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Physiological Endpoints for Potential SSRI Interactions in Fish

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Selective serotonin reuptake inhibitors (SSRIs) are among the pharmaceutical compounds frequently detected in sewage treatment plant effluents and surface waters, albeit at very low concentrations, and have therefore become a focus of interest as environmental pollutants. These neuroactive drugs are primarily used in the treatment of depression but have also found broader use as medication for other neurological dysfunctions, consequently resulting in a steady increase of prescriptions worldwide. SSRIs, via inhibition of the serotonin (5-hydroxytryptamine, 5-HT) reuptake mechanism, induce an increase in extracellular 5-HT concentration within the central nervous system of mammals. The phylogenetically ancient and highly conserved neurotransmitter and neurohormone 5-HT has been found in invertebrates and vertebrates, although its specific physiological role and mode of action is unknown for many species. Consequently, it is difficult to assess the impact of chronic SSRI exposure in the environment, especially in the aquatic ecosystem. In view of this, the current knowledge of the functions of 5-HT in fish physiology is reviewed and, via comparison to the physiological role and function of 5-HT in mammals, a characterization of the potential impact of chronic SSRI exposure on fish is provided. Moreover, the insight on the physiological function of 5-HT strongly suggests that the experimental approaches currently used are inadequate if not entirely improper for routine environmental risk assessment of pharmaceuticals (e.g., SSRIs), as relevant endpoints are not assessed or impossible to determine.

Keywords antidepressants, environmental risk, fish serotonergic system, HPG axis, HPI axis, 5-hydroxytryptamine, immune system, SSRIs

INTRODUCTION

Antidepressants in the Aquatic Environment

In recent years, the issue of pharmaceutical residues in the aquatic environment has received increasing attention. Many compounds have been found to be not or only partially biologically degraded in sewage treatment plants (STPs).[1] Consequently, some of these bioactive substances are continuously discharged into receiving waters, potentially leading to chronic exposure of aquatic species.[2] Although environmental concentrations of pharmaceuticals are extremely low (nanograms per liter) when compared to other pollutants, such as pesticides, household and personal care products, aquatic organisms may be affected at a subtoxic level, as has been reported for estrogens.[3] Subtle effects may result in long-term changes that may become manifest at the individual and population level, only to be detected following several generations of a given species in an ecosystem.[2–4] Such subtle effects, and not the routinely determined overt toxicity at extremely high concentrations, are causally related to a highly specific mode of action. This obviously pertains to pharmaceuticals, which are tailored toward interaction with a specific molecular target or system, and thus a specific mode of action is desired in order to demonstrate efficacy in patients at relatively low concentrations of the pharmaceutical. However, as the pharmacological targets in humans, for example, neurotransmitter systems in the brain, are often phylogenetically highly conserved across numerous kingdoms and phyla, including fish, a potential for adverse effects in environmental species, even at the low concentrations observed in the environment, may be hypothesized.[5] When potential subtle effects in various species are considered, it is quite obvious that the standardized ecotoxicological tests employing acute exposure to pharmaceutical concentrations several orders of magnitude above those observed in the environment are incapable of delivering useful data for environmental risk assessment of pharmaceuticals.

Selective serotonin reuptake inhibitors (SSRIs) are best known for their antidepressive properties, but are also

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administered in the treatment of other psychatric disorders, e.g., anxiety, eating disorders, and obsessive-compulsive disorder in connection with Tourette's syndrome. Since fluoxetine (Prozac™, Fontex™, Seromex™, Seronil™, Sarafem™) was marketed as one of the first SSRIs available for clinical application in the 1980s,[6] the use of SSRIs in human therapeutics has increased steadily. The number of SSRI prescriptions in Canada rose from 8.7 million to 15.7 million between 1999 and 2003, representing an increase of 80.3%.[7] A similar trend is apparent in the United States, where the number of prescriptions for fluoxetine rose from 7.3 million in 2000 to 23.0 million in 2005.[8] Paxil™ (paroxetine hydrochloride) headed the RxList of the Top300 prescriptions for 2005, based on the number of U.S. prescriptions dispensed.[9] Comparable developments have been observed in Australia and in many European countries.[10] In Norway, there has been a 320% increase in SSRI consumption since 1996.[11] Consequently, the issue of SSRIs and their metabolites as micropollutants in the aquatic environment has received more and more attention over the past years. As these drugs require chronic administration to elicit the desired therapeutic effect in patients, it can be assumed that when the current prescription trend is sustained and the per capita wastewater treatment effluent to surface water ratio steadily increases, the environmental concentrations of these pharmaceuticals will also increase. It should also be taken into account that there may be seasonal variations, that is, the number of SSRI prescriptions may peak during the fall and winter, caused by a higher incidence of anxiety and depression ("winter depression").

Fluoxetine and several other SSRIs have been detected in sewage water as well as in surface waters (Table 1). Paroxetine and sertraline were detected in raw sewage effluents from a psychiatric hospital in Tromsø (Norway) at concentrations of 20 ng/L and 100 ng/L, respectively.[12] In a subsequent study, water samples from three different STPs and a pump station were analyzed for the presence of the five most common SSRI formulations: citalopram, sertraline, paroxetine, fluoxetine, and fluvoxamine.[11] The highest levels were found for citalopram, with 612 ng/L and 382 ng/L in the Hamna STP influent and effluent, respectively, demonstrating the limited retention and biological degradation of SSRIs in STPs. Fluoxetine was also detected in U.S. streams at an estimated maximum concentration of 12 ng/L[13] and in STP effluents at concentrations of up to 540 ng/L[14]; however, the latter value is rather high compared to other analytical data and most likely not representative for the SSRI levels in U.S. STP effluents. In Canada, the distribution of fluoxetine in surface waters near STPs demonstrated maximum concentrations of 99 ng/L and 46 ng/L in effluent and receiving water, respectively.[15] The pharmacologically active

TABLE 1
SSRI detection in the aquatic environment. *Note* LOD, limit of detection; n.a., not available; STP, sewage treatment plant

Compound	Concentration (ng/L)	Sampling site	Number of samples, detection method (recovery)	LOD (ng/L)	Reference
Fluoxetine	50 ± 5	Peterborough STP effluent	n= 3, LC-ESI-MS/MS	5–20	15
	38 ± 3	Burlington STP effluent			
	99 ± 7	Little River STP effluent			
	13 ± 1	Hamilton Harbour			
	46 ± 4	Little River			
Fluoxetine	12 (estimated)	U.S. streams	n= 84, LC-ESI-MS, (≤60 %)	18	13
Fluoxetine	540	STP effluent	n = 3? LC-ESI-MS, (79–82 %)	n. a.	14
Fluoxetine	17	STP influent	n = 6, LC-ESI-MS/MS	0.1	16
	25	STP effluent			
	2.6	Las Vegas Wash			
Norfluoxetine	9.9	STP influent	n = 6, LC-ESI-MS/MS	0.1	16
	3.9	STP effluent			
	1.3	Las Vegas Wash			
Paroxetine	20	Åsgård sewer	n= 1, semiquantitative LC-ESI-MS/MS	n. a.	12
Sertraline	100	Åsgård sewer	n= 1, semiquantitative LC-ESI-MS/MS	n. a.	12
Citalopram	32.7	Sjølund pump station	LC-ESI-MS	n. a.	11
	13.0	Langnes STP influent			
	9.2	Langnes STP effluent			
	145	Breivika STP influent			
	62.0	Breivika STP effluent			
	612	Hamna STP influent			
	382	Hamna STP effluent			

fluoxetine metabolite norfluoxetine (see next section) was either not determined[13] or not detected[14,15] in these studies due to methodological restrictions.[16] However, some information is available from a most recent study where norfluoxetine was reported in water samples (STP influent and effluent, surface water, drinking water) with the highest concentration of 9.9 ng/L in STP influent.[16] However it must be noted that the available analytical data on environmental SSRI levels are somewhat biased, as most data stem from sampling sites specifically chosen for SSRI contamination, i.e., downstream of STPs of intensely urbanized areas[13] or in the vicinity of STPs receiving hospital effluents.[11,12] Moreover, in order to properly evaluate the environmental contamination and bioavailability of SSRIs, the ready dissipation of SSRI (e.g., fluoxetine) from the aqueous phase to sediments has to be accounted for.[17] Indeed, information on the presence of SSRIs in sediments and therefore aqueous to sediment partitioning and the respective environmental consequences is yet lacking.

Despite rapid analytical developments, there is still a dearth of knowledge regarding the environmental half-life and bioavailability of norfluoxetine in the aquatic environment. In addition to ambient exposure, norfluoxetine may be produced via metabolic conversion of fluoxetine in aquatic organisms. In a preliminary study, Brooks and coworkers[18] examined three different fish species, bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), and black crappie (*Pomoxis nigromaculatus*), from an effluent-dominated stream in northern Texas. Brain, liver, and muscle tissue samples were analyzed for the presence of fluoxetine, sertraline, and their respective metabolites, norfluoxetine and desmethylsertraline. Unfortunately, the study failed to provide data on the concentrations of these compounds in the ambient water at the sampling sites. Nonetheless, the target compounds were found in all tissues of the species examined at levels greater than 0.1 ng/g homogenate, with a detection limit of 0.01 ng/g. In all three species, the highest concentrations were detected in brain (mean $\pm$ standard deviation: fluoxetine 1.58 $\pm$ 0.74 ng/g; norfluoxetine 8.86 $\pm$ 5.9 ng/g; sertraline 4.27 $\pm$ 1.4 ng/g; desmethylsertraline 15.6 $\pm$ 14.3 ng/g) and liver (fluoxetine 1.34 $\pm$ 0.65 ng/g; norfluoxetine 10.27 $\pm$ 5.73 ng/g; sertraline 3.59 $\pm$ 1.67 ng/g; desmethylsertraline 12.94 $\pm$ 10.45 ng/g). The physiological consequences of these concentrations in exposed fish are yet unclear, as presently almost no information is available on SSRI pharmacokinetics and pharmacodynamics in fish species.

Uptake of lipophilic xenobiotics ($\log K_{ow} > 3$) into the bloodstream in fish primarily occurs across the gills; that is, the amount of uptake via ingestion appears to be negligible in view of the normally low feeding rates and water intake of fish.[19] In contrast to the situation in mammals (intestinal uptake), xenobiotics absorbed via fish gills are distributed systemically and thus are not subject to a "first-pass effect" in the liver—that is, metabolic conversion and partial excretion prior to systemic distribution.[20] The latter considerations are important when mammalian data are employed in the evaluation of fish pharmacokinetics and exposure levels. Nonetheless, mammalian toxico- and pharmacodynamics and metabolism of SSRIs may serve as a basis to estimate the behavior and potential adverse effects of these pharmaceuticals in fish.

Physicochemical Properties and Pharmacokinetics of SSRIs

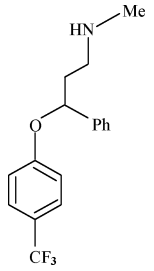
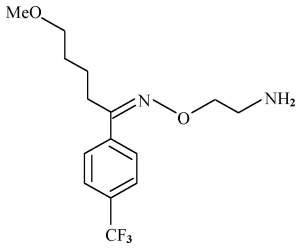
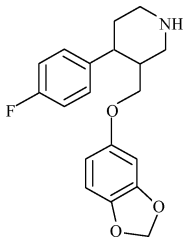
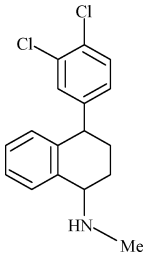
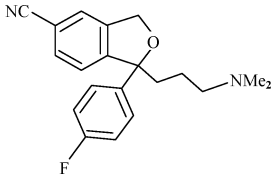
Compounds classified as SSRIs share a common primary mode of action as antidepressants, i.e. inhibition of the specific serotonin transporter (5-hydroxytryptamine transporter, 5-HTT) in the central nervous system. However, the pharmacologically active substances display some considerable differences in their chemical structure, pharmacokinetic properties, and metabolism[21] (Table 2).

Although all of the SSRIs presented in Table 2 are moderately to highly lipophilic, fluoxetine has by far the largest volume of distribution (up to 100 L/kg body weight), indicating extensive tissue accumulation.[6] Fluoxetine also appears to represent a special case with regard to pharmacological activity as its metabolic conversion, mainly *N*-demethylation catalyzed by the cytochrome P-450 (P450) isoenzyme CYP2D6[22] in the human liver, results in the production of the equally active metabolite norfluoxetine. Under steady-state conditions, the concentration of norfluoxetine usually exceeds the concentration of fluoxetine.[6] The pharmacological half-life ($t_{1/2}$) of fluoxetine ranges between 1 and 4 days, while norfluoxetine is even more stable in human plasma ($t_{1/2}$ = 7–15 days). The half-lives of the other SSRIs range between 24 and 36 h (Table 2). As a consequence of the high volume of distribution and thus long $t_{1/2}$ of fluoxetine, treatments of 1 to 22 months are necessary to achieve steady-state conditions in human patients. The primary excretion route for all SSRI formulations and their metabolites is via urine.[21]

The various SSRIs often not only are substrates for specific cytochrome P-450 isoenzymes but also display inhibitory activity and may either regulate their own metabolism via a negative feedback mechanism or block other P450 isoenzymes; therefore, a common feature of all formulations is their potential for drug–drug interactions with other classes of pharmaceuticals, as reviewed by Hiemke and Härtter.[6] For example, clinical pharmacokinetic studies demonstrated that fluoxetine, as well as its main metabolite norfluoxetine, may affect the biotransformation pathways of other pharmaceutical compounds, such as the tricyclic antidepressant clomipramine[21] and the sedative midazolam[23] metabolized by CYP2D6 and CYP3A4, respectively, thereby impairing the clearance of these substances. Fluvoxamine was shown to be an inhibitor of several CYP subtypes, including CYP1A2, CYP2D6, and CYP3A4,[6] and may also interfere with the metabolism of CYP2C9 substrates.[24] Further potential interactions of SSRI formulations with various drugs through P450 enzyme inhibition have been summarized by Park and colleagues.[25]

As mentioned earlier, norfluoxetine and the sertraline metabolite desmethylsertraline were detected in different tissues

TABLE 2
Physicochemical and pharmacokinetic properties of commonly prescribed SSRIs.[201] *Note*. bw., body weight; conc., concentration; Me, methyl group; n.a., not available; Ph, phenyl group

Generic name (CAS number)	Structure	Chemical formula, MW (g mol^{-1}), log K_{ow}	Therapeutic dose, V_d (L/kg bw.)[6], bioavailability[21], plasma conc. (ng/ml)	Protein binding[21], $t_{1/2}$, P450 isoenzyme (kinetics type)[6]
Fluoxetine (54910-89-3)		$C_{17}H_{18}F_3NO$ 309.33 1.57[14]–5.37[202]	20–60 mg/day[22] 14–100 <90% 50–480[22] (NFX: 50–450)	>95% 1 to 4 days CYP2D6 CYP2C9 (nonlinear)
Fluvoxamine (54739-18-3)		$C_{15}H_{21}F_3N_2O_2$ 318.34 3.63[202]	150 mg/day[21] ∼25 ∼50% n.a.	77% 15.6 h CYP1A2 CYP2D6 (nonlinear)
Paroxetine (61869-08-7)		$C_{19}H_{20}FNO_3$ 329.37 3.369[202]–4.74[203]	20 mg/day[21] 2–12 ∼50% 10.7[204]	∼95% ∼24 h CYP2D6 (nonlinear)
Sertraline (79617-96-2)		$C_{17}H_{17}Cl_2N$ 306.23 5.29[205]–5.567[202]	50 mg/day[21] >20 n.a. n.a.	>95% 26 h CYP2B6 CYP2C19 CYP2C9 (linear)
Citalopram (59729-33-8)		$C_{20}H_{21}FN_2O$ 324.39 3.74[205]–4.222	40 mg/day[21] 14[21]–20[206] ∼80% n.a.	80% 35 h CYP2C19 (linear)

of several fish species[18] and may at least partially be produced by enzymatic conversion. Presumably, the enzymes responsible for SSRI metabolism in fish also belong to the cytochrome P-450 superfamily, as these key metabolizing enzymes are highly conserved among vertebrates and may display a similar substrate specificity.[26] In the Japanese puffer fish (*Takifugu rubripes*), also known as fugu, orthologous nucleotide sequences from 17 of 18 mammalian P450 families were detected.[27] Even though McArthur et al.[28] observed that teleost and mammalian P450 genes belonging to the CYP3A subfamily have undergone

independent diversification in the course of gene duplication events that may have coincided with the acquisition of new functions, it can be expected that SSRIs may interact with and inhibit some P450 isoenzymes in fish. This would be of particular concern with regard to the interaction with P450 isoenzymes responsible for steroid metabolism and thus hormonal homeostasis in fish and in view of the presence of heterogeneous pharmaceutical mixtures in the aquatic environment.

In order to assess the potential risk of SSRI exposure to aquatic wildlife, i.e. fish species, a better understanding of the involved physiological and pharmacological mechanisms would be advantageous. As serotonin is a phylogenetically ancient neurotransmitter, it is highly conceivable that SSRIs, active in humans and other mammals, could display similar pharmacological activites in fish. However, due to the complexity of the serotonergic system and, in particular, its interactions with other neurotransmitter and hormone systems, the downstream effects of SSRI action on known pharmacological target molecules in fish may differ considerably. In addition, SSRIs may affect other signal transduction pathways in fish that are either not present or differently regulated in humans.[2] As a consequence, standardized acute exposure experiments investigating the ecotoxic potential of SSRIs are most likely not sufficient to assess the risk of chronic exposure to low environmental concentrations.

It is beyond the scope of this review to address the potential cumulative/synergistic/additive effects of mixtures of SSRIs or other pharmaceutical agents acting on the serotonergic system (tricyclic antidepressants [TCAs], monoamine oxidase [MAO] inhibitors). Nevertheless, the possible occurrence of these interactions under environmental exposure conditions should be kept in mind. In order to evaluate the potential impacts of chronic SSRI exposure in the aquatic environment and in view of the potential interactions of SSRIs with fish P450 enzymes, 5-HT receptors, and 5-HTT, the currently known anatomical features and physiological functions of the mammalian and fish serotonergic system are compared.

THE SEROTONERGIC SYSTEM

Mammals

In addition to its role as vasoconstrictive agent in blood vessels, serotonin (5-hydroxytryptamine, 5-HT) is best known as a neurotransmitter of the central nervous system. The analysis of various mammalian species, representing different phyla, with methods such as immunohistofluorescence and radiography, revealed that the anatomical organization of the vertebrate serotonergic system is highly conserved.[29] A characteristic accumulation of 5-HT-positive cells occurs in the midline raphe region of the mammalian brain, projecting numerous fibers into different areas of the telencephalon.[29] In the mammalian brain, serotonergic neurons generated during embryonic development of the organism represent one of the most ramified neuronal networks.

In chemical terms, 5-HT is a hydrophilic indoleamine derived from the amino acid tryptophan. It has been recognized that the key enzyme for 5-HT synthesis is the tryptophan hydroxylase (TPH), which converts tryptophan to 5-hydroxytryptophan (Figure 1), a process limited by tryptophan availability.[30]

5-HT is stored in presynaptic vesicles or in storage cells to be released in response to a stimulating signal. As neurotransmitter,

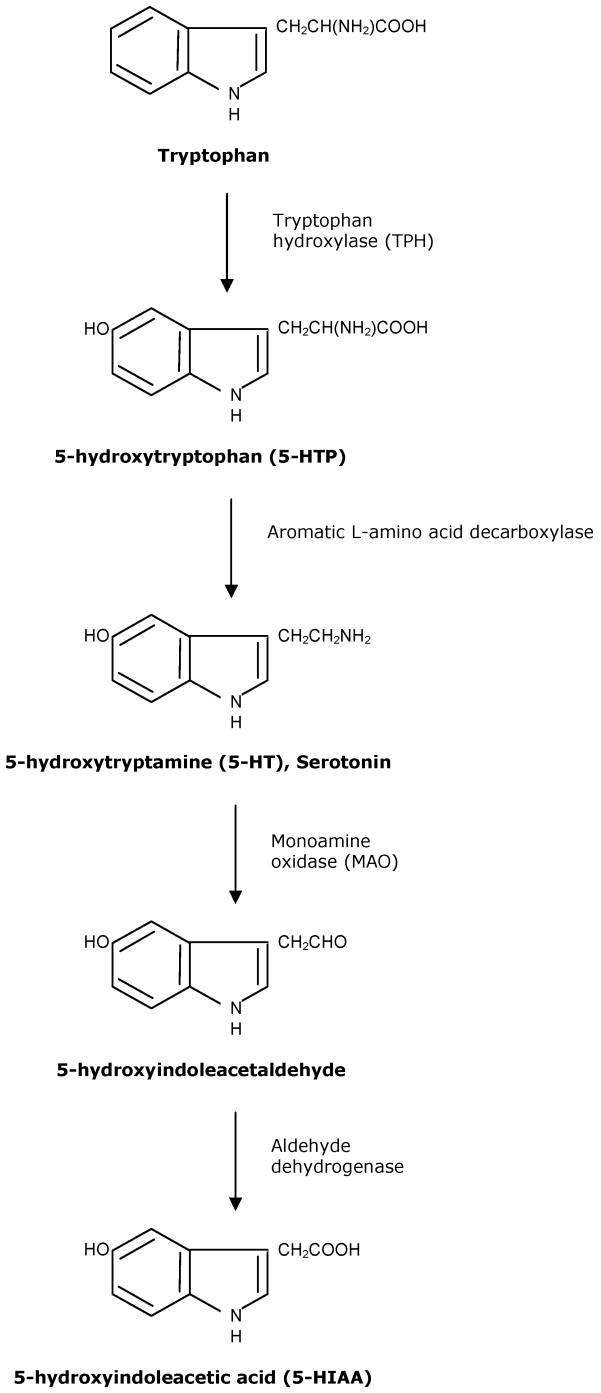


FIG. 1. Serotonin metabolism, modified from Fuller.[197]

TABLE 3

5-HT receptor subtypes and signaling pathways in various species, classification based on pharmacological profile, second messenger system and nucleotide sequence homologies.[32,60,207,208] *Note*. AC, adenylate cyclase; PLC, phospholipase C; a) predicted, based on mRNA fragments; b) $G_{i/0}$, G_s, and G_q are G protein subtypes; c) 5-HT_{1B} (rodents)/5-$HT_{1D\beta}$ (human): species homologues (97% sequence similarity)[32]; d) no functional protein detected.

Family	Transduction pathway [b)]	Subtypes	Species
5-HT_1	$G_{i/0}$ inhibition of AC, stimulation of K^+efflux	5-HT_{1A}	Human, rodents (mouse, rat), other mammals (cat, cow, dog), chicken[a)], frog, fish (Arctic charr, fugu, tilapia, trout), sea urchin[a)]
		5-HT_{1B}/ 5-$HT^{c)}_{1D\beta}$	Human, rodents (mouse, rat, guinea pig, hamster), other mammals (cow, pig, oppossum), chicken[a)], fish (trout, zebrafish[a)]), sea urchin[a)]
		5-HT_{1D}/ 5-$HT_{1D\alpha}$	Human, rodents (mouse, rat, guinea pig), other mammals (cow, pig), fish (fugu, tilapia)
		5-HT_{1E}	Human, rodents (mouse, rat, guinea pig), other mammals (cow, dog[a)])
		5-HT_{1F}	Human, rodents (mouse, rat, guinea pig), pig
5-HT_2	G_q stimulation of PLC, inhibition of K^+ efflux	5-HT_{2A}	Human, rodents (mouse, rat, guinea pig), other mammals (cow, dog, pig, rhesus monkey), fish (zebrafish[a)])
		5-HT_{2B}	Human, rodents (mouse, rat, guinea pig), other mammals (cow, dog, pig), fish (zebrafish)
		5-HT_{2C}	Human, rodents (mouse, rat), other mammals (chimpanzee, cow, pig), fish (zebrafish[a)]), sea urchin[a)]
5-HT_3	ligand-gated ion channels	5-HT_{3A}	Human, rodents (mouse, rat), other mammals (dog, ferret)
		5-HT_{3B}	Human, rodents (mouse, rat), rabbit
		5-HT_{3C}	Human
		5-HT_{3D}	Human
		5-HT_{3E}	Human
5-HT_4	G_s stimulation of AC	?	Human, rodents (mouse, rat), cow, fish (zebrafish[a)])
5-$ht^{d)}_5$	$G_{i/0}$?	5-ht_{5A}	Human, rodents (mouse, rat, guinea pig), fish (zebrafish[a)])
		5-ht_{5B}	Rodents (mouse, rat[a)])
5-HT_6	G_s	?	Human, rodents (mouse, rat), chimpanzee, fish (swordtail, zebrafish[a)])
5-HT_7	G_s	?	Human, rodents (mouse, rat, guinea pig, golden hamster), fruit fly, fish (zebrafish[a)])

5-HT binds to presynaptic and postsynaptic receptors resulting in activation of intracellular messenger systems. By activating somatodendritic autoreceptors, 5-HT has an inhibitory effect on its own release from serotonergic neurons in the dorsal raphe region.[31] On the basis of structural and functional properties, seven families of 5-HT receptors have been recognized in mammals to date, with some containing several subtypes[32], resulting in a total of at least 15 members.[33] With the exception of the 5-HT_3 family, which is a member of the ligand-gated ion channel receptor superfamily, all known 5-HT receptors are coupled to a G-protein[32] (Table 3).

The 5-HT receptor class represents one of the most versatile neurotransmitter receptor classes known to date with most likely more subtypes being identified in the future.[34] Moreover, the occurrence of multiple splice variants, RNA edited isoforms or receptor homo- and heterodimerizations cannot be excluded.[32] As integral elements of serotonergic signal transduction, 5-HT receptors represent the primary drug target sites. Consequently, pharmaceutical development has provided for various substances with agonistic or antagonistic properties applicable to research and for medicinal purposes (Table 4).

Following interaction with and consequently activation of postsynaptic receptors, 5-HT is removed from the extracellular space, thereby terminating neurotransmission (Figure 2). Removal occurs by means of active transport into presynaptic nerve endings, where 5-HT is stored in vesicles via an unspecific vesicular monoamine transporter (VMAT).[35] Vesicular 5-HT may be recycled or degraded by a monoamine oxidase (MAO) and an aldehyde dehydrogenase to the main product 5-hydroxyindole acetic acid (5-HIAA). In terrestrial vertebrates, two MAO forms have been described.[36] MAO A is mainly responsible for deamination of serotonin and norepinephrine and can be inhibited by

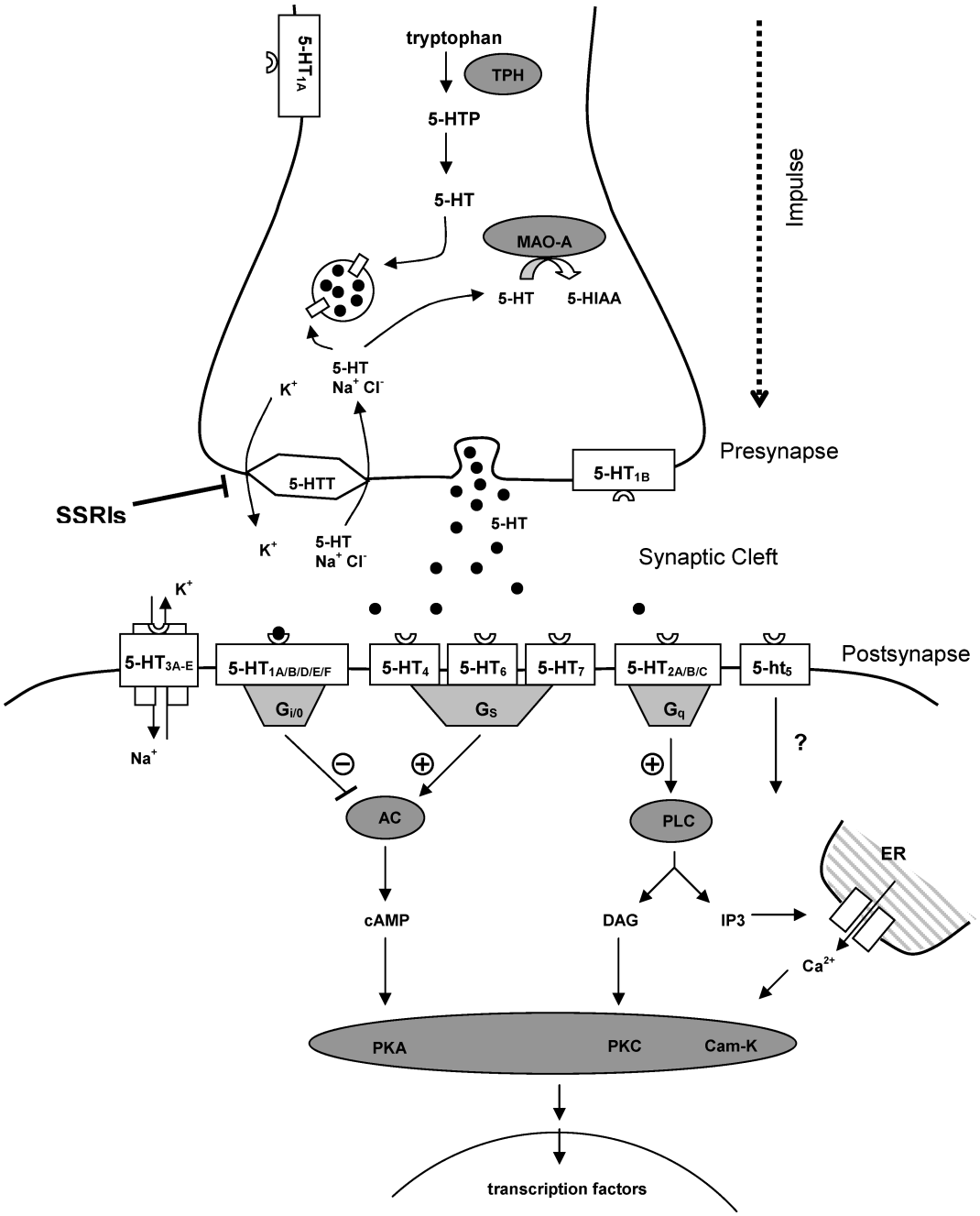


FIG. 2. Serotonergic neurotransmission in mammals. AC, adenylate cyclase; Cam-K, Ca^{2+}/calmodulin-dependent protein kinase; DAG, diacylglycerol; ER, endoplasmatic reticulum; IP3, inositol triphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; ⊣ = inhibition; → = stimulation; 5-ht_5= predicted 5-HT receptor subtype 5, based on mRNA fragments.

harmaline and clorgyline, while MAO B primarily deaminates benzylamine and phenylethylamine and is inhibited by deprenyl and pargyline.[37]

The specific 5-HT transporter (5-HTT)—representing the main target for the pharmacological action of SSRIs—is located in the presynaptic plasma membrane (Figure 2) and is primarily responsible for the synaptic clearance of 5-HT after neurotransmitter release.[38] In contrast to the numerous 5-HT receptors reported, as yet only one 5-HTT protein has been identified.[34] As a monoamine transporter, 5-HTT belongs to the solute-linked carrier 6 (SLC6) family, a diverse set of Na^+/Cl^--dependent neurotransmitter transporters comprising five subfamilies.[39] Three

TABLE 4
Selected 5-HT receptor agonists and antagonists

Function	Substance	5-HT receptor subtype
Agonist	2-methyl-5-HT	3
	8-OH-DPAT	1A
	α-methylserotonin	2
	Chlorophenyl piperazine	1
Antagonist	Cyproheptadine	2A
	Ketanserin	2A
	LY53857	2
	Metergoline	1
	Methiothepin	1
	Methysergide	1 + 2
	Metoclopramide	3
	Mianserin	2
	Ritanserin	2A
	Spiperone	1A

of these subfamilies, including the monoamine transporters, are thought to share a common structural motif, namely, 12 transmembrane domains with a larger extracellular loop containing potential glycosylation sites between domains 3 and 4, and several cytoplasmic phosphorylation sites[40,41] (Figure 3). The mammalian 5-HTT has been described in the central and peripheral nervous system and also in nonneuronal tissue such as platelet, placental, and pulmonary membranes.[42,43] The process of 5-HT uptake by the 5-HTT is not yet fully understood but it appears to be a two-step mechanism, i.e. symport of 5-HT with Na^+ and Cl^- ions into the cell and subsequent export of K^+.[40] Apparently, there are several mechanisms involved in the regulation of 5-HTT activity. Short-term changes in transporter activity may be induced by posttranslational modifications such as phosphorylation.[44] Potential target sites for phosphorylation are located at the cytoplasmic NH_2 and COOH termini of the 5-HTT molecule (Figure 3). It has been shown that activation of protein kinase C (PKC) causes internalization of 5-HTT followed by a decrease of 5-HTT-mediated 5-HT uptake in the human cell line HEK-293 *in vitro*.[45] In a subsequent study, inhibitors of protein phosphatases 1 and 2 as well as PKC activators were observed to diminish 5-HT transport activity in the same cell model.[41] Therefore, direct phosphorylation of 5-HTT as well as unbalanced protein kinase activity may play a role in the regulation of 5-HT reuptake.

SSRIs block the presynaptic 5-HTT and prevent the clearance of 5-HT from the synaptic cleft (Figure 2), thereby causing an elevation of extracellular 5-HT concentrations.[46] In human therapeutic regimens, chronic treatment with SSRIs is required to achieve the desired pharmacological effect. This is at least partially due to an autoinhibitory effect of 5-HT on its own release via presynaptic 5-HT_{1A} autoreceptors. Furthermore, long-term modulation of serotonergic signaling by SSRIs is most likely not purely based on their direct inhibitory action on the 5-HTT but also mediated by alterations in 5-HTT gene expression levels.[44]

Fish

The distribution of serotonergic neurons in the central nervous system has been examined in several fish species, and 5-HT-containing cells and serotonergic fibers have been found in different parts of the brain. Kah and Chambolle[47] identified 5-HT fibers and perikarya in various regions of the goldfish (*Carassius auratus*) brain via immunohistochemistry. The telencephalon was found to be highly innervated by serotonergic fibers from other brain areas, though it did not contain any immunoreactive perikarya. 5-HT immunoreactive fibers were also detected in parts of the pituitary and the midbrain tegmentum. Serotonergic

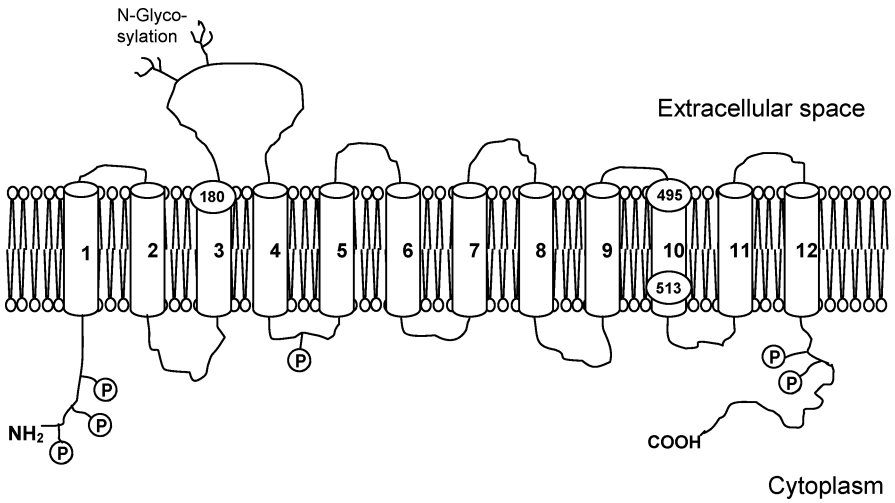


FIG. 3. Structure of the mammalian 5-HTT with 12 transmembrane domains, redrawn from Mortensen et al.,[62] with permission. (P) phosphorylation sites; (180), (495), (513), protein residues affecting SSRI potency.

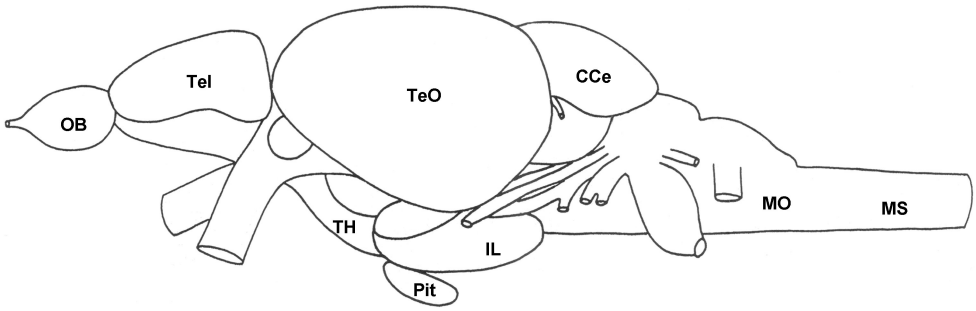


FIG. 4. Lateral view of the zebrafish brain, modified from Wulliman et al.[198] Cce, *corpus cerebelli*; IL, inferior lobe; MO, *medulla oblongata; MS, medulla spinalis*; OB, olfactory bulb; Pit, pituitary; Tel, telencephalon; TeO, *tectum opticum*; TH, tuberal hypothalamus.

perikarya were localized in the posterior hypothalamus region of the diencephalon, in the ventral *medulla oblongata* and in an area between midbrain and medulla oblongata, called isthmus (*nucleus isthmi*). The isthmus is homologous to the raphe region in terrestrial vertebrates and seems to be phylogenetically ancient. A similar arrangement of serotonergic neurons has been found in the brain of zebrafish (*Danio rerio*)[48] (Figure 4). In addition to serotonergic fibers in the forebrain—consisting of olfactory bulb and telencephalon—and in the *tectum opticum*, 5-HT neurons were detected in the pineal stalk, hypothalamus, posterior tuberculum, and a large cluster in the tegmentum forming a medial raphe nucleus.

To complement studies on the anatomical organization of the 5-HT neuronal network, efforts to detect the various proteins that are involved in the function of the serotonergic system have resulted in the identification of several key components of the 5-HT metabolism and signal transduction system in fish, including enzymes, metabolites, receptors, and transporter mechanisms known from mammalian studies. The presence of TPH or TPH subtypes has been detected in different organs of several fish species, including the pineal organs of adult rainbow trout (*Oncorhynchus mykiss*) and pike (*Esox lucius*),[49] the hypothalamus of adult Atlantic croaker (*Micropogonias undulatus*),[50] and the brain and retina of zebrafish embryos.[51,52] In addition, the catabolic enzyme MAO has been found in fish species such as perch (*Perca fluviatilis*),[37] goldfish,[53] rainbow trout,[54,55] carp,[55] pike,[56] catfish (*Ictalurus melas*),[57] Atlantic croaker,[50] and zebrafish.[58] However, in contrast to the serotonergic system of terrestrial vertebrates, which features the two MAO types A and B, only one form of MAO has been detected in fish.[36,55,56] While the MAO protein in some fish species has been reported to be distinct from both MAO A and MAO B,[36] evidence for pharmacological and structural similarities between fish MAO and mammalian MAO, especially MAO A, have been described in goldfish[53] and zebrafish.[58]

To date, knowledge on the presence of functional 5-HT receptors in fish is still limited. Using molecular cloning techniques, 5-HT_1 receptor genes were identified in fugu by Yamaguchi and coworkers,[59] and three genomic clones, named F1Aα, F1Aβ, and F1D, were isolated. Following alignment of the deduced amino acid sequences, the highest similarity between fugu and human 5-HT1 receptor genes was observed in transmembrane regions, with 88.5% for F1Aα and 86.2% for F1Aβ when compared to the human 5-HT1A receptor gene and 78.6% for F1D and the human 5-HT1D receptor. Furthermore, it was determined that all physiologically essential amino acid residues that have been identified for human 5-HT1 receptors were conserved in fugu, indicating ligand binding properties that are similar to the equivalent human receptors. However, the occurrence of additional phosphorylation sites in fugu 5-HT receptor molecules suggested that the regulation of fugu 5-HT1 receptors may be different from their human homologues. The presence of several 5-HT receptor types has also been predicted for zebrafish on the basis of sequence similarities between zebrafish mRNA fragments and human mRNA (Table 3).[60] Despite the suggestive evidence just described, it has to be taken into account that similarities in nucleotide sequences may not directly translate into protein expression, let alone functional analogy as a 5-HT receptor with equivalent signal transduction.

The same caveats apply for the investigation of putative 5-HTTs. Although recognizable 5-HTT homologues, based on nucleotide sequences, have been found to be expressed near-ubiquitously in animal phyla,[39] the regulation and properties of the transporter protein may be diverse. Therefore, functional experiments are required to characterize the 5-HT transport mechanism in different species. In zebrafish, mRNA fragments have been detected that show similarities to the mRNA sequence of the human 5-HTT.[60] Recently, Wang and colleagues[61] cloned two 5-HTT cDNAs—*serta* (DQ285098) and *sertb* (DQ285099)—from zebrafish that demonstrated an amino acid sequence similarity of 66–69% and 75%, respectively, compared to other vertebrate 5-HTTs. The expression patterns of both 5-HTT genes were determined by *in situ* hybridization. While the gene product of *serta* was detected in raphe nuclei, ventral posterior tuberculum, and pineal organ, *sertb* was found to be expressed in the medulla oblongata and in the retina. In order to examine the functionality of the gene product, *serta* cDNA was cloned into a vector and transfected

into human embryonic kidney cells (HEK293 cell line) along with an enhanced green fluorescent protein expression vector. Fluorescent colonies were then used to perform 5-HT transport assays with [^{3}H]-5-HT, which confirmed the occurrence of 5-HT uptake. Furthermore, 5-HT transport was inhibited by fluvoxamine maleate with an inhibiting concentration (IC_{50}) of 183.4 nM (79.7 μg/L). This IC_{50} was comparable to values obtained for human and bovine 5-HTT yielding IC_{50} values of 176 ± 18 nM (76.5 ± 8 μg/L) and 138 ± 9 nM (59.9 ± 4 μg/L), respectively.[62]

The presence of a 5-HTT-like transporter was also revealed in various brain areas, e.g., in preoptic and diencephalic regions, of Japanese medaka (*Oryzias latipes*) by an *in vitro* autoradiographic binding assay.[63] The iodinated cocaine analog [^{125}I]-RTI-55, which was used as radioligand as it has been reported to bind to the 5-HTT in mammals, was completely displaced by fluoxetine hydrochloride at a concentration of 10 μM (3.46 mg/L). This provides support for the classification of this transporter as 5-HTT and suggests that 5-HT transport may be similar in mammals and fish. However, ligand binding to the 5-HT transporter-like protein alone does not prove that actual transport takes place. Therefore, further studies are needed to confirm the functional capability of this putative 5-HTT in medaka and to determine whether it may be susceptible to SSRI inhibition.

In summary, several components of the serotonergic system as known from mammalian studies have been identified in fish to date. 5-HT has been detected as neurotransmitter not only in the fish brain, but also in other fish organs and tissues where it seems to be involved in the modulation of regulatory processes, e.g., an endocrine/paracrine factor in enterochromaffin cells lining the gut, gill vasculature, specialized cells of the retina, several types of immunocompetent cells, and possibly in ovarian follicles (Krieger, personal communication). Accordingly, 5-HTT has been identified in zebrafish retina[61] and was also found in ovarian follicles of zebrafish (Krieger, personal communication). Therefore, as the molecular composition of the serotonergic system in fish appears to be comparable to the mammalian system, it is reasonable to assume that environmental SSRI exposure may affect serotonergic signaling in fish by increasing 5-HT availability via 5-HTT inhibition. Nevertheless, the understanding of 5-HT function in fish physiology and the regulation of 5-HT uptake via 5-HTT is still rather limited.

PHYSIOLOGICAL EFFECTS OF 5-HT IN SELECTED TISSUES

5-HT in the Gills

Using immunohistochemical methods, 5-HT immunoreactive neurons innervating the proximal part of the efferent arterial vasculature, the filament epithelia, and the venous sinus of the gills of rainbow trout, perch, black bass (*Micropterus dolomieui*), eel (*Anguilla anguilla*), and catfish have been identified.[64] In addition to serotonergic neurons, nonneuronal 5-HT immunopositive cells, namely, neuroepithelial cells (NECs), have been found in the efferent part of teleost gill filaments.[65] NECs displaying 5-HT immunoreactivity were detected in all fish species examined, including sturgeon (*Acipenser baeri*), a nonteleost, while in rainbow trout an additional 5-HT immunopositive cell type was observed and described as polymorphous granular cells.[64] In trout gill filaments, these two cell types have been designated as possible sites for indoleamine synthesis based on immunohistochemical experiments using relevant polyclonal antibodies, which also revealed the intracellular presence of 5-HT as well as the metabolite 5-methoxytryptamine. Furthermore, since NECs in fish were found to be morphologically similar to mammalian oxygen chemosensors, e.g., carotid bodies, it was suggested that these cells might have a comparable function.[66,67] Supporting this hypothesis, 5-HT immunoreactive NECs in the gills of zebrafish were found to respond to hypoxic conditions by exhibiting hypertrophy measured as increased projection area and higher proliferation rates, accompanied by a decrease in K^+ current.[67] Based on these findings, it has been proposed that the reaction to hypoxia that seems to be initiated by inhibition of background K^+ conductance may involve serotonergic signal transmission. However, whether or to what extent 5-HT is involved in the process of oxygen chemoreception in fish is still a matter of discussion. In a previous study on isolated rainbow trout gills, Burleson and coworkers[68] examined the influence of several neurochemicals, including 5-HT, on the activity of afferent nerves innervating oxygen-sensitive chemoreceptors in the first gill arch. They reported that 0.1 ml injections of 1 mM 5-HT caused a weak stimulation of chemoreceptor activity followed by a weak inhibition. This slight effect compared to the strong stimulation elicited by 100 nM acetylcholine or nicotine indicates that despite 5-HT having some modulating influence, 5-HT does not appear to be the major neurochemical effector in hypoxia reaction. However, 5-HT may affect gas exchange in fish in another way, as it seems to regulate the blood flow in the gills by constricting the arterio-arterial branchial vasculature.[65] In an *in vivo* study with rainbow trout, intra-arterial 5-HT injections (50–250 nmol /kg) caused a decrease of blood pressure in the dorsal aorta and an increase of ventral aortic blood pressure, presumably by constricting arteries and arterioles in the gill filaments.[69] Concomitantly, the arterial oxygen tension declined while the arterial carbon dioxide tension increased, accompanied by a reduction in extracellular pH. Taken together, these observations can be seen as indications for an impaired gas transfer associated with 5-HT-induced vasoconstriction. Pretreatment with the 5-HT receptor antagonist methysergide can reverse these effects of 5-HT on trout gills, indicating the involvement of either the 5-HT_1 or the 5-HT_2 receptor subtype.[69,70] The role of 5-HT_2 receptors in mediating branchial vasoconstriction in fish gills was confirmed for the bald notothen (*Pagothenia borchgrevinki*), an Antarctic fish, using specific pharmacological agonists and antagonists.[71] The prebranchial injection of the 5-HT_2 receptor agonist α-methylserotonin caused an increase of branchial vascular resistance and ventral aortic pressure, thereby mimicking the action of 5-HT, while treatment with the 5-HT_1 receptor agonist piperazine had no effect. Again, branchial vasoconstriction

caused by 5-HT could be blocked with methysergide, while vasoconstriction induced by α-methylserotonin was completely abolished by treatment with the specific 5-HT_2 receptor antagonist LY53857 maleate. All of the results just described suggest the vasoconstrictive actions of 5-HT in the bald notothen to be mediated by receptors of the 5-HT_2 subtype. In accordance with the aforementioned findings of Fritsche et al.[69] in rainbow trout, a methysergide-sensitive reduction of arterial oxygen partial pressure was also observed by Sundin and coworkers[71] in the bald notothen as a consequence of 5-HT-induced (0.1 nmol/kg) branchial vasoconstriction in the arterio-arterial pathway. The effect of 5-HT on arterial oxygen tension was thought to be caused by a redistribution of the blood flow within the gill. This hypothesis was confirmed in a subsequent study with rainbow trout, which demonstrated that, despite a continuous cardiac output, the blood flow rate in the distal part of the gill filaments was clearly reduced after 5-HT treatment with 100 nmol/kg.[72] Such a redirection of the blood caused by constriction of the efferent filamental artery sphincter[64] could also explain the increased flow rate through the arterio-venous system observed in Atlantic cod (*Gadus morhua*) after 5-HT injection.[73]

As well as serotonergic neurons, other neurotransmitter systems innervating the gill vasculature have been identified, i.e., cholinergic and adrenergic neurons.[65] These three systems seem to interact in regulating blood flow and oxygen transfer in the gills. Indeed, serotonergic neurons have been detected in synaptic contact with catecholaminergic nerve fibers in trout gill.[64] Furthermore, 5-HT-mediated release of catecholamines was observed in this system.[69] Circulating catecholamines elicited dilation of the filament arterial vasculature through activation of β-adrenoreceptors, thereby representing a mechanism to reverse the vasoconstriction induced by 5-HT,[69] while acetylcholine exerted vasoconstrictive effects analogous to those of 5-HT.[64]

This complex regulation system controlling gill blood circulation and gas transfer in fish may be affected by exogenous factors. Fish gills are directly and permanently exposed to the aquatic environment and may therefore be particularly susceptible to nonspecific effects of pharmaceutical substances in the surrounding water. So far, studies on the impact of SSRIs on fish gills or studies testing gill tissue for accumulation of these drugs are lacking. However, as Brooks and colleagues[18] found fluoxetine, sertraline, and their metabolites norfluoxetine and desmethylsertraline in fish brain, liver, and, in small amounts, muscle tissue, it is likely that SSRIs may also be found in gills. SSRIs may reach the gills via blood circulation potentially in concentrations that could modulate serotonergic signaling, i.e., may specifically induce gill filament vasoconstriction through increased availability of 5-HT. Thereby, continuous SSRI exposure may lead to a decrease of blood flow and a subsequent impairment of gas exchange in certain areas of the gills and, consequently, to sustained chronic stress. The latter may be of higher relevance in the summer months of temperate regions and during the dry seasons of tropical regions of the world, where generally lower oxygen saturation and reduced water volumes and flow rates coupled with unmitigated STP effluent volumes are encountered.

Involvement of 5-HT in Light/Dark Adaptation and Circadian Rhythm

5-HT appears to play a regulatory role in sensory perceptions, e.g., visual perception, and related circadian rhythmicity. High levels of endogenous 5-HT have been detected in the retinas of various nonmammalian species, with the highest reported concentrations found in teleost retina.[74] Similar to the observations of Tornqvist et al.,[75] who detected serotonergic amacrine cells in the goldfish retina, Jaffé and coworkers[76] localized endogenous 5-HT in amacrine cells of the retina of striped mojarra (*Eugerres plumieri*) using biochemical and immunohistochemical techniques. In the same fish species, possible modulatory interactions between 5-HT, dopamine and noradrenaline were studied.[77] The fish were injected with different 5-HT concentrations (1, 5, 10, 20 μM) in a 10-μl injection volume *in vivo* and the retinas were isolated 3 h later for catecholamine analysis via high-performance liquid chromatography. 5-HT was found to significantly reduce the dopamine content in the retina at the lowest applied concentration of 1 μM, with the maximum reduction occurring at 10 μM. The reverse effect was observed for noradrenaline, as 5-HT increased the retinal noradrenaline levels.

In addition to 5-HT, a 5-HT uptake mechanism has been found in the retina of different fish species. Prior to the recent detection of the *sertb* gene product in the inner nuclear layer of zebrafish retina,[61] the 5-HTT was identified and characterized with radioligand binding assays in isolated goldfish retina.[78] 5-HT uptake was found to be temperature- and sodium-dependent and showed an allosteric regulation, suggesting the presence of a stimulatory as well as an inhibitory site in the transporter molecule. Blockade of the 5-HT reuptake mechanism in the retina of striped mojarra by injecting 10 μl of 5 μM imipramine did not abolish the previously observed decreasing and increasing effect of 5-HT on dopamine and noradrenaline content, respectively.[77] Thus, the action of 5-HT is apparently not connected to changes in 5-HT uptake but rather is mediated at the level of the 5-HT receptors. Indeed, experiments with the 5-HT receptor antagonists methysergide, metergoline, and cyproheptadine demonstrated that the opposing effects of serotonergic modulation on dopamine and noradrenaline content in the retina could be explained by activation of two different 5-HT receptor subtypes.[77] As the diminishing effect of 5-HT on dopamine levels was significantly inhibited by methysergide and metergoline, but not by cyproheptadine (Table 4), it seems likely that it was mediated by 5-HT_1 receptors. In contrast to the effect on dopamine, 5-HT-induced changes in noradrenaline levels could be blocked by all three antagonists, indicating the involvement of both 5-HT_1 and 5-HT_2 receptors in this modulatory mechanism.

5-HT has been reported to be involved in the control of retinomotor movement.[79] In lower vertebrates, photoreceptors and retinal pigment epithelium show specific movement patterns

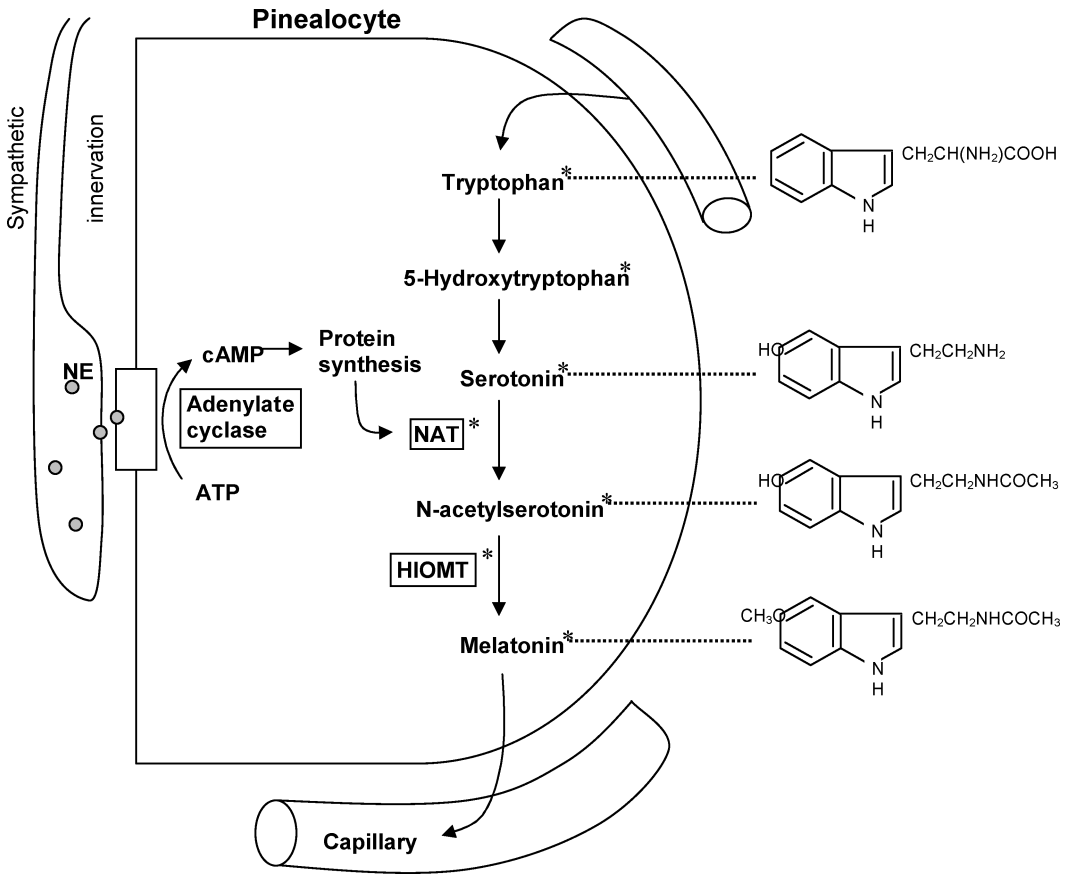


FIG. 5. Schematic of the pineal gland associated with tryptophan to melatonin conversion, modified from Heldmaier.[199] NAT, serotonin *N*-acetyltransferase; NE, norepinephrine; HIOMT, hydroxyindole *O*-methyltransferase; asterisk indicates detected in fish.

as a reaction to changing light conditions or in response to an endogenous circadian signal. In a study with isolated retinas of the green sunfish (*Lepomis cyanellus*), Dearry and Burnside[79] demonstrated that 0.1 mM 5-HT had an influence on the dopaminergic regulation of cone contraction by stimulating dopamine release, thus inducing light adaptation of isolated dark-adapted retina. The latter data are suggestive of a neurotransmitter function of 5-HT in the regulation of circadian rhythmicity. In addition, 5-HT is the precursor of melatonin, which is synthesized in photoreceptor cells of the retina and in the pineal organ (Figure 5) and is involved in the regulation of light/dark adaptation. Lima and Schmeer[78] reported an increased uptake of [^{3}H]-5-HT into isolated retinal cells of dark-adapted compared to light-adapted goldfish and suggested that this effect may be related to the production of melatonin. Serotonin-*N*-acetyltransferase (NAT), a key enzyme of melatonin synthesis, has been found in the pineal gland (Figure 5) and retina of rainbow trout[80,81] and of embryonic and adult zebrafish.[82] The production of NAT is initiated by norepinephrine (NE) signaling.

Recurring changes in the activity of NAT, with peak efficiency during the dark in the pineal gland, result in varying levels of circulating 5-HT and, consequently, melatonin.[80] In rainbow trout, 5-HT levels in the retina[74] and in the pineal organ[80] were found to exhibit daily fluctuations, with patterns changing under different photoperiodic conditions. It was suggested that, with a shortened photoperiod, an increased 5-HT production during the night may be necessary to ensure 5-HT availability for optimal melatonin synthesis in the pineal organ.[80] Interestingly, as for vertebrates, atypical activity of NAT has been observed in fish.[81] While it is generally assumed that the vertebrate retina and pineal organ both display high NAT activity in the dark, melatonin production in trout retina was actually shown to increase during the day.

Provided that SSRIs reach the retina and pineal organ in fish and affect the local 5-HT reuptake, chronic SSRI exposure may cause an alteration of normal fluctuation patterns of 5-HT and melatonin. Consequently, behavioral processes such as hunting, feeding, and reproductive behavior that are related to certain photoperiodic conditions may be affected. In salmonids, initiation and regulation of smolt transformation, referring to the change from territorial feeding behavior to downstream migration, largely depends on photoperiodic signals.[83] Taking into account that NAT-dependent melatonin production in fish showed

some unusual patterns,[81] the consequences of higher 5-HT levels caused by reuptake inhibition in fish may not be directly comparable to the effects in other vertebrate species. In order to elucidate whether SSRIs may have an influence on the circadian rhythm and associated behavior in fish, further investigation will be required.

SEROTONERGIC MODULATION OF STRESS RESPONSE AND BEHAVIOR

5-HT in Chromaffin Tissue and Modulation of Catecholamine Release

In addition to the aforementioned functions at the individual organ level, 5-HT may also influence various regulation systems such as stress response and correlated behavior patterns at the organism level.

Many studies with mammals have focused on investigating the mechanisms involved in acute stress reactions and it has been demonstrated that there are interactions between the adrenergic and the serotonergic system. In this context, the level of extracellular 5-HT appears to be an important factor. In 5-HTT knockout mice, several abnormal phenotypes have been observed that show similarities to clinical features of mood and anxiety disorders in humans, including increased anxiety-like behaviors and exaggerated plasma adrenaline responses to stress, as reviewed by Holmes and colleagues.[84]

It has been recognized that release of the catecholamines adrenaline and noradrenaline from chromaffin cells in fish is a reaction to an acute physiological challenge such as hypoxia.[85] Chromaffin cells containing catecholamines have been found in the head kidney and in the walls of the posterior cardinal veins of rainbow trout.[86,87] In contrast to the well-defined medulla of the adrenal gland in other vertebrates, chromaffin cells in fish form loose aggregations termed chromaffin tissue.[87] The cells are mostly innervated by sympathetic cholinergic fibers, which release acetylcholine in response to stimulation, thereby triggering the exocytosis of catecholamines.[86] It has been proposed that 5-HT may be part of the regulation regime controlling catecholamine release. Reid and colleagues[87] detected 5-HT-like immunoreactivity in chromaffin cells within the posterior cardinal vein of European eel and Atlantic cod. In rainbow trout, 5-HT was not present in chromaffin cells, but was found in a distinct population of cells located in kidney tissue. These findings provide evidence that 5-HT may act as a paracrine factor on catecholamine-releasing cells. Additionally, in an *in vivo* study with rainbow trout, intra-arterial injections of different 5-HT doses (50–250 nmol/kg) caused dose-dependent increases in plasma noradrenaline and adrenaline levels.[86] In the same study, 5-HT treatment (250 nmol/kg) also induced the release of noradrenaline and adrenaline from chromaffin cells of the head kidney *in situ*.

Based on the observations in several fish studies, it is assumed that 5-HT plays a regulatory role during the acute stress reaction to conditions of low oxygen levels,[69,71,88] resulting in catecholamine release from chromaffin cells. In both rainbow trout[69] and bald notothen,[71] a reduction of arterial oxygen partial pressure was observed after intra-arterial 5-HT injections. In the gill filament of developing zebrafish embryos, it was observed that the formation of 5-HT-containing NECs coincided with an increased reaction to hypoxic conditions, suggesting a contribution of serotonergic neurotransmission to oxygen chemoreception.[88] However, the involvement of 5-HT in hypoxia response is controversial. In an *in vivo* study with rainbow trout, an increase of plasma catecholamine levels was observed under hypoxic conditions, after desensitization of nicotinic receptors.[89] The authors attempted to characterize the underlying mechanism and found that treatment with the $5\text{-HT}_1/5\text{-HT}_2$ receptor antagonist methysergide had no effect. Therefore, it was assumed that serotonergic receptor activation was not involved in the direct stimulation of catecholamine release during acute hypoxia. Instead, the renin–angiotensin system, a regulator of cardiovascular function in fish, seemed to be activated in hypoxic trout without functional nicotinic receptors and to induce an increase in catecholamine levels. Similarly, Sundin and Nilsson[90] observed that hypoxia-induced branchial vasoconstriction in rainbow trout was apparently not mediated by serotonergic signaling, as it was unaffected by methysergide. Nevertheless, it remains to be determined whether the potential modulating effect of 5-HT during hypoxia may be mediated by receptor subtypes other than 5-HT_1 and 5-HT_2.

Overall, the results of several studies indicate that 5-HT plays a role in the control of catecholamine release from chromaffin cells; however, the regulation of the specific reaction to hypoxic conditions appears to be very complex, involving a variety of modulatory factors. Consequently, the importance of potential effects of SSRIs on catecholamine release and subsequent processes are at present difficult to assess.

Serotonergic Influence on the Corticotropic System

While catecholamines are secreted during acute stress conditions, corticosteroids produced by the adrenal gland play a major role in responses to chronic stress. The regulation of these reactions appears to be quite complex. There are indications of a correlation between the brain serotonergic system and the hypothalamus-pituitary-adrenal (HPA) axis in mammals[91] or in fish the hypothalamus-pituitary-interrenal (HPI) axis, which is recognized as homolog of the HPA axis. Numerous stressors seem to increase the synthesis and turnover of 5-HT, possibly mediated by stress-related corticosteroid production. Reciprocally, 5-HT appears to be involved in the regulation of corticosteroid release as several studies have demonstrated treatment with 5-HT precursors or 5-HT receptor agonists to cause an elevation of plasma glucocorticoid concentrations in humans, as reviewed by Dinan.[92]

Evidently, 5-HT may exert its effects at different levels of the HPA axis. As one possible mechanism, it has been proposed that 5-HT induces the secretion of ACTH from the

pituitary and, ultimately, the release of corticosteroids by stimulating the release of corticotropin releasing hormone (CRH) in the hypothalamus.[91,92] 5-HT and CRH immunopositive cells have been detected in corresponding areas in rat brain with direct synaptic contact between serotonergic nerve terminals and CRH neurons.[93] In addition, an *in vitro* study with explanted rat hypothalami confirmed the stimulating effect of the serotonergic system on CRH secretion.[94] This action of 5-HT is probably mediated through 5-HT_{1A} and 5-HT_2 receptors, as it could be blocked by metergoline, ketanserin, and ritanserin and mimicked by 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) (Table 4). Furthermore, a direct serotonergic stimulation of ACTH release in the pituitary has been suggested. Again, this action of 5-HT appears to be associated with an activation of postsynaptic 5-HT_{1A} and 5-HT_2 receptors.[91] 5-HT_2 receptors have also been implicated in mediating a paracrine effect of 5-HT on the HPA axis in rats, namely, the direct triggering of corticosteroid release at the adrenocortical level.[95]

In regard to a potential interaction of SSRIs with the HPA axis, it is interesting to note that knockout mice lacking the 5-HTT showed a disproportionate plasma adrenocorticotropic hormone (corticotropin, ACTH) response to stress.[84] Therefore, blockage of the 5-HT reuptake by SSRIs and consequential prolonged serotonergic signaling may have similar consequences, albeit to a lesser extent, as not all existent 5-HTT molecules will be completely inhibited.

Like the HPA axis, the HPI axis in fish mediates the stress response of the organism by activating the corticotropic system with the key substances CRH, ACTH, and cortisol[96] (Figure 6), although slight differences in anatomy and regulation of the pathway have been noted. In mammals, hypothalamus and pituitary are interconnected by a median eminence, thereby providing an interface for the transfer of hypothalamic factors.[97] In fish, such a blood portal system is lacking. Instead, there is a direct connection formed by CRH-positive neurons that innervate the anterior region of the pituitary. With regard to regulatory

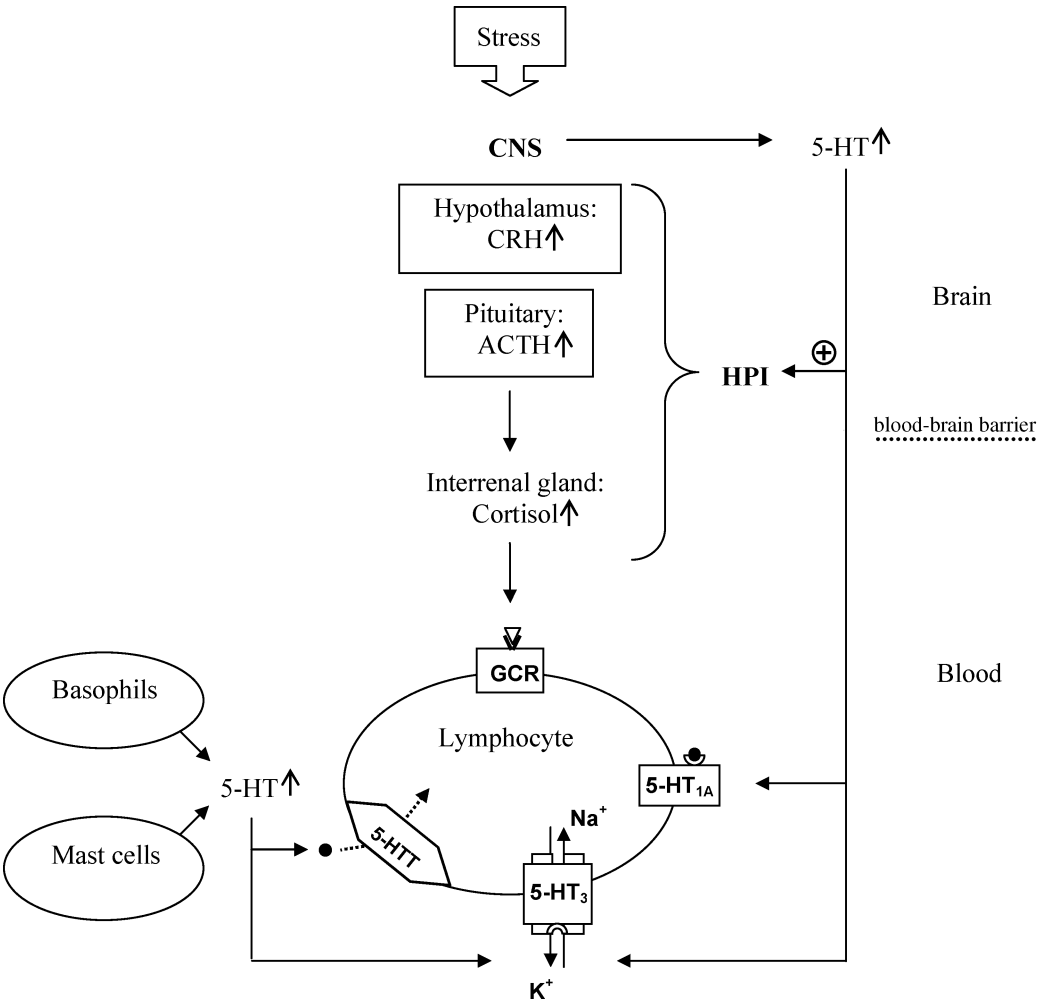


FIG. 6. Schematic of serotonergic interaction with the HPI axis and immune system in fish. GCR, glucocorticoid receptor; ● = 5-HT; ∇ = cortisol; ·····▶ = transport.

mechanisms, it has been found that the release of the major corticosteroid in fish, cortisol, is apparently controlled not only by ACTH but also by α-melanophore-stimulating hormone.[96] Nevertheless, as in mammals, 5-HT seems to play a regulatory role in the piscene HPI axis[98,99]; therefore, the corticotropic system represents a potential target for SSRI action in fish during environmental exposure.

The assumption that 5-HT modulates cortisol release is supported by the observation that serotonergic and corticotropic systems in fish appear to exist in close physical proximity to each other, and indeed in some areas actually overlap. CRH-positive neurons were detected in the preoptic region and in the basal hypothalamus of rainbow trout,[100,101] brain areas that were both also found to be innervated by numerous serotonergic fibers.[102] In addition to morphological studies, physiological experiments have provided evidence for an interaction between 5-HT and the cortisol secretion system in fish. Using an *in vitro* radioimmunoassay approach, Pepels and colleagues[103] found that 5 μM (1.1 mg/L) 5-HT stimulated CRH release from superfused telencephalic tissues of tilapia (*Oreochromis mossambicus*). Furthermore, 5-HT also seems to exert an influence on the periphery of the HPI axis, as it does in the mammalian HPA axis. An *in vivo* study with rainbow trout demonstrated that different doses of the 5-HT_{1A} receptor agonist 8-OH-DPAT (1, 10, 40 μg/kg), injected through a catheter into the dorsal aorta, caused an increase in plasma cortisol levels compared to controls injected with saline.[99] It was observed that cortisol concentrations were significantly elevated after application of 10 and 40 μg/kg 8-OH-DPAT. These results show that 5-HT may affect the HPI axis via activation of 5-HT_{1A} receptors, thereby triggering cortisol release from the interrenal tissue. Consequently, by increasing the availability of 5-HT over a prolonged period of time, SSRIs could have a similar effect on cortisol levels in fish. However, long-term fluoxetine treatment of chinook salmon (*Oncorhynchus tshawytscha*) did not reveal any changes in plasma cortisol concentrations when measured after a prolonged stress period caused by behavioral testing.[104] The latter finding indicates that it may be difficult to assess whether potential molecular changes during SSRI exposure will have repercussions for the whole organism, as, comparable to the situation in mammals,[91,95] the regulatory interaction between 5-HT and the HPI axis in fish seems to be reciprocal—that is, there are indications for the influence of chronic stress on internal 5-HT concentrations. In a study with Arctic charr (*Salvelinus alpinus*), Winberg and coworkers[105] demonstrated that stress induction by daily handling over a period of 4 weeks resulted in an elevated concentration of the main 5-HT metabolite 5-HIAA and increased 5-HIAA/5-HT ratios; these two parameters are considered to be indicators for 5-HT turnover in the fish brain.

Social stress in a population hierarchy also seems to affect the serotonergic system, as higher 5-HIAA concentrations and 5-HIAA/5-HT ratios were detected in subordinate fish which experience higher stress levels than dominant individuals.[98,105,106] In contrast, a higher dopaminergic activity, represented by the ratio of dopamine to the primary metabolite 3,4-dihydroxyphenylacetic acid, may counter the stress-induced increase in plasma cortisol concentration and 5-HT activity, thereby inducing social dominance.[107] Evidently, the dopaminergic system in fish brain interacts with the serotonergic system in an antagonistic manner. In this context, low concentrations of SSRIs may have the potential to exert subtle effects, in this case by inducing an imbalance between these two neurotransmitters.

The biomolecular changes in connection with social status may be accompanied by behavioral inhibitions. It was observed that subordinate Arctic charr displayed a lower locomotor activity than dominant fish,[108] while subordinate status in rainbow trout was marked by decreased food intake.[109] Additionally, subordination in fish appears to be characterized not only by an increased serotonergic activity in the brain and moderated behavioral reactions but also by higher plasma cortisol concentrations than in dominant individuals,[110] presenting an additional link between the serotonergic system and the HPI axis. Indeed, when investigating the effect of cortisol treatment on social status and brain 5-HT levels in juvenile female rainbow trout, DiBattista et al.[110] showed that elevation of plasma cortisol concentrations appeared to predispose the fish towards subordination. As brain monoaminergic activity was changed as a result of cortisol administration, this effect may have been mediated by an antagonistic activity of the serotonergic and dopaminergic systems. It was shown that the effects of cortisol treatment were converse in hypothalamus and telencephalon, with higher serotonergic and lower dopaminergic activity in the telencephalon and the opposite effect in the hypothalamus. Based on the latter observations, the authors suggested that the decrease in hypothalamic serotonergic activity in juvenile rainbow trout after cortisol administration was due to a negative feedback mechanism abating cortisol secretion. However, in a study with sexually mature male Arctic charr, aimed at examining the effects of inter-male competition on concentrations of brain monoamines and endocrine stress responses, brain 5-HT activity did not appear to be correlated with plasma cortisol levels.[111] The contrasting results demonstrate that interactions may not only be species- and gender-specific but may also depend on the reproductive status of the respective animals. These parameters must be considered in the evaluation of the relationship between serotonergic activity and HPI axis as well as in the ecotoxicological risk assessment of SSRIs as potentiators of 5-HT signaling.

Serotonergic Modulation of Behavior Patterns

As described earlier, the physiological reaction to social stress in connection with hierarchical ranking is often accompanied by distinct changes in behavior patterns that may be mediated by 5-HT. In general, the serotonergic system has been implicated in the modulation of feeding, sexual, and aggressive behavior in various animal phyla, including coelenterates, platyhelminths, nematodes, molluscs, annelids, crustaceans, echinoderms, and chordates, as reviewed by Weiger.[112] In most vertebrate species examined so far, increased serotonergic activity

has been associated with an inhibition of aggressive behavior.[107] For instance, the antiaggressive effect of 5-HT has been noted in rat, mink, and pigeon, as well as in several fish species[107] such as the bluehead wrasse (*Thalassoma bifasciatum*). To increase serotonergic neurotransmission, male bluehead wrasses, representing a behaviorally dominant phenotype, were injected intraperitoneally with either a single acute fluoxetine dose of 10 μg/g in a field experiment or with a daily fluoxetine dose of 6 μg/g in a chronic 14-day laboratory test.[113] The fish from both experiments were then subjected to resident–intruder tests. Acute fluoxetine treatment resulted in a significant reduction of aggression against the intruder compared to controls, regarding chase frequency and chase duration. Chronic fluoxetine administration similarly reduced aggressive behavior. Additionally, males treated chronically with fluoxetine displayed lower activity levels before intruder introduction. This appeared to be a distinct effect, as there was no correlation observed between activity and aggression in individual fish.

It has also been proposed that the pineal hormone melatonin (Figure 5), a derivative of 5-HT that is involved in the synchronization of the circadian rhythm, may modulate aggressive behavior and may therefore be responsible for the antiaggressive effect of 5-HT treatment observed in fish.[114] However, this hypothesis was not supported by the results of a recent study by Lepage and colleagues.[115] These authors conducted resident–intruder tests to evaluate the level of aggression in 2-year-old rainbow trout and subsequently treated the fish either with tryptophan, the SSRI citalopram, or melatonin. Fish of the first two treatment groups were fed with special experimental food that was supplemented with tryptophan or citalopram (100 μg/kg). In the third group, melatonin was administered via intraperitoneally implanted capsules. While tryptophan as well as citalopram treatment resulted in decreased aggression and lower plasma cortisol levels compared to controls, melatonin treatment had no significant effect on either parameter. These results indicate 5-HT itself to be the antiaggressive agent in fish.

In summary, 5-HT has been recognized as a regulatory factor of stress reactions mediated by the HPI axis and, furthermore, as a modulator of behavior patterns associated with social status. It has also been shown that the SSRIs fluoxetine and citalopram may be able to modulate aggressive behavior in fish. As there are fish species, e.g., salmonids, that require aggressive interactions to define social relationships between individuals[116] and predominance in reproduction,[117] chronic exposure to SSRIs could affect established dominance hierarchies in fish populations, especially during sensitive periods in the reproductive cycle. This may result in a higher reproduction rate of formerly subordinate fish with an unfavorable genetic predisposition that would normally be subdued by dominant—and arguably healthier—individuals. Therefore, long-term SSRI-induced imbalance of social hierarchy could possibly impair the genetic balance in a given population. Furthermore, rank-dependent differences in physiological processes such as ion regulation have been observed in salmonid populations that show distinctive hierarchical relationships.[118] These physiological variations between fish of different dominance status have toxicological implications insofar as specific social ranks may be more strongly affected by aquatic contaminants. Therefore, alterations in 5-HT levels by SSRIs and a subsequent shift in social position may render an individual susceptible to toxicant exposure. Such potential consequences at the population level are, however, impossible to predict by simple toxicity tests.[118]

Apart from the rather extensively studied relationship between the serotonergic system and aggression-related behaviors in fish, there are some other behavioral patterns that seem to be modulated by 5-HT via the corticotropic pathway and may also be affected by SSRIs. As already mentioned, subordinate social status, which is associated with stress and higher 5-HT levels, may lead to repressed feeding and locomotor activity. In goldfish, intracerebroventricular injections of 5-HT (10 μg/μL) caused a significant reduction in food intake. This inhibitory effect could be partially abolished by co-administration of a CRH antagonist, indicating an influence of the HPI axis.[119] Decreased locomotor activity was observed in juvenile chinook salmon after chronic (20 days) treatment with a fluoxetine dose of 2.5 mg/kg, repeatedly administered by intraperitoneal injection, in comparison to fish given long-term saline injections. The increased activity in the saline-treated fish was attributed to the stress induced by handling and injections.[104]

In addition to affecting behavior through the HPI axis, 5-HT may mediate its control in conjunction with other neurotransmitter systems. In salmonids, the serotonergic and the dopaminergic system seem to play a role in the onset of migration behavior, which is a fundamental part of their specific life cycle.[120] In coho salmon (*Oncorhynchus kisutch*), brain 5-HT content and dopamine levels were found to increase significantly during smolt transformation. Examination of the brains of fluoxetine-treated male bluehead wrasses from chronic experiments (6 μg/g daily over 14 days)[113] revealed evidence for the involvement of the arginine vasotocin (AVT) system in mediating serotonergic effects on aggressive behavior and social status.[121] The fish brains were sectioned and analyzed for AVT mRNA abundance in the preoptic area of the hypothalamus using *in situ* hybridization. It was observed that the amount of AVT mRNA was significantly lower in bluehead wrasse males treated with fluoxetine than in control fish. The results of a previous study conducted by the same research group had already provided evidence for a link between decreased AVT mRNA expression and subordinate behavior in bluehead wrasse.[122] Therefore, it has been suggested that serotonergic effects on aggressive behavior and related social status may be mediated by this neuropeptide system in fish. In goldfish, 5-HT enhanced the presynaptic release of the inhibitory factor glycin at the synaptic connection to specialized neurons, which coordinate locomotor activity.[112] These examples illustrate that even slight changes in extracellular 5-HT levels, as potentially caused by environmental SSRI exposure, may alter behavioral output by modulating other signaling cascades. In other words, potential SSRI-induced increases in 5-HT

availability could result in abnormal behavior pattern and a disruption of established social hierarchies in fish. As an additional unfavorable factor, low social status may cause chronic stress,[116] and heightened cortisol release under chronically stressful conditions has been implicated in the decrease of antibody production and the reduction of the number of antibody-producing cells in fish, thereby impairing the immune response.[96] The serotonergic system has been proposed to act as a mediator between HPI axis and the immune system, but may furthermore modulate the immune response directly.[123]

5-HT AND THE IMMUNE SYSTEM

It has long been recognized that stress affects the immune response in mammals[124,125] and that 5-HT may be involved as a modulating factor.[126] Indeed, blockade of 5-HT synthesis using the TPH antagonist *p*-chlorophenylalanine resulted in the inhibition of interleukin-2-stimulated human T cell proliferation.[127] Administration of a selective 5-HT_{1A} receptor antagonist had the same effect, suggesting that this 5-HT receptor subtype is present in human lymphocytes and involved in the signal transduction process. In addition, several other 5-HT receptor subtypes and a high-affinity 5-HT uptake mechanism have been identified in human lymphocytes and macrophages, as reviewed by Mössner and Lesch.[34,128] The concentration-dependent and saturable 5-HT transport system of lymphocytes displayed characteristics similar to the 5-HTT in neuronal tissues, in that it was shown to be dependent on temperature, sodium, and chloride and could be inhibited by the 5-HT reuptake inhibitors fluoxetine ($IC_{50} = 45 \pm 7.5$ nM), fluvoxamine ($IC_{50} = 27 \pm 4.3$ nM), and clomipramine ($IC_{50} = 3.5 \pm 0.56$ nM).[128] Furthermore, there are numerous studies dealing with serotonergic effects on different components of the mammalian immune system.[34] Reciprocally, the immune response may influence serotonergic signaling; e.g., interleukin-4 was found to cause a decrease of 5-HT uptake into human B-lymphocytes.[129]

In stressed fish, the metabolism of 5-HT and catecholamines (noradrenaline, dopamine) was found to be elevated. Since monoamines are able to pass the blood–brain barrier in teleost fish, the stress response of the organism may also cause an increase in peripheral monoamine concentrations[123] (Figure 6). It is assumed that circulating 5-HT also exerts an immunomodulatory effect in fish by activating specific receptors on lymphocyte membranes.[123] Using classical receptor binding studies with 2-methyl-5-HT as competing ligand, 5-HT_3 receptors were identified in lymphocytes of rainbow trout based on the displacement of [^{3}H]5-HT.[130] Further evidence for the presence of 5-HT_3 receptors in trout lymphocytes was provided by Ferrière and colleagues.[131] As increases in intracellular Ca^{2+} levels have been associated with an induction of leukocyte proliferation in catfish,[132] the main objective of their experiments was to examine the effects of 5-HT on calcium signaling in peripheral blood lymphocytes of rainbow trout. Isolated trout lymphocytes were exposed to 25 μM and 50 μM 5-HT and cytosolic Ca^{2+} concentrations were calculated based on fluorescence measurements.[131] In Ca^{2+}-replete cell culture medium, 5-HT exposure resulted in increased intracellular Ca^{2+} levels. In contrast, no serotonergic effect was observed in Ca^{2+}-free medium, suggesting a 5-HT-induced mobilization of Ca^{2+} from extracellular sources. Neither the 5-HT_{1A} receptor agonist 8-OH-DPAT (25 or 50 μM) nor the 5-HT_2 receptor antagonist ketanserin (10 μM) had any effect on intracellular Ca^{2+} concentrations, whereas treatment with 2-methyl-5-HT (20 μM) resulted in elevated Ca^{2+} levels in the cells. Moreover, this stimulating effect could be blocked by administration of 20 μM metoclopramide, indicating that 5-HT may mediate an immunomodulatory effect in rainbow trout via activation of 5-HT_3 receptors. In a related study conducted by Meyniel and coworkers,[130] it was shown that increasing concentrations of 5-HT (0, 62.5, 125, 250 nM) inhibited the phytohemagglutinin-induced proliferation of T cells, presumably via 5-HT_3 receptors, as 2-methyl-5-HT had a similar effect in a concentration range of 0.5 to 100 μM. Analysis of cell cycle progression revealed that 5-HT and 2-methyl-5-HT prevented the transition of activated T cells from G_0/G_1—to S-phase during the cell cycle.

Even though 8-OH-DPAT had no effect on intracellular Ca^{2+} concentrations in trout lymphocytes,[131] 5-HT_{1A} receptors may still be involved in mediating an immunosuppressive effect of 5-HT on T- as well as on B-lymphocytes[133] via different signaling pathways. The inhibiting influence of 5-HT (250 nM) on lymphocyte proliferation was mimicked by 8-OH-DPAT (100 nM) in rainbow trout, while the 5-$HT_{1A/1B}$ receptor antagonist spiperone reversed this effect.[133] These results suggested that trout lymphocytes express functional receptors of the 5-HT_{1A} subtype, at least after mitogenic stimulation with lipopolysaccharides and phytohemagglutinin.

In addition to 5-HT receptors, fish lymphocytes have also been found to express a transporter for 5-HT that may be regulated via 5-HT_{1A} receptors.[134] Transport of 5-HT into isolated trout lymphocytes was shown to require the presence of Na^+ ions in the extracellular medium, which corresponds to the Na^+-dependent transport mechanism found in mammals. In the same study, it was observed that the uptake rate of the trout 5-HTT was adjusted to changing levels of intracellular 5-HT. To investigate the underlying processes, isolated lymphocytes were incubated with substances known to increase intracellular cyclic adenosine monophosphate (caMP) concentrations, including cholera toxin and forskolin. All agents had a stimulatory effect on 5-HT transport into the cells, suggesting that modulation of the 5-HTT may be mediated by 5-HT_{1A} receptors, which are negatively coupled to adenylate cyclase and cAMP production (Table 3). Since a monoamine and a specific 5-HT uptake mechanism with distinct pharmacological profiles had been described, it was attempted to determine which transporter was responsible for 5-HT uptake in trout lymphocytes. While the 5-HTT located in the plasma membrane is susceptible to inhibition by SSRIs such as fluoxetine, the unspecific vesicular monoamine transporter, which was isolated from bovine chromaffin granules, has been found to be specifically blocked by the antihypertensive

drug reserpine.[135] As reserpine did not alter 5-HT transport in isolated rainbow trout lymphocytes, it has been suggested that the vesicular monoamine transporter may not exist in fish lymphocytes.[134] In contrast, exposure to the SSRIs fluoxetine, paroxetine, and sertraline at concentrations of 5, 10, and 20 μM (fluoxetine: 1.73, 3.46, and 6.92 mg/L) caused a decrease of 5-HT uptake into the immune cells. These findings seem to confirm the presence of the plasma membrane-bound type of 5-HTT in trout lymphocytes, although this hypothesis needs to be corroborated as the concentrations employed were exceedingly high. In addition to the identification and characterization of the 5-HT transport mechanism in peripheral blood lymphocytes of rainbow trout, a potential serotonergic effect on the proliferation of isolated lymphocytes was examined in the same study. Following a 96 h exposure to 0.1, 1, and 10 μM of the SSRI formulations alaproclate, zimelidine, fluoxetine, paroxetine, and sertraline (fluoxetine: 0.03, 0.35, and 3.46 mg/L), the incorporation of [^{3}H]thymidine was assessed. Fluoxetine, paroxetine, and sertraline significantly blocked the phytohemagglutinin-induced proliferation of trout lymphocytes in a concentration-dependent manner, starting at 0.1 μM, while alaproclate and zimelidine only had a significant effect at the highest concentration of 10 μM.

The above-mentioned results have demonstrated that 5-HT may play a role as modulator of the immune response in fish and that SSRIs may mimic these immunomodulatory effects. It has been shown that isolated fish lymphocytes may express a functional 5-HTT that can be blocked by SSRIs, thereby inhibiting 5-HT uptake into the cells. Moreover, 5-HT as well as SSRIs appear to inhibit the PHA-induced proliferation of T-cells in rainbow trout.[134] In view of these findings and despite the difficulties associated with extrapolating from effects observed following acute exposure to potential chronic scenarios, the presently available data suggest that continual exposure to environmental concentrations of SSRIs may have an effect on the immunocompetence of fish.

ROLE OF 5-HT IN REPRODUCTIVE PROCESSES

The potential role of the serotonergic system in the modulation of reproductive processes has been addressed extensively in invertebrate studies primarily evaluating descriptive rather than mechanistic endpoints either using 5-HT or SSRIs (Table 5). In invertebrate species, including clams, mussels, crustaceans, echinoderms, and worms, 5-HT was demonstrated to regulate spawning and oocyte maturation. Fong and colleagues[136–138] showed that SSRIs may affect the spawning process in mollusc species. Treatment of zebra mussels with fluoxetine resulted in an induction of spawning at concentrations of 100 nM ($\sim$30 μg/L) in males and 1 μM in females.[137] Fluvoxamine proved to be even more potent than fluoxetine, as it induced spawning in male zebra mussels at concentrations as low as 1 nM (318 ng/L). The latter findings were corroborated by similar observations by Honkoop et al.[139] and Krantic et al.[140] in marine clams. In the crustacean *Daphnia magna*, subchronic (30 days) fluoxetine exposure to 36 μg/L significantly increased fecundity.[141] In most invertebrate studies (Table 5), the concentrations of 5-HT and SSRIs employed were well above the levels measured in the environment and thus the results should be interpreted with caution as to their relevance for environmental risk assessment.

In contrast to the situation in invertebrates, more mechanistic, albeit limited, data are available on the function of 5-HT and the potential influence of SSRIs on aquatic vertebrate reproduction (Table 6). The endocrine regulation of reproduction in vertebrates is mediated via the hypothalamus-pituitary-gonad (HPG) axis. Like the HPA/HPI axis, the HPG axis displays a hierarchical organization with the hypothalamus representing the highest-ranking level. The main hypothalamic factors controlling this system are gonadotropin-releasing hormones (GnRHs). In mammals, GnRHs are secreted into the blood portal system located in the neurohypophyseal median eminence and transported to the anterior pituitary,[142] where they stimulate the release of gonadotropic hormones, namely, follicle-stimulating hormone (FSH) and luteinizing hormone (LH).[142] 5-HT appears to play a modulatory role in the regulation of gonadotropin release. Indeed, electron-microscopic autoradiographic observations confirmed the presence of 5-HT fibers in the medial preoptic region of the rat brain, with several of these in synaptic contact with LHRH-immunoreactive neurons.[143] It has also been shown that 5-HT in the preoptic region of the rat brain has a stimulatory effect on LH secretion, probably mediated by 5-HT$_1$ and 5-HT$_2$ receptors.[144] Moreover, modulation of HPG signaling by 5-HT may not be restricted to the brain level, but may also be directly effective in the gonads. Key components of the serotonergic system including 5-HT, TPH (TPH-1), and the specific 5-HT transporter (SLC6A4) were localized in mouse cumulus oophorous granulosa cells, oocytes, and early embryos by means of reverse-transcription polymerase chain reaction analysis and immunofluorescence confocal microscopy.[145] 5-HT and SLC6A4 were detected in both ovarian cell types as well as in embryonic tissue, while TPH-1 expression was restricted to cumulus cells. The latter observations indicate that ovarian cells may be the local source of 5-HT as a paracrine agent. The same authors demonstrated that 5-HT caused a dose-dependent increase of cAMP and Ca^{2+} levels in cumulus cells, presumably via 5-HT$_7$ and 5-HT$_{2A}$/5-HT$_{2B}$ receptors, respectively.[146] Furthermore, 5-HT was shown to modulate gonadotropin-induced steroid secretion of human granulosa cells *in vitro*.[147]

In fish, the functional relationship of hypothalamus and pituitary regions varies between different species, but in general, the formation of neurological interfaces during phylogeny resulted in increased hypothalamic control of pituitary function.[148] This is accompanied by a stronger influence of the pituitary on gonad development. The HPG axis regulates reproductive processes including gonad differentiation and maturation in fish. As in mammals, GnRHs are mainly responsible for the stimulation of gonadotropic hormone (gonadotropin, GTH) release from the

TABLE 5
Effects of 5-HT and SSRIs on reproduction-related processes in aquatic invertebrates. *Note*. 5-HT, 5-hydroxytryptamine; bw, body weight; Conc., concentration; d, days; (f), female; FX, fluoxetine, FLV, fluvoxamine; (m), male; n.a., not available

Invertebrate species	Substance	Conc./dose	Application, duration	Pathway/endpoint	References
Molluscs					
Zebra mussel (*Dreissena polymorpha*)	5-HT	10 mM	*in vivo*, 30 min	Induces oocyte maturation and germinal vesicle breakdown	136
		100 μM	*in vitro*, 80 min	Induces germinal vesicle breakdown	
Zebra mussel (*Dreissena polymorpha*)	FLV	1 nM (m)	*in vivo*, 4 h	Induces spawning	137
		100 nM (f)			
	FX	50 nM (m) 5 μM (f)	*in vivo*, 4 h	Induces spawning	
	PX	1 μM (m)	*in vivo*, 4 h	Induces spawning	
Fingernail clam (*Sphaerium striatinum*)	FLV	10 nM	*in vivo*, 4 h	Stimulate parturition	209
	PX	10 μM	*in vivo*, 4 h		
	FX	5 μM	*in vivo*, 12 h	Amplifies 5-HT-induced stimulation of parturition	
Baltic clam (*Macoma balthica*)	FX	1 ppm	*in vivo*, 30 min	Induces spawning (extension of spawning season)	139
Surf clam (*Spisula solidissima*)	5-HT	1 nM–100 μM (EC_{50}= 0.55 μM)	*in vitro*, 30 min	Induces germinal vesicle breakdown	140
Crustaceans					
Water flea (*Daphnia magna*)	FX	36 μg/L	*in vivo*, 30 d	Increases fecundity	141
Red swamp crayfish (*Procambarus clarkii*)	5-HT	15 μg/g bw	*in vivo*, 15 d (injection at 1, 5, 10 d)	Increases ovarian index and oocyte size	210
	FX	15 μg/g bw	*in vivo*, 15 d (injection at 1, 5, 10 d)	Increases ovarian index and oocyte size, amplifies 5-HT-induced effect	
	5-HT	15 μg/g bw	*in vitro*, 24 h	Stimulates release of ovary-stimulating hormone	211
Fiddler crab (*Uca pugilator*)	5-HT	125 nmol/crab (2.5 m*M*)	*in vivo*, 7 d (injection at 1, 3, 5 d)	Stimulates ovarian and testicular development	212
Echinoderms					
Starfish sp. (*Patiria pectinifera* + *Aphelasterias japonica*)	5-HT	10–100 μM	*in vitro*, n.a.	Amplifies maturation-inducing effect of 1-methyladenine in oocytes	213
Nemertea					
Milky ribbon worm (*Cerebratulus lacteus*) Salmon white headed ribbon worm (*Micrura alaskensis*)	5-HT	1 μM	*in vitro*, 2–3 h	Stimulates oocyte maturation via cAMP increase	214
Nematoda					
Roundworm (*Caenorhabditis elegans*)	5-HT	5 mg/ml	*in vivo*, 60 min	Induces egg laying	215
	FX	0.5 mg/ml	*in vivo*, 60 min	Induces egg laying	

TABLE 6
Effects of 5-HT and SSRIs on reproduction-related processes in aquatic vertebrates *Note* 5-HT, 5-hydroxytryptamine; bw, body weight; Conc., concentration; DiOH-p, 17α, 20β-dihydroxy-4-pregnen-3-one; FX, fluoxetine; GnRH, gonadotropin releasing hormone; ip, intraperitoneal; LHRHa, luteinizing hormone-releasing hormone analog; max., maximal; n.a., not available; PAH, preoptic anterior hypothalamus

Vertebrate species	Substance	Conc./dose	Application, duration	Pathway/endpoint	Reference
Amphibians					
African clawed frog (*Xenopus laevis*)	5-HT	1.2 μM	*in vitro*, n.a.	Inhibits progesterone-induced oocyte maturation	[213]
European green toad (*Bufo viridis*)					
Fish					
Goldfish (*Carassius auratus*)	5-HT	10 μM	*in vitro*, 30 min	Stimulates GnRH release in mature females	[159]
	5-HT	2.5 μg/g bw (f) 5 μg/g bw (m)	*in vivo*, 30 min (ip injection)	Stimulates release of serum gonadotropin in sexually mature females (f) and males (m)	[157]
	FX + 5-HT	10 μg/g bw + 2.5 μg/g bw	*in vivo*, 90 + 30 min (ip injection)	Amplifies 5-HT-induced increase in serum gonadotropin	
Atlantic croaker (*Micropogonias undulatus*)	5-HT	20 μg/g bw	*in vivo*, 60 min (ip injection)	In conjunction with LHRHa, increases gonadotropin level	[153]
	FX + 5-HT	10 μg/g bw + 20 μg/g bw	*in vivo*, 90 + 60 min (ip injection)	Amplifies 5-HT-induced increase in gonadotropin level	
Mummichog (*Fundulus heteroclitus*)	5-HT	50 nM (10.6 μg/L)	*in vitro*, max. 72 h	Inhibits steroid-induced oocyte maturation	[163]
Medaka (*Oryzias latipes*)	5-HT	10 μg/L	*in vitro*, 10 h	Stimulates oocyte maturation and production of 17β-estradiol and DiOH-p	[164]
Sea bream (*Pagrus major*)	5-HT	1 μM	*in vitro*, 30 min	Induces GnRH secretion from PAH	[155]

fish pituitary (Figure 7). Additionally, GnRHs have been found to induce ovulation and increase plasma concentrations of the steroid hormones cortisol, progesterone, and testosterone, while reducing the plasma estradiol level in catfish (*Heteropneustes fossilis*).[149] As fish do not possess a blood portal system connecting the hypothalamus with the pituitary, GnRHs appear to reach the anterior pituitary by direct innervation,[97,150] stimulating gonadotrope cells to produce GTHs. To date, two different forms of GTH have been described in fish. GTH I and II have been identified as functionally homologous to the mammalian FSH and LH, respectively.[151] GTH synthesis and release from the pituitary are apparently controlled by the interaction of several neurotransmitters and neuropeptides (Figure 7), which are produced in the hypothalamus and the preoptic area of the fish brain.[152] Several substances, among them 5-HT and gamma-aminobutyric acid (GABA), display a stimulatory influence on gonadotrope cells, while the dopaminergic system appears to maintain an inhibitory function.

It has been demonstrated in several studies that 5-HT induces the release of GTH-II at different levels of the fish HPG axis.[153–159] Khan and Thomas[154] examined the brain of mature male and female Atlantic croaker *in vitro* to localize 5-HT and GnRH immunoreactive elements and found similar neuronal distribution patterns, especially in the preoptic-anterior region of the hypothalamus and in the olfactory bulb. Evidence for interaction between 5-HT and GnRH was found by studying the effects of 5-HT on GnRH release in mature female goldfish ex vivo, as 10 μM and 100 μM 5-HT significantly stimulated GnRH secretion from the preoptic-anterior hypothalamus and the pituitary.[159] Dopamine had the reverse effect, inhibiting GnRH

TABLE 7
SSRI ecotoxicological data *Note*. CP, citalopram; EC, effective concentration; FX, fluoxetine; FL, fluvoxamine; LC, lethal concentration; LOEC, lowest observable effect concentration; NOEC, no effect concentration; PX, paroxetine; ST, sertraline; d, days.

Species	Substance	Endpoint	Concentration/dose	Reference
Invertebrates (free-swimming)				
Water flea (*Ceriodaphnia dubia*)	FX	LC_{50} (48 h)	0.756 μM (0.23 mg/L)	185
Water flea (*Ceriodaphnia dubia*)	CP	LC_{50} (48 h)	3.90 $\pm$ 0.27 mg/L	216
	FL		0.84 $\pm$ 0.41 mg/L	
	FX		0.51 $\pm$ 0.07 mg/L	
	PX		0.58 $\pm$ 0.13 mg/L	
	ST		0.12 $\pm$ 0.05 mg/L	
Water flea (*Ceriodaphnia dubia*)	CP	NOEC (8 d)	0.80 mg/L	216
		LOEC (8 d)	4.00 mg/L	
	FL	NOEC (8 d)	0.366 mg/L	
		LOEC (8 d)	1.466 mg/L	
	FX	NOEC (8 d)	0.089 mg/L	
		LOEC (8 d)	0.447 mg/L	
	PX	NOEC (8 d)	0.22 mg/L	
		LOEC (8 d)	0.44 mg/L	
	ST	NOEC (8 d)	0.009 mg/L	
		LOEC (8 d)	0.045 mg/L	
Water flea (*Daphnia magna*)	FX	LC_{50} (48 h)	2.65 μM (0.82 mg/L)	185
Water flea (*Daphnia magna*)	CP	EC_{50} (48 h)	20 $\pm$ 4.0 mg/L	217
	FL		13 mg/L	
	FX		6.4 $\pm$ 1.3 mg/L	
	PX		6.3 mg/L	
	ST		0.92 $\pm$ 0.17 mg/L	
Water flea (*Daphnia magna*)	PX	EC_{50} (48 h)	2.5 mg/L	218
Invertebrates (sediment-dwelling)				
Midge (*Chironomus tentans*)	FX	LC_{50} (48 h)	15.2 mg/kg	185
Scud (*Hyalella azteca*)	FX	LC_{50} (48 h)	>43 mg/kg	185
Vertebrates				
African clawed frog (*Xenopus laevis*)	FX	EC_{10} (96 h)	3.0 mg/L	183
		EC_{50} (96 h)	4.9 mg/L	
		LC_{10} (96 h)	7.1 mg/L	
		LC_{50} (96 h)	7.5 mg/L	
	PX	EC_{10} (96 h)	3.6 mg/L	
		EC_{50} (96 h)	4.1 mg/L	
		LC_{10} (96 h)	4.4 mg/L	
		LC_{50} (96 h)	5.12 mg/L	
	ST	EC_{10} (96 h)	3.0 mg/L	
		EC_{50} (96 h)	3.3 mg/L	
		LC_{10} (96 h)	3.6 mg/L	
		LC_{50} (96 h)	3.9 mg/L	
Fathead minnow (*Pimephales promelas*)	FX	LC_{50} (48 h)	2.28 μM (0.71 mg/L)	185
Japanese medaka (*Oryzias latipes*)	FX	LC_{50} (48 h)	>28.9 μM (8.9 mg/L)	185

release in these brain regions at concentrations of 10 μM and 100 μM. In sea bream (*Pagrus major*), 5-HT concentrations of 1 μM significantly induced GnRH secretion from the preoptic-anterior hypothalamus of juvenile and adult fish ex vivo.[155] This action seemed to be mediated by 5-HT_2 receptors, since it could be blocked by ketanserin (Table 4). Reciprocally, GnRH appears to influence the serotonergic system in fish. The intra-peritoneal application of 0.15 μg GnRH analog per gram body weight resulted in an increase of 5-HT and noradrenaline concentrations in the hypothalamus and pituitary of female

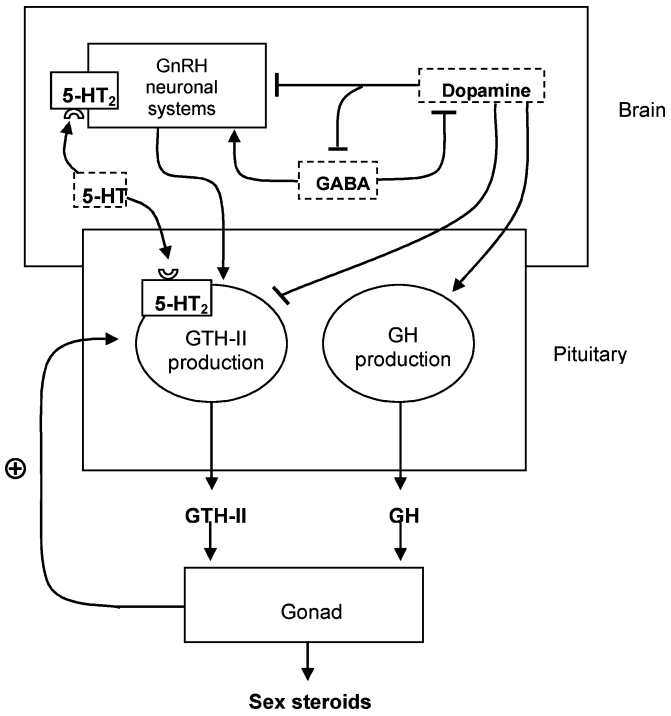


FIG. 7. Simplified schematic of HPG axis in fish. GABA, gamma-aminobutyric acid; GH, growth hormone; ⊣ = inhibition; → = stimulation.

catfish.[149] Simultaneously, dopamine concentrations decreased, again revealing a counterbalancing relationship between these neurotransmitters.

In addition to its suggested stimulation of GnRH secretion, it has been assumed that 5-HT may be able to increase serum concentrations of GTHs by directly acting on gonadotrope cells in the pituitary. Somoza and colleagues[157] examined the effects of 5-HT on the serum GTH-II concentration in male and female goldfish at different maturational stages *in vivo*. In both sexes, intraperitoneal administration of different doses of 5-HT (2.5, 5, 10, 20 μg/g body weight) resulted in a dose-dependent increase of the GTH-II concentration. Pretreatment with 10 μg/g fluoxetine enhanced this effect, while 10 μg/g of ketanserin blocked the stimulating action of 5-HT. In contrast, injection of different 5-HT doses (5, 10, 20 μg/g) in the preoptic brain area had no effect on serum GTH-II concentrations, suggesting that 5-HT exerts its effects on the HPG axis in goldfish specifically at the pituitary level. In a similar study with Atlantic croaker, it was shown that the effect of 5-HT on the HPG axis may depend on the reproductive status of test animals.[153] Indeed, intraperitoneal injections of 20 μg/g 5-HT in 1-year-old females within their first reproductive cycle did not change GTH-II plasma concentrations, while cotreatment with a GnRH analog (LHRHa, 20 ng/g) induced a stimulation of GTH-II release. Administration of 10 μg/g fluoxetine prior to cotreatment with 5-HT and LHRHa amplified the effect of 5-HT, corroborating the observations by Somoza and coworkers[157] in goldfish. In older female Atlantic croaker (2–3 years of age), treatment with 5-HT alone was sufficient to increase plasma GTH-II levels.[153] Interestingly, the regulatory system controlling GTH-II secretion in Atlantic croaker appears to be organized differently than in other fish species studied so far, as dopamine had no inhibitory effect on GTH-II release. *In vitro* experiments with perfused pituitary fragments of goldfish[156,158] and Atlantic croaker[153] confirmed that 5-HT stimulates the release of GTH-II from the pituitary. Again, this effect could be blocked by the antagonists ketanserin[156] or methiothepin and mianserin[158] (Table 4).

In the yellow snapper (*Lutjanus argentiventris*), considerable fluctuations of 5-HT, dopamine, and noradrenaline concentrations in both hypothalamus and pituitary were noticed during gonadal development.[160] Indications for serotonergic modulation of the HPG axis at the hypothalamus level were also found in rainbow trout. While no changes of either 5-HT or 5-HIAA concentrations in connection with reproductive processes could be detected in the pituitary,[161] the observed monoamine levels in the telencephalon and hypothalamus of trout varied depending on the developmental stage.[162] In both brain regions, 5-HT turnover increased during the periovulatory phase, whereas dopaminergic activity declined in both pituitary and hypothalamus. In contrast, the situation was reversed during vitellogenesis, with the serotonergic activity decreasing in the telencephalon and the preoptic-anterior hypothalamus and dopamine concentrations rising in the hypothalamus.[162] These observations suggest that the relative concentrations of these hormones, i.e., their overall balance, may be important for the regulation of GTH-II release, either indirectly by modulating GnRH secretion in the hypothalamus or directly via the pituitary. Therefore, specific increases of 5-HT availability caused by SSRI action may affect the 5-HT/dopamine ratio in the fish brain and lead to a disruption of this interaction regulating reproductive processes via the HPG axis. Apart from its influence on the hypothalamus and pituitary level, 5-HT also seems to have an effect at the gonad level. This hypothesis is supported by the observation of 5-HT in mid- to late-vitellogenic follicles of zebrafish using immunohistochemical methods (Krieger, personal communication). Based on an *in vitro* study with mummichog (killifish, *Fundulus heteroclitus*), Cerdà and coworkers[163] reported that the meiotic maturation of oocytes, stimulated by maturation-inducing steroid (MIS), was blocked by adding 0.05 μM 5-HT hydrochloride (10.6 μg/L) to the cell culture medium. Furthermore, treatment with 100 μM 5-HT hydrochloride induced an increase of cAMP concentrations inside the follicles. This indirectly indicates the presence of 5-HT_4, 5-HT_6, or 5-HT_7 receptors on killifish follicles, as these 5-HT receptor subtypes are thought to be positively coupled to adenylate cyclase in mammals[32] (Table 3). The definitive nature of the receptor types involved in this signal transduction pathway remains to be elucidated. In contrast to the observed inhibitory influence in killifish, 10 μg/L and 100 μg/L of 5-HT had a stimulatory effect on oocyte maturation in Japanese medaka *in vitro*

by inducing synthesis of estradiol and MIS in granulosa cells, resulting in germinal vesicle breakdown.[164] Overall, these studies demonstrate the complexity of serotonergic actions involved in the control of the HPG axis in fish. Moreover, the modulatory function of 5-HT appears to be largely dependent on reproductive status and may vary considerably in different fish species. These factors also make it very difficult to determine whether exposure to environmental concentrations of SSRIs will affect the reproductive system to an extent that may have consequences at the individual or population level. It must be taken into account that in most fish studies using SSRIs to influence serotonergic transmission the agents were administered by injection and at rather high doses, e.g., in the milligrams per kilogram range.[157] As a result, the substances are more likely to reach a specific target site, while the application via tank water, simulating environmental exposure, depends on the stability of the compounds in water and their absorption via the gill and, to a lesser extent, the gastrointestinal tract. Furthermore, when pharmaceutical substances are absorbed from water they become subject to metabolism that may lead to their degradation and deactivation. In the case of fluoxetine, however, metabolic conversion leads to its active demethylated form, norfluoxetine. It is feasible to assume, however, that SSRIs may exert adverse effects on fish reproduction, especially during vitellogenesis, when under normal physiological conditions 5-HT levels should be low. Chronic exposure to SSRIs may induce chronically elevated 5-HT concentrations, altering normal seasonal and daily variations and thus resulting in a subtle but decisive hormonal imbalance, which could influence regular gametogenesis and reproductive behavior. Consequently, SSRI environmental risk assessment demands specific ecotoxicological tests with well-defined and mechanistically plausible endpoints to discern possible subtle effects on reproduction.

SEROTONERGIC REGULATION OF EMBRYO DEVELOPMENT

In connection with the modulatory influence on reproductive processes, 5-HT is also involved in the regulation of invertebrate[165,166] and vertebrate embryo development.[165,167] To date, it has been reported that oocytes of starfish, fish, amphibians, and mammals contain 5-HT, and 5-HT receptors have been detected on the cell surface of oocytes as well as follicular cells of various species.[168] Even prior to the presence of differentiated serotonergic neurons releasing neurotransmitter, 5-HT is involved in the regulation of basic developmental processes related to early embryogenesis, including cell proliferation, migration, differentiation, and morphogenetic cell movements during gastrulation and postgastrulation.[165] By controlling morphogenesis, for instance, 5-HT of maternal origin appears to influence the development and maturation of the mouse brain.[169] Furthermore, it has been shown that 5-HT induces neurogenesis and neuronal differentiation, which includes neurite outgrowth and synaptogenesis, through 5-HT1A receptors in other mammals.[170,171] In several *in vitro* studies using both vascular and nonvascular cells of various mammalian species, 5-HT has been recognized to elicit a mitogenic effect, either mediated by extracellular receptors or by internalization of 5-HT via the 5-HTT.[172] This may be connected to the stimulatory effect of 5-HT on neuroembryogenesis.[170]

The reuptake of 5-HT via active transport and thus termination of 5-HT signaling appears to be an important cue in early embryonic stages; therefore, SSRIs may have the potential to interfere with normal embryo development. In a review by Lauder,[173] it was reported that exposure of cultured mouse embryos to 5-HT uptake inhibitors including fluoxetine, in low micromolar concentrations that did not result in general embryotoxic effects, caused craniofacial malformations, reduced cell proliferation, and increased cell death in craniofacial and cardiac mesenchyme. Blockade of the 5-HTT with 1 μM of paroxetine also decreased proliferation of rat fetal heart cells *in vitro*.[174] In another study, the presence of 5-HTT mRNA was detected in the developing rat brain.[175] Transient expression of the 5-HTT in some brain areas during embryogenesis suggested that, in mammalian ontogeny, there may be critical periods for the regulation of brain 5-HT levels, during which the embryo may be particularly susceptible to SSRI exposure. Developing invertebrate and amphibian embryos may reach an SSRI-sensitive stage when genomic activity switches from transcription of maternal to zygotic genes during the so-called mid-blastula transition.[165] In sea urchins, the binding of 5-HT agonists and antagonists to intracellular or extracellular serotonergic receptors may disrupt mid-blastula transition and lead to increased incidences of malformations, as reviewed by Buznikov and colleagues.[165]

One particular role of 5-HT and the pre-nervous serotonergic system during embryogenesis may be a modulatory influence on the development of the invariant left–right (LR) asymmetry of heart, viscera (liver, spleen, gut), and brain[167] (Figure 8). Since 5-HT is a relatively small charged molecule, it fits the profile of a molecular determinant that may be distributed through gap junctions in the early embryo. Therefore, 5-HT appears to represent an ideal candidate to participate in the initiation of LR axis formation, which involves the breaking of bilateral symmetry.[176–178] It is known that deviations in LR determination at this stage result in abnormal laterality phenotypes that are generally classified as either complete inversion (*Situs inversus*) or heterotaxia (*Situs ambiguus*), i.e., displaying a combination of discordant normal and abnormal LR asymmetries.[179] In some cases, normally asymmetrical organs may be duplicated or missing. In mammals, errors in laterality development are often associated with complex heart defects.[179]

Indications for serotonergic signaling as an early step in the patterning of the LR axis have been found in studies with chick and frog embryos.[176,177] In *Xenopus laevis* embryos, 5-HT was localized asymmetrically in specific cells of early cleavage stages.[176,180] When early *Xenopus* embryos were exposed to subtoxic concentrations of pharmacological agents blocking

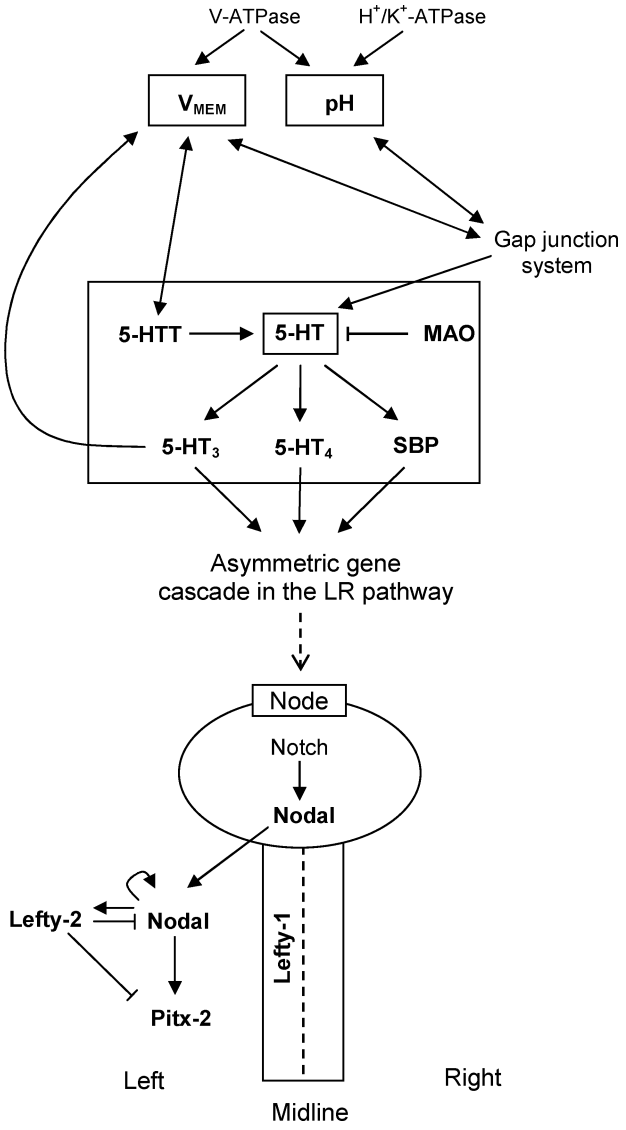


FIG. 8. Proposed interaction of ion flow, serotonergic system and the regulation of LR asymmetry development via the *nodal* pathway during early embryogenesis, modified from Levin[167] and Schier.[200] LR, left-right; SBP, serotonin binding protein; V_{MEM}, membrane voltage.

5-HT_3 or 5-HT_4 receptor subtypes or the two isoforms of the 5-HT-degrading enzyme MAO, a randomization of visceral organ position was observed; i.e., the incidence of heart, gut, and gallbladder heterotaxia was significantly increased.[176] In addition, blocking the already-mentioned components of the 5-HT signaling pathway altered the normal left-sided expression pattern of the earliest known asymmetrically transcribed *nodal*-related gene *xnr-1*. These data suggest that serotonergic actions occur upstream of the early asymmetric gene cascade (Figure 8). It is assumed that the 5-HT-gated ion channel 5-HT_3, which is permeable for potassium ions, may interact with H^+/K^+-ATPase in the regulation of ion flow during the initial phases of LR asymmetry. Fukumoto and colleagues[177] tested the hypothesis that the two known 5-HT transporters, the specific 5-HTT and the unspecific VMAT, may play a role in LR patterning events in *Xenopus*. Directly after fertilization, *Xenopus* embryos were exposed to low micromolar concentrations of different SSRIs including fluoxetine and citalopram or VMAT inhibitors (e.g., reserpine) from fertilization to stage 16 (∼18 h) and evaluated for visceral organ laterality. All pharmacological inhibitors were shown to induce heterotaxia, primarily during cleavage stages. Moreover, fluoxetine and reserpine induced bilateral expression of the normally left-sided *xnr-1*. Although pharmacological experiments alone are insufficient to prove the involvement of the target molecule, the results of this study strongly support the assumption that 5-HT transport is an important process for the establishment of LR asymmetry. Concurrently, it was demonstrated that the normal development of LR asymmetry may be disrupted by SSRIs. The involvement of 5-HTT and VMAT in LR-relevant regulation was also confirmed in chick embryos. In view of the considerable differences in the gastrulation process of birds compared to amphibian species, the latter finding points to a stringent evolutionary conservation of these developmental mechanisms.

As most of the studies investigating the serotonergic actions in early embryo development of lower vertebrates have been conducted with amphibian or chick embryos, there is a dearth of information on the function of 5-HT and potential effects of SSRIs during fish embryogenesis. Nevertheless, considering that developmental processes in vertebrate embryos are generally very similar and that the LR determination pathway appears to be highly conserved, it can be assumed that LR asymmetry in fish embryos may also be affected by SSRIs. As a consequence, fish may exhibit organ dysfunctions, e.g., heart defects, or show behavioral aberrations as a result of abnormal brain laterality.[181] Whether exposure to environmental SSRI concentrations has an influence on fish ontogeny remains to be investigated.

PAST AND CURRENT EFFORTS TO DETECT ECOTOXICOLOGICAL EFFECTS OF SSRIs

To date there is only limited information available regarding the ecotoxicological potential of SSRIs, especially under chronic exposure conditions. Most of the standardized testing methods that are currently employed in environmental risk assessment of pharmaceuticals including SSRIs examine acute effects in systems *in vitro* or *in vivo*. Using cultures of primary hepatocytes from immature rainbow trout (PRTH) and the PLHC-1 cell line, derived from a hepatocellular carcinoma in topminnow (*Poeciliopsis lucida*), the toxicity of fluoxetine was determined by assessing cell viability, activity of cytochrome P450 1A enzyme, and generation of reactive oxygen species (ROS).[182] Both cell types were exposed to fluoxetine concentrations up to 140 μM (48.4 mg/L) for 24 h. The cell cultures were then analyzed with 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyltetrasodium bromide tetrazolium (MTT) and 7-ethoxyresorufin-*O*-deethylase (EROD) assays. The MTT assays showed effective concentration (EC_{50}) values of 5 μM (1.73 mg/L) and 66 μM (22.8 mg/L) for PLHC-1 and PRTH, respectively, and the EROD assay resulted in an EC_{50} value of 77 μM (26.6 mg/L) for PRTH. Changes in ROS levels were only determined for PLHC-1 cells. Fluoxetine exposure had only a marginal stimulatory effect on ROS production (a significant increase was found only at 40 μM [13.8 mg/L]), thus demonstrating limited cytotoxicity, as would be expected from a pharmaceutical designed for chronic therapeutic intervention. It has to be emphasized that the fluoxetine concentrations employed in this cytotoxicity study were several orders of magnitude higher than detected environmental levels.

Potential embryotoxic effects of fluoxetine, paroxetine and sertraline were tested by Richards and Cole[183] using the acute (96-h) Frog Embryo Teratogenicity Assay *Xenopus* (FETAX).[184] SSRIs demonstrated a teratogenic potential only at high concentrations with malformations and lethality detected at concentrations above 2 mg/L. All three SSRIs induced skeletal and muscular tail flexures. Fluoxetine exposure also induced facial malformations that may be interpreted as being the result of serotonergic regulation of craniofacial morphogenesis in *Xenopus* embryos. These results are in agreement with the observation that neither fluoxetine nor fluvoxamine caused any effects in *Xenopus* embryos up to a concentration of 1 mg/L using the FETAX system (Kreke, unpublished data). However, the endpoints examined with the FETAX are not specific enough to detect potentially subtle effects of SSRIs on embrogenesis.

Brooks and colleagues[185] conducted *in vivo* tests to examine the acute effects of fluoxetine exposure on different phyla including algae, daphnids, and fish species (Japanese medaka and fathead minnow). Juvenile fathead minnows (*Pimephales promelas*) and medaka (*Oryzias latipes*) were exposed to nominal concentrations of fluoxetine (1.8, 3.6, 7.2, 14.5, 28.9 μM) for 48 h. The LC_{50} values for fathead minnow, intrapolated from two tests with 11- and 14-day-old fish, were rather high, i.e., 2.22 μM and 2.88 μM, respectively. Medaka were evidently not susceptible to fluoxetine exposure, as their survival rate was unaffected at all fluoxetine treatment levels. These findings are not surprising, as acute effects would not a priori be expected, but rather confirm the notion that pharmaceuticals designed for chronic therapeutic intervention are not acutely toxic in any species. In view of the pharmacological characteristics and the current understanding of SSRIs in mammals and other species, i.e., their potential influence on normal physiological function, and in view of the prevailing environmental conditions, further *in vivo* experiments focusing on the potentially subtle effects of low SSRI levels (ng/L) in fish over an extended period of time are required. Long-term effects may occur at much lower concentrations and follow different toxicodynamic mechanisms than extrapolated from acute exposure studies.[186] Indeed, some indications for subtle effects of SSRIs on behavioral and reproductive processes in nontarget organisms were found in several studies with aquatic invertebrates (Table 5), even though these investigations often had an economic rather than an ecological purpose, e.g., yield increase in mussel farming.[2] Nevertheless, these investigations represent a starting point for the understanding of potential impact of SSRIs in the aquatic environment. Interestingly, it was also discovered in studies with invertebrates that lower concentrations of SSRIs may have a more pronounced effect than higher concentrations. In exposure experiments with the marine snail *Ilyanassa obsoleta*, injections of 1 μM fluoxetine caused a higher stimulation of larval metamorphosis than a concentration of 100 μM.[187] However, these findings need further confirmation, as many of the studies investigating the impact of SSRI exposure on invertebrates were conducted with a limited degree of replication such that reporting of false positive (chance) effects cannot be excluded.

In fish, SSRIs such as fluoxetine have been used as pharmacological agents to manipulate and characterize serotonergic action. It has been observed that fluoxetine had a stimulating influence on GnRH and GTH-II release[157,159]; therefore, SSRIs may interfere with fish reproductive processes by altering endogenous 5-HT concentrations. Foran and colleagues[188] evaluated the reproductive capabilities of adult Japanese medaka following a 4-week exposure to nominal fluoxetine concentrations of 0.1, 0.5, 1, and 5 μg/L. After 14 days of treatment in single-sex groups, fish were paired in separate exposure tanks to assess reproductive endpoints, including number of eggs produced (total and per day), spawning frequency, percentage of fertilized eggs, hatching success, and proportion of malformed hatchlings. Additionally, somatic and physiological parameters such as condition factor, gonadal somatic index, hepatic vitellogenin content, and circulating steroid concentrations were measured. No changes in fecundity, fertility, spawning rate, and hatching success were observed. A slightly higher frequency of developmental abnormalities compared to embryos of control animals was noted in all fluoxetine-treated groups; however, these effects did not appear to be concentration-dependent. The only physiological endpoint that appeared to be affected by fluoxetine exposure was the concentration of circulating estradiol, which was elevated in females exposed to 0.1 μg/L. However, as this increase of estradiol levels was only significant at the lowest fluoxetine treatment concentration and no dose-response relationship was observed, these findings have to be considered with caution. The lack of significant response to higher fluoxetine concentrations may be due to saturation effects and/or feedback inhibition. Therefore, the current data suggest that fluoxetine does not elicit an effect on reproductive function in Japanese medaka even following 4 weeks of exposure. Nevertheless, the endpoints chosen in this study may not have been sufficiently specific, i.e., not incorporating the known mode of action of fluoxetine, to detect a potentially subtle influence of this SSRI under prolonged exposure conditions. It should also be considered that these findings refer to a single test organism and that medaka may be less sensitive to fluoxetine than other fish species; indeed, species-specific differences regarding susceptibility to chemical exposure have

already been observed in a study using medaka compared to tests with fathead minnow.[189]

One problem of the environmental risk assessment of pharmaceutical substances is that even when the known mode of action is considered in the selection of ecotoxicity tests and endpoints, the existence of other molecular targets and associated pathways cannot be excluded.[2] Indeed, although SSRIs are propagated as having almost no nonspecific effects in humans, also implied by the term "selective" in the naming of this pharmaceutical class, there are some indications that these compounds may not only interact with the 5-HTT but also with MAO enzymes and certain 5-HT and even acetylcholine receptors.[190–193] The three SSRIs fluoxetine, fluvoxamine, and citalopram, for instance, demonstrated a competitive inhibitory activity toward rat brain MAO in the micromolar range.[190] In an *in vitro* study with *Xenopus laevis* oocytes expressing cloned rat 5-HT_{2C} receptors, Ni and Miledi[191] observed that micromolar concentrations of fluoxetine blocked the responses of these receptor previously elicited by 5-HT. Using the same model system, fluoxetine was also shown to block both muscle and neuronal nicotinic acetycholine receptors (nAChR) in a noncompetitive and voltage-dependent manner.[192] Similarly, sertraline and paroxetine displayed a noncompetitive inhibitory effect on two human and one chick nAChR subtypes in a low to intermediate micromolar concentration range.[193] It has to be emphasized, however, that the SSRI levels used in the latter studies are not environmentally relevant; nor are they applicable to a chronic exposure situation. In addition to the already-mentioned enzymes and receptors, nontarget organisms may possess yet other signal transduction pathways, not common in humans or other mammals, that may be affected by SSRIs. In this context, biochemical and cellular responses may serve as early warning signals that exposure to SSRIs may have deleterious effects at the organism level.[194] In addition, the analysis of defined behavioral endpoints, especially associated with reproductive processes, may be advantageous.

Theoretical models aiming to predict the environmental impact of SSRIs are inconclusive when they are not supported by experimental data. So far, these models primarily rely on parameters such as physicochemical properties of the compound in question, pharmacological data obtained in mammalian studies, measured or predicted environmental concentrations,[195] and the limited amount of ecotoxicological data. Ideally, such a model could be used to evaluate the ecotoxic potential of SSRI formulations. However, there may be some problems with the implementation in actual risk assessment as long as analytical data sets are inconsistent—an example is the high variability of the fluoxetine log K_{ow} (Table 2)—and information on environmental toxicology is unavailable. In a recent survey in Switzerland, the ecotoxicological hazard potential of fluoxetine and citalopram along with other pharmaceuticals and their metabolites was estimated.[196] The theoretical analyses were based on Swiss sales data, human metabolism, and a baseline toxicity that was derived from acute *Daphnia* toxicity tests. For the two SSRI formulations, no indications for an ecotoxicological risk in Swiss wastewater were found. However, the authors reported that the proposed screening method showed some limitations as specific toxicological effects or chronic exposure conditions were not considered in the calculations, because the required experimental data were lacking. Furthermore, the model employed did not take into account specifically acting metabolites of baseline toxic parent compounds, which may be critical regarding substances such as fluoxetine and its bioactive metabolite norfluoxetine. This clearly illustrates that an "easy" model to estimate the potential ecotoxicity of SSRIs as a pharmaceutical class is not available.

For an adequate risk assessment of SSRIs, a combination of theoretical and practical approaches would be advantageous, incorporating physicochemical parameters, known physiological interactions, and specific endpoints, as well as respective experimental data that should preferably be derived from chronic studies with different fish species commonly used in environmental monitoring and laboratory research. However, instead of conducting life-cycle tests with standardized endpoints, a more mechanistic approach incorporating the characteristics of the pharmacological target system is required. Although analytical data on the presence of SSRIs in the aquatic environment are scarce and should be considered with caution as they may not be representative, the results of the preliminary study conducted by Brooks and colleagues[18] who detected SSRIs and metabolites in fish tissues should not be ignored. Therefore, analogous to the suggestions of Owen and colleagues[20] concerning the potential interactions of β-blockers with the fish adrenergic system, a better understanding of the serotonergic system in fish species, including 5-HT receptor and transporter function, would be advisable to define suitable evaluation parameters and biomarkers for SSRI ecotoxicology testing. The information gained in physiological investigations could then also be used to estimate the potential impact of other pharmaceutical agents modulating 5-HT availability such as tricyclic antidepressants and MAO inhibitors.

CONCLUDING REMARKS

The presence of pharmaceutical residues in the aquatic environment has become an issue of public, political, and also scientific concern. Neuroactive drugs such as SSRIs may affect nontarget organisms due to the presence of phylogenetically highly conserved signaling mechanisms with similar functions. By summarizing the currently available information, it is demonstrated that the serotonergic system plays a modulatory role in several physiological processes in fish and that 5-HT signal transduction may be mediated by neuronal, endocrine, and paracrine pathways. The influence of 5-HT on different target organs and tissues appears to be species-specific and may also depend on gender and/or the developmental and reproductive status of the individual. Because of the complexity of the serotonergic system and in view of the limited amount of experimental data on the effects of SSRIs, it is difficult to assess the potential consequences of continuous SSRI exposure for

fish populations. Clearly, current experimental approaches used for routine environmental risk assessment of pharmaceuticals, including SSRIs, are inadequate if not entirely inappropriate, especially as relevant endpoints are not assessed or impossible to determine in the experimental designs employed. Future toxicology testing of environmentally relevant compounds must encompass and reflect the known pharmacological effects of those substances and should therefore focus more strongly on specific molecular targets, rather than being simply descriptive. In the case of SSRIs, this will primarily comprise the 5-HT transporter and receptors; however, the interactions of 5-HT-specific pathways with other hormonal and neurotransmitter systems should not be ignored.

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