Discharging dopamine

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

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GENERAL DISCUSSION

Targeting cAMP- and cGMP-dependent pathways to increase tyrosine hydroxylase activity as a novel symptomatic treatment for Parkinson's disease

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Introduction

The progressive degeneration of midbrain dopaminergic neurons in Parkinson's disease (PD) is causing decreased dopamine release into the striatum, which results in a variety of motor dysfunctions. As tyrosine hydroxylase (TH) is the rate-limiting enzyme in dopamine synthesis, increasing endogenous TH activity in PD would result in the replenishment of decreasing levels of endogenous dopamine, which improves neuronal dopamine signaling and possibly alleviates initial motor symptoms. A variety of protein kinases regulate TH activity by phosphorylation of serine residues located in the regulatory domain of the enzyme. The serine at position 40 (Ser40) is the main phosphorylation site that increases activity of TH. It does so by lifting the inhibitory interaction of dopamine with the catalytic region within the enzyme. As such, we aimed at increasing Ser40 phosphorylation specifically in dopaminergic neurons to increase TH enzyme activity. After we revisited upstream cAMP-dependent regulation of Th Ser40 phosphorylation in dopaminergic MN9D cells and in the mouse striatum, we tested the exogenous application of L-DOPA on the phosphorylation state of Th. Here, we found that acute L-DOPA application leads to downregulation of Ser40 phosphorylation, which could be reversed by enhancing cAMP signaling. In our search for upstream targets that are able to increase TH activity in midbrain dopaminergic neurons, we demonstrate a parallel route of Ser40 phosphorylation through cGMP-dependent pathways. This was done by targeting the specifically expressed guanylyl cyclase 2C (Gucy2c) receptor (GC-C) in midbrain TH-positive neurons. We show that using GC-C specific ligands, Th Ser40 phosphorylation levels are upregulated. Most interestingly, using custom peptides that are designed to activate GC-C, we increased Ser40 phosphorylation levels in dopamine cells and in the striatum. These data suggest new therapeutic options for PD, where an increase in TH activity specifically in dopamine deprived nigrostriatal neurons, through targeting cAMP and cGMP-dependent pathways, can be achieved. After a short summary of each chapter, our proposed route to increase TH activity in the clinic is elucidated and discussed.

cAMP-dependent activation of Th and hierarchical phosphorylation in vitro

In **Chapter 2**, we revisited Th phosphorylation by cAMP-dependent signaling in dopaminergic MN9D cells. These routes increase Ser40 phosphorylation, while Ser31 phosphorylation is downregulated. The increase in Ser40 phosphorylation was found to be mediated by PKA, whereas crosstalk with Erk1/2, an upstream kinase of Ser31, was suggested to mediate the downregulation of Ser31 phosphorylation. In subsequent experiments, we briefly demonstrated that additional kinases signal upstream of Ser40 and Ser31 phosphorylation, such as p38MAPK, CDK5, PI3K and GSK3. Next, we investigated previous claims on hierarchical phosphorylation of the serine residues, in which it is proposed that phosphorylation of one serine site affects the extend of the other serine site's phosphorylation by its kinase. In literature, Ser31 and Ser19 phosphorylation were found to indirectly stimulate the rate of Ser40 phosphorylation [1–4]. For this reason, we thoroughly tested this hypothesis using an unphosphorylated and phosphomimetic Th mutant approach of Ser08, Ser19, Ser31 and Ser40 in transfected Neuro2A cells. Strikingly, our results contradict with the previously published hypothesis as in our model Ser40 phosphorylation is required for Ser31 to be phosphorylated and not the other way around. In conclusion, we propose that Ser40 phosphorylation is the rate-limiting step in TH-activity and targeting this site may have great therapeutical relevance.

cAMP-dependent activation of Th ex vivo

Next, we investigated cAMP-dependent modulation of Th phosphorylation in an ex vivo pharmacology model. **Chapter 3** discusses the design and specificity of this ex vivo method and revisits cAMP-dependent signaling on Th phosphorylation in the striatum. We learned that in the striatum, the phosphorylation of Th Ser40 and Ser31 are also affected through increased cAMP signaling, in which Ser40 phosphorylation is increased, while Ser31 phosphorylation is downregulated, previously shown in MN9D cells. We suggest that the cAMP-dependent downregulating effect of Ser31 phosphorylation is mediated through a crosstalk mechanism, as results in MN9D cells suggested this hypothesis as well. We demonstrate a trend towards PKA being the kinase upstream of Ser40 phosphorylation, and Erk1/2 is found upstream Ser31 phosphorylation. Additionally, we briefly demonstrated that additional kinases affect Ser40 and Ser31 phosphorylation, such as p38MAPK, CDK5, PI3K and CAMKII. This further shows that our ex vivo model can be used for testing novel compounds that mediate Th phosphorylation as the upstream components are available.

The influence of L-DOPA treatment on Th activity

TH is subjected to end-product feedback inhibition via catecholamines inhibiting its activity, resulting in the suppression of the dopamine biosynthesis pathway [1,5–18]. In **Chapter 4**, we investigated the effect of L-DOPA on the endogenous dopamine machinery as PD patients are subjected high exogenous levels of L-DOPA [19–21]. We demonstrated that L-DOPA downregulates the phosphorylation level of Th Ser40. This inhibition of Ser40 is suggested to be mediated through L-DOPA derived dopamine as dopamine downregulates Ser40 phosphorylation in a similar manner. Possibly this inhibition of Ser40 is mediated by autoregulatory feedback inhibition through D2R-Gi signaling. Consequently, the observed inhibitory effects of catecholamines on Ser40 phosphorylation could be rescued by enhancing cAMP signaling.

cGMP-dependent activation of Th

It has been suggested in literature that cGMP-dependent protein kinase G (PKG) signaling is a parallel route next to cAMP-dependent signaling upstream of Ser40 phosphorylation [22–27]. Therefore, we investigated cGMP signaling on Th Ser40 phosphorylation in dopamine cells (**Chapter 5**). In the search to identify specific pathways that regulate cGMP signaling in the mouse dopaminergic system, we addressed pathways and receptors upstream of PKG and compared them to PKA routes as a reference. We investigated GC-C, a member of the guanylyl cyclase receptors, that generates cGMP that is found to be specifically expressed in mouse midbrain dopaminergic neurons [28–30]. With this insight, we investigated if activation of this receptor in dopamine cells and in the striatum increases phosphorylation of Th Ser40. We demonstrated that receptor activation by endogenous peptide ligands increased the phosphorylation of Ser40 Th in both models. Next, a *GUCY2C* gain-of-function mutant *R792S*, which generates elevated levels of cGMP [31], demonstrated enhanced ligand potency by increasing the phosphorylation of Ser40 to a higher level compared to the WT receptor, emphasizing the importance of cGMP signaling in TH regulation. Additionally, we show that a subset of custom peptides was able to increase Ser40 phosphorylation and thus may act as potent GC-C activators. These findings demonstrate proof of principle for developing a novel therapeutic for pharmacological interventions in early Parkinson's disease, through increasing Th Ser40 phosphorylation specifically in nigrostriatal neurons mediated through upstream cGMP-dependent signaling routes.

Proposed therapeutic

Pre-synaptic targeting: TH phosphorylation at Ser40

To alleviate the loss of dopamine, we aim to increase TH activity specifically in nigrostriatal neurons to enhance dopamine production. We propose to increase TH enzyme activity through activation of upstream cAMP and/or cGMP routes that facilitate the phosphorylation of Ser40. Our proposed symptomatic therapeutic for PD is aimed to enhance specifically expressed nigrostriatal receptors or enzymes that regulate the localization, duration, and amplitude of these cyclic messengers. Increasing cAMP and/or cGMP signaling in turn will activate upstream kinases of Th Ser40 phosphorylation that are endogenously available. The aim of targeting pathways specifically expressed in nigrostriatal neurons is of particular interest, as this allows potential side-effects to be reduced.

Adenylyl and guanylyl cyclase targets

In the search for approaches to manipulate cAMP- and cGMP-dependent routes as a therapeutic we documented alternative targets of interest. The potential of endogenous ligands and their receptors is dependent on the selective expression of the receptor, evidence of their effect on second messenger stimulation and ligand pharmacokinetics regarding passage of the blood brain barrier (BBB). Crosstalk between the cAMP- and cGMP-dependent signaling routes occur at multiple nodes of the pathway. For example, proteins PKA and PKG share similar substrates [27]. Endogenous ligands and receptors that may act on TH activity via cAMP-dependent signaling in the SNpc are the pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), peptide histidine valine (PHV), peptide histidine isoleucine/methionine (PHI/PHM) that act on the PAC1 and VPAC receptors, Secretin receptors, gastric inhibitory peptide (GIP) receptors, glucagon, glucagon-like peptide I and II receptors, GRF receptors, adenosine receptors (caffeine) and nicotinic acetylcholine receptors (nAChRs) [32–55]. Ligands and receptors that may act on TH activity via cGMP-dependent pathways in the SNpc are the natriuretic peptides atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) that act on their respective pGCs, and nitric oxide (NO), carbon monoxide (CO) and protoporphyrin IX (PPIX) that act on their respective sGC [27,56–68].

Targeting GUCY2C

GUCY2C is strongly and selectively expressed throughout the mouse Ventral Tegmental Area (VTA), SNpc and striatum [28–30] and proposed to be expressed in human TH- positive SNpc neurons as well [30]. As shown in literature and in the previous chapter (Fig. 2 and Fig. 6), binding of the receptor by a GUCY2C ligand, cGMP is rapidly produced [69]. We additionally show that using GC-C specific ligands, Th Ser40 phosphorylation is increased in dopamine cells and in the striatum. Therefore, targeting GC-C could be effective to increase endogenous TH activity in nigrostriatal neurons. In this thesis, sources such as Allen Institute for Brain Science (www.brain-map.org) and GenePaint (www.genepaint.org) were used to assess the expression of Gucy2c in mouse midbrain tissue. However, the limited amount of data of GC-C expression in human SNpc [30], should be further investigated for this target to be implemented in a human PD model.

cAMP- and cGMP-dependent pathways: PDEs

A second approach to enhance cAMP and cGMP signaling, is to inhibit the degradation of cyclic messengers that is mediated by cyclic nucleotide phosphodiesterases (PDEs). These PDEs comprise a group of enzymes that degrade the phosphodiester bond of cAMP and cGMP and show to be specifically expressed within tissues [70]. Accordingly, PDEs are able to regulate local levels of intracellular second messengers.

There is crosstalk among the cAMP- and cGMP-dependent pathways mediated by these PDEs. PDE-facilitated feedback mechanisms are complex and dependent on the substrate preference of PDEs [71,72]. The family of PDEs are classified into 11 members, namely PDE1-PDE11. Those PDEs have different substrate specificities, as some are cAMP-selective hydrolases (PDE 4, 7 and 8); cGMP-selective (PDE 5, 6, and 9) or dual-specific (PDE 1, 2, 3, 10, and 11) [70]. Phosphorylation of PDEs, mediated by protein kinases also influence the activity of feedback inhibition [71,72]. Additionally, in some cases cAMP may be able to bind to the allosteric cGMP-preferring regulatory segments GAF subdomains on PDEs, increasing their activity [64]. Generally, the basal cAMP concentration is higher than that of cGMP in cells. Therefore, an increase in the intracellular cGMP level leads to more competition between cAMP and cGMP for the binding site at the dual-specific PDEs and thus results in a reduction in cAMP hydrolysis by these PDEs and an increase in intracellular cAMP [64,72].

Moreover, it has been shown that PDEs are tightly connected to different physiological functions and also to pathological conditions [70,73,74]. To that end, isoform selective PDE inhibitors have been developed [70,75–77]. Dependent on their specificity, PDE inhibitors could be used as a drug to block one or more PDE subtypes, and thereby regulating cAMP and or cGMP availability [70,76,78]. Immediately after the discovery of PDEs, an effective PDE-inhibitor, caffeine was found [75,79]. Subsequently, caffeine analogs such as theophylline, have been developed to use as therapeutic agents [50]. Nowadays, various selective and non-selective PDE inhibitors are known. Recent therapeutic and commercial success of drugs such as the selective PDE5 inhibitor sildenafil (Viagra), are widely used. BAY 73-6691, another example of a selective PDE9 inhibitor that is in preclinical development for Alzheimer's disease treatment, shows to exhibit neuroprotective effects [80–83]. Accordingly, a specifically expressed PDE within nigrostriatal neurons could be a target to develop small molecule inhibitors for that PDE, to reduce the degradation of cAMP or cGMP messengers, increasing upstream Th Ser40 phosphorylation signaling.

Combinatory treatment

The effects of GC-C activation on Th Ser40 phosphorylation that we observed are considered marginal. As can be extrapolated from the gain-of-function mutant *R792S* results in the previous chapter that elicited a 1.5-fold greater response in Ser40 phosphorylation levels compared to *WT-GUCY2C*, it clearly demonstrates that there is room for improvement regarding the effect size on Th phosphorylation. To that end, we suggest a synergistic mode of targeting multiple nodes of the upstream signaling pathways that affect the Ser40 phosphorylation state. Synergistic effects of ligands on upstream pathways of TH have been described before, for example the effects of vasoactive intestinal polypeptide (VIP) and nicotine on TH activity and gene expression [84]. VIP or nicotine alone where devoid of an effect on TH activity, while synergistic application using the same concentration enhanced TH activity and expression. Thus, synergistic treatment application opens up new therapeutic opportunities. Combined

application of a ligand that induces cyclic nucleotide production, such as a GC-C ligand, together with a therapeutic that inhibits degradation of nucleotides such as a PDE inhibitor, can enhance and prolong second messenger signaling and thus increase the effect size of treatment. As such, the therapeutic threshold needed to reach a therapeutic effect may be reduced. Fig. 1 demonstrates the proposed effects of targeting upstream cAMP- and cGMP-dependent signaling routes on the activation of Th at different nodes in a dopamine neuron.

Drug administration

Compounds that do not cross the blood brain barrier (BBB) can only reach the target in the brain when delivered directly by invasive and precarious methods such as intraventricular infusion, intracerebral implantation and BBB disruption [85]. Therefore, using endogenous compounds to cross the BBB that employ pharmacological or physiological based transport processes, such as using lipid carriers, liposomes and receptor-mediated transport systems are beneficial [85]. Using peptide shuttles that cross the BBB could be exploited in our purpose for delivering the drug to the striatal terminals. Fusing the peptide to a shuttle peptide using a peptide linker are novel methods to improve drug delivery [86]. Also, the application of the drug can be accomplished by a variety of methods, such as injection of the drug, oral administration and nasal inhalation to reach the central nervous system, which has proven to be successful in the clinic [87,88]. In that event, the suggested route of administration of our guanylin peptides is likely via inhalation through the nose using a nose spray, where the drug passes the nose epithelium and reaches the brain [89]. However, there are uncertainties that these peptides pass the BBB [90], which could be accomplished by chemical modification of the peptide, further facilitating passage of the barrier.

Future perspectives

We aim to set out the most suitable approaches to manipulate the identified cAMP- and cGMP-dependent routes that result in increased phosphorylation of TH at Ser40. In parallel, we are interested in the extent of Th phosphorylation leading to dopamine content and signaling in the mouse striatum. Guanylyl cyclase 2C (GUCY2C or GC-C), was found to present an interesting target for boosting TH activity specifically in nigrostriatal neurons, via cGMP-dependent mechanisms resulting in the phosphorylation of Th at Ser40. Subsequent approaches are aimed to discover expression of GC-C protein in the SNpc of human post-mortem material, preferably in midbrain and striatal PD brain tissue. Expression profiles of additional upstream targets that modulate cyclic nucleotide signaling, such as PDEs or adenylyl/guanylyl cyclases and investigating the interactome of TH could help us to develop a novel therapeutic. Next, we advance by testing the potency of custom peptides and small molecules on TH phosphorylation and activity and determine the ability of the drugs to penetrate the BBB. Finally, we would test these compounds in an in vivo model and assess behavior responses and motor activity, preferably in a PD model, compared with the L-DOPA therapeutic. In summary, boosting endogenous tyrosine hydroxylase activity as a novel treatment for PD would be a tremendous step forward to alleviate initial motor symptoms with reduced side-effects, enhancing the quality of life of patients suffering from this debilitating disease.

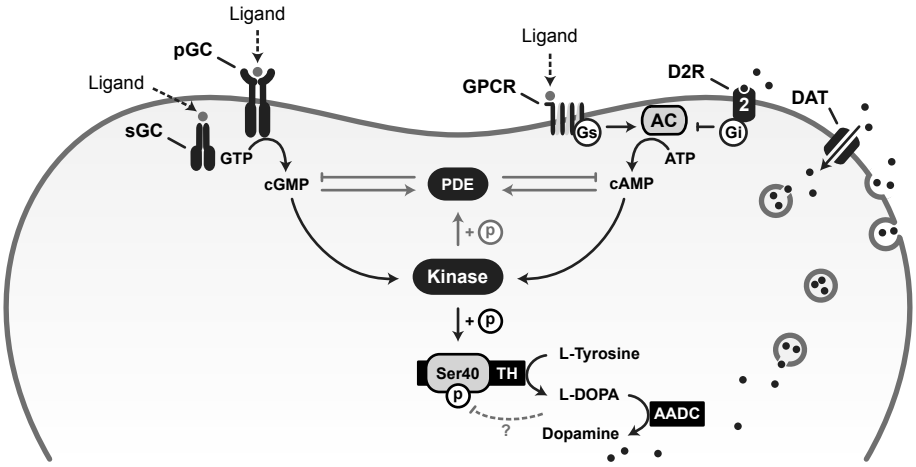

Figure 1: cAMP- and cGMP-dependent routes to increase Th activity in nigrostriatal neurons

Illustration of cAMP and cGMP-mediated phosphorylation of Th Ser40 in a striatal terminal. Activation of a sGC, pGC or an AC-coupled GPCR induces cGMP or cAMP catalysis, which subsequently activates respective PDEs and kinases. PDEs regulate the bioavailability of cAMP and cGMP within the cell, and kinases are able to phosphorylate TH and PDEs to regulate activity. Increased TH Ser40 activity, may lead to increased dopamine production, which elevates the dopamine content. L-DOPA or L-DOPA derived dopamine may regulate the activity of TH by inhibitory feedback mechanisms, directly as a substrate or indirectly by G-protein coupled Gi inhibitory D2R activation once released in the synaptic cleft. *Abbreviations: AADC, aromatic amino acid decarboxylase; AC, adenylyl cyclase; D2R, Dopamine 2 Receptor; DAT, Dopamine transporter; pGC, particulate guanylyl cyclase; sGC, soluble guanylyl cyclase; G-protein-coupled receptor (GPCR); Gi, large guanine-nucleotide-binding regulatory protein α inhibitory subunit; Gs, large guanine-nucleotide-binding regulatory protein α stimulatory subunit; PDE, phosphodiesterase; TH, Tyrosine hydroxylase*.

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