Academic dissertation

Zebrafish as a model of **Parkinson's disease**

Ville Sallinen, M.D.
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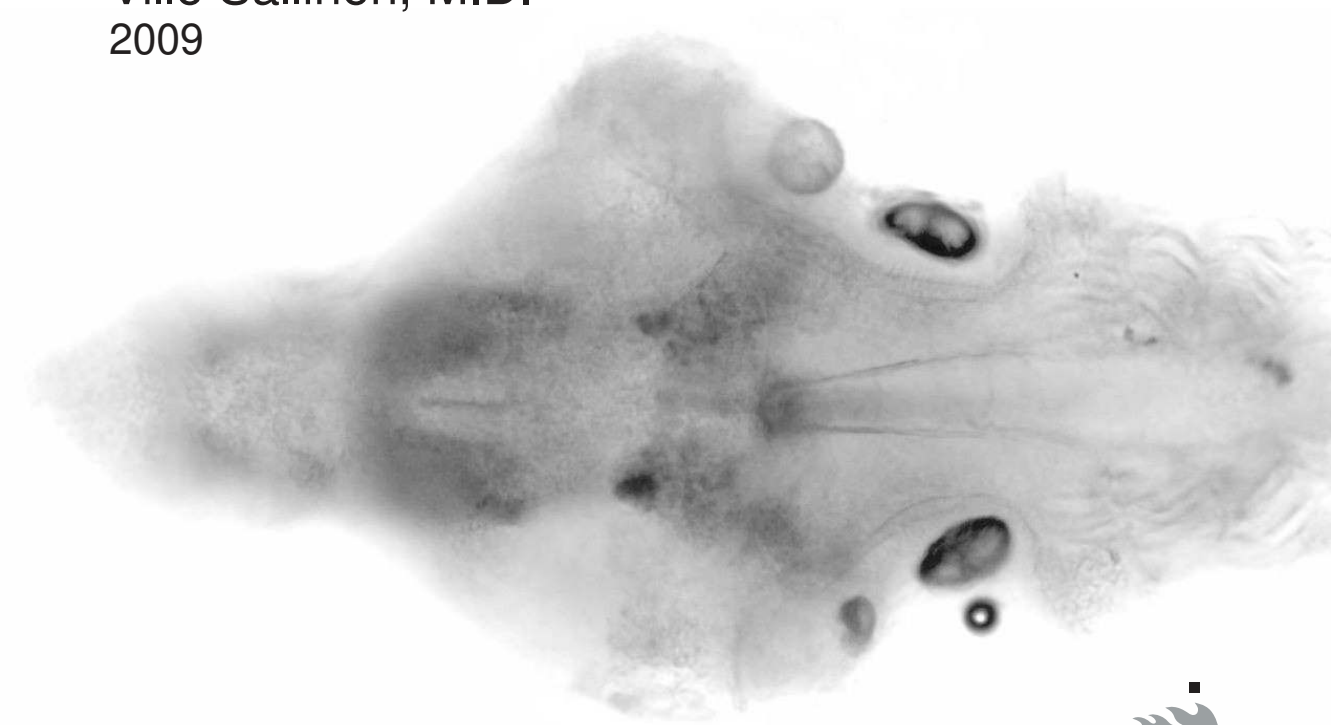

UNIVERSITY OF HELSINKI

Zebrafish as a model of Parkinson's disease

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&

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Academic Dissertation
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To k

”Essentially, all models are wrong, but some are useful”

-George EP. Box, Norman R. Draper (1987).

Empirical Model-Building and Response Surfaces, p. 424, Wiley.

Table of contents

List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-IV).

I. **Anichtchik O, Sallinen V, Peitsaro N, Panula P.** Distinct structure and activity of monoamine oxidase in the brain of zebrafish (Danio rerio). *J Comp Neurol* 498:593-610 (2006)

II. **Sallinen V, Sundvik M, Reenilä I, Peitsaro N, Khrustalyov D, Anichtchik O, Toleikyte G, Kaslin J, Panula P**. Hyperserotonergic phenotype after monoamine oxidase inhibition in larval zebrafish. *J Neurochem*, Epub Feb 13th 2009

III. **Sallinen V, Torkko V, Sundvik M, Reenilä I, Khrustalyov D, Kaslin J, Panula P.** MPTP and MPP+ target specific aminergic cell populations in larval zebrafish. *J Neurochem* 108:719-731 (2009), Epub Nov 27th 2008

IV. **Sallinen V*, Kolehmainen J*, Priyadarshini M, Chen YC, Panula P.** Lack of Pink1 affects distinct dopaminergic neurons and predisposes larval zebrafish to toxicity of MPTP. Submitted.

* equal contribution

Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disease among the elderly. Its etiology is unknown and no disease-modifying drugs are available. Thus, more information concerning its pathogenesis is needed. Among other genes, mutated PTEN-induced kinase 1 (PINK1) has been linked to early-onset and sporadic PD, but its mode of action is poorly understood. Most animal models of PD are based on the use of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is metabolized to MPP+ by monoamine oxidase B (MAO B) and causes cell death of dopaminergic neurons in the substantia nigra in mammals. Zebrafish has been a widely used model organism in developmental biology, but is now emerging as a model for human diseases due to its ideal combination of properties. Zebrafish are inexpensive and easy to maintain, develop rapidly, breed in large quantities producing transparent embryos, and are readily manipulated by various methods, particularly genetic ones. In addition, zebrafish are vertebrate animals and results derived from zebrafish may be more applicable to mammals than results from invertebrate genetic models such as *Drosophila melanogaster and Caenorhabditis elegans*. However, the similarity cannot be taken for granted.

The aim of this study was to establish and test a PD model using larval zebrafish. The developing monoaminergic neuronal systems of larval zebrafish were investigated. We identified and classified 17 catecholaminergic and 9 serotonergic neuron populations in the zebrafish brain. A 3-dimensional atlas was created to facilitate future research. Only one gene encoding MAO was found in the zebrafish genome. Zebrafish MAO showed MAO A-type substrate specificity, but non-A-non-B inhibitor specificity. Distribution of MAO in larval and adult zebrafish brains was both diffuse and distinctly cellular. Inhibition of MAO during larval development led to markedly elevated 5-hydroxytryptamine (serotonin, 5-HT) levels, which decreased the locomotion of the fish. MPTP exposure caused a transient loss of cells in specific aminergic cell populations and decreased locomotion. MPTP-induced changes could be rescued by the MAO B inhibitor deprenyl, suggesting a role for MAO in MPTP toxicity. MPP+ affected only one catecholaminergic cell population; thus, the action of MPP+ was more selective than that of MPTP. The zebrafish *PINK1* gene was cloned in zebrafish, and morpholino oligonucleotides were used to suppress its expression in larval zebrafish. The functional domains and expression pattern of zebrafish PINK1 resembled those of other vertebrates, suggesting that zebrafish is a feasible model for studying PINK1. Translation inhibition resulted in cell loss of the same catecholaminergic cell populations as MPTP and MPP+. Inactivation of PINK1

sensitized larval zebrafish to subefficacious doses of MPTP, causing a decrease in locomotion and cell loss in one dopaminergic cell population.

Zebrafish appears to be a feasible model for studying PD, since its aminergic systems, mode of action of MPTP, and functions of PINK1 resemble those of mammalians. However, the functions of zebrafish MAO differ from the two forms of MAO found in mammals. Future studies using zebrafish PD models should utilize the advantages specific to zebrafish, such as the ability to execute large-scale genetic or drug screens.

Abbreviations

3-MT	3-methoxytyramine
3-OMD	3-O-methyldopa
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine, serotonin
5-HTP	5-hydroxytryptophan
6-OHDA	6-hydroxydopamine
A	adrenaline
A1–A17	noradrenergic and dopaminergic cell populations
aa	amino acid
AADC	aromatic L-amino acid decarboxylase (EC 4.1.1.28)
ADH	alcohol dehydrogenase (EC 1.1.1.1)
ADP	adenosine-5'-diphosphate
ALDH	aldehyde or aldose dehydrogenase
AR	adrenoreceptor
ATP	adenosine-5'-triphosphate
B1-9	serotonergic cell populations
C1-3	adrenergic cell populations
CNS	central nervous system
COMT	catechol O-methyltransferase (EC 2.1.1.6)
COX-2	cyclooxygenase-2 (EC 1.14.99.1)
DA	dopamine
DAB	diaminobenzidine
DAT	dopamine transporter
DBH	dopamine beta hydroxylase (EC 1.14.17.1)
DDC	DOPA decarboxylase (a.k.a. AADC) (EC 4.1.1.28)
DHPG	3,4-dihydroxyphenylglycol
DIL	diffuse inferior lobe
DNA	deoxyribonucleic acid
DOPAC	dihydrophenylacetic acid
dpf	day(s) post-fertilization
E1-5	histaminergic cell populations
EDAC	1-ethyl-3,3(dimethyl-aminopropyl)-carbodiimide
EDTA	ethylenediaminetetraacetic acid
ENU	N-ethyl-N-nitrosurea
FGF8	fibroblast growth factor 8
Gbx2	gastrulation brain homeobox 2
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
HA	histamine
HDC	histidine decarboxylase (EC 4.1.1.22)
hpf	hour(s) post-fertilization
HPLC	high-performance (or pressure) liquid chromatography
HVA	homovanillic acid
iNOS	inducible NOS
ir	immunoreactivity
LC	locus coeruleus
L-DOPA	3,4-dihydroxyphenylalanine
MAO A, B	monoamine oxidase A, B (EC 1.4.3.4)
MET	metanephrine
MHB	mid-hindbrain boundary
MHPG	3-methoxy-4-hydroxyphenylglycol

MO,mo	morpholino
MPP+	1-methyl-4-phenylpyridium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
NA	noradrenaline
NAT	noradrenaline transporter
NMET, NMN	normetanephrine
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase (EC 1.14.13.39)
nTPOC	nucleus of the tract of the postoptic commissure
ONOO-	peroxynitrate
Otx2	orthodenticle homeobox 2
PBS	phosphate-buffered saline
PBS-T	PBS containing 0.3% Triton X-100
PCPA	p-chlorophenylalanine
PCR	polymerase chain reaction
PD	Parkinson's disease
PEA	phenylethylamine
PFA	paraformaldehyde
PGZ	periventricular gray zone
PINK1	PTEN-induced kinase 1
PINK1-ir	PINK1-like immunoreactivity
PMT	photomultiplier tube
PNMT	phenylethanolamine N-methyltransferase (EC 2.1.1.28)
PTEN	phosphatase and tensin homolog
QPCR	real-time (quantitative) PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcriptase PCR
SERT	serotonin reuptake transporter
Shh	sonic hedgehog
SNpc	substantia nigra pars compacta
SO_4	sulfate
SS	serotonin syndrome
SSRI	selective serotonin reuptake inhibitor
TH	tyrosine hydroxylase (EC 1.14.16.2)
TH-ir	TH immunoreactivity
TMN	tuberomamillary nucleus
TPH1,2	tryptophan 5-hydroxylase isoform 1, 2 (EC 1.14.16.4)
VLA	vanillactic acid
VMA	vanillylmandelic acid
VMAT 1,2	vesicular monoamine transporter 1, 2
VTA	ventral tegmental area
WT, wt	wild type
ZFN	zinc finger nuclease

Introduction

The human brain carries out remarkably complex tasks that are essential for the survival and successful functioning of an individual. These functions result from the cooperation of roughly 100 billion (10^{11}) neurons. Neurons communicate with each other by secreting neurotransmitters, which regulate ion fluxes through the cell membrane. Some types of neurotransmitters are directly involved in mediation of synaptic signal transduction, while others are used for more subtle regulation of neuronal excitability. Amine neurotransmitters belong to the latter category and are therefore commonly called modulatory neurotransmitters.

Monoaminergic transmitters are involved in various physiological processes such as mood, appetite, locomotion, reward, memory, and learning. They have also been implicated in several human diseases or pathologial conditions, including Parkinson's disease (PD), Huntington's disease, schizophrenia, addiction, serotonin syndrome, anxiety, depression, aggression, and migraine.

PD is the second most common progressive neurodegenerative disease after Alzheimer's disease. PD is characterized by difficulties in movement control. Its classic symptoms include resting tremor, inability to start movements, rigidity, and difficulties in maintaining gait. PD results from the slow degeneration of dopaminergic neurons in the pars compacta of the substantia nigra and subsequent reduction of the modulatory actions of dopamine in the striatum. The etiology of PD remains unknown and no disease-modifying drugs are available. Thus, the need for more information on the pathogenesis of PD is obvious.

Zebrafish has been widely used as a model organism for developmental biology, and many important discoveries concerning vertebrate development have been made by using this organism. Zebrafish have many favorable properties as experimental animals. The embryos are small and transparent, and as adults they are inexpensive and easy to maintain, develop rapidly, and breed in large quantities. Furthermore, manipulation of zebrafish is relatively easy with a variety of methods, including reverse and forward genetics as well as physical, chemical, and pharmacological interventions. Zebrafish are vertebrate animals and thus in many respects relatively similar to other vertebrates, including humans. This has allowed the use of zebrafish to model several human diseases. Nevertheless, prior to drawing conclusions from a zebrafish model of human disease, careful validation is needed, and the similarities and differences in the basic functions involved in the modeled phenotype must first be established.

The aim of this study was to establish and test modeling PD using larval zebrafish. Little was known about the aminergic neuronal systems in larval zebrafish. Therefore, the developing monoaminergic neurotransmitter systems and one of their main metabolizing enzymes, MAO, were first characterized. The next

step was to characterize the effects of the neurotoxins MPTP and MPP+ on larval zebrafish. Then, the toxin-induced PD model was used to study the role of a PD susceptibility gene, *PINK1*.

1. Review of the literature

1.1 Modulatory neurotransmitter systems

1.1.1. Synaptic transmission in the brain

The human brain, approximated to consist of over 100 billion (10^{11}) neurons, has a remarkable capability to carry out complex tasks. A substantial property of a neuron is its ability to fire an action potential upon excitation. An action potential is a pulse-like wave of voltage that travels along an axon (or other plasma membrane). To be able to work together, neurons need a way to transmit this potential to one another. Ramón y Cajal was the first to propose that neurons communicate with each other by means of specialized junctions, synapses (a term coined by Charles Sherrington in 1897). Two types of synapses exist: electrical synapses and chemical synapses. At electrical synapses, currents flow through gap junctions that form pores in the plasma membrane of two adjacent cells. These pores permit diffusion of a variety of substances, including ions and second messengers, between the neurons' cytoplasms. At chemical synapses by contrast, the neurons are not in direct contact, but the signal is transmitted by chemical compounds called neurotransmitters. Whereas in electrical synapses the transmission can be bidirectional, in chemical synapses the transmission is unidirectional, from the presynaptic neuron to the postsynaptic neuron. As the action potential invades the presynaptic terminal, calcium channels open and Ca^{2+} flow causes the release of neurotransmitters from the presynaptic terminal into the synaptic cleft, a space between the pre- and postsynaptic neurons. These neurotransmitters subsequently bind to specific receptors located on the postsynaptic membrane and regulate its ion fluxes. The ensuing currents result in excitatory or inhibitory postsynaptic potentials that modify the excitability of the postsynaptic neuron. Most of the excitatory neurons release glutamate, while the vast majority of inhibitory neurons use gamma-aminobutyric acid (GABA) or glycine as neurotransmitters. However, a large number of different molecules can serve as neurotransmitters.

1.1.2. Amine neurotransmitters

Amine neurotransmitters are small molecules sharing an amine group ($-NH_2$). There are five main amine neurotransmitters in mammals: dopamine, noradrenaline, adrenaline, serotonin, and histamine. Dopamine, noradrenaline, and adrenaline share a catechol moiety and are called catecholamines. Dopamine, noradrenaline, adrenaline and serotonin are monoamines, whereas histamine is a diamine. See Figure 1 for chemical structure of amines and Figure 2 for a schematic view of the

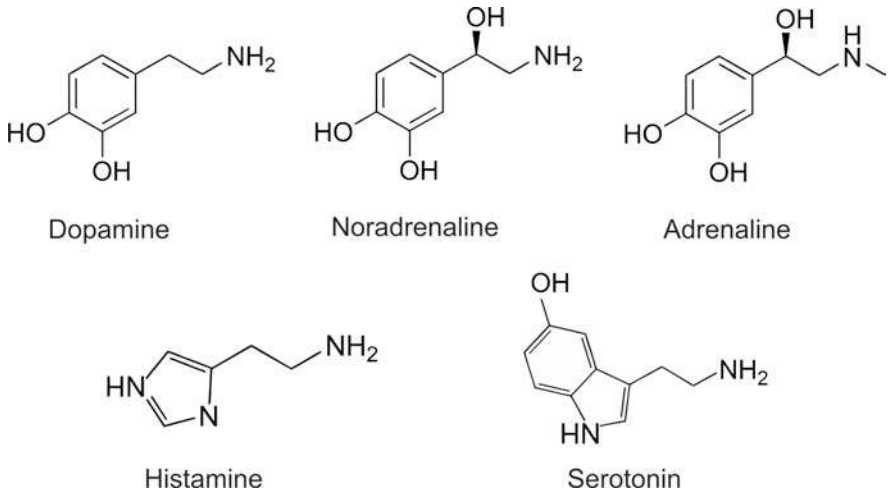

Figure 1.
Chemical structures of biogenic amine neurotransmitters.

synthetic and metabolic pathways of amines. Amine neurotransmitters mainly function via metabotropic G-protein coupled receptors (except 5-HT_3 receptor) that modulate other receptor systems. Instead of inducing large postsynaptic potentials, activation of most amine receptors modulates the processing of information in the neurons by affecting their intracellular biochemical processes. Hence, they are called modulatory neurotransmitters.

1.1.2.1. The catecholaminergic system

1.1.2.1.1. History

3,4-dihydroxyphenylethylamine (dopamine) was initially found to be a sympathomimetic, adrenaline-like substance[1]. At the discovery of dopa-decarboxylase (DDC)[2] and the synthetic pathways of noradrenaline and adrenaline, 3,4-dihydroxyphenylethylamine was considered only as a metabolic intermediate[3, 4]. Nearly 40 years after its first discovery, in 1952, Sir Henry Dale proposed the name dopamine because it was an amine produced from the precursor L-DOPA (3,4-dihydroxyphenylalanine)[5]. Soon thereafter, the specific functions of dopamine in blood pressure regulation were discovered[6, 7]. Dopamine was also found in the brain[8, 9], with high concentrations in the striatum,[10] leading to the suggestion that dopamine is involved in the regulation of movement[11]. However, the turning point in dopamine research was the discovery of dopamine deficiency in the striatum of patients with PD[12] and the ”miraculous” attenuation of their sympoms upon administration of L-DOPA[13]. Soon after this, catecholaminergic neurons and fibers

were histochemically demonstrated for the first time[14]. Dopamine was later also suggested to be involved in schizophrenia[15].

1.1.2.1.2. Catecholamine synthesis, storage, and degradation

All catecholamines are synthesized from a common precursor, tyrosine, which is obtained from the diet. The rate-limiting step in the synthesis of catecholamines is the hydroxylation of tyrosine to DOPA by the enzyme tyrosine hydroxylase (TH, EC 1.14.16.2)[16], which can be detected in all catecholaminergic neurons and thus is used as a marker for these cells[17]. DOPA is decarboxylated by dopa decarboxylase (DDC, also known as L-aromatic amino acid decarboxylase, AADC, EC 4.1.1.28) to yield dopamine[2]. In noradrenergic and adrenergic cells, dopamine β-hydroxylase (DβH, EC 1.14.17.1) converts dopamine to noradrenaline[18]. In adrenergic cells, phenylethanolamine N-methyltransferase (PNMT, EC 2.1.1.28) further converts noradrenaline to adrenaline[19]. In the brain, all catecholamines are concentrated from the neuronal cytoplasm into storage vesicles by vesicular monoamine transporter, (VMAT2)[20, 21] and released into the synaptic cleft upon arrival of an action potential. VMAT1 exists in the adrenal gland, but not in neurons[22]. However, some neurons exhibit VMAT2, but no enzymes for biogenic amines[23]. On the other hand, other neurons, such as the dopaminergic interneurons in the olfactory bulb[24], express the synthesizing enzymes for transmitter amines, but lack VMAT2[22]. The former situation may represent uptake and usage of ”borrowed transmitters”, while in the latter case, a transmitter could be released without storage in vesicles[25].

Dopamine is degraded via two converging routes in mammals. First, monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT) degrade dopamine to dihydrophenylacetic acid (DOPAC) or 3-methoxytyramine (3-MT), respectively. DOPAC or 3-MT are then degraded to homovanillic acid (HVA) by COMT or MAO, respectively. Thus, both MAO and COMT are needed in the metabolism of dopamine[26, 27]. Dopamine levels in the brain are increased after inhibition of MAO or COMT. Indeed, MAO B inhibitors are used in the treatment of PD (see Section 1.2.).

Similar to dopamine degradation, noradrenaline is degraded by MAO and COMT via two routes. Dopamine is metabolized to normetanephrine (NMN) by COMT or to 3,4-dihydroxyphenylglycol (DHPG) by MAO. DHPG and NMN are further degraded to 3-methoxy-4-hydroxyphenylglycol (MHPG) by COMT and MAO, respectively. By the action of alcohol dehydrogenase (ADH, EC 1.1.1.1), MHPG is degraded into 3-methoxy-4-hydroxymandelic (vanillylmandelic) acid (VMA), which is then excreted[26-28]. Adrenaline follows the same pathway as noradrenaline, with the exception of production of metanephrine (MN) instead of NMN. The synthesis and metabolism of monoamines are summarized in Figure 2.

Another important inactivation route of synaptic amine neurotransmitters is the reuptake system[29]. Dopamine is taken up by the presynaptic neuron via dopamine transporters (DAT)[30, 31]. The mechanism of action of cocaine is inhibition of DAT, leading to accumulation of excess dopamine in the synaptic cleft[32]. The action of amphetamine is even more interesting; it gains access inside the cell by being a substrate for DAT, causes the release of monoamines from vesicles into the cytoplasm, and further promotes reverse transport of the monoamines via DAT to the extracellular space[33]. Like dopamine, a high-affinity reuptake system also exists

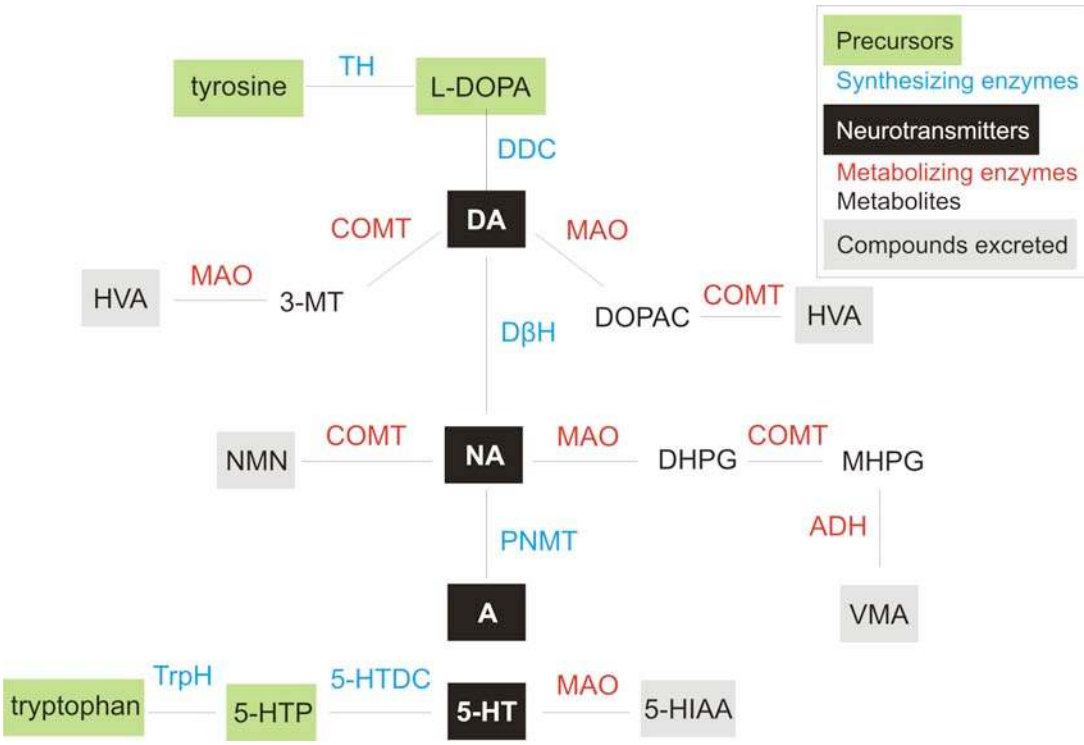

Figure 2.
Illustration showing the synthesis and metabolism of monoamine neurotransmitters. Abbreviations: 3-MT - 3-methoxytyramine, 5-HIAA - 5-hydroxyindoleacetic acid, 5-HT - 5-hydroxytryptamine, 5-HTDC - 5-hydroxytryptophan decarboxylase, ADH – alcohol dehydrogenase, COMT - catechol-O-methyl transferase, DA - dopamine, DβH - dopamine beta-hydroxylase, DDC - DOPA decarboxylase, DHPG - 3,4-dihydroxyphenylethylene glycol, DOPAC - dihydroxyphenylacetic acid, A - adrenaline, HVA - homovanillic acid, L-DOPA - 3,4-dihydroxy-L-phenylalanine, MAO - monoamine oxidase, MHPG - 3-methoxy-4-hydroxyphenyethylene glycol, NA - noradrenaline, NMN - normetanephrine, PNMT - phenylethanolamine N-methyltransferase, TH - tyrosine hydroxylase, TrpH - tryptophan hydroxylase, VMA - 3-methoxy-4-hydroxymandelic acid.

for noradrenaline (noradrenaline transporter, NAT)[34, 35]. Certain commonly used antidepressants, such as venlafaxine and duloxetine, inhibit NAT in addition to the serotonine transporter (SERT)[36]. Dopamine and noradrenaline can also use each other's uptake transporters[37].

1.1.2.1.3. Anatomy and development of catecholaminergic systems

Formaline-induced fluorescent methods (developed by Eränkö[38] and later improved by Falck and Hillarp[39]) allowed visualization and mapping of the monoamines in the brain[40]. The original methods did not discriminate between the catecholamines, and the reaction was strong for noradrenaline and dopamine, whereas adrenaline stained only faintly. The diamine histamine was not detected with this method and was long neglected (see Section 1.1.2.3.).

In 1964, Dahlström and Fuxe[40] described 12 catecholaminergic cell groups in the rat brain using formaline-induced fluorescence. Later reanalyses using antibodies against the biosynthetic enzymes (TH, DBH, PNMT, see above) revealed a total of 17 noradrenergic (A1-A7) or dopaminergic (A8-17) cell groups and 3 adrenergic cell groups (C1-3) in the rat brain[41]. Cells that contain TH and DBH are defined as noradrenergic, whereas cells containing only TH are considered dopaminergic. Adrenergic neurons are defined by the expression of all three enzymes: TH, DBH, and PNMT. The numbering of the cell populations follows a roughly caudorostral direction.

The noradrenergic cell groups A1-A7 are located caudally relative to the dopaminergic A8-A17 groups. The A1 cell group is present in the ventromedial medulla oblongata at the level of the area postrema and continues caudally into the spinal cord. The A2 cell group is in the nucleus tractus solitarius-dorsal vagal motor nucleus complex. The A3 cell group is only detected with the formaline-induced fluorescence method, and thus, largely neglected. The A4 and A6 groups form the locus coeruleus and innervate essentially the whole central nervous system (CNS) with ascending and descending projections. The A5 and A7 groups are located in the pons.

Dopaminergic neurons are more widespread. The midbrain dopaminergic cell populations (A8-10) are the most studied ones due to their roles in important pathological processes in the human brain. The dopaminergic cell populations A8-A10 are located in the ventral mesencephalon. A8 and A10 form the ventral tegmental area (VTA), and A9 is located in the substantia nigra pars compacta (SNpc) (Figure 3A). The nigrostriatal pathway, which connects the SNpc with the striatum, is affected in PD. Mesolimbic and mesocortical pathways connect dopaminergic neurons in the VTA with the ventral striatum and frontal cortex, respectively. The currently predominant understanding of schizophrenia postulates that the negative symptoms of the disease are caused by hypoactive mesocortical

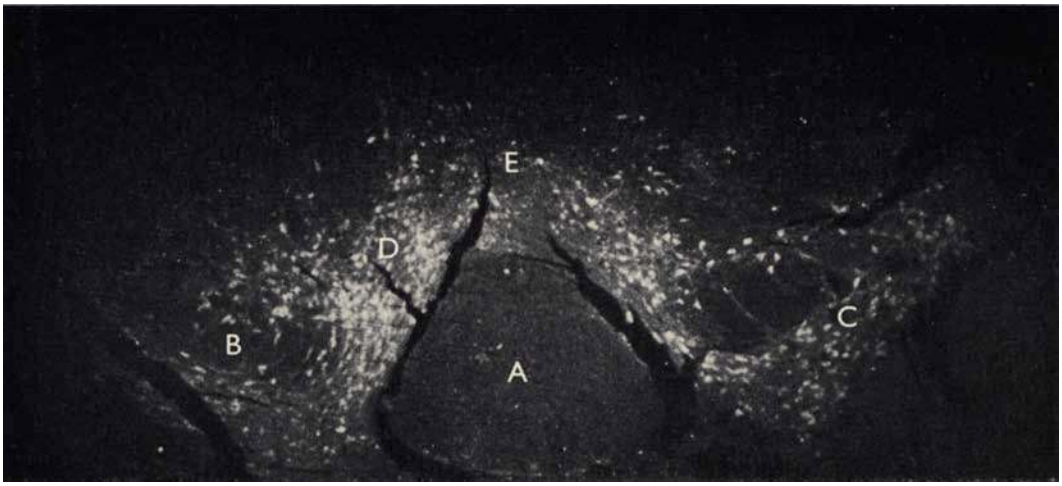

Figure 3A

Groups A9 and A10. Normal rat. Transverse section. A large number of small to medium-sized, round to oval nerve cells (A10) of medium green fluorescence are present just laterally and dorsolaterally of the nuc. inperpeduncularis (A), mainly within an area (D) corresponding to the nuc. parabrachialis pigmentosus in the cat. Many cells belonging to A9 are present within the zona compacta (C) ventral to the lemniscus medialis (B), in which a few fluorescent cells are present. A number of fluorescent nerve cells are present dorsal to the nuc. linearis (E). x 40. Reprinted from Acta Physiol Scand., Vol. 64, Suppl. 232 Dahlström and Fuxe, Evidence for the Existence of Monoamine-containing Neurons in the Central Nervous System I. Demonstration of Monoamines in the Cell Bodies of Brain Stem Neurons, Copyright (1964), with permission from Wiley-Blackwell.

connections, while the positive symptoms are due to hyperactive mesolimbic projections[42].

The A11-A15 populations are found in the diencephalon. Large multipolar dopaminergic cells of the A11 population are located in the caudal diencephalon medial to the fasciculus mammillothalamicus and give rise to the diencephalospinal tract. A12 cells are principally located in the arcuate nucleus and in the nearby periventricular nucleus and project to the neurohypophyseal complex and the pars intermedia of the pituitary[43]. The A13 population is located in the dorsal hypothalamus, mostly in the zona incerta. A14 neurons are located adjacent to the anterior recess of the third ventricle in the rostal periventricular hypothalamus. The A13 and A14 groups together give rise to the incertohypothalamic dopamine projection system. Cell bodies of the large and complex A15 population are located above the optic chiasm and extend caudally to the posterior hypothalamus.

A16 dopamine cells are located in the olfactory bulb, principally in the glomerular layer, but a few cells are also found in the outer plexiform layer. The majority of A17 dopaminergic cells are amacrine cells located in the inner nuclear

or inner plexiform layer of the retina[44, 45] and function in the processing of visual information.

Ventral C1 and dorsal C2 adrenergic cell groups are situated at the rostral end of the A1 and A2 noradrenergic cell groups in the medulla oblongata. However, there is overlap and intermingling between these adrenergic and noradrenergic cells, especially in the A1 area. C3 cells are found in a complex in the dorsal midline areas, within and dorsal to the medial longitudinal fascicle, near the ventral border of the fourth ventricle[46].

For a review of the catecholaminergic systems in the vertebrates, see Smeets and González[47]. A schematic drawing of the vertebrate catecholaminergic cell populations is shown in Figure 3B.

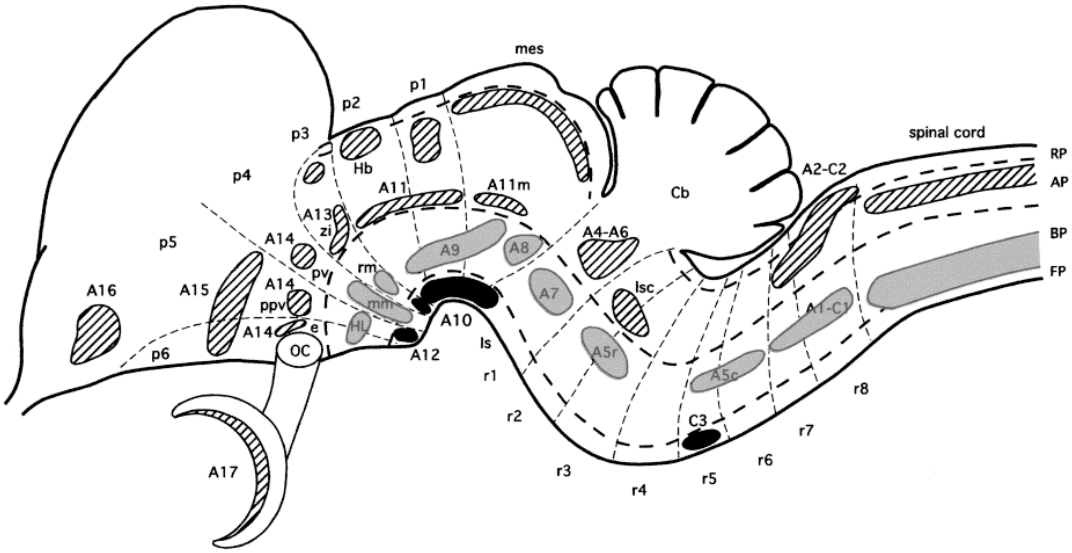

Figure 3B

Schematic drawing of a hypothetical vertebrate brain in which the segmental arrangement of the CA cell groups has been summarized for all vertebrate classes. The classical A1–A17/C1–C3 nomenclature as proposed by Hökfelt et al.[43, 48] is used in combination with new terms that have been recently introduced by Puelles and Verney[49]. The longitudinal and neuromeric subdivisions are indicated by dashed lines in this drawing. Note that some CA cell groups extend over several segments and that cell groups indicated as dorsal and ventral subdivisions of a cell group present rather rostral and caudal portions. Abbreviations: AP, alar plate; BP, basal plate; Cb, cerebellum; e, epichiasmatic population; FP, floor plate; HB, habenula; HL, lateral hypothalamic cell group; mes, mesencephalon; mm, mammillary cell group; OC, optic chiasm; ppv, preoptic periventricular nucleus; pv, paraventricular nucleus; p1–p6, prosomeres 1–6; rm, retromammillary area; RP, roof plate; r1–r8, rhombomeres 1–8; zi, zona incerta. Reprinted from Brain Research Reviews, vol. 33, Willhelmus J.A.J. Smeets and Agustin González, Catecholamine systems in the brain of vertebrates: new perpectives through a comparative approach, pp. 308-379, Copyright 2000, with permission from Elsevier. Licence number 2164911486771.

The anatomy of adult zebrafish catecholaminergic cell groups corresponds well with the mammalian one, the largest difference being the lack of midbrain dopaminergic neurons[50, 51]. The homologs of the substantia nigra and ventral tegmental area in the teleost, particularly in zebrafish, are currently unclear. For further discussion of zebrafish catecholaminergic cell populations, see Section 5.2.
Several factors affecting the development of catecholaminergic neurons have recently been discovered, and remarkably many of these studies were performed using zebrafish. Midbrain dopaminergic neurons have been of particular interest due to their role in schizophrenia, addiction, and PD[52].

Noradrenergic locus coeruleus neurons are among the earliest neurons in the brain, arising from the dorsal isthmus of the hindbrain at E9 in the mouse[53]. Little is known about the differentiation of other noradrenergic cell groups. Differentiation of noradrenergic LC neurons (i.e. expression of TH and DBH) requires sequential activation of BMP[54], Mash1[55], Phox2a[54, 56], and Phox2b[57]. Rnx is also needed[58], but its position in this cascade is unclear at the moment.

The first dopaminergic neurons in the mouse arise rostral to the midbrain-hindbrain boundary (MHB a.k.a. midbrain-hindbrain organizer, MHO) or isthmus, near the floor plate at E10[59]. Since the floor plate and isthmus are the two main organizing centers that pattern the neural plate and tube, the precursor cells of midbrain dopaminergic neurons are dependent on sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF8), respectively, secreted from the centers[60-62]. Second-line transcription factors are needed for the regionalization of the mid- and hindbrain. These include Pax2[63], Lmx1b[64], Wnt1[65] as well as Engrailed-1, Engrailed-2[66], and Pax5[67]. At the time neurons become postmitotic and start to express TH, several transcription factors are expressed that are responsible for the survival of the midbrain dopaminergic neurons. Nurr1[68] is expressed throughout life[69], controlling dopamine synthesis[70], vesicle packaging, and reuptake by regulating the transcription of TH[71], VMAT2[72], and DAT[73], respectively. Interestingly, mutated Nurr1 is associated with familial PD[74, 75], and Nurr1 expression modulates susceptibility of midbrain dopaminergic neurons to the neurotoxins MPTP[76], MPP+, and 6-OHDA[77] (see Section 1.2.3.), providing a link between the developmental factors and mature-onset disease. Slightly after Nurr1 expression, Pitx3 is expressed exclusively in the midbrain dopaminergic neurons[78]. Like Nurr1, Pitx3 is also expressed throughout life. Pitx3 mutant mice (Aphakia, founded originally as a blind mouse line in 1968[79]) display a specific absence of dopaminergic neurons in the substantia nigra[80, 81]. Lmx1b was previously proposed to regulate Pitx3 expression, and thus being involved in the maintainance and survival of midbrain dopaminergic neurons[82]. However, it now seems that lack of Pitx3 expression in Lmx1b-deficient embryos is due to the requirement of Lmx1b

for the expression of Wnt1 and FGF8 and subsequent failure in patterning and regionalization[52, 83]. Nurr1 and Lmx1b affect the development of diencephalic dopaminergic neurons in zebrafish[84, 85]. Yet Lmx1b and Pitx3 are not expressed in dopaminergic neurons in zebrafish[84]. The role of Pitx3 in the development of dopaminergic neurons in zebrafish is currently unclear. The factors determining other catecholaminergic cell groups are not well known, with the exception of the dopaminergic A11 group, which was recently reported to be dependent on the expression of the Orthopedia (Otp) homeodomain protein[86, 87].

1.1.2.1.4 Catecholamine receptors and functions

Noradrenaline (and adrenaline) mediate cellular signaling through G-protein-coupled adrenoceptors (ARs). They were originally divided into two subtypes, α- and β-ARs, by the potency of different agonists[88]. Later, based on their anatomical distributions, α-AR were further divided into α1- and α2-ARs[89]. Upon cloning, new receptor subtypes emerged, and now three α1-ARs (α_{1A}, α_{1B}, α_{1D}[90-92]), three α2-ARs (α_{2A}, α_{2B}, α_{2C}[93-96]), and three β-ARs (β_1, β_2, β_3[97-99]) have been identified in mammals.

As no truly subtype-specific drugs are available, the functions of the AR subtypes have been studied using genetically altered animals. Although α_1-ARs are the most abundant form in the CNS, their functions in the brain are the least understood. α_1-ARs are suggested to be involved in locomotion[100] and control of motor activity[101] as well as cognitive functions[102, 103]. Hypotension, sedation, analgesia, and hypothermia are mainly mediated by the α_{2A}-AR subtype. α_{2C} seems to be involved in several CNS processes such as stress responses, locomotion, memory[104], the startle reflex[105], aggression[105], and modulation of the dopaminergic and serotonergic systems.

β-ARs are mainly located in the cerebral cortex, nucleus accumbens, and striatum. Physiologically, β-ARs are involved in various memory functions[106-108] and in respiratory[109], cardiovascular[110], and renal[111] sympathetic nervous control. Pathophysiologically, β-ARs have been suggested to be involved in glial proliferation after injury[112, 113] and in various psychiatric conditions (e.g. stress[114, 115], depression[116], schizophrenia[116, 117]). Altered expression of β-ARs has been found in PD[118], Alzheimer's disease[119, 120], and Huntington's disease[121].

In addition, ARs have multiple diverse functions outside the CNS, most prominently within the cardiovascular system, but these discussed here.

Orthologs for mammalian α_{2A}-, α_{2B}-, and α_{2C}–ARs in zebrafish have been identified and pharmacologically characterized[122]. α_2-AR activation modulates locomotion and skin color (through melanophore aggregation) in zebrafish[123]. Two additional AR subtype genes, designated as α_{2Da} and α_{2Db}, were also identified in

zebrafish, but these do not have mammalian orthologs[124]. The localization of α_2-ARs in the brain of zebrafish has been described in a tentative fashion[123, 125]. Genes similar to α_{1B}-AR (Gene ID 558460) and two β-AR genes (Gene IDs 100007610 and 571980) are found in the zebrafish genome, but no studies on these have been published.

Five types of dopamine receptors have been discovered in mammals (D_1-D_5). They are all G-protein-coupled receptors and are classified as D_1-like (D_1 and D_5) or D_2-like (D_{2-4}) based on their amino acid composition, pharmacological characteristics, G-protein coupling, and other functional properties[126, 127]. D_1 and D_5 receptors couple with Gs/olf-type G-proteins to increase cAMP prodution by adenylyl cyclases, while D_2, D_3 and D_4 couple with Gi/o proteins to decrease cAMP production and to activate K^+ channels and inhibit Ca^{2+} channels. Dopamine receptors in the CNS are involved in the control of locomotion, cognition, reward, emotions, and neuroendocrine secretion[127, 128]. According to the classical model of dopamine modulation[129], D_1 and D_2 receptors have a major role in modifying the activity of the striatum in the motor loop (see Section 1.2., Figure 5): the medium-sized spiny neurons of the ”direct pathway” are excited by dopamine via D_1 receptors, while the medium-sized spiny neurons of the ”indirect pathway” are inhibited by dopamine via D_2 receptors. The overall effect of dopamine is the activation of the thalamus, regardless of the pathway. Receptors of the D_1 type are the most abundant dopamine receptors in the CNS and are widely expressed in the mammalian brain[130, 131]. Classical antipsychotics, such as chlorpromazine and haloperidol, are D_2 receptor antagonists, although they are generally nonselective and bind to several other types of neurotransmitter receptors as well. However, the extrapyramidal side-effects resulting from overdoses of these substances are mediated via dopamine receptors. On the other hand, dopamine agonists, such as bromocriptine and cabergoline, are used in the treatment of PD, and although these substances are agonists of D_2 receptors, they do act on other subtypes as well.

D_3 receptors are concentrated in the limbic region of the striatum, the nucleus accumbens, suggesting that D_3 receptors function mainly in the mesolimbic system[132]. For this reason, the D_3 receptor subtype has been considered as an attractive target for antipsychotic drug actions[133, 134].

Unlike the other dopamine receptor subtypes, which are concentrated in the striatal region, D_4 receptors are mainly located in limbic structures, such as the amygdala and hippocampus, in addition to the frontal cortex and mesencephalon[135-137]. The atypical antipsychotic drug clozapine has 20 times higher binding affinity to D_4 receptor than the other types of dopamine receptors, and thus, the D_4 receptor subtype seems to be relevant for the action of clozapine[138].

Currently, no drugs discriminating between D_1-like receptors (D_1,D_5) exist, and D_5 receptor function is the least understood of all dopamine receptors. The D_5 receptor exhibits ten-fold higher affinity for dopamine than D_1[139]. D_5 receptor knockout mice have increased sympathetic tone and are hypertensive[140]. Modulation of hippocampal acetylcholine release has been linked to D_5 receptors [141].

Genes for D_1-D_4 dopamine receptors have been identified in zebrafish[142-144]. However, their pharmacological profiles and functions are poorly known. Their expression has been studied in larval zebrafish using whole-mount *in situ* hybridization and light microscopy[142-144]. However, the exact anatomical localizations of the dopamine receptor subtypes have not been reported in zebrafish. Also, no expression analysis of the adult zebrafish brain exists. Zebrafish D_1 receptors are expressed in the larval zebrafish brain in the diencephalon, hypothalamus, and the lateral domains of rhombomeres 3, 4, 6, and 7[142]. Zebrafish D_2 and D_3 receptors are expressed in larval fish in specific nuclei of the telencephalon, diencephalon, and hindbrain[143]. D_4 receptors are expressed widely in the larval zebrafish brain and are found in the telencephalon, diencephalon, midbrain, and spinal cord[144].

1.1.2.2. The serotonergic system

1.1.2.2.1. History

Serotonin (5-HT) was first isolated from bovine serum and named to indicate its source (serum) and function (increase of the vascular tone)[145]. Later, it was also detected in the brain, among other tissues[146]. A hypothesis[147, 148] that 5-HT might be involved in brain functions arose from the observation that a hallucinogenic compound (lysergic acid diethylamide, LSD)[149] antagonized the actions of 5-HT in peripheral tissues. However, evidence has since accumulated that the hallucinogenic effects of LSD are due to partial agonism of 5-HT_{2a} receptors[150]. Although already in 1911 Ramón y Cajal had seen clusters of large multipolar neurons in the midline of the brain stem[151], it was Dahlström and Fuxe in 1964 who described that these cells in the raphe nuclei contained serotonin[40]. These breakthroughs allowed further studies of the brain serotonergic systems and are the basis of our current knowledge.

1.1.2.2.2. 5-HT synthesis, storage, and degradation

The synthesis, storage, and degradation of serotonin share many common features with catecholamine chemistry. Serotonin is synthezised in two steps and has many features in common with the catecholamine synthesis pathway (Figure 2 in Section 1.1.2.1.2.). First, tryptophan is hydroxylated by tryptophan hydroxylase (TPH, EC

1.14.16.4) to produce 5-hydroxytryptophan, which is a rate-limiting step in the synthesis[152]. Two forms of TPH exist in mammals, TPH1 and TPH2, the former being expressed mainly in the periphery and the latter mainly in the brain[153]. The second step is the decarboxylation of 5-hydroxytryptophan by AADC (a.k.a. DDC) to produce 5-HT [154]. Thus, AADC/DDC is a shared enzyme in catecholamine and serotonin synthesis and can be detected in both catecholaminergic and serotonergic cells.

Like catecholamines, serotonin is packed into vesicles by VMAT2, transported along the axon, and eventually released. Serotonin is inactivated by transporting it back into the producing neuron by the serotonin reuptake transporter (SERT)[155] or by degradation via MAO (mainly MAO A) to produce 5-HIAA (see Section 1.1.3.). SERT is the target of selective serotonin reuptake inhibitor (SSRI) antidepressants.

1.1.2.2.3. Anatomy and development of the serotonergic system

Serotonergic populations were first described and classified (B1-9) in the rat brain by using a formaline-induced fluorescence method[40] and later confirmed with immunohistochemistry by using antibodies against 5-HT and TPH[156-158] (much later it was found that most TPH antibodies detect both TPH1 and TPH2, TPH2 being the dominant form in the rat brain[153]). In mammals, most of the serotonergic neurons are located in the raphe nuclei, while some are found more caudally, sparsely distributed in the reticular formation. The boundary between these two groups is difficult to determine. The raphe nuclei are divided developmentally into a rostal raphe (B5-9), which gives rise to ascending projections to most areas of the brain, and a caudal raphe (B1-4), which sends descending innervation to the brain stem and spinal cord[159]. In addition, transient serotonergic cells have been reported in the hypothalamus during the development[160], and some nerve cells of the adult hypothalamus are also capable of transporter-mediated uptake of 5-HT[161].

Rostral and caudal raphe cell populations are also present in fish. In addition and contrary to mammals, particularly prominent serotonergic populations are found in the hypothalmus of several fish species[51, 162-176] and also in the pretectum/thalamus and epiphysis of zebrafish[51].

Compared with studies concerning the development of midbrain dopaminergic neurons, knowledge of the development of serotonergic (raphe) cell populations is scarce. Similarly to the catecholaminergic cell populations, precursor cells of the serotonergic raphe populations are dependent on secreted factors FGF8 and Shh from the two main organizing centers, the isthmus and floor plate, respectively[60-62]. A target of Shh, Nkx2.2, is necessary for the development of serotonergic neurons[177]. Nkx2.2 downregulates Phox2b, whose maintainance would prevent a switch from a motor neuron phenotype to a serotonergic neuron

phenotype[178]. In the very first serotonergic neurons (in rhombomere r1), Phox2b is irrelevant since it is never expressed. Serotonergic dorsal raphe nuclei also develop independently of Nkx2.2 expression.

Nkx2.2 and Ascl1/Mash1 are suggested to function upstream of and activate in parallel GATA-3, Lmx1b, and Pet-1, which are expressed in post-mitotic developing serotonergic neurons[179-183]. GATA-3 is involved in the development of serotonergic neurons in the caudal raphe, but not in the rostral raphe[179]. (Mis)expression of Nkx2.2, Lmx1b, and Pet-1 is both sufficient and necessary to induce 5-HT neurons[182]. In addition, GATA-2 is necessary for the development of serotonergic neurons[184].

1.1.2.2.4. 5-HT receptors and functions

In mammals, at least 14 serotonin receptor subtypes exist. They are classified into seven families ($5HT_1$ – $5\text{-}HT_7$). Serotonin receptors are members of the G-protein-coupled receptor family, except for $5\text{-}HT_3$ receptors, which are ligand-gated ion channels. Serotonin is involved in many physiological and pathophysiological states such as memory and learning, anxiety, drug abuse, depression, schizophrenia, and migraine. The relationship of selected receptors to different neurological functions is briefly introduced here.

The **$5\text{-}HT_{1A}$** receptor subtype was the first one to be cloned and characterized[185, 186]. It is located both pre- and postsynaptically and is best known for its involvement in anxiety and depression. $5\text{-}HT_{1A}$ receptor-deficient mice display an anxiety-like phenotype[187], which seems to be caused by developmental effects since conditional receptor gene knockout in the adult animal does not cause similar alterations in behavior[188]. $5\text{-}HT_{1A}$ agonists (such as the partial agonist buspirone) have antidepressive effects similar to SSRIs[189], and this receptor type is therefore thought to be involved in the effects of SSRI-type antidepressants. Triptans that are used in the treatment of migraine are **$5\text{-}HT_{1B/1D}$** receptor agonists. They stop or prevent the migraine attack by 1) constricting intracranial and meningeal blood vessels via $5\text{-}HT_{1B}$ receptors on the smooth muscle cells of the vessels[190, 191], and 2) inhibiting the release of vasoactive substances and nociceptive activity in the trigeminal ganglia via $5\text{-}HT_{1D}$ receptors[192-194]. The **$5\text{-}HT_{2A}$** receptor subtype is believed to be a key mediator of the hallucinogenic effects of LSD[150]. Functions of **$5\text{-}HT_{2C}$** receptors have been linked to suicides[195], schizophrenia[196], anxiety, depression[197], spatial memory, and obesity. **$5\text{-}HT_3$** receptors are located in the spinal trigeminal nucleus, the area postrema, and the nucleus of the solitary tract. Their best-known clinical significance is related to mediation of emesis[198]. Indeed, $5\text{-}HT_3$ antagonists (such as ondansetron) are widely employed as antiemetics[199]. **$5\text{-}HT_4$** receptors are located in the basal ganglia, substantia nigra, and hippocampus and are important in learning and locomotion. High levels of **5-**

HT_6 receptor expression are detected in the cortex, and these receptors are involved in regulation of cholinergic neurotransmission and possibly in learning, anxiety, schizophrenia, and obesity. 5-HT_6 receptor antagonists are currently undergoing clinical trials for the treatment of Alzheimer's disease [200]. **5-HT_7** receptors are believed to be involved in the regulation of sleep and mood.

Three zebrafish 5-HT receptor genes have been identified recently. Two genes found encoding orthologs of the human 5-HT_{1A} receptor, were designated Htr1aa and Htr1ab, and one gene probably orthologous to both human 5-HT_{1B} and 5-HT_{1D} was designated Htr1bd[201]. Identification of these receptors is based on sequence analysis, and little is known about their pharmacologial properties and function. The expression of zebrafish 5-HT receptors has been studied in some detail in both larval and adult zebrafish brain[201]. Zebrafish 5-HT receptors are expressed widely in the larval and adult brain and are found in the superior raphe nucleus, retina, ventral telencephalon, optic tectum, thalamus, posterior tuberculum, cerebellum, hypothalamus, and reticular formation. Additionally, autoreceptor activity is found in the pretectal diencephalic cluster, superior raphe nucleus, paraventricular organ, and caudal zone of the periventricular hypothalamus[201].

1.1.2.3. The histaminergic system

1.1.2.3.1. History

Beta-iminazolylethylamine (histamine) was recognized as an agent with the potential to cause constriction of smooth muscle cells in the gut and relaxation of vascular smooth muscle (vasodilation)[202]. It was subsequently renamed histamine since it was an amine derived from histidine and occurred in tissues. Histamine was soon discovered to stimulate gastric acid secretion[203], mediate inflammation, and cause anaphylaxis[204]. Secretion of gastric acid was later found to be mediated through H2-type histamine receptors[205]. The sedative actions of histamine receptor inverse agonists (antihistamines) suggested a role for histamine in the central nervous system. However, it was not until 1943 that histamine was detected in neuronal tissues; first in the peripheral nerves[206] and then in the brain[207] of several animal species. It was subsequently shown that histamine is produced in the brain, mostly in the hypothalamus[208]. Further evidence for the existence of a histaminergic neuron system was provided by lesion studies[209, 210]. However, there was a long debate whether a functional histaminergic system actually existed in the mammalian brain, until it was directly visualized in the tuberomamillary nucleus of the posterior hypothalamus using anti-histamine and anti-HDC antibodies[211-213]. The existence of histamine-producing neurons was later confirmed in studies using *in situ* hybridization with probes against HDC mRNA[214, 215].

1.1.2.3.2. Histamine synthesis, storage, and degradation

Histamine is synthesized by histidine decarboxylase (HDC, EC 4.1.1.22) from histidine[216]. Contrary to other neurotransmitter amines (see above), the limiting step in its synthesis is the availability of the precursor, histidine. Histamine is packed into vesicles by vesicular monoamine transporter type 2 (VMAT2)[20, 21, 217]. Histamine is degraded to an inactive form, tele-methylhistamine (t-MHA), mainly by histamine N-methyltransferase (HNMT, EC 2.1.1.8) in the brain. Tele-methylhistamine is further metabolized to t-methyl-imidazoleacetic acid by monoamine oxidase B (MAO B)[218, 219]. The optional oxidative inactivation route executed by diamine oxidase (EC 1.4.3.6) is important in the periphery and can also be activated in the brain[220]. Unlike other neurotransmitter amines, no high affinity reuptake system for histamine has been found in the brain.

1.1.2.3.3. Anatomy and development of the histaminergic system

In all studied mammalian species and in most other investigated vertebrates, the brain histaminergic neurons are exclusively found in the tuberomamillary nucleus (TMN) located in the posterior hypothalamus, from where they send projections to all regions of the CNS.The TMN is subdivided into five anatomical parts (E1-5)[213, 221, 222]. Histaminergic cells in the TMN are detected from E20 in the rat [223, 224] and from 3 dpf in zebrafish[225]. A transient histaminergic system is present in the ventral mesencephalon and rhombencephalon on E13-E20 in the rat[223]. The functions of the transient system are currently unknown, but its strong conservation suggests an important role during development. However, the transient histaminergic system has not been detected in zebrafish[225].

1.1.2.3.4. Receptors and functions

Histamine has traditionally been better known for its functions in inflammation, allergy, and gastric acid secretion than for its functions in the CNS. The effects of histamine are mediated through four different G-protein-coupled receptor types (H_1-H_4) in vertebrates and through ionotropic histamine receptors in invertebrates[226, 227]. H_1[228, 229] and H_2 receptors[205, 230] are located in both the CNS and peripheral tissues, while H_3 receptors[231, 232] are mostly confined to the CNS and H_4 receptors[233, 234] to peripheral tissues. The main functions of peripheral histamine are to mediate inflammatory responses, especially allergic reactions (H_1 receptor), and to stimulate gastric acid secretion (H_2 receptor). Although H_1 receptor inverse agonists (antihistamines) have been useful in treating symptoms of allergy, trials treating other inflammatory processes have proven ineffective. The H_4 receptor was discovered only recently[233-236], and several reports suggest an important role in inflammation[237-241].

H_1 receptors are expressed in most brains regions[242, 243], and their functions are related to memory[244, 245], feeding and energy metabolism[246, 247], endocrine control[248], and behavioral states[249]. H_3 receptors, autoreceptors located on presynaptic terminals of histaminergic neurons, inhibit histamine release[231]. H_1 and H_3 receptors are postulated to be involved in disorders of sleep[249], memory[245], eating[250, 251], alcoholism[252-254], and PD[255-257]. H_2 receptors are also expressed widely[258], are involved in learning and memory[244], and have been suggested to be involved in schizophrenia[259, 260].

Genes for H_1-H_3–like histamine receptors in zebrafish have been identified[261, 262], but their pharmacological profiles and functions are poorly understood

1.1.3. Monoamine oxidase (MAO)

1.1.3.1. History

Three enzymes metabolizing amines, tyramine oxidase[263], adrenaline oxidase[264], and aliphatic amine oxidase[265] were originally found independently. These three enzymes were soon recognized to actually be the same enzyme, capable of oxidative deamination of primary, secondary, and tertiary amines, and hence named amine oxidase[266]. Later, amine oxidase was distinguished from diamine oxidases by its ability to oxidate monoamines, but not di- or polyamines, and thus renamed monoamine oxidase (MAO, EC 1.4.3.4)[267]. The next turning point was the accidental discovery of the antidepressive potential of an antituberculosis drug, isoniazide, a potent inhibitor of MAO[268, 269]. However, many of the early MAO inhibitors were soon associated with a serious side-effect, the cheese reaction*, which largely prevented their use. Another crucial finding was the existence of two isoforms of MAO – A and B – with distinct substrate and inhibitor specificities[270], followed by the finding that these two forms were differently distributed in the mammalian brain[271]. MAO B was found in the basal ganglia and was important for the metabolism of dopamine. On the other hand, it was already known that patients with PD had dopamine deficiency in their striatum[12] and that their symptoms could be relieved by increasing dopamine levels by administration of L-DOPA[13]. Experimental work suggested that dopamine levels could be elevated also by inhibiting MAO B[272]; this was followed by the success of clinical trials using deprenyl to treat PD patients[273]. Today, many different types of MAO inhibitors exist and are used to treat depression and PD. MAO has also been linked to several other physiological and pathophysiologial processes (see Section 1.1.3.6.).

* Normally, MAO in the gut and liver metabolize dietary amines and prevent them from entering the systemic circulation. If this MAO is inhibited so that it cannot metabolize these amines, tyramine (and other amines) may induce release of noradrenaline from peripheral adrenergic neurons, leading to severe hypertension {{1160 Hutchison,J.C. 1964; }}. Tyramine is found in cheese (hence the name), but also in beer and wine.

1.1.3.2. Structure and enzymatic reaction

MAO enzymes contain covalently bound flavin adenine dinucleotide (FAD), and are thus classified as a flavoproteins. The two isoforms of MAO, A and B, are encoded by different genes[274], located side by side at the Xp11.23 in humans[275, 276]. They consist of 15 exons with identical exon-intron organizations, suggesting a local evolutionary duplication of an ancestral MAO gene[277]. Amino acid sequences of MAO A and B are 70% identical. The active site of MAO B contains an entrance cavity and hydrophobic substrate cavity, while the active site in MAO A is a single hydrophobic cavity. The active site is formed by Tyr60, Phe168, Leu171, Cys172, Tyr188, Ile198, Ile199, Gln206, Tyr326, Phe343, Tyr398, and Tyr435 residues in human MAO A and Tyr69, Phe177, Ile180, Asn181, Tyr197, Ile207, Phe208, Gln215, Ile335, Phe352, Tyr407, and Tyr444 residues in human MAO B [278, 279] (for complete sequences of human and zebrafish MAOs, see Section 4.2.). MAO catalyzes the oxidative deamination of several biogenic and exogenic amines in the brain and peripheral tissues, among which are many important neurotransmitters, such as serotonin, dopamine, and noradrenaline (Figure 4).

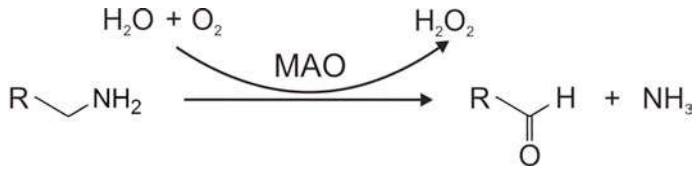

Figure 4.

Monoamine oxidases catalyze oxidative deamination reactions where oxygen is used to remove an amine group, forming a corresponding aldehyde in addition to ammonia and hydrogen peroxide. R denotes an arbitrary group.

The products of the reaction are potentially toxic. The aldehyde formed from dopamine is cytotoxic, and normally detoxified *in situ* by aldehyde dehydrogenase. Levels of aldehyde dehydrogenase are decreased in PD[280]. Hydrogen peroxide and ammonia are also toxic at higher concentrations.

The two isoforms of mammalian MAO, A and B, were originally classified on the basis of their substrate and inhibitor affinity profiles[270]. MAO A has a higher substrate affinity for serotonin, dopamine, and noradrenaline and the inhibitor clorgyline, whereas MAO B has a higher substrate affinity for phenylethylamine and benzylamine and the inhibitor deprenyl (Table 1). In humans dopamine can be deaminated by both forms of MAO[281], while in rodents it is mainly deaminated by MAO A[282].

Table 1.

Substrate	MAOA			MAOB		
	K_m (μM)	V_{max} (pmol min^{-1} mg protein^{-1})	V_{max}/K_m (μmol M^{-1} min^{-1} mg protein^{-1})	K_m (μM)	V_{max} (pmol min^{-1} mg protein^{-1})	V_{max}/K_m (μmol M^{-1} min^{-1} mg protein^{-1})
Adrenaline	125 ± 42	379 ± 54	3.03 ± 1.11	266 ± 9	465 ± 61	1.75 ± 0.23
Dopamine	212 ± 33	680 ± 123	3.21 ± 0.77	229 ± 33	702 ± 158	3.07 ± 0.82
5-Hydroxytryptamine	137 ± 24	228 ± 31	1.66 ± 0.37	1093 ± 20	6.6 ± 1.3	0.006 ± 0.001
Noradrenaline	284 ± 17	561 ± 42	1.98 ± 0.19	238 ± 30	321 ± 13	1.35 ± 0.18
2-Phenylethylamine	140 ± 22	20 ± 8	0.14 ± 0.06	4 ± 2	309 ± 24	77.3 ± 39.1
Tryptamine	35 ± 6	58 ± 5	1.66 ± 0.32	35 ± 8	108 ± 2	2.84 ± 0.60
Tyramine	127 ± 18	182 ± 28	1.43 ± 0.30	107 ± 21	343 ± 48	3.21 ± 0.77

Substrate specificities of the monoamine oxidases in the human cerebral cortex. The table shows that amines are substrates for both forms of MAO, but some are preferred over other. Km – Michaelis-Menten constant, Vmax – maximum rate. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Neuroscience], Youdim et al., 2006: The therapeutic potential of monoamine oxidase inhibitors, Nature Reviews Neuroscience 2006 Apr;7(4):295-309 Copyright 2006. Licence number 2112380747433.

1.1.3.3. Distribution of MAO

MAO is tightly bound to the outer membrane of mitochondria by transmembrane alpha-helices in its C-terminal region[283]. The distribution of MAO in the brain of different mammalian species is highly similar. MAO A is located mostly in catecholaminergic neurons and MAO B in serotonergic and histaminergic neurons and glia[284]. The striatum and hypothalamus have the highest activity, while the cerebellum and neocortex have low levels of MAO activity[285]. In humans, MAO A is found in the noradrenergic cells of the locus coeruleus[286]. In the rat, MAO A is also present in the interpeduncular nucleus[287]. Human MAO B expression is widespread, and mRNA is detected in raphe neurons, tuberomamillary histaminergic neurons and dentate gyrus granule cells[286]. High activity of rat MAO B is found in the epiphysis.

MAO is also present in almost all peripheral tissues. In the liver, lungs, placenta, intestine, and kidney, it seems to protect the body from exogenous amines by preventing their entry into the circulation or by removing them. MAO B is found in the blood-brain barrier (BBB) and thought to act as a metabolic barrier. Levels of MAO in the BBB vary greatly between mammalian species[288] and may cause different susceptibility of different species to exogenous toxins[289] (see Section 1.2.3.2.).

1.1.3.5. Physiological roles of MAO in the brain

MAO metabolizes exogenous amines, terminates the action of endogenous amines, and controls intra- and extracellular levels of neurotransmitter monoamines. MAO B is found in serotonergic neurons, but it does not prefer serotonin as a substrate. MAO B in these cells probably metabolizes other amines and prevents their

Table 2.

Phenotypical features		MAO A KO	MAO B KO	MAO AB KO
Neurotransmitter levels	5-HT (serotonin)	200% ↑	–	700% ↑
	NE (norepinephrine)	130% ↑	–	200% ↑
	DA (dopamine)	110% ↑	–	200% ↑
	PEA (phenylethylamine)	–	700% ↑	1400% ↑
Locomotor activity	Open field	↓	–	↓↓
Startle reactivity		↓	?	?
Aggression	Resident–intruder aggression	↑↑	–	↑
	Aggression in familiar environment	↑	–	↑
Anxiety and fear	Plus-maze anxiety	–	–	↑
	Open-field anxiety (periphery/center ratio)	–	–	↑↑
	Fear conditioning	↑	?	?
Depression	Immobility time in forced swim test	↓	↓	?
	Immobility time in tail-suspension test	↓	?	?

Behavioral and neurochemical findings in MAO A, MAO B, and MAO AB knockout mice (compared with wild-type mice). Keys: ↑ - increase, ↓ - decrease, - - no change, ? – unknown. Reprinted from Advanced Drug Delivery Reviews, Vol. 60, Bortolato et al., Monoamine oxidase inactivation: From pathophysiology to therapeutics, pp. 1527-1533, Copyright (2008), with permission from Elsevier. Licence number 2112390135640.

presence in the synaptic vesicles. Glial MAO metabolizes the monoamines secreted into synaptic cleft.

Knockout mice have revealed different *in vivo* properties of the two MAO enzymes (Table 2). MAO A-deficient mice[290], and actually men also[291], have increased brain levels of serotonin and noradrenaline and display more aggression. MAO B- deficient mice, on the other hand, do not express abnormal aggression and show only elevated levels of phenylethylamine (PEA) in the brain. In MAO A and B double knockout mice, the brain levels of 5-HT, NA, and PEA were increased more than in single knockout mice, suggesting that both MAO A and MAO B can compensate for a deficiency in the other[292].

1.1.3.6. Pathophysiological roles of MAO

MAOs are involved in several pathological conditions, including PD, serotonin syndrome, and such psychiatric disorders as depression, addiction, and aggression. The most recent link to a pathological process, revealed as MAO A downregulation, was found in several different types of neoplasms in various animal species (including zebrafish), indicating an important role for MAO also in cancer [293]. Depression is nowadays largely treated with SSRIs or other amine uptake-modifying drugs, but MAO A inhibitors are particularly effective in treating depression in the elderly[294] or atypical depressions[295]. Levels of MAO B but not of MAO A increase with age in both mice and humans[296, 297], and MAO B is also upregulated in PD[298, 299]. Whether the upregulation is a reaction to the dopaminergic neurodegeneration or the cause of it is unclear. While the MAO B inhibitor

deprenyl has been found to be anti-apoptotic and neuroprotective via mechanisms unrelated to MAO inhibition[300-303] and to delay the progression of clinical symptoms[304-308], it does not halt the disease progress[309-311]. However, a recent publication reported that elevation of MAO B activity in adult mouse astrocytes is sufficient to produce Parkinson-type neuropathology[312]. This suggests that MAO B inhibition might be neuroprotective if it commences sufficiently early. In fact, tobacco smoke contains MAO inhibitors[313], the activities of MAO A and MAO B are lowered in the brains of smokers[314], and smokers have a lowered incidence of PD[315].

1.2. Parkinson's disease

1.2.1. Epidemiology, clinical symptoms, and treatment

Parkinson's disease (PD) is a progressive neurodegenerative disorder. It is named after James Parkinson, who first described the symptoms of five patients in 1817[316]. The incidence of PD increases after the age of 50 and rapidly increases after the age of 75[317]. Onset of PD is clearly age-related, and its prevalence will increase as the population ages. The main symptoms of PD include resting tremor, postural instability, bradykinesia (slow movements), hypokinesia (reduced movement), akinesia (lack of movement), and rigidity. These symptoms are caused by the progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNpc) and loss of dopaminergic input into the striatum[318] (Figure 5).
Several studies suggest that the striatal dopaminergic nerve terminals are the primary targets of degeneration and subsequently cause the degeneration of the cell somata[319, 320]. The neuropathological hallmarks of PD are loss of nigrostriatal dopaminergic neurons and presence of intracellular alpha-synuclein, parkin, and ubiquitin contained in Levy body (LB) inclusions[321].

The treatment of PD is currently symptomatic, as no preventive, curative, or even disease-modifying treatment is available. L-DOPA, a precursor of dopamine, is the oldest and most efficacious drug for treating PD. However, long-term use of L-DOPA produces motor fluctuations and dyskinesias and thus, treatment is usually started with other medications. These include amantadine, monoamine oxidase B (MAO B) inhibitors, anticholinergics, and dopamine receptor agonists. Neurosurgical options exist, such as deep brain stimulation of the subthalamic nucleus, which may be used after symptoms can no longer be managed with medication[323]. There is an urgent need for new, disease-modifying treatment options for PD.

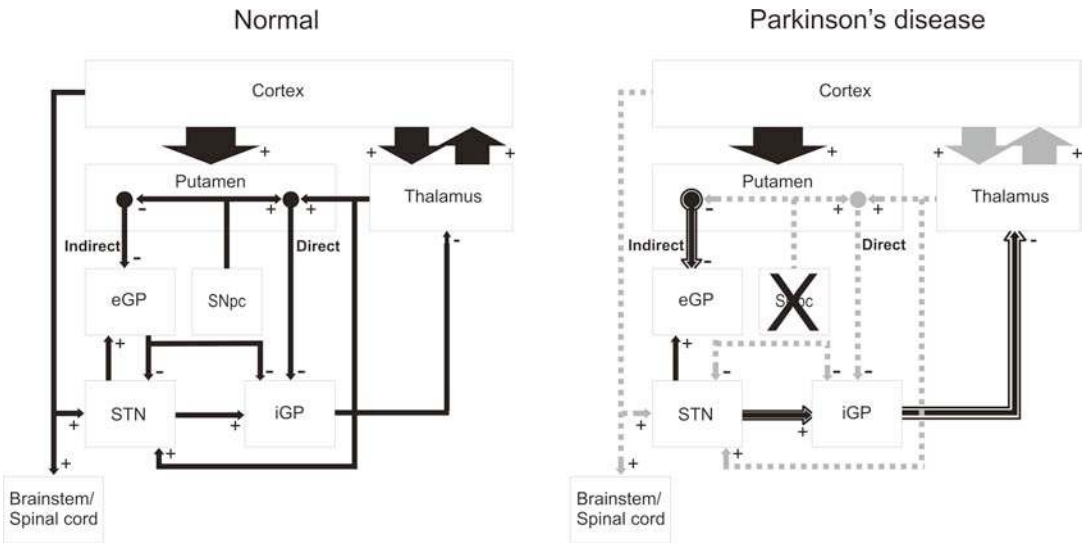

Figure 5.
Simplified illustration of the basal ganglia circuits involved in movement control in the normal condition (left) and in Parkinson's disease (right). Input from the cortex to the basal ganglia terminates in specific regions, which in turn project to specific regions in the thalamus. These regions in the thalamus project back to the same regions of the cortex from which the loop originates[322]. In Parkinson's disease, the degeneration of dopaminergic neurons in SNpc leads to dopamine deficiency in the striatum. This affects the direct and indirect pathways in a way that leads to activation of the internal globus pallidus. Inhibition of the thalamus by activated and tonically active iGP is increased. Thus, the loss of dopamine in the striatum ultimately leads to decreased input into the brainstem and spinal cord. Abbreviations: eGP – external globus pallidus, iGP – internal globus pallidus, SNpc – substantia nigra pars compacta, STN – subthalamic nucleus. Modified with permission from Circuits and Circuit Disorders of the Basal Ganglia by DeLong and Wichmann in Archives of Neurology, Vol. 64, Issue 1, pp 20-24, Copyright © (2006) American Medical Association. All rights reserved.

1.2.2. Etiology

The etiology of PD is currently unknown. It is believed to be caused by an interplay of genetic and environmental factors. 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP) was first produced as a side-product of MPPP (a type of synthetic heroin) synthesis and identified as a cause for parkinsonism in a college student in 1976 and in several heroin addicts in 1982[324]. Pesticides have been suspected of causing PD because of their mechanism of action – causing dysfunction in the respiratory chain of mitochondria. Meta-analyses of epidemiological data suggest that a positive association exists between pesticide exposure and PD[325, 326]. In addition, the pesticide rotenone is used to produce experimental parkinsonism (see Section 1.2.3.). Somewhat surprisingly, cigarette smoking is inversely associated with the

risk of PD[315], which may be due to the inhibition of MAO (see 1.1.3.6. Pathophysiological roles of MAO).

Insights into the pathogenesis of PD have been achieved via recent findings of new mutated genes causing hereditary forms of PD. Investigation of affected families has led to the identification of six genes involved in PD pathogenesis. These include alpha-synuclein[327] and leucine-rich repeat kinase 2[328], both of which are inherited in an autosomal dominant manner and Parkin[329], DJ-1[330] and PINK1[331], which are all inherited in an autosomal recessive manner (see next Section). Additionally, a very rare cause of autosomal dominant PD has been linked to UCHL1[332]. Studies of these genes have suggested that PD is caused by mitochondrial dysfunction (PINK1, Parkin), oxidative stress (DJ-1, Parkin), and protein aggregation (Parkin, alpha-synuclein, UCHL1).

1.2.2.1. PINK1

Although originally found in some forms of cancer[333], mutated PTEN-induced kinase 1 (PINK1) has recently been demonstrated to cause early-onset autosomal recessive familial PD [331]. The human *PINK1* gene contains a mitochondrial targeting signal and encodes a 581-amino-acid protein, which has a serine/theorine kinase domain[331]. Both mitochondrial localization and kinase activity have been shown [334]. Confusingly, PINK1 has been reported to be located in the inner mitochondrial membrane[334-337], the intermembrane space[334, 336, 338], the outer mitochondrial membrane[335] and even the cytoplasm[339-342]. Recently, PINK1 has been suggested to span the outer mitochondrial membrane, with the kinase domain facing the cytoplasm[343].

PINK1 is present in several organs. In the brain, PINK1 expression is widespread. Immunohistochemical methods show signals in both neurons and glial cells, whereas *in situ* hybridization reveals expression only in neurons[335, 344]. The physiological functions of PINK1 remain incompletely understood. PINK1 has been found to be neuroprotective by decreasing cytochrome c release[345]. On the other hand, loss of functional PINK1 leads to increased lipid peroxidation and decreased function of complex I of the mitochondrial respiratory chain[346]. Knocking out PINK1 in *Drosophila* leads to dopaminergic cell death, which can be prevented with antioxidants, suggesting that PINK1 is essential in preventing neurons from undergoing oxidative stress and subsequent death[347]. This protection against oxidative stress is mediated by the mitochondrial chaperone TNF receptor-associated protein 1 (TRAP1) via phosphorylation by PINK1[336]. Cytoplasmic PINK1 activity has been proposed to protect neurons from the toxicity of MPTP[340].

Inactivation of PINK1 in *Drosophila* leads to dopaminergic cell death, muscle degeneration, photoreceptor loss, and male sterility, which are accompanied by mitochondrial dysfunction. These effects were prevented by overexpressing

another PD susceptibility gene, Parkin, but not vice versa, suggesting that Parkin functions downstream of PINK1[348-350]. Other components of this pathway remain obscure.

Results from *in vitro* studies suggest that Omi/HtrA2 works downstream of PINK1, with PINK1 positively regulating Omi/HtrA2 by phosphorylation[338]. Based on G399S mutations found in sporadic PD patients, Omi/HtrA2 was designated as the PARK13 locus[351]. However, recent *in vivo* studies carried out on *Drosophila* do not support these suggestions, instead showing no significant role of Omi/HtrA2 in PD[352]. Yet another study carried out in *Drosophila* suggested that Omi/HtrA2 functions downstream of PINK1, but independently of Parkin[353]. Furthermore, Omi/HtrA2 G399S mutations are found in equal frequencies in PD patients and normal individuals[354, 355]. Due to conflicting results, the role of Omi/HtrA2 in PD and its relationship to PINK1 are unclear.

In *Drosophila,* PINK1 regulates the morphology of mitochondria by promoting fission over fusion by interacting with Drp1[356-358]. Fission and fusion have been implicated in cell death regulation [359, 360].

Much of the research regarding the pathways and interactions of PINK1 has been carried out in cell culture or on the invertebrate *Drosophila*. In contrast to these results, PINK1 knockout/silenced mice do not exhibit dopaminergic cell loss or muscle degeneration[361, 362], but have a deficit in releasing dopamine in the striatum[362]. No gross changes in the ultrastructure of mitochondria were detected in these mice, although the number of large mitochondria was increased, mitochondrial respiration was impaired, and the mice were more susceptible to oxidative stress[363]. Whether the role of PINK1 is similar in invertebrates and vertebrates remains to be elucidated.

1.2.3. Toxin-induced animal models of PD

Four toxic substances are commonly used to produce experimental parkinsonism. **6-hydroxydopamine (6-OHDA)** is a catecholaminergic neurotoxin that was introduced more than 40 years ago[364]. It gains access to catecholaminergic neurons via dopamine (DAT) and noradrenaline (NAT) transporters and destroys these cells by the effects of reactive oxygen species and quinones[365]. 6-OHDA does not cross the blood-brain barrier (BBB), and thus, it is usually administered intracerebrally to produce a unilateral lesion of the substantia nigra. It can be directly injected into the substantia nigra to start neurodegeneration within 24 hours[366, 367], or into the striatum to cause a more protracted retrograde degeneration of the SNpc within 1-3 weeks[368]. 6-OHDA has also been reported to produce gliosis[369], but not formation of Levy bodies (LBs). As described earlier, **MPTP** is a potent neurotoxin causing parkinsonism in several species, including humans. It easily crosses the BBB and in the brain it is converted into its toxic form MPP+ by MAO B[370]. MPP+ enters

dopaminergic cells via DAT[371] and interferes with the mitochondrial respiratory chain by inhibiting complex I[372]. Conversion into MPP+ and uptake by DAT are critical steps, and the toxicity of MPTP is abolished if these steps are inhibited[370, 373]. Although the MPTP model mimics PD quite accurately, it does not produce LBs or cell loss in other monoaminergic nuclei such as the locus coeruleus[374, 375] (see more details about MPTP in the next Section). **Rotenone** is widely used as a pesticide. It readily gains access to all organs, including the brain, where it binds to and inhibits mitochondrial complex I – similarly as MPP+. Contrary to the MPTP and 6-OHDA models of PD, rotenone exposure produces LB-like inclusions in the remaining dopaminergic neurons of the substantia nigra[376]. It was initially thought that it produces specific neurodegeneration of the substantia nigra alone[376], but more recent work has demonstrated inconsistent loss of nigral dopaminergic neurons and also loss of striatal neurons[377-379]. Thus, the correlation of this model to PD must be done with caution.

1.2.3.1. MPTP model of PD

The neurotoxicity of MPTP was first discovered in humans in the early 1980's, when several drug addicts demonstrated an acute onset of clinical symptoms indistinguishable from PD. MPTP was produced inadvertently during an illicit synthesis of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), an analog of meperidine[324]. MPTP produces a severe irreversible PD-like syndrome in humans and monkeys, which is characterized by all typical symptoms of PD. Furthermore, being indistinguishable from PD, the response of MPTP-exposed humans and monkeys to levodopa and the development of dyskinesias during long-term medical treatment are identical to those seen in patients with "idiopathic" PD. The MPTP model of PD has been used in several species, including primates, rodents, cats, goldfish, and even worms[380]. Interestingly, among rodents, only certain strains of mice are susceptible to MPTP neurotoxicity[381], whereas rats are show no sensitivity[382].

1.2.3.2. Mechanism of action of MPTP

The BBB prevents access of many neurotoxic substances into the brain. The barrier consists of elaborate tight junctions between the vascular endothelial cells and close associations of astrocytes and their foot processes with the endothelial basal lamina[383]. MPTP is a lipophilic substance that readily crosses the BBB. MPTP *per se* is not toxic, but instead needs to be converted into a toxic metabolite, MPP+, by MAO B. MAO B exists in the endothelial cells of the BBB and the levels of MAO B correlate with the levels of neuronal loss induced by MPTP[288, 289]. Since MPP+ cannot cross the BBB, the conversion at the BBB provides the first line of defense against MPTP toxicity. The high expression of MAO B in the BBB of rats and

certain mouse strains explains their lack of susceptibility to MPTP as MPTP is metabolized to MPP+ outside of the brain and cannot thereafter cross the BBB[384, 385].

In the brain, MAO B is located in serotonergic, histaminergic, and glial cells, but not in dopaminergic cells[284, 386]. MPTP toxicity has been shown to be attenuated by blocking the entry of MPTP into glial cells, but not by destroying the serotonergic cells. Thus the conversion of MPTP into MPP+ is commonly thought to take place in astrocytes[387]. However, MAO B also exists in the histaminergic neurons of the tuberomamillary nucleus, where it also represents a possible platform for the conversion. This step is critical for the toxicity of MPTP, as demonstrated by abolishment of toxicity by inhibition of MAO B activity[370]. In astrocytes, MPP+ upregulates, via different cytokines, inducible nitric oxide synthase (iNOS)[388]. iNOS is minimally expressed in the normal brain, but is upregulated in the microglia of the substantia nigra in PD patients and in MPTP-treated mice[389, 390]. iNOS produces lipophilic and uncharged nitric oxide (NO) in large quantities, which may then easily enter neurons in the vicinity. On the other hand, polar MPP+ cannot freely exit the astrocyte, thus needing to be actively transported to the extracellular space. Such a transporter for monoamines exists, and this transporter may be responsible for moving MPP+ out of the astrocyte[391]. However, the exact mechanism is unclear.

After its release into the extracellular space, MPP+ is taken up into dopaminergic neurons by DAT[371]. The importance of DAT in MPTP toxicity is indicated by studies where DAT-knockout mice were protected from toxicity[373, 392]. Within the neuron, MPP+ can be actively driven into mitochondria by a membrane electrical gradient[393]. However, MPP+ can also be taken up into vesicles by VMAT2[20, 394], thus preventing MPP+ access into mitochondria. In fact, a high ratio of VMAT2/DAT has been found to be neuroprotective[395].

Once inside mitochondria, MPP+ inhibits complex I of the mitochondrial respiratory chain, leading to a reduction of cellular ATP[372]. This depletion of energy results in formation of superoxide radicals[396, 397]. It has been hypothesized that NO produced by the glia cells and superoxide radicals interact, producing peroxynitrate (OONO-)[398], a highly oxidizing molecule that could nitrate a number of cellular components, ultimately leading to cell death. A schematic view of the steps of MPTP toxicity is shown in Figure 6.

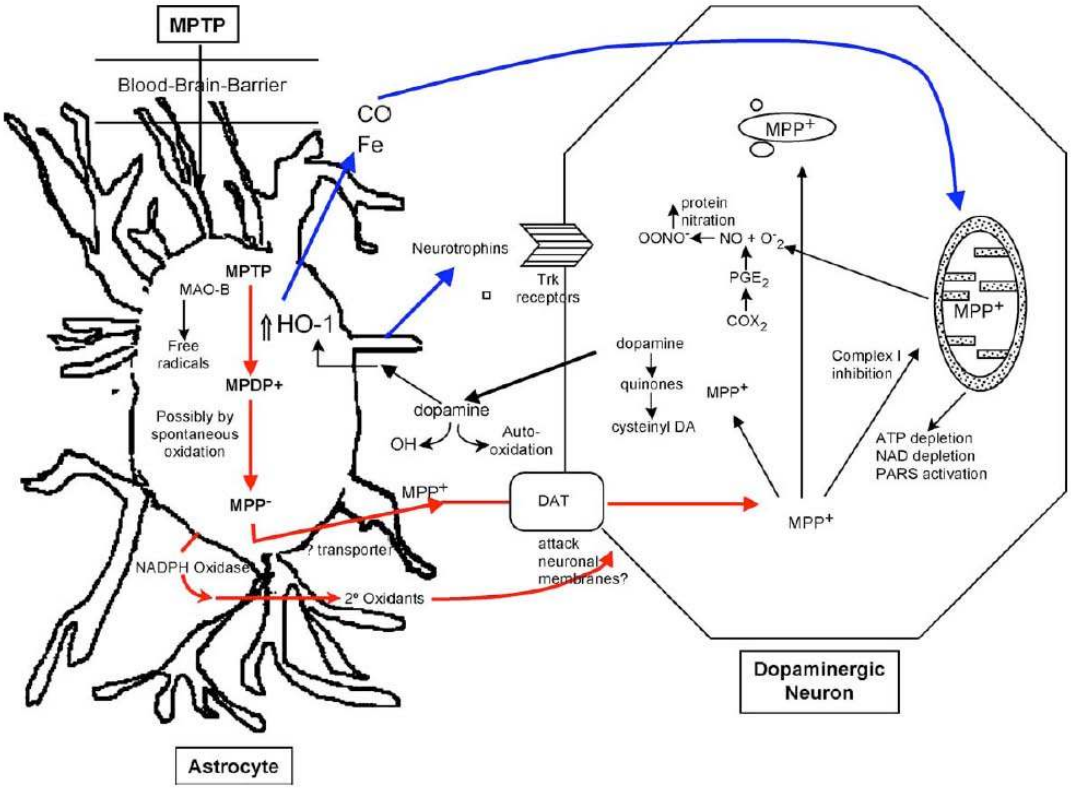

Figure 6.
Schematic representation of the mechanism of action of MPTP (and MPP+). MPTP readily crosses the BBB and is converted to MPP+ by glial MAO B. MPP+ gains access to dopaminergic neurons via DAT. Inside the neuron's mitochondria, MPP+ inhibits complex 1 of the respiratory chain, leading to a decrease in ATP and formation of ROS. The cascade ultimately leads to cell death. Red arrows represent the initial (toxification) role of glial cells. Blue arrows represent the second (neuroprotective) role of glial cells. Reprinted from Molecular Brain Research, Vol. 134 issue 1, Smeyne RJ and Jackson-Lewis V, The MPTP model of Parkinson's disease, pp. 57-66, Copyright (2005), with permission from Elsevier. License number 2120320844874.

1.3. Zebrafish

Zebrafish are small freshwater fish originating from the tropical waters of South-East Asia. They are commonly kept as aquarium fish, as they are easy to maintain. Zebrafish were first introduced for biomedical research purposes by George Streisinger in 1981. Streisinger chose zebrafish, among other species, due to their ideal combination of properties.

1.3.1. Advantages and disadvantages of zebrafish

Zebrafish develop *ex utero,* making them particularly suitable for studying early development, as observation is easy. The first large-scale mutation screens picked over a thousand mutants based on their early gross developmental phenotype [399, 400]. *Ex utero* development allows early manipulation by a variety of methods – morpholino knockdown, RNA overexpression, and transplantation as well as chemical, physical, and pharmaceutical interventions. Largely due to these advantages, zebrafish were initally recognized as a model for developmental biology.

Organogenesis is rapid in zebrafish larvae, and major organs, such as the heart, vascular system and intestine have developed by 2 days post-fertilization (dpf). Organogenesis and functional organs can easily be visualized since zebrafish larvae can be kept transparent up to 5 dpf, by which time the larvae swim[401], eat, sleep[402, 403], and capture prey[404-406]. Transparency not only allows observation of the phenotype by eye, but also makes it possible to observe development at the single-cell level *in vivo*, through the advantage of fluorescent dyes and especially green fluorescent protein (GFP)-transgenic methods. Confocal imaging of whole-fish systems is possible without the need for time-consuming sectioning. As zebrafish is a relatively novel model organism, only a few tested antibodies against zebrafish proteins are available. However, zebrafish has high genetic similarity to mammals, and many antibodies that work on mammals will work also on zebrafish. Indeed, careful characterization and appropriate controls are essential before making conclusions on their specificity in zebrafish. The zebrafish research community maintains a database of antibodies that are proven for zebrafish (zfin.org). Due to the lack of characterized antibodies, *in situ* hybridization has been used extensively to study the expression of genes. Earlier, hybridization signals were commonly detected by the use of dyes for light microscopy. This did not allow exact anatomical localization of the signals without sectioning the sample. However, fluorescent *in situ* hybridization methods have recently been developed to allow exact 3D localization at high resolution[407].

The small size of zebrafish enables cost-efficient maintenance of numerous adult individuals. Large numbers of progeny result in statistically

significant sample sizes, even from a relatively small-scale unit. Pharmacological manipulation of thousands of individuals is relatively easy since drugs are readily absorbed into the fish from the water.

The zebrafish genome is nearly completely sequenced and the genome assembly is accessible (ftp.emsembl.org/pub/assembly/zebrafish/Zv7release), allowing efficient reverse genetics. However, at the time when the fish lineage diverged from land vertebrates more than 300 million years ago, the ancestral fish genome underwent a third full-scale duplication[408]. Therefore, even after many of the duplicated genes have been lost due to redundancy in the course of fish evolution, zebrafish possess two copies of many mammalian genes (ca. 20%)[409], rendering these genes more difficult to study. Several genetic manipulation methods are, however, available to investigate their functions. One of the most commonly used – the morpholino technique - is a relatively rapid, easy, and economical way to lower the expression of a gene-of-interest (knockdown)[410]. It is based on modified oligonucleotides carrying morpholine residues that are resistant to degradation and bind to specific mRNA sequences to block their translation. Advantages notwithstanding, the morpholino technique has several disadvantages. It is efficacious for only 2–5 dpf, depending on the expression level and the distribution of gene expression, allowing only studies on embryonic and early larval zebrafish[411, 412]. Furthermore, it was recently shown that some morpholinos induce p53 activation and subsequent neuronal apoptosis[413], rendering the interpretation of results from morpholino experiments more difficult (see Section 5.1.5.).

Targeted gene knockout methods are not available in zebrafish due to the lack of stem cell culture techniques. However, other methods producing (loss-of-function) mutations in specific genes-of-interest have been developed. TILLING (target induced local lesions in genomes) uses N-ethyl-N-nitrosurea (ENU) mutagenesis to produce random mutations, and fish are subsequently selected by screening for mutations in genes-of-interest, resulting in maintenance of zebrafish lines with a mutated (and possibly nonfunctional) gene-of-interest[414]. An alternative approach is to use retroviral insertional mutagenesis[415, 416]. Yet another method is to use engineered zinc-finger nucleases (ZFNs). ZFNs are engineered restriction enzymes customized to cut the DNA sequence at a specific site. The cleavage of a double-stranded DNA may lead to mutations, as these breaks are repaired by error-prone nonhomologous end-joining[417].

Another powerful tool to facilitate research on specific genes-of-interest is transgenesis. Expression of reporter genes, such as GFP, in a controlled tissue-specific manner is relatively easy in zebrafish[418]. Injections of plasmid DNA or bacterial artificial chromosomes (BACs) at the one-cell stage are the most widely used methods to produce transgenic zebrafish[419]. Transgenic zebrafish expressing

GFP under the control of a promotor-of-interest allows 3D imaging and manipulation at the cellular level in transparent live larval zebrafish[420, 421].

The possibility of carring out efficient forward genetic experiments, such as mutagenesis screens, is perhaps the biggest advantage of the use of zebrafish in genetic research. Their size, economical cost, generation time, and rapid development allows large-scale screening, while the relative similarity to higher vertebrates renders the results obtained widely applicable (see Section 1.3.2.).

Additionally, from an ethical point of view, there is a drive towards animal models with lower CNS levels. It is already possible to replace some studies now performed on mammalians with studies on zebrafish. Nevertheless, functional similarity of zebrafish physiology to other vertabrates must not be taken for granted, and the fundamentals of basic mechanisms in zebrafish need to first be confirmed, and only then should one proceed to study new mechanisms. Relevant novel findings should be confirmed with other vertebrate animals before making general conclusions.

1.3.2. Zebrafish models of human diseases

A wide range of animal species – from worms to monkeys – has been used to model human diseases. All animal models have advantages and disadvantages. One of the key elements of a human disease model is similarity to the modeled human condition. Classical genetic animal models, such as *Drosophila melanogaster* and *Caenorhabditis elegans,* can readily be genetically engineered, have large numbers of progeny, and are inexpensive to maintain. However, they are invertebrates and are evolutionarily, pharmacologically, developmentally, and anatomically distinct from vertebrates. On the other hand, rodents and primates are highly similar to humans in many respects, but they are more difficult and expensive to maintain – ruling out efficient forward genetics. Zebrafish share some of the advantages of invertebrate models (short generation time, large numbers of progeny, inexpensive maintenance, genetic engineering), but belong to vertebrates and are in many ways similar to mammalians – allowing effective modeling of human diseases. To date, a number of different pathological entities have been characterized in zebrafish. Disease models vary from genetic disorders to cancer and cardiovascular diseases. Some complex neurological disorders have also been successfully modeled in zebrafish. Examples of diseases modeled using zebrafish are shown in Table 3. Although anatomical, genetic, and functional similarity has been demonstrated in many cases, one must bear in mind that before proceeding to specific large-scale screens the basic physiological and pathophysiological mechanisms should undergo sufficient validation. Also, genetic similarity does not necessarily mean functional similarity.

Table 3.

Cardiovascular diseases	**Hematological diseases**
Cardiac fibrillation[422]	Congenital sideroblastic anemia[448]
Cardiomyopathy[423, 424]	Severe congenital anemia[449]
Long-QT syndrome[425, 426]	**Muscular diseases**
Thrombosis[427-429]	Duchenne muscular dystrophy[450, 451]
Developmental disorders	Spinal muscular atrophy[452]
Cranio-lenticular-sutural dysplasia[430]	**Nephrologic disorders**
Fetal alcohol syndrome[431]	Acute renal failure[453]
Fragile X syndrome[432]	Nephrotic syndrome[454]
Endocrine disorders	Polycystic kidney disease[455, 456]
Glucocorticoid-induced osteoporosis[433]	**Neoplasms**
Obesity[434]	Acute lymphoblastic leukemia[457]
Porphyria[435, 436]	Liver cancer[458]
Gastrointestinal diseases	Neuroendocrine carcinoma[459]
Familial adenomatous polyposis[437]	Rhabdomyosarcoma[460]
Hirschsprung's disease[438]	**Neurologic or psychiatric disorders**
Steatohepatitis[439, 440]	Addiction[461]
Genetic syndromes	Alzheimer's disease[462]
Bardet-Biedl syndrome[438]	Anxiety[463]
Barth syndrome[441]	Epilepsy[464]
Hermansky-Pudlak syndrome[442]	Huntington's disease[465]
Joubert syndrome[443]	Parkinson's disease[466-469]
Lenz microphtalmia[444]	Schizophrenia[470]
Matthew-Wood syndrome[445]	Sleep disorders[402]
Menkes disease[446]	**Others**
Shwachman-Diamond syndrome[447]	Deafness[471, 472]
	Eye diseases[473]
	Immune and infectious diseases[474]

Examples of human disease models in zebrafish. Note that many of the studies were published recently.

2. AIMS OF THE STUDY

The aim of this study was to establish and test an animal model of PD using larval zebrafish and to use the model to investigate the function of PD-associated protein PINK1. This necessitated some initial studies on the basic mechanisms involved, including the development of aminergic neuron populations, the metabolism of amine neurotransmitters, and the characterization of zebrafish MAO enzyme. The MPTP-induced model of PD was chosen to be characterized since it is widely used in other animal species and is considered to have high validity[380].

Specific aims were as follows:

1. To map and characterize the developing catecholaminergic and serotonergic neuron populations of zebrafish (II,III)
2. To identify and characterize zebrafish MAO gene(s), confirming the presence of MAO activity in zebrafish (I)
3. To evaluate the substrate and inhibitor properties of zebrafish MAO (I)
4. To describe the expression of MAO activity and mRNA expression in the adult zebrafish brain and in the development of embryonic and larval zebrafish (I,II)
5. To characterize the effects of MAO inhibition during development on the aminergic neurotransmitter systems and locomotor activity (II)
6. To characterize the effects of MPTP on larval zebrafish aminergic systems and behavior (III)
7. To clarify the role of MAO in the neurotoxicity of MPTP in zebrafish (III)
8. To establish the presence of *PINK1* in zebrafish (IV)
9. To investigate the expression of cloned PINK1 in adult and larval zebrafish (IV)
10. To characterize the neurotransmitter system phenotype of PINK1-knockdown (IV)
11. To assess the role of PINK1 in MPTP toxicity (IV)

3. Materials and methods

3.1. Experimental animals

Larval and adult zebrafish (*Danio rerio*) from the Turku line and the AB strain were used. The Turku line was originally obtained from a local source and maintained in the laboratory for over 10 years. It has been used in several earlier studies in our laboratory[51, 225, 261, 466, 475, 476]. Fish were kept in aged oxygenated tap water in normal glass aquarium tanks or in a PP-module stand-alone unit (Aqua Schwarz, Göttingen, Germany) under a 14:10 h light/dark cycle. Water temperature was kept at 26-29°C and pH at 6.5-7.5. Fish were fed twice a day with artemia and once a day with dry food (Pet Dry) during working days and once a day with artemia on weekends.

Zebrafish embryos were collected shortly after spawning and transferred onto a Petri dish with E3 medium (mQ water containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$). 1-phenyl-2-thiourea (Sigma, St. Louis, MO, USA) 0.2 mM was added to the media of embryos to be studied before 3 dpf to prevent pigment formation[477, 478].

Appropriate permits for conducting the experiments were obtained from the Office of the Regional Government of Southern Finland in agreement with the ethical guidelines of the European convention.

3.2. Preparation of tissues

Fish were killed by decapitation after immersion in ice-cold water. Larval fish were fixed whole, and brains of 3 dpf or older larval fish were dissected under a stereomicroscope after fixation. Brains of adult fish were dissected directly after sacrifice and subsequently immersion-fixed. Fixation was done at 4°C overnight (o/n). 4% 1-ethyl-3,3(dimethyl-aminopropyl)carbodiimide (EDAC) in 0.1 M phosphate buffer (PB), pH 7.4, was used for MAO enzyme histochemistry and histamine immunostaining. For other staining purposes, 2-4% paraformaldehyde (PFA) fixation was used.

For cryostat sectioning, the samples were immersed in 20% glucose in PB o/n, mounted in embedding medium (M-1 Embedding Matrix #1310, Lipshaw, Pittsburgh, PA, USA) and quick-frozen on solid carbon dioxide. Samples were stored at –20°C prior to sectioning with a Leitz 1720 digital cryostat (Wetzler, Germany) or a Leica CM3050 S cryostat (Leica Microsystems, Mannheim, Germany) at 16-20 µm onto poly-L-lysine-coated slides (Menzel-Gläser, Gerhard Menzel, Glasbearbeitungswerk GmbH & Co. KG, Brunswick, Germany). The slides were stored at –20°C.

3.3. MAO histochemistry

Samples were washed in 0.05 M phosphate-buffered saline (PBS) for 30 min at room temperature (RT) and incubated for 2 h in darkness in 0.05 M Tris-HCl, pH 7.6, containing 0.8 mg of diaminobenzidine tetrahydrochloride (DAB), 10 mg of horseradish peroxidase, 60 mg of nickel sulfate and 10 mg of tyramine in 10 ml of buffer. The color reaction is based on the production of hydrogen peroxide (H_2O_2), resulting from the oxidation of tyramine by MAO. In the presence of H_2O_2, horseradish peroxidase catalyzes the oxidation of DAB. Oxidized DAB forms insoluble brown precipitates. Nickel sulfate improves the sensitivity of the staining by binding to the DAB precipitate and produces a bluish-black precipitate[479]. The reaction was stopped by washing the embryos in 0.05M Tris-HCl for 2 x 5 min. Embryos were post-fixed in 2% PFA o/n. They were then cleared in 70% glycerol in PBS and mounted on microscope slides. All reagents were from Sigma. Negative control samples, in which the substrate was omitted, did not exhibit any signal.

3.4. MAO activity assays

Adult brains or batches of 100 pooled whole larval zebrafish were homogenized in homogenizing buffer (10 mM K_2HPO_4, 1 mM EDTA, pH 7.6) using an ultrathin Ultra-Turrax T8 mechanical homogenizer (IKA®-Werke, Staufen, Germany). The homogenates were centrifuged at 5000 rpm for 5 min and the supernatant was separated and used immediately or stored at -70°C until use. Assays were performed on 96-well plates; each well contained 10 mg/ml of protein (3-5 µl of the brain homogenate), 100 µl of amine substrate (different concentrations), and 50 µl of chromogenic solution (1 mM vanillic acid, 500 µM 4-aminoantipyrine, 4 U/ml horseradish peroxidase type II in 0.2 M potassium PB, pH 7.6). 5-HT, DA, PEA, HA, GABA, tyramine, and MPTP were used as substrates. Substrate or homogenate was omitted in control samples. When MAO antagonists (deprenyl or clorgyline) were used to assess the inhibition of zebrafish MAO enzyme, brain homogenates were incubated with different concentrations of antagonists for 30 min prior to the assay incubation. The assay plates were incubated at 28°C for 0.5-2 h, and the reaction results were read on a Multiscan EX microplate reader (Labsystems, Helsinki, Finland) using a 492-nm filter. The method is based on the determination of the amount of H_2O_2 produced during the oxidation of amines by MAO. In the presence of H_2O_2, horseradish peroxidase catalyzes the oxidation of 4-aminoantipyrine, which condenses with vanillic acid to produce a red quinoneimine dye. All reagents were from Sigma.

3.5. Cloning of a zebrafish MAO gene

A 5-µg sample of RNA extracted from the brain of adult zebrafish was reverse-transcribed with a First-Strand Synthesis Kit (Invitrogen BV, Groningen, The Netherlands) using the forward primer 5'-ACC ATG ACT GCC AAC GC-3' and the reverse primer 5'-TGC TGC CAG AAA TGA GGA C-3', which were designed based on the complete coding sequence information found in GenBank (accession no. AY185211). The PCR product was analyzed on agarose gel electrophoresis, purified with the Minelute Gel Extraction Kit (Qiagen, Hilden, Germany), ligated into the pGEM-Teasy plasmid (Promega, WI, USA), and sequenced.

3.6. In situ hybridization

The distribution of MAO mRNA in zebrafish was investigated using digoxigenin (DIG)-labeled RNA sense and antisense probes synthesized using the linearized plasmid pGEM-Teasy containing the complete coding sequence of zebrafish MAO as a template. The plasmid was linearized with SalI for T7 polymerase (sense) and with Bst120I for SP6 polymerase (antisense). A DIG RNA labeling kit (Roche, Mannheim, Germany) was used to perform the synthesis reactions. The probes were purified using Quick Spin RNA columns (Roche) and checked on an ethidium bromide-containing agarose gel.

Whole-mount samples were first washed with PBS containing 0.1% Tween-20 (Fluka, Buchs, Switzerland) (PBS-Tw) for 2 x 5 min at RT and then incubated in methanol at –20°C for 30 min. Rehydration was done by incubating the samples for 5 min in 1:1 methanol:PBT-Tw and 2 x 5 min in PBT-Tw at RT. Samples were permeabilized with 1x proteinase K treatment in PBS-Tw for 5-12 min depending on the developmental stage of the fish, and the reaction was stopped by washing the samples with 2 mg/ml glycine in PBS-Tw for 2 x 2 min. This was followed by refixation in 4% PFA in PB for 30 min at RT and washing in PBS-Tw for 3 x 5 min. Samples were prehybridized in Hyb+ solution (50% deionized formamide (Amresco, Solon, OH, USA), 5 x SSC (saline-sodium citrate buffer), 0.1% Tween-20, 0.5 mg/ml torula yeast RNA, and 50 µg/ml heparin (Sigma) at 68°C for 2 h. The hybridization was carried out in Hyb+ solution containing the DIG-labeled probe in a dilution of 1:100 at 68°C for 16-20 h. Posthybridization washing was done at 68°C in the following solutions: 5 min in Hyb- (as Hyb+, but without tRNA and heparin), 3 x 5 min in 25% Hyb- in 2 x SSC with 0.1% Tween-20 (SSC-Tw), 5 min in 2 x SSC-Tw, and 2 x 30 min in 0.2 x SSC-Tw. This was followed by washing at RT in the following solutions: 5 min in 1:1 0.2 x SSC-Tw:MAB-Tw (100 mM maleic acid, 150 mM NaCl, 0.1% Tween-20) and 5 min in MAB-Tw. After washing, the samples were incubated in blocking solution (2% blocking reagent (Roche) in MAB-Tw) for 1 h and then in blocking solution containing anti-DIG-antibody conjugated to alkaline phophatase (Roche) in dilution 1:4000. After

incubation, samples were washed in MAB-Tw 4 x 15 min, followed by coloration reaction with BM purple (Roche) at RT until purple staining was visible, typically 10-20 h. The reaction was stopped by washing in PBT-Tw for 2 x 5 min, followed by refixation with 4% PFA o/n at 4°C. The samples were infiltrated with 80% glycerol in PBS and mounted between two coverslips.

In situ hybridization of tissue sections was carried out essentially as described for whole mounts. However, no methanol, rehydration, or proteinase K treatments were performed. For coloration, sections were incubated with 5-bromo-4-chloro-3-indolylphosphate-4-toluidine salt and 4-nitro blue tetrazolium chloride (Roche) in a buffer containing 100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20 instead of BM purple.

3.7. Immunohistochemistry of whole-mount samples

Samples were fixed depending on the primary antibody used. After overnight fixation, the samples were kept in 0.1 M PB for up to 2 weeks. Samples were then washed in PBS containing 0.3% Triton-X (PBS-T) for 3 x 30 min at RT, followed by preincubation in PBS-T containing 1% dimethylsulfoxide (DMSO) and 4% normal serum o/n at 4°C. Endogenous peroxidase activity was blocked in samples destined for DAB development by incubation in methanol containing 0.3% H_2O_2 for 10 min. Incubations with primary antibodies were done in PBS-T containing 2% normal goat or swine serum o/n at 4°C. Samples were then washed for 10 min + 3 x 30 min in PBS-T at RT. Incubation with secondary antibodies was done in PBS-T containing 2% normal serum o/n at 4°C, followed by washing for 10 min in PBS-T and 3 x 30 min in PBS. Samples were infiltrated with 80% glycerol in PBS o/n and then mounted between two 0.17-mm (standard no. 1.5) microscope coverslips (Menzel-Gläser). Glass spacers of the same thickness were inserted to prevent compression and subsequent distortion artifacts.

3.8. Immunohistochemistry of cryosectioned samples

Frozen sectioned samples were first air-dried at RT for 5 min, followed by washing in PBS for 2 x 5 min. Incubations with primary antibodies were in done in PBS-T containing 2% normal goat or swine serum o/n at 4°C. Samples were then washed for 3 x 5 min in PBS-T. Incubation with secondary antibodies was done in PBS-T for 1 h at RT. Samples were then washed for 3 x 5 min in PBS. They were mounted using Permount (Fisher, Fair Lawn, NJ, USA) or 80% glycerol in PBS and covered using 0.17-mm (standard no. 1.5) coverslips (Menzel-Gläser).

3.9. Primary antibodies

The following primary antibodies were used: mouse anti-tyrosine hydroxylase (diluted 1:1000, Diasorin, Stillwater, MN, USA, samples fixed with 4% PFA), rabbit anti-serotonin (diluted 1:1000, Sigma, St. Louis, MO, USA, samples fixed with 4% PFA), rabbit anti-histamine[480] (diluted 1:5000, samples fixed with EDAC), goat anti-ChAT (diluted 1:200, Chemicon, Temecula, CA, USA, samples fixed with 4% PFA), ZRF-1 (diluted 1:5000, ZIRC, 4 % PFA), ORX-A (diluted 1:1000, Chemicon, 4% PFA), and NPFF[481] (diluted 1:2000, 4% PFA).

For immunofluorescence, goat or donkey anti-mouse or anti-rabbit Alexa fluor® (488, 561, or 633) -conjugated secondary antibodies were used in dilution 1:1000. For light microscopy, secondary labeling was carried out by using the Universal Vectastain Elite Kit, which is based on the biotin-streptavidin method (Vector Laboratories, Burlingame, CA, USA). This was finished by the coupled oxidation reaction in a solution containing 0.025% DAB, 0.01% H_2O_2, and 0.3% $NiSO_4$ in 0.05 M Tris-HCl buffer, pH 7.6, until a bluish-black product was visible, typically after a couple of minutes. For double staining purposes, $NiSO_4$ was omitted, resulting in brown staining.

3.10. High-performance liquid chromatography (HPLC) analysis

Fifteen to twenty-five whole fish were pooled and homogenized in 150 µl of cold 0.4 M perchloric acid containing 0.1% $Na_2S_2O_5$ and 0.1% EDTA. The homogenate was centrifuged at 27000 g at 4°C for 20 min and filtered through Acrodisk syringe filters (13 mm) with a 0.45-µm polyvinylidene difluoride membrane.

Concentrations of dopamine, noradrenaline, and their metabolites were measured using an HPLC system consisting of a Waters pump, model 515 (Waters Corporation, Milford, MA, USA), Autosampler 717Plus (Waters), and ESA Colochem 5100A electrochemical detector (ESA, Chelmsford, MA, USA), equipped with a conditioning cell, model 5021 (potential +0.5 V) and an analytical cell, model 5011. Reversed-phase chromatography was performed with Merck 50943 LichroCART100 RP-18 column (125 x 4 mm, 5 µm) with a Waters Symmetry precolumn cartridge (3.9 x 20 mm, 5 µm). The mobile phase contained 0.05 M H_3PO_4, 50 mM citric acid, 0.15 mM EDTA, 2 mM octanesulfonic acid sodium salt and 5% methanol (w/v). pH was adjusted to 3.8 with 10 M NaOH.

Serotonin levels were measured using an HPLC system consisting of a Waters pump, model 510, Autosampler 717Plus, and Concorde electrochemical detector (Waters). Reversed-phase chromatography was performed with a C18 column (Waters Symmetry, 4.6 x 150 mm, 5 µm) with a precolumn cartridge (Waters Symmetry). Mobile phase was similar to that described above, but pH was 2.2.

Both HPLC systems were used in an isocratic mode, with a flow rate of 1.0 ml/min. All determinations were done at RT.

3.11. Microscopy and image analysis

Light microscopic analysis was performed using two different microscope systems: Olympus AX70 light microscope (Olympus Corporation, Tokyo, Japan) connected through an Olympus DP71 camera to AnalySIS or Cell^{A} software (Olympus Soft Imaging System GmbH, Münster, Germany) and Leica DM IRB inverted microscope (Leica Microsystems) connected through a Leica DFC 490 Color Digital Camera to Leica Application Suite 2.7.0 R1 software.

Fluorescence microscopy was carried out using a Leica TCS SP2 confocal microscopy system with an Argon laser with a 488-nm laser line, a green diode laser with a 561-nm laser line, and a Helium-Neon laser with a 633-nm laser line. The following coverslip-corrected objectives were used: Leica HCX PL APO 63x (1.3 NA, glycerol immersion), Leica HC PL APO 20x (0.70 NA, dry), and Leica HC PL APO 10x (0.40 NA, dry). All double-, or triple-stained samples were scanned sequentially to reduce cross-talk between channels. Images were obtained in 1024 x 1024 pixel format. Stacks of images were taken at 0.2- to 1.2-μm intervals. They were later compiled to produce maximum-intensity projection images using Leica Confocal Software or ImageJ 1.37a software (National Institutes of Health, Bethesda, MD, USA). They were further processed using Paint Shop Pro 7 (Jasc Software, Eden Prairie, MN, USA), and figures were compiled using CorelDraw 10 – 13 software (Corel Corporation, Ottawa, Canada). Animations and 3D-modeling were done using Imaris 6.0 software (Bitplane AG, Zurich, Switzerland). Animations were further processed using QuickTime 7.4.1 Pro software (Apple Inc., Cupertino, CA, USA). Image processing was performed using MacBook Pro (Apple Inc.) equipped with 2.2 GHz Intel Core 2 Duo with 4 GB 667 MHz DDR2 SDRAM. 3D-image rendering was done using a Hewlett-Packard PC workstation (Hewlet-Packard, Palo Alto, CA, USA) equipped with a Dual-Core AMD Opteron™ Processor 2222 3.00 GHz and 16 GB of RAM running on Microsoft Windows XP Professional x64 Edition.

3.12. Drug treatment protocols

Zebrafish larvae were transferred into the wells of a 6-well plate shortly after spawning. Up to 30 fish were raised per well containing 3 ml of E3 solution. Larvae were briefly transferred to a Petri dish for manual dechorionation at 24 hpf. Solutions were replaced daily with prewarmed E3 solution followed by addition of the drugs. Depending on the experiment, larval fish were exposed to the following drugs: MPTP (Sigma) 50, 100, or 1000 μM on days 1-4 post-fertilization; MPP+ (Sigma) 100, 500, or 1000 μM on days 1-4 post-fertilization; deprenyl (Sigma) 1,

10, or 100 μM on days 0-4, 0-5, or 0-7 post-fertilization or 2 h prior to testing; fluvoxamine 100 μM on days 0-5 post-fertilization or 2 h prior to testing; and PCPA 150 or 1500 μM on days 1-5 post-fertilization. Control groups received no drugs. Experiments were carried out independently three times, and the results were combined. MPTP and MPP+ exposures were carried out with extreme caution in accordance with current safety protocols[482].

3.13. Behavioral analysis

Larvae were placed in individual hemisphere-shaped wells (diameter 4 cm, volume 12 ml) or in the wells of a 48-well plate. They were automatically tracked using a CCD camera with a sampling rate of 5 samples/s for 10 min. A higher sampling rate of 25 samples/s produced essentially similar results. Tracks were analyzed and total distance moved (cm) and movement (percentage of time spent moving) were calculated from the acquired coordinates using the Ethovision 3.1 software (Noldus Information Technologies, Wageningen, The Netherlands). Only fish exhibiting a normal gross phenotype were included in the analysis. Movements of less than 0.2 cm were considered system noise and filtered. A track was excluded if the system could not detect the sample more than 10% of the time analyzed. Tracks exhibiting reflection artifacts were also excluded from the analysis. Fish that did not move at all during the 10-min tracking period were also excluded (only in study IV).

3.14. Morpholino knockdown technique and RNA rescue

Two morpholino oligonucleotides targeting the PINK1 mRNA were used; one targeting the 5-utr-site (-9 - +16 from the translation start site, GAGAGGAAATCTGAAGGCTT TTACG) and the other targeting the splice region of exon 3 and intron 3 (TCACAACCTAC CCGTTCAAAGTCAG). For control purposes, a morpholino oligonucleotide with 5 mismatch nucleotides (GACAGCAAATCTCAAGGGTTTTAGG) was used.

Morpholino oligonucleotide solution (4 nl) was injected into the yolk of an embryo at 1-4 cell stage by using a micromanipulator (Narishige, Tokyo, Japan). The morpholino oligonucleotide solutions contained 25% phenyl red and 0.25-3 g/l of morpholino oligonucleotide diluted in sterile distilled water. Some of the morpholino oligonucleotides were tagged with a fluorescent tag (fluorescein), and on such occasions embryos were examined quickly under fluorescent light to discard individuals not exhibiting uniform distribution of the morpholino oligonucleotide. Efficacy of the morpholino knockdown was studied using RT-PCR and QPCR. Specificity of the morpholino oligonucleotides was confirmed using a 5-base pair mismatch morpholino oligonucleotide, two morpholino oligonucleotides targeting different sites (translation-inhibition, splice-inhibition) of the same target

mRNA, determination of the delta113 p53 isoform, and coinjection of a p53-morpholino oligonucleotide and RNA rescue.

3.15. Statistical analysis

Quantitative data were analyzed using either Student's t-test (for single comparisons) or analysis of variance (ANOVA) followed by Bonferroni's, Dunnett's, or Newman-Keuls' post-hoc test. GraphPad Prism 2.01 - 4.01 software (GraphPad Software Inc., San Diego, CA, USA) was used for the analyses. The significance level was set at 0.05 prior to sampling. The results are given as means ± SEM. All experiments were performed at least three times.

4. Results

The main findings are presented here. For comprehensive details, the reader is encouraged to consult the original publications (I-IV).

4.1. Development of catecholaminergic and serotonergic systems (II-IV)

Prior to using an animal model to study human pathology, the fundamentals of normal anatomy and physiology of the model must be understood. Earlier studies concerning the development of catecholaminergic and serotonergic cell populations in zebrafish have been published. While the first studies reported the timed appearance of catecholaminergic populations, they failed to distinguish between separate cell populations in the adult fish – mostly because they used *in situ* hybridization with a color dye, yielding diffuse signals[483, 484]. Another report finely dissected the diencephalic catecholaminergic cell populations in sections, but lacked a time scale and a description of the nondiencephalic populations[485]. Furthermore, sections only provide us with assumptions made from 2D data. However, these classifications have been used in several subsequent studies, without exact knowledge of the complexity of distinct cell populations, their 3D anatomy, or their time of appearance. To accurately describe the developing cell populations, we investigated the timed appearance of catecholaminergic and serotonergic populations by using 3D confocal microscopy at 0-7 dpf. Cell populations were assigned numbers to indicate their rostrocaudal distribution in the adult zebrafish brain, allowing comparisons of embryonic, larval, and adult cell populations[51]. A 3D model was created and an atlas presented to augment future studies on these systems (Figure 7 and III Supplementary information).

The timed appearance of catecholaminergic cell populations is presented in Table 4 (see Section 5.2.). The first aminergic cells were detected in close proximity to the nucleus of the tract of the post-optic commissure (nTPOC) just before 24 hpf. Both serotonergic and catecholaminergic cells were detected in this region, and some cells exhibited a double phenotype, being immunoreactive for both tyrosine hydroxylase and serotonin. Such colocalization was not detected in later development or in adult fish. Cells of this early aminergic population showed prominent descending axons that projected towards the spinal cord. These early catecholaminergic cells are morphologically similar to those found later in population 12, thus most likely corresponding to this population. Serotonergic cells in this region are persistently detected, but show a different morphology later. They most likely correspond to the anterior paraventricular cell population (population 2).

The number of catecholaminergic cells increased rapidly as new cell populations emerged. By 48 hpf, catecholaminergic cell populations were detected in the olfactory bulb and telencephalon (populations 1 and 2), anterior

paraventricular organ (population 8), caudal hypothalamus (population 12), locus coeruleus (population 14), internal reticular formation (population 15), vagal lobe (population 16), area postrema (population 17), and postoptic/thalamic/posterior tuberculum (population 5,6,11). At 60 hpf, populations in the pretectum (population 7) and posterior paraventricular organ (population 10) emerged. The cells of the intermediate paraventricular organ emerged last, at 72 hpf (population 9). All populations appeared in their final place and immediately showed adult-like morphology. No apparent migration of cells was detected.

Serotonergic cell populations were detected in the rostral and caudal raphe (populations 5-7) at 48 hpf, pretectum (population 1), and intermediate and posterior paraventricular organ (populations 3 and 4) at 60 hpf, vagal lobe (population 9) at 72 hpf, and finally in the reticular formation (population 8) at 96 hpf. Serotonergic cells in the posterior paraventricular organ intermingled with catecholaminergic cells (population 10), but no colocalization was found in this region. Serotonin immunoreactivity was also detected in the epiphysis from 36 hpf onwards.

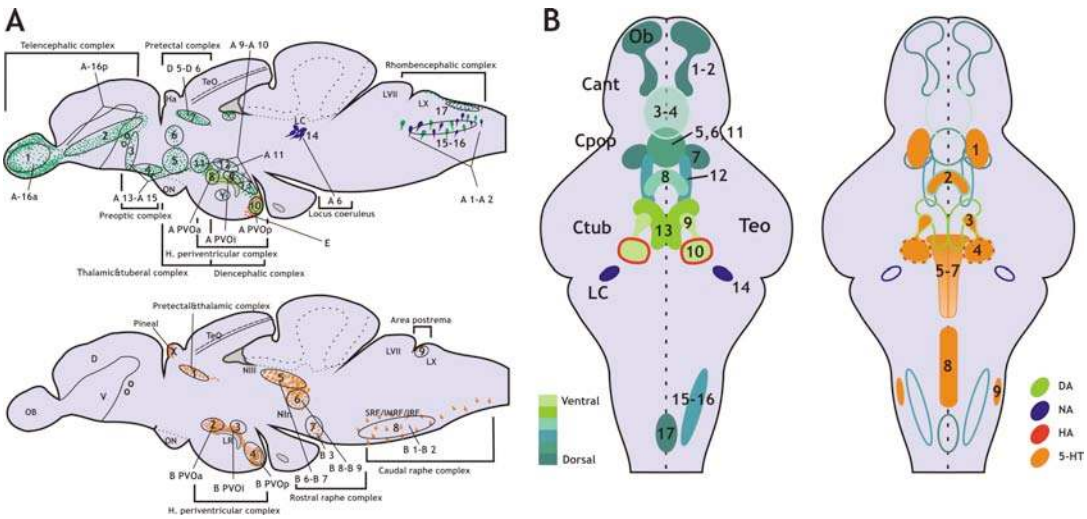

Figure 7.

Schematic illustration showing the adult (A) and larval (B) aminergic cell populations of the zebrafish brain. (II, III). Abbreviations: Cant – anterior commissure, Cpop – postoptic commissure, Ctub – the commissure of the posterior tuberculum, D – dorsal telencephalic area, E – histaminergic cell group, Ha – habenula, IMRF – intermediate reticular formation, IRF – inferior reticular formation, LC – locus coeruleus, LR – lateral recesses of the diencephalic ventricle, LVII – facial lobe, LX – vagal lobe, NIII – oculomotor nucleus, NIn – interpeduncular nucleus, OB – olfactory bulb, ON – optic nerve, PVOa, i, p – anterior, intermediate and posterior parts of the paraventricular organ, SRF – superior reticular formation, V – ventral telencephalic area, TeO – optic tectum. Modified from Studies II and III, with permission from Wiley-Blackwell.

Two genes encoding tyrosine hydroxylase found in the zebrafish genome are designated *th* (Gene ID: 30384) and *th2* (Gene ID: 414844). *In situ* hybridization was also carried out using *th* and *th2* probes. *th* mRNA expression was similar to TH immunoreactivity. *th2* mRNA expression was detected in the anterior, intermediate, and posterior parts of the paraventricular organ and in the preoptic region.

4.2. Structure, activity, and function of zebrafish MAO (I,II)

Contrary to mammals that have two *MAO* genes, only one gene related to *MAO* was found in the zebrafish genome. Zebrafish MAO had 67-69% nucleotide and protein identity with mouse, rat, and human MAO A and B. The number and length of exons of zebrafish *MAO* were identical to human *MAO A* and *B*. 12 amino acid residues form the active site of human MAO A and B. In zebrafish MAO, 8 of these twelve amino acids were identical to both human MAO A and B, two were identical to human MAO A (Asn181, Phe208), and two were mismatches to both MAO A and B – a result suggestive of a more MAO A-like active site in zebrafish MAO (Figure 8).

Zebrafish MAO substrate specificity was tested with adult brain homogenates using a MAO activity assay, which is based on formation of H_2O_2. Tyramine was found to be the best substrate for zebrafish MAO, followed by serotonin, PEA, and MPTP. Hardly any activity was detected when dopamine was used as a substrate. Negative controls (GABA, histamine, substrate omitted) did not produce measurable activity, as expected. Inhibitor specificity of zebrafish MAO was tested by preincubation of the brain homogenate or live fish with either

Figure 8.

Comparison of human MAO A and MAO B peptide sequences with the deduced zebrafish MAO peptide sequence. Similar amino acids are indicated in bold and underlined; gray shading represents amino acids involved in the formation of the active center of MAO. The encircled residue is suggested to be responsible for inhibitor specificity[283].

clorgyline or deprenyl, which are irreversible and selective inhibitors of human MAO A and B, respectively. Up to 30-40% inhibition of tyramine or serotonin catabolizing activity was detected when deprenyl and clorgyline were used separately or together. In larval fish treated with deprenyl, the activity of MAO was decreased by 36-74% at 3-7 dpf, depending on the fishes' age.

To further study the *in vivo* substrate properties of zebrafish MAO, HPLC analysis of monoamine neurotransmitters and their metabolites was performed on larval zebrafish treated with deprenyl (Figure 9). Serotonin concentrations were dramatically increased, while concentrations of the MAO-dependent metabolite of serotonin, 5-HIAA, were below the detection limit after deprenyl exposure, suggesting metabolism of serotonin via a MAO-dependent pathway. Dopamine, noradrenaline, or adrenaline concentrations, by contrast, were unchanged after deprenyl exposure, suggesting that their metabolism in zebrafish is not MAO-dependent to a large extent.

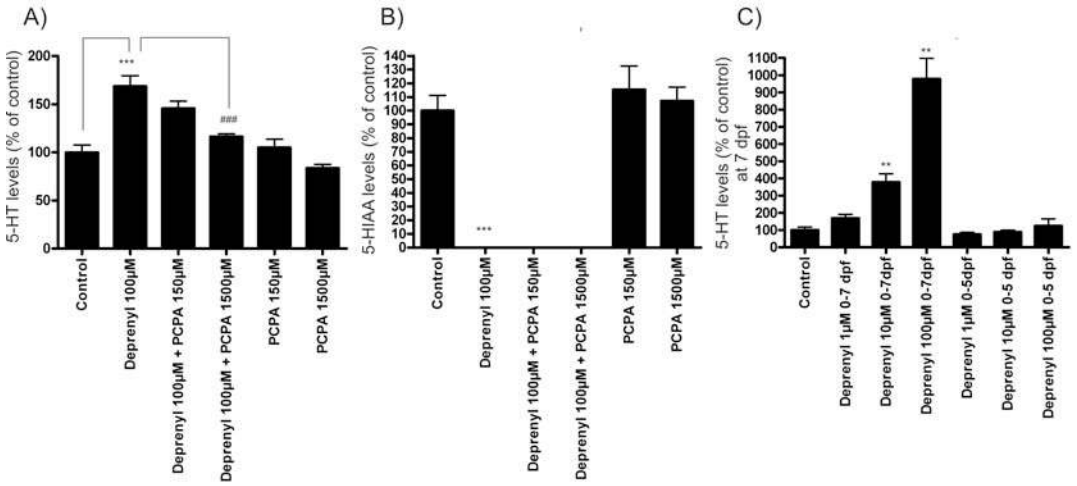

Figure 9.

HPLC analysis of 5-HT and 5-HIAA concentrations in zebrafish treated with deprenyl and/or PCPA. The age of the fish is 5 dpf in A and B and 7 dpf in C. Deprenyl and PCPA exposures were for 0-5 dpf. 100% equals mean of control. **A:** Deprenyl increases 5-HT levels and PCPA 1500 uM restores the elevated levels. **B:** 5-HIAA levels were below the detection limit after deprenyl treatment, indicating that MAO inhibition was effective and no or little 5-HT metabolite was formed. PCPA treatment did not affect 5-HIAA levels. **C:** 5-HT levels are even more strongly elevated after 0-7 days' treatment with deprenyl. 5-HT levels returned back to basal levels in 2 days after cessation of deprenyl treatment. ** $p<0.01$, *** or ### $p<0.001$. n = 3-4 pooled samples. Comparisons were made using one-way ANOVA, followed by Bonferroni's post-hoc test for selected pairs in A and B. In C, Dunnet's post-hoc test was used. Selected pairs were the following: control vs. deprenyl 100 µM, control vs. PCPA 150 µM, control vs. PCPA 1500 µM, deprenyl 100 µM vs. deprenyl 100 µM + PCPA 150 µM, deprenyl 100 µM vs. deprenyl 100 µM + PCPA 1500 µM. Reprinted from Study II, with permission from Wiley-Blackwell.

4.3. Localization of MAO in adult and developing zebrafish (I-III)

Expression of MAO mRNA in different zebrafish tissues was examined using RT-PCR and QPCR. The strongest *MAO* expression was detected in the brain, followed by the intestine, liver, heart, and gills. Negligible *MAO* expression was detected in the eyes, muscles, and spleen.

Localization of MAO expression in the brain was studied in detail using enzyme histochemistry and *in situ* hybridization. Tyramine was used as a substrate for histochemistry since other substrates did not produce sufficient reaction for morphological demonstration. Deprenyl produced complete inhibition of the reaction already at 10 μM, while clorgyline produced only partial inhibition even at 100 μM. Staining was often diffuse and without clear cellular or fiber-like appearance. However, some MAO-positive cells were detected in distinct clusters (see study I, Figure 9 for a schematic presentation).

Telencephalon. Olfactory bulbs were virtually devoid of MAO activity and mRNA. Cells exhibiting MAO activity were detected near catecholaminergic cells in the preoptic area from 4 dpf onwards. However, no clear colocalization was found at this site.

Diencephalon. A population of MAO-positive cells was detected in the telencephalon that continued caudally into the diencephalon and was located near the catecholaminergic cells in the suprachiasmatic nucleus. Small stellate cells exhibiting intense MAO activity and mRNA were detected in the epiphysis and habenula. Habenular MAO activity and mRNA were detected from 5 dpf onwards, but no MAO activity or mRNA was detected before 7 dpf. Diffuse MAO activity was detected at the site of the catecholaminergic and histaminergic cells in the periventricular hypothalamus, but in adults, due to the intense staining, colocalization was difficult to judge. MAO mRNA was also detected at these sites. However, in larval zebrafish, localization of MAO activity was seen in histaminergic cells and in the caudal cells of catecholaminergic cell population 13.

Mesencephalon. The optic tectum showed layered distribution of MAO activity and mRNA. Small cells exhibiting MAO activity and mRNA were detected in the periventricular gray zone.

Rhombencephalon. Strong MAO activity was detected in the adult zebrafish in an area lateral and caudal to the nucleus interpeduncularis and ventral to the medial raphe. This area is not well defined in current anatomical literature. However, this area was also apparent in larval fish at 5 dpf. Catecholaminergic cells of the locus coeruleus exhibited MAO activity from 2 dpf and mRNA from 1 dpf onwards. MAO activity was detected in the aminergic cells in the internal reticular formation and vagal lobe.

Serotonergic cells in the raphe nuclei showed both MAO activity and mRNA. The cerebellum was devoid of MAO activity in adult and larval zebrafish.

However, mRNA was detected in the adult fish in the cerebellar corpus, but not in larval zebrafish.

4.4. Alteration of behavior and 5-HT-immunoreactivity after MAO inhibition (II)

MAO inhibition by deprenyl during development dramatically increased the levels of 5-HT, but left DA, NA, and A levels intact. 5-HT levels were increased to 170% of control levels after 0-5 dpf treatment with 100 µM deprenyl. Treatment at 0-5 dpf with 10 µM and 100 µM deprenyl increased 5-HT levels to 380% and 980% of control levels, respectively (Figure 9A,C). The levels of 5-HT normalized in 2 days after stopping the treatment (Figure 9C). The increase in 5-HT was accompanied by decreased spontaneous locomotion, swimming preference on the surface, and increased heart rate (Figure 10A). Increased 5-HT-ir in the ventral hypothalamus was also detected, along with ectopic 5-HT-ir cells after deprenyl treatment (Figure 10C). In addition, 5-HT-ir was decreased in the cell somata of the 5-HT cell populations. Ectopic 5-HT-ir cells were not detected if the larvae were treated with fluvoxamine (an inhibitor of 5-HT uptake) 2 h prior to sacrifice, suggesting that these cells accumulate 5-HT by uptake via SERT (Figure 10D). The levels of 5-HT and behavioral alterations were normalized by treatment with the serotonin synthesis inhibitor, PCPA, suggesting causality between the increased levels of 5-HT and the behavioral effects.

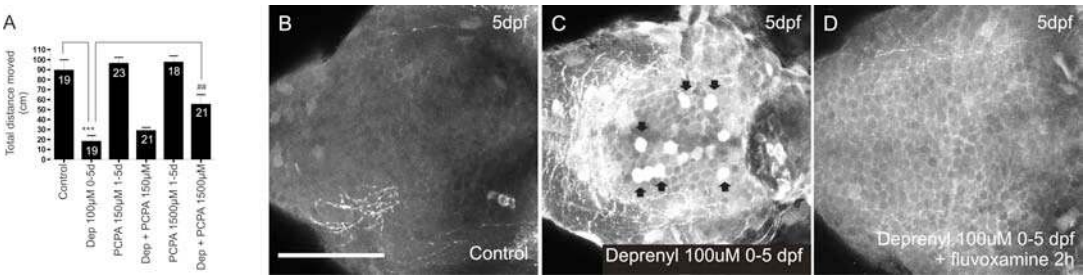

Figure 10.

A: Effects of deprenyl and PCPA on the locomotor activity of 5 dpf larval zebrafish. Deprenyl exposure decreased locomotion, while PCPA alone had no effect. PCPA rescued locomotion after deprenyl treatment. *** $p<0.001$, ## $p<0.01$ **B:** 5-HT-ir in the normal 5 dpf larval zebrafish hypothalamus. **C:** 5-HT-ir in the hypothalamus after deprenyl treatment. Note that 5-HT-ir is increased and ectopic 5-HT-ir cells are detected (arrows). **D:** Ectopic 5-HT-ir cell were not detected in deprenyl-exposed fish that were also treated with fluvoxamine, suggesting that the ectopic cells accumulate 5-HT by SERT-mediated uptake. Modified from Study II, with permission from Wiley-Blackwell.

4.5. Effects of MPTP and MPP+ on aminergic systems and behavior (III)

Larval zebrafish were exposed to MPTP or MPP+ at 1-4 dpf, and amine levels were determined using HPLC at 5 dpf. Exposure to 1000 μM MPTP decreased the levels of dopamine, noradrenaline, and serotonin significantly, and this could be rescued with MAO inhibition by deprenyl exposure. MPP+ decreased only dopamine and noradrenaline levels.

The effects of MPTP and MPP+ on specific catecholaminergic and serotonergic cell populations were determined using immunohistochemistry and counting the absolute number of cells from a 3D image stack at 5dpf. MPTP caused dramatic loss of TH-ir cells in the pretectal (7) and diencephalic (5,6,11) populations followed, by the preoptic (3-4) and hypothalamic (13) populations. MAO inhibition alleviated the MPTP-induced loss of cells. Cells in these populations were also counted at 7 dpf, and at that time, no loss of cells was detected. MPTP also caused loss of 5-HT-ir cells in the serotonergic intermediate paraventricular organ (3) at 5 dpf. MPP+ caused only loss of TH-ir cells in the diencephalic population (5,6,11) at 5 dpf.

Both MPTP and MPP+ caused a transient decline in locomotor activity at 5-6 dpf, which returned to basal levels at 7 dpf. Simultaneous deprenyl exposure partially restored the activity.

4.6. Zebrafish PINK1 gene and protein structure and expression (IV)

One zebrafish *PINK1* gene (*zfPINK1*) residing on chromosome 23 was identified in the zebrafish genome database (Zv8 assembly (September 2008) at the Pre Ensembl Genome browser). The *zfPINK1* gene contains 8 exons and shows approximately 61% similarity to the human and mouse *PINK1* genes. The genomic organization was similar between zebrafish, mouse, and human *PINK1* genes, each containing 8 exons of similar sizes.

The *zfPINK1* is predicted to encode a protein containing 574 amino acids (aa). The predicted zfPINK1 protein is 58% and 54% identical to human and mouse protein sequences, respectively. The functional domain structure of PINK1 is highly conserved across species. A ser/threonine kinase domain was predicted at 143–493 aa using the Pfam program (http://pfam.sanger.ac.uk/). The subdomains included an ATP moiety anchoring site at 149-157 aa, a protein kinase active site at 346–358 aa, and a chelation site at 372-372 aa predicted using the ScanProsite program (http://www.expasy.ch/tools/scanprosite). The leader signal, which is cleaved in mitochondrial targeting, was predicted to consist of 84 aa from the translation start site (using MitoProtII). An APE region was predicted at the C-terminus at 402-404 aa, and a motif containing the arginine residue binding to the APE motif at 483-495 aa.

zfPINK1 mRNA expression in zebrafish tissues was investigated using semi-quantitative RT-PCR. The highest expression levels were detected in the forebrain and midbrain. zfPINK1 mRNA expression was detected from 3 hpf until the adult stage, with minor temporal variations in expression levels. PINK1 mRNA expression was detected ubiquitiously in the brain, eyes, heart, liver, and lateral line.

PINK1-like immunoreactivity (PINK1-ir) was detected widely in the brain gray matter. PINK1-ir was detected in all zones of the telecenphalon and in the preoptic, suprachiasmatic, thalamic, habenular, and hypothalamic regions of the diencephalon. In the optic tectum, PINK1-ir was distributed in a layered fashion. PGZ and DIL were densely packed with PINK1-ir cells. PINK1-ir cells were found in the Purkinje cell layer of the cerebellum. The distribution of *PINK1* mRNA was highly similar to the PINK1-ir distribution.

PINK1-ir was colocalized with TH-ir and 5-HT-ir, but not with the fibrillar astrocyte marker ZRF-1. PINK1-ir was detected in similar locations also in larval zebrafish.

Western blotting using the PINK1 antibody produced two bands of 33 and 66 kDa. Preabsorption of the PINK antibody by a blocking peptide obtained from the manufacturer abolished the Western blot staining completely.

4.7. Phenotype of PINK1 knockdown fish (IV)

A *PINK1*-MO targeting the splice site of exon 3 and intron 3 was primarily used. Efficacy of the MO was confirmed by detection of a band shift in the RT-PCR gel and by a decrease in the level of QPCR signal. A dose of 8 ng was chosen due to lack of gross morphological disturbances, yet production of a knockdown of 75 ± 5.4% in the transcript levels at 5 dpf. Possible off-targeting activation of p53 ^{413} was ruled out by using QPCR to detect normal levels of p53 and delta113 isoform mRNA.

PINK1-MO injected fish were morphologically indistinguishable from their WT siblings. Spontaneous locomotor activity was not altered by *PINK1* knockdown.

TH1 and *TH2* transcript levels were decreased by 82 ± 2.6% and 83 ± 4.3%, respectively ($p<0.01$, t-test, n=3). However, the levels of *DAT* or *HDC* transcripts were unaltered by *PINK1* knockdown. *TH1* mRNA expression was lowered in the ventral diencephalic cluster at 3 dpf, and a decreased expression in the pretectal cell population was also detected at 5 dpf. A decline in *TH2* mRNA expression was detected in the posterior part of the paraventricular organ at 3 dpf. We also detected a loss of TH-ir cells in TH-ir population 5,6,11. The loss of TH-ir cells in this population was further confirmed by using another MO targeted at the 5'-UTR

region of *zfPINK1*. This loss of TH-ir was rescued by PINK1 mRNA injection. 5'-utr-PINK1-mo injection also caused loss of TH-ir cells in the pretectal population, but this was not rescued by the *PINK1* RNA injection.

No major differences in the immunoreactivity for 5-HT, orexin, calretinin, ZRF-1, or NPFF were detected between *PINK1* knockdown individuals and their controls.

4.8. Effects of PINK1 knockdown on MPTP toxicity (IV)

As PD is thought to be caused by an interplay between genetic susceptibility factors and environmental stressors, we wanted to see whether *PINK1* knockdown would render zebrafish more susceptible to neurotoxicity induced by MPTP exposure. Subeffective doses of MPTP (50 μM) did not produce detectable alterations in the gross morphology, spontaneous locomotor activity, or TH-ir in the brain of normal zebrafish. However, *PINK1* knockdown fish exposed to 50 μM MPTP showed decreased spontaneous locomotor activity. Additionally, TH-ir loss was detected in the pretectal cell population, which was not observed in *PINK1* knockdown fish not exposed to MPTP. TH-ir loss that was detected in the population 5,6,11 after PINK1 knockdown was not exacerbated after MPTP 50 μM exposure.

5. Discussion

5.1. Evaluation of methods

5.1.1. Turku line of zebrafish

Fish of the Turku line were mainly used because of their better yield of viable individuals (compared with e.g. the AB line). The Turku line originates from our laboratory and was bred from a large pool of fish obtained originally from a local commercial supplier. The ultimate exact origin of the fish is unknown. The line has been used in several studies concerning brain neurotransmitter anatomy[51, 225, 261, 466, 475, 476], and thus, the line is fairly well characterized. However, the line in not commonly used outside our laboratory, and discrepant in the results from other laboratories may be partly explained by line differences. Very few zebrafish lines are well characterized.

5.1.2. MAO enzyme activity determination and histochemistry

We used a peroxidase-linked colorimetric MAO activity measurement assay, which is based on the production of hydrogen peroxide. Tyramine, 5-HT, PEA, and MPTP all produced concentration-dependent increases in the optical absorbance of the reaction. Dopamine was also used as a substrate. However, dopamine is highly unstable and rapidly auto-oxidized, producing hydrogen peroxide. For this reason, we used an anti-oxidant, ascorbic acid, in the solution. The dopamine-induced increase in optical absorbance was hardly detectable, suggesting that dopamine is not readily metabolized by zebrafish MAO. However, the presence of ascorbic acid in the solution might affect the development of the staining reaction. Other neurotransmitters that are not mammalian MAO substrates were also tested; GABA and histamine produced no detectable activity, as expected.

Deprenyl (a mammalian MAO B inhibitor) and clorgyline (a mammalian MAO A inhibitor) were used to determine the inhibitor specificity of the zebrafish MAO. Both drugs similarly decreased the activity of zebrafish MAO partially, but not completely. Others have reported complete inhibition of zebrafish MAO with 5-HT and PEA as substrates and deprenyl and clorgyline as inhibitors [486]. This discrepancy might be related to differences in experimental conditions. The experiments of Setini *et al.* [486] were carried out at 37°C, which is the physiological body temperature of mammals, but not relevant for poikilothermic zebrafish. Indeed, we have observed a decrease in MAO activity with increasing temperature (data not shown). We used a temperature of 28°C to more accurately mimic the physiological conditions. The observed partial inhibition with deprenyl and clorgyline remains unexplained (see Sections 4.2. and 5.3.1.). We cannot exclude the contribution of other enzymes to the measured MAO activity.

Tyramine was used as a substrate for histochemical MAO detection since 5-HT, dopamine, or MPTP did not produce sufficient reactions for morphological description. Deprenyl abolished the histochemical reaction already at the 10 μM concentration, while clorgyline only partially inhibited the reaction even at such a high concentration as 100 μM. The differences in inhibition levels between the enzyme activity assay and histochemistry might be attributable to temperature since histochemistry was carried out at RT (20 – 22 °C). However, the fixation used might also affect the activity of zebrafish MAO. Fixation with PFA abolished zebrafish MAO activity completely (data not shown), and therefore, the samples were fixed using the bifunctional fixative EDAC. Although fixation with EDAC did not abolish MAO activity, it might have decreased it.

MAO histochemistry suffers from poor resolution in whole-mount zebrafish brain samples – a problem consistent in all stainings of whole-mount samples examined under the light microscope. None of the commercially available MAO antibodies tested produced any signal in the zebrafish brain (data not shown). Future efforts should be aimed at producing zebrafish MAO antibodies and/or developing a fluorescent method for the detection of MAO activity in tissues.

5.1.3. Immunohistochemistry

In Study II, we showed colocalization of TH- and 5-HT-ir in some of the early neurons. Colocalization is difficult to prove reliably using immunofluorescence due to possible bias in the specificity of the primary and secondary antibodies and bleed-through of the fluorescence. Primary antibodies against TH and 5-HT have been characterized in previous work and seem to be highly specific[51]. We used anti-rabbit or anti-mouse Alexa Fluor 488 and 568 secondary antibodies. To minimize the false binding of secondary antibodies, we used highly cross-absorbed antibodies that had been absorbed against bovine IgG, goat IgG, mouse IgG (for anti-rabbit ab), rat IgG, human IgG, and rabbit IgG (for anti-mouse ab). We used an Argon laser with a 488-nm laser line and green diode laser with a 561-nm laser line. The images were scanned sequentially between frames with moderate PMT gains (<800 V) and conservative filter settings (500-550 nm and 600–750 nm). The emission spectrum of Alexa 488 extends to near 700 nm, and thus if excited, may be detected with the filter setting used. However, excitation of Alexa 488 fluor is virtually nonexistent using the 561-nm laser line (Figure 11), and no bleed-through from the 488 channel to the 568 channel should occur. On the other hand, the 488-nm laser line does excite Alexa 568 fluor slightly, but the emission spectrum of Alexa 568 does not exceed 550 nm (Figure 11). Thus, bleed-through from the 568 channel to the 488 channel should not occur either. Lack of bleed-through is further supported by the existence in samples of both TH-positive but 5-HT-negative cells and 5-HT-positive but TH-negative cells.

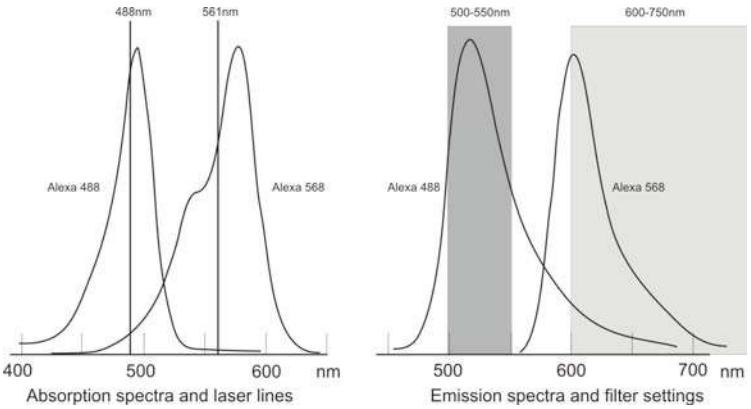

Figure 11.
Illustration showing the absorption and emission spectra of Alexa 488 and 568 fluors, laser lines, and filter settings.

5.1.4. Behavioral analysis

We used an automated video tracking system to evaluate the behavior of larval zebrafish in a hemi-sphere-shaped chamber or in the wells of a 48-well plate. The experimental setting is similar to the open-field settings used in experiments with other animal species. This is a robust method to evaluate locomotion and yields quantitative information about speed, activity/rest periods, and swimming patterns. However, the interpretation of the cause of any observed alterations is arbitrary since conditions from intricate psychiatric disorders (e.g. anxiety) to gross morphological deficits may have an effect. Although, experimental settings for the evaluation of addiction[487, 488], anxiety[463], and sleep[402, 403] have been introduced (for review, see Ninkovic and Bally-Cuif[461]), few methods are available to assess the fine motor functions of zebrafish.

Zebrafish are schooling fish, and analysis of the behavior of individual fish might affect the results. Analysis of schools of fish would be more 'natural', but due to technical limitations (inability to track individual fish among a school of fish), this was impossible.

From a technical point of view, the detection and tracking of the small larval zebrafish was challenging. To obtain reliable results, several exclusion criteria had to be implemented. Due to the small size of larval zebrafish, the system had difficulties in detecting the sample, and thus, tracks where the sample size was less than 90% of the track of the maximum sample size were excluded. In addition, the system occasionally switched the tracking point within one fish (e.g. from the head to the trunk), creating false interpretation of small movement. This was controlled by setting an input filter of 2 mm for minimum distance moved. Although constant and stable illumination was used and the surfaces of the 48-well plates were covered with antireflective material (parafilm), light reflections from the edges were

sometimes misinterpreted as the sample by the computer. Therefore, tracks with apparent reflection artifacts were excluded.

In addition to technical exclusion criteria, a few exclusion criteria based on the experimental animals were implemented. First, fish exhibiting abnormal gross phenotypes were excluded to minimize the influence of developmental disorders, which also occur in WT fish. Second, fish that had not moved at all during the 10-min tracking period were interpreted as having an acute stress reaction and were excluded (in Study IV).

A sampling rate of 5 samples / s is a balance between the amount of data obtained and reliable detection of fish movement. However, one might have concerns whether the sampling rate was sufficient to track larval zebrafish, which might burst-swim rapidly. A sampling rate of 25 samples/s was compared with 5 samples/s in pilot studies, and no significant differences were detected. Therefore, 5 samples/s was considered adequate.

The fact that 96 fish can be tracked simultaneously and the results analyzed automatically render the video tracking system feasible for the use in high-throughput screening.

5.1.5. Translation inhibition with morpholino oligonucleotides

At the introduction of morpholino oligonucleotide (MO) technology, a mismatch morpholino injection to control the specificity of the MO was deemed sufficient. However, recent findings have modified the opinion of sufficient controls for morpholino specificity. Up to 18% of investigated MOs have had off-target effects, such as cell death in the brain[413, 489], rendering usage of MOs to investigate neurodegenerative diseases challenging. Many off-target effects are sequence-specific and mediated via a p53-dependent pathway[413]. The sequence specificity of these effects is apparent in cases where the 5-base mismatch MO does not trigger off-target effects. However, it is not currently known how the sequence specificity is formed, what kind of sequences will trigger off-target effects, or how these effects could be avoided. Thus, controls considered sufficient at the moment include the following: 1) injection of a mismatch MO to control for nonspecific effects, 2) use of a second MO against the same target to confirm phenotype specificity, 3) co-injection of p53-MO to control sequence-specific off-target effects or exclusion of these by analyzing the levels of delta113, a fingerprint for p53-mediated off-target effects, 4) monitoring of the knockdown efficiency by PCR or antibody-mediated methods, and 5) rescue of the phenotype by co-injecting mRNA not recognized by the MO[411]. Some researchers have also suggested additional controls for a splice-inhibiting MO to include 1) sequencing of the splice products, 2) testing of the function of the modified protein, and 3) RNA *in situ* hybridization to determine possible retained pre-mRNA in the nucleus[490]. The dramatic increase of

experiments examine phenotype specificity has rendered the MO technique more demanding. However, new methods for reverse genetics for zebrafish are being developed[411].

5.2. Development of catecholaminergic and serotonergic systems

Catecholaminergic and serotonergic cell populations in the adult zebrafish brain have been described in detail elsewhere[51]. Reports on the development of these cell populations in zebrafish have been published earlier[174, 483-485]. Guo *et al.*[483] and Holzschuh *et al.*[484] elegantly described the timed appearance of catecholaminergic cell groups, but failed identify the diencephalic complex as distinct cell populations. Rink and Wullimann[485] nicely dissected these populations, but did not describe the timed appearance of these populations (for comparison, see Table 4). Our analysis essentially fills these gaps, as we present both the exact 3D location and the timed appearance of the cell populations. We present a novel nomenclature in which cell populations are assigned numbers by their rough rostrocaudal orientation in the adult zebrafish brain. The nomenclature is compatible with the earlier description of adult cell populations[51]. Contrary to earlier descriptions, we found that all catecholaminergic cell populations can be detected already at 72 hpf, although they are more readily separable at 5 dpf. However, populations 5, 6, and 11 were not yet separately distinguishable in subpopulations at thia timepoint.

The development of catecholaminergic and serotonergic systems followed a very similar spatiotemporal sequence of appearance, as in other reported teleosts [163, 164, 491]. First 5-HT-ir and TH-ir cells were detected in the nucleus of the tract of the postoptic commissure (nTPOC). Colocalization of TH and 5-HT was seen in some of these early neurons, but their fate with respect to the adult populations is unclear. Colocalization is not detected later in development, and it has not been reported before. Otherwise, the appearance of the 5-HT neurons corresponded well with that described for TphD1 and TphD2 [172].

The aminergic systems are highly conserved in vertebrates, and thus, zebrafish represents a feasible model for further studies of these systems. However, some differences exists. Although the serotonergic raphe nuclei are highly conserved in vertebrates, the pretectal and paraventricular cell populations are not present in higher vertebrates. Moreover, the homolog of midbrain dopaminergic neurons in (zebra)fish is currently unknown. These differences need to be taken into account when using zebrafish as a model to study these systems.

Table 4.

Area	Population	Guo *et al.*[483]	Holzschuh *et al.*[484]*	Rink and Wullimann[485]	(Study III)
		Time of origin (first detected at hpf)			
nTPOC	**	22	18	<48	20-24
Telencephalon / Olfactory bulb	1-2	72	48	<48	48
Preoptic area	3-4	<96	<96	<120	60
Postoptic / Thalamic / Posterior tuberculum	5, 6, 11	48	?	<48	48
Pretectum	7	96	60	<120	60
APVOa	8	?	?	<48	48
APVOi	9	?	?	<120	72
APVOp	10	?	?	<120	60
PVO, PTN	12	?	?	<48	<48
Hc d, PTN	13	48	<48	<48	<48
LC	14	30	24	<48	24
LX, IRF	15-16	72	36	?	36
Area postrema	17	?	<96	?	36

Time of origin of the developing catecholaminergic cell populations in zebrafish and comparison with other publications. * Note that Holzschuh *et al.*[484] used *in situ* hybridization to demonstrate the catecholaminergic cells, explaining the earlier appearance of cells in nTPOC. ** Catecholaminergic cells in nTPOC putatively develop to form population 12.

Teleosts (zebrafish among them) have two tyrosine hydroxylase genes[492]. In addition to differences in their coding region, these two genes have differences in their promoter regions, predicting different tissue distribution patterns. Despite their sequence differences, their predicted tertiary structures are highly similar, suggesting functionality of both enzymes. In teleost barramundi, TH1 was found in the brain and kidney, while TH2 expression was restricted to the brain[492]. However, no detailed analysis of TH2 in any species has been reported. We found expression of TH1 in similar cell populations as when using the TH-antibody. By contrast, TH-ir and *TH2* mRNA expressions seemed completely distinct. *TH2* mRNA expression was found in the anterior, intermediate, and posterior parts of the paraventricular organ and in the preoptic region. 5-HT cell populations (and TphD1) are detected near to or at these sites. It is unclear whether TH2 and TphD1 are colocalized.

5.3. MAO in the brain of zebrafish

5.3.1. A single form of MAO in zebrafish: MAO A-type substrate specificity, but non-A-non-B-type inhibitor specificity

The largest difference between zebrafish MAO and mammalian MAOs is the lack of isoforms in the former; thus, the presence of a single form in zebrafish. This seems to be typical of fish. A single form of MAO has been reported in carp (*Cyprinus carpio*)[493], rainbow trout (*Salmo gairdneri*)[493], pike (*Esox lucius*)[494], trout[495], and catfish (*Parasilurus asotus*)[496]. Carp[497] and trout[495] have a non-A-non-B form of MAO, whereas catfish MAO is pharmacologically relatively similar to mammalian MAO A[496]. On the other hand, deprenyl (a MAO B inhibitor) has been shown to be protective against the toxicity of MPTP in goldfish[498-500]. Recently, a single form of MAO was reported in zebrafish[486]. Analysis of the structural composition of the substrate binding site suggested a more MAO A-like form for zebrafish MAO[486]. Our *in vitro* and *in vivo* studies support this hypothesis; however, zebrafish MAO also has properties of MAO B, such as inhibition by deprenyl. A recent report suggested that the inhibitor specificity is determined by the amino acid residue Ile-335 (for MAO A) and Tyr-326 (for MAO B)[283]. The corresponding residue in zebrafish MAO is Leu, suggesting a non-A-non-B type of inhibitor specificity. In line with this, we found similar partial inhibition with both MAO A and MAO B inhibitors. On the substrate side, however, zebrafish MAO seems to be more closely related to mammalian MAO A than B. The highest activity was detected with tyramine (substrate of both forms in mammalians), followed by 5-HT and PEA (prefers MAO A substrates in mammals). Moreover, after *in vivo* inhibition of zebrafish MAO, the levels of 5-HT were strongly elevated, while the levels of NA and DA were unaltered, further suggesting a MAO A type of substrate specificity.

At the discovery of mammalian MAO A and B sequences, it was noted that their exon-intron organization was identical[277]. This led to a suggestion of a common ancestral gene that had been duplicated [277]. Zebrafish MAO has an exon-intron organization identical to that of the mammalian genes, but its sequence is equally different from both forms of mammalian MAO, suggesting that the duplication of the ancestral mammalian *MAO* gene leading to A and B isoforms occurred later in evolution than the divergence of the fish and mammalian lineages. Ray-finned fish (Actinopterygii) have apparently separated from the ancestors of tetrapods (Sarcopterygii, lobe-finned fish) about 420 million years ago[501]. Zebrafish is a teleost fish, among 20,000 other species. It is, therefore, possible that fish express the “original” single form and “precursor” of mammalian MAOs. However, it is also possible that the second form of MAO has been lost in (zebra)fish.

Interestingly, all terrestrial vertebrate animals studied thus far exhibit two forms of MAO[502], while among amphibians, only anurans have two forms[503]. This might reflect the need for metabolism of a wider range of exogenous amines, but also a more intricate modulation of endogenous amine metabolism.

The metabolic routes of DA and NA are unclear in zebrafish. Our results imply that they are not metabolized by MAO to a large extent. Another route for DA and NA metabolism in higher vertebrates is COMT (see Section 1.1.2.1.2.). We detected the presence of 3-MT in zebrafish, which is a metabolite produced by COMT in mammalians, thus suggesting the presence of COMT activity in zebrafish. However, COMT has been completely uninvestigated in zebrafish to date, and future studies are needed to elucidate the relationships between COMT and catecholamine metabolism in this species.

5.3.2. Distribution of zebrafish MAO is similar to mammalian MAO A and B

The expression patterns of zebrafish *MAO* released in ZFIN.org as part of an expression analysis of a large number of zebrafish genes demonstrated a uniform distribution in the brain[504, 505]. Contrary to these findings, we detected a very nonuniform distribution of MAO activity and mRNA expression in the adult and larval zebrafish brain. Two distinct staining patterns were observed, cellular and diffuse in the neuropil.

Colocalization of cellular MAO with 5-HT-ir and TH-ir was studied. Noradrenergic cell groups in the locus coeruleus (TH-ir population 14) and vagal lobe (A1-A2, TH-ir population 16) had strong MAO activity. Also rostral cells of TH-ir population 13 contained MAO activity, but other dopaminergic cells were devoid of MAO activity. Histaminergic cells contained MAO activity. Serotonergic cells in the rostral raphe, habenula, and internal reticular formation displayed MAO activity, while the hypothalamic paraventricular 5-HT cell populations did not coexpress MAO. Localization of MAO A in the locus coeruleus and MAO B in the raphe nuclei and histaminergic cells of primates have been reported[284, 286, 506]. In dopaminergic cells of the substantia nigra of humans, only a few neurons contain MAO B[298]. Taken together, MAO in the zebrafish brain is present within noradrenergic, serotonergic, and histaminergic neurons, rather than within dopaminergic neurons, an observation similar to the distribution of MAO in mammalian brains. It is of note that the serotonergic cell populations not present in the mammalian brain (hypothalamic populations) contain no MAO in zebrafish. However, MAO activity is adjacent to these populations and at their projection sites in the ventral hypothalamus.

MAO mRNA expression and MAO activity during zebrafish development corresponded well with the expression pattern of mouse *MAO A*[507]. The *MAO* mRNA was detected approximately one day earlier than the corresponding MAO

activity. This might reflect the time required for the translation and posttranslational processing to produce a functional enzyme. Thus, one must keep in mind that detection of mRNA is not a guarantee of a functional enzyme.

MAO mRNA expression and activity were only partially overlapping, and in several regions, in both adult and larval fish, we found discrepancies between the histochemistry and *in situ* hybridization signals. For example, the cerebellum showed strong mRNA expression, while no MAO activity was detected. On the other hand, the internal reticular formation and vagal lobe displayed strong cellular MAO activity in larval zebrafish, but no mRNA expression. This raises concern about the specificity of the stainings. However, the controls suggested specific reactions; omitting the substrate in the histochemistry abolished the staining completely, and using a sense probe in *in situ* hybridization produced no reaction. Several potential reasons can be found for these phenomena, including turnover and transport of the enzyme and posttranscriptional control. Discrepancies between the MAO protein and mRNA expression have also been reported in non-CNS human tissues[508].

5.3.3. Hyperserotonergism after MAO inhibition

We found ten-fold increases in the levels of 5-HT following deprenyl treatment, but no alterations in the levels of DA, NA, or A. This is contrary to findings in mice, in which deprenyl treatment increases levels of DA, but not 5-HT[509, 510]. However, increased 5-HT was in line with studies on MAO A knockout mice[290], suggesting MAO A-type *in vivo* substrate preference. Ectopic cells were also found, and 5-HT-ir was diminished in the somata of 5-HT cells. Deprenyl has been reported to reduce the 5-HT synthesis in the serotonergic cell somata, but not in the terminals[511]. Similar mechanisms might be causing the diminished 5-HT-ir that we found. The mechanism by which ectopic cells gain 5-HT-ir can be either synthesis or uptake of 5-HT. TpH has not been reported in these locations[172], but we cannot exclude the possibility of TpH expression after deprenyl treatment. On the other hand, they have been reported not to express SERT either[512]. The increased 5-HT-ir was abolished after fluvoxamine (an SSRI) treatment, favoring the uptake hypothesis.

The acute and the long-term effects of increased levels of 5-HT on the locomotor activity of zebrafish seem to be the opposite. Injection of 5-HT into the pericardium increases locomotion[513], while 24-h bath exposure to the 5-HT uptake inhibitor fluvoxamine decreases locomotion[514]. We found decreased locomotion and swimming closer to the surface after 0-5 dpf exposure to deprenyl. The vertical placement difference might be related the swim bladder being innervated by 5-HT fibers[515].

In mammals, exposure to drugs that increase the levels of 5-HT may produce a potentially lethal state called the serotonin syndrome (SS). SS is

characterized by restlessness, agitation, hyperthermia, and sympathetic activation[516]. Zebrafish treated with deprenyl exhibit hugely increased levels of 5-HT, but lack the characteristic features of SS, except for an increase in heart rate, which may be a sign of sympathetic activation. Contrary to restlessness or agitation, spontaneous locomotion decreased. Zebrafish are poikilothermic, so hyperthermia is not even theoretically possible. Even higher increases in the levels of 5-HT might be needed to produce SS features in zebrafish. Alternatively, zebrafish may respond differently to increased levels of 5-HT than mammals, and thus, similar diagnosic criteria for SS may not be applicable.

All in all, the deprenyl-induced model of zebrafish hyperserotonergism may be useful in future research on serotonin-modifying genes or drugs.

5.4. MPTP

5.4.1. Neurochemical changes after MPTP exposure in larval zebrafish

MPTP exposure leads to dopaminergic cell death in the substantia nigra of mammals. The zebrafish dopaminergic population corresponding to the mammalian substantia nigra is still a matter of debate. The strongest candidate at the moment is the posterior tuberculum (adult population 11, larval indiscernible population 5,6,11), based on immunohistochemical and tracer studies[50, 51, 517]. The posterior tuberculum sends connections to the subpallium, which is the putative zebrafish striatum[50, 517]. However, the corticostriatal pathway regulated by dopamine is poorly characterized in fish.

While our experiments were conducted, MPTP was reported to cause cell loss in the pretectal population at 5 dpf[469], putative posterior tuberal nucleus at 2 dpf[468] and diencephalic dopaminergic neurons at 5dpf[467]. Such variation in the affected populations reflects the difficulty in distinguishing separate cell populations and quantifying their cell loss. In fact, only in the study by Bretaud *et al.*[467] were the cells actually counted. The method of quantitation raises concerns, though. The TH-positive neurons were divided into three populations; telencephalic, diencephalic, and locus coeruleus. The "diencephalic cell population" was reported to diminish from approximately 40 cells to 30 cells following 250 µM MPTP application. Presumably (judging from the published images), this population is a combination of the populations 5,6,11, 10, 12, and 13. From this viewpoint, 40 cells seems quite low as we detected ca. 70 cells in population 13 alone. This discrepancy between cell counts might be explained by different staining techniques; we used immunofluorescence, while Bretaud *et al.*[467] used *in situ* hybridization. *In situ* hybridization may be less sensitive, thus with fewer cells detected. In addition, *in situ* hybridization staining might more blurred, preventing reliable cell counting.

In other previous reports, the cell loss was judged by calculating 2D area occupied by the population[469] or merely by subjectively evaluating the images[468]. Although the 2D area of the cell population might correspond to the number of cells, this is subject to several types of bias. First, the populations are 3D structures, and thus, they should be treated as volumes instead of areas. Second, alterations in cell density do not necessarily affect area (or volume), but greatly influence the number of cells. Lam *et al.*[468] describe MPTP-induced cell loss as early as 2-3 dpf, but analysis of such young fish presents issues that need to be considered. First, many of the dopaminergic neuron populations (i.e. target populations) have not yet developed, among them the putative substantia nigra, population 11 (larval combined population 5,6,11). Although the early diencephalic TH-positive cells in the nTPOC putatively develop to form the posterior tuberal nucleus (population 12), this is not certain. Second, MAO activity is needed to convert MPTP into its toxic form, MPP+. We showed that MAO activity is very low before 2 dpf, suggesting that MPTP conversion to MPP+ is not yet fully developed at that time.

Two further studies were published while our results were in the editoral process[518, 519]. Wen *et al.*[518] used VMAT2-GFP transgenic fish to demonstrate MPTP-induced cell loss in several VMAT2-positive neuron clusters, presumably corresponding to TH-ir populations 5,6,11, 7, 12, and 13 as well as the 5-HT-ir populations 2, 3, and 4. Instead of the 5-HT-ir cell populations, the VMAT2-positive cell populations might correspond to the TH2-positive cell populations. Thirumalai et al.[519] reported loss of dopaminergic fibers, but not cells, after exposure to a low concentration of MPTP (10 μM).

In accordance with the results of others, we detected TH-ir cell loss in the pretectum (7) and hypothalamus (13) following MPTP exposure. However, contrary to others, but in line with Wen et al., we found prominent TH-ir cell loss in the population 5,6,11. Additionally, we detected TH-ir cell loss in the preoptic population (3, 4), which has not been reported earlier.

The results concerning the 5-HT cell populations are conflicting. While Lam *et al.*[468] reported no effects on 5-HT-ir populations, Wen *et al.*[518] described cell loss in all hypothalamic 5-HT neuron populations (2, 3, and 4). We found dramatic loss of cells in the intermediate part of the paraventricular organ (2) following MPTP, but no significant loss in other populations.

To confirm the observations seen at the cellular level, we analyzed the levels of catecholamines and serotonin after MPTP exposure using HPLC. Levels of DA, NA, and 5-HT were decreased significantly following MPTP exposure – in line with the cellular data.

A summary of our findings relative to previously reported findings can be found in Table 5.

5.4.2. Mechanism of action of MPTP in zebrafish – roles of MAO and DAT

Even if MPTP exerts its effects via its toxic metabolite MPP+ in mammals, this might not be true for zebrafish. We therefore analyzed the potential of deprenyl (an established mammalian MAO B inhibitor) to rescue the neurochemical changes induced by MPTP. Deprenyl rescued the affected TH-ir cell populations and also restored the concentrations of DA, NA, and 5-HT, suggesting that MPTP exerts its toxic effects via its metabolite MPP+. Rescue by deprenyl has been reported for the pretectal population[469] and hypothalamic cells[468], but not for other populations or amine levels.

We then tested the effects of MPP+. We hypothesized that its effects would be stronger and more widespread than those of MPTP since MPP+ can spread into and affect all parts of the brain, while MPTP may only be metabolized to MPP+ in locations that contain MAO activity. To our surprise, the effects of MPP+ were more specific and less widespread than those of MPTP. MPP+ caused TH-ir cell loss only in the population 5,6,11 and decreased the levels of NA and DA. 5-HT-ir cells populations or levels of 5-HT were unchanged after MPP+ exposure. This suggests that MPTP could have effects unrelated to MPP+ and MAO activity.

On the other hand, MPP+ gains access inside the cells via DAT, and therefore, expression of DAT could regulate the susceptibility. Pretectal (7) and hypothalamic (13) TH-ir cell populations contain DAT, while the preoptic (3-4) populations do not seem to contain it[484]. DAT expression in population 5,6,11 is difficult to judge due to different classification of the cell populations in Holzschuh *et al*.[484]. However, an area named PT resembles this population. DAT-positive, but TH-negative cells are reported at this site, along with TH-positive but DAT-negative cells. Interestingly, knockdown or pharmacological inhibition of zebrafish DAT has been reported to rescue MPTP-induced cell loss in the pretectum and the "whole diencephalon"[469].

It is noteworthy that zebrafish DAT is identified based on its predicted amino acid sequence similarity to human DAT. The predicted zebrafish DAT protein has 76% identity and 9% similarity to human DAT, and 63% identity and 14% similarity to human NAT[484]. However, the functional properties of zebrafish DAT, most importantly substrate specificity, have not been investigated and are unknown. On the other hand, no homolog for NAT has thus far been reported for zebrafish. Therefore, zebrafish could be speculated not to have distinct transporters for noradrenaline and dopamine, but instead a hybrid transporter for both. This is supported by the finding that some of the cells in the noradrenergic locus coeruleus express putative zebrafish DAT, although many noradrenergic populations do not express it[484]. Further studies are needed to uncover the functions of putative zebrafish DAT.

Taken together, the population 5,6,11 was targeted by both MPTP and MPP+, and the MPTP-induced cell loss in this population could be prevented by MAO inhibition. Population 5,6,11 thus resembles in this respect (susceptibility to MPTP/MPP+) the mammalian substantia nigra. These results further suggest that the corresponding cell population to the mammalian substantia nigra could be 5,6,11.

5.4.3. Behavioral changes after MPTP and MPP+ exposure in larval zebrafish

The zebrafish locomotor network develops through several steps beginning with spontaneous tail coiling at approximately 17 hpf and reaching a mature swimming pattern at 5 dpf[401]. We wanted to analyze behavior after the aminergic neurotransmitter systems had developed and the locomotor network had reached maturity. We therefore chose to analyze the locomotion of 5-7 dpf larval zebrafish after MPTP or MPP+ exposure.

Exposure to 1000 μM MPTP on days 1-4 post-fertilization produced a delayed and transient decrease in locomotor activity, which was maximal at 6 dpf. Although the neurochemical changes after MPP+ exposure were milder, and the brain serotonergic system was not affected significantly, the behavioral changes after MPP+ exposure were similar to those after MPTP exposure. Thus, the behavioral changes might be caused by the neurochemical changes common to MPTP and MPP+ exposures. Furthermore, deprenyl restored the locomotion of MPTP-exposed fish at 5 dpf, and also prevented the decrease in amine levels. Since deprenyl inhibits MAO, which forms MPP+ from MPTP, our observation further suggests that the alterations in behavior are mediated via MPP+. However, deprenyl may have effects of its own on behavior and neurotransmitter levels that might interfere with the interpretation of the rescue effects. Therefore, the effects of deprenyl were assessed. Deprenyl elevated the brain levels of 5-HT, but did not affect the levels of DA or NA. Furthermore, the elevated 5-HT levels caused a decrease in spontaneous locomotion, indicating that the deprenyl *per se* does not rescue the locomotion deficit cause by MPTP – a finding further supporting the role of MPP+ in the locomotor alteration.

Others have found slowness in the tail touch response at 3 dpf[468], immobility at 4 dpf[469], and decreased locomotion at 7 dpf[467]. The behavioral alterations before 5 dpf reported by others are difficult to compare with our results since the locomotor network is still under development and locomotion patterns are different. However, we have also noticed immobility directly after high-dose MPTP exposure (1000 μM), which might be caused by acute toxicity. We did not observe any behavioral alteration after 100 μM MPTP, while others have reported decreased locomotion at 7 dpf after 42.9 μM MPTP exposure on days 1-5 post-fertilization[467].

These discrepancies might be due to differences in the lots of MPTP or fish lines used. However, it is noteworthy that in our study 100 µM MPTP did not affect catecholamine or serotonin levels. Interestingly, very low doses of MPTP (10 µM) have been reported to increase movement in 3-day-old larval zebrafish, which was associated with a loss of descending TH-ir fibers[519]. Rescue of locomotion by deprenyl has not been reported earlier in zebrafish.

In line with our results, similar transient declines in locomotor activity have been observed in adult zebrafish after MPTP or 6-OHDA exposure, along with decreased catecholamine levels[466].

Table 5.

	Bretaud *et al.*[467]	Lam *et al.*[468]	McKinley *et al.*[469]	Wen *et al.*[518]	Thirumalai and Cline[519]	Study III
DA populations affected	7, 13	13	(3-4?), 7, 8, 13	7, 5,6,11, 12, 13	None, loss of fibers	3-4, 5,6,11, 7, 13
Behavioral change	Decrease in locomotion at 7dpf	Slowness in tail touch response at 3dpf	Immobile at 4dpf	N/A	Increased movement at 3dpf	Decrease in locomotion at 5-7dpf
Concentration and administration protocol	1-5dpf 9mg/l (42.9 µM) & 45mg/l (214.6 µM)	1-2-3dpf 800 µM	1-4dpf 5-20 µg/ml (23-95 µM)	1-5dpf 10-40µg/ml (46-190µM)	1-3dpf 10 µM	1-4dpf 100 µM and 1000 µM
Effect of deprenyl	N/A	Rescue of population 13	Rescue of population 7	N/A	N/A	Rescue of affected cell populations, DA, NA and 5-HT levels and behavior
Markers used for detecting dopaminergic cells	TH *in situ* hybridization	TH *in situ* hybridization	TH *in situ* hybridization	GFP under VMAT2 promoter	TH immunostaining	TH immunostaining
5-HT population affected	N/A	None	N/A	2, 3, 4	N/A	3

Comparison of published studies on MPTP-induced PD models in larval zebrafish. N/A – not available.

5.5. PINK1

We produced a genetic model of PD by knocking down *PINK1* in larval zebrafish. The zebrafish PINK1 gene and protein structure and their distributions were highly similar to other studied vertebrates, rendering zebrafish a feasible model for investigation the functions of PINK1. *PINK1* knockdown produced a significant decline in the number of TH-ir cells in the population 5,6,11 – the same population that is susceptible to the parkinsonian toxins MPTP and MPP+. Subeffective doses of MPTP produced a decrease of TH-ir cells in the pretectum (population 7) in *PINK1* knockdown fish – the same population that is the other main target of effective doses of MPTP. Furthermore, spontaneous locomotion was decreased in *PINK1* knockdown fish exposed to subeffective doses of MPTP. This suggests that pathological pathways of PINK1 and MPTP converge at some point and PINK1 deficiency increases sensitivity to oxidative stress.

5.5.1. Distribution of PINK1 in zebrafish

PINK1 mRNA expression and immunoreactivity have been studied in detail in rodents and humans. *PINK1* mRNA expression is widespread and the highest levels are detected in the hippocampus, substantia nigra, and cerebellar Purkinje cells[344, 520]. While glial cells do not express *PINK1* mRNA, they are PINK1-immunoreactive[335, 521]. Potential reasons have been sought and protein transportation and antibody nonspecificity have been suggested. We detected PINK1-ir and mRNA in the gray matter, but the white matter was devoid of staining. Double immunostainings of PINK1 and the fibrillar astrocyte marker ZRF-1 suggested that PINK1-ir is not located in glial cells in zebrafish.

Psychiatric disorders, such as psychosis and depression, have been described in human homozygotes carrying nonsense *PINK1* mutations along with early-onset parkinsonism[522]. The mechanisms by which PINK1 deficiency causes mental symptoms are unclear, as no post-mortem studies on *PINK1* mutation carriers have been reported. No studies concerning the expression of *PINK1* in aminergic cells of the brain have been published to date. We detected PINK1-ir in all catecholaminergic and serotonergic neurons in the zebrafish brain. This suggests that PINK1 deficiency may cause disturbances in both catecholaminergic and serotonergic systems in a cell autonomous manner.

PINK1 mRNA was expressed along the lateral line in larval zebrafish. The function of PINK1 in these cells is obscure, but possible roles include functions in mechanosensation, development, and mainteinance of sensory cells. Interestingly, sensory abnormalities have been reported in both homozygous and heterozygous *PINK1* mutation carriers [523].

5.5.2 PINK1 knockdown and MPTP toxicity

The number of TH-ir cells in the catecholaminergic cell population 5,6,11 was decreased in *PINK1* knockdown fish. As no gross morphological alterations were detected and the other neurotransmitter systems seemed intact, the effect is likely specific. However, the phenotypes of PINK1 deficiency are quite variable across the species. Human homozygotes carrying mutated *PINK1* develop PD, but show no pre-morbid gross phenotype or developmental defects. PINK1-deficient mice do not have a gross morphological phenotype, nor do they exhibit loss of TH-ir cells[361, 362]. On the other hand, PINK1 deficiency in *Drosophila* leads to dopaminergic cell loss, muscle apoptosis, male sterility, and mitochondrial dysfunction [348-350]. Recently, zebrafish *PINK1* knockdown has been reported to cause severe developmental defects along with massive neurodegeneration[524]. We found a similar dramatic phenotype with one of our MO; (5'-utr-PINK1-MO), with severely altered development and a small brain. In further examination, the levels of p53 and delta-113 were found to be highly elevated, suggesting massive off-target effects (see Section 5.1.5.). The expression levels of p53 and delta-113 were not studied by Anichtchik et al.[524].

The *PINK1* knockdown phenotype that we observed is more similar to that detected in the mouse and human than that in *Drosophila*. The severe phenotype observed in *Drosophila* suggests that the functions of PINK1 differ between invertebrates and vertebrates. Thus, findings obtained with *Drosophila* models should be confirmed using vertebrates. There might be other genes to compensate the loss of PINK1 in vertebrates.

An external stressor may be needed for the neurodegeneration of dopaminergic neurons in PINK1-deficient mammals[525]. We observed increased susceptibility to MPTP in *PINK1* knockdown fish in terms of locomotion and dopaminergic cell loss. Loss of TH-ir cells in TH-ir population 5,6,11 was also observed in *PINK1* knockdown fish. Population 5,6,11 has been suggested to correspond to the mammalian substantia nigra, based on immunohistochemical and tracer studies[50, 51]. We found that it is also a target of MPTP and MPP+. The results in *PINK1* knockdown fish further support the hypothesis of population 5,6,11 corresponding to the mammalian substantia nigra.

Since the mechanisms of PINK1 and MPTP seem to be similar to those of other vertebrates, zebrafish offer a promising model for investigating the mechanisms of dopaminergic degeneration and pathogenesis of PD. In addition to genetic, functional, and anatomical similarities to higher vertebrates, zebrafish allow the possibility for carrying out efficient forward and reverse genetic studies.

6. Conclusions and future directions

The main conclusions derived from the present series of investigations are as follows:

1. Seventeen catecholaminergic and 9 serotonergic developing neuron populations were mapped in 1-7 dpf zebrafish and compared with the corresponding adult cell populations. A 3D atlas was created to facilitate future studies.
2. Zebrafish possesses only one gene encoding MAO.
3. Zebrafish MAO shows MAO A type substrate specificity and non-A-non-B-type inhibitor specificity.
4. Zebrafish MAO activity and mRNA expression are both diffuse and distinctly cellular in the zebrafish brain. MAO activity is localized in noradrenergic neurons, but also in some dopaminergic and serotonergic nuclei. Most zebrafish brain MAO activity is diffusely distributed in the neuropil and possibly of glial nature.
5. Inhibition of MAO during the development of zebrafish results in increased levels of serotonin. Increased levels of serotonin decrease the locomotion of fish and lead to detection of ectopic 5-HT-containing cells in the hypothalamus that putatively take up, but do not synthesize, 5-HT.
6. MPTP administration leads to transient loss of TH-ir cells in specific aminergic cell populations and decreased locomotion
7. Effects of MPTP could be restored with the MAO inhibitor deprenyl, indicating the involvement of MAO in MPTP toxicity. MPP+ caused similar behavioral alteration as MPTP, but affected only one catecholaminergic cell population.
8. One *PINK1* gene was found in the zebrafish genome. Structural analysis revealed a high degree of similarity between the functional domains of zebrafish PINK1 and human PINK1 proteins.
9. *PINK1* is expressed widely in the brain of zebrafish. PINK1 is localized in all cells presenting TH- or 5-HT-ir. PINK1 distribution in the brain is similar to that described for the human and rodent brain.
10. *PINK1* knockdown did not lead to any gross morphological defects. However, *TH1* and *TH2* transcript levels were decreased and loss of TH-ir cells was noted in one population.
11. *PINK1* knockdown rendered zebrafish larvae susceptible to low doses of MPTP, which was demonstrated by a locomotion defect and TH-ir cell loss in one population. This suggests that PINK1 is important in regulation of oxidative cell damage. The finding may have relevance for uncovering the genetic or environmental causes of PD.

Future directions:

1. Another important enzyme in the metabolism of modulatory neurotransmitter amines, COMT, is currently uninvestigated in zebrafish. Cloning, expression analysis, and pharmacological characterization of zebrafish COMT will provide new insights into the metabolism of biogenic amines in zebrafish.
2. All studies on zebrafish tyrosine hydroxylase have so far concentrated on the TH1 isoform. TH2 is uncharacterized and its role is unknown. Double-labeling studies using other markers for aminergic neurotransmitters could reveal the potential functional roles of TH2. Several mutants affecting the development of TH1 cell populations have been reported. Whether these mutants will affect the development of TH2 cell populations also remains to be seen. The susceptibility of TH2 populations to MPTP/MPP+ should be investigated.
3. Zebrafish α2-adrenoreceptors have been cloned and characterized at pharmacological and functional levels[122]. Similar characterization of other adrenoceptor subtypes and already identified receptors for DA, 5-HT, and HA in zebrafish is encouraged prior to conducting behavioral studies. Results from such experiments may otherwise be difficult to interpret, as it is unknown how drugs characterized only in rodents or humans affect zebrafish receptors.
4. Zebrafish DAT has been cloned and its expression analyzed[484]. However, many questions still remain. What are its substrates and their affinities? Is zebrafish DAT exclusively expressed in dopaminergic neurons? Double labelings with markers of DA or NA cells (TH, DBH) should be carried out.
5. More sophisticated methods for analyzing the motor functions of zebrafish should be developed and implemented.
6. Hyperserotonergism and MPTP/MPP+ models of PD in zebrafish could be used in genetic and/or drug screens to obtain novel knowledge.

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Zebrafish as a model of Parkinson's disease

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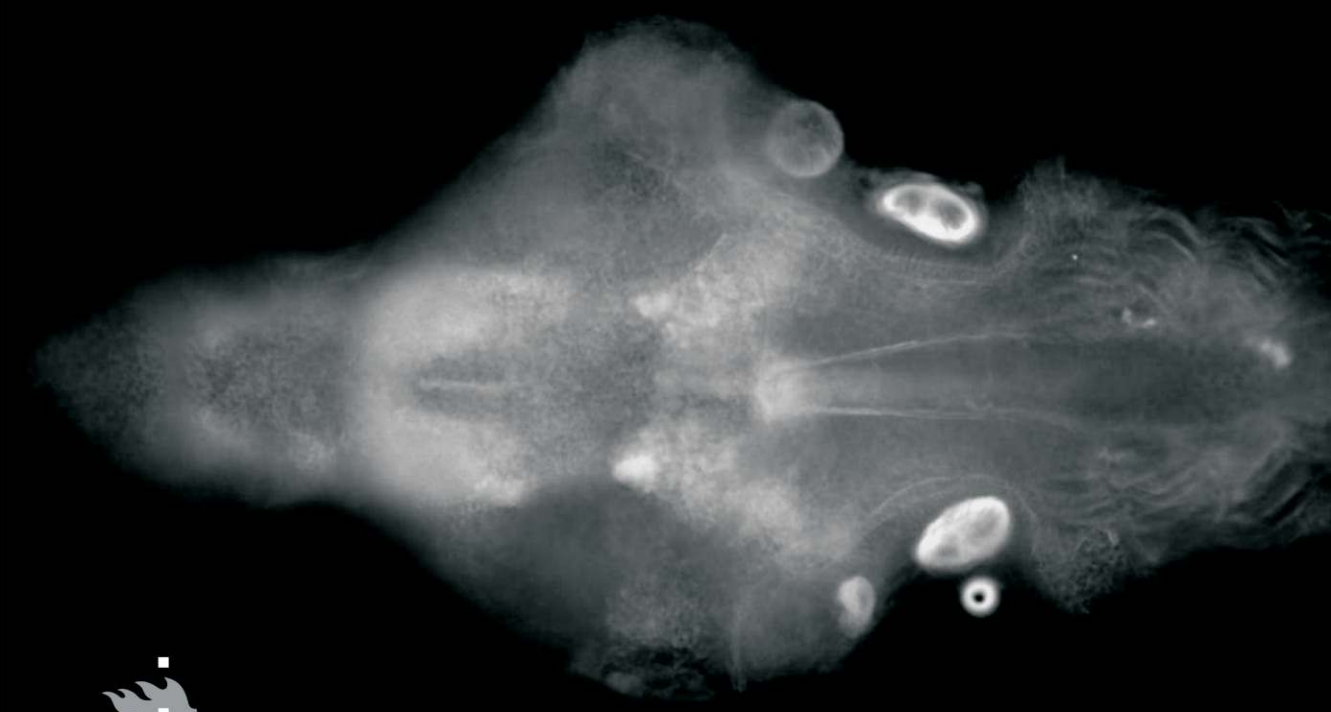

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