Pathophysiological Roles of Brain Aquaporins in Parkinson's Disease and Aging

by

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Katja Stahl
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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized by selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), leading to severe motor dysfunction (4). To date, the etiology of PD remains unknown, thus impairing the development of therapeutic interventions that may halt the progression of the disease. Aging is thought to be the most prominent risk factor, given the increased prevalence rate with age (5, 6). Further, epidemiological as well as toxin-based studies have pointed to a role of environmental toxins in the pathogenesis (7-9), although the mechanism by which these toxins selectively target dopaminergic neurons in the SNpc remains to be elucidated.

Brain aquaporins (AQPs) are water and solute channels in the brain. Although not previously related to PD, several lines of evidence argue for a link between these. AQP9 is an aquaglyceroporin with particularly broad substance permeability, transporting water, glycerol, monocarboxylates and arsenite, among others (10-14). This water channel has recently been found to be selectively expressed in dopaminergic plasma membranes as well as mitochondrial inner membranes in the SN (15-17), indicating that it may serve as a toxin influx route into these cells, thereby accounting for an increased susceptibility to cell death. AQP4, on the other hand, is an orthodox aquaporin exclusively permeable to water. It is localized in astrocytic cell membranes and plays a crucial role in water- and osmoregulation, and hence in maintaining a homeostatic microenvironment required for neuronal function (18-21). To date, however, the microenvironment surrounding the dopaminergic neurons in the SNpc has been largely neglected, although excess extracellular water in the SNpc has been demonstrated in patients with PD (22), thereby suggesting a role of AQP4 in PD. This thesis investigates the selective vulnerability of dopaminergic neurons in the SNpc by focusing on these two novel target molecules in the pathogenesis of PD as well as aging.

Using mice deficient in *Aqp9*, we investigated loss of dopaminergic cells in the SNpc in normal aging and in toxin-based models of PD mediated by MPP+, the toxic metabolite of MPTP known to selectively target dopaminergic neurons in the SNpc. Initially, MPP+ permeability through AQP9 was confirmed in *Xenopus* oocytes expressing AQP9. We further established a reliable *in vitro* organotypic culture model of PD for evaluation of unilateral dopaminergic cell death and survival, where nigral dopaminergic cell death was induced by means of 6-hydroxydopamine (6-OHDA) in ventral midbrain slices, and cell survival was confirmed following concomitant exposure to the growth factors brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF). We exploited this model to evaluate dopaminergic cell loss in ventral midbrain slices from *Aqp9*$^{-/-}$ and WT littermates unilaterally exposed to MPP+. A corresponding *in vivo* model of unilateral intrastriatal injections of MPP+ in *Aqp9*$^{-/-}$ and WT littermates was then employed for stereological estimates of dopaminergic cell loss in the SNpc and evaluation of its subsequent effects. These models demonstrated a significant reduction of dopaminergic cell loss in the SNpc of *Aqp9* deficient mice compared to WT littermates following exposure to MPP+. *In vivo*, the reduced dopaminergic cell loss was accompanied by improved motor outcomes and higher levels of dopamine and its metabolites in the striatum, which was not attributable to genetic up- or down-regulation of dopamine transporters, dopamine receptors, apoptotic factors or antioxidants. These findings demonstrate that AQP9 contributes to the selective vulnerability of the dopaminergic neurons in the SNpc and thus to the pathogenesis of PD. Moreover, aged *Aqp9*$^{-/-}$ mice were significantly more resistant to

dopaminergic cell death in the SNpc than WT littermates at 12 months but not 6 months, as analyzed by stereological quantifications. This indicates that AQP9 is involved age-dependent dopaminergic cell loss in the SNpc, thus directly linking AQP9 to the main risk factor for developing PD.

To evaluate the role of AQP4 in PD, we first characterized the expression of this protein in the SN under physiological conditions in mice, using confocal imaging and quantitative immunogold labeling. We found significantly higher expression of AQP4 in perivascular membranes as well as in the neuropil compared to the neocortex, indicating that AQP4 may play an important role in the SN under physiological conditions. In a sub-acute MPTP model of PD, where the toxin was injected subcutaneously, we found significant upregulation of AQP4 in perivascular and neural endfeet as well as in the neuropil compared to saline controls, as quantified by immunogold labeling. These results were replicated in an acute MPP+ model of PD, and further confirmed by qRT-PCR. These findings are in line with studies showing water accumulation in the SN in patients with PD, further suggesting that deletion of *Aqp4* may serve a protective role in PD. We evaluated the role of AQP4 in PD using *Aqp4* deficient mice and WT littermate controls in an acute MPP+ model of PD, and found significantly reduced dopaminergic cell loss in the SNpc in $Aqp4^{-/-}$ mice compared to WTs, as quantified by stereology. These results points to a detrimental role of AQP4 in the pathogenesis of PD.

In conclusion, this thesis has identified AQP9 and AQP4 as two novel molecules involved in the pathogenesis of PD by selectively increasing the vulnerability of dopaminergic neurons in the SNpc, either by a affecting the inherent characteristic of these neurons or by altering the microenvironment surrounding these cells. These novel molecules may eventually open new avenues for the treatment of PD.

LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AADC	Amino Acid Decarboxylase
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
AQP	Aquaporin
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
cAMP	Cyclic Adenosine Monophosphate
CNS	Central Nervous System
COMT	Catecol-O-methyltransferase
CSF	Cerebrospinal Fluid
DA	Dopamine
DAB	3,3'-Diaminobenzidine
DAPC	Dystrophin-Associated Protein Complex
DAT	Dopamine Transporter
DIV	Days In Vitro
DJ-1	Protein Deglycase
DOPAC	3,4-Dihydroxyphenylacetic Acid
ETC	Electron Transport Chain
GDNF	Glial Cell-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GP	Globus Pallidus
GPe	Globus Pallidus, external segment
GPi	Globus Pallidus, internal segment
GSH	Gluthatione
HD	Huntington's Disease
HPLC	High-Performance Liquid Chromatography
HVA	Homovanillic Acid
ICAM-1	Intercellular Adhesion Molecule 1
IL-1β	Interleukin-1 β
IFN-γ	Interferon-γ
IRE	Insulin Response Element
Kir4.1	Inwardly Rectifying Potassium Channel 4.1
KO	Knockout
LB	Lewy Body
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
L-Dopa	3,4-dihydroxyphenylalanine
LRRK2	Leucine-Rich Repeat Kinase 2
MAO-B	Monoamine Oxidase B
MHC	Major Histocompatibility Complex
MnSOD	Superoxide Dismutase
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple Sclerosis
NMO	Neuromyelitis Optica
NO	Nitric Oxide
PD	Parkinson's Disease
PINK1	PTEN-Induced Putative Kinase 1
qRT PCR	quantitative Real Time Polymerase Chain Reaction
ROI	Region of Interest
ROS	Reactive Oxygen Species
RRF	Retrorubral Field
RVD	Regulatory Volume Decrease
siRNA	small interference RNA
SN	Substantia Nigra
SNpc	Substantia Nigra pars compacta
SNpl	Substantia Nigr pars lateralis
SNpr	Substantia Nigra pars reticulata
SRS	Systematically Random Sample
STN	Subthalamic Nucleus
TH	Tyrosine Hydroxylase
TRPV4	Transient receptor potential cation channel subfamily V member 4
TNF-α	Tumor Necrosis Factor α
VMAT2	Vesicylar Monoamine Transporter 2
VTA	Ventral Tegmental Area
WT	Wild Type

LIST OF PAPERS INCLUDED IN THE THESIS

Cytoprotective effects of growth factors: BDNF more potent than GDNF in an organotypic culture model of Parkinson's disease

Stahl K, Mylonakou MN, Skare Ø, Amiry-Moghaddam M, Torp R. *Brain Research*. 2011;10(1378):105-118

Targeted deletion of AQP9 protects against MPP^+ induced loss of nigral dopaminergic neurons in mice

Stahl K, Rahmani S, Prydz A, MacAulay N, Mylonakou MN, Torp R, Skare Ø, Berg T, Ottersen OP, Leergaard TB, Amiry-Moghaddam M. *Submitted.*

Reduced age-dependent dopaminergic cell loss in AQP9-deficient mice

Stahl K, Leergaard TB, Amiry-Moghaddam M. *Manuscript*.

Subcellular expression of aquaporin-4 in substantia nigra of normal and MPTP-treated mice

Prydz A, Stahl K, Puchades M, Davarpaneh N, Nadeem M, Ottersen OP, Gundersen V, Amiry-Moghaddam M. *Submitted*.

Reduced dopaminergic cell loss in AQP4-deficient mice subjected to intrastriatal injections of MPP+

Stahl K, Prydz A, Rahmani S, Skare Ø, Amiry-Moghaddam M. *Manuscript*.

LIST OF PAPERS NOT INCLUDED IN THE THESIS

Generation of high purity neuronal progenitor cells and oligodendrocyte progenitor cells from blastocyst-derived human embryonic stem cells and human induced pluripotent stem cells

Wedemeyer M*, Stahl K*, Ferguson D, Frame J, Cruz C, Harness J, Nistor G, Okano H, Yamanaka S, Keirstead H. *Manuscript.* *Both authors have contributed equally

Organotypic cultures as a model of Parkinson s disease. A twist to an old model.

Stahl K, Skare Ø, Torp R. *ScientificWorldJournal.* 2009;11(9):811-21.

THE CANDIDATE'S EXPERIMENTAL CONTRIBUTIONS

Paper I: Cytoprotective effects of growth factors: BDNF more potent than GDNF in an organotypic culture model of Parkinson's disease

- Organotypic cultures, toxic injections of slices and growth factor treatment
- Immunofluorescence and confocal imaging
- Cell quantifications
- Real-time PCR
- Writing, editing and submitting the manuscript

Paper II: Targeted deletion of AQP9 protects against MPP^+ induced loss of nigral dopaminergic neurons in mice

- Breeding and maintenance of *Aqp9*$^{-/-}$ and WT colonies
- Organotypic cultures and toxic injections of slices
- Cell quantification in organotypic slices
- Stereotaxic surgery and injections of MPP+/saline
- Post-operative care and behavioral assessments of animals
- Perfusion fixation and tissue preparation for immunocytochemistry
- Immunocytochemistry for stereology
- Stereological quantifications
- Dissections for HPLC and qRT-PCR analysis
- Statistical analysis of organotypic culture quantifications, stereological quantifications, and HPLC data
- Writing, editing and submitting the manuscript

Paper III: Reduced age-dependent dopaminergic cell loss in AQP9-deficient mice

- Breeding and maintenance of *Aqp9*$^{-/-}$ and WT colonies
- Perfusion fixation and tissue preparation for immunocytochemistry
- Immunocytochemistry
- Stereological quantifications
- Statistical analysis
- Writing and editing the manuscript

Paper IV: Subcellular expression of aquaporin-4 in substantia nigra of normal and MPTP-treated mice

- Perfusion fixation for light- and electron microscopic analysis of the untreated substantia nigra
- Immunofluorescence and confocal microscopy of the untreated substantia nigra
- Prepared figure 1, wrote parts of the manuscript and edited the manuscript

Paper V: Reduced dopaminergic cell loss in AQP4-deficient mice subjected to intrastriatal injections of MPP+

- Breeding and maintenance of *Aqp4*$^{-/-}$ and WT colonies
- Stereotaxic surgery and injections of MPP+/saline
- Post-operative care and behavioral assessments of animals
- Perfusion fixation and tissue preparation for immunocytochemistry
- Immunocytochemistry
- Stereological quantifications
- Confocal microscopy
- Dissection for qRT-PCR analysis
- Statistical analysis of stereological quantifications
- Writing and editing of the manuscript

INTRODUCTION

PARKINSON'S DISEASE

Epidemiology

Parkinson's disease (PD) was discovered in 1817 by James Parkinson (23), who named it "The Shaking Palsy". To date, PD is one of the most common neurodegenerative disorders, affecting 1-2% of the population over 50 years, with a mean onset age of 65 years (4). The prevalence increases steadily with age, with more than 2.6% affected patients from 65-69 years, and 4.8% over 85 years (5, 6). Along with a demographical shift with a progressively larger proportion of elderly in the population, the incidence rate of PD is expected to escalate, resulting in increased demands on healthcare resources.

The prevalence rate of PD is unevenly distributed among genders and geographical locations. PD is more common in men than in women (24), and has been shown to be more prevalent in Western countries (25). PD has also been shown to have a higher prevalence in rural areas and areas associated with farming (26).

Clinical Symptoms and Diagnosis

Clinically, the cardinal features of PD include tremor at rest, bradykinesia and muscle rigidity, resulting in considerable motor disability for PD patients (27). Tremor in PD is defined by a low frequency resting tremor, usually starting in unilateral extremities and spreading bilaterally as the disease progresses (28). Rigidity is characterized by stiffness of limbs and resistance to movement, resulting in cogwheel-like movements. Bradykinesia is seen as slow or small movements and difficulties in initiating movements (akinesia), in addition to small handwriting (micrographia) and decreased facial expression (masked face). As the disease progresses, patients may develop postural instability, often leading to balance problems and falls. This symptom is less responsive to treatment (27).

In addition to the motor dysfunction, patients may also suffer from non-motor symptoms, including autonomic dysfunction, loss of olfaction, speech changes, constipation, sleep disturbances, fatigue, depression and dementia, the latter of which occur in 30% of the patients. These symptoms are generally resistant to intervention by classical PD treatment (27, 29). Large clinical heterogeneity is seen among PD patients in regard to clinical symptoms. Most diagnoses are made presumptively by confirming most of the early symptoms and by ruling out other conditions that may produce similar symptoms, such as a tumor or stroke. For final diagnosis, two of the four cardinal symptoms must be present. However, a conclusive diagnosis can only be made post mortem (30).

Dopamine and Its Pathways in the Brain

The clinical symptoms seen in PD can mainly be attributed to loss of dopaminergic cells in the substantia nigra pars compacta (SNpc) in the midbrain, which project to the striatum of the basal ganglia through the

nigrostriatal pathway. The basal ganglia are composed of a complex network of several synaptically interconnected subcortical structures receiving input from a wide range of dopaminergic as well as non-dopaminergic cells. From the basal ganglia, signals are further projected to the thalamus, which relays signals to the cerebral cortex, including the motor cortex. This intricate and reciprocal network allows fine tuning of signals, which enables goal-directed psycho-motoric behavior (31, 32). Loss of dopamine in the SNpc thus causes alterations in these intricate pathways, ultimately leading to motor dysfunction.

Dopamine

Dopamine (DA) is a member of the catecholamine family, comprising DA, norepinephrine and epinephrine. The classical pathway for DA biosynthesis (33) starts with conversion of the amino acid tyrosine to 3,4-dihydroxyphenylalanine (L-dopa) by the enzyme tyrosine hydroxylase (TH), the rate-limiting step of the synthesis. L-dopa is then catalyzed to DA by aromatic amino acid decarboxylase (AADC). DA can further be converted to norepinephrine and epinephrine by dopamine β-hydroxylase and phenylethanolamine *N*-methyltransferase, respectively, which are not present in the ventral midbrain (34, 35). After synthesis, DA is stored in presynaptic vesicles via vesicular monoamine transporter 2 (VMAT2), where the oxidation prone DA is stabilized by a slightly acidic pH (36), thus preventing oxidative stress in the cytosol (37).

Under normal conditions, DA is released into the synapse in a tonic manner by L-dependent Ca^{2+} channels (38), and binds to five G-protein receptors. The D1 receptor class comprises D1 and D5, and act excitatory by increasing cytosolic cyclic AMP (cAMP). The D2 class includes subtype D2, D3 and D4, and acts inhibitory by depressing cAMP (39). Following exocytosis, DA is taken up by the membrane-bound dopamine transporter (DAT) and re-used through VMAT2-uptake, or metabolized to 3,4-Dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase B (MAO-B) located in the outer mitochondrial membrane of neurons and glial cells. Alternatively, DA is metabolized via glial cells by catecol-O-methyltransferase (COMT) to 3-methoxy-4hydroxy-phenylacetic acid (HVA) (40).

Dopaminergic Neurons and Pathways

There are four main dopaminergic pathways in the brain; the nigrostriatal, mesolimbic and mesocortical originating from the ventral mesencephalon and the tubero-infundibular originating from the hypothalamus (31). More than 90% of the dopaminergic cell bodies in the brain are located in the ventral mesencephalon (31), where DA is produced in the substantia nigra (SN), ventral tegmental area (VTA) and retrorubral field (RRF). These areas are largely continuous with each other, with SN located laterally, VTA medially, and the RRF dorsally. The SN is subdivided into the substantia nigra pars compacta (SNpc) and the substantia nigra pars reticulata (SNpr), in addition to substantia nigra pars lateralis (SNpl), a lateral extension of the SNpc. Each of these regions primarily contains dopaminergic cells with small groups of GABAergic interneurons and projection neurons in VTA and SNpr.

Dopaminergic neurons in the ventral midbrain are divided into three distinct groups, A8 (RRF), A9 (SNpc) and A10 (VTA) in addition to a scarce dopaminergic population in the SNpr (41). These dopaminergic cell groups have slightly different characteristics and projection patterns, innervating the striatum in a topographic manner (Figure 1). Dopaminergic neurons of the ventral tier of the SNpc projects to the patch compartment of the dorsolateral striatum (mainly putamen), through the nigrostriatal pathway (3, 31), and is involved in in motor control (42). The dorsal part of the SNpc projects to the matrix of the ventromedial striatum (mainly caudate nucleus) through the mesocortical pathway and is associated with cognition, whereas the VTA projects to the matrix of the ventral striatum (nucleus accumbens) and is involved in reward (43, 44). Clustered dopaminergic neurons of the dorsal tier of the SNpr mainly target the striatal patch compartment. Moreover, the matrix-directed dopaminergic neurons express calbindin immunoreactivity, whereas dopaminergic neurons projecting to the patches do not (3). The remaining 10% of dopaminergic cells are located in the hypothalamus (A11-A15) (35) and the olfactory bulb (A16), involved in autonomic and endocrine functions, and olfaction, respectively (45). The close association between these pathways generates some of the non-motor symptoms seen in PD.

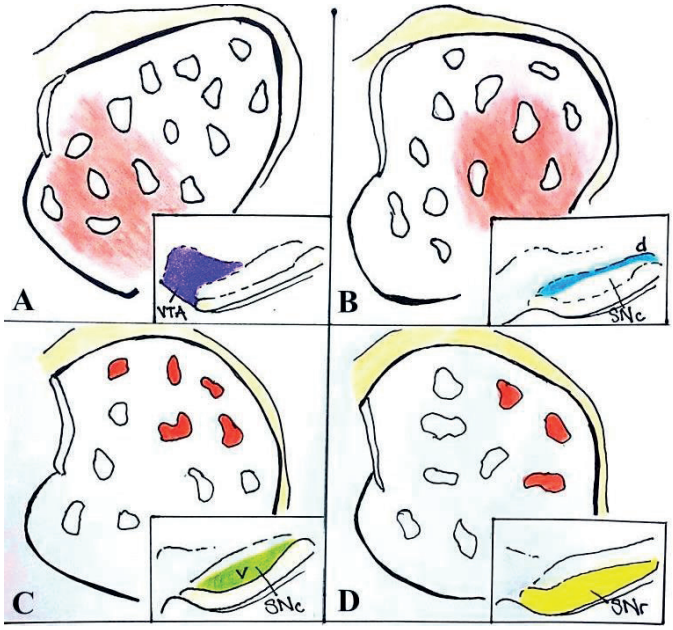

Figure 1. Projection patterns from the ventral midbrain to the striatum

The VTA projects to the patch departments of ventral striatum (A). The dorsal tier (d) of the SNc mainly targets the matrix of the medial part of the striatum (B), whereas the ventral tier (v) of the SNc targets patches of the dorsal striatum (C). The SNr projects to lateral patch departments of striatum (D).

D, dorsal; SNc, Substantia Nigra pars compacta; SNr, Substantia Nigra pars reticulata; V, ventral; VTA, ventral tegmental área.

Adapted from Gerfen *et al*., 1987 (3)

The main role of DA projecting from the SNpc through the nigrostriatal pathway is to modulate motor input converging in the basal ganglia, comprising the caudate nucleus and putamen (striatum), globus pallidus (GP), which is subdivided into an external (GPe) and internal part (GPi), and subthalamic

nucleus (STN) in the forebrain, and substantia nigra (SN) in the midbrain. Thus, SN is reciprocally connected with the basal ganglia. Striatum is the main input area, and receives massive and topographic input from the cerebral cortex, the thalamus and the brainstem. For motor control, GPi is the main output source, targeting the ventral lateral nucleus of thalamus, which in turn projects to primary and supplementary motor cortices. Taken together, these projections comprise a loop system running from cortex to the striatum, through the GP and thalamus and back to the cortex, modulated by the SN (31, 46, 47) (Figure 2).

The nigrostriatal pathway running from SNpc to striatum can be separated in a direct and indirect pathway. The direct pathway innervates putamen and the inner segment of the GP (GPi) whereas the indirect pathway innervates putamen, the outer segment of the GP (GPe) and the STN. The direct pathway excites the putamen through D1 receptors but inhibits GPi through GABAergic projections. The indirect pathway, on the other hand, inhibits putamen through D2 receptors. The two first projections of the indirect pathway from the putamen to the GPe and from the GPe to the STN are GABAergic, whereas the final projection from STN to GPi is glutamatergic. Thus, release of DA ultimately leads to inhibition of the GPi through both pathways, resulting in low inhibition of thalamus via GABAergic projections, and ultimately stimulation of motor cortices through glutamatergic projections (31, 46-49) (Figure 2A). PD is characterized by a selective loss of dopaminergic cells the SNpc, primarily in the ventral tier that densely innervates the sensorimotor part of the striatum (50). This cell loss results in altered activity in the nigrostriatal pathway that spreads through the motor loop, characterized by increased excitation of the indirect pathway and decreased inhibition of the direct pathway, both leading to an increased stimulation of the GPi and enhanced inhibition of thalamus. The overall result is a decreased stimulation of motor cortices, leading to hypokinesia (46, 48) (Figure 2B).

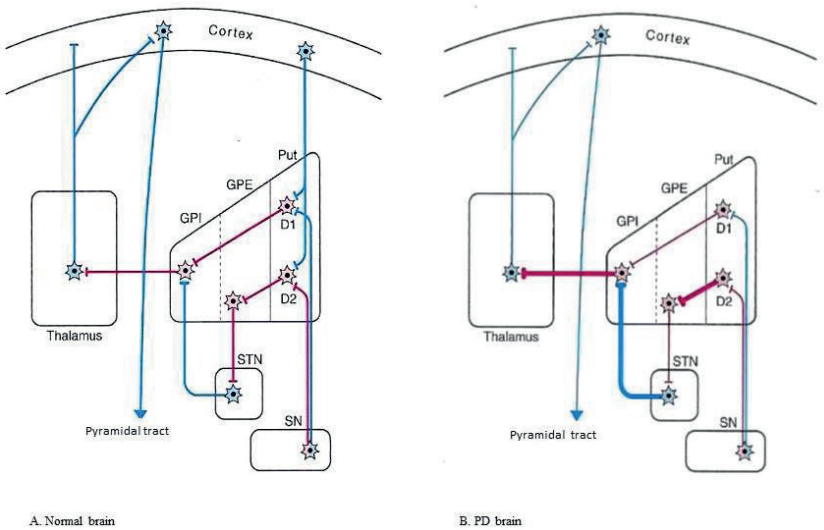

Figure 2. The cortico-striato-thalamo-cortical loop in physiological conditions and Parkinson's disease

Excitatory projections are shown in blue, inhibitory projections in red. D1, dopamine receptor 1; D2, dopamine receptor 2; GPe, Globus Pallidus external; GPi, Globus Pallidus internal; PD, Parkinson's disease; SN, Substantia Nigra; STN, nucleus subthalamicus.

Adapted from Helseth *et al*., 2007 (1)

Etiology

The etiology of PD is largely unknown. It is estimated that approximately 5-10% of the cases are caused by inheritable monogenetic mutations (51, 52). The remaining 95% of diagnosed PD cases are of idiopathic origin. Genetic studies on monozygotic twins show that idiopathic PD cannot be considered as an inherited disease (53), as a familial history is only seen in 20% of the cases (54). These studies strongly suggest that environmental factors are important in the etiology of PD, where environmental toxins has been demonstrated to be involved (4, 7, 8)

Genetic Factors

To date, at least 15 loci (PARK1-15) and 11 genes for PARK loci are associated with PD or parkinsonism in an autosomal dominant, autosomal recessive or X-linked inheritance pattern (54, 55). Among these, mutations in the genes coding for α-synuclein, leucine-rich repeat kinase 2 (LRRK2), parkin, PTEN-induced putative kinase 1 (PINK1) and protein deglycase (DJ-1), have been shown to cause PD almost indistinguishable from the idiopathic form. Mutations in these genes are associated with protein aggregation, reduced protein clearance, mitochondrial dysfunction and oxidative stress (56).

Dominantly inherited PD-causing mutations have been identified in the genes coding for α-synuclein and LRRK2, and are usually associated with late onset and formation of Lewy bodies (LBs). A mutation in α-synuclein was the first genetic mutation associated with PD, known to cause aggregation of α-synuclein and formation of LBs (57-61). LRRK2 represents the most significant cause of familial PD cases (62-64). It encodes a kinase present in synaptic vesicles in the cytosol and the outer mitochondrial membrane, and is likely related to dysregulation of vesicles and mitochondrial dysfunction (65, 66). PINK1, parkin and DJ-1 are autosomal recessive forms of PD causing early onset PD without formation of LBs, and are all associated with mitochondrial dysfunction and oxidative stress. PINK1 encodes a kinase in the inner mitochondrial membrane protecting against mitochondrial dysfunction (67-69), while Parkin codes for an E3 ubiquitin ligase involved in protein clearance by marking proteins for degradation (70), and they normally work together to govern mitochondrial quality control (71). DJ-1 is a mitochondrial protein involved in the protection against oxidative stress, and forms a complex with parkin and PINK1 to maintain mitochondrial function in the presence of an oxidative environment (72).

Environmental Factors

While aging remains the main risk factor for development of PD, environmental factors acting on a predisposing genetic background are thought to be tightly linked to the etiology. Environmental neurotoxins have long been suspected to be linked to PD but the causative mechanisms are not fully understood. The first conclusive demonstration of this link came from drug users after administration of the false heroin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), causing irreversible parkinsonism indistinguishable from idiopathic PD (73, 74). The symptoms were reproduced in primate (75, 76) and

rodent (77) models of PD, and the toxin was later shown to cause PD by inhibiting complex I of the electron transport chain in dopaminergic cells via transportation through DAT (78).

Epidemiological case-control studies as well as prospective studies have supported the hypothesis of a link between environmental toxins and PD. Several studies have reported an increased risk of developing PD in areas coupled to farming, rural living, well water drinking of water contaminated with arsenite and manganese, and to pesticide exposure, such as the insecticide rotenone, the herbicide paraquat and the fungicide maneb (79-83). However, other studies have failed to find a correlation (84-86), and the field has thus been widely debated. Recent meta-analyses of numerous epidemiological studies have revealed a significant association between pesticides and PD (7-9), suggesting that the discrepancy can be attributed to case ascertainment and control selection, study size, diagnostic criteria and assessment of exposure to pesticides.

The link between PD and environmental toxins is further supported by experimental studies on pesticides. Rotenone has been shown to induce parkinsonism by inhibition of complex I, both *in vitro* (87) and *in vivo* (88). Furthermore, paraquat is structurally similar to 1-methyl-4-phenylpyridinium (MPP+) (89), the active metabolite of MPTP, and has been demonstrated to induce nigrostriatal damage in animal models, further strengthening the link between pesticide exposure and PD (90-92).

Taken together, a link between neurotoxins and PD is clearly demonstrated through animal models as well as epidemiological analyses. However, the mechanism by which these toxins selectively target dopaminergic cells remains to be elucidated.

Pathogenesis

Pathological Hallmarks

The main pathological hallmark of idiopathic PD is a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) in the midbrain. The cell loss is associated by the presence of intraneural protein aggregates, called Lewy bodies (LBs), as well as neuroinflammation.

LBs are eosinophilic inclusions found in neuronal soma and neuritis. They are mainly composed of misfolded α-synuclein (93), a presynaptic protein with unknown function, in addition to ubiquitin (94) and neurofilaments (95). Dysfunction in protein metabolism appears to be important for their formation. However, it is unclear whether they are pathogenic and mechanistically cause neuronal death (96), or neuroprotective by sequestering potentially toxic proteins (97). Regardless of their nature, LBs are neither restricted to the CNS nor to PD. They can be found in the periphery (98) as well as in other diseases, such as dementia with LBs (99), and in normal aging (100, 101). Furthermore, many of the monogenic mutations do not lead to development of LBs, a feature that also may be observed in sporadic PD, as well as after administration MPTP. These observations demonstrate that neurodegeneration can occur without the presence of LBs, both in familial and sporadic forms of PD (102, 103).

Loss of nigral neurons follows a specific pattern with a more susceptible area in the lateral part of the ventral tier of the SNpc (50) projecting to the patch compartment of the striatum (3) (Figure 1). The cell loss results in severe DA depletion in the sensorimotor striatum in the dorsal putamen, accounting for the motor symptoms (46). Clinical symptoms of PD emerge when as much as 60-80% of the nigral DA neurons are lost (104, 105). Despite a clear association between degeneration of dopaminergic neurons in the SN and the pathology of PD, loss of neurons have been observed in the dopaminergic VTA (106), the noradrenergic locus coeruleus (107), the cholinergic Meynert basalis nucleus (108), the olfactory bulb (100), and in the vagus nerve and sympathetic ganglia (100), possibly accounting for some of the non-motor symptoms. However, selective loss of dopaminergic cells in the SNpc is identified as the key player of PD, making it crucial to understand the underlying vulnerability of these cells.

Selective loss of dopaminergic neurons is also evident in normal aging (109-114), however not sufficient to cause PD given the high rate of cell loss at clinical onset. Of notice, however, a district micro-anatomical pattern has been found in age-related dopaminergic cell loss in PD, where aging has been associated with a higher cell loss in the dorsal tier and the medial part of the SNpc projecting to the striatal matrix compartment, demonstrating an opposite pattern compared to PD within the SNpc (109) (Figure 1). There is also evidence for a higher cell loss in the VTA with aging (115). On the other hand, more recent and thorough studies in primates have provided support for the fact that PD represents an exaggerated version of aging by demonstrating accumulation of similar markers for dopaminergic neuron demise in both conditions (116-119). To date, it is unknown whether different mechanisms are separating the two conditions, or whether idiopathic PD represents an acceleration of aging.

Finally, neuroinflammation is a feature seen in the PD pathogenesis (120-124), although not specific to PD nor neurodegenerative diseases. It is unknown whether neuroinflammation is a cause or effect of the degenerative process, which will be discussed in detail later.

Selective Vulnerability of Dopaminergic Neurons in the SNpc: A9 Phenotypic Characteristics

The A9 group of dopaminergic cells in the SNpc displays several characteristics not present in other dopaminergic populations. A9 neurons have a distinctive cellular morphology, with long, thin axons that are poorly myelinated and massively arborized (100, 125-127). This morphology has been demonstrated to increase metabolic demands and the risk of oxidative stress in A9 cells compared to other dopaminergic cell populations (128-130). Long axons require more energy to accommodate transport machinery to and from soma, while the low diameter impairs motility and further increases the metabolic demands (131, 132). Further, lack of nodes of Ranvier elevates Na^+/K^+ activity due to a less efficient action potential mechanism (133). Massive arborization with many synapses is energy demanding, as mitochondria are concentrated around voltage gated Ca^{2+} sites (132, 134). Taken together, these cells require a highly dispersed mitochondrial network with an increased metabolic demand associated with production of reactive oxygen species (ROS), while having reduced transportation mechanisms for mitophagy.

Another characteristic of A9 cells is their pacemaker activity, generated by a by Cav 1.3, a rare type of L-type Ca^{2+} channel (38, 134). This self-generated activity makes these cells highly metabolically active and increases the production of ROS. However, it has recently been demonstrated that this channel is not solely responsible for the increased oxidative stress seen in these cell (130). A9 cells further lack calbindin, a protective Ca^{2+} buffering protein (135). Hence, these cells rely heavily on mitochondria to supply energy for Ca^{2+} pumps and buffer high levels of cytosolic Ca^{2+} directly, with an increased risk to stress the mitochondrial system due to calcium overloading (132). Although a link between calbindin and neuroprotection is reported (136), other studies have demonstrated that it is not causally related to the vulnerability of these cells (137). Moreover, the SNpc is particularly enriched in MAO-B (138), which is involved in generation of ROS, as described below. Finally, A9 neurons express high levels for DAT (31), which could lead to higher influx for toxins, including endogenous DA that accumulates and oxidizes in the cytosol, and potential exogenous toxins. However, expression of DAT is not limited to the A9 cells.

Despite all these characteristics of A9 dopaminergic neurons, none of them have proven to solely account for the selective vulnerability of these cells in PD, thereby stressing the importance of identifying novel potential selective vulnerability factors.

The Role of Mitochondria and Oxidative Stress

Familial forms of PD and neurotoxins leading to PD have provided valuable insight to the pathology of the disease. In both sporadic and familial PD, oxidative stress is thought to be a common underlying mechanism that leads to cellular dysfunction and eventually cell death. Oxidative stress is intimately linked to mitochondrial dysfunction, as mitochondria are the primary production site of ROS.

Mitochondria are the main source of ATP, generated by oxidative phosphorylation where electrons are transferred through complexes in the electron transport chain (ETC) located in the inner mitochondrial membrane. During this process, electrons can leak from the chain, especially from complex I and III, and react with oxygen to form detrimental ROS, starting with O_2^- (superoxide), which can be converted to the reactive H_2O_2 (hydrogen peroxide) (139). Under physiological conditions, ROS production is relatively low, and removed from mitochondria by antioxidants, such as manganese superoxide dismutase (MnSOD), which converts O_2 to H_2O_2, which is further converted to H_2O by gluthatione (GSH). Dysfunction in this process, either by blockage of electron movement along the chain or imbalanced antioxidant levels, lead to an increase in ROS that may cause damage to cellular components, such as DNA, RNA, lipids and proteins. Oxidative stress is identified as a main driver to cell death in the SNpc in PD (139, 140) as well as in aging (141). Imbalanced antioxidant defenses are observed in the SNpc of PD patients, with increased MnSOD, leading to elevated levels of H_2O_2 (142, 143), and reduced levels of GSH, leading to a decline in cellular capability to inactivate H_2O_2 (144). Furthermore, the activity of the mitochondrial complex I is reduced in SN of PD patients (140, 145), while complex I deficiency and lipid peroxidation are observed in post-mortem SNpc PD brains (140, 146). The cell death can be executed by apoptotic, necrotic or autophagic pathways, all of which are associated with PD experimentally as well as in post-mortem analyses of PD patients (147-151).

A key player of the excessive oxidative stress seen in dopaminergic cells is DA metabolism itself (152, 153). Non-vesicular DA is an unstable molecule and is easily oxidized to produce free radicals, toxic quinones and melanin (154). DA forms reactive metabolites when enzymatically metabolized by MAO and aldehyde dehydrogenase to DOPAC, forming H_2O_2 in the process. MAO-B expression is particularly high in the SN (138), and it has been shown that MAO-B activity is increased with age and doubled in patients with PD (138), where it correlates with dopaminergic cell loss (155). Second, auto-oxidation of the catechol ring of DA forms ROS and highly reactive DA quinones that can modify protein structure (154). This process is potentiated in the presence of iron (Fe^{3+}), which also catalyzes a reaction between DA quinones and H_2O_2 to form 6-hydroxydopamine (6-OHDA), a neurotoxin known to cause oxidative stress and mitochondrial dysfunction (156), and formation of melanin. The latter accumulates progressively in dopaminergic neurons with age in humans (157) but not in rodents, and probably has a neuroprotective role by binding iron to prevent further generation of ROS (158). The black appearance of these melanin-containing cells is the basis for the name substantia nigra, meaning black substance. In experimental conditions, DA is toxic in a variety of neuronal and non-neuronal cells both *in vitro* (159) and *in vivo* (160). However, DA metabolism is specific to dopaminergic neurons, but not exclusive to the A9 group.

The Role of Glia Cells and Inflammation

Astrocytes

Despite being the most abundant cell population within the human CNS (161), astrocytes have gained little attention in PD**.** Initially, they were merely thought to provide scaffolding for neurons, but it is now widely recognized that they make numerous essential contributions to the functioning of the healthy brain, including maintenance of brain homeostasis by regulation of fluids, pH, ions, transmitters and metabolites, and integrity of the blood brain barrier (BBB). Further, they are involved in neuronal survival by stimulating synaptogenesis, neurogenesis and release of signaling molecules such as transmitters, antioxidants and neurotrophic factors, including glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF). Astrocytes further secrete factors that can stimulate both cell survival and death, such as cytokines and inflammatory molecules, and ROS (162-171). To execute all these functions, astrocytes form neurovascular units, where their terminal processes, endfeet, contact the vasculature and other processes enwrapping neuronal synapses (163, 172). Their highly elaborated processes are organized in distinct domains with minimal overlap (173) while being connected by gap junctions for transfer of ions and signaling molecules that enable functioning as a syncytium (174). Astrocytes are highly heterogeneous cells present throughout the CNS (175, 176), with protoplasmic astrocytes in gray matter enveloping neuronal cell bodies, fibrous astrocytes in white matter involved in myelination, and radial glia present during development and in the retina and cerebellum (177). An archetypical morphological feature of all astrocytes, however, is their expression of intermediate filaments, which form the cytoskeleton, including glial fibrillary acidic protein (GFAP) and vimentin (178).

In response to CNS injury, such as trauma, ischemic damage, neuroinflammation or neurodegeneration, astrocytes can activate and undergo morphological and molecular changes (179). Reactive gliosis is a gradual process ranging from upregulation of GFAP and hypertrophy to proliferation and finally formation of glial scars (180, 181). Molecular changes include production of antioxidants, and in prolonged cases, release of pro-inflammatory cytokines and production of ROS (180). Thus, reactive astrogliosis is a process of both neuroprotective and detrimental character for the surrounding neuronal and non-neuronal cells, depending on context, duration and severity of the insult.

Although it is widely known that astrocytes form a heterogeneous population, specific regional and interregional properties of astrocytes remains to be elucidated in certain brain areas, including the SN. It has been shown that the density of astrocytes is low in the SN compared to VTA, RRF and the neocortex (182), making the metabolically active and vulnerable cells in SN less protected against DA metabolism by MAO-B and COMT (40) and ROS detoxification by GSH, which is present in mesencephalic astrocytes and neurons (183, 184), and less supported by astrocytic neurotrophic factors. This limited astroglial environment might be a susceptibility factor for dopaminergic cells in the SN. In many but not all cases of PD and animals models of PD, a mild increase in the number of astrocytes and GFAP expression has been observed, with a more pronounced expression in experimental models (182, 185-187). The amount of GFAP-positive astrocytes has been shown to correlate inversely with the amount of dopaminergic cell death (182), whereas the amount of α-synuclein-positive astrocytes correlates with the severity of cell death in PD (188). These observations suggest that dopaminergic neurons in an area with less astrocytes are more susceptible to the degenerative process.

Several lines of evidence suggest that astrogliosis occurs after neuronal cell death in PD, indicating that it is not a primary cause of cell death. In the MPTP model, astrocytic activation parallels the time course of dopaminergic cell death in the SNpc and striatum, and GFAP-expression remains upregulated after most dopaminergic neurons are lost (124, 189, 190). Nevertheless, astrocytes may become reactive in face of an inflammatory environment, being highly sensitive to pro-inflammatory cytokines, such as interleukin-1 β (IL-1β) and tumor necrosis factor α (TNF-α) (191), and the inflammatory sustainer interferon-γ (IFN-γ), all of which are shown in the SN of patients with PD (120, 192). Furthermore, astrocytes in the SN are found to express particularly high levels of intercellular adhesion molecule 1 (ICAM-1), which mediates sustained inflammation (193), and overproduction of this molecule has been found in reactive astrocytes in the SNpc in post mortem PD brains (194). In this way, astrocytes may be linked to propagation of the disease, potentially accelerated by generation of nitric oxide (NO) (186, 195). NO produced in astrocytes forms reactive intermediates that results in elevated oxidative stress (195), and NO overproduction and radicals in are shown in post mortem PD brains (196).

In conclusion, the limited expression of astrocytes in the SN seem to provide less protection against pathological processes, such as protein aggregation, oxidative stress and degeneration, while promoting progression of the disease in face of long-term inflammation. It is crucial to identify specific characteristics of the population of astrocytes in the SN that might contribute to the protection or propagation of the cell loss seen in the pathogenesis of PD.

Microglia

Like astroglial activation, the role of microgliosis is dual; beneficial or detrimental, depending on the situation. Several studies have demonstrated a deleterious role of microglia in the pathology of PD (197-199). Microglia are resident immunocompetent and phagocygotic cells in the CNS (197). In response to a variety of pathological events, such as infection, inflammation, trauma, ischemia, and neurodegenerative diseases, they become activated and act as scavengers (198, 200). The resting microglia then quickly migrate towards the lesion site, proliferate, become hypertrophic, change the expression of receptors and enzymes, and produce immune response molecules and toxic molecules, such as pro-inflammatory cytokines, prostaglandins and complement proteins, and ROS (197, 201-203). If the damage persists and cell death occurs, reactive microglia adhere to cells and undergo further transformation to phagocytes (197).

The SN contains the highest density of microglia compared with other brain regions (204). Moreover, the SN is particularly vulnerable to microglial inflammation, as induced by lipopolysaccharide (LPS) injections (205, 206). Extensive microgliosis, pro-inflammatory cytokines and oxidative stress-mediated damage is found in post mortem brains of PD patients (120-123, 196, 207, 208) as well as in animal models of PD (124, 190, 208, 209), including upregulated expression of major histocompatibility complex (MHC) molecules and pro-inflammatory cytokines, including TNF-α and IL-1β (120, 122-124, 190) and reactive oxygen- and nitrogen species (190, 196, 208). These features were co-localized with microglia and were increased in areas most affected by dopaminergic cell death, suggesting a significant role of microglia and inflammation in the degenerative process (153). In mice intoxicated with MPTP, a strong microglial reaction precedes a complete dopaminergic cell death (124, 190, 208), suggesting a participation of microglia in the neurodegenerative process. In a chronic pathological event, such as for PD, stressed dopaminergic neurons may activate microglia and release factors that further damage neurons and adhere microglia, causing a vicious cycle of neuroinflammation and neurodegeneration by phagocytosis (210).

It remains elusive whether neuroinflammation is a cause of PD pathogenesis or secondary stress response. Regardless of the origin, inflammation is clearly involved in the pathogenesis of PD. It must be emphasized, however, that both microgliosis and astrogliosis are unlikely to be specific for PD, as neuroinflammatory processes contribute to several neurodegenerative disorders, such as Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and progressive supranuclear palsy (197, 198, 210).

PD as a Prion Disease

Following a series of transplantation of cells from human fetal ventral mesencephalon into the striatum of PD patients, where the implanted cells developed Lewy bodies, (211-215), it was suggested that α-syntrophin can act in a prion-like fashion (100, 216). A prion is an abnormally folded protein propagating by transmitting a misfolded state by acting as a template to guide misfolding of more proteins into prion form, typically tightly packed β-sheets. These newly refolded prions can further convert more proteins,

leading to an exponential growth. In this way, misfolded α-synuclein may act as a template for the conversion of the native α-helix form to a pathogenic β-sheet structure (217), a reaction that may occur spontaneously or due to inherited amino acid exchanges in the prion protein (218, 219). *In vitro*, several studies have shown that aggregation of α-synuclein occurs by a mechanism resembling prions. However, it is unclear to what extent they are self-propagating in a prion-like manner (220-224). *In vivo* studies of intracerebral injections of aggregated α-synuclein in transgene animal models with α-synuclein mutations have further shown accelerated occurrence of protein aggregation, followed by nigral loss and motor deficits, not present to the similar extent in wild type (WT) animals (225-227). Thus, exogenous seeds of α-synuclein may accelerate the pathogenesis of a genetically predisposed α-synuclein aggregation disease.

In conclusion, several candidate factors are suggested to be implicated in the selective vulnerability of dopaminergic cells in the SNpc. Despite extensive research in the field, none of these factors have been found to solely account for the dopaminergic death known to cause PD. Thus, the factors providing a link between the etiology and the pathogenesis are still missing.

Current Treatment

Despite intense research in the PD-field, no efficient long-term treatment that halts or reverses the progression of the disease has been developed. The absence of a curing therapy is tightly linked to the unresolved understanding of the inherent selective vulnerability of dopaminergic cells in the SNpc. Thus, most of the current therapies remain aimed at alleviating the symptoms by affecting the existent level of DA in the brain. Several options exist for such symptomatic treatment of PD, including the DA precursor L-Dopa, DA agonists, and prevention of metabolic breakdown of DA by MAO-B- and COMT inhibitors. Finally, surgical intervention with deep brain stimulation may interfere with nerve signals generated along the nigrostriatal pathway to reduce motor deficits. Despite the initial success in relieving the motor symptoms, pharmacological interventions have limited therapeutic benefit as the disease progresses, which can be attributed to the fact that clinical symptoms emerge when as much as 60-80% of the nigral DA neurons are lost (104, 228). Thus, the beneficial effects wear off after 4-6 years of treatment, leading to development of motor fluctuations (increased parkinsonian symptoms when the medication effect ends) and dyskinesias (drug-induced involuntary movements) (229, 230).

With the possible exception of MAO-B inhibitors (231), none of symptomatic therapies modify the progressive course of the disease. Stem cell therapy offers a potential way to replace the nigral cell loss. However, an obstacle is differentiation into homogenous and stable A9 cells, thus presenting a potential risk of teratoma development (232, 233). Clinical attempts to replace the dopaminergic neurons with human fetal ventral mesencephalon tissue have been carried out in double-blind placebo-controlled trials, where cultivated dopaminergic cells were transplanted bilaterally into the putamen. These studies resulted in no or minor improvements in the intervention groups, and to development of graft-induced dyskinesia in a sub-group of patients (234, 235). Despite initial success, successful grafting and release of dopamine from the grafted cells for more than a decade, the implanted neurons developed Lewy bodies and were prone to cell death in similar ways as the endogenous dopaminergic cells in the SNpc, demonstrating that the disease can propagate from host to graft cells (211-213, 215). These observations highlight the

importance of understanding the underlying mechanisms responsible for the selective vulnerability of these cells in order to develop a therapy that halts the progression of the disease.

NEURONAL AND GLIAL CONTRIBUTIONS TO PARKINSON'S DISEASE

Since the discovery of dopaminergic cell death in the SNpc of patients with PD, research has focused on inherent factors of these neurons contributing to the selective cell death. The discovery that MPTP (73, 74) and monogenic mutations (57, 59-62, 67) lead to parkinsonism almost indistinguishable from idiopathic PD, has provided valuable insight to the etiology as well as pathogenesis of the disease, leading to extensive research on mitochondrial dysfunction, oxidative stress and protein aggregation. Yet so far, however, none of the proposed candidates that discriminate the A9 phenotype from other dopaminergic populations as well as other neuronal cells have been found to exclusively account for the selective vulnerability of these cells in the face of an endogenous stressed environment or exogenous toxins. This highlights the need for identifying novel molecules involved in the selective vulnerability in order to bridge the gap between the etiology and pathogenesis, and potentially offer therapeutic targets.

Historically, Parkinson's disease has almost exclusively been studied in a neurocentric perspective. However, neurons do not exist in a vacuum but are surrounded by a network of glial cells that maintain a homeostatic environment crucial for neuronal function (162, 178). Although a wide variety of astroglial functions is starting to unravel, their distribution, function and heterogeneous nature have not yet been completely uncovered in several brain areas, including the SN. Today, we are slowly beginning to elucidate a role of glial cells in the SN as well as in PD. It is crucial to understand the glial contributions to the environment in which the dopaminergic cells in the SNpc are living, and complete our understanding of the pathogenesis of PD.

This thesis offers insight both to the neuronal and glial contributions to the disease by focusing on novel molecules in the context of Parkinson's disease; aquaporins (AQPs). The focus is directed towards brain-specific aquaporins, AQP9 and AQP4, located primarily in neurons (AQP9) and astrocytes (AQP9 and AQP4).

AQUAPORINS

In 1992, Agre and co-workers discovered that red blood cells contained a membrane protein that conferred high water permeability (236), later called aquaporin-1 (AQP1). It was shown that this protein belongs to a superfamily of water channel proteins allowing passive, bidirectional transportation of water across the plasma membrane, thus increasing the velocity of transmembrane water transportation compared to diffusion or carriage by co-transporters across the phospholipid bilayer (19). Aquaporins are highly conserved throughout evolution, and more than 150 types are identified in plants, microbes, invertebrates, non-mammal vertebrates and mammals (237, 238).

In humans, 13 subtypes are identified, AQP0-AQP12, ubiquitously distributed in tissues (2). Based on sequence homology, aquaporins can be divided into subgroups, comprising orthodox aquaporins, aquaglyceroporins and super-aquaporins (Figure 3) (163, 239, 240). Ortodox aquaporins, comprising AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8, are highly selective for water, whereas aquaglyceroporins, comprising AQP3, AQP7, AQP9 and AQP10, show permeability to some neutral solutes as well, including glycerol and urea (239, 240). Aquaglyceroporins also show sequence homology to the bacterial glycerol facilitator protein (GlpF) (241). The final group is super-aquaporins, to which AQP11 and AQP12 belongs. They are localized in cytoplasm, involved in intracellular water transport, organelle volume and intra-vesicular homeostasis, but their permeability and function are not fully understood (242).

Molecularly, the aquaporin superfamily is identified by six transmembrane α-helical domains connected by five loops that form the extracellular and cytoplasmic vestibules, with an intracellular carboxyl and amino terminal (239, 243, 244). Two connecting loops, each containing the consensus motif Asn-Pro-Ala (NPA), overlap in the center of the lipid bilayer to form a pore with a central constriction that allows water movement (239, 244). They are composed of ~300 amino acids and have a molecular weight around 30 kDa (245). Most but not all aquaporins are inhibited by mercury, which depends on the presence of cysteines at certain positions within the aquaporin structure (246). In biological membranes, aquaporins form homotetramers containing four independent monomers, in which each serve as a pore (247, 248).

In orthodox aquaporins, selective water permeability is determined by three specializations (2, 249-251). First, the narrowest constriction of each monomer, located close to the extracellular entrance, has a diameter of ~2.8 Å, identical to the diameter of a water molecule (252). Second, this constriction site contains a positively charged arginine site that serves to block the entrance of protonated water and cations (251). Third, positively charged dipoles near the center of the channel cause formation of hydrogen bonds, which reorient the water molecules in the two halves of the channel and prevent proton flow (251). Despite the strict water selectivity, it has been shown that the central pore of some orthodox aquaporins is capable of transporting ions and gases, including O_2 and CO_2 and nitric oxide (253-255). In aquaglyceroporins, the arginine constriction is wider, with a pore size of ~7 Å, demonstrated by studies on the crystal structure of GlpF (256) and AQP9 (248). It is also less polar due to replacement of histidine by a glycine, which makes room for an aromatic side chain from a neighboring tyrosine or phenylalanine (257, 258). These features enable passage of a broader selection of solutes. However, the central constriction region of the pore is conserved in all aquaporins, indicating that they use the same proton exclusion mechanism (248).

Aquaporins in the Brain

To date, eight aquaporins have been described in rodent brain cells, AQP1, AQP2, AQP3, AQP4, AQP5, AQP8, AQP9 and AQP11 (18, 259-265). Most of them are studied in cell cultures and only three of them are demonstrated in brain at both protein and mRNA level, namely AQP1, AQP4 and AQP9 (15, 163). AQP1 is located on ependymal cells of the choroid plexus (259) and is involved in formation of

cerebrospinal fluid (CSF) (266). It will not be further discussed in this thesis. AQP4 is also expressed in ependymal cells of the ventricles but is primarily associated with astrocytic cell membranes (20). Subpopulations of astrocytes also express AQP9 in plasma membranes as well as in inner mitochondrial membranes (16). Moreover, AQP9 is the only aquaporin expressed in neurons, where it is located in the plasma membrane and the inner mitochondrial membrane (16). AQP9 and AQP4 are discussed in detail below.

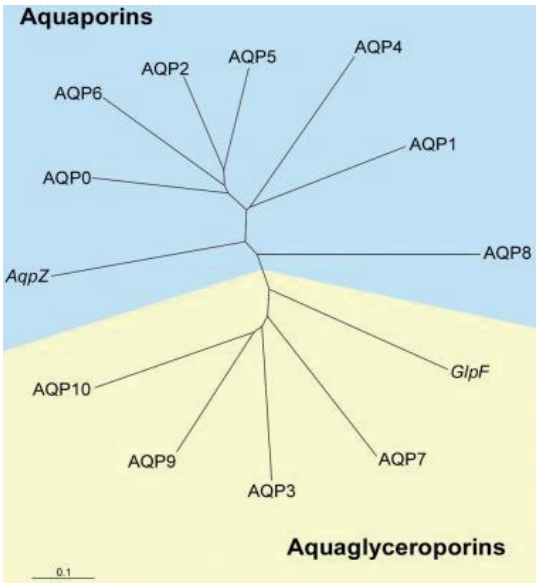

Figure 3. Human aquaporin gene family

A phylogenetic tree illustrates the genetic homology between the various members of the aquaporin family. Water permeable aquaporins (ortodox aquaporins) and glycerol permeable aquaporins (aquaglyceroporins) are separated as the two major subgroups. The figure also includes the *E. coli* homologues (AqpZ and GlpF). Super-aquaporins (AQP11 and AQP12) are not included. The scale bar represents genetic distance between homologues.

AQP, aquaporin; GlpF, glycerol uptake facilitator protein.

From Agre *et al*., 2002 (2).

Aquaporin-9: The Neuronal Contribution

Molecular Characterization

AQP9 shares sequence homology with the aquaglyceroporins AQP3 (48%), and AQP7 (45%) (10), and further shares similarities with AQP10 and bacterial GlpF (241) when clustered in a phylogenetic tree, indicating a common ancestral gene (Figure 3). Its gene consists of six exons and five introns distributed over ~25 kb (12), with a negative insulin response element (IRE) in the promoter region (267). Human AQP9 has been found to share 76% amino acid sequence with rat AQP9 and to share similar permeability profiles (10), indicating that its characteristics are evolutionary conserved.

An Aquaglyceroporin with Broad Substance Permeability

Despite close similarities to other aquaglyceroporins, AQP9 is unique among aquaglyceroporins due to a particularly broad permeability to structurally unrelated solutes. When expressed in *Xenopus* oocytes, it is shown to be permeable to water, polyols (glycerol, mannitol, sorbitol) carmabides (urea, thiourea) purines (adenine) and purimidines (uracil and the chemotherapeutic agent 5 fluorouracil), monocarboxylates (lactate, B-hydroxybutyrate) (10-12) and ammonia (13) in a mercury- and phloretin sensitive manner (12). The AQP9-mediated transportation of monocarboxylates increases significantly in acidic conditions with pH <5.5, suggesting that these agents are primarily permeating in their protonated form (11, 268). Other solutes have been demonstrated not to be transported through AQP9, including ions, cyclic sugars (D-glucose, D-mannose and myoinositol), the nucleosine uridine, glutamine and glycine (11). The particularly high permeability through AQP9 can be attributed to a an oval shaped pore with dimensions 7x12 Å compared to a round pore in GlpF with a diameter of 7 Å. This shape and enlargement can be assigned to a lower pore density towards the hydrophobic face of the pore (248), allowing transportation of bulkier solutes compared to other aquaglyceroporins.

Notably, AQP9 has also been shown to be permeable to the metalloid arsenite, the most toxic form of arsenic (14), which has been linked to PD in epidemiological studies as well as experimentally (79, 80, 83). Thus, it is not unlikely that other toxins may be transported through AQP9 as well. MPP+ and paraquat are two candidates that potentially may permeate AQP9, as they structurally resemble puridines, which are readily transported through AQP9 (11, 89). These toxins are tightly associated with PD (7-9, 73, 74, 88-92), making AQP9 a potential mediator of transportation of toxins associated with PD.

Distribution of AQP9 in the Brain

Using homology cloning, AQP9 was first described in rat liver (11), then in human liver (12). Subsequent protein studies confirmed abundant expression in liver, where it was shown to be localized in hepatocytic plasma membranes facing the sinusoids (264, 269-271). AQP9 protein and mRNA has also been demonstrated in several other organs and cells, including testis (264, 271) spleen (264), leukocytes (12, 272), lung (12), bone marrow (12), retina (273), adipose tissue (274, 275), small intestine and brain (17, 264, 269).

In the brain, *Aqp9* mRNA was first found in rat astrocytes (11) and confirmed by immunohistochemical studies in the rodent brain (17, 264, 269, 270). In these initial studies, Elkjaer and colleagues (264) reported expression of AQP9 in certain glial cells, including ependymal cells lining the ventricles and tanycytes of the mediobasal hypothalamus, but not in astrocytes or neurons, which was reproduced by others (270). Using PCR, immunocytochemistry and Western blotting, Badaut and colleagues (269) reported the presence of AQP9 in endothelial cells of pial vessels and in astrocyte cell bodies and processes in mice, with high intensity in glia limitans and white matter, as well as in the subfornical organ and cerebellar Bergmann glia (17). In rats, they further reported a prominent expression of AQP9 in catecholaminergic cell bodies and proximal fibers in the brain stem and hypothalamus but not the habenula or in other monoaminergic cell groups (17). Amiry-Moghaddam *et al.* (16) further demonstrated

that AQP9 was present in rat plasma membranes and inner mitochondrial membranes of dopaminergic neurons and astrocytes in the SN, VTA and arcuate nucleus, using immunogold electron microscopy.

A study by Rojek and colleagues (276) reporting unspecific brain AQP9-labeling in *Aqp9*$^{-/-}$ mice, opened a debate in the field with regard to brain-specific AQP9, as previous studies demonstrating its expression in brain were primarily based on antibody-dependent techniques. Conclusive evidence for the presence of AQP9 in the brain was provided by Mylonakou *et al*. (15), using *Aqp9*$^{-/-}$ controls and *in situ* hybridization to demonstrate that *Aqp9* mRNA positive cells were the same as those where AQP9 immunoreactivity was observed, with predominant expression in the SN. A blue native analysis further confirmed its tetrameric and thus functional expression. Finally, they demonstrated that AQP9 was significantly more abundant in the liver compared to the brain, with only 3% and 0.5% of that of the liver in rats and mice, respectively. The subtle expression together with the species differences may have accounted for the discrepancies seen in previous studies. Authentic expression of astrocytic AQP9 was further demonstrated by small interference RNA (siRNA) directed against AQP9 in astrocytic cultures, which resulted in decreased expression of AQP9 (277). Following the confirmation of AQP9 in the rodent brain, the presence of AQP9 was also demonstrated in the primate brain, where the distribution was in line with previous findings, showing expression in astrocytes and catecholaminergic neurons. In addition, AQP9-positive labeling was also found in neurons of insula cortex, primary motor cortex, and the hippocampal granule cell layer in dentate gyrus (278), which was in agreement with previous findings of *Aqp9* mRNA but not protein found in the rodent cortex and hippocampus (15). Thus, the distribution of AQP9 in the primate brain is in line with the expression in the rodent brain, with somewhat more widespread neuronal labeling, indicating that a similar pattern may be found in humans. However, this antibody-based primate study must be interpreted with caution due to cross-reactivity of the antibody with GFAP and an unknown mitochondrial matrix-protein.

One study performed in rats has shown that AQP9 is expressed as two isoforms, one ~30 kDa isoform equivalent to the liver isoform anchored in the cell membrane of dopaminergic neurons and astrocytes, and a larger pool of a ~25 kDa isoform found in the inner membrane of mitochondria of these cells (16). The shorter isoform was detected by Western blotting of rat brain mitochondrial inner membranes, and a sequence analysis of PCR gels revealed that the short isoform had a shorter open reading frame with a start codon associated with perfect Kozak consensus site, likely obtained by alternative splicing. These results were later confirmed by a quantitative immunogold electron microscopy study, demonstrating that unlabeled mitochondria outnumbered the labeled ones in the SN of *Aqp9*$^{-/-}$ mice, whereas the opposite was true for WTs (15). These results indicate that mitochondria may contain an authentic pool of AQP9. The presence of mitochondrial expression of aquaporins is in line with previous findings, which have demonstrated expression of AQP8 in the inner mitochondrial membrane (279, 280). This pool, however, has not shown a functional significance, as measured by water and glycerol permeability (281).

To date, AQP9 is the only known neuronal aquaporin. Its selective localization on dopaminergic cells in the SN, where it is expressed in the plasma membrane as well as in the inner mitochondrial membrane, makes it an ideal candidate for the selective vulnerability of these neurons in PD. Possibly, AQP9 may serve as a gateway for toxins into dopaminergic neurons and their inner mitochondrial membranes, where they can interfere with the electron transport chain to induce oxidative stress and cell death.

Physiological and Pathophysiological Roles of AQP9

To date, no functional studies have been performed on brain AQP9, and its physiological role in the brain thus remains elusive. However, liver AQP9 has been widely studied. Although speculative, some of these findings may provide clues for its physiological role in the brain.

Metabolism and Diabetes Mellitus

Given the transportation of energy substrates through AQP9, such as glycerol and monocarboxylates, AQP9 in liver is thought to play a role in metabolism, particularly in face of scarce glucose resources and low concentrations of plasma insulin. In hepatocytes, AQP9 may serve as a major route for uptake of glycerol released during lipolysis, which can be used by the liver as a substrate for gluconeogenesis during fasting periods (267, 268, 276). This has been demonstrated by higher glycerol blood concentrations in *Aqp9*$^{-/-}$ mice than WT controls (276) and a dramatic increase of AQP9-expression following fasting, returning to baseline levels upon re-feeding (268). This diet-dependent expression of AQP9 further indicates that AQP9 is negatively regulated by an increase in the blood insulin concentration, presumably attributed to a negative insulin response element in the AQP9 promoter (267, 268). This was demonstrated by a significant increase of hepatic AQP9 mRNA and protein in a model of diabetes mellitus (268) and insulin resistance (267), indicating that AQP9 may play a role in the pathophysiology of diabetes mellitus.

It has been suggested that AQP9 may serve a similar role in the brain as a glucose-lactate channel. Using a model of diabetes mellitus in rats, AQP9 over-expression was reported selectively in neurons of catecholaminergic nuclei, and decreased in brain stem slices after insulin exposure (282). Further, using siRNA in astrocytic cultures, the same group demonstrated reduced glycerol uptake and changes in astrocytic energy metabolism (277). These findings indicate that AQP9 may be involved in brain metabolism, although the idea requires more experimental support. The brain relies heavily on a stable energy balance. Thus, in face of scarce glucose levels, it has been shown that the brain can exploit other metabolites, including glycerol (283-285), lactate and ketone bodies (283, 286-288), all of which permeate brain AQP9 and rely on astrocyte-neuron interaction (285, 288). The diffusion of monocarboxylates is pH-dependent, with increased permeability in acidic conditions, further supporting the idea of increased dependence on AQP9 in lack of glucose (11, 268). AQP9-positive neurons are known to overlap with glucose-sensitive neurons, which are present in the brain stem and hypothalamus (17, 289, 290), further supporting a link to brain metabolism in these cells. Given the highly metabolic environment seen in dopaminergic cells in the SN, it is not unlikely that these cells are equipped with mechanisms supporting alternative energy substrates to sustain their activity levels and a non-toxic environment.

Water and Metabolite Homeostasis in Brain Ischemia

Like all aquaporins, AQP9 also transports water, and may therefore be involved in brain water homeostasis. The presence of AQP9 in astrocytes of glia limitans bordering the subarachnoid space and in tanycytes in the ependymal lining of the cerebral ventricles points to a possible role in water regulation between cerebrospinal fluid and brain parenchyma (264, 269), while its location in white matter and hypothalamic nuclei suggests that it may be involved in extracellular water homeostasis. This putative role has been demonstrated in a mouse model of transient focal cerebral ischemia, showing an increase in AQP9 expression in the infarct border zone (269), similarly to AQP4 (291, 292), possibly contributing to edema formation. Given the lactic acidosis that follows an ischemic stroke, lactate buffering through AQP9 may also be important in this pathological condition, supported by the fact that lactate permeability increases significantly during acidic conditions (11). Upregulated expression of AQP9 in reactive astrocytes in the pre-infarct border zone following ischemic stroke thus argues for a role of AQP9 in lactate-buffering and water homeostasis (269).

Parkinson's Disease

A putative role of AQP9 in PD has been suggested by our group (15, 16), but the link has not been investigated experimentally. Given the selective localization of AQP9 in dopaminergic cells in the SN, together with its broad substance permeability, including neurotoxins, AQP9 is a highly attractive candidate in the pathogenesis of the disease by potentially accounting for the selective vulnerability of the cells which are degenerating in PD. In support of the link between AQP9 and PD, epidemiological studies have demonstrated a correlation between PD and diabetes mellitus (293-296). The IRE located in the AQP9 promoter (267) and several studies indicating upregulation of AQP9 in models of diabetes mellitus (267, 268) suggest that AQP9 might be the third variable underlying the correlation between these pathological conditions. Finally, it has been demonstrated that there is a higher expression of AQP9 in male livers compared to females (270). Although this gender-based expression is not investigated in the brain, it is interesting to note that PD is more common in males than in females (24, 25).

In conclusion, several lines of indications are linking AQP9 to PD, but the connection lacks experimental support.

Aquaporin-4: The Glial Contribution

Molecular Characterization

Cloning and molecular characterization of brain *Aqp4* at the mRNA level was reported by two independent studies (18, 297). The gene was reported to have two different initiation sites that gives rise to two functional proteins, one 301 and the other 323 amino acids long, corresponding to the two isoforms M23 (AQP4c) and M1 (AQP4a), respectively (18, 297). When expressed in *Xenopus* oocytes, both the

M1 and the M23 isoforms produce an increase in osmotic water flux identical to that of AQP1 (18, 19), and when compared to other aquaporins, AQP4 shows the highest water permeability coefficient, followed by AQP1 (298). More recently, four new isoforms of AQP4 (AQP4b, d, e, f) have been described (299), in which one isoform (AQP4e) is expressed in astrocytic plasma membranes and mediate water transportation (300). Hence, AQP4 isoforms a, c and e are orthodox aquaporins transporting water in a mercury-insensitive manner (18). The remaining isoforms (AQP4b, d, f) are obtained by alternative splicing. They are located intracellularly in Golgi apparatus and late endosomal degradation compartments and do not mediate water transportation, and their function remains to be specified (300).

Distribution of AQP4 in the Brain

AQP4 has been described in several tissues and organs, including kidney, retina, inner ear, skeletal muscle, glandular epithelia, intestines, and lungs (18, 297, 301). The predominant site of expression, however, is the brain, where it is found to be the most abundantly expressed aquaporin (18). By means of specific antibodies, it has been widely demonstrated that AQP4 is expressed throughout the CNS, especially in astroglial cells bordering blood vessels and the subpial surface, subpopulations of ependymal cells lining the ventricles, and in osmosensory areas, including the supraoptic nucleus and subfornical organ (20), AQP4 is expressed in both gray and white matter but has not been reported in neurons, oligodendrocytes or microglia (278).

Immunogold cytochemistry has revealed that AQP4 is particularly pronounced in astrocytic endfeet abutting brain microvessels (perivascular pool) and found to a much lesser extent in non-endfeet processes contacting synapses (perineural pool) (20, 302, 303). The polarized expression of AQP4 in astrocytic endfeet can be attributed to intracellular anchoring of AQP4 to the dystrophin-associated protein complex (DAPC), a large membrane assembly that connects the cytoskeleton to the extracellular matrix (304, 305). This has been demonstrated in *mdx* mice lacking dystrophin, showing loss of AQP4 from striated muscle membranes, ependymal lining, perivasculary and in subpial membranes, while AQP4 pools in kidney, lungs and gastrointestal tract were unaffected (304). Further, a near selective depletion of the perivascular pool of aquaporins was seen after removal of α-syntrophin in mice, a cytoskeletal component of the complex, indicating that α-syntrophin is responsible for the anchoring mechanism (305-307). Freeze fracture immunogold labeling has revealed that AQP4 tetramers are organized into specialized square structures referred to as orthogonal arrays of particles (square arrays) in astrocytic membranes (308). The size of these structures is determined by the ratio of the isoforms M1 and M23, as the latter is critical to stabilize the structure (251, 308). The function of this organization remains unknown, but could potentially secure a very high capacity for water transport across the endfoot membrane (21).

The distribution of astrocyte AQP4 differs significantly within the brain, suggesting that AQP4 has multiple physiological roles (20, 21, 309). To date, this heterogeneity is not well described. It is crucial to characterize its expression in order to reveal the physiological roles of AQP4 within specific areas.

Physiological and Pathophysiological Roles of AQP4

Control of Water Flux and Brain Edema

The specific localization of AQP4 to brain-blood and brain-CSF interfaces, where AQP4 is highly polarized to astrocytic endfeet facing vascular systems, suggests a role for AQP4 in cerebral water balance (292). This role has been demonstrated in studies where *Aqp4* or components of the DAPC are deleted (292, 306, 310). Moreover, glial-conditional *Aqp4* knockouts demonstrate delayed postnatal reabsorption of water, resulting in slightly higher brain water content in *Aqp4* knockout animals (164). Although removal of AQP4 itself does not result in impaired water homeostasis (292, 311, 312), AQP4 has been demonstrated to play a key role in several pathological conditions associated with water accumulation in brain tissue, known as brain edema. As the cranium forms a physical constriction to swelling, increased intracranial pressure is lethal. Brain edema is a frequent consequence of several pathological conditions, including stroke, head injury, hyponatremia, brain tumor and inflammation. The role of AQP4 in controlling water flux between the blood and brain was first demonstrated in a model of ischemic stroke, where post-ischemic cerebral edema was significantly reduced in *Aqp4*$^{-/-}$ mice (292). This role can primarily assigned to the perivascular pool of AQP4, demonstrated by reduced post-ischemic edema formation in *mdx* animals (310) and *α-syntrophin*$^{-/-}$ mice (305, 306), confirming that the perivascular pool of AQP4 facilitates edema formation. A glial conditional *Aqp4*$^{-/-}$ (164) further confirms that this pool resides in astrocytes and not in endothelial cells.

Potassium Homeostasis, Neural Excitability and Epilepsy

Water movement in the brain is indirectly involved in ionic homeostasis (313, 314), which is intimately linked to neuronal activity. Glia cells are crucial for clearance of K^+ from the synaptic space following neuronal activity, where the inwardly rectifying potassium channel 4.1 (Kir4.1) serves to redistribute K^+ from areas of high synaptic activity to remote liquid compartments, referred to as potassium spatial buffering (315). Kir4.1 is found to co-localize with AQP4 in astrocytic endfeet (316), and mice lacking α-syntrophin (313) or AQP4 (317), were found to display a delayed clearance of extracellular K^+ following high-frequency synaptic activation (313). Furthermore, these animals showed prolonged and more severe epileptiform seizures than WT controls (313, 317). Other studies on mesial temporal epilepsy have demonstrated mis-localization of perivascular AQP4 in patients (314), as well as in an animal model of this condition induced by kainate (318). These data indicate that the perivascular pool of AQP4 anchored to α-syntrophin may have an important role in spatial potassium buffering and neural excitability, although a clear functional relationship between AQP4 and Kir4.1 is debated (319).

Water and Waste Clearance and Protein Aggregation

Astrocytic AQP4 has also been associated with water and waste clearance from the brain by means of bulk flow through the glymphatic system, which is hypothesized to correspond to a lymphatic system of

the brain (320-322). Here, changes in extracellular volume induces water and waste clearance by a paravascular route driven in part by cardiac pulsations, where water moves from arteries to veins via the interstitial space, facilitated by AQP4 (323). This route has been demonstrated by two-photon imaging, where fluorescent tracers were injected into the subarachnoid space (320, 321). *Aqp4*$^{-/-}$ mice exhibited reduced tracer flow into the brain and delayed clearance of intraparenchymally injected tracers, including tagged amyloid-β (320). Moreover, the glymphatic system showed reduced activity in a model of AD, where the glymphatic failure preceded significant amyloid-β deposits (324) Thus, AQP4-mediated water flow along vessels may not only be relevant for clearing brain interstitial water, but also waste and soluble proteins. Although the hypothesis of the glymphatic system has strengthened extensively by recent preclinical research, considerable gaps remain in our knowledge with regard to its existence in the human brain (325).

Cell Volume Regulation and Brain Tumors

AQP4 plays an important role in regulation of cell volume. Cells have the ability to prevent detrimental swelling in response to hypoosmotic stress by means of regulatory volume decrease (RVD), which brings the cell back towards baseline levels by releasing osmolytes into the extracellular space, which in turn leads to obligatory efflux of water (326, 327). This mechanism is mediated by a complex consisting of AQP4 and transient receptor potential vanilloid 4 (TRPV4) in astrocytes (328). Given the anatomical proximity of astrocytes to fluids, such as blood and CSF, astrocytes are the first cells to be exposed to osmotic changes, and counteracts the dynamic changes via water efflux through AQP4 (163).

Cell volume regulation is also an important feature for invading glioma cells, as it enables accommodation of narrow spaces as they invade (329). AQP4 is significantly upregulated in glioblastomas compared to low grade gliomas and normal brain tissue (329, 330), and is highly expressed in the leading edge of invading tumor cells (329). It has been shown to play a role in migration and invasion of glioma cells (331, 332), although other studies have shown that it is more important for adhesion (329, 331). A role of AQP4 in astroglial cell migration has also been found in wound healing *in vitro* and *in vivo*, where *Aqp4*$^{-/-}$ mice showed impaired migration and glial scar formation towards a cortical stab injury (333). So far, an adhesive role of AQP4 in physiological conditions has not been found (334).

AQP4 in Inflammation

AQP4 is thought to be related to the pathogenesis and progress of various neuroinflammatory diseases, including brain edema, head trauma, tumor, meningitis, malaria, neuromyelitis optica (NMO), multiple sclerosis (MS), as well as chronic neurodegenerative diseases. Although the molecular mechanisms of AQP4 in the inflammatory process are still unknown, it has been demonstrated that brain AQP4 is upregulated in astrocytes in acute inflammatory models induced by injections of lysophosphatidylcholine (LPC) (335) or lipopolysaccharide (LPS) (336) and by pro-inflammatory cytokines (337), whereas deletion of *Aqp4* results in reduced production of pro-inflammatory cytokines compared to WT controls

in astrocyte cultures (338). The possible link between neuroinflammation and AQP4 was first suggested in NMO, where serum antibodies were found to recognize astrocytic AQP4 (339). This link was further strengthened in models of MS, where the absence of *Aqp4* in astrocyte cultures reduced the production of pro-inflammatory cytokines compared to WT controls (338). Upregulation of brain AQP4 has also been described in a model acute bacterial meningitis, where the CSF contained inflammatory products (340). In this model, *Aqp4*$^{-/-}$ mice showed reduced edema, improved neurological status and increased survival. On the other hand, reduction of AQP4 has also been described in inflammatory diseases, including cerebral malaria (341), where *Aqp4*$^{-/-}$ exhibited more severe signs of cerebral malaria as well as brain edema, demonstrating a protective role of AQP4. These data clearly point to a role of AQP4 in neuroinflammation, although it remains unclear how it mediates this process and whether it is beneficial or detrimental. AQP4 has also been shown to be involved in neuroinflammation in several chronic neurodegenerative diseases, which will be outlined in the following section.

AQP4 and Chronic Neurodegenerative Diseases

AQP4-induction has been shown in several chronic neurodegenerative diseases, including AD, ALS, spongiform encephalopathy as well as PD. Although little is known about AQP4 in the pathogenesis of these diseases, several lines of evidence point to neuroinflammation as a key process involved.

Several studies have reported upregulated expression of AQP4 and GFAP around senile plaques in AD (342, 343) although others have found a reduction in the expression (344) or no alteration (345). This contradiction can possibly be explained by a study showing upregulation of AQP4 in the neuropil surrounding plaques and loss of AQP4 from endfoot membranes at sites of perivascular amyloid deposits (346), indicating a redistribution of AQP4 from endfoot membranes to non-endoot domains. Furthermore, low concentration of amyloid-β induced AQP4-expression in astrocytic cell cultures, whereas higher concentrations reduced the expression (347). Finally, deletion of AQP4 decrease amyloid-β induced gliosis in astrocytic cultures, indicating that AQP4 is involved in astrocyte activation following amyloid-β exposure (347). Another link between AQP4 and AD has been proposed through the glymphatic system, in which *Aqp4*$^{-/-}$ mice exhibited delayed clearance of intraparenchymally injected amyloid-β (320).

AQP4 has also been linked to ALS, where AQP4 mRNA and protein are specifically overexpressed in astrocytes of the gray matter at end stage of the disease in a *SOD-1*$^{-/-}$ model (348). Moreover, a marked increase of AQP4 expression has been shown in spongiform encephalopathy in a model of the disease as well as in affected cattle, where the expression correlated with GFAP immunoreactive astrocytes and prion depositions (349).

In PD, the role of AQP4 is less explored, although some studies point to a link between these. In a model of PD induced by an intracerebral injection of 6-OHDA, a significant upregulation of *Aqp4* mRNA expression as well as *Gfap* was observed in the lesioned SN, indicating reactive gliosis (350), whereas other studies have found reduced *Aqp4* mRNA in blood samples of PD patients (351). Acute and chronic MPTP models of PD have further demonstrated reduced gliosis and augmented microglial activation, in addition to increased dopaminergic cell loss in the SN of *Aqp4*$^{-/-}$ mice (352-355). The same authors have

further demonstrated that AQP4 deficiency resulted in equal vulnerability to MPTP between dopaminergic neurons in the SN and VTA in acute and chronic models of PD (354), thus indicating that the susceptibility differences between SN and VTA may be attributed to differences in astrocytes of these regions. These authors have pointed to a role of inflammatory factors mediated by AQP4, supported by a more pronounced inflammatory profile of AQP4 deficient mice than WTs in a chronic MPTP model of PD (355).

Taken together, these data indicate that AQP4 plays a role in several degenerative diseases. However, the literature is contradictory, and it remains to be clarified whether this role is beneficial or detrimental.

HYPOTHESES AND AIMS OF THE THESIS

This thesis evaluates the pathophysiological roles of brain aquaporins in PD and aging, focusing on selective vulnerability of dopaminergic neurons in the SNpc. This vulnerability is elucidated by investigating neuronal and glial contributions to the pathogenesis, through AQP9 and AQP4, respectively.

The Neuronal Contribution: AQP9 in PD and Aging

Background: PD is a neurodegenerative disease where aging represents the most prominent risk factor (5, 6). The etiology of PD is intimately linked to environmental toxins, as shown through epidemiological studies demonstrating a correlation between pesticides and PD (7-9), as well as by administration of neurotoxins leading to PD (73-77, 79). However, the mechanism in which these toxins cause selective degeneration of dopaminergic cells in the SNpc remains unknown. AQP9 has been demonstrated to be selectively expressed in dopaminergic neurons in the SN (15-17). Further, this aquaglyceroporin has particularly wide substance permeability to structurally unrelated molecules, including toxins, such as arsenite (10-14).

Hypothesis: We hypothesize that AQP9 can serve as a gateway for neurotoxins to enter dopaminergic cells in the SNpc, thereby participating in the selective cell death of this region seen in PD, as well as during aging.

Aims: We seek to reveal whether AQP9 is permeable to the PD-associated neurotoxin MPP+. Further, we aim to determine whether *Aqp9*$^{-/-}$ animals are less susceptive to MPP+ than WT animals in mouse models of PD *in vitro* as well as *in vivo*. Finally, we aim to investigate whether AQP9 is involved in age-related cell death in the SNpc using *Aqp9*$^{-/-}$ and WT controls of different age groups.

The Glial Contribution: AQP4 in PD

Background: Astrocytes play a crucial role in maintenance of micro-environmental homeostasis, and are thus intimately linked to neuronal function (18-20). It has been demonstrated that the expression of astrocytes is particularly low in the SNpc (182), although their role in the pathogenesis of PD remains largely unexplored. AQP4 is shown to play a role in several chronic neurodegenerative diseases, where its expression is upregulated and potentially linked to neuroinflammatory processes (335, 342, 348, 350). However, its expression in PD requires further support, and it remains to be elucidated whether it plays a beneficial or detrimental role.

Hypothesis: We hypothesize that astrocytes may influence the selective vulnerability of dopaminergic cell death, and that expression of AQP4 in these cells may mediate the neurodegenerative process in PD.

Aims: We aim to unravel the expression and distribution of AQP4 in the SNpc under physiological conditions and in mouse models of PD. Further, we seek to determine whether AQP4 is beneficial or detrimental in a mouse model of PD using *Aqp4*$^{-/-}$ and WT animals.

SUMMARY OF THE PAPERS

Paper I: Cytoprotective effects of growth factors: BDNF more potent than GDNF in an organotypic model of Parkinson's disease

Background: Organotypic slice cultures of the ventral mesencephalon have been used to model dopaminergic cell loss in PD by means of incubation with neurotoxins targeting dopaminergic cells, with the advantage of closely resembling an *in vivo* situation, as the micro-anatomy is kept intact within the slices (356, 357). However, pronounced rostro-caudal variation of dopaminergic cell number in the SN may bias the results when comparing slices exposed to factors promoting cell death and survival to control slices. We have previously developed a method for unilateral toxic application which generates an internal control within each slice (358). This model can be exploited to perform accurate quantitation of cell death and survival. A well-studied candidate for promotion of cell survival and regeneration is neurotrophic factors, where glial-cell derived neurotrophic factors (GDNF) and brain-derived neurotrophic factors (BDNF) are shown to have the most pronounced effects (359-362). It is unclear however, which of these factors that are most potent.

Aims: In this study, we aimed to apply our previously developed model for unilateral toxic application in organotypic cultures to study dopaminergic cell survival and regeneration in the substantia nigra provided by GDNF and BDNF. We aimed to unravel whether the internal controls generated by this method could provide unbiased estimates of their effects on cell survival following toxic stimulation, and hence serve as a reliable model for PD using other toxins and survival factors.

Methods: Organotypic cultures were generated from the ventral mesencephalon of Wistar rats (P5), and kept for 8 days in vitro (DIV). After 2 days, slices were unilaterally exposed to 200 μm 6-OHDA or saline at three sites of each hemisphere by means of glass electrodes and air force. Slices were exposed to 100 ng GDNF, BDNF or both prior to toxic application (DIV 0), simultaneously as the toxin (DIV 2) or 24 hrs after the toxic exposure (DIV 3). Effects of the toxin and neurotrophic factors were evaluated as dopaminergic cell survival, regeneration of these cells, and enhancement of the phenotypic expression, as measured quantitatively by TH-positive cell counts and TH mRNA levels, and qualitatively by confocal imaging of TH, VMAT2 and MAP2ab labeling, and electron microscopy of TH labeling.

Results: Organotypic cultures with unilateral application of 6-OHDA provided ipsilateral cell death without diffusion to the contralateral hemisphere, thereby generating an internal control. Cytoprotection promoted by neurotrophic factors showed that pre-, co-, or post-treatment with BDNF, GDNF or both significantly protect dopaminergic cells in the SN from unilateral cell death induced by 6-OHDA, promoted dendritic sprouting as well as an upregulation of the dopaminergic phenotype, all restricted to the hemisphere exposed to the toxin. The effect was more pronounced with BDNF alone, particularly when provided prior to the toxic exposure

Conclusions: In this study, we have taken advantage of a unilateral toxic application technique in an organotypic culture model of PD, and enabled a precise comparison of cytotoxic and cytoprotective effects on dopaminergic cells within its micro-anatomical network, not confounded by rostro-caudal variations. This technique may serve as a validating step prior to *in vivo* studies with unilateral stereotaxic surgery when modeling PD and studying cell survival.

<u>Paper II:</u> Targeted deletion of AQP9 protects against MPP^+ induced loss of nigral dopaminergic neurons in mice

Background: It is well established that neurotoxins, such as MPTP, can induce selective cell death of dopaminergic cells in the SNpc, thereby causing PD (73-76, 363). How these toxins selectively target and enter the cells, however, remains to be elucidated. AQP9 is a candidate for such toxin transportation, given is selective expression in catecholaminergic neurons as well as its broad permeability range, including neurotoxins correlated with PD (10-14). Its role in the selective vulnerability of dopaminergic cells in the SNpc and hence PD remains to be established.

Aims: We aimed to investigate whether AQP9 is permeable to MPP+. Further, we aimed to determine whether *Aqp9*$^{-/-}$ mice are less susceptible to dopaminergic cell death in the SNpc caused by MPP+ than WT littermates by utilizing our previously developed organotypic culture model, and by establishing a corresponding *in vivo* mouse model for unilateral and intrastriatal injections of MPP+ to cause dopaminergic cell death in the ipsilateral SNpc with minimal mortality rates.

Methods: MPP+ permeability was evaluated by exposing *Xenopus* oocytes injected with *Aqp9* cRNA to radioactivity-labeled ^{3}[H]MPP+ as well as the control agent ^{14}[C]urea. Control oocytes were either un-injected or injected with *Aqp4* cRNA. Nigral susceptibility to MPP+ exposure was then evaluated in *Aqp9*$^{-/-}$ and WT littermates in an *in vitro* organotypic culture model of PD with unilateral injections of MPP+, as well as in an *in vivo* model with unilateral, intrastriatal injections of MPP+. Dopaminergic cell death in the SNpc, SNpr and VTA was analyzed by stereological quantifications of corresponding sequential sections through the SN, using the contralateral side as an internal control. Indirect measurements of dopaminergic cell death included motor function assessments, HPLC analysis of DA and its metabolites as well as qRT-PCR analysis of potential up- or downregulation of relevant genes.

Results: ^{3}[H]MPP+ and ^{14}[C]urea permeated AQP9 oocytes to a significantly higher degree than AQP4 oocytes. Functional AQP4 oocytes were ensured by increased uptake of radioactively labeled water compared to non-injected oocytes. Using organotypic cultures, we demonstrated reduced dopaminergic cell death in the ipsilateral SN following unilateral exposure to MPP+ in AQP9 deficient mice compared to WTs. Further, we established an *in vivo* model of PD where unilateral intrastriatal injections of MPP+ caused ~65% cell death in the ipsilateral SNpc without affecting the VTA, and with <15% mortality rate. Using the contralateral hemisphere as an internal to control in stereological quantifications, we demonstrated significantly reduced dopaminergic cell loss in the SNpc of *Aqp9*$^{-/-}$ compared to WT littermates, with ~46% and ~65% cell death, respectively. Furthermore, *Aqp9*$^{-/-}$ performed better on motor behavioral test, showed higher levels of DA and its metabolites in the ipsilateral striatum, and no compensatory up- or downregulation of dopamine transporters, dopamine receptor 2, or antioxidants.

Conclusions: We conclude that AQP9 is permeable to MPP+, and that deletion of *Aqp9* results in reduced selective loss of TH-positive neurons in the SNpc in MPP+ animal model of PD *in vitro* as well as *in vivo*. These results show that AQP9 contributes to the selective vulnerability of dopaminergic cells in the SNpc seen in PD by serving as a gateway for toxins into these cells.

<u>Paper III</u>: Reduced age-dependent dopaminergic cell loss in AQP9-deficient mice

Background: Aging is by far the most prominent risk factor of PD, with an exponentially increasing incidence rate after 50 years (5, 6). As for PD, normal aging is associated with reduced motor function and selective loss of dopaminergic cells in the SNpc, although far less severe (109, 110). As clinical symptoms of PD are not present until 60-80% of the dopaminergic cells are lost (104, 105), similar pathological mechanisms may mediate aging and PD to induce selective cell loss. Although the cause for this cell loss remains unknown, it is widely accepted that exogenous as well endogenous toxins contribute to the pathogenesis (79-83, 152-154, 156, 157). We have previously demonstrated that AQP9 is selectively expressed in dopaminergic cells in the SNpc, is permeable to neurotoxins known to induce PD, and that mice deficient in AQP9 show reduced loss of dopaminergic cells in the SNpc following exposure to MPP+. Whether AQP9 can influence dopaminergic cell death in the SNpc in normal aging, however, remains to be determined.

Aims: We aimed to unravel whether AQP9 is involved to age-related dopaminergic cell death in the SNpc.

Methods: To investigate whether AQP9 is involved in age-related dopaminergic cell loss in the SNpc, we used unbiased stereological quantifications of tyrosine hydroxylase immunoreactive cells (TH-ir) in the ventral midbrain, where dopaminergic cell density was estimated from corresponding sequential sections through this region. ROIs for the SNpc, SNpr and VTA were delineated for each section to account for morphological heterogeneity along the anterior-posterior axis, which enabled evaluation of the density at specific points along the axis. The density of dopaminergic cells was quantified in the SNpc, SNpr and VTA of *Aqp9*$^{-/-}$ and WT mice of different age groups, 6 months and 12 months.

Results: A significant difference was found between TH-ir cell densities in the SNpc of *Aqp9*$^{-/-}$ and WT mice for older animals (12 months), showing a less dense compacta in WT mice. This pattern was also evident in the VTA, but not in the SNpr. For 6 months old animals, no difference was observed between the genotypes in any of the regions. When investigating dopaminergic cell loss along the anterior-posterior-axis of the SNpc, we found a significantly higher TH-ir cell loss in the anterior part. Here, a significant difference was seen between *Aqp9*$^{-/-}$ and WT mice of 6 months, and a very strong trend is present between the genotypes in the 12 month group. No significant differences were observed between any groups in the posterior SNpc.

Conclusion: We conclude that the density of dopaminergic cells is reduced in the SNpc as well as in the VTA with aging. Further, we conclude that this cell loss is mediated by AQP9, as animals lacking this channel show less dopaminergic cell loss than WT littermates with increasing age. The AQP9-mediated cell loss is more pronounced in the anterior part of the SN than the posterior part, evident at 6 months as well as 12 months.

Paper IV: Subcellular expression of aquaporin-4 in substantia nigra of normal and MPTP-treated mice

Background: The selective vulnerability of dopaminergic neurons in the SNpc in PD has mainly been investigated by searching for inherent mechanisms of these neurons. The astrocytic microenvironment surrounding these cells, however, has been largely neglected, although these cells are crucial for the maintenance of homeostasis as well as active regulation of the environment via gliosis (292). It is demonstrated that the number of astrocytes in the SNpc is particularly low (182), although the phenotypic expression of these astrocytes is not well described, including the expression of AQP4. This aquaporin has been shown to be implicated in several chronic neurodegenerative diseases, where up- and downregulation as well as loss of endfoot polarization have been described (342, 343, 345, 346, 348-350). The expression pattern of AQP4 in PD remains largely unexplored, however.

Aims: In this paper, we aim to characterize the expression of AQP4 in the SN under physiological conditions. Further, we aim to investigate its expression pattern in the SN in an MPTP model of PD.

Methods: PD was induced in a subacute MPTP model, where mice receive four systemic injection of MPTP or saline every 2 hrs in one day. AQP4 expression in the SNpc of MPTP and saline treated controls, as well as untreated animals, was investigated by means of quantitative immunogold electron microscopy and qualitative immunofluorescence confocal microscopy.

Results: Under physiological conditions (untreated animals), we found a significantly higher expression of AQP4 in the SN compared to the cerebral cortex by means of quantitative immonogold labeling. The labeling was highly pronounced in perivascular membranes as well as in the non-endfeet domains corresponding to the neuropil, in contrast to the neocortex where the labeling was more restricted to perivascular membranes. This pattern was supported by confocal images. MPTP treatment in a subacute model of PD induced significant loss of TH-positive cells in the SN, as compared to saline controls. The loss was accompanied by a significant upregulation of AQP4 expression, evident in perivascular and perineural membranes, as well as in the neuropil, as revealed by quantitative immunogold labeling and confocal imaging. Moreover, significant upregulation of AQP4 was also seen in the neocortex following MPTP treatment, although not evident by confocal imaging. Finally, MPTP-treated mice showed a considerable increase in the level of GFAP compared to untreated mice as well as saline controls, as shown by confocal imaging.

Conclusion: We conclude that AQP4 is highly expressed in the SN under physiological conditions, and that the expression is pronounced in both perivascular membranes and the neuropil. Further, we conclude that this AQP4 expression is significantly upregulated in a subacute model of PD, following the same pattern of expression, with increased expression around microvessels, perineural membranes as well as in the neuropil, in addition to increased expression in the neocortex. The upregulation is accompanied by astrogliosis in the SN.

<u>Paper V</u>: Reduced dopaminergic cell loss in AQP4-deficient mice subjected to intrastriatal injections of MPP+

Background: We have previously shown a significant upregulation of AQP4 in a subacute model of PD, indicating that AQP4 may be involved in the pathogenesis of PD. Several studies on chronic neurodegenerative diseases have linked AQP4 to neuroinflammation (342, 343, 345, 346, 348-350, 364, 365), and MPTP models of PD have shown increased dopaminergic cell death and elevated activation of inflammatory factors in AQP4 deficient mice compared to controls (352-355). However, the link between AQP4 and PD requires further experimental support in order to demonstrate whether AQP4 plays a beneficial or detrimental role in PD.

Aims: In this study, we aim to confirm our results from paper IV by investigating the expression of AQP4 in PD in an acute MPP+ model of PD. Further, we aim to unravel whether AQP4 plays a beneficial or detrimental role in PD in terms of dopaminergic cell death.

Methods: We used our previously established acute model of PD with unilateral intrastriatal injections of MPP+ to model PD, and saline injections as controls. Expression of AQP4 was evaluated by confocal imaging, quantitative immunogold electron microscopy, and qRT-PCR. The beneficial/detrimental role of AQP4 in PD was determined by using *Aqp4*$^{-/-}$ animals and WT littermates in this model, and analyzed by means of stereological quantifications of TH-ir cell density in the SNpc, SNpr, and VTA, according to the method established in paper II and III. Indirect measurements of the role of AQP4 were carried out through behavioral studies on motor function in these animals.

Results: We found significantly increased upregulation of *Aqp4* mRNA in the ipsilateral hemisphere of WT mice following unilateral treatment with MPP+, not present in saline controls. The pattern followed a similar but un-significant trend on protein level, with upregulation in the neuropil compared to the contralateral hemisphere and saline controls, and to a less extent in perivascular and perineural membranes, as quantified by immunogold labeling. The pattern was not present in the VTA. The results were confirmed by confocal imaging, which further indicated increased GFAP expression following MPP+ treatment. Further, *Aqp4*$^{-/-}$ animals showed significantly reduced dopaminergic cell loss in the SNpc compared to WT littermates, with ~40% and ~64% TH-ir loss, respectively, not present in the VTA or in saline controls. Behavioral assessments showed no difference in motor function between the genotypes, but a significant difference between animals injected with MPP+ and saline controls, thus verifying our behavioral tests.

Conclusions: We confirm that AQP4 mRNA and protein is upregulated in the SNpc also in an acute model of PD. The upregulation is seen in the neuropil and to a less extent in perivascular and perineural membranes, and is paralleled by astrogliosis. Further, upregulation of AQP4 mediates the selective death of dopaminergic neurons in the SNpc, as deletion of *Aqp4* significantly reduced cell death in this area. We conclude that AQP4 may play a detrimental role in PD.

METHODOLOGICAL CONSIDERATIONS

Modeling PD: From *in vitro* Organotypic Cultures to *in vivo* Stereotaxic Injections

Although *in vivo* experiments most closely resemble the effect of the experimental interventions, a general issue related to these experiments is lack of control of undefined variables that may confound the results, given that the entire organism is affected. When modeling PD *in vivo*, genetic mutations may influence the expression of related proteins, as discussed below, or the introduction of toxins may affect other cell types or brain as well. *In vitro* models offer the potential to study the isolated effects of these interventions but eliminate the possibility to evaluate the effect of the intervention in a biological system. In the studies presented in this thesis, we have followed a path from *in vitro* to *in vivo* studies to account for these potential caveats.

Organotypic slice cultures present a merge between *in vitro* and *in vivo* studies, as the three-dimensional cytoarchitecture of the region of interest is kept intact. The preserved neuron-glial interaction provides a microenvironment that facilitates cellular differentiation and enables long-term survival of neurons, making the method ideal for studies of cell death and survival (356, 357, 360). Nevertheless, axotomy presents a limitation to the method, as most striatal dopaminergic innervations are established already from embryonic day 17, but continues three weeks postnatally (366). On the other hand, studying an age-related disease in neonatal animals brings forward a question of validity. Thus, early postnatal animals (P5) are thought to represent a compromise between established cytoarchitecture and axotomy. A second limitation when using organotypic slice cultures is the pronounced variation in dopaminergic cell density along the rostro-caudal axis. This inter-slice variation may confound the results when comparing experimental slices to control slices. We have overcome this obstacle by developing a model for unilateral application of toxins, thereby generating an internal control (i.e., the SN on the contralateral side) that increases the reliability of the model (358, 367). Here, we have applied this model to investigate dopaminergic cell death and survival in the SN (paper I), and exploited this study as a methodological platform for further *in vivo* investigations (paper II). The refined organotypic culture method closely resembles *in vivo* stereotaxic models where toxins are unilaterally injected into the striatum or the SN to produce ipsilateral lesions for comparison to cell survival in the contralateral hemisphere, and hence increases the representativeness of the model.

Transgene Animal Models

Transgene animal models represent a vast benefit for functional studies on proteins, as deletion or insertion of selective genes enable studies on the isolated effect of the protein knocked out or in. Knock-out (KO) models have further enabled validation of antibodies by introducing negative controls, thereby improving antibody-based techniques substantially.

In this thesis, *Aqp9*$^{-/-}$ was generated by replacement of neomycin phosphotransferase expression cassette in exon 2 (276), whereas for *Aqp4*$^{-/-}$, a flippase recognition target (FRT)-neomycin-FRT-LoxPD validated cassette was inserted downstream of exon 3, and a LoxP site was inserted upstream of exon 1 (368). In

paper II and paper IV, we confirm that the KO-animals are devoid of *Aqp9* and *Aqp4* mRNA (<0.2 copy numbers relative to saline contralateral reference gene), respectively, by means of qRT-PCR. This is particularly crucial for validation of paper II and III, as antibodies against AQP9 results in unspecific labeling. Replacement of a sequence in one of the initial exons makes it highly unlikely that any functional form of these proteins can be translated, correctly folded and integrated into any membrane. Thus, it is doubtful that the mutated proteins can fulfill their normal functions as integral membrane proteins to any degree. Nevertheless, the *Aqp9* promoter and regulatory sequences are likely to be intact in the *Aqp9*$^{-/-}$ animals, although the levels of the mutated transcript have not been examined quantitatively in the original publication (276). Moreover, as the brain is a plastic organ, compensatory mechanisms may counterbalance the loss of proteins by upregulating other proteins with similar functions. In the context of transgene brain aquaporin models where *Aqp4* or *Aqp9* are knocked out, it cannot be excluded that other brain aquaporins are upregulated to compensate for the loss of function or that other channels are upregulated in a similar way as formation of astrocytic connexins are increased following *Aqp4* knockout, as shown by Katoozi *et al*. (369). Another possibility is that other proteins may be co-downregulated with the knockout of the target proteins (370). Indeed, siAQP4 treatment in astrocyte cultures has been shown to induce a slight but not significant decrease in AQP9 expression (277). Such effects may mask or bias the results in a systematically manner. Regardless, transgene animal models offer an invaluable way to study the function of a protein of interest.

Neither *Aqp9*$^{-/-}$ nor *Aqp4*$^{-/-}$ mice exhibit phenotypic characteristics, as both show normal development, survival, weight and function as well as gross brain anatomy (276, 292, 311, 312), unless they are stressed. The lack of gross phenotypic disturbances after knocking out a protein may question the physiological importance of the target protein. It is not unlikely that brain aquaporins mainly play a role in face of a stressed environment, such as disrupted water and energy balance or head trauma, which is in line with human mutations in *Aqp1* and *Aqp2* that did not produce any major phenotypic disturbances besides reduced urinary-concentrating functions (371, 372). Nevertheless, less evident alterations do in fact occur following knockout of these genes, such as retinal abnormalities and hearing deficits, postnatal retention of water as well as enlarged extracellular space in *Aqp4*$^{-/-}$ (21, 164, 373), demonstrating the widespread consequences of knocking out single genes. The fact that aquaporins are highly conserved throughout evolution clearly stresses their importance (21, 237, 238).

It is crucial to bear in mind that the expression of aquaporins may differ from rodents to humans, thus questioning the translational validity of the knockout models used in our studies. AQP4 has been reported to be more abundant in humans than in rodents (374), and the relative number of astrocytes increases dramatically with phylogeny and brain complexity (178), along with the complexity of these cells (175). Expression of AQP9 also shows differences between species, with higher expression in the rat brain compared to mice, and more pronounced expression in primates, both on mRNA and protein level (15, 278). To date, however, brain AQP9 has not been described in humans. Notably, both AQP4 and AQP9 follow the same pattern of distribution but with a more pronounced expression in primates, indicating that studies on brain AQP4 and AQP9 can be translated into humans and that they are highly relevant.

Animal Models of Parkinson's Disease

Translating animal models of PD into the human disease opens an important question of validity. This can primarily be attributed to the fact that rodents do not develop PD naturally. It is crucial to remember that none of the animal models used to model PD are complete animal models of PD, meaning that neither the pathogenesis nor clinical symptoms can be completely reproduced in rodents. Instead, they mimic specific features of PD, and are rather models of parkinsonism. Further, PD is a chronic disease that develops over decades, whereas models of PD induce dopaminergic cell loss in a day, or sometimes weeks or months. These rather acute models potentially bring other pathological components along, such as widespread inflammation and a necrotic cell death rather than apoptosis, potentially displaying a skewed picture of the pathogenesis. Nevertheless, the effects of these models can be studied separately to elucidate basic mechanisms.

Several models can be used to mimic PD, including monogenic mutations and neurotoxins known to cause parkinsonism. Although several monogenic mutations produce a reliable induction of dopaminergic cell death in the SNpc, familial PD only accounts for 5% of cases (51) and does not reflect the typical nature of the disease. A specter of toxins is used to model PD, in which MPTP, 6-OHDA and rotenone are the most common. The pesticide rotenone is highly lipophilic and readily crosses the BBB, where it induces dopaminergic cell death in the SN by blocking mitochondrial complex I (87, 88). However, reports on selectivity for neurotoxicity are variable (88).

6-OHDA Model of PD

Historically, 6-OHDA has been extensively used to model PD. This DA analogue does not cross the BBB, but is readily taken up by DAT when administered intracerebrally. The toxin induces catecholaminergic cell death by generation of ROS via intra- and extracellular auto-oxidation, hydrogen peroxide formation induced by MAO activity or direct inhibition of the mitochondrial respiratory chain (375). Interestingly, 6-OHDA has also been demonstrated as a physiological endogenous neurotoxin (156, 375). Although 6-OHDA produce dopaminergic cell death in the SNpc, it is also shown to affect the ventral tegmental area (376).

MPTP Model of PD

Since the discovery in the 1980s that MPTP causes parkinsonism in humans (73, 74), non-human primates as well as various other mammalian species, this toxin has been extensively used as a model of PD (75-77). MPTP selectively targets dopaminergic neurons in the SNpc. Studies on the mechanisms of MPTP have revealed that it acts as a lipophilic pro-toxin that crosses the BBB and is converted to the toxic metabolite MPP+ in astrocytes and serotonergic neurons (377) by MAO-B (378). MPP+ is then released into the extracellular space and accumulated by DAT into dopaminergic neurons (379), causing a

bilateral lesion of the nigrostriatal tract. MPP+ finally produces neurodegeneration through blockade of complex I and III of the electron transport chain (78).

In our *in vivo* studies, MPTP was administrated in a sub-acute model, where mice received four subcutaneous injection of MPTP every second hour for one day or control injections of saline. The evident benefit of MPTP administration is the induction of selective dopaminergic cell death in the SNpc almost identical to that seen in PD (105), where the VTA remains largely unaffected (380, 381), and degeneration of dopaminergic nerve terminals is greater in the putamen than in the caudate nucleus (382). These features were also evident in our model. However, it should be noted that MPTP shows affinity, although much lower, for DAT located on other cell groups as well as other monoamine receptors (228, 383) potentially inducing non-specific cell death, particularly in acute models (228, 384). As the sections for immunogold quantifications of AQP4 are only labeled with this antibody, we cannot rule out that unspecific cell loss has occurred in our model and that AQP4 is upregulated around these cells and included in our analysis. Nevertheless, as the sections are obtained from the substantia nigra it is highly unlikely that other monoaminergic cell groups are included. Another benefit of the MPTP model is that it opens for the ability of administrating multiple injections to better mimic the degenerative process seen in PD, although not comparable to the length of degeneration in PD. Using multiple injections, we potentially decrease an acute toxic reaction causing less specific cell death (381). However, a chronic model with implanted osmotic pumps delivering constant small amounts of the toxin would have been a more optimal model to mimic the disease (385). Despite prolonged administration, however, MPTP does not generate Lewy bodies (76), although it has been described in a few cases (386, 387). In line with this, intracytoplasmic inclusions were not observed in our model. Although a pathological hallmark, it should be noted that LBs are not evident in all cases of PD and thus not a criteria.

One of the main drawbacks of MPTP is its induction of highly variable results due to variable susceptibility rates with age, gender, strains and vendors (77, 381, 385, 388-391). Furthermore, MPTP is associated with a high mortality rate, sometimes reaching 90%, which mainly can be attributed to cardiac failure from the systemic injections (385). In our model, we observed a mortality rate of ~50%. The systemic injections further induce a bilateral damage, which reduces the animal's ability to move and thus eat. Finally, from a technical point of view, working with MPTP requires strict safety procedures as it readily crosses the BBB.

The high mortality rate as well as the lack of an internal control due to the bilateral damage encouraged us to directly infuse the active metabolite MPP+ intracerebrally to model PD.

MPP+ Model of PD

For inducing cell death in the SNpc, we chose a model of retrograde neurodegeneration by targeting the striatal nerve terminals. To mimic the pattern of neurodegeneration as it occurs in PD, with more severe dopaminergic cell loss in the ventral tier (50), we targeted the dorso-lateral striatum corresponding to the putamen, the input area from the ventral tier of the SNpc (3) (Figure 1). It should be noted that there is no clear separation between the caudate nucleus and putamen by capsula interna in rodents, however, the topographical projection pattern is maintained in a comparable manner (392). 95% of the projections are

ipsilateral, leading to unilateral cell death in the ipsilateral hemisphere, while the contralateral hemisphere represents an internal control. We cannot exclude, however, that the remaining 5% of the nigrostriatal fibers affect the contralateral hemisphere. Thus, controls receiving injections of saline were included. These animals also controlled for a potential mechanical disruption of the nigrostriatal pathway caused by the intrastriatal injection. An alternative way would be to target the SN directly, but his method risks severe tissue destruction in the target area, thereby confounding the results. A final option is to target the medial forebrain bundle. However, this method is better suited to model the nigrostriatal pathway rather than dopaminergic neurons *per se* (393).

Using the MPP+ model, we decreased the mortality rate to <15% and produced a relatively stable dopaminergic cell loss around 65% despite variations in age and gender. Intensive post-operative care, with daily injections of analgesia, saline and glucose further contributed to an increased health condition of the animals. Animals were able to move around in the cage as the motor problems were confined to one hemisphere, which also enabled precise quantification of motor dysfunction in standardized behavior tests. In this model, we showed that the dopaminergic cell loss was primarily confined to the SNpc, without affecting the VTA. Furthermore, the contralateral hemisphere was used to accurately quantify the dopaminergic cell loss within each animal, while lack of differences in saline controls verified the model by excluding potential damage caused by the mechanical injury as a potential dependent variable. A dopaminergic cell loss of ~65% corresponds to the clinical onset of PD, where the symptoms become apparent. Our model thus mirrors an early phase of the disease. This relatively low degree of cell death may be attributed to the concentration of the injected toxin of 7.5 µg. As higher doses produced higher mortality rates, we kept the concentration at a minimum while keeping the dopaminergic cell loss within an acceptable range. A potential way to increase the cell death without increasing the dose would be to keep the animals alive for a longer period, from 2-3 weeks. In this regard, it should be noted that it has been reported that the dopaminergic cell loss is temporal in rodents, in contrast to permanent loss of these cells seen in primates (394, 395).

A limitation of the MPP+ model is the acute damage caused by a single injection, which does not resemble the progressive degenerative process in PD. This acute and coarse model may potentially mask some of the effects produced by a chronic and progressive degeneration. Although we have controlled for mechanical tissue destruction by pure striatal saline injections, the toxin itself produced massive tissue destruction in the striatum, potentially interfering with striatal functions related to both motor and limbic functions, in addition to generating widespread inflammation and astrogliosis, as indicated by gross macro-anatomical destruction in the ipsilateral striatum, neocortex and white matter, and upregulation of *Gfap*. Thus, widespread striatal tissue destruction might interfere with our behavior studies, where our results likely reflect striatal tissue damage to a similar degree as nigral dopaminergic cell loss. Loss of nigrostriatal terminals likely account for the ipsilateral turning behavior observed following apomorphine administration, as the dopaminergic nerve terminals are lost and cannot be super-sensitive to stimulation by a DA agonist (396-400). This is supported by our qRT-PCR-results showing very low levels of *D2* in the injected hemisphere for both genotypes. Other genes in the qRT-PCR analysis further reflect striatal damage in the injected side, including upregulated *Bax* and downregulated *Bcl-2*, indicating apoptosis. Finally, our HPLC analysis is mainly based on measurements of striatal DA and its metabolites. It is not unlikely that our results do not reflect the true nigral dopaminergic cell loss, as the effect is masked by striatal damage. Finally, damage to striatal axons may itself induce retrograde cell death, thereby inducing

unspecific dopaminergic cell loss in the SN. In conclusion, our behavior studies, qRT-PCR- and HPLC results must be interpreted with caution.

Using MPTP/MPP+ in mice, significant species differences in regard to MPP+ sensitivity must be taken into account. Mice are far less susceptible to MPP+ than primates, which again are less susceptible than humans (385, 401). Thus, unknown underlying mechanisms render humans more susceptible to PD-toxins, possibly contributing to the lack of development of PD in rodents. Moreover, it has been demonstrated that MPP+ in rodent but not primates generates a temporal reduction of the dopaminergic phenotype, expressed as loss of TH, without permanent loss of dopaminergic neurons (394). Due to our relatively short treatment span before euthanization of animals and the lack of TH/Nissl co-localization stains, we cannot exclude this possibility.

In general, the MPTP/MPP+ model of PD is the model that most reliably reproduces features of human PD. Chronic infusion of MPTP by osmotic pumps most closely resembles the disease, but does not provide an internal control in one hemisphere. Regardless of how the toxin is introduced, it is important to keep in mind that rodents are significantly less sensitive to MPP+ than other mammals and primates. This indicates that unknown physiological and/or pathophysiological mechanisms are separating their degenerative nature from ours.

Immunocytochemistry, Confocal Microscopy and Electron Microscopy

In order to determine the pattern and amount of dopaminergic cell loss, distribution and upregulation of AQP4, it was crucial to identify the location and abundance of these proteins. For these purposes, we used 3,3'-Diaminobenzidine (DAB) immunocytochemistry for bright field light microscopic analysis, immunofluorescence for confocal microscopy, and immunogold cytochemistry for electron microscopy. While light microscopy permits visualization on a cellular level, electron microscopy increases the resolution considerably and enables visualization of subcellular compartments. These techniques are all based on immunocytochemistry, which use antibodies directed against specific epitopes in order to detect proteins of interest. Using indirect immunocytochemistry, in which the primary antibody is connected to a species-specific secondary antibody, the sensitivity and strength of the signal increases, and has therefore been done in the present studies. The secondary antibody is covalently attached to a signal molecule, such as DAB, a fluorescent dye or a gold particle, thus enabling visualization in a bright field microscope, confocal microscope or electron microscope, respectively.

Antibody-based techniques offer an invaluable tool for detection and visualization of specific proteins *in situ*. However, certain limitations follow these methods, primary linked to specificity. Although antibodies are designed to target specific epitopes in a protein of interest, the tissue fixation process may crosslink proteins of interest to neighboring molecules, resulting in unspecific binding or lack or binding, causing false positives or negatives, respectively. Antibodies may also cross-react with other similar epitopes to create unspecific binding. This issue is a problem for antibodies targeting brain AQP9. Thus, this protein was not analyzed by immunohistochemical methods in our studies. For AQP4, this potential problem was addressed by including *Aqp4*$^{-/-}$ mice, and the specificity of the antibody against TH is

already well documented. The specificity of the secondary antibody was verified by including sections where the primary antibody was omitted.

Immunocytochemistry analyzed by confocal or bright field microscopy provides a means to detect the location and relative abundance of specific proteins on a cellular level. Immunofluorescence enables labeling of several proteins within the same section, making it possible to visualize the localization of proteins in relation to each other. We took advantage of this when characterizing the expression of AQP4 in relation to dopaminergic cells and astrocytes, visualized by TH and GFAP, respectively. A common problem with this technique, however, is photo-bleaching due to photochemical destruction of a fluorophore, thus limiting the available time to visualize the section. The problem of photo-bleaching is bypassed by DAB-staining. This method is thus ideally suited for stereological cell quantifications, where the sections are illuminated over longer time intervals. However, one (maximally two) protein(s) of interest can be stained using this method, making it difficult to visualize the protein of interest in its context.

In paper IV and V, we aimed to characterize and quantify the distribution and subcellular location of AQP4 before and after induction of dopaminergic cell loss in two models of PD. For this purpose, we used electron microscopy, which is a powerful tool for obtaining precise topological data. By applying contrasting agents, it is possible to distinguish subcellular compartments. Furthermore, the localization of single molecules can be determined by means of immunogold cytochemistry, where colloidal gold particles are coupled to secondary antibodies. As the gold particles are electron dense, they appear as circular dots that are easy to localize and quantify. However, lateral resolution must be considered, as the distance from the center of a gold particle of 15 nm to the target is ~30 nm due to the primary-secondary complex (402), reducing the preciseness of the localization. In addition, the complex may potentially block free underlying epitopes, thereby masking some of the target proteins. As we aimed to quantify the expression of AQP4 at specific locations, the tissue was post-embedded in resin, only enabling detection of antigen molecules on the surface of the tissue. Consequently, the antibodies will have equal access to epitopes in different compartments, making this method ideal for quantification compared to pre-embedding. However, this surface-restricted staining explains why immunogold microscopy requires higher antibody affinity than light microscopic methods, as the latter allow the antibodies to diffuse into the whole tissue specimen. Using the well-characterized and widely used antibody against AQP4, this issue did not present a problem in our studies. Finally, the resin Lowicryl was chosen due to its good preservation of antigens (403), further optimizing the quantification. However, the post-embedding technique is subject to unspecific labeling. To control for this phenomenon, *Aqp4*$^{-/-}$ animals were used as negative controls.

Despite the clear advantages when identifying the location of specific proteins, the high magnification power also represent a navigational challenge within the tissue. In our sections, this problem is evident when trying to distinguish between the sub-regions of the ventral midbrain. As our tissue was not double-labeled with TH, it was not possible to discriminate dopaminergic neurons in the SNpc and VTA from GABAergic neurons in the SNpr. However, double-labeling with TH would not enable discrimination between SNpc and VTA. Due to this navigational problem, our quantifications of AQP4 are rather an effect of the ventral midbrain than the SNpc in paper IV. In paper V, we optimized the protocol by dissecting out specific sub-regions of the ventral midbrain prior to embedding; the SNpc, SNpr and VTA.

Still, however, we cannot completely rule out that other neuronal types are included as the dissections are made by hand. In conclusion, supplying this method with light microscopy is crucial to complete the total picture, as we have done in both paper IV and V.

Stereological Cell Quantification

Stereology is method for performing systematic, random sampling of a region of interest (ROI) to provide unbiased and quantitative data within that region. The method enables estimation of cell density in a three-dimensional volume using two-dimensional serial tissue sections.

The two commonly used methods for unbiased number estimation in neuroscience are the optical fractionator method (404) and the VRef xNV method (405). The optical fractionator is considered to be the gold standard because it does not require estimates of the global volume of the ROI (404) , and the estimates obtained have been found to be more precise than corresponding estimates obtained with the VRef xNV method (406). In addition, when tissue shrinkage is a significant factor, which is the case following staining and dehydration, the optical fractionator is preferred to the VRef xNV method, which is sensitive to shrinkage effects (407).

Optical Fractionator uses thick sections (15-30 µm post-processing) and estimates the total number of cells from the number of cells sampled with a Systematic Randomly Sampled (SRS) set of unbiased virtual counting spaces covering the entire region of interest with uniform distance between counting spaces in directions X, Y and Z. The cell counts are unbiased in that they are not influenced by the size, shape, spatial orientation, and spatial distribution of the cells under study.

Using this method, the tissue must be thick to allow many focal planes through the dissector and to permit guard zones to avoid sampling of cutting artifacts. Thus, our tissue sections of the midbrain were cut at 40 µm to due to shrinkage to ~25 µm following tissue processing. We choose every 3rd section for a sequential representation of the SN. In paper III, 6 sequential sections were included in the analysis (Bregma -3.08 to -3.68), excluding the peripheral and less dense regions of the SN. As significant differences were found in the cell density along the anterior-posterior axis in this study, we decided to include 10 sections to cover the entire nigra (Bregma -2.70 to -3.80) in paper II and V, while potentially increasing the statistical power. From paper III to paper II and V we further optimized the method by reducing the size of the counting frame as well as the SRS grid layout to better cover the central parts of the ROIs. The counting frame was estimated to include a recommended cell number of 1-6 cells in the SNpc. Although optimal for the SNpc, the frame size was not ideal for the SNpr, containing too few cells, or for the VTA, containing too many cells. A potential solution could have been to adapt each frame to each ROI, which would have been a time-consuming procedure. Using this sampling procedure, we estimated a total number of ~3000 TH positive neurons/hemisphere of WT control hemispheres with Gundersen's coefficients of errors <10%, indicating low variance. These numbers are in line with the literature, which shows a range between 5200-23,670 TH+ neurons bilaterally, with a mean value of 12,000 neurons (408). This wide range indicates that the underlying method highly affects the quantifications, and that the ratio between the two hemispheres is a more accurate measurement.

An essential principle of the methodological validity in stereology is to find a reproducible way to delineate a brain region of interest. Using anatomical landmarks to identify the SNpc, SNpr and VTA is extremely important as TH stains all catecholaminergic cell groups equally, meaning that the TH-positive cells in the A9 SNpc cannot be separated from the A10 cells in the VTA or the A8 cells in the RRF. It is not likely that other catecholaminergic cells are present and influence our quantification, as the enzymes converting dopaminergic neurons into noradrenergic or adrenergic neurons are not present in this region (34, 35). To separate between these ROIs, the tractus opticus was used as an anatomical landmark in anterior sections, while the medial lemniscus was used in posterior sections. In animals treated with MPP+ (paper II and V), TH-positive cells were largely abolished, and the anatomical landmarks less identifiable. In order to quantify the cell density in this hemisphere using the same volume size, the contralateral hemisphere served as a template and was copied onto the corresponding ipsilateral side, thereby making an internal control. In paper III, the same method was used by choosing one side as the template in order to keep the volume identical between corresponding hemispheres. In the latter study, we did not know which side was left or right. Thus, the quantified density of the two hemispheres was summarized upon statistical analysis.

Another important principle, particularly when studying cell death, is to clearly define criteria for included cells. We included cells in which the nucleus was clearly visible and focused and the cell membrane was intact and clearly separated from the nucleus as well as the extracellular environment. However, both the cut-off for inclusion of cells as well as the definition of ROIs are subject to individual bias. Thus, despite being the most unbiased method of cell quantification, a subjective component is not completely removed.

The most powerful way of increasing statistical power is to increase the *n* rather than the number of sections. In some cases, such as for saline-treated animals (paper II and V) and the aging study (paper III), increasing the *n* could potentially have provided less deviations and more powerful data.

Quantitative Real Time PCR

Quantitative Real Time polymerase chain reaction (qRT-PCR) is an assay for making accurate quantifications of a specific mRNA expression by detecting and amplifying targeted DNA copies in an exponential way. In qRT-PCR, the amplified DNA is detected as the reaction progresses and not only in the end, as for conventional PCR, thereby reducing variability and resulting in a quantitative relationship between the amount of starting target sequence and amount of PCR product accumulated at any particular cycle. For our studies, TaqMan probes were chosen as they are known to increase the sensitivity of detection.

mRNA isolated from tissue was converted to cDNA to prevent degradation using the enzyme reverse transcriptase. The target cDNA was then detected and amplified by two gene-specific primers situated closely together. In addition, a gene-specific TaqMan probe containing a quencher and a fluorescent reporter is targeted to a position between the two PCR primers. Following annealing, polymerization of a new DNA strand is initiated from the primers, and once the polymerase reaches the probe, its 5'-3-

exonuclease degrades the probe, physically separating the fluorescent reporter from the quencher, resulting in an increase of fluorescence. Fluorescence is detected and measured in a real time PCR thermocycler and the exponential increase of the product is used to determine the quantification cycle (C_q) in each reaction. For standardization and internal control, a reference gene is used, such as *Gapdh* or *β-actin*. The amount of mRNA generated from the control housekeeping gene is compared to the target gene, enabling quantification of relative mRNA level. For reliable calculations, it is crucial that the reference gene is stable. Thus, we included several reference genes; *Tbp*, *Gapdh*, β-*actin*, *Hprt1*. Using NormFinder, *Gapdh* and *Hprt1* proved to be the most stable and were chosen for ensuring consistent results. Alternatively, the absolute DNA copy numbers can be quantified, which reflects the exact mRNA copy number. However, this method requires calibration of DNA standard curves to which the target DNA is compared to, which is time consuming and requires that the PCR of the sample and the DNA standard have the same amplification efficiency.

The main advantage of TaqMan is accurate and concurrent fluoremetric quantification of PCR product during PCR. In contrast, conventional RT-PCR includes PCR followed by electrophoretic separation of products and finally quantification of PCR products by densiometric analysis. One possible pitfall upon PCR is amplification of genomic DNA. We have reduced this risk to a minimal by using primers targeting exon-exon boundaries, in addition to pre-treating the RNA samples with DNase. It should be noted, however, that the latter procedure may be deleterious if only small amounts of RNA are present that are partially destroyed as well (409)**.** A second pitfall lies in the PCR step, as one cannot assure that all mRNA are equally reverse-transcribed, quantitatively speaking (410).

Although qPCR is a reliable and unbiased way of measuring the expression of specific genes present in the cell at a particular time point, it is important to keep in mind that the mRNA level of a gene not necessarily reflects its protein level. It has been estimated that only about 40% of cellular protein levels can be predicted from mRNA measurements, as measured by mass spectometry (411-413) as well as *in vivo* (414, 415). Transcription, mRNA decay, translation, and protein degradation are key processes determining steady state protein concentrations (411). The cell can also hold some mRNA in a pool for later use, as a stress response. Generally, abundant housekeeping genes have stable mRNAs and stable proteins compared to transcription factors, signaling genes, chromatin modifying genes, and genes with cell-cycle-specific functions (412). These facts indicate that mRNA results must be interpreted with caution and should be supported by protein measurements.

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a technique used to separate, identify, and quantify specific organic molecules and ions. It is based on a mobile phase, involving movement of a liquid solvent containing the sample mixture through a column by means of high pressure. The column is filled with a solid adsorbent material, known as the stationary phase. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the various components. This leads to separation of the molecules as they flow through the column, which is quantified by a

detector as they exit the column. We used this method to detect the level of dopamine and it metabolites after treatment with MPP+ in the striatum in order to indirectly evaluate the level of activity in the dopaminergic cells in the SN projecting through the nigrostriatal pathway. We also measured the levels in the SN and used the cerebellum as a negative control.

The key benefit of HPLC is its high degree of sensitivity and preciseness, allowing accurate and unbiased quantifications of chemical compounds. The main limitation is its time consuming aspect and the need for large amounts of control tissue to control for the method as well as positive controls (untreated striatum) and negative controls (untreated cerebellum) to control for variations seen between parallel measurements, thus increasing the number of animals considerably (416). Our measurements are further limited by the fact that injections are performed directly into the striatum, causing a massive tissue disruption that likely masks our results. Potentially, inclusion of saline controls could have provided information on damage caused by the needle *per se*. Based on our findings from stereological quantifications, however, cell death is induced by the toxin and not the injection. Further, the measurements from the SN do not provide a true image of dopaminergic cell functioning in the SN, as only a small amount of the projections are internal. Finally, the results from the SN may be masked by inclusion of the entire midbrain in the analysis, also including DA and DA metabolites from the VTA and RRF.

Aquaporin-Expression in *Xenopus laevis* Oocytes and Permeability Measurements by Radio-Isotopes

Due to low endogenous water permeability*, Xenopus laevis* oocytes are widely used as an expression system for aquaporins, achieved by cytoplasmic microinjection of mRNA (236). Oocytes expressing water channels develop a significant increase in osmotic water permeability (417). The oocyte uptake of specific molecules can be measured by radio-isotopic methods, where an atom in a chemical compound is replaced by a radioactive isotope. As radioactive decay is much more energetic than chemical reactions, the radioactive isotope can be present in low concentration and its presence detected by sensitive radiation detectors, thus enabling precise quantification of solute permeability through the expressed aquaporin. In paper II, *Aqp4* or *Aqp9* cRNA was injected into *Xenopus* oocytes, followed by exposure to radioactive water, known to permeate both, radioactive urea, known to diffuse through AQP9, and radioactive MPP+. In addition, naïve control oocytes were included to confirm the lack of baseline permeability of solutes.

Despite the low water permeability of *Xenopus* oocytes, native oocyte expresses many ion channels and transporters. Moreover, the overexpression of exogenous membrane proteins may change the expression of native oocyte proteins. If these proteins are not present in significant amounts in the control oocyte, it may be difficult to determine which properties can be attributed to a heterologously expressed protein and which are due to a change in the activity of endogenous protein (417).

DISCUSSION

PD is characterized by a selective degeneration of dopaminergic cells in the SNpc. The lack of identification of the factors causing the susceptibility of these neurons hinders the development of therapeutic agents that may halt the degeneration, leaving patients with a purely symptomatic treatment. Differential susceptibility of specific subsets of neurons within a region is not a unique feature for the midbrain. Hippocampal CA1 neurons are more vulnerable than CA3 neurons to a variety of adverse conditions, including global cerebral ischemia, toxins, chronic epileptic seizures, aging, oxidative stress and early stages of AD (418-421). Thus, similar neuronal populations with regional proximity may have unique vulnerability profiles. In these cases, cell death cannot be attributed to global or regional effects but is closely associated with local micro-anatomical mechanisms. This thesis has investigated factors contributing to this selective vulnerability of dopaminergic neurons in the SNpc by focusing on two novel target molecules residing in dopaminergic neurons and the surrounding astrocytes; AQP9 and AQP4, respectively.

Selective Vulnerability Mediated by AQP9

Several phenotypic characteristics of the A9 dopaminergic cell group have been pointed out as potential susceptibility candidates, although none of these candidates have solely been shown to account for the susceptibility of the A9 dopaminergic cells in the SNpc. In paper II and III, we provide evidence that AQP9 plays a role in the selective susceptibility of these cells by demonstrating that deletion of this gene results in selectively increased survival of dopaminergic neurons in the SNpc in MPP+ models of PD *in vitro* and *in vivo* (paper II) as well as in normal aging *in vivo* (paper III). Reliable *in vitro* modeling of dopaminergic cell death and survival (paper I) served as a methodological platform for further *in vivo* modeling and understanding of the link between AQP9 and PD.

The involvement of AQP9 in PD and aging must be understood within the context of environmental toxins. Since the discovery of MPTP (73, 74), toxins have been intimately linked to PD, and have further gained support from epidemiological studies showing a correlation between environmental toxins and PD (7-9, 79-83). To date, however, the mechanism to which these toxins selectively targets dopaminergic cells in the SNpc has been unknown. AQP9 has a particularly broad substance permeability, given its wide and less polar pore compared to orthodox aquaporins (248, 250, 256-258), and a lower pore density towards the hydrophobic face than other aquaglyceroporins, allowing permeation of larger and bulkier substances (250). Permeable substances include the toxin arsenite as well as pyridines, the latter of which structurally resemble MPP+. In paper II, we show that the toxin MPP+ also permeates AQP9 in *Xenopus* oocytes expressing AQP9, demonstrating that AQP9 is permeable to toxins directly associated with PD-inducing toxins (Figure 4). We do not know however, the relative contribution of toxin permeability accounted for by AQP9 as compared to other relevant toxin transportation channels, primarily DAT.

The permeability of PD-related toxins through AQP9 is relevant for the selective vulnerability of dopaminergic neurons because AQP9 is shown to be specifically localized in TH-positive neurons in the SNpc. Several immunohistochemical studies have identified the presence of AQP9 in various neurons and

astrocytes, including various groups of catecholaminergic neurons in midbrain and hypothalamic nuclei (16), and astrocytes in both gray and white matter (16, 17, 269, 278). However, these results must be interpreted with caution as studies using *Aqp9*$^{-/-}$ animals have demonstrated an unspecific cross-reaction of AQP9 antibodies with GFAP and an unknown mitochondrial matrix protein (15, 276). The only reliable study demonstrating AQP9 localization in the brain using *in situ* hybridization and qPCR have provided evidence for the expression of *Aqp9* in the SN, as well as hypothalamus and cortex, shown to be translated into protein in the SN in dopaminergic cell membranes and inner mitochondrial membranes by means of immunocytochemical studies on *Aqp9*$^{-/-}$ animals compared to WTs (276). Furthermore, a study using si*Aqp9* in primary astrocyte cultures has provided support for the presence of AQP9 in astrocytes (277), but does not provide any information on regional location. In paper II, we confirm that *Aqp9* mRNA is highly expressed the mouse midbrain and to a lesser extent in the cortex, using qRT-PCR. Moreover, we find a high expression of *Aqp9* mRNA in the striatum, although significantly lower than the midbrain. This pool could reflect *Aqp9* mRNA expression in striatal GABAergic neurons or astrocytes, or *Aqp9* in nerve terminals of dopaminergic cells residing in the SN. Traditionally, the cell body has been considered the exclusive source of axonal protein, but accumulating evidence suggests that local translation may occur in axons (422). This implies that AQP9 may be expressed in nerve terminals and serve as a toxin channel in a similar way as DAT transports DA and MPP+ (379), causing retrograde degeneration of dopaminergic cells in the SNpc. Studies on mRNA localization may determine its exact localization within the striatum. Finally, studies on protein expression are needed to reveal whether striatal *Aqp9* mRNA is translated into protein. Notably, no studies have provided conclusive evidence for the selective presence of AQP9 in A9 dopaminergic neurons, as neither the VTA nor the RRF are studied with proper techniques. Thus, we cannot rule out that AQP9 is expressed in other dopaminergic cell groups in the midbrain or other neuronal or astrocytic populations. Furthermore, reliable studies on AQP9 have only been carried out in rodents, and are not explored in the human brain, raising a question of validity. Given the evolutionary conserved expression of aquaporins (237, 238), as well as the presence of *Aqp9* mRNA in the primate whole brains (278), it is reasonable to assume that a similar pattern of expression is seen in humans. Given the lack of specific antibodies in a vast amount of studies, however, it is crucial to stress that the total picture of AQP9 localization is not yet completed.

Having demonstrated the permeability of MPP+ through AQP9 and the location of AQP9 to the midbrain, we provided evidence that deletion of *Aqp9* resulted in increased cell survival of dopaminergic cells in the SNpc, both in an *in vitro* and *in vivo* MPP+ model of PD (paper II) as well as in aged animals (paper III). In line with the *in vitro* results, the in vivo MPP+ model showed significantly reduced dopaminergic cell loss in the SNpc of *Aqp9*$^{-/-}$ mice, with ~46 % and ~65 % loss in *Aqp9*$^{-/-}$ and WT littermates, respectively, whereas significant dopaminergic cell loss was not seen in the VTA in any of the genotypes. These results provide evidence that AQP9 contributes to the selective cell death of dopaminergic neurons in the SNpc induced by the PD-specific toxins MPP+, thus indicating that AQP9 is involved in the pathogenesis of PD. The reduced cell death in *Aqp9*$^{-/-}$ is further supported by higher levels of DA and its metabolites in the striatum and better motor function in the *Aqp9*$^{-/-}$ animals, in addition to no evident up- or downregulation of relevant genes related to the dopaminergic phenotype, such as *Dat* and *Drd2*. Of notice, we also found significant cell death in *Aqp9*$^{-/-}$ animals, which can be attributed to MPP+ uptake through DAT in both genotypes (379). This feature is of particular importance, as it demonstrates that AQP9 cannot exclusively account for the toxin uptake and subsequent cell death, indicating that AQP9 is one vulnerability feature in a multifactorial pathogenesis. Furthermore, given the location of AQP9 to both neurons and astrocytes

in the SN, we are not able to discriminate the relative contribution of each cell type. Potentially, cell death is not primarily induced by direct neuronal uptake but through secondary mechanisms mediated by astrocytic toxin-uptake.

Having established a contribution of AQP9 to the pathogenesis of PD in toxin-based based models of PD, we aimed to understand the role of AQP9 in dopaminergic cell death in the SNpc in face of the most prominent risk factor of PD, namely aging (paper III). In the absence of toxin exposure, we found an increasing cell death with age in WT controls, with a significantly higher dopaminergic cell death at 12 months but not 6 months in WTs compared to *Aqp9*$^{-/-}$. Significant dopaminergic cell loss in the SNpc with increasing age is in line with a pattern seen in humans (109, 110), primates (112, 113, 115) and mice (114). In elderly individuals without clinically defined PD, a mild to severe neuronal loss within the SN is seen in 1/3 with an estimated rate of 4.7-9.8 % loss per decade (109, 111). Considerable age-related cell loss has also been shown in other dopaminergic populations, including the VTA and the RRF (115, 423), which is not seen to the same extent in PD (106). This is in line with our findings in paper III and II, where we demonstrated a significant age-related decline in the SNpc as well as the VTA in WTs compared to *Aqp9*$^{-/-}$, whereas the dopaminergic cell loss was restricted to the SNpc without affecting the VTA significantly in the MPP+ *in vivo* model.

Further, we found a significant difference in dopaminergic cell loss along the rostro-caudal axis, with higher loss in the anterior part than the posterior in WTs compared to *Aqp9*$^{-/-}$ at both 6 and 12 months, which was not reproduced in the MPP+ *in vivo* model. These results could potentially reflect a difference in the pattern of cell death in the two conditions. More pronounced dopaminergic cell loss has been reported in the caudal part of the SNpc in PD (424), whereas other studies have reported a more uniform cell death along the rostro-caudal axis (104). A similar opposite pattern in PD and aging has been reported for the dorso-ventral axis, in which PD is associated with severe cell death in the ventral tier of the SNpc compared to a more pronounced loss in the dorsal tier in aging (104, 109, 113, 115, 424, 425). However, it is not unlikely that our findings rather reflect a methodological limitation, as the peripheral anterior sections with lower cell density are excluded in this study, whereas the MPP+ *in vivo* study included more animals as well as more sections throughout the SN, thereby increasing the statistical power.

Finally, we found a significant reduction of dopaminergic neurons in the SNpr in both genotypes following MPP+ injections, not evident with increasing age without toxin-application. It has been shown that the sensorimotor striatum located in the dorso-lateral striatum receives input both from the ventral tier of the SNpc and the scarce population of dopaminergic neurons in the SNpr (3, 426, 427) (Figure 1). This striatal region corresponds to our site of injection and may thus induce cell death in these neurons through DAT and/or AQP9, potentially reflecting a PD-specific pattern. Alternatively, it may reflect a side effect of the acute model, which may induce more unspecific cell loss compared to chronic models (228, 363, 384). In regard to the age-related differences between the models, it should be noted that the animals included in the HPLC analysis range from 5-10 months, which may potentially influence our results. As no significant differences were observed at 6 months, animals in the stereological and qRT-PCR analysis (2-4 months) are not expected to show age-related differences between the genotypes.

In conclusion, selective dopaminergic cell death is a hallmark of PD as well as aging. Our findings in paper II and III indicate slightly different micro-anatomical patterns of cell death, which is in line with the

literature (50, 109, 115, 423), but might also reflect side-effects of the acute toxic insult by MPP+ as well as a higher statistical power in the MPP+ *in vivo* model. To date, it is unknown whether there are underlying differences between dopaminergic cell death in aging and PD or whether PD represents an accelerated version of aging. Although early studies pointed out a difference in the micro-anatomical pattern of dopaminergic cell loss within the SNpc (109, 113, 115, 425), recent studies have demonstrated that markers of likely contributors to dopaminergic degeneration in PD accumulate with age in the SNpc, including α-synuclein and ubiquitin, markers of oxidative and nitrative stress, dopamine transporters and glial reactions (117-119), indicating that the underlying pathology is fundamentally the same. This hypothesis is supported by epidemiological studies, showing that age is by far the most prominent risk factor of PD with an exponential incidence rate with age (5, 6). In this view, cellular changes exist along a continuum where aging actively produces a vulnerable pre-parkinsonian state that can exaggerate by a combination of genetic and environmental factors, thereby resulting in PD (428). In paper II and III, we provide evidence that AQP9 represents a significant vulnerability factor that impacts the selective dopaminergic cell death in the SNpc in PD as well as in aging.

Studying cell death mediated by AQP9 during aging in laboratory animals raises an important question on the role of environmental toxins. In contrast to humans, laboratory animals live in a relatively clean and controlled environment, without exposure to exogenous toxins. Toxins transported through AQP9 do not need to derive from external sources, but may originate from the endogenous microenvironment. As outlined in the introduction, dopaminergic neurons are exposed to a particularly high endogenous toxic environment through DA metabolism, which generates elevated levels of reactive oxygen and nitrogen species (152-154, 156, 157). An imbalance in ROS production and antioxidative mechanisms is a general feature of aging (141), increasing the rate of oxidative stress in neurons as well as astrocytes. The free radicals generated through DA metabolism can give rise a harmful environment by reacting with several components of cells, including free nucleosides, thereby generating purine and pyrimidine radical cations, such as 8-hydroxyguanine and 8-hydroxyadenine (429-431). Given the permeability of purines and pyrimidines through the bidirectional AQP9 (11), it is not unlikely that these toxic nucleosides or other endogenous toxic compounds may be imported into dopaminergic neurons from the microenvironment, thereby increasing the vulnerability of these cells (Figure 4). Moreover, ROS and highly reactive quiones generated from DA metabolism can further be converted to 6-OHDA, particularly due to the high levels of iron in the SN (156), thus forming a neurotoxin widely used to model PD (159, 376, 432). AQP9 may potentially allow diffusion of this neurotoxin. Finally, DA itself serves as a toxic agent (152, 153). In line with high levels of DAT in the SN as a vulnerability factor, AQP9 could potentially facilitate DA uptake and increase cytosolic DA to further escalate the oxidation (40). A hypothesis of a toxic microenvironment fits well with the fact that the extracellular space is reduced with aging (433) and that toxic waste molecules are less cleared in aging animals (434), easily allowing diffusion into the surrounding cells provided that toxin-permeable channels are present.

An important question rising from our findings in paper II and III is *why* selective expression of AQP9 in the SNpc could be beneficial for A9 cells. Although disadvantageous in some conditions, AQP9 may be beneficial in others. In several models of ischemia, A9 cells are shown to be more resistant to ischemia in contrast to more vulnerable neurons in the SNpr and striatum (419, 435), showing delayed histopathological neuronal damage. In a model of transient middle cerebral artery occlusion of ischemic stroke, the expression of AQP9 immunolabeling was shown increase significantly in astrocytes (269).

However, given the cross-reactivity of AQP9 antibodies with GFAP, these results may merely reflect gliosis surrounding the ischemic insult and must be interpreted with caution. Regardless, AQP9 may play a role in ischemia by promoting clearance of extracellular lactate resulting from lactic acidosis during ischemia. This hypothesis is supported by the fact that lactate permeability increases 4-fold when pH decreases to 5.5 (11), suggesting that lactate is mainly transported in its protonated form. Given the expression of AQP9 in the inner mitochondrial membrane, it has further been suggested that lactate transportation through mitochondrial AQP9 may be involved in reduction of ROS production through mild uncoupling of the ETC (16). When lactic acid is exposed to the high pH of the matrix, it will become deprotonated and serve to reduce the proton gradient across the inner mitochondrial membrane. An alteration in the proton gradient driving the ETC induces a mild uncoupling, as it allows protons to bypass F_0F_1-ATP synthase (complex V), thus slightly depolarizing the inner membrane (436). A modest depolarization is known to reduce formation of free radicals in the matrix (437). Hence, these cells will be less susceptible to ischemic insults.

Dopaminergic neurons in the SNpc are further known to be glucose-sensitive. Although no functional studies are performed on AQP9 in the brain, AQP9 in the liver is shown to be intimately linked to metabolism by transportation of the alternative energy sources glycerol and ketone bodies, and by regulating its expression in correlation to plasma insulin levels due to an IRE in the promoter (267). In this way, AQP9 may contribute to survival of dopaminergic neurons in face of scarce energy resources. It is essential to keep in mind that PD is an age-related disease. Our life expectancy has increased significantly during the past decades due to modern medicine, also bringing new age-related diseases into the picture. In an evolutionary perspective, it has probably been more essential to protect against ischemia and metabolic deprivation potentially occurring at an early stage in life to ensure survival, rather than preventing induction of neurodegenerative diseases.

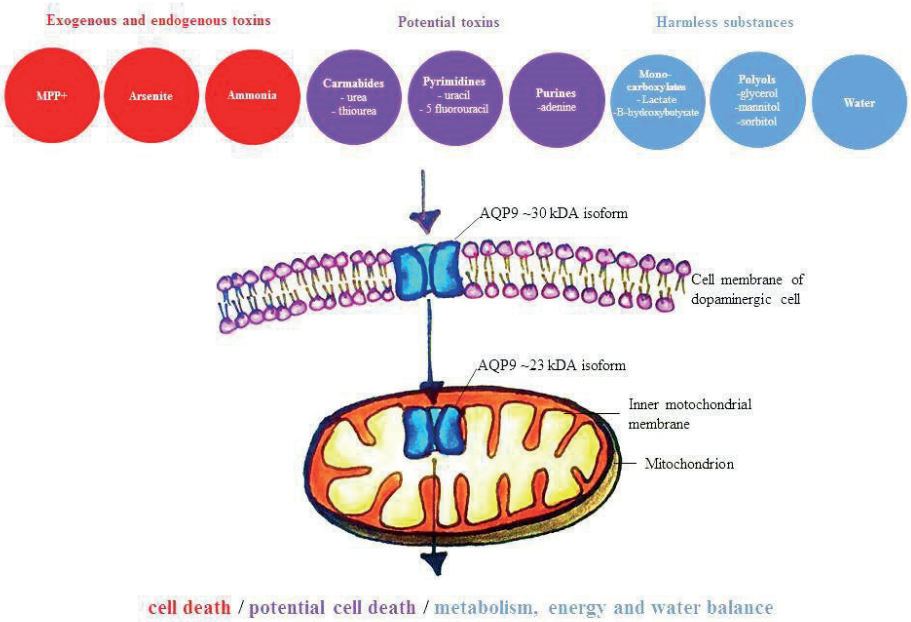

Figure 4. Proposed mechanisms of AQP9-mediated dopaminergic cell death and physiological functions.

Selective Vulnerability Mediated by the Glial Microenvironment and AQP4

In addition to intrinsic cellular factors involved in the selective vulnerability of dopaminergic neurons in the SNpc, this thesis has also focused on extrinsic factors in the microenvironment as contributors to the pathogenesis of PD – a field that has been largely neglected. It has previously been shown that implanted human fetal ventral mesencephalon into the putamen of PD patients developed Lewy bodies and were prone to cell death in similar ways as the endogenous dopaminergic cells in the SNpc (211-215). Involvement of glia in the disease progression of neurodegenerative diseases has further been demonstrated in ALS, where ALS-patient derived neural progenitor cells differentiated to astrocytes induced loss of motor neurons in mice (438). These studies strongly suggest that the local microenvironment contributes to the selective vulnerability of the degenerative process. In paper IV and V, we demonstrate that AQP4 localized on astrocytes surrounding the dopaminergic cells in the SN can be a vulnerability factor contributing to selective cell death. We demonstrate that AQP4 is highly expressed in the SN and is upregulated in a sub-acute as well as an acute model of PD, and that deletion of this gene increases the survival of dopaminergic neurons in the SNpc.

Resident astrocytes surround neurons are crucial to maintain homeostasis and sustain neuronal function (162-171), as described in detail in the introduction. In the SNpc, astrocytes are particularly important to balance the highly oxidative environment by providing anti-oxidative mechanisms, to maintain ionic homeostasis to sustain pacemaker activity and to activate in face of pathological insults and support survival through release of neurothrophic factors (162, 165, 167, 179, 183). Although the literature on astrocytes in the SN is scarce, it has been demonstrated that the SNpc contains a particularly low number of astrocytes compared to the neighboring regions VTA and RRF (182), possibly representing a vulnerability factor. Our confocal images of the untreated substantia nigra in paper IV confirm a relatively low expression in the SNpc compared to SNpr and neocortex, although no quantifications were performed. It should be noted, however, that the quantitative study referred to above as well as our own observations were based on GFAP immunoreactivity, which only stain ~15% of astrocytes (178). Thus, other astrocytic phenotypes may be present. A more complete analysis of astrocytic expression in the SNpc and the bordering regions is needed to uncover the role of astrocytes in the SN under physiological as well as pathophysiological conditions.

Homeostatic regulation by astrocytes is to a large extent carried out by passive, bidirectional water flow through AQP4, which redistributes water according to osmotic gradients (18, 163, 297). AQP4 is polarized to the perivascular endfeet and pial membranes to distribute water flow to/from the vasculature, and to a lesser extent in perineural membranes enwrapping synapses (20, 163, 172, 302, 303). Like astrocytes, AQP4 also displays large regional heterogeneity, with higher expression in the cerebellum and inferior colliculus and lower expression in the neocortex (439). In the SN, the expression of *Aqp4* mRNA has been described to be relatively scarce, as shown by *in situ* hybridization (440). However, a quantitative comparison of AQP4 protein expression in the SN in relation to other brain regions has not been performed so far. In paper IV, we found significantly higher expression of AQP4 in the SN compared to the neocortex, with pronounced expression in perivascular membranes as well as in the neuropil, as shown by quantitative immunogold labeling. These findings indicate that AQP4 plays a crucial role in the SN under normal physiological conditions, which can be attributed to several factors. First, the morphology of the dopaminergic neurons in the SNpc as well as their pacemaker activity make these neurons particularly metabolically active (38, 100, 125-134), with higher oxygen consumption rates

and elevated mitochondrial energetic metabolism than dopaminergic neurons in the VTA as well as in the olfactory bulb in primary cultures (130). The elevated energetic requirements produce large amounts of metabolic water, as each glucose molecule generate 42 water molecules from glycolysis to oxidative phosphorylation (441). Thus, the high expression of AQP4 could potentially reflect a linear relationship between metabolic activity and clearance of metabolic water. Further, the unmyelinated axons increase the density of nodes of Ranvier (133) and thus the levels of extracellular K^+ following the constant generation of action potentials as a result of pacemaker activity (38, 134). As AQP4 is intimately linked to Kir4.1 (315, 316), this could explain the highly expressed perineural pool seen in the SN. The high expression of AQP4 in SN could alternatively be related to the oxidative environment with eminent levels of ROS generated through DA metabolism (152-154, 156) and high levels of MAO-B (138). It has been demonstrated that the central pore of some orthodox aquaporins, including AQP4, is permeable to gases and cations, such as CO_2, O_2 and NO (253-255), although it is currently unknown to what extent this level of permeability is biologically relevant. Potentially, the large amount of free radicals in the SN can flow through the central pore to clear the toxic environment. AQP4 could also have a more indirect role in clearance of metabolic water and waste, free radicals and protein disposals, such as α-synuclein, through the glymphatic system. This model proposes that enlargement of the extracellular space induces water and waste clearance by a paravascular route, where water moves from arteries to veins via the interstitial space, facilitated by AQP4 (320-322). This route could potentially dilute and clear waste and toxins to optimize the microenvironment.

Several neurodegenerative disease models have shown loss of perivascular AQP4, loss of polarization or upregulation of AQP4, including AD (342, 343, 345, 346, 364), HD (350), ALS (348, 365) and spongiform encephalopathy (349) as well as normal aging (434). However, its expression pattern in PD is sparse. Based on several studies on AQP4 in neurodegenerative diseases as well as the high level of AQP4 in the SN under physiological conditions, we investigated the expression levels and micro-anatomical pattern of AQP4 in the SN under pathophysiological conditions in various *in vivo* models of PD, using a sub-acute MPTP model (paper IV) and an acute MPP+ model (paper V). Both models produced a significant loss of TH-positive cells (442), and confocal imaging revealed that loss of TH positive cells in the SN was paralleled by a considerable upregulation of AQP4 perivascularly and diffusely in the tissue after toxin-treatment, compared to saline controls and the contralateral hemisphere in the MPP+ model. In paper IV, immunogold labeling revealed significant upregulation of AQP4 expression in the SN following treatment with MPTP compared to saline, evident in perivascular- and perineural membranes, as well as in non-endfeet membranes likely corresponding to the diffuse labeling seen by confocal imaging. In paper V, intrastriatal injections of MPP+ resulted in a similar trend, although not significant. Here, the upregulation was primarily seen in in non-endfeet membranes. When compared to saline controls, only minor differences were observed, although it should be noted that the number of saline controls was low and thus a source of variance. On the other hand, qRT-PCR analysis in paper V revealed a significant upregulation of *Aqp4* mRNA compared to the contralateral hemisphere as well as saline controls, indicating similar trends in the two models. The discrepancy in AQP4 protein expression in the two models may be attributed to several factors. First, the MPTP model more closely simulates a neurodegenerative process, whereas the acute MPP+ model induces a severe toxic insult. As indicated by our qRT-PCR results, an ongoing upregulation of *Aqp4* is indeed taking place after MPP+ treatment. Unlike AQP2 in kidneys that can be rapidly upregulated from an intrinsic pool (443), upregulation of AQP4 is a more lengthened process that probably relies on phosphorylation and

intracellular signaling cascades (444). Potentially, extending the time interval between injection and euthanization would permit translation into protein in the MPP+ model. Alternatively, the acute insult prevents translation into protein by the extremely toxic environment introduced by MPP+, such as ROS damage to the DNA, RNA or free nucleobases (429-431). Another potential factor accounting for the discrepancy is the age-difference between the two models, with animals of 6-8 weeks in the MPTP study and 5-8 months in the MPP+ study. In fact, a substantially lower baseline level of AQP4 was found in the older animals in the MPP+ study compared to the young animals in the MPTP study, with ~60% lower expression of AQP4 in MPP+ saline controls and contralateral hemispheres compared to MPTP saline controls in perivascular and perineural membranes as well as non-endfeet domains, thus strongly indicating an age-related decline of AQP4. This observation is in line with the literature. Nedergaard's group (434) found a dramatic decrease in AQP4 polarization with advancing age, primarily confined to the perivascular pool of AQP4. This could explain why we found the most pronounced effect in AQP4 upregulation in the neuropil following MPP+ treatment in older animals. The same authors demonstrated an age-dependent upregulation of AQP4 in tissue surrounding arterioles, in line with upregulation of brain AQP4 with age described by others (445), which likely is due to elevated levels of reactive astrocytes with age (446, 447). Thus, although AQP4 expression is assumed to increase with age, the perivascular pool seems to decline, possibly masking some of our results in paper V. Noteworthy, PD is an age-related disease, thus questioning utilization of young animals to model the disease in the first place. Using older animals in a chronic model would potentially increase the validity of the results.

Loss of AQP4 polarization has been found in several disease models, including stroke (448), epilepsy (318) AD (346) and aging (434), and is known to compromise astrocyte function. In contrast, we find an increased expression of AQP4 in perivascular as well as perineural membranes, in addition to the neuropil, accompanied by elevated expression of GFAP, following neurotoxic treatment. This pattern is in line with several disease models, including ischemic stroke (449), brain tumors (329, 450, 451), bacterial meningitis (340), MS (338), as well as several chronic neurodegenerative diseases, such as AD (342, 343, 345, 346, 364) HD (350), ALS (348, 365) and spongiform encephalopathy (349). As these chronic neurodegenerative diseases share similarities with PD, they provide an indication of AQP4 expression in relation to regional, progressive cell death. In patients with AD, upregulation of AQP4 and GFAP has been reported in cortical astrocytes, along with increased *Aqp4* mRNA in a mouse model of AD (343, 345, 364). In line with our findings in paper IV, AQP4 upregulation has been found to be strong in the neuropil in AD (342, 346). Further, AQP4 expression is largely studied in relation to senile plaques, where AQP4 is reported to be lost in vessels associated with such plaques in a mouse model of AD (346) and in the dense core of plaque bodies in human neocortex but enhanced at the borders of the plaque (342), whilst another study shows that AQP4-ir corresponds to plaques and is increased in perivascular membranes (343). Potentially, a PD model including LBs would have painted a different picture on AQP4 distribution in PD than our models, as astrocytes are known to accumulate α-synuclein (452). Further, in models of ALS, a slight upregulation of perivascular AQP4 and GFAP has been described in mice (365), whereas vast upregulation of AQP4 mRNA and protein has been shown in rats, where expression was increased around vessels together with GFAP, and in motoneuron perikaya (348). These results also indicate that differences between species may be present, which has also been found between bovine and mice in spongiform encephalopathy, with more pronounced expression and earlier upregulation of AQP4 in bovine (349). As we have used mice in both our models, we cannot exclude that the pattern would have been different or more pronounced in other species. In line with our results in paper IV and V, the same

study found that affected cattle showed pronounced upregulation of mesencephalic AQP4 in astrocytic membrane domains as well as in the neuropil, along with increased GFAP-ir (349). In a model of HD, intracerebral injections of quinolinic acid induced upregulation of *Aqp4* mRNA in reactive astrocytes (350). However, saline injections produced similar results, indicating that the upregulation may be attributed to BBB disruption. The same study reported upregulation of *Aqp4* mRNA in the SN following intrastriatal 6-OHDA treatment but not after medial forebrain bundle axotomy (350). However, these models were not controlled by saline injections. Thus, we are the first to characterize protein expression of AQP4 in the SN in PD, and find an overlapping pattern with other chronic neurodegenerative diseases, with increased expression of AQP4. Our next step was to determine whether AQP4 plays a beneficial or deleterious role in the pathology of PD.

In paper V, we investigated the role of AQP4 in the pathogenesis of PD, using *Aqp4*$^{-/-}$ animals and WT littermates treated with unilateral intrastriatal injections of MPP+. Deletion of *Aqp4* resulted in a pronounced and significant reduction of dopaminergic cell loss in the SNpc, with ~40% loss compared to ~64% in WT littermates. No significant dopaminergic cell loss was observed in the in the VTA or in saline controls in any genotypes, indicating that the toxin-induced cell death was specific to the SNpc. These results demonstrated that AQP4 contributes to the selective vulnerability of dopaminergic cells in the SNpc. *How* AQP4 mediates this cell death is not clear from our results. It has been demonstrated that deletion of *Aqp4* results in increased formation of astrocytic gap junctions (369), which connect astrocytes to a syncytium (174). A high expression of these channels would allow redistribution of waste and harmful components generated by the degenerating cells, thereby reducing the toxic gradient in the microenvironment. Moreover, several lines of evidence have linked AQP4 to neuroinflammation. AQP4 is involved or upregulated in several neuroinflammatory diseases, such as NMO (339), MS (338), acute bacterial meningitis (340), pathological inflammation induced by LPC- (335) and LPS injections (336) as well as conditions that induce secondary inflammation, including stroke (292, 453), tumor (329, 450), in addition to chronic neurodegenerative diseases (342, 343, 345, 346, 348-350, 364, 365). Moreover, it has been shown that pro-inflammatory cytokines can serve as signal molecules for upregulation of astrocytic AQP4 *in vitro* (337). Many of these studies have shown a direct link between AQP4 and neuroinflammation by deletion of *Aqp4*, which reduced the formation of pro-inflammatory cytokines in a model of MS (338), reduced cytotoxic edema in a model of bacterial meningitis (340) hyponatremia (454) and focal ischemia (292), and reduced levels of cytokines as well as astrocyte swelling in brain edema and BBB disruption in a model of global ischemia (453). Furthermore, *Aqp4* deficient primary astrocyte cultures showed reduced cytokine release compared to WT astrocytes (338). In an *in vivo* model of inflammation induced by LPS injections, *Aqp4*$^{-/-}$ mice showed reduced inflammation compared to WTs, indicated by higher number of microglia and leukocytes in the latter group (338). Although it has been reported that AQP4 expression is induced in reactive microglia after LPS injections (440), others have demonstrated that microglia do not co-localize with AQP4 following LPS injections (338, 355), indicating that it is unlikely that AQP4 directly regulates microglial inflammatory responses.

In summary, these studies argue for a pro-inflammatory role of AQP4 expressed in astrocytes. Upregulation of AQP4 may be a direct reflection of astrogliosis, a feature seen in several chronic neurodegenerative diseases (342, 345, 346, 348-350, 364, 365, 434). This is in line with our findings, showing increased GFAP expression co-localized with AQP4 following toxic treatment, although the protein expression and co-localization was not quantified.

The pro-inflammatory mechanism of AQP4 has been linked to release of cytokines, and is thought to involve AQP4-dependent astrocyte water permeability and consequent cell swelling followed by release of cytokines. Support for this hypothesis has been demonstrated by increased cytokine release following astrocytic and non-astrocytic cells transfected with AQP4 and AQP1 compared to naïve astrocytes (338). In this way, the pro-inflammatory role of AQP4 involves a positive feedback cycle of local brain swelling (cytotoxic edema) and secretion of pro-inflammatory cytokines, which at the molecular level rely on AQP4-dependent osmotic water permeability and astrocyte swelling (338). Evidence for involvement of aquaporins in an analogous process, secretory vesicle exocytosis, has been reported (455, 456), though the biophysical mechanisms remain speculative on how aquaporin water transport facilitates fusion of secretory vesicles with the cell plasma membrane. The hypothesis of AQP4-mediated cytokine release fits well with PD, which is intimately linked to inflammation and associated with elevated levels of cytokines, both in post mortem PD brains as well as in animal models (120-124). A second potential pro-inflammatory and pro-degenerative role of AQP4 in PD is astrocyte migration and glial scar formation, which involves AQP4-facilitated water transport in the lamellipodia at the leading edge of migrating cells (329, 338). *Aqp4*-null astrocytes migrate much slower than WT astrocytes *in vitro* (333) and *in vivo* (457), and glial scarring is reduced in *Aqp4*$^{-/-}$ (333) indicating that AQP4 may inhibit survival of injured dopaminergic neurons by scar formation. However, evidence for changes in the number or astrocyte reactivity in postmortem brains of PD patients is inconsistent (185, 187), and gliosis has been shown to be more prominent in models of PD than in patients (186). Notably, upregulation of AQP4 is not necessarily co-localized with GFAP expression, as shown in a mouse model of ALS (348), and GFAP upregulation is not automatically accompanied by an increase in AQP4, evident in a model of cerebral malaria (341). This indicates that AQP4 expression is not linearly related to GFAP.

Despite the convincing literature on AQP4 as a mediator on inflammation and cell death, AQP4 has also been found to protect against inflammation as well as cell death in some disease models. *Aqp4*$^{-/-}$ mice have exhibited reduced survival in vasogenic edema associated with tumor, cold brain injury and persistent ischemia compared to WTs (451, 458, 459), more severe signs of cerebral malaria (341), and less clearance of amyloid-β by the glymphatic system (320), indicating that AQP4 may contribute to plaque formation in AD. In four studies by the same group, *Aqp4*$^{-/-}$ mice were more prone to MPTP-induced neurotoxicity in acute and chronic models of PD (352-355). *Aqp4* deficiency after MPTP exposure further resulted in reduced astroglial proliferation and GDNF protein synthesis (352), elevation of pro-inflammatory cytokines (354), hyperactive microglial and astroglial inflammatory responses in the SN (354) as well as in thymus, spleen and lymph nodes leading to loss of regulatory T-cells (353). On the other hand, they reported reduced leukocyte activation in *Aqp4*$^{-/-}$, in line with others who found a protective role of *Aqp4* deletion (338). Moreover, they demonstrated that AQP4-deficient co-cultures of astrocytes and microglia exposed to MPP+ promoted activation of microglia and pro-inflammatory cytokines (355). Hence, these studies indicate that AQP4 is involved in neuroinflammation, probably through a cross-talk between astrocytes and microglia. The authors attribute the augmented dopaminergic cell loss in *Aqp4*$^{-/-}$ mice to reduced proliferation and neurotrophic support from astroglia, as well as release of cytokines from astroglia (352-355). Of note, none of these findings have been replicated by other groups. Moreover, their acute and chronic MPTP model produces a dopaminergic cell loss between 36.8-42% in WT animals (352-355), which is not compatible with a model of PD ranging from 60-80% loss of dopaminergic cells (104, 105). The authors further describe elevated expression of GFAP-positive cells, microglia and cytokines in *Aqp4*$^{-/-}$ mice after treatment in the control condition saline (355).

Notably, these *Aqp4*$^{-/-}$ animals are reported to show structural brain abnormalities, including altered BBB integrity (460). Such abnormalities are not in line with other studies on *Aqp4* knockout animals (292, 311, 312). Although AQP4 potentially could increase cell survival, in line with the glymphatic system theory (320), it is unlikely to be responsible for reduced neuroinflammation as the CNS diffusion of inflammatory cells and soluble factors seems to be increased, rather than reduced, by AQP4 (338).

The fact that AQP4 plays different pro-inflammatory roles in various pathological conditions may be attributed to regional heterogeneity of AQP4, being highly expressed in the midbrain and to a less extent in the neocortex (439). Thus, more knowledge is needed on the heterogeneity and phenotypic characteristics of glial cells to unravel their sub-regional roles. In conclusion, we demonstrated that AQP4 facilitates dopaminergic cell death in the SNpc in an MPP+ model of PD. Whether this is mediated by release of cytokines and sustained inflammation, by astrocytes, microglia or both, remains to be determined. Including microglial markers in paper V would have provided information on whether AQP4 involved in their activation and/or sustenance.

Brain Aquaporins in PD

Knocking out *Aqp9* and *Aqp4* in an animal model of PD resulted in reduced dopaminergic cell death in the SNpc to a very similar extent. This questions whether the cell protection is exerted by deletion of a brain aquaporin in general, rather than selective effects exerted by AQP9 and AQP4 alone. The fact that transfection of astrocytes and non-astrocytic cells with both AQP4 and AQP1 resulted in secretion of cytokines argues for a role of brain aquaporins in neuroinflammation (338). Furthermore, both AQP4 and AQP1 overexpression is reported in AD (345). Thus, it is likely that AQP9 may contribute to neuroinflammation as well, although not investigated. However, AQP9 expression is found in leukocytes (264), and has been found to be upregulated in peripheral blood derived mononuclear cells from patients with inflammatory diseases (461-463). Nevertheless, both AQP4 and AQP1 are orthodox aquaporins only permeable to water, whereas AQP9 shows a broad permeability specter, suggesting that AQP9 may serve other roles as well. Moreover, both AQP4 and AQP9 are associated with astrocytes, which are crucial inflammatory mediators. So far, AQP9 is the only aquaporin identified in neurons and in dopaminergic cells in the SN in particular, suggesting a direct link to the inherent vulnerability of these neurons. *Aqp4*/*Aqp9* double knockout mice in a model of PD would provide answers to whether the neuroprotection would be increased, thereby indicating a potential separate effect of the two aquaporins.

Therapeutic Implications

Identification of novel molecules that contribute to the selective vulnerability of dopaminergic cells in the SNpc opens for a new therapeutic future in PD, although several questions remain to be elucidated. By blocking AQP9, one could potentially halt the progressive nature of the disease. AQP9 antagonists are identified but are known to affect other aquaporins as well (11). Global blockade of specific proteins is not unproblematic, however, and may cause unknown side effects as aquaporins are expressed in several organs. To date, brain-specific aquaporin antagonists are not identified. However, transgene animal

models provide clues that global removal of AQP9 does not cause phenotypic side effects. It is crucial to note that AQP9 is not the only factor promoting dopaminergic cell death, as shown by a significant amount of cell death also in *Aqp9*$^{-/-}$ animals. Thus, aquaporin antagonists would not enable a complete obstruction of the degenerative process, or regenerate new cells to compensate for the cell loss.

For AQP4, there are no identified blockers. Nevertheless, blocking of this channel would reduce transmembrane water flux in the brain. It is unlikely that water flux is mediating cell death in PD, given the chronic nature of this disease. Hence, it is crucial to identify the underlying mechanisms mediating the neuroprotection to evaluate the potential therapeutic implications of AQP4 in PD.

Finally, one of the main obstacles for treatment of PD in relation to a potential halting treatment is the lack of accepted definitive biomarkers for the disease (464). At the time of clinical onset, 60-80% of the dopaminergic cells are already lost (464). Nevertheless, the discovery of AQP9 and AQP4 as vulnerability factors for selective cell death in the SNpc in PD may open new paths in the field of PD.

CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis has investigated the selective vulnerability of dopaminergic neurons in the SNpc in PD by focusing on the neuronal and glial contributions to the pathogenesis of PD through AQP9 and AQP4, respectively. We have found that deletion of these molecules gives partial neuroprotection in experimental models of PD.

We provide evidence that AQP9 may increase the selective vulnerability of the SNpc by serving as a gateway for external toxins into these cells. Although we reproduce the results *in vitro* and *in vivo*, our findings result from models causing a severe acute insult. Thus, these findings should be replicated in other and more chronic models of PD for a better simulation of the degenerative process, where selective AQP9 blockers may be tested to evaluate a potential neuroprotective effect. It is further crucial to stress that the complete picture of localization of AQP9 in the brain is not yet painted. It is of particular interest to determine the exact location of AQP9 within the midbrain, in dopaminergic neurons as well as astrocytes, to better understand its role in the selective vulnerability of A9 neurons. Due to the lack of proper antibodies, laser cutting followed by micro mRNA analysis could serve as a method to determine its intra-regional pattern of expression. Moreover, conditional *Aqp9* knockouts would provide evidence to the relative contribution of AQP9 from neurons and astrocytes to neuroprotection. Finally, it is crucial to characterize the expression of AQP9 in the human brain to validate the translational application of the results.

We further provide evidence for upregulated AQP4 expression in two animal models of PD, where deletion of *Aqp4* resulted in increased selective neuroprotection of dopaminergic neurons in the SNpc. Whether this neuroprotective effect is a secondary consequence of the vast upregulation of this protein seen in PD, or a direct cause resulting from simultaneous alteration of other proteins, remains to be elucidated.

In conclusion, this thesis has identified two novel molecules that contribute to the selective vulnerability of dopaminergic neurons in the SNpc, and may serve as future target molecules in the therapy of PD to halt the progression of the disease.

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APPENDIX I-V