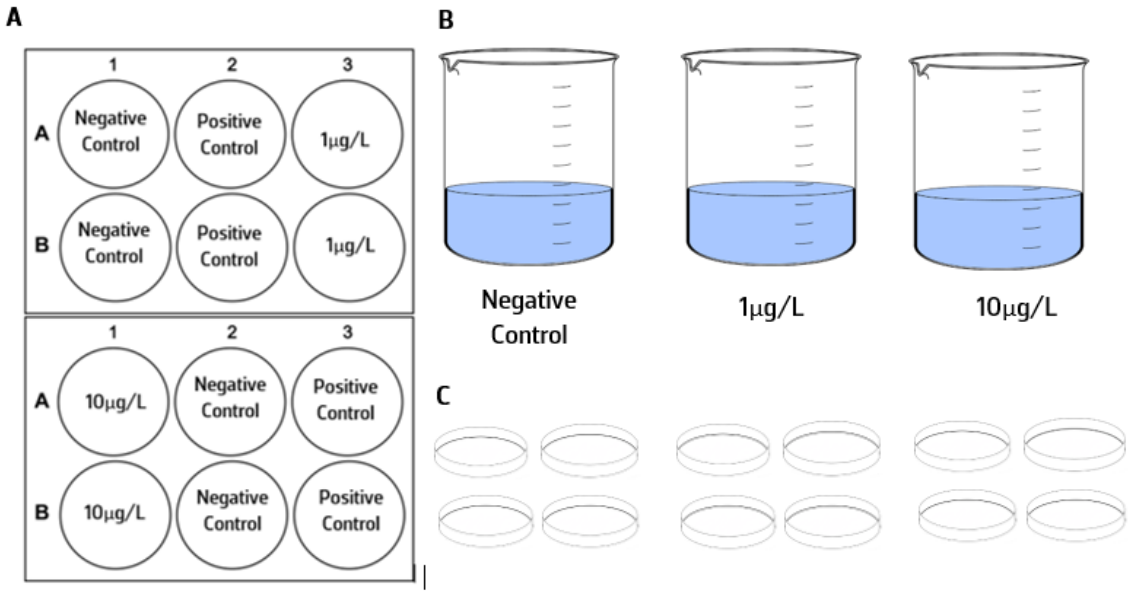

Figure 7. Representative scheme of the experimental design. **A:** 6-well plates in which 25 embryos would be placed in 10mL of medium in each well. These plates were repeated, making a total of 4 plates. Negative control composed of dechlorinated water; Positive Control for 4mg/L of 3,4-dichloroaniline and then the respective concentrations of venlafaxine. **B:** Beakers with 80mL of medium in which 200 embryos were placed in each. **C:** Petri dishes with 20mL of medium in which 50 embryos were placed. Only scheme A was used for observations under the inverted optical microscope, and then the embryos were collected at different times at random from the 6-well plates, beakers, and petri dishes.

6.5.5. Observations And Collection Of Embryos And Larvae

As previously mentioned, observations were made at 8, 24, 48, 72, 96 and 144hpf according to a table provided. However, embryo and larvae were collected at 32, 56, 72, 96 and 144 hpf for eppendorfs, placed in RNAlater and stored at -80°C. The collection of material was performed at these hours due to the analysis of the time scale in which the selected genes present in Table 4 were expressed in the retina.

6.6. Methods

6.6.1. Morphometric Analyzes

The embryos will be observed at 8, 24, 48, 72, 96 and 144hpf under an inverted microscope (Nikon Eclipse TS100) to assess their viability, images of each embryo were captured using Nikon's NIS-Elements D program. In non-hatched embryos, the perimeter of the surface of the chorion and eyes were measured, as well as the largest and smallest radius, Figure 8. Using this same program, all measurements of the eyes were performed - largest radius, smallest radius and perimeter - and animal size -Figure 9.

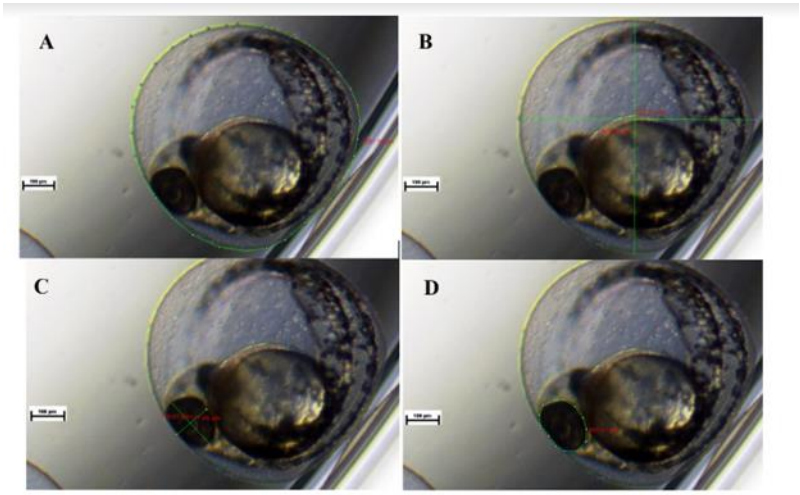

Figure 8. Exemplification of the measurement of the perimeter of the chorion (A), average rays of the chorion (B), average rays of the eyes (C) and ocular perimeter (D). (From: (324))

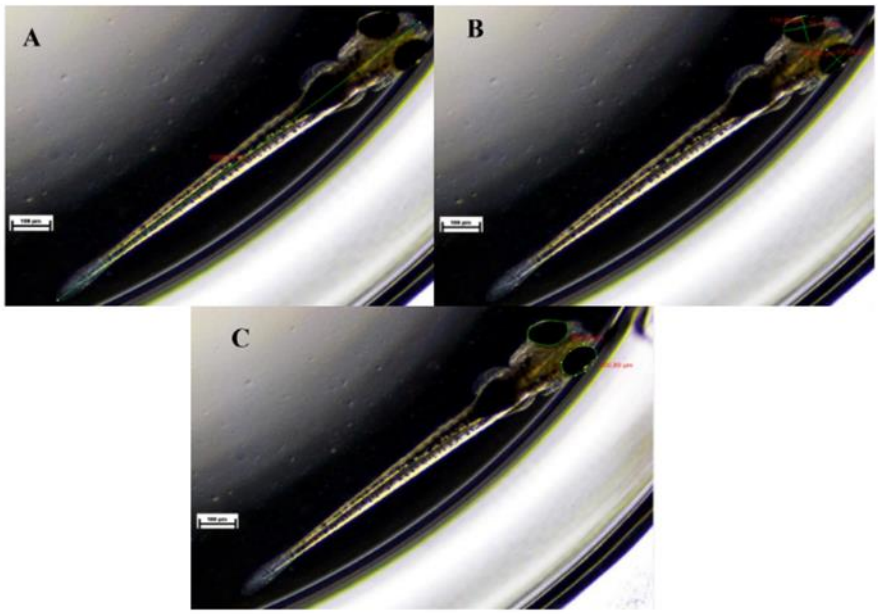

Figure 9. Exemplification of the measurement of the larval length (A), medium rays of the eyes (B) and ocular perimeter (C). (From: (324))

6.6.2. Tissue Sampling

From 24 to 144 hpf the total embryos would be collected and stored in RNAlater for further evaluation of gene expression. However, second tests would be carried out in which the embryos would grow to 144hpf (without any collection before) and only then would they be collected to remove the eyes. Thus, these zebrafish larvae would be submerged in ice for 15 minutes until they are anesthetized. Then, each embryo would be transferred to a Petri dish and an incision would be made on the dorsal side of the head from the forehead. Subsequently, the medial side of the eyes would be exposed, and a second incision would be made in the posterior area of the eyes, causing their separation, Figure 10. The fish would be sacrificed by rupture of the spine and the entire procedure would be performed using the binocular loupe.

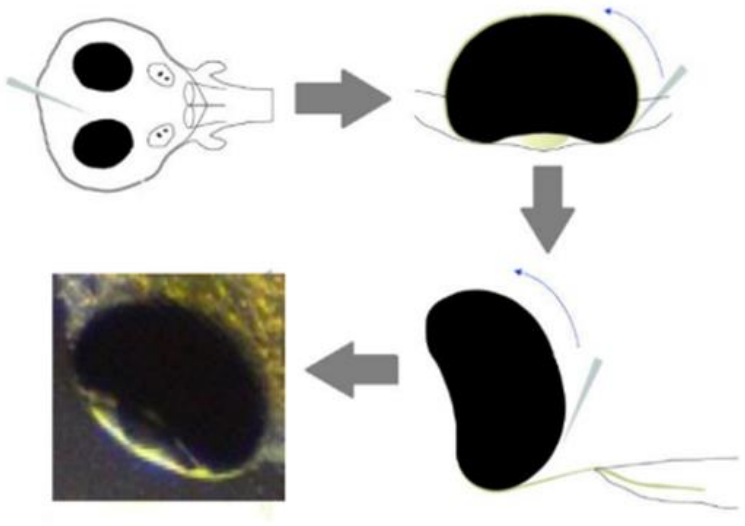

Figure 10 . Zebrafish eye dissection procedure. (Adapted from: (325))

6.6.3. Genetics Analyses

Although there was no possibility of reaching this part of the work, the following protocol would be similar to the one presented below.

6.6.4. Primers

Here are some primers for genes expressed on the zebra fish retina described in the literature. Two reference genes are also referred to for later comparison. Table 5shows the sequences found for the interest genes.

Table 5. Primers found in literature for retina genes of zebrafish.

Gene	Forward Primer	Reverse Primer	Source
pde6c	5'-TTGGCCTCTGGAATACTGGCTCTC-3'	5'-GTTTGACCAGAACCCGGAAG-3'	(265)
pax6a	5'-GGAACCGTGCGTCTCATAAC-3'	5'-GAAGTGGCACTATCCCCGTA-3'	(326)
pax6b	5'-TTCACTGTTTTGCTCGGAAG-3'	5'-GTCCAGCTTCTCGCTCAGTC-3'	
rho	5'-GGCTTCACCACCACCATGTA-3'	5'-GTGGTGATCATGCAGTGACG-3'	(327)
six3a	5' - AGTTTCCCCTGCCTAGAACC-3'	5'-AAACCAATTTCCGACCTGTG-3'	(328)
six3b	5'-TCAACAAGCACGAGTCCATC-3'	5'-GCAGCTTCTCTGCTTCTTGG-3'	
rx3	5'-AGTCAATTCTGGGATTTAAGGGAGAGA-3'	5'-GCTGACCATTACAGTAGACCTACAAAC-3'	(329)
atoh7	5'-CCAGAGACCCGGAGAAGTTTGAGAGT-3'	5'- CCAGTCTTTCTGAGGATCCACGTGTC-3'	
vsx1	5'-TCAGGGAACTCTCAAAAGAGGAAAAA-3'	5'-ACCTGTATCCTGTCCTCTGGTAGCTCT-3'	
vsx2	5'-CCTGGAGGTATTCCGTCTTTCTACAGT-3'	5'-CTGGCT CAGTGAAGACTTTGACATTTT-3'	
Reference Genes			
ef1	5'-GGACACAGAGACTTCATCAAGAAC-3'	5'-ACCAACACCAGCAACGT-3'	(330)
actb1	5'-TCCCAAAGCCAACAGAGAGAAG-3'	5'-GTCACACCATCACCAGAGTCC-3'	

6.6.5. RNA Isolation And cDNA Synthesis

Embryos from the toxicological and accumulation assays, preserved in RNAlater, would be used to isolate total RNA. Briefly, total RNA would be isolated using Illustra RNAspin Mini RNA Isolation kit (GE Healthcare), according to the manufacturer's protocol. RNA quality would be verified by electrophoresis in agarose gel and by the measurement of the ratio of optical density at 260/280 nm. RNA would be quantified using Quant-IT RiboGreen RNA Reagent and Assay Kit (Invitrogen) using a fluorescent microplate reader (Fluoroskan Ascent, Labsystems). One microgram of total RNA would be subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen) and synthesis of cDNA would be executed using Iscript cDNA Synthesis (Biorad). For example, extractions of six different pools of embryos per treatment could be performed (330).

6.6.6. Quantitative Real-Time PCR (qRT-PCR)

Gene expression of genes that would be selected later could be assessed by means of quantitative real time PCR (qRT-PCR). Primer pairs for each target gene could be selected from existing literature or designed using Primer 3 software available in http://www.ncbi.nlm.nih.gov/, based on available sequences in GeneBank. Genebank accession numbers of target sequences were given in Table 4. Identities of the amplicons would be confirmed by cloning and sequencing of the DNA fragments. To determine the efficiency of the PCR reactions, standard curves would be made, with 6 serial dilutions of the template (concentrations range from 0.05 to 50 ng/l), and the slopes and regression curves would be calculated. Reactions for qRT-PCR would be conducted in an iQ5 BioRad, with 10L of SYBR Green Supermix (BioRad), 2L of each primer (final concentrations ranging from 0.001 M to 0.6 M) and 2L of cDNA, in a total volume of 20L, in duplicate. Conditions, in general, would be as follows (noticed that some adjustments could be done): 95ºC for 3 min, followed by 40 cycles of 95ºC for 10 s, and 72ºC for 30s. At the end of each run a melting curve analysis was done (from 55 to 95ºC) to determine the formation of the specific products. Gene expression would be quantified by normalization with multiple reference genes (for example: *elongation factor 1 (ef1)* and *actin β1 (actb1)*) using Normfinder algorithm. The relative expression ratio could be calculated considering the efficiency of each gene using the Pfaffl mathematical model. To calculate ΔΔct, the control group comprised the average of Cts of the controls from each group. Data would be presented as mean of mRNA level in relation to the reference genes (330).

6.6.7. Statistical Analyses

Differences in mRNA expression and the differences among treatments in accumulation assays could be evaluated by means of a one-way ANOVA, followed by a multiple comparison test (Dunnett's test) at a 5% significance level. Data could be log (mRNA expression and enzyme activities) or square root (accumulation assays) transformed in order to fit ANOVA assumptions. All tests could be performed with Statistica 7 (Statsoft, Inc). Differences in the frequency of each type of abnormality among treatments could be analyzed in the toxicological assays at 5% significance level. Tests would be performed with SPSS. Data would be presented as mean ± standard error.

6.7. Expected Results

6.7.1. Mortality, Gross Malformations And Morphometric Analyzes

Regarding mortality, a rate close to 100% can be expected at the end of 144hpf for the positive control, as this is well studied with regard to the induction of toxicity in fish species in their initial stages of life (331). For the negative control, a mortality rate of no more than 20% should be expected, since the embryos are only in dechlorinated water and as such there is no toxic compound that can harm their development. Eggs degenerate because of unsuccessful fertilization or development failures. The quality of the batch of eggs seems to depend on the female fish, as some females consistently produce good quality eggs, others never will. Also the development rate and the rate of hatching vary from one batch to another (320). For venlafaxine concentrations, slightly higher mortality compared to the negative control may occur. An increase rate of embryo malformations can be found and altered dark pigmentation in embryos and larvae, related to melanocytes, can be the most frequent anomaly found. However, venlafaxine could cause an increase in yolk egg malformations and spinal deformities (332). Besides that, an acceleration in early development and an increase in hatching rate to 48hpf could happen for venlafaxine (156). For perimeter and length, in principle, changes to these parameters will not be expected (171).

6.7.2. Gene Expression

There is little information in the literature regarding the influence of antidepressants, especially venlafaxine, on the expression of visual genes, making it difficult to make possible predictions about the results. However, taking into account some of the studies, a down-regulation or up-regulation of the rhodopsin *(rho)* gene may occur, because these outcomes depend heavily on the modes of action of the tested antidepressants. If there are changes in this gene, there will be a disturbance in the ocular development of the animals (236). Regarding the *pax6* gene, it is expected to occur down-regulation, because this event was verified in a study in which the zebrafish was exposed to venlafaxine (242). In the case of the visual homeobox gene *(vsx1)* an enrichment in its expression can be observed (263). Some studies report a kind of interconnection between the *atoh7* gene and the *pax6* gene and, as such, if changes in the expression of *pax6* occur, it may also affect the expression of *atoh7* and, thus, the development of the retina will be affected (278).

Chapter 7 . Conclusions

7. Conclusions

This review provides the reader with the current knowledge about the impact of neuroactive drugs on non-target organisms living in aquatic environment more specifically in visual system. The implications of chronic, and low concentration, exposure to pharmaceutical compounds in aquatic organisms and humans remains partially unknown. Currently, most of the studies focus on estrogens effects at the environmental level; however, various types of compounds (including pharmaceutical and personal care products) are found in the aquatic environment. As shown by several studies, antidepressants and their biologically active metabolites are present in municipal wastewater and in river water downstream of WWTPs, constituting a potential for modest accumulation of these compounds into fish that inhabit these locations.

Antidepressants are correlated with neuroplasticity in hippocampus and prefrontal cortex where there is stimulation of neurogenesis, gliogenesis, dendritic arborization and new synapse formation. In the case of nervous system these pharmaceuticals have the aptitude to directly affect the CNS and disrupt neuro-endocrine signaling. As the visual system is an extension of CNS it is expected that the structures of the eye may be very sensitive to the action of this type of drugs, observing similar effects. Thus, as mentioned throughout this review, antidepressants can cause changes in the expression of some genes involved, for example, in phototransduction, in light receptors, development of the lens and layers of the retina. Consequently, aquatic animals with alterations in these structures may develop changes in predator–prey relationships, reproduction patterns, food-web dynamics, survival, decrease visual acuity of prey to a point at which they cannot avoid predators approach thereby increasing their probability of capture and foraging activities. This can potentially lead to population declines and community-level changes.

These data reveal that the biologically active metabolites of emerging pollutants should be incorporated in any evaluation of the risks to the environment, aquatic organisms and to human health as a result of the release of these substances into the aquatic environment. For better understanding of possible effects, a mechanism-based approach focused on target tissues, molecules and organs should yield more meaningful results and insights than traditional acute toxicity testing. Disturbances of the reproductive system and hormone system, neurobehavioral changes, to name some key targets, may have far reaching effects on the population level.

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