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REVIEW

Tryptophan 2,3-dioxygenase, a novel therapeutic target for Parkinson's disease

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ABSTRACT

Introduction: Alterations in the activity of tryptophan 2,3-dioxygenase (TDO) cause imbalances in the levels of serotonin and other neuroactive metabolites which can contribute to motor, psychiatric, gastrointestinal, and other dysfunctions often seen in Parkinson's disease (PD). TDO is a key enzyme of tryptophan metabolism at the entry of the kynurenine pathway (KP) which moderates production of neuroactive compounds primarily outside the central nervous system (CNS). Recent data from experimental models indicate that TDO modulation could have beneficial effects on PD symptoms not targeted by traditional dopamine substitution therapies.

Areas covered: Based on data available in PubMed and ClinicalTrials databases up until 1 August 2021, we summarize current knowledge of KP alterations in relation to PD. We overview effects of TDO inhibition in preclinical models of neurodegeneration and discuss findings of the impact of enzyme inhibition on motor, memory and gastrointestinal dysfunctions, and neuronal cell loss.

Expert opinion: TDO inhibition potentially alleviates motor and non-motor dysfunctions of PD. However, data suggesting harmful effects of long-term TDO inhibition raise concerns. To exploit possibilities of TDO inhibitory treatment, development of further selective TDO inhibitor compounds with good bioavailability features and models adequately replicating PD symptoms of systemic origin should be prioritized.

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1. Introduction – Parkinson's disease etiology, symptoms, and present-day treatment

Parkinson's disease (PD) is the second most common neurodegenerative disease and is among the leading causes of disability worldwide [1]. It is characterized by the loss of dopaminergic neurons in the *substantia nigra*, leading to the appearance of the characteristic motor symptoms: rigidity, bradykinesia, and tremor. Motor dysfunctions in PD are frequently accompanied by gastrointestinal symptoms such as obstipation, dyspepsia, and abnormal salivation. Patients' past medical history often reveals that disorders of the digestive system precede the manifestation of motor signs, indicating the involvement of the enteric nervous system in advance of loss of neurons in the central nervous system (CNS) [2]. Psychiatric symptoms such as depression, mood, and sleep disorders are also frequently present in PD, which further aggravate the burden caused by the illness [3]. Realization of the manifold symptoms of the disease and identification of pathological changes outside of the CNS as well [2] have led to the development of a more holistic view of PD, recognizing the involvement of various organs and tissues of the body rather than attributing the disorder solely to CNS dysfunctions.

It is generally acknowledged that PD is a multifactorial disease: lifestyle, environmental, and genetic factors either increase or decrease the risk of the development and progression of the disorder [4]. Due to the intensive research focusing on elucidating the genetic alterations leading to PD, up until today genetic variability of approximately 90 genomic loci which are proven or proposed to contribute to the disease has been identified [5]. Genes located in these loci encode proteins that have roles in various cellular functions, such as protein and vesicle transport, autophagy and mitochondrion maintenance, among others [6]. Genetic variations of only a handful of these genes (some of which are also termed as 'PARK' genes, indicating their relationship with the disease) have been found to have a direct, causal relationship with PD. These, so-called pathogenic gene variants cause both sporadic and familial cases, among which the latter are significantly rarer. The majority of PD cases are termed 'idiopathic.' For these there is no clearly identified genetic cause behind the disease. However, evidences are accumulating steadily suggesting that variations in one or more disease-related genes are present in the majority of idiopathic PD cases too [6].

Variants of specific PD-related genes are often linked to specific disease phenotypes such as earlier symptom onset, faster disease progression, and presence of more severe psychiatric symptoms [7]. Still, distinction of the different genetic PD forms both from each other and also from idiopathic cases often poses a great challenge. Although PD-related genes fulfil diverse cellular roles, the similarities in disease manifestation suggest that the impairments of various cellular functions eventually merge into a 'common route,' leading to PD development.

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Article highlights

- Via alteration of TDO activity Trp metabolism and by that serotonin and neuroactive KP metabolite levels can be modulated both within and outside of the CNS.
- In different models of neurodegenerative diseases inhibition of TDO showed beneficial effects in increasing neuroprotection, decreasing proteotoxicity, protecting against memory loss, and diminishing motor and gastrointestinal dysfunctions.
- Due to the active site structure and the shared substrate with IDO, however, selective inhibition of TDO is a challenge and differences have been observed in responses to enzyme inhibition in different disease models and also in disease versus non-disease states.
- Results of disease models forecast that progress in development of TDO inhibitor therapy may offer treatment for disease symptoms originating outside of the CNS, for this however, improved inhibitor specificity, bioavailability and accurate models of systemic PD effects are required.

This box summarizes key points contained in the article.

As a result of the intensive research focusing on a better understanding of PD, impairment of various cellular functions has been identified as culprits of the disease. These are related to mitochondrial electron transport disturbances and consequent decrease in energy generation, excitotoxicity and excessive production of reactive oxygen species, misfolded protein aggregates, and defective protein clearance (reviewed [8]). Despite the great effort put into unveiling the underlying causes the molecular pathomechanism of PD is not fully understood yet.

To this day no curative pharmacological treatment is available for PD, either for preventing or delaying disease progression [9]. All known treatments are palliative aiming at alleviating motor and non-motor symptoms of the disease [10]. Here we give a brief overview of current therapeutic approaches with highlights on difficulties and potential pitfalls in order to call attention to gaps in the approaches for PD treatment that could be filled by new therapeutic developments. For a detailed review of currently available PD therapy see [10].

Today available treatment for the motor symptoms of the disease is focused on reestablishing dopamine-related functions, mainly by the use of dopamine agonists, substitution of the neurotransmitter by its precursor (levodopa), and/or decreasing dopamine degradation *via* the inhibition of MAO B and/or COMT enzymes. Though pharmaceutical treatments by these approaches are beneficial in alleviating motor symptoms, vegetative side effects, such as orthostatic hypotension and worsening of psychiatric symptoms (e.g. impulse control disorders) are not uncommon. Moreover, with the progression of the disease levodopa induced dyskinesia can emerge, further complicating the already complex management of the symptoms.

As more advanced treatment methods for motor symptoms, deep brain stimulation (DBS), MRI-guided focused ultrasound, and application of enteral levodopa-carbidopa suspension are available. However, selection of patients who are eligible for these treatments requires great care as consideration of the cognitive status of the individual, risks of surgical procedures, possible postoperative complications is required together with compliance of the patients, which is crucial for the long-term treatment success. Furthermore, when planning on implementing DBS treatment, the presence of genetic risk factors and/or causal mutations should also be taken into consideration since there are reports indicating that the success of DBS on alleviating PD symptoms is also affected by the presence of certain gene variants [11]. The outcome of DBS treatment is likely to be promising in the case of patients bearing the most common LRRK2 mutation, G2019S, and variants of the *PRKN* gene. Unfortunately the effects of such intervention seem to be less favorable in those carrying the R1441G mutation of the aforementioned *LRRK2* gene [12].

In addition to the modulation of dopamine levels for the alleviation of motor symptoms, treatment of non-motor symptoms such as dementia, depression, psychosis, insomnia, and vegetative nervous system dysfunctions requires modulation of various other neurotransmitters. Drugs for the treatment of these should be selected carefully. Many medications used for the treatment of non-PD patients carry the potential of serious side effects in PD patients because of the imbalance of neurotransmitters due to the nature of the disease.

Considering the complexity of the disease and the limitations of currently available therapy, there is an urgent need to find new means by which both motor and non-motor symptoms of PD could be treated. Besides the improvement of palliative treatment, there is also a pressing need for the development of therapeutic approaches capable of curing PD, or at least slowing disease progression.

2. Attempts to widen the range of targets for PD treatment – targets in the kynurenine pathway

Despite that the kynurenine pathway (KP) was first discovered in the mid-19th century [13], nearly a century passed until all the enzymes involved in Trp metabolism were identified [14,15]. For now, alterations of the pathway have been linked to various human diseases, particularly to neurodegenerative and psychiatric disorders and diseases involving autoimmunity and malignancies [15].

Due to the various effects of kynurenine metabolites and the potential that modulation of Trp metabolism holds, in the recent decades the KP gained particular interest in search for possible novel therapeutic targets. In the following sections we give a brief summary on the KP, highlighting key enzymes and metabolites possessing neuroactive properties. Following this, an overview of data is given on the alterations of the KP in relation with PD, as it has been learned from *in vitro* and *in vivo* models of the disease and from analysis of human samples.

2.1. The kynurenine pathway

Trp, one of the nine essential amino acids, in addition to protein synthesis is also indispensable for the formation of hormones and neurotransmitters such as melatonin and serotonin, respectively. However, for this only a small percentage

of the dietary intake of Trp is utilized and the majority of the amino acid is metabolized *via* the KP [16] (Figure 1).

The first step of the KP is the conversion of Trp into N-formyl-L-kynurenine in a reaction catalyzed by indoleamine 2,3-dioxygenase 1 and 2 (IDO1 and IDO2) and tryptophan 2,3-dioxygenase (TDO) enzymes. N-formyl-L-kynurenine is then metabolized into L-kynurenine (KYN) by formamidase. KYN is situated at an important branch point of the KP, since it can be converted into i) kynurenic acid (KYNA) by kynurenine aminotransferases (KATs), ii) anthranilic acid (AA) by kynureninase (KYNU), and iii) 3-hydroxykynurenine (3-HK) *via* a reaction catalyzed by kynurenine 3-monooxygenase (KMO). 3HK can further be metabolized into xanthurenic acid (XA) by KATs or can form 3-hydroxyanthranilate (3-HAA), which is further converted into 2-amino-3-carboxymuconate semialdehyde (ACMS) in a reaction catalyzed by 3-hydroxyanthranilate 3,4-dioxygenase. ACMS can be further processed by aminocarboxymuconate-semialdehyde decarboxylase (ACMSD) for the synthesis of 2-aminomuconate semialdehyde, which is then further metabolized into picolinic acid (PIC). ACMS can also undergo non-enzymatic cyclization and form quinolinic acid (QUIN), which is then ultimately metabolized into nicotinamide adenine dinucleotide (NAD^+), a crucial molecule for cellular energy production and metabolism.

Along the pathway various metabolites, collectively referred to as kynurenines, are generated, among which several ones possess neuroactive properties (for a detailed description of the pathway see [17]). Among these KYNA is well known for its neuroprotective effects mainly due to its N-methyl D-aspartate (NMDA) receptor antagonist, ionotropic glutamate receptor inhibitor, and G-protein-coupled receptor (GPR35) and aryl hydrocarbon receptor (AHR) activator properties (reviewed in [18]). On the contrary, QUIN along with the oxidative stress and free radical generator 3-HK and 3-HAA

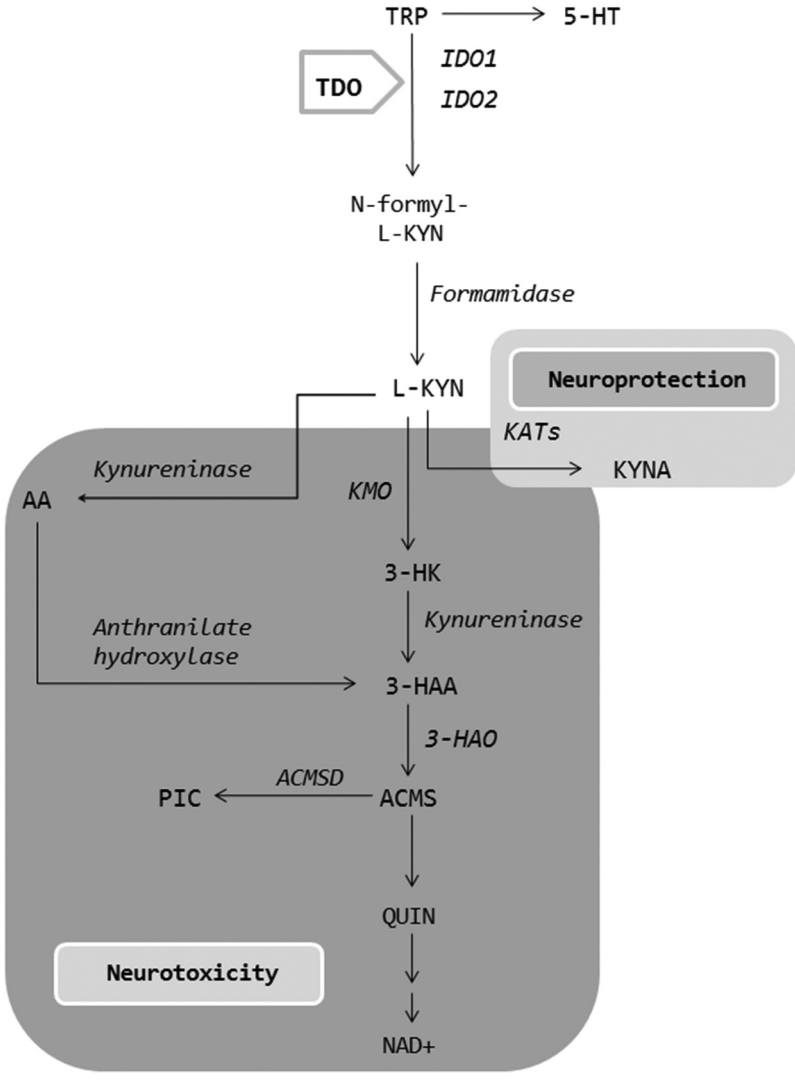

Figure 1. Schematic illustration of the kynurenine pathway. With TDO enzyme (indicated in bold) inhibition not only the formation of kynurenine metabolites of both the neurotoxic (dark gray background) and neuroprotective (light gray background) branch of the pathway can be affected, but also modulation of Trp and 5-HT levels can be obtained.

Abbreviations: TRP: Tryptophan; 5-HT: serotonin; TDO: tryptophan 2,3-dioxygenase; IDO1: indoleamine 2,3-dioxygenase 1; IDO2: indoleamine 2,3-dioxygenase 2; L-KYN: L-kynurenine; KATs: kynurenine aminotransferases; KMO: kynurenine 3-monooxygenase; AA: anthranilic acid; 3-HK: 3-hydroxy-kynurenine; 3-HAA: 3-hydroxyanthranilic acid; 3-HAO: 3-hydroxyanthranilate 3,4-dioxygenase; ACMS: 2-amino-3-carboxymuconate semialdehyde; ACMSD: 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase; PIC: picolinic acid; QUIN: quinolinic acid; NAD+: nicotinamide adenine dinucleotide.

[16], is a member of the group of neurotoxic kynurenine metabolites [17]. QUIN is a known excitotoxin due to its NMDA receptor agonist effect [19].

Since imbalances of the KP have been linked to several human disorders, modifying the activities of enzymes involved in Trp metabolism seems to be an appealing therapeutic approach. In this respect IDO1 and TDO enzymes are among the most attractive targets, since both catalyze the first and rate limiting step of the KP, the conversion of Trp into KYN. However, despite catalyzing identical reactions, IDO1 and TDO differ in multiple aspects (for more on this see below, and for a detailed review in [20]). One of these is that while IDO1 is expressed mainly by immune cells and plays a major role in immune regulation by maintaining and adjusting kynurenine metabolite levels in the local microenvironment, TDO is expressed primarily in the liver and is responsible for the regulation of systemic Trp levels [21]. Under physiological conditions, the vast majority of Trp is degraded by hepatic TDO, while IDO1 activity in extrahepatic tissues is significantly smaller [16]. Based on this, modulation of TDO activity seems to be a more straightforward approach for the modulation of systemic Trp and kynurenine metabolite levels. Here we aim to overview what potential of TDO modulation might have in respect of PD treatment. Findings regarding modulation of other KP enzymes are beyond the scope of this review, and those interested in that can find excellent reviews on the topic in [17] and [22].

2.2. KP alterations in PD

The relationship between KP and PD was first reported by Ogawa and coworkers in 1992 [23], and since these findings alterations of KP metabolites and enzyme activities have been repeatedly reported in PD and also in other neurodegenerative disorders. In the following section we summarize data obtained from studies involving disease models and findings based on analysis of human PD samples. We highlight results that led to and underpin the notion of TDO activity manipulation being a promising therapeutic approach.

Studies from the early 1990s showed that KYNA administered directly into the CNS alleviated parkinsonian symptoms in rat and primate toxin models of the disease [24,25] and was protective against QUIN induced nigrostriatal dopaminergic neuronal cell loss [26]. The protective effects of KYNA were attributed to its NMDA receptor inhibitory properties [24–26]. A possible mechanism by which KNYA could reverse parkinsonism symptoms was proposed *via* blocking excitatory amino acid transmission in the medial segment of the globus pallidus [25]. KYNA was also found to be protective against another neurotoxin, MPP+ (1-methyl-4-phenylpyridinium). Lee and colleagues reported that pretreatment of SH-SY5Y and SK-N-SH cells with KYNA inhibited mitochondrial apoptotic processes induced by MPP+ [27].

In light of the findings that KYNA has protective effects against various neurotoxins, results showing that the widely used dopamine precursor levodopa reduces extracellular KYNA levels in rat striatum suggest a seemingly contra productive effect of the prodrug [28]. However, simultaneous administration of 6-OHDA and levodopa treatment did not cause observable decrease in striatal KYNA levels. Based on their results Wu and coworkers proposed that the decrease in striatal KYNA levels in this rat model is a consequence of enhanced dopaminergic activity resulting from elevated dopamine concentration in the CNS, rather than due to a secondary peripheral effect of levodopa [28].

Analysis of human PD samples indicate upregulated activity of the KP, resulting from the enhanced metabolism of Trp both in the CNS and the periphery. The ratio of KYN/Trp (an indicator of IDO1 and TDO activity) was elevated in both serum and cerebrospinal fluid (CSF) samples of PD patients compared to controls, while the Trp level was decreased in PD sera. These changes were most prominent in patients with highest disease activity which the authors defined as stage 5 on the Hoehn and Yahr scale [29]. Since increased Trp degradation reflects activation of the immune system, these findings underpin the importance of systemic inflammation in the advanced disease state and makes the KP a potential target for intervening this process. Since in the periphery primarily TDO is responsible for Trp metabolism, increased KYN/Trp ratio in patients' sera suggests increased TDO activity [29]. Further analysis of peripheral tissues of PD patients revealed that the activity of KAT1 and KAT2–which are responsible for the formation of KYNA from KYN – were decreased in plasma samples; however, in red blood cells increased KAT2 and consequently elevated KYNA levels were observed [30].

In a more recent study Oxenkrug *et al*. observed lower Trp and higher KYN, AA, and KYNA levels and increased KYN/Trp ratio in plasma of PD patients compared to control individuals [31]. Changes in KP metabolite levels in PD could be the consequence of systemic subclinical chronic inflammation that promotes dysfunction of the KP both in the periphery and in the CNS. Based on their results Oxenkrug and colleagues proposed that impaired kynurenine metabolism could serve as a link between PD and features of metabolic syndrome, which are often present in the early stages of the disease [31].

In line with findings of levodopa therapy modulating the levels of KNYA in an animal model of the disease [28], Havelund and colleagues observed significant differences in KP metabolites when comparing groups of PD patients who had received or not levodopa treatment. In the plasma of patients with levodopa-induced dyskinesia, the 3-HK/KYNA ratio was significantly increased, while AA levels in both plasma and CSF of the same group were significantly decreased. These observations led the authors to conclude that the 3-HK/KYNA ratio could serve as a potential biomarker of dyskinesia linked to levodopa treatment in PD patients [32].

Recently Heilman and colleagues reported significantly higher levels of 3-HK and lower levels of 3-HAA in the plasma of PD subjects compared to control individuals [33]. Since 3-HAA is generated by the conversion of 3-HK by KYNU, an increase in the 3-HK/3-HAA ratio suggests a diminished activity of the enzyme. The levels of plasma 3-HK were also proportional with the duration of the disease and severity of symptoms. In CSF samples of the same PD patients the levels of the neuroprotective KYNA and KYNA/KYN ratio were significantly lower, while the QUIN/KYNA ratio was increased compared to non-PD controls [33]. The decrease in KYNA

level was proposed to be a consequence of Trp metabolism shifting toward the QUIN producing branch of the KP, leading to diminished KYNA, and decreased 5-HT production. These changes of KP metabolite levels in the CSF were found to be associated with disease phenotype: higher levels of QUIN were associated with more severe disease symptoms, while olfactory dysfunction was linked to lower levels of KYNA [33].

Findings of changes in the levels of Trp and its metabolites both in the CNS and periphery of PD patients, and the beneficial effects of KYNA administration in animal models of the disease strengthen the notion that the KP can be a potential therapeutic target in PD treatment. Associations between disease symptoms and changes in particular KP metabolite levels suggest that restoring the imbalances of these could be beneficial by lessening PD symptom severity. A straightforward way of achieving this could be *via* the modulation of the activity of the enzymes responsible for the generation and transformation of the metabolites. Considering that changes in KP metabolite levels have been reported repeatedly both in the CNS and periphery, TDO, an enzyme playing a major role in regulating Trp levels in peripheral tissues and in the CNS, is an appealing candidate for such enzyme modulator therapies.

3. TDO, a key enzyme at the entry point of the KP

3.1. Expression, structure, activity

TDO is encoded by the tryptophan 2,3-dioxygenase (*TDO2*) gene spanning over 16,000 nucleotides on the long arm of chromosome 4. Despite TDO and IDO catalyzing the same reaction, the sequences and genomic structures of the genes encoding them differ, suggesting that they evolved separately [20]. TDO is a 406 amino acid protein with a molecular mass of 47 kDa. It forms homotetramers, containing a heme-cofactor at each active site. In contrast to IDO, which shows broad substrate tolerance, TDO is highly specific to Trp [34]. The two enzymes also differ in their localization: while IDO is present throughout all tissues of the body except liver [35], TDO is mainly localized in the liver, but is also expressed in the CNS [36]. Under pathological conditions however, TDO mRNA has also been detected in various cancerous tissues (see below) [37]. While IDO1 is activated by inflammatory signals and plays an important role primarily during immune activation [38], TDO is in charge of modulating systemic and brain Trp and serotonin levels [39]. In mammals TDO is likely to be the enzyme that is mostly responsible for the metabolism of Trp toward NAD+ production [20]. TDO expression and enzyme activity can be modulated by various mechanisms (for a review see [38]). Glucocorticoid responsive elements (GREs) in the promoter of *TDO2* enable activation of the gene by glucocorticoids at transcriptional level [36,40–44]. Modes of TDO regulation include activation by its substrate, Trp itself [45,46], and its cofactor heme. Results of *in vitro* studies suggest NAD(P)H to be an allosteric modulator of TDO acting as an inhibitor through a feedback mechanism [47,48].

3.2. Alterations in TDO function in disease states

Upregulation of TDO has been reported in various diseases states. In the following section we summarize data available on the involvement of the enzyme in different human diseases with an emphasis on neurodegenerative disorders, and among them in particular on PD and disorders often accompanying PD, such as anxiety and depression. Since many of these studies involve genetic and/or pharmacological inhibition of the enzyme, some of the TDO inhibitors are also mentioned in this section. However, a detailed overview of TDO inhibitors applied in PD research will be given in a separate section.

Since the discovery of upregulated TDO expression in various human cancers [49] modulation of the activity of the enzyme became a hot topic in research focusing on finding ways to combat tumors. In 2011 Opitz and colleagues identified that the TDO-derived KYN is an AHR agonist, and that activation of the receptor suppresses antitumor immune responses consequently enhancing tumor cell survival. In line with this, the TDO-AHR pathway was found to be active in human brain tumors and showed association with disease progression and severity of disease outcome [50]. Despite intensive research focusing on understanding the ways by which TDO promotes tumor development and progression, the exact mechanisms are not fully understood yet [51]. Preclinical studies involving the P815 tumor mouse model (a model which was generated by injecting DBA/2 mice derived P815 mastocytoma cells into the peritoneal cavity of naïve syngeneic DBA/2 mice, resulting in solid tumor growth [52]) showed that pharmacological enzyme inhibition promoted rejection of TDO expressing tumors. This observation supports the notion of favorable effects of TDO inhibition in cancer treatment and warrants further research in the area [49].

Considering that Trp is crucial for the synthesis of serotonin, it is not surprising that alterations in TDO expression and enzyme function have been related to depression and anxiety as well. Kanai and colleagues reported that *Tdo-/-* mice showed less anxiety in anxiety-related behavior tests, which was attributed to the increased levels of Trp and its metabolites both in the plasma, hippocampus, and midbrain of the animals [53]. Lack of TDO also resulted in enhanced adult neurogenesis as indicated by the increase of neuronal progenitors in the hippocampus and the subventricular zone of adult animals [53]. In line with this, rats exposed to repeated stress exhibited an increase in circulating corticosterone concentrations and consequent TDO up-regulation, resulting in decreased Trp and increased kynurenine levels [54]. The link between TDO and depression is not restricted to regulating the amount of available Trp for serotonin synthesis by the enzyme: there is a growing body of evidence for the imbalance of neuroprotective and neurotoxic kynurenines in depression [16]. It is proposed that this is a consequence of the upregulation of IDO1, TDO, and/or KMO enzymes due to systemic inflammatory changes and stress [16].

Supports the notion of TDO inhibition as a therapeutic approach for depression treatment that several drugs used in the treatment of depression were found to have TDO inhibitory effects (reviewed in [16]). Among them tianeptin, a

serotonin reuptake enhancer antidepressant itself, has TDO inhibitory properties [55,56].

TDO inhibition was found to be protective also in ischemic stroke. In a mouse model of ischemic stroke, increased mRNA expression of *TDO2*, but not *IDO1* and *IDO2* were observed in the core and peri-infarct regions of middle cerebral artery occlusion (MCAO) animals. As a consequence, L-KYN levels were increased in the brain tissues of affected mice, leading to the activation of endogenous AHR, thus aggravating neuronal damage. However, the TDO inhibitor 680C91 proved to be neuroprotective in this model: treatment with the drug decreased L-KYN production and Trp accumulation, resulting in decreased AHR activation and reduction of infarct volume [57].

3.2.1. TDO and neurodegenerative diseases

Changes in TDO expression have been reported in various models of neurodegenerative diseases (Table 1). Campesan and colleagues conducted a study on the involvement of the KP in Huntington's disease (HD) with the use of a fruit fly (*Drosophila melanogaster*) model. Flies expressing mutant huntingtin (mHtt) presented increased 3-HK/KYNA ratio and decrease in the number of photoreceptor neurons (rhabdomeres), a hallmark of neurodegeneration in this model. Genetic inhibition of TDO or KMO in mHtt expressing animals led to a decrease in the 3-HK/KYNA ratio, signifying a shift toward the production of KYNA, thus creating a more neuroprotective environment. In line with this, TDO or KMO knockout in mHtt expressing animals led to a significant rescue of neurons [58]. The beneficial effects of the genetic inhibition of KMO could be abolished by feeding the flies with 3-HK, suggesting a causative role of restoring the imbalance of neuroprotective and neurotoxic kynurenine metabolites in the observed neuroprotection [58]. Pharmacological inhibition with 680C91 also resulted in a significant reduction of neurodegeneration [59]. In animals lacking the TDO enzyme, restoring the physiological concentrations of 3-HK was not sufficient for abolishing neuroprotection (that could be only achieved by higher concentrations of 3-HK) [59]. Similarly, as seen in the case of TDO inhibition, feeding the animals with Trp also resulted in reduced 3-HK/KYNA ratio, further supporting the causal role of KYNA in neuroprotection in this model [59]. A possible explanation to the above could be that 3-HK-toxicity in primary neurons depends on its uptake by neutral amino acid transporters, the same transporters by which Trp is transported into cells. One can assume that in the presence of excessive Trp, the amino acid inhibits 3-HK toxicity by

Table 1. Effects of TDO inhibition in models of neurodegenerative diseases.

Disease modeled	Model	TDO inhibition	Effects of TDO inhibition	Reference
HD	mHtt expressing Drosophila melanogaster	Genetic inhibition	1. Establishment of a more neuroprotective environment *via* decreasing the 3-HK/KYNA ratio. 2. Decreased neuron loss.	[58]
HD	mHtt expressing Drosophila melanogaster	Pharmacological inhibition by 680C91	1. Shift in the KP toward the synthesis of KYNA. 2. Reduced neurodegeneration.	[59]
PD	Human α-syn expressing Drosophila melanogaster		1. Shift in the KP toward the synthesis of KYNA. 2. Enhanced crawling behavior. 3. Improvement in climbing activity.	
AD	Human Aβ42 peptide expressing Drosophila melanogaster		4. Amelioration of otherwise shortened life span.	
AD	APPSwe-PS1E9 mouse model	Hippocampal *TDO2* down-regulation by COX-2 inhibitor ibuprofen and pharmacological TDO inhibition by 680C91	Prevention of spatial memory deficits.	[62]
AD	APP23 amyloidosis mouse model	Pharmacological inhibition by 680C91	1. Reversal of recognition memory deficits. 2. No measurable changes in kynurenine metabolite levels in the CNS. 3. No effect on behavior related to spatial learning and memory, and anxiety.	[63]
PD	α-syn toxicity Caenorhabditis elegans model	Genetic inhibition	1. Decreased toxicity of α-syn and other aggregation-prone proteins such as beta-amyloid peptide and polyglutamine. 2. Suppression of age-related decline of motility. 3. Extension of chronological lifespan and reproductive span.	[65]
SM	Mouse experimental autoimmune encephalomyelitis	Genetic inhibition	1. No influence on myelin-specific T-cells, myelin degradation, leukocyte infiltration in the CNS and overall clinical disease activity. 2. Increased neuronal survival in the spinal cord.	[60]
PD	Rotenone-induced mouse model	Pharmacological inhibition by NTRC 3531–0 and LM10	1. Decreased cell loss and neuroinflammation in the *substantia nigra*. 2. Improvement of motor functions. 3. Decrease in spatial memory recognition loss. Diminished glial fibrillary acidic protein and α-syn expression in the colon. 4. Restoration of intestinal dysfunction (indicated by diminished rotenone-induced colon length decrease and enhancement of intestinal transit).	[66]

Abbreviations: HD: Huntington's disease; 3-HK: 3-hydroxykynurenine; KYNA: kynurenic acid; KP: kynurenine pathway; α-syn: alpha-synuclein; *TDO2*: Tryptophan 2,3-dioxygenase gene; TDO: Tryptophan 2,3-dioxygenase protein; COX-2: cyclooxygenase-2; CNS: central nervous system.

competitively blocking the uptake of the metabolite on the common transporters [59].

The strong neuroprotective effect of TDO inhibition in HD fly model encouraged researchers to investigate the effects of TDO inhibition in other neurodegenerative diseases. Genetic inhibition of either TDO or KMO in both Alzheimer's disease (AD) and PD *Drosophila* models enhanced crawling behavior and improved climbing ability of the animals [59]. An improvement in climbing activity was also observed in animals in which pharmacological TDO inhibition was produced by 680C91. In addition to the improvement of motor functions, both KMO and TDO inhibition mitigated the otherwise shortened life span. These protective effects were accompanied by changes in kynurenine metabolite levels: the ratio of 3-HK/KYNA was dramatically reduced, however, no significant changes were observed in Trp levels. Based on these findings neuroprotection was attributed to the shift toward the formation of the neuroprotective KYNA [59].

The involvement of TDO in AD was further strengthened by results of studies involving transgenic AD mice and human AD brain samples [61]. Upregulation of TDO was observed both in the mouse model and in human brain samples. In mice the elevated TDO expression was accompanied by an elevated level of the neurotoxic QUIN, displaying an age- and brain region-dependent accumulation. Based on these results it was concluded that elevated expression of TDO is causally linked to enhanced production QUIN and/or PIC, metabolites which are likely to contribute to the neurodegeneration observed in AD [61].

In recent reports Woodling *et al*. and Sorgdrager and colleagues reported that pharmacological inhibition of TDO improved cognitive performance of APP23 and APP-PS1 AD mice by reversing recognition memory deficits of the animals [62,63]. However, anxiety and cognitive dysfunction provoked with lipopolysaccharide (LPS) stress were not prevented by pharmacological TDO inhibition in this mouse model [64]. These seemingly contradictory findings call attention to the possibly varying effects of TDO inhibition in different disease states and also to the importance of the choice of disease model. Regarding the molecular and biochemical differences underlying these opposing results a straightforward explanation could be that AD mice and LPS treated animals show differences regarding the responsiveness for TDO inhibitory treatment. However, TDO inhibition did not affect kynurenine metabolite levels either in brains of APP23 mice or LPS-treated animals [63,64]. Thus further research is needed in order to clarify the exact mode of action of TDO inhibition on cognitive functions.

For a better understanding of TDO involvement in the pathomechanism of PD, van der Goot *et al*. used a *Caenorhabditis elegans* model [65]. In this model the detrimental effects of α-syn accumulation are indicated by age-related progressive decline in the motility of the animals. TDO knockdown decreased α-syn toxicity and similarly toxicity of other aggregation-prone proteins such as β-amyloid peptide and polyglutamine, suggesting a general role of TDO in the process [65]. Genetic inhibition of TDO also extended both the chronological lifespan and the reproductive span of the animals and suppressed age-related motility decline. Neither inactivation of downstream KP enzymes nor feeding the animals with KYN protected against α-syn toxicity, suggesting that variations of KP metabolite levels are not responsible for the protective effects of TDO inhibition [65]. On the contrary, feeding the worms with increasing amounts of Trp led to a dose-dependent suppression of α-syn toxicity. The findings of van der Goot and colleagues point to a further mechanism by which the KP can affect neurodegeneration: *via* regulating age-related protein toxicity [65]. The data suggest that this is achieved through Trp, likely by the amino acid itself or its derivatives acting on other signaling molecules and pathways involved in protein toxicity. Another mechanism by which Trp can be protective was outlined above: blocking 3-HK toxicity by competing for transporter to cellular uptake [59]. These findings indicate that modulation of Trp levels *via* inhibition of TDO could help by delaying the age-related decline of protein homeostasis thus could be a valuable tool in the treatment of aging-associated neurodegenerative disorders, such as PD.

Besides *C. elegans* and *D. melanogaster* models of neurodegenerative diseases, studies using models of higher organisms also yielded promising results on the neuroprotective effect of TDO inhibition. Using mouse experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis, Lanz and colleagues showed upregulated TDO expression in the liver, but not in the CNS of the animals [21]. Though genetic inhibition of TDO did not have an impact on myelin-specific T-cells, myelin degradation, leukocyte infiltration in the CNS and overall clinical disease activity, an increase in neuronal survival in the spinal cords of TDO-/- animals was observed. The authors concluded that manipulation of systemic Trp levels can contribute to the creation of an overall neuroprotective milieu [21], thus modulation of TDO activity could be an applicable mean of promoting neuroprotection in neurodegenerative diseases.

In a recent study Perez-Pardo examined the effect of pharmacological TDO inhibition in a rotenone-induced model of the disease [66]. In these studies, a small-molecule, brain-penetrable selective TDO inhibitor NTRC 3531–0 was used. Inhibition of TDO either by NTRC 3531–0 or LM10 in the rotenone-induced mouse model significantly improved rotarod test evaluated motor functions and diminished spatial recognition loss caused by the toxin treatment [66]. The number of tyrosine hydroxylase positive cells in the *substantia nigra* indicated that NTRC 3531–0 and LM10 treatment decreased rotenone-induced cells loss revealing the neuroprotective effect of TDO inhibition. Besides neurodegeneration, neuroinflammation was also diminished, shown by the decrease of both the mean volume and space occupied by microglia in the *substantia nigra* of rotenone-infused animals upon TDO inhibitor treatment. Beneficial effects of NTRC 3531–0 and LM10 treatment were observed also in the periphery: rotenone led to an increased expression of the enteric glial cell marker, glial fibrillary acidic protein (GFAP) and to an enhanced accumulation of α-syn in the enteric plexus of the animals. In contrast with that, TDO inhibition partially reversed both GFAP and α-syn up-regulation in the colon. In line with

these, both NTRC 3531–0 and LM10 treatment diminished rotenone-induced colon length decrease and enhanced impaired intestinal transit, indicating that TDO inhibition could be a promising tool for the restoration of intestinal dysfunction related to PD [66].

3.3. Effects of TDO inhibitors in models of neurodegenerative disorders

The growing body of evidence on the involvement of TDO in various diseases over the past decades promoted the search for TDO inhibitors. This led to the development of inhibitory compounds of various chemical classes, out of which, however, only the group of indol-3-yl derivatives emerged as selective for TDO [36]. A detailed discussion on the various forms of inhibitors affecting both IDO and TDO or selectively the latter one is beyond the scope of this review, for this see the overview of Kozlova *et al*. [36]. Instead, we summarize data on TDO inhibitors studied on neurodegeneration, especially in studies focusing on PD. The following section summarizes findings of preclinical research involving *in vitro* and *in vivo* models of neurodegeneration. To the best of our knowledge currently no selective TDO inhibitor is in clinical trial yet.

Fluoroindole 680C91:

The fluoroindole compound 680C91 (Figure 2(a)) was developed by Salter and colleagues (Wellcome Research Laboratories [67]) with the aim of producing a selective TDO inhibitor for the treatment of depression. Since then, the compound has been utilized in various studies modeling neurodegeneration and other CNS injuries. As mentioned in a previous chapter, inhibition of TDO by 680C91 protected against neurodegeneration in a fruit fly model of HD [59], decreased infarct volume in a murine model of ischemic stroke [57] and improved recognition memory deficits in a mouse model of AD [63]. Results of Woodling and coworkers however call attention to potential dangers of TDO inhibition by 680C91 [62]. These authors reported that in their study involving a mouse model of AD, treatment of AD mice with 680C91 for one month improved the cognitive functions of the animals, however, the same treatment regimen resulted in worsening performance in novel object recognition tests in control animals. This result suggests that TDO inhibition under physiological conditions might have adverse effect (s) [62].

680C91 induced TDO inhibition was also found to have different effects on memory function of genetic models of AD mouse and in LPS-treated animals (as discussed in the previous chapter) [62–64], calling attention to the importance of specific circumstances in various disease states which can influence whether inhibition of the enzyme is beneficial, detrimental, or has no impact on a specific disorder/symptom.

6-fluoro-indole substituted indol-3-yl derivative LM10:

In vitro studies of Pilotte and coworkers showed that treatment with 680C91 led to a complete blockage of Trp degradation. Though results of enzyme assays proved this to be a consequence of direct TDO inhibition, the applicability of the compound was questioned since *per os* implemented 680C91 in the P815 mouse tumor model showed poor bioavailability presumably due to its poor solubility [49]. This led to an extensive structure – activity study on a series of 3-(2-(pyridyl)ethenyl)indoles in order to overcome the obstacle and improve water solubility, hence bioavailability of the compound. A result of this work is the development of LM10. Similarly to 680C91, LM10 shows selectivity toward TDO, however, presents higher oral bioavailability [68]. LM10 (Figure 2(b)) belongs to the class of indol-3-yl derivatives, with a 6-fluoro-indole substitution in the 3-position by a tetrazolyl-vinyl side chain [49]. *Per os* treatment by LM10 of a mouse tumor model promoted tumoral immune rejection, marking use of the compound as a promising new therapeutic approach for cancer treatment [49]. Regarding neurodegenerative diseases, treatment of an AD mouse model with LM10 improved cognitive dysfunction of the animals [62].

A brain penetrable TDO inhibitor NTRC 3531–0:

In a very recent study Perez-Pardo and colleagues introduced a small molecule size, brain penetrable TDO inhibitor, NTRC 3531–0 (Figure 2(c)). The effect of NTRC 3531–0 was evaluated in parallel with that of LM10 in a rotenone-induced mouse model of PD [66]. While both compounds resulted in an improvement of motor, cognitive, and gastrointestinal dysfunctions, prominent differences were observed between the two inhibitors regarding their effects on Trp levels. In contrast to NTRC 3531–0, treatment with LM10 did not result in a prominent rise in either plasma or brain Trp levels despite the high level of the inhibitor in the plasma of animals. The lack of expected Trp increase in the periphery upon LM10 application was explained by the results of cellular assays which indicated noticeably lower cellular potency of the molecule. The absence of an increase in brain Trp levels was explained as the

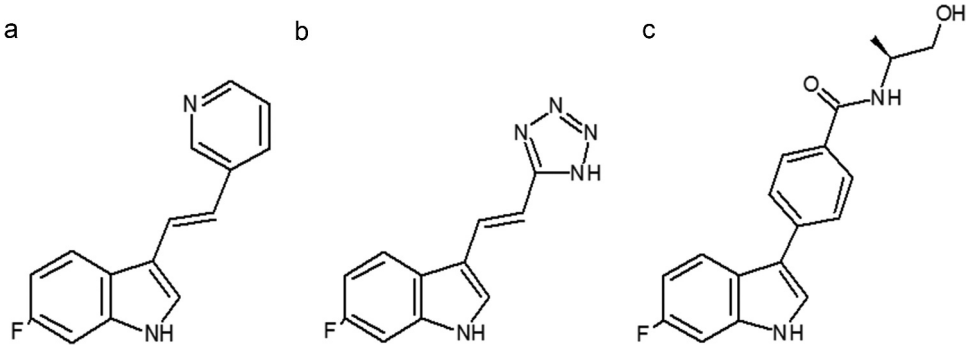

Figure 2. Chemical structure of TDO inhibitors 680C91 (a), LM10 (b) and NTRC 3531–0 (c) [66–68].

consequence of poor brain penetration of LM10 [66]. NTRC 3531-0 in contrast was found to show higher brain penetration. Based on their findings Perez-Pardo and coworkers concluded that NTRC 3531–0 is the most promising compound for studying both CNS and peripheral functions of TDO [66].

Besides molecules resulting from searches for specific TDO inhibitors, there is a handful of drugs used in everyday medicine that also possess TDO inhibitory effects. In addition to medications used in the treatment of depression and other mood disorders (discussed in section 'Alterations in TDO function in disease states'), recently a widely used non-steroidal anti-inflammatory drug, ibuprofen was shown to inhibit TDO. With the use of an AD mouse model, Woodling *et al*. demonstrated that treatment with ibuprofen down-regulated hippocampal TDO expression and improved cognitive functions of the animals [62].

The development of adequate inhibitors of TDO poses challenges and pitfalls which need to be overcome. One of these is the selectivity of the molecules: exhibiting inhibition on TDO, but not on IDO. A further difficulty is the relatively small size and the lipophilic nature of the active site of the TDO enzyme, which greatly narrow the spectrum of chemical structures of molecules that can be utilized [36,68]. A hopeful development in TDO inhibition research could be the recent description by Lewis-Ballester *et al*. of an L-Trp exo binding site which, in contrast to the active site of the enzyme, does not affect the catalytic activity of the protein; however, it was found to be critical in the cellular stability and half-life of the enzyme. Together with the recently described crystal structure of the protein [69], these data widen the possibility of extending the field of potential TDO activity modulating compounds [36].

4. Conclusion

Various reports on alterations of KP metabolite levels in peripheral tissue samples of PD patients strengthen the notion of existing links between TDO function and the pathomechanism of the disease. These results make TDO enzyme an appealing new therapeutic target in this neurodegenerative disorder. TDO inhibition indeed alleviated PD symptoms in various models of the disease and enzyme modulation resulted in manifest neuroprotection, giving high hopes for such therapeutic approaches in the clinical practice as well. However, no TDO inhibitor has reached the clinical phase so far. In order to facilitate the development of such therapies, further preclinical studies are necessary. In light of the promising results of recent preclinical studies, further research for developing novel selective TDO inhibitors and trials involving the use of those in treatment of PD are highly warranted.

5. Expert opinion

Despite intensive research aiming at understanding better the molecular mechanisms underlying PD, the exact pathomechanism of the disease is still not fully elucidated. It is clear that the motor symptoms of the disorder are the consequences of the loss of dopaminergic neurons in the *substantia nigra*. However, there is a growing body of evidence supporting the notion that PD is not restricted to the CNS and more and more data shed light on the involvement of the peripheral nervous system and systemic inflammation in the development of accompanying non-motor symptoms.

Trp metabolism has a direct effect on serotonin synthesis and *via* the KP it produces several neuroactive metabolites, thus providing potential targets for the treatment of both primary and the often present accompanying non-motor symptoms of PD.

Inhibition of TDO, a key enzyme at the entry of the KP which has a major role in determining Trp and KP metabolite levels, can exert beneficial effect on PD development and progression *via* various mechanisms. It has been reported in the early 1990s that the concentration of serotonin is diminished in PD and shows negative correlation with disease severity [70]. Increasing serotonin level *via* TDO inhibition holds the potential of improving cognitive function and also alleviating depressive symptoms, which are often present in PD [18,66]. TDO inhibition results in a shift toward the production of the neuroprotective KYNA [58,59] and also holds the potential of modulating the levels of downstream neurotoxic kynurenine metabolites such as the free radical generator 3-HK and the NMDA receptor agonist QUIN [19]. Indeed, intrastriatal administration of QUIN in mice led to an increase in the levels of phosphorylated α-syn, and as recent findings of Tavassoly and colleagues indicate QUIN directly promotes the formation of α-syn aggregates [71]. Another possible mechanism of neuroprotection by TDO inhibition is blocking 3-HK toxicity *via* elevating Trp level, since the amino acid and the neurotoxic kynurenine metabolite compete for the same transporter for cellular uptake [59].

The key role of TDO within the KP pathway together with its localization within the human body should be taken into consideration when evaluating the therapeutic potentials of TDO inhibition: hepatic TDO is mainly responsible for regulating systemic Trp and consequently, kynurenine metabolite levels. However, while under physiological conditions the activity of IDO1 in extrahepatic tissues is significantly lower [16], under stress, systemic inflammation, and immune activation, IDO1 is highly upregulated. As systemic inflammation/immune activation might be in a causal relationship with PD, implementation of TDO and IDO1 inhibitor combinational therapy should be considered.

The question whether the beneficial effects of TDO inhibition are due to modulation of the enzyme activity in the CNS, the periphery or both is also crucial when developing TDO inhibitor drugs [66]. Synthesis of molecules that show high bioavailability and are also capable of crossing the blood-brain-barrier often poses a great challenge. However, central TDO inhibition might not be required for achieving disease symptom modification. This notion is supported by the increasing amount of data indicating the involvement of peripheral tissue and vegetative nervous system in the course of PD (such as chronic intestinal inflammation, alterations of the gut microbiome, α-syn aggregates spreading *via* the vagal

nerve [72]). The observed advantageous effects of a non-brain penetrant KMO inhibitor on the phenotype of AD mouse model support this notion [73]. Research using tissue-specific TDO knockout animals could help elucidating this question.

Due to the complexity and diverse symptoms of PD, none of the currently available models of the disease is perfectly able to recapitulate all aspects of the disorder [74]. Moreover, the use of different disease models can sometimes lead to confusing results. Well represents this the contrasting findings of Kanai *et al*. [53] and Imbeault and colleagues [64] on the anxiolytic effects of TDO inhibition. To interpret these seemingly contradictious findings the different disease models used should be taken into consideration. In the study of Kanai *et al*. TDO knockout mice were used and anxiety-related behavior tests were performed *per se*, without any stressors implemented. On the contrary, Imbeault and colleagues evaluated anxiety-related behavior in a mice model in which LPS-induced neuroinflammation was used as a stressor and the animals were treated with 680C91 TDO inhibitor *per os*. The mode of TDO inhibition should also be taken into consideration when evaluating results of these studies. According to Pilotte *et al*., although treatment with 680C91 led to a complete blockage of Trp degradation and direct TDO inhibition by the compound was detected in *in vitro* studies, results of experiments involving the P815 mouse tumor model revealed poor bioavailability of *per os* implemented 680C91 [49].

In summary, based on currently available data TDO inhibition seems to be beneficial in PD by various mechanisms: i) by inducing a shift in the production of neuroprotective and neurotoxic KP metabolites; ii) increasing Trp levels thus a) promoting serotonin synthesis and b) blocking 3-HK toxicity and iii) *via* protecting against proteotoxicity [65].

It is an interesting question whether the mode of action and/or effects of TDO inhibition vary between different genetic types and idiopathic PD cases. The diversity of genes/proteins and by these the cellular functions being affected in the disease raises the question whether the molecular causes leading to mitochondrion dysfunction, protein aggregation, neuroinflammation, and further dysfunctions can all be looked at as parts of one or several pathways which eventually 'meet' in a common pathomechanism and lead to loss of dopaminergic neurons. This question becomes even more intriguing if we consider the multi-system nature of PD, which is being recognized more generally nowadays. It is clear that the loss of dopaminergic neuronal structures in the CNS plays a direct role in the development of some of the most characteristic PD symptoms, but it is also suspected that alterations in the peripheral nervous system and even in the intestinal microbiome might have previously unnoticed contribution to this. The complexity and yet unclarified pathomechanism underlying the disease make it particularly difficult to determine exactly how does TDO inhibition exert beneficial effects in PD.

Moreover, TDO inhibition in PD might hold risks as well. In their study Cuartero and colleagues identified L-KYN as an AHR agonist, thus playing a deleterious role in cerebral ischemia in a mouse model of stroke [57]. They found that diminishing L-KYN levels by the use of the TDO inhibitor 680C91 led to inhibition of AHR activation which resulted in decreased infarct volume. On the other hand, recent results in PD suggest a protective role of AHR activation in the disease *via* inducing Parkin expression that leads to a decrease of α-syn production [75]. Carbidopa, a widely used anti-PD drug, is also an AHR activator and recently dopamine itself has been shown to possess AHR agonist effects as well [76]. These results well demonstrate the complexity and differences of possible pathomechanisms underlying various neurological disorders and imply a potential pitfall of TDO inhibition in PD. Results on worsening/decline in cognitive functions of wild-type mice upon long term (4 weeks) TDO inhibitor treatment might also raise concerns [62].

Despite the controversies and cautions, considering the various beneficial effects TDO inhibition holds, conducting further preclinical studies and pharmacological developments which would enable testing TDO inhibitors in clinical studies are highly warranted. This requires the identification and development of preclinical models which correctly reflect the symptoms of PD patients. In order to achieve this identification of the primary molecular mechanism(s) leading to the loss of dopaminergic neurons is essential. Elucidating the pathomechanism underlying PD would give us a better understanding of the disease and would help answer the question whether TDO modulation is indeed a feasible mean for affecting the development of the disease by interfering levels of KP metabolites.

Abbreviations

PD: Parkinson's disease
CNS: central nervous system
DBS: deep brain stimulation
KP: kynurenine pathway
NAD^+: nicotinamide adenine dinucleotide
KYNA: kynurenic acid
NMDA: N-methyl D-aspartate
QUIN: quinolinic acid
3-HK: 3-hydroxykynurenine
3-HAA: 3-hydroxyanthranilic acid
IDO1: indoleamine 2,3-dioxygenase 1
TDO: tryptophan 2,3-dioxygenase
KYN: N-formylkynurenine
MPP+: 1-methyl-4-phenylpyridinium
CSF: cerebrospinal fluid
KAT: kynurenine aminotransferase
AA: anthranilic acid
KYNU: kynureninase
TDO2: tryptophan 2,3-dioxygenase gene
GRE: glucocorticoid responsive element
AHR: aryl hydrocarbon receptor
MCAO: middle cerebral artery occlusion
HD: Huntington's disease
mHtt: mutant huntingtin
AD: Alzheimer's disease
PIC: picolinic acid
LPS: lipopolysaccharide
GFAP: glial fibrillary acidic protein

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants, or patents received or pending, or royalties.

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