Discharging dopamine

*Boosting endogenous tyrosine hydroxylase activity as a treatment for Parkinson's disease*

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**Publication date**

2023

**Citation for published version (APA):**


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GENERAL INTRODUCTION

Targeting tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis as a treatment for Parkinson’s disease

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Parkinson’s disease

Parkinson’s disease (PD) is the second most prevalent neurodegenerative disease worldwide \(^1\) that affects 1-2 individuals per 1000 humans of the population in Europe \(^2\). The prevalence of PD increases with age to 1\% of patients that are above 60 years old \(^3,4\). The symptoms are caused by progressive degeneration of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc), located in the midbrain \(^5\). Human SNpc neurons contain the dark pigment neuromelanin, and loss of SNpc neurons, marked by depigmentation in PD patients is clearly visible at neuropathological examination \(^6\) (Fig. 1, upper section). SNpc dopamine neurons project to the corpus striatum \(^7,8\), one of the forebrain centers in charge of motor control (Fig. 1, upper section). The functional connectivity between both structures is referred as the nigrostriatal pathway and depends on the release of the catecholamine dopamine \(^9\). Due to the neuronal degeneration in the SNpc, there is less projection of dopaminergic fibers from the SNpc to the striatum (Fig. 1, lower section), resulting in a decreased dopamine release.

**Figure 1. Nigrostriatal degeneration of dopaminergic neurons**

Illustration of the functional connectivity between the SNpc in the midbrain (cell bodies) and the striatum (terminals) that is called the nigrostriatal pathway. Additionally, the mesolimbic pathway, the connectivity between the VTA that projects towards the ventral striatum/NAc and prefrontal cortex is illustrated. Both pathways depend on the release of dopamine and are known to be involved in PD pathology. Coronal midbrain section of normal healthy versus PD state of the SNpc (dark gray). Loss of pigmentation of neuromelanin in PD patients is clearly visible, in which approximately 70\% of SNpc neurons already have been lost. Degeneration of nigrostriatal and mesolimbic dopaminergic neurons in the midbrain causes a reduction in the amount of dopamine neurons, functional dopamine neuron terminals and dopamine transmission. **Abbreviations:** NAc, Nucleus Accumbens; PD, Parkinson’s disease; SNpc, Substantia nigra pars compacta; VTA, Ventral Tegmental Area.
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Studies reported that additionally but to a lesser extent the connectivity from the ventral tegmental area (VTA) to the ventral striatum, called the mesolimbic pathway is affected as well. To date there is no cure to halt the observed neurodegeneration.

When patients are diagnosed, PD is characterized by a range of motor symptoms, such as bradykinesia, akinesia, muscle cramps and tremor at rest. PD is also featured by a variety of non-motor symptoms, such as cognitive dysfunction, depression, anxiety, delusion, sleep abnormalities, hallucination, and impulsive and compulsive behaviors, suggestively partly influenced by given therapy. Strikingly, clinical symptoms only emerge at an advanced disease stage, in which a substantial amount of approximately 70% of SNpc neurons has already been lost and is numbers are still declining. Before clinical diagnosis and obvious PD-like motor symptoms, the patients go through a so-called prodromal phase. Prodromal PD symptoms include loss of smell, constipation, mood disruptions, and problems sleeping. Interestingly, the prodromal symptoms can occur as early as 20 years before the onset of motor symptoms. Thus, improving diagnosis would be beneficial to start treatment as early as possible that could delay the onset of clinical symptoms and improve the patient’s quality of life.

Only around 10% of PD cases have a monogenetic cause, and the remaining 90% of cases are sporadic, where the chance of developing PD increases with age. Several mutations in genes, such as glucocerebrosidase (GBA), leucine-rich repeat kinase 2 (LRRK2), Parkin (PARK2), UCHL1 (PARK5), DJ-1 (PARK7), PTEN-induce putative kinase 1 (PINK1), α-synuclein (SNCA), vacuolar protein sorting-associated protein 35 (VPS35) and other genes have been associated with increased risk for PD. Environmental factors such as toxins, pesticides, intake of heavy metals, dairy products and head trauma are all found to increase PD risk.

Treatment opportunities

L-DOPA: the current therapy for Parkinson’s disease

Dopamine in dopaminergic midbrain neurons is synthesized by the enzyme Aromatic amino acid decarboxylase (AADC) from the precursor L-3,4-dihydroxyphenylanine (L-DOPA). Prior, L-DOPA is synthesized by tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis (Fig. 2A).

To replenish the decreased level of dopamine, and as soon as a PD patient exhibits motor symptoms, they are treated orally with L-DOPA, which is considered the gold standard in the treatment of PD. L-DOPA is administered together with the AADC inhibitor carbidopa, that does not pass the blood brain barrier (BBB) to prevent peripheral conversion of L-DOPA to dopamine. Absorption of L-DOPA takes place in the small intestine, through the L-amino acid transport (LAT) system, sharing uptake with other essential large amino acids. Only 30% of the oral L-DOPA supplementation reaches the circulation intact of which merely 1% enters the brain. L-DOPA is effective at plasma concentrations of 2 μg/mL, however effectiveness is unpredictable when disease state progresses.

From the circulation, L-DOPA reaches the brain via cerebral arteries, passes the BBB via active transport via LAT, and is dispersed all over the brain. After an initial period of 5-10 years of effective levodopa treatment (honeymoon period), patients develop fluctuations in...
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their therapeutic response, leading to a ‘wearing-off’ effect, in which the therapeutic effect is shortened in duration of each consecutive L-DOPA dosage \(^{47-49}\). Accordingly, L-DOPA treatment requires regular incremental adjustments in dosage and dosages per day. Interestingly, it is suggested that the presence of L-DOPA reduces the uptake of L-Tyrosine \(^{50-52}\). This possibly further down scales the endogenous dopamine production and elevates the dependence of exogenous L-DOPA delivery.

The production of dopamine from L-DOPA by the enzyme AADC, is however not restricted to dopamine neurons in the midbrain, but it also facilitates additional enzymatic reactions in other neuronal cell type systems. These AADC-positive systems include the brain serotonin system, the noradrenergic system, trace amine neuromodulator systems, and glial cells \(^{45,53-55}\) (Fig. 2B). The expression of AADC in serotonin neurons is suggested to underlie one of the major side-effects observed after long-term levodopa treatment: Levodopa-induced-dyskinesia (LID). LiDs are suggested to be the result of AADC-mediated biosynthesis of dopamine that is derived from exogenous L-DOPA in serotonergic neurons \(^{56,57}\). These afferent nerves will additionally serve a role for storage and release of L-DOPA-derived dopamine \(^{58}\). Therefore, dopamine will be released from non-dopaminergic afferents, resulting in uncontrolled, excessive swings in dopamine release. Serotonin transporter blockade with selective serotonin reuptake inhibitors (SSRIs) was recently shown to counteract L-DOPA-induced dyskinesias in 6-hydroxydopamine (6-OHDA)-lesioned rats \(^{59-62}\). Also, lesions of the serotonin fibers or activating serotonergic auto-receptors with receptor agonists blocked the development of LID in lesioned animals \(^{63-66}\).

Post-synaptic targeting: Medium Spiny Neurons
To completely bypass presynaptic dopamine synthesis, Dopamine receptor agonists already are implemented as a therapy for PD \(^{67-69}\). Dopamine receptors in the post-synaptic membrane of medium spiny neurons (MSNs) are targeted to enhance the neuronal signaling action potential, increasing its sensitivity. Results demonstrate improved mobility and a prolonged motor response mediated by L-DOPA in PD patients, suggesting a long-lasting effect of treatment. However, their complicated mode of action is proposed controversial, with lower efficacy and high incidence of adverse effects \(^{70-73}\). One of the issues is that these agonists are not specific, because of the wide distribution of dopamine receptors within different brain regions \(^{74-77}\).

Recently, several gene therapy methods for PD went into clinical trials \(^{78-80}\). Some methods that are included are AADC-TH-GCH gene therapy, viral vector-mediated gene delivery, RNA interference-based therapy, and CRISPR-Cas9 gene editing \(^{80,81}\). In AADC-TH-GCH gene therapy, AADC, TH and glutamic acid decarboxylase (GCH), enzymes that are crucial in dopamine biosynthesis are used \(^{82}\). The MSNs, that are not affected in PD are targeted to synthesize dopamine and showed improved motor performance and sustained expression of the genes in phase 1 human trials and primates \(^{83-88}\). They found that gene delivery indeed resulted in increased levels of dopamine \(^{89,90}\). Nevertheless, it has been debated whether MSNs are the right target at all because they lack the ability to store and release dopamine properly \(^{78}\), leading to unregulated expression levels that can lead to harmful levels of dopamine and its metabolites \(^{91-93}\).
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Figure 2. Biosynthesis of dopamine

The biosynthetic pathway of dopamine. A | TH has a predominant role in the biosynthesis pathway of dopamine. TH uses oxygen (O2), the cofactor Tetrahydrobiopterin (BH4), and a ferrous iron (Fe2+) atom in the active site to facilitate the catalytic reaction of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). In turn, aromatic amino acid decarboxylase (AADC) decarboxylates L-DOPA to dopamine. B | Illustration describing the dopamine supplementation therapy by L-DOPA for PD patients. L-DOPA, which can pass the blood-brain barrier, is usually administered peripherally in combination with a peripheral AADC inhibitor. It reaches the brain via cerebral arteries and passage of the blood-brain barrier. Then, through activity of AADC, L-DOPA is converted to dopamine in the brain. The presence of AADC is, however, not limited to dopamine-synthesizing neurons. Accordingly, in addition to the original treatment target area, L-DOPA reaches various AADC-containing neuronal cell types in diverse brain regions. Consequently, dopamine is synthesized in AADC-containing neurons that normally synthesize neurotransmitters or modulators other than dopamine, for example serotonin neurons in the raphe nuclei.

Replacement therapies: Cell reprogramming and transplantation

For almost half a century cell transplantation therapies have been researched to commemorate the loss of dopamine neurons and catecholamine signaling in PD. However, efficacy was dramatically low and severe side effects occurred, also in later studies. Gladly, clinical cell therapy for PD has been picked up again for the last decades. Cells that are mainly used as a therapy are human embryonic stem cells (hESCs), human induced pluripotent stem (iPS) cells, and human fetal mesencephalic tissue. hESCs have some beneficial properties such as unlimited self-renewal capacity and potential to differentiate into specialized cells. Kriks and other researchers described successful conversion of hESCs to dopaminergic neurons, showing long survival, no tumor growth and an increase in dopamine production. Allografts of human fetal ventral mesencephalic (VM) tissue is currently suggested to be the most effective cell replacement therapy for PD patients. These allografts contain developing midbrain dopamine neurons and their precursors. Some successful open-lab trials that exist in this area reported improved motor symptoms in a selection of PD patients. In the last decade, induced pluripotent stem cells (iPSCs), are also used for reprogramming into dopamine cells. Hallett and colleagues reported that iPSC reprogrammed dopaminergic neurons, transmitted into the striatum of a monkey PD model, showed survival of transplanted...
neurons and motor progression. The benefits of using this iPSC model are that patient-specific cells can be acquired, which reduces immune reactions and ethical issues associated compared to other transplantation therapies. However, these techniques still comprise of a great deal of challenges. Apart from the obvious complexity of the procedure, problems that occur such as the purity of the material injected, the risk of tumorigenesis, immune reactions, ethical issues, pathology and a variety of other challenges should first be resolved.

**Halting neurodegeneration: Blocking mitochondria-dependent apoptosis**

Halting or slowing down the progressive degeneration of dopaminergic neurons in the Substantia pars compacta (SNpc) would be a breakthrough in the treatment of PD. First, the molecular mechanism underlying dopaminergic cell death in PD should be elucidated, exposing the intrinsic vulnerability of these neurons. Promising results in pre-clinical cell and animal PD models demonstrated degrees of neuroprotection, however clinical attempts to achieve neuroprotection have been disappointing. Interestingly, in PD pathology, accumulating evidence aims towards mitochondria-dependent apoptosis underlying neuronal loss. Blocking mitochondria-dependent apoptosis may therefore be a therapeutic to prevent the loss of dopamine neurons. However, the components of the apoptotic pathway have to be identified first in midbrain dopamine neurons. To ensure proper development and maintenance of the dopaminergic system, it has been hypothesized that a set of transcription and growth factors orchestrate specific dopaminergic pro- and anti-apoptotic factors that determine cell fate. Nevertheless, early detection of PD pathology is necessary to prevent further loss of midbrain dopamine neurons for these therapeutics to be effective.

**Proposed treatment**

**TH: the rate-limiting enzyme in dopamine biosynthesis**

As previously discussed, TH is the rate-limiting step in dopamine biosynthesis. TH is a member of the Aromatic amino acid hydroxylases (AAAHs), that are involved in the biosynthesis and signaling of monoamine neurotransmitters and hormones. Other members of AAAH enzymes are phenylalanine hydroxylase (PAH) and tryptophan hydroxylase 1 and 2 (TPH1, TPH2). They catalyze the hydroxylation of their respective amino acids. AAAH enzymes require the co-factors tetrahydrobiopterin (BH4), oxygen and iron for this reaction to occur. AAAHs are conserved tetrameric structures with identical subunits, however PAH can also exist as a dimer. The subunits consist of a 3-domain organization including the N-terminal regulatory domain, central catalytic domain and C-terminal oligomerization domain (Fig. 3A). The AAAHs display high-sequence identity, with 293 amino acid residues of catalytic domains, that demonstrate approximately 65% sequence identity across the sequence. AAAH’s are therapeutic targets for many diseases within neurology, psychiatry and cardiology. An example of this is the recessive disease phenylketonuria (PKU), associated with mutations in PAH, leading to loss of enzyme activity. Symptoms that arise are progressive intellectual impairment, autism, seizures, motor deficits and rash. It is characterized by the accumulation of phenylalanine and its degradation products, which is very toxic to the brain. Treatment options are administration of large neutral amino acids to prevent phenylalanine entry into the brain or oral BH4 co-factor application to increase PAH activity. Oral BH4 treatment is used for several disorders, such as hypercholesterolemia, diabetes mellitus, and cardiovascular disorders. However, this is not considered for PD. Another disease regarding...
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TH, called Tyrosine hydroxylase Deficiency (THD) is characterized by L-DOPA-responsive dystonia and/or infantile parkinsonism. This rare autosomal recessive disorder is largely caused by TH missense mutations and treated with L-DOPA supplementation. TH, called Tyrosine hydroxylase Deficiency (THD) is characterized by L-DOPA-responsive dystonia and/or infantile parkinsonism. This rare autosomal recessive disorder is largely caused by TH missense mutations and treated with L-DOPA supplementation.

Regulation of TH expression and enzyme activity
TH is present in the brain, retina, sympathetic nervous system, gut and adrenal medulla, and predominantly found in the cytoplasm. In all species, TH is coded by a single gene and in human, TH is found on locus 11p15.5. TH has a molecular weight of approximately 60 kDa and forms a functional homotetramer of 240 kDa. Interestingly, the human TH gene contains 14 exons that encode four isoforms of TH, hTH1, hTH2, hTH3 and hTH4, as a result of alternative splicing of the gene. The roles of the different isoforms in TH regulation are unclear. The TH protein found in other vertebrates is comparable to the human TH isoform hTH1. Isoforms hTH2, hTH3 and hTH4 have an additional 4, 27 or 31 (27+4) amino acids inserted on the N-terminus. In the caudate and putamen, hTH1 and hTH2 are expressed equally and account for 95% of the TH protein present.

In the adrenal medulla and locus coeruleus, stress or chronic drug treatments induced TH gene transcription and increased mRNA stability, indicating that TH is being regulated on the transcriptional level. However, in midbrain neurons, only modest or insignificant alterations in TH mRNA levels are observed during stressful events. In addition, more than 50 single nucleotide polymorphisms (SNPs) in the human TH gene have been described that occur in 1/3 of the population. These SNPs have been associated with a variety of uncommon movement disorders, such as L-DOPA-responsive dystonia, Parkinsonism and progressive infantile encephalopathy with L-DOPA-nonresponsive dystonia.

Besides regulation of TH via transcription, it can also be regulated via post-translational modifications such as phosphorylation and dephosphorylation, leading to changes in enzyme stability and feedback inhibition. The regulatory domain of TH harbors several conserved phosphorylation sites that are important for the regulation of its enzymatic activity. Conserved over species, the regulatory domain contains three serine residues (position 19, 31 and 40 in hTH1; Fig. 3B) that can be phosphorylated. It has been suggested that Ser8 in rodents and threonine 8 in human (Thr8) may be phosphorylated, however the function is never shown.

TH serine phosphorylation by a variety of protein kinases
Serine phosphorylation of TH harbors several functions. TH Ser40 phosphorylation augments enzymatic activity by altering the conformation of TH, leading to an increased dissociation of inhibitory catechols and increasing the affinity for its cofactor BH4. TH Ser31 phosphorylation promotes activity as well, although to a considerably lower extent than Ser40 and is involved in enzyme stability and localization. Ser19 phosphorylation is involved in enzyme stability and there is little evidence that Ser19 phosphorylation has a direct effect on TH activity. There is no evidence that Ser8 phosphorylation increases TH activity.
Ser40 phosphorylation

Many protein kinases are known to phosphorylate TH at Ser40. It has been widely described that protein kinase A (PKA) can phosphorylate TH at Ser40 \(^{164,172,174,177,179,181,184,191–206}\). Also, activation of protein kinase C (PKC), in response to phorbol esters or phospholipase C, leads directly or indirectly to an increase of TH phosphorylation at Ser40 \(^{207–209}\). Third, Calcium calmodulin-dependent protein kinase (CAMKII) was also confirmed as an in vitro TH Ser40 kinase \(^{210}\). Interestingly, a role for protein kinase G (PKG) on TH phosphorylation was suggested \(^{211,212}\). In bovine adrenal chromaffin cells increased PKG activity, produced significant increases in phosphorylation of TH. Inhibition of PKG, confirmed that the increased phosphorylation was mediated by PKG. When both PKA and PKG signaling routes were activated in this experiment, an additive effect on TH phosphorylation was not observed, suggesting that PKA and PKG phosphorylates the same serine residue. However, the target serine site is never shown directly \(^{212}\). PKG shares the same consensus phosphorylation motif with PKA \(^{212–214}\), and such it is in general hard to discriminate between PKA and PKG substrates. It is therefore highly likely that PKG may also control TH Ser40 phosphorylation in a parallel route.
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Ser31 phosphorylation
The extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and cyclin-dependent kinase 5 (CDK5) are known to phosphorylate TH at Ser31 both in vitro and in situ. The direct effects of TH phosphorylation at Ser31 on enzyme activity are modest and the increased TH activity is believed to due to a decrease in Km value for the cofactor BH4 rather than the dissociation of inhibitory CAs. In addition, TH stability was reported to be decreased by inhibition of CDK5-induced Ser31 phosphorylation, whereas additional data show that Ser31 phosphorylation targets TH to vesicles for transport along microtubules from the soma to the distal parts of neurites.

Ser19 phosphorylation
Calcium calmodulin-dependent protein kinase (CAMKII) has been reported to not only phosphorylate TH at Ser40 but also Ser19 in situ. Also, p38-regulated/activated protein kinase (PRAK) was found to phosphorylate TH Ser19. Next, stability has been correlated with the phosphorylation state of Ser19, which allows binding of 14-3-3, followed by decreased proteolysis by trypsin. Binding of 14-3-3 also promotes TH activity, by protecting TH from being dephosphorylated.

Figure 4 demonstrates all described protein kinases in various experimental set-ups that increase phosphorylation of its specific TH serine site.

Hierarchical phosphorylation
Next to the role of each individual TH serine residue has, they can affect the extent of phosphorylation of the other serine residues. This interdependence of phosphorylation sites has been explored in a number of studies and has resulted in a hypothesis describing hierarchical phosphorylation. Overall, Ser19 and Ser31 phosphorylation has been shown to increase the phosphorylation rate of Ser40.
Lehmann and colleagues (2006) were the first to describe hierarchical phosphorylation. They increased phosphorylation of Ser19 and Ser31 by angiotensin II (AngII) of purified TH, which increased the rate of phosphorylation of Ser40. This was evident when the reaction did not include dopamine. Therefore, they suggest that the role of Ser19 and Ser31 phosphorylation in dopamine-free TH is to increase the rate of rephosphorylation of Ser40 of TH after it has been dephosphorylated. Also in adrenal chromaffin cells, pre-incubation with AngII increased Th Ser40 phosphorylation by forskolin almost 2-fold. Added to these findings, downregulation of Ser31 phosphorylation by using an ERK1/2 inhibitor decreased basal Ser40 phosphorylation by 50% 165.

Thus, Ser19 and Ser31 are suggested to indirectly stimulate TH catalytic activity by increasing the rate of Ser40 phosphorylation by its upstream kinase 222.

**Upstream of PKA: adenylyl cyclases and G-protein coupled receptors**

In short, TH activity is regulated by kinase phosphorylation of serine residues in the regulatory domain. Out of the upstream kinases, PKA is the most abundant described Ser40 kinase 174,175,191. Inactive PKA is a heterodimer of 2 regulatory subunits bound to 2 catalytic subunits. The regulatory subunit possesses a pseudo-substrate which occupies the substrate binding pocket of the catalytic subunit, preventing activity towards other substrates. The key second messenger required for activation of PKA, cyclic adenosine monophosphate (cAMP), is produced by the conversion of adenosine triphosphate (ATP) to cAMP by adenylyl cyclases (AC) 226. Upon binding of cAMP to the regulatory subunit, the regulatory and catalytic subunit dissociate. The free catalytic subunit can now phosphorylate a wide array of downstream targets 227–229. In mammals, ACs can be divided into AC1-10 and are expressed throughout the brain 230–234, and peripheral regions 234. Besides activating PKA, cAMP can interact with multiple additional downstream effectors such as exchange protein activated by cyclic AMP (Epac), cAMP-regulated cyclic nucleotide phosphodiesterases (PDEs) and cyclic nucleotide gated (CNG) channels 227–229,235,236.

Most ACs, except soluble AC (AC10 or sAC) are coupled to G-protein coupled receptors (GPCRs) which can either stimulate or inhibit AC activity. The GPCRs mode of action is primarily activating heterotrimeric G proteins composed a complex of α, β, and γ subunits. GTP binding leads to a conformational change in Gα, promoting dissociation of the Gα and Gβγ subunits. Each of these units can in turn modulate the activity of effector proteins. Gα proteins are coupled to adenylyl cyclase. Gβγ subunits can play a role to recruit proteins to the membrane or modulate the activity of kinases, ion channels, or phospholipases 237,238. The Gα can be stimulatory or inhibitory (Gs and Gi, respectively) and therefore increase AC activity or inhibit AC activity. Gβγ can indirectly modulate AC activity, however this is much lower extend as compared to Gα 238. GPCRs can be modulated by a wide range of regulatory signals, including hormones and neurotransmitters 240–242. The most obvious GPCRs in the brain dopamine system are dopamine receptors D1-D5 77. D1-like receptors (D1 and D5) are Gαs coupled, whereas D2-like receptors (D2, D3 and D4) are Gai coupled. Of these dopamine receptors, D1-D5 are present post-synaptic, whereas D2-like receptors are the only present pre-synaptic 76. Importantly, pre-synaptic stimulation of D2 of the nigrostriatal neuron inhibits AC activity, thereby downregulating the phosphorylation and activity of TH in an autoregulatory feedback loop 243,244.
Next to L-DOPA treatment, dopamine receptor agonists are used as a therapy for PD ⁶⁷–⁶⁹. These agonists directly stimulate dopamine receptors on the post-synaptic membrane of medium spiny neurons (MSNs), bypassing the presynaptic dopamine synthesis, and act on the D2-class dopamine receptors and D1-class receptors activated by dopamine (and rotameric), phenylbenzazepines, tetracyclis and bicyclics ²⁴⁵. Originally, they were used as an adjunctive therapy in advanced PD, however they are now implemented in early stages of the disease. However, their use is still a complicated and sometimes a controversial issue because it involves difficult pharmacokinetics, lower efficacy and high incidence of adverse effects ⁷⁰–⁷³. These adverse effects are explained by the wide distribution of dopamine receptor expression within different brain regions, making it not restricted to the striatal neurons that are the main target in PD ⁷⁴–⁷⁷.

A second group of interesting AC-coupled GPCRs found in the brain are members of GPCR family B or secretin receptor-like GPCRs. These GPCRs are activated by a superfamily of structurally related peptide hormones which include pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP) secretin, peptide histidine isoleucine/methionine (PHI/PHM), peptide histidine valine (PHV) and glucagon-like peptides ²⁴⁶–²⁴⁹. Some of these peptide hormones are found to increase TH activity in various experiments ²⁰⁴,²⁴⁸–²⁵⁹. Therefore, a specifically expressed GPCR in a dopamine neuron that is positively coupled to an AC or is a potential therapeutic target as it may lead to increased TH activity (Fig. 5, right segment).

**Parallel route: natriuretic peptide receptors**

Interestingly, besides the well described cAMP-PKA pathway, a parallel pathway involving the natriuretic peptide receptor family has been shown to control TH activity. Instead of signaling via cAMP, it is mediated through alterations of second messenger cyclic guanosine monophosphate (cGMP) ²⁶⁰.

In mammals, there are three known natriuretic peptide receptors (NPRs), namely NPR-A/GC-A, NPR-B/GC-B, and NPR-C/Clearance receptor that are bound by Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), respectively. The other members of the family are GC-C, a receptor for the intestinal guanylin family of peptides, and Ret-GC-1 and Ret-GC-2, receptors in the retina that regulate the photoreceptor dark cycle ²⁴²,²⁶¹–²⁶⁶. These receptors, except from NPR-C are unique in the sense that they have an extracellular ligand binding domain that is directly coupled to an intracellular guanylate cyclase domain. NPR-C does not possess intrinsic enzyme activity as it lacks the intracellular guanylate cyclase domain. Upon activation of the cyclase, cGMP is catalyzed from GTP regulating activity of a variety of intracellular effector targets including PKG, Cyclic nucleotide-gated (CNG) channels and cGMP-dependent PDEs ²⁶⁷,²⁶⁸. Besides these so-called particulate guanylyl cyclases (pGC), the guanylate cyclase domain can also be expressed in the cytoplasm without an extracellular ligand binding domain, the soluble guanylate cyclases (sGC). Ligands for sGC include nitric oxide (NO) and carbon monoxide (CO). Activation of NPRs, that are present in the brain, have been described to be associated with increased TH activity when induced by natriuretic peptides ²¹²,²⁶⁹,²⁷⁰. Thus, a specifically expressed pGC or sGC in a dopamine neuron is another potential therapeutic target as it may lead to increased TH activity (Fig. 5, left segment).
Figure 5: Cyclic nucleotide-dependent activation of TH

Proposed signaling routes on TH activation after activation of AC-coupled GPCRs, pGCs or sGCs. The targets are activated by an exogenous ligand, which leads to intracellular catalysis of secondary messengers cAMP or cGMP. Elevated levels of cAMP or cGMP directly activate its respective protein kinase, that phosphorylates TH at Ser40, boosting TH activity and dopamine biosynthesis. Abbreviations: AADC, aromatic amino acid decarboxylase; AC, adenylyl cyclase; pGC, particulate guanylyl cyclase; sGC, soluble guanylyl cyclase; G-protein-coupled receptor (GPCR); Gs, large guanine-nucleotide-binding regulatory protein α stimulatory subunit; TH, Tyrosine hydroxylase.

Scope and aim of this thesis

In this chapter, we discussed the major drawbacks of current therapies used in movement disorders such as PD. Progressive degeneration of dopamine neurons leads to loss of dopamine signaling in the basal ganglia and substantia nigra. Dopamine production is targeted by exogenous application of L-DOPA and is successful for the early stages of the disease. However, L-DOPA therapy leads to severe side-effects, such as L-DOPA induced dyskinesias and atypical extranigral actions. Also, the therapeutic window in which the therapeutic response is effective narrows in later stages of the disease. Subsequently, the use of dopamine agonists has some major drawbacks, as the efficacy of these therapeutics diminishes when disease progresses and aspecificity of expression towards different brain areas, such as the cortical regions. To increase dopamine bioavailability and release to commemorate the loss of dopamine signaling and relieve symptoms, we propose a different route. We aim to target TH, which is the rate-limiting enzyme in dopamine synthesis. As TH is the limiting enzyme in the reaction, and dopamine interacts with the catalytic region of TH to decrease its activity, increasing the activity of the enzyme could accelerate dopamine biosynthesis in an endogenous manner. We propose that increasing endogenous enzyme activity specifically in nigrostriatal neurons by activation of upstream cAMP/cGMP routes, will lead to enhanced local dopamine production in the target area of interest, which reduces potential side effects and counteracts the dopamine depletion.

In chapter 2, we first revisited the AC-cAMP-PKA signaling route on Th Ser40 and Ser31 phosphorylation in dopaminergic MN9D cells. We confirm that various cAMP analogs increase Th Ser40 phosphorylation. Interestingly, Ser31 phosphorylation was downregulated in response to the same second messengers.
Using small chemical kinase inhibitors, we show crosstalk between the upstream kinases of Ser40 and Ser31 pathway. Finally, we investigated hierarchical phosphorylation of Th, as it is proposed that phosphorylation of one serine could affect the rate of phosphorylation of the other serine. With the use of phospho-mimetic mutants, we show that phosphorylation of Th at Ser40 is required for phosphorylation of Ser31, whereas we find no evidence to support the reported claim this is the other way around. This suggests Th Ser40 could be the most relevant phosphorylation site increasing the enzyme’s activity as well as function.

In the 3rd chapter, we describe an ex vivo approach to investigate Th phosphorylation in microdissected striatal or midbrain mouse brain slices. This was performed in order to provide a more detailed picture on upstream signaling routes of Th in an ex vivo setting. The distribution of Th Ser31 and Ser40 phosphorylation in these micro-dissected areas were examined, and cAMP-dependent routes on Th phosphorylation were explored. Using our ex vivo model, we demonstrated abundant expression of (phosphorylated) Th protein present in the corpus striatum, and the ability to modulate Th Ser40 phosphorylation via cAMP-dependent routes. We first revisited PKA and ERK1/2 crosstalk in the mouse striatum. Using a library of kinase specific small inhibitors, we demonstrated a trend towards downregulated Ser40 phosphorylation levels mediated by PKA. However, we show that ERK1/2 is upstream of Th Ser31 phosphorylation. In sum, we were able to increase Th Ser40 in microdissected mouse striatal slices by inducing cAMP-dependent signaling routes.

Chapter 4 was focused on the effect of catecholamines L-DOPA and dopamine on Th phosphorylation. The influence of increased levels of L-DOPA and dopamine was investigated on Th Ser40 phosphorylation in dopamine terminals in the mouse striatum. L-DOPA downregulates Ser40 phosphorylation, suggesting an inhibitory feedback mechanism on Th activity. Possibly, these effects are caused by an autoregulatory feedback inhibition route mediated by D2R. Indeed, dopamine application downregulated Ser40 phosphorylation as well. Strikingly, the negative effects of L-DOPA on Th phosphorylation could be reversed by activating cAMP signaling. Altogether, we propose that an endogenous approach to boost dopamine synthesis by targeting upstream signaling pathway routes of Th phosphorylation could especially be effective in early PD stages and may even boost the effectiveness of L-DOPA therapy.

In chapter 5, we investigated the guanylyl cyclase receptor (GC-C, or GUCY2C gene) as a potential upstream target for Th phosphorylation in dopaminergic neurons. Using a set of endogenous peptide ligands, together with custom peptide analogs, we demonstrate that targeting the GC-C receptor is a successful method for increasing Th Ser40 phosphorylation in dopaminergic neurons. As GC-C is specifically and exclusively expressed in dopaminergic midbrain neurons, targeting this receptor in vivo may be considered as a possible therapeutic target to increase dopamine production in PD.

Finally, the main findings from our experimental chapters are summarized and implications are discussed in chapter 6.
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References

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