Sandeep Mittal *Editor*

Targeting the Broadly Pathogenic Kynurenine Pathway

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This book is dedicated to my mother,
Maya Devi Mittal, and father,
Dr. Khyali Ram Mittal, for their continued
inspiration and wisdom; to my wife,
Monika, for her endless support;
and to our three children, Priya, Aadesh,
and Anisha who are the joy of my life.

Foreword

Kynurenines: Poor Relatives No More

More than 160 years have passed since Justus von Liebig isolated "fine, colorless, and silky crystals" from dog urine and named the substance kynurenic acid (Greek: *kynos*, dog + *ouron*, urine). At the turn to the twentieth century, Frederick G. Hopkins identified tryptophan and realized that this amino acid could not be manufactured in the body but had to be supplied with the diet. Another 30 years later, Yukio Kotake isolated kynurenine from rabbit urine, setting the stage for the successive decoding of the cascade of metabolic events, which became known as the kynurenine pathway of tryptophan degradation. By the 1960s, most pathway enzymes and several of their control mechanisms had been elaborated using classic biochemical approaches. As no exceptionally impressive physiological functions of pathway metabolites were found at the time, "kynurenines" were largely viewed as rather innocuous bioprecursors en route to NAD^+, and the focus of most leading investigators in the field shifted to other, more appealing, tryptophan metabolites such as serotonin and melatonin.

As illustrated in chapter after chapter in this book, however, we have now reached an era where the roles of kynurenines in physiology and pathology have become too obvious to overlook. Exciting, and often accidental, discoveries have begun to alert basic scientists and clinical investigators alike that "attention must be paid" to these tryptophan metabolites, whose dysfunction in diseases affecting millions of patients worldwide had been ignored for too long. The timing is right since advances in genetics and the related emphasis on precision medicine, increasingly sophisticated analytical and surgical methods, as well as novel non-invasive procedures and devices, now enable us to conceptualize and address biological questions in ways that were unimaginable even a generation ago. In the medical realm, these developments allow us to design targeted interventions and envision distinct clinical benefits based on our new insights into the pathophysiologically relevant features of the kynurenines.

Not surprisingly, almost every article in the following compendium contains an essentially identical schematic version of the kynurenine pathway and points out that, at least in mammals, the vast majority of dietary tryptophan is first broken down oxidatively and then further degraded by this catabolic cascade. For good reason, therefore, many contributors emphasize the importance of the initial event, i.e., the oxidative opening of tryptophan's pyrrole ring, which is catalyzed by the highly—and distinctly—regulatable enzymes indoleamine 2,3-dioxygenase (IDO) 1 and 2, and tryptophan 2,3-dioxygenase (TDO). Although the term "rate-limiting" should be used only contextually, i.e., not to the exclusion of other pathway enzymes such as the pivotal kynurenine 3-monooxygenase, IDO and TDO are indeed of exceptional interest for their critical involvement in the function and dysfunction of immune and endocrine systems. Related to the large number of inspiring discoveries regarding their putative roles in physiology and pathology, these oxygenases have so far understandably also received preferred attention in attempts to modify kynurenine pathway metabolism by genetic or pharmacological means. A number of exciting developments in this regard, as well as their translational ramifications in areas as diverse as oncology and depressive disorders, are highlighted in this volume.

Several fundamentally new, remarkable discoveries have recently been made in kynurenine biology but are necessarily somewhat de-emphasized in most chapters due to an explicit focus on clinically relevant phenomena. Probably most interesting, the physiological actions and chemical properties of individual pathway metabolites turn out to be far more intricate than previously assumed. The realization that l-kynurenine is a potent ligand of the aryl hydrocarbon receptor (and may thus mediate tumor immunity), that kynurenic acid also activates this receptor as well as GPR35, and that the often neglected metabolites xanthurenic acid and cinnabarinic acid selectively affect metabotropic glutamate receptors (and in the case of xanthurenic acid also vesicular glutamate uptake) has obvious functional ramifications, especially in the areas of oncology and neurobiology. Maybe even more far reaching is the ability of most kynurenines to participate actively in intra- and intercellular processes involving reduction/oxidation phenomena and in the generation or scavenging of reactive free radicals. Although debated for many years, this area of investigation has accelerated markedly in recent years. For example, it turns out that both 3-hydroxykynurenine and 3-hydroxyanthranilic acid, which are increasingly recognized as key players in the metabolic cascade, have pro- as well as antioxidative qualities and can form but also eliminate short-lived, biologically active radicals. The circumstances favoring one or the other of these properties and effects in an in vivo environment, the interdependent fate and functions of these two metabolites, and possible implications for pathological situations are far from being understood. One of the lessons to be learned from these and related considerations is that both basic and clinical studies involving kynurenines will always benefit from a holistic approach to pathway metabolism.

A corollary of the commendable overall focus on clinically relevant topics in this book is the wealth of novel information derived from studies in humans. Although open to the usual criticisms based on patient selection (disease heterogeneity, genetic variance, lack of concern for drug effects, comorbidities, etc.), these articles are indispensable for several reasons. They not only contribute to existing databases concerning disorders that are known to be causally linked to immune system dysfunctions but also provide evidence for less-appreciated roles of kynurenines in the pathogenesis of respiratory and cardiovascular diseases. Maybe most consequentially, we are reminded that intestinal microorganisms are capable of degrading tryptophan along the kynurenine pathway and thus influence the status of kynurenines in the host. In classic bedside-to-bench fashion, these clinical studies therefore generate etiologically relevant new hypotheses, which can now be readily tested in appropriate experimental situations and also contribute invaluable data for investigations of the physiological role of kynurenines in both animals and humans. Moreover, though considerable care needs to be taken to prevent overinterpretation, especially with regard to brain processes, this volume offers the interested reader an excellent state-of-the-art guide regarding the use of circulating kynurenines as biomarkers in both physiology and pathology.

The final chapters, too, provide ample food for thought, highlighting the promise of treatments with pharmacological agents that specifically target individual kynurenine pathway enzymes. However, though significant progress has been made during the past years—again with major emphasis on IDO and TDO inhibitors—and initial clinical trials are underway, kynurenine-directed efforts in medicinal chemistry and drug discovery are clearly still in their early stages. Based on the impressive preclinical and clinical evidence detailed here, there can be little doubt that normalization of impairments in kynurenine pathway metabolism has significant therapeutic potential in a wide range of pathological conditions. It is therefore hoped that appropriately selective drugs for hypothesis testing and subsequent clinical use will become available in the not too distant future.

Although kynurenines do not face the danger of being relegated to the ash heap of history a second time, many challenges remain. As also alluded to by several authors here, we still know far too little about the genetic—and epigenetic—mechanisms that determine the intricate features of the kynurenine pathway in health and disease, lack appropriate imaging techniques to assess its function and dysfunction noninvasively, and, as mentioned above, need to rapidly develop additional specific agents, which are capable of accurately manipulating kynurenines in vitro and especially in vivo. All authors, and especially the volume editor, Dr. Mittal, should be thanked for their superb summary and clarification of the current status of the field and for providing an outstanding rationale for many exciting research directions.

Baltimore, MD, USA Robert Schwarcz, Ph.D.

Preface

My introduction into the world of tryptophan metabolism and the kynurenine pathway evolved from my desire to find new avenues to better understand brain tumor biology and immune suppression with the hope of identifying new treatment targets. As my own research progressed, I began to explore the tryptophan field for potential research collaborators, reagents, and resources, and I was astonished to note that tryptophan catabolism plays a major role in not only health but also a wide range of human diseases. In fact, I was quite amazed to learn that the kynurenine pathway was recognized as a prominent player in several ailments including inflammation, cardiovascular disease, respiratory illnesses, psychiatric disorders, neurodegenerative diseases, and stem cell biology. Although the kynurenine pathway in the context of brain tumors is still in its infancy, the landscape of tryptophan metabolism field is quite mature. This sparked the idea to bring together, in a single volume, leading authorities from across the globe to share their thoughts and ideas on the common topic.

This book highlights the known associations between kynurenine pathway and the various pathologies, as well as examines the current status of drug development and clinical trials of compounds known to alter tryptophan metabolism. The research represented here provides a distinctive bridge between basic mechanistic understanding of the kynurenine pathway and clinically relevant translational applications. It explores the indications that tryptophan metabolism is a potential biomarker of disease activity, can contribute to local and possibly systemic immune suppression in cancer, and is an attractive target for a variety of inhibitors which are readily available. When considering the sheer number of people that suffer from one (or more!) of the diseases and disorders listed below, the potential clinical impact of drugs targeting the kynurenine pathway enzymes is immeasurable.

In order to facilitate the understanding of the different biological contexts of the kynurenine pathway, the book is divided into six parts. Part I (6 chapters) provides a broad overview of tryptophan metabolism and the kynurenine pathway and discusses its importance in early brain development, aging, mood, behavior, and cognition. Part II (8 chapters) addresses the role of the kynurenine pathway in some of the most common systemic diseases including inflammation, allergy, HIV/AIDS,

cardiovascular, respiratory and gastrointestinal diseases, insulin resistance, and infections. Part III (5 chapters) outlines the critical role of tryptophan metabolism in neurodegenerative diseases, epilepsy, stroke, pain, and migraines, and various neuropsychiatric conditions. Part IV (4 chapters) focuses on oncology including stem cell biology, cancer biology, neuro-oncology, and hematologic malignancies. Part V (5 chapters) explores development of animal models to study the kynurenine pathway as well as molecular imaging techniques. Finally, Part VI (3 chapters) highlights the different inhibitors of the kynurenine pathway, the ongoing clinical trials targeting the kynurenine pathway, and discusses the future of tryptophan metabolism research.

Overall, my goal of bringing together experts from a wide range of disciplines has come to fruition and far exceeded my initial vision. This book project could not have been successfully completed without the hard work and dedication of its contributing authors. I am also particularly indebted of my colleagues, students, and lab members, as well as the team at Springer for their support, help, and patience during the entire process. I hope readers will gain the same appreciation for the breadth and depth of the tryptophan metabolism field as I have in the preparation of this volume.

Detroit, MI, USA Sandeep Mittal

Contents

Part I
Overview

Chapter 1
Overview of the Kynurenine Pathway of Tryptophan Metabolism

Sharon K. Michelhaugh, Anthony R. Guastella, and Sandeep Mittal

Abstract Tryptophan (TRP) is an essential amino acid that plays a critical role in synthesis of a host of modulatory biomolecules including serotonin, melatonin, tryptamine, and kynurenine (KYN). TRP can either be incorporated into proteins, converted to the neurotransmitter serotonin (5-hydroxytryptamine), or metabolized to kynurenine. The majority of dietary TRP is metabolized via the kynurenine pathway (KP). The initial and rate-limiting step in the KP involves one of three enzymes, namely, the two isoforms of indoleamine 2-3-dioxygenase (IDO1 and IDO2) and tryptophan 2,3-dioxygenase (TDO). In this chapter, we provide a broad overview of the KP and explore the gene regulation of the key enzymes involved.

Keywords Kynurenine • Tryptophan • Kynurenine pathway • Indoleamine 2,3-dioxygenase • Tryptophan 2,3-dioxygenase • Serotonin • Gene promoter

List of Abbreviations

3-HK	3-Hydroxykynurenine
AhR	Aryl hydrocarbon receptor

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GR	Glucocorticoid receptor
IDO	Indoleamine 2,3-dioxygenase
KAT	Kynurenine aminotransferase
KYNA	Kynurenic acid
KYN	Kynurenine
KMO	Kynurenine 3-monooxygenase
KP	Kynurenine pathway
QUIN	Quinolinic acid
TRP	Tryptophan
TDO	Tryptophan 2,3-dioxygenase
STAT	Signal transducer and activator of transcription

Introduction

Tryptophan (TRP) is an essential amino acid that, after consumption, is either incorporated into proteins via new protein synthesis, converted to the neurotransmitter serotonin (5-hydroxytryptamine) in select populations of neurons by TRP hydroxylase, or metabolized to kynurenine (KYN) by one of the trio of 2,3-dioxygenase enzymes. The rate-limiting conversion of TRP to KYN may be mediated by either of two forms of indoleamine 2,3-dioxygenase (IDO) or by tryptophan 2,3-dioxygenase (TDO). The general schema of the kynurenine pathway (KP) is shown in Fig. 1.1. The KYN may be further metabolized to kynurenic acid (KYNA; can antagonize glutamate receptors), to 3-hydroxykynurenine (3-HK), or to anthranilic acid and, further downstream, quinolinic acid (QUIN; glutamate receptor agonist) [1]. However, a recent study has shown that in the context of glioma (and will likely be relevant not only to other brain tumor types, but many other cancers), KYN directly activates the aryl hydrocarbon receptor (AhR; also known as the dioxin receptor) [2], which has a well-established history of pathogenesis [3].

Gene Regulation of the Kynurenine Pathway Enzymes

As mediators of the rate-limiting step of KYN production, enzyme activity of IDO1, IDO2, and TDO has a broad scope in the context of KYN-related disorders. Therefore, an assessment of factors regulating gene expression of the enzymes will be beneficial to understand pathogenic mechanisms of the KYN pathway. In silico-generated promoter maps for all three human genes are shown in Fig. 1.2. Overall, the three gene promoters have very little in common.

The IDO1 promoter is the most well studied of the three dioxygenase genes. IDO1 gene expression is well known to be regulated by interferon-γ-mediated activation of the interferon-responsive factor 1, which may act in concert with or independently from signal transducer and activator of transcription (STAT) factors

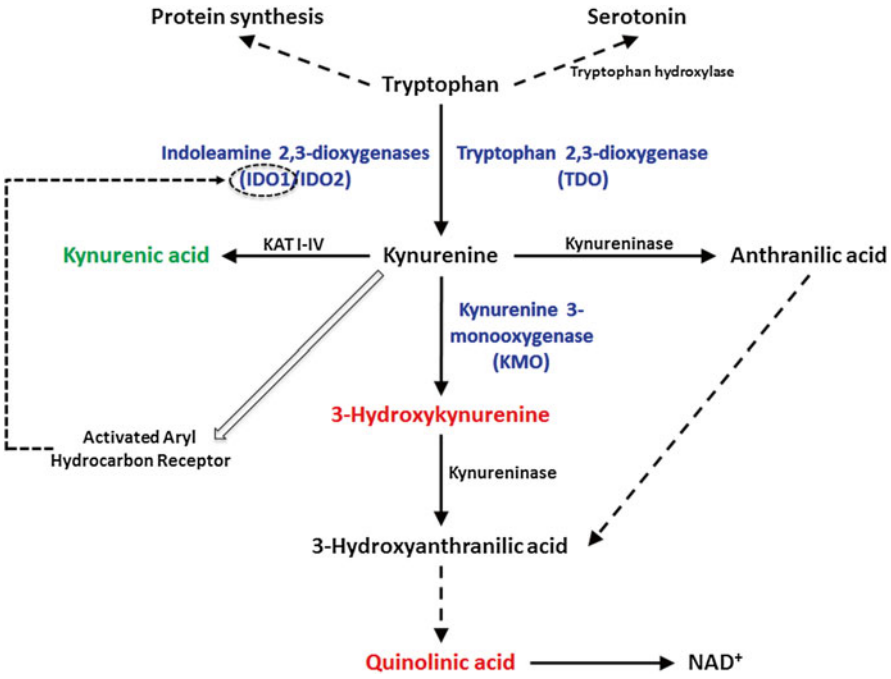

Fig. 1.1 Overview of the kynurenine pathway (KP). Tryptophan has three predominant metabolic pathways in the body: incorporation into proteins, serotonin production, or the KP. Some KYN metabolites are neurotoxic (QUIN, which activates glutamate receptors), while KYNA is neuroprotective (due to antagonism of glutamate receptors). KYN was also recently shown to be an AhR receptor agonist in the context of human glioma

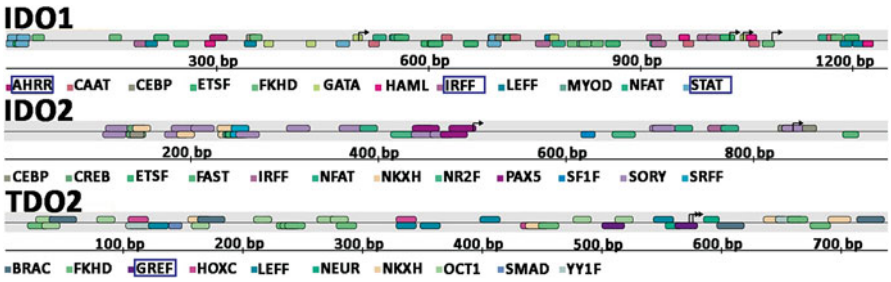

Fig. 1.2 IDO1, IDO2, and TDO gene promoters. In silico analyses of the three gene promoters were performed with Genomatix MatInspector using the ElDorado 12-2013 database and the Matrix Family Library Version 9.1 [4]. Potential regulatory transcription factor binding sites with a matrix similarity greater than 0.9 are shown. Transcriptional elements that have been demonstrated experimentally to regulate gene expression (as determined by publications indexed in PubMed) are indicated with a *blue box*. *Arrows* indicate the transcriptional start sites of bona fide transcripts

[5–7]. Studies have also shown that IDO1 expression can be upregulated by treatment with AhR ligands [8–10]. In light of the recent discovery that KYN is an activating ligand for the AhR [2], this implies a feed-forward loop that has the potential to generate persistent, high levels of IDO1 expression and warrants further study. Interestingly, the IDO1 promoter also contains a binding site for the forkhead family of transcription factors (of which forkhead box P3 [FOXP3] is a member), but studies exploring IDO1 regulation via this binding site are lacking in the literature.

Recent studies of the TDO promoter have revealed that the glucocorticoid receptor (GR) can regulate TDO expression in a cell type-dependent manner through binding to the GR receptor binding sites (noted as GREF in Fig. 1.2). In the liver, where TDO is prominently expressed, GR upregulates TDO expression [11], contrary to glioblastoma cells in which GR receptor activation represses the TDO gene promoter [12].

An examination of the literature reveals very little information about the regulation of the genes encoding the KYN-modifying enzymes kynurenine 3-monooxygenase (KMO), kynureninase (KYNU), or kynurenine aminotransferase (KAT) I-IV. In silico analyses are shown in Fig. 1.3. These gene promoters have some elements in common with the dioxygenase enzymes (such as the forkhead family and interferon response elements) giving rise to the intriguing thought of coordinated regulation of these KP-member genes. Clearly, further study of the regulation of the KP gene promoters may provide new insights into the potential interrelationships of the individual enzymes.

Kynurenine Pathway: Role in Disease

The KP has two main responsibilities in regard to TRP in the body: deplete serum levels and convert it to its biologically active metabolites. These metabolites, along with the enzymes responsible for their production, have implications in a plethora of disease states. IDO has been connected to various types of infections, such as the hepatitis B and C virus [13], malaria [14], and bacteremic patients [15], as well as cancer and the immune escape often observed in tumors [16]. Under normal physiological conditions, TDO mediates the metabolism of TRP in mature hepatocytes [17], and although TDO has not been found to be influenced by immunological responses, constitutive expression modulates the immune system in some cancers [18, 19]. TDO and KMO have been shown to play a role in adult and fetal neurogenesis, respectively [20, 21]. The downstream metabolites QUIN and KYNA have been shown to be neurotoxic and neuroprotective, respectively, and as such, a fine balance in the QUIN:KYNA ratio is observed within the brain [22]. Although KYNA is neuroprotective, increased levels have been observed in patients with schizophrenia [23], whereas increased levels of QUIN are associated with Huntington's disease [24]. These few examples shed light on the intricate role of the KP in human physiology and that therapeutics targeting the enzymes of the KP will be of great clinical significance.

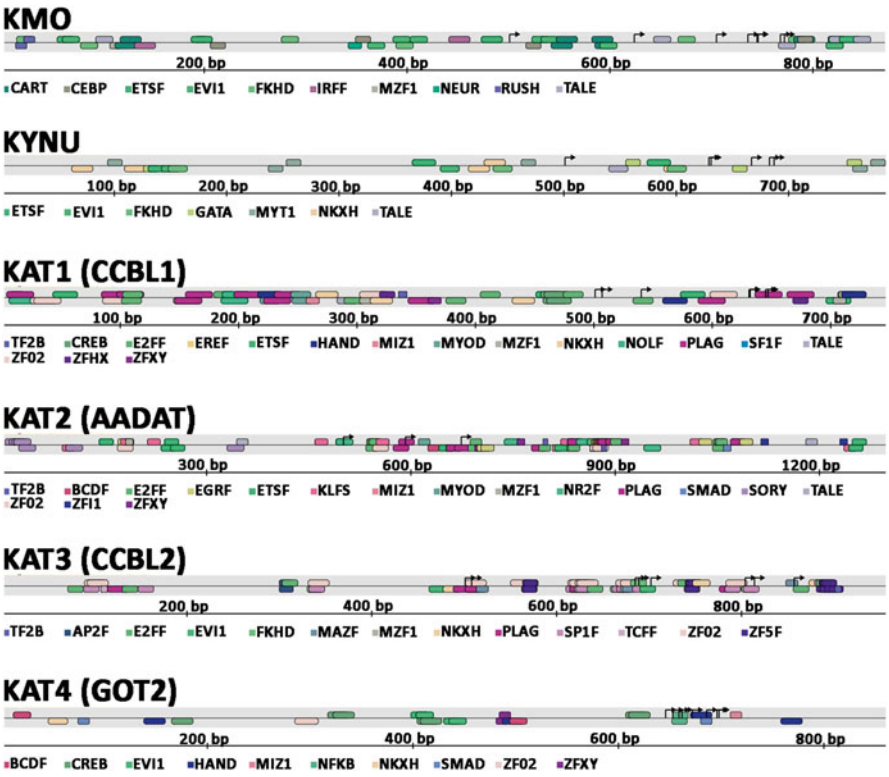

Fig. 1.3 Gene promoters of KYN-modifying enzymes. In silico analyses of the gene promoters for KMO, KYNU, and KAT I–IV were performed with Genomatix MatInspector using the ElDorado 12-2013 database and the Matrix Family Library Version 9.1 [4]. Potential regulatory transcription factor binding sites with a matrix similarity greater than 0.9 are shown. No published studies have assessed the regulation of these gene promoters

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Chapter 2
Tryptophan Metabolism and the Hepatic Kynurenine Pathway in Health and Disease

Abdulla A.-B. Badawy

Abstract Tryptophan (TRP) metabolism and disposition are reviewed with particular emphasis on the hepatic kynurenine pathway (HKP) in health and disease. Over 95 % of dietary TRP is metabolized in the HKP, which is controlled mainly by tryptophan 2,3-dioxygenase (TDO) and produces important metabolites affecting functions in the brain and periphery. TDO is regulated by glucocorticoids, the substrate TRP, and the cofactor heme and by feedback inhibition by reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H). TDO activity determines the rate of TRP degradation and hence its availability and that of its kynurenine (KYN) metabolites for various functions. TDO controls synthesis of heme in liver and serotonin (5-HT) in brain. TDO plays a central role in alcoholism, which exerts multiple effects on the HKP. The 5-HT deficiency in major depressive disorder (MDD) is due to a high TDO activity and antidepressant drugs act in part as TDO inhibitors. Only the HKP contains all the necessary enzymes for nicotinamide adenine dinucleotide (NAD+) synthesis and so plays the central role in pellagra. Criteria for assessing TRP oxidation are proposed. TDO may play a central role in the hepatic porphyrias by utilizing the regulatory heme pool. Maternal TRP availability is enhanced throughout pregnancy by TDO inhibition and altered TRP disposition. Immune activation does not play a role in TRP disposition during pregnancy nor in the 5-HT deficiency in MDD. Liver TDO inhibition is a potential strategy for treatment of depression, hepatic porphyrias, and cancer in view of emerging evidence of the role of TDO in cancer biology.

Keywords Alcoholism • Depression • Indoleamine 2,3-dioxygenase • Kynurenine pathway • Nicotinic acid • NMDA receptors • Pellagra • Porphyria • Pregnancy • Serotonin • Tryptophan • Tryptophan 2,3-dioxygenase

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List of Abbreviations

AA	Anthranilic acid
BCAA	Branched-chain amino acids
CAA	Competing amino acids
FAD	Flavin-adenine dinucleotide
3-HAA	3-hydroxyanthranilic acid
5-HIAA	5-hydroxyindole-3-acetic acid
3-HK	3-hydroxykynurenine
HKP	Hepatic kynurenine pathway
5-ALAS	5-aminolaevulinate synthase
5-HT	5-hydroxytryptamine or serotonin
5-HTP	5-hydroxytryptophan
IDO	Indoleamine 2,3-dioxygenase
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
KA	Kynurenic acid
KP	Kynurenine pathway
KYN	Kynurenine
MDD	Major depressive disorder
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
NAD(P)H	Reduced nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acids
NMDA	*N*-methyl-*D*-aspartate
PA	Picolinic acid
PLP	Pyridoxal 5´-phosphate
QUIN	Quinolinic acid
TRP	Tryptophan
TDO	Tryptophan 2,3-dioxygenase
TTOX	Total tryptophan oxidation
TTOXF	Total tryptophan oxidation relative to plasma-free tryptophan

Introduction

The essential amino acid *L*-tryptophan (TRP) is unique because of its multiple activity. The International and Regional Societies for Tryptophan Research exist to promote TRP research across many scientific disciplines and medical specialties, and the diversity of chapters in this publication is a testimony to involvement of TRP and its metabolism in a wide array of clinical conditions. In this chapter, a brief overview of TRP metabolism will precede a detailed account of the biochemistry of the hepatic kynurenine pathway (HKP) in health and disease.

The Tryptophan Metabolic Pathways

At most, only 1 % of dietary TRP is utilized for protein synthesis, because, in a person in nitrogen equilibrium, the amount of protein synthesized matches exactly that which is degraded [1]. The bulk of dietary TRP is therefore available for metabolism, via four pathways, the quantitatively most important of which is the HKP, accounting for >95 % of TRP degradation [1, 2]. Though minor, the other three pathways (Fig. 2.1) produce physiologically important metabolites, including serotonin (5-hydroxytryptamine; 5-HT) and melatonin (in the pineal), tryptamine, indole-3-pyruvic acid, and indole-3-acetic acid. The HKP will be described separately below, but the following is a brief description of the other three pathways (for additional details, see [1, 2]).

5-HT synthesis is a 2-step process, with TRP hydroxylation being rate-limiting. However, decarboxylation of 5-hydroxytryptophan (5-HTP) to 5-HT could be limit-

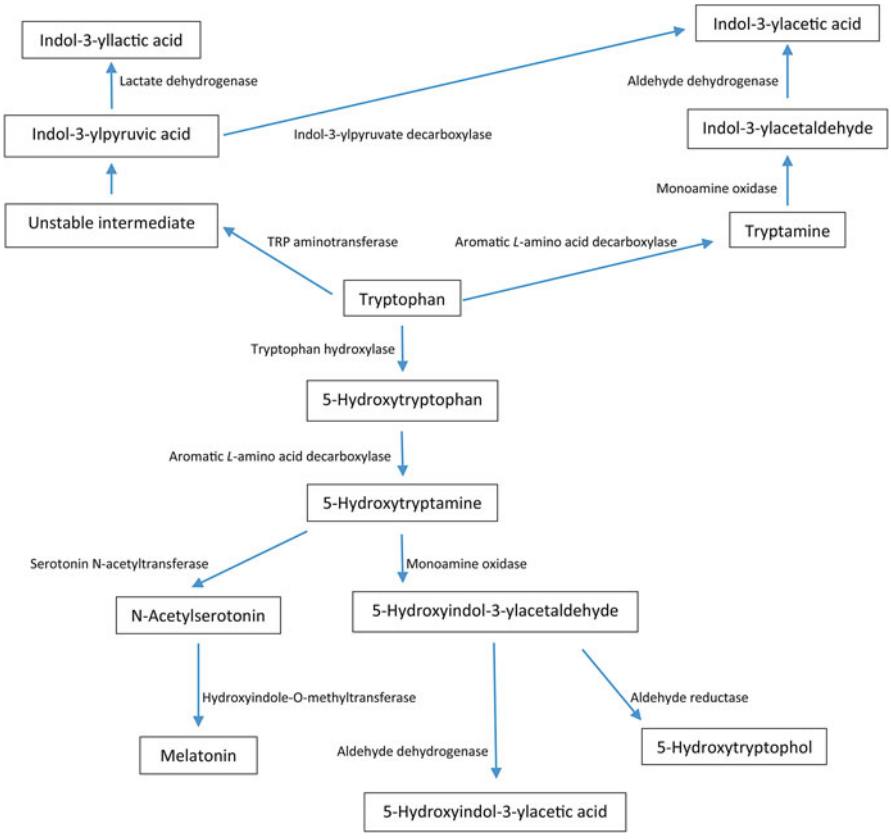

Fig. 2.1 The decarboxylation, transamination, and hydroxylation pathways of tryptophan (TRP) degradation

ing under conditions of decreased availability of the pyridoxal 5´-phosphate (PLP) cofactor caused by nutritional, pharmacological, or "functional" vitamin B_6 deficiency. As the brain cannot synthesize TRP, it has to rely on peripheral sources. Three factors control TRP availability to the brain. At the primary level, liver TDO controls circulating [TRP] [2]. At the secondary, but more immediate level, are plasma TRP binding, which determines levels of free TRP (the form immediately available for tissue uptake), and competition with TRP for cerebral uptake from a number of competing amino acids (CAA), notably the branched-chain amino acids (BCAA) leucine, isoleucine, and valine, and the aromatic amino acids tyrosine and phenylalanine. TRP availability to the brain and hence changes in brain [TRP] can be assessed from, and are best expressed as, the [free TRP]/[CAA] and [total TRP]/[CAA] ratios.

Urinary excretion of the major 5-HT metabolite 5-HIAA (5-hydroxyindole-3-acetic acid), as an index of whole body 5-HT synthesis and degradation, represents only 1 % of total urinary TRP metabolite excretion in humans [3], with the cerebral 5-HT pathway being a small fraction of the 1 %, whereas the decarboxylation and transamination pathways are relatively greater quantitatively [4, 5]. As the HKP metabolizes most of dietary TRP, it is clear that minor fluctuations in its activity can exert major changes in the other pathways, especially the cerebral 5-HT pathway.

Plasma Tryptophan Disposition

Plasma TRP is largely bound to albumin, with only ~5–10 % free and hence available for tissue uptake [6]. TRP binding is expressed as the percentage free TRP (100×[free TRP]/[total TRP]. As equilibration between the free and bound fractions is rapid, it is important to measure both fractions. Ultrafiltration is the best procedure for preparation of free TRP and should be performed using freshly isolated plasma or serum, as frozen storage increases TRP binding, thus resulting in a falsely low free [TRP]. This and other methodological factors in free TRP determination are detailed in [6]. Table 2.1 lists the various conditions that can affect plasma TRP disposition. Non-esterified fatty acids (NEFA) are the physiological displacers of albumin-bound TRP. Thus, changes in TRP binding can be traced to changes in albumin and NEFA levels and also to chemicals that directly displace TRP from binding sites, e.g., salicylate. If displacement is strong and sustained, the increase in plasma free [TRP] is accompanied by a decrease in total [TRP], due to increased tissue uptake and the above rapid equilibration. Binding can also be decreased when TRP is displaced by NEFA and lipolytic agents, such as catecholamines, sympathomimetic agents, and phosphodiesterase inhibitors, or if albumin is decreased, e.g., in pregnancy or liver cirrhosis [6, 7]. Binding is increased when lipolysis is inhibited by insulin, nicotinic acid, and other antilipolytic agents [6].

Most investigators do not measure plasma free TRP, but it is important that, in assessing the TRP disposition status, both free and total (free + albumin-bound) TRP are measured in the first instance and, if necessary, albumin and NEFA levels,

Table 2.1 Plasma tryptophan disposition

Condition/Mechanism	Free TRP	Total TRP	% Free TRP
Basal (fasting)	6.0	60	10
TDO/IDO induction	4.2	42	10
TDO inhibition	8.0	80	10
Decrease in albumin ≥19 %	9.0	60	15
Displacement by NEFA or drugs	9.0	60	15
Sustained displacement	12.0	45	27
Inhibition of lipolysis	3.0	60	5

TRP Tryptophan

Values are arbitrary figures in μM or % based on experimental observations

and information on whether subjects are receiving antilipolytic drugs or agents that can displace albumin-bound TRP is obtained. Failure to measure free TRP has led to incorrect interpretation of the TRP status in pregnancy, on the basis of which the TRP depletion concept is based (see below). Measuring both free and total [TRP] is also important to establish if TRP degradation in the kynurenine pathway (KP) is enhanced or inhibited and also whether increased production of kynurenine (KYN) metabolites is the result of activation or induction of the rate-limiting enzymes of the pathway, namely hepatic tryptophan 2,3-dioxygenase (TDO; formerly tryptophan pyrrolase) and extrahepatic indoleamine 2,3-dioxygenase (IDO), or simply due to altered flux of TRP down the pathway. Thus, in the absence of information on plasma free TRP levels, increased production of KYN and its metabolites cannot be attributed to induction of TDO or IDO.

As outlined in Table 2.1, TDO or IDO induction results in proportionate decreases in both free and total [TRP] without altering TRP binding, whereas TDO inhibition increases both free and total [TRP], also without altering TRP binding. Thus, assessment of TRP disposition along the above lines can establish the baseline TRP metabolic status and its biological determinants [6, 7].

The Hepatic Kynurenine Pathway

Description and control of the HKP

As well as accounting for >95 % of dietary TRP metabolism, the HKP is unique in containing all the enzymes necessary for synthesis of nicotinic acid (niacin) and subsequent intermediates leading to NAD^+ and complete oxidation to CO_2 and water (Fig. 2.2). As will be seen in this and other chapters throughout this publication, KYN and 8 of its metabolites, as well the important redox cofactors $NAD^+(P^+)$ and their reduced forms NAD(P)H, possess important biological properties. The HKP is controlled by the first enzyme, TDO, which catalyzes the oxidative cleavage of the pyrrole moiety to form *N´*-formylkynurenine. This is rapidly hydrolyzed to

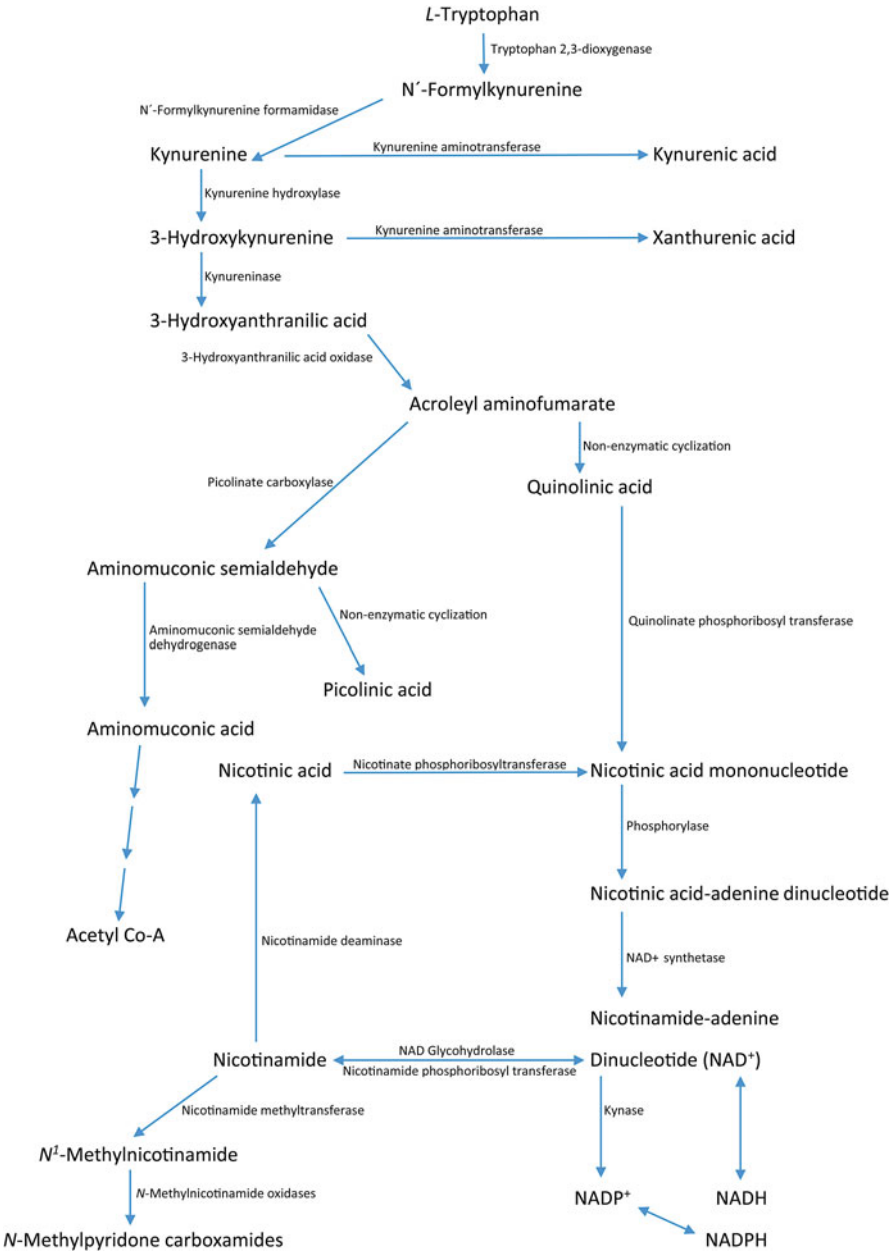

Fig. 2.2 The hepatic kynurenine pathway (reproduced from [23])

KYN by the abundant *N´*-formylkynurenine formamidase. The major route of KYN degradation involves oxidation to 3-hydroxykynurenine (3-HK) and beyond. Transamination of KYN is a minor route under normal conditions, as the K_m's of KYN (and 3-HK) for kynurenine aminotransferase (KAT) are much higher than those for kynurenine hydroxylase (KMO) and kynureninase (KYNU), with hydroxylation of KYN proceeding at a rate at least three times that of its transamination [1]. However, transamination becomes quantitatively significant when [KYN] is elevated, e.g., after KYN or TRP loading or induction (or activation) of TDO or IDO. The increased transamination of KYN (and of 3-HK) after TRP loading causes a "functional" vitamin B_6 deficiency, defined as decreased availability of the PLP cofactor, thereby undermining activities of KYNU and PLP-dependent enzymes in other metabolic pathways, with important clinical consequences. A major feature of this functional B_6 deficiency after TRP loading is increased formation and urinary excretion of kynurenic acid (KYNA), xanthurenic acid (XA), KYN, and 3-HK in this decreasing order (for references, see [1]). With TRP loading or increased plasma (free) [TRP] by other causes, the flux of TRP down the pathway is increased simply by the law of mass action, without any change in TDO activity, but if this is accompanied by TDO (or extrahepatic IDO) induction, the flux will be greater. It is therefore important when considering the TRP disposition status to establish if increased formation of KYN metabolites is due to increased flux of TRP or net induction of TDO or IDO.

KYN is thus preferentially hydroxylated to 3-HK by kynurenine hydroxylase, a mitochondrial NAD(P)H-dependent enzyme also requiring the active form of vitamin B_2 (riboflavin), flavin-adenine dinucleotide (FAD). Transamination of 3-HK to XA is also normally negligible. 3-HK is thus mainly oxidized to 3-hydroxyanthranilic acid (3-HAA) by the PLP-dependent KYNU. 3-HAA is the next immediate precursor of quinolinic acid (QUIN) produced by nonenzymatic cyclization of the product of the 3-HAA oxidase reaction, acroleyl aminofumarate. This latter compound occupies a central position at the 2 arms of the HKP, one leading to synthesis of niacin and NAD^+ dinucleotides and the other to complete oxidation to CO_2 and water. The flux of TRP favors dinucleotide synthesis and dietary TRP is more effective than dietary niacin in increasing the synthesis of these pyridine dinucleotides [8]. Picolinic acid (PA) levels are normally very low, and significant amounts can be formed only when the flux of metabolites is so great that the dehydrogenase competing with the nonenzymatic cyclization of aminomuconic semialdehyde is saturated with its substrate. Picolinate carboxylase is thought to be the second rate-limiting enzyme of the HKP after TDO [9].

Regulation and Functions of Tryptophan 2,3-Dioxygenase

TDO Regulation

As TDO is the rate-limiting enzyme of the HKP, it is important to discuss briefly its functions and regulation of its activity (Table 2.2). TDO is regulated by at least 4 mechanisms: hormonal induction by glucocorticoids, substrate activation and stabilization by TRP, cofactor activation by heme, and feedback inhibition by NAD(P)H [1, 2]. In studying TDO regulation, the choice of the experimental animal model is important. In man, rat, mouse, and some, but not all, other animal species, TDO exists in two forms: the active heme-containing holoenzyme and the inactive heme-free apoenzyme, in roughly equal proportions [10]. Extent of TDO saturation with heme is expressed as the heme-saturation ratio (holoenzyme activity/total enzyme activity) or as a % thereof. With mice, there are significant strain differences in TDO activity and biological (including immune) responses [7]. Species lacking the free apoenzyme include the cat, frog gerbil, golden (Syrian) hamster, guinea pig, ox, rabbit, and sheep [10]. In my opinion, the Wistar rat should be the experimental model of choice for TRP studies.

Glucocorticoid induction involves enhanced de novo TDO synthesis at the transcriptional level. Species lacking the free apoenzyme do not possess the glucocorticoid mechanism [10]. In species possessing both forms of TDO, only one half of the newly synthesized apoenzyme following glucocorticoid induction becomes heme-saturated, thus leaving the heme saturation ratio unaltered. Some hormones such as insulin, glucagon, dibutyryl c-AMP, catecholamines, and sex hormones modulate TDO activity by enhancing or suppressing glucocorticoid induction, releasing glucocorticoids, activation via TRP, or direct enzyme inhibition [1, 2].

By contrast with glucocorticoid induction, substrate activation by TRP involves increased saturation of the apoenzyme with heme and stabilization against degradation. In rats, a 50 mg/kg dose of TRP stabilizes, but does not activate TDO [11]. Larger doses will additionally activate TDO, and it is therefore important in acute TRP loading studies in humans that a 2 g dose (~29 mg/kg) is used in preference to larger doses. Although TRP does not enhance TDO synthesis, its activation of the enzyme is blocked by the translational inhibitors cycloheximide and puromycin, but not by the transcriptional inhibitor actinomycin D. This paradox can be explained

Table 2.2 Regulation and functions of hepatic tryptophan 2,3-dioxygenase (TDO)

Regulation mechanism (*effector*)	Function
Glucocorticoid induction (*cortisol*)	Disposal of excess TRP
Substrate activation and stabilization (*TRP*)	Synthesis of kynurenines
Cofactor activation (*heme*)	Control of tryptophan availability
Feedback inhibition [*NAD(P)H]*	Control of hepatic heme biosynthesis

TRP Tryptophan

NAD(P)H Reduced nicotinamide adenine dinucleotide (phosphate)

by TRP enhancing synthesis of another protein involved in TDO activation [11]. 5-Aminolaevlinate dehydratase and possibly also subsequent enzymes in the heme-biosynthetic pathway may be involved [12]. Cofactor activation of TDO is a direct effect of heme. However, heme itself has also been suggested [13] to mediate glucocorticoid induction of TDO mRNA transcription and translation.

Feedback control of the HKP is achieved by end-product inhibition of TDO by NAD(P)H, which act by an allosteric mechanism [2]. TDO activity can also be inhibited by a variety of agents, including allopurinol, antidepressant drugs, hydrazine compounds, estrogens, phenolic compounds, progesterone, and salicylate. Inhibitors of TDO of anti-proliferative potential are considered elsewhere in this publication.

TDO Functions

By controlling the HKP and hence extent of TRP breakdown, TDO performs a number of important functions within and outside the liver. The properties and functions of KYN and its metabolites will be described briefly below and in various contributions in this publication.

Intrahepatically, TDO controls the synthesis of KYNs and nicotinic acid and hence that of the important redox cofactors $NAD^+(P^+)H$ and the rate of heme biosynthesis. An inverse relationship exists between the level of heme saturation of TDO and activity of the rate-limiting enzyme of heme synthesis 5-aminolaevulinate synthase (5-ALAS) [14], thus rendering this saturation a sensitive marker of changes in cytosolic heme levels. Hepatic heme biosynthesis is controlled by end product repression of 5-ALAS exerted by a small pool of "free," "unassigned," or "readily exchangeable" heme in the hepatic cytosol of a $\sim 10^{-7}$ M concentration, which is utilized by TDO. Heme utilization by TDO may therefore have important implications in acute hepatic porphyria and its treatment (see below).

Extrahepatically, TDO functions are related to control of plasma TRP availability for protein synthesis, cerebral 5-HT synthesis, and disposal of TRP. As stated above, control of TRP availability to the brain is determined primarily by TDO activity. Disposal of TRP and hence prevention of its accumulation at undesirable levels is achieved by glucocorticoid induction of TDO. This is achieved in rat, man, and species possessing both forms of TDO. By contrast, species lacking the free apoenzyme and its glucocorticoid induction mechanism are sensitive to the toxic effects of excess TRP [10]. In a partial attempt to mitigate these effects, the TDO of these latter species degrades TRP at lower substrate levels and at a faster rate than the former species [2, 15].

Physiological Functions of Metabolites of the HKP

Only the key functions of KYN metabolites are outlined here, with detailed accounts being given elsewhere in this publication. Key functions include formation of the pellagra-preventing factor NA (nicotinic acid), insulin inactivation (XA), NAD^+ synthesis (NA), NMDA antagonism (KYNA) and agonism (QUIN), suppression of T cell responses (KYN, 3-HK, 3-HAA), and zinc binding (PA).

Differences Between TDO and IDO

As much of the content of this publication is concerned with the role of extrahepatic IDO, particularly in relation to immune activation and disease, it is important to describe the differences between this enzyme and hepatic TDO and also as a prelude to a discussion of the specific role of TDO in disease. Differences are summarized in Table 2.3. Some comments are noteworthy. Although TDO is of hepatic origin, it can be expressed elsewhere, e.g., in rodent brain [16] and many cancers [17]. In this latter regard, many compounds have been developed [18] or confirmed [19] as potent TDO inhibitors and shown to possess antitumor activity [19]. The importance of TDO in carcinogenesis and its therapy is considered elsewhere in this publication. Although 1-methyl tryptophan (1-MT) is traditionally considered a specific IDO inhibitor, recent evidence suggests that it is a poor inhibitor of purified

Table 2.3 Main differences between tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO)

Characteristics	TDO	IDO
EC Number	1.13.11.11	1.13.11.17
Location	Liver	Extrahepatic
Richest source	Liver	Placenta, lung, intestine
Activity level vs TDO	100 %	5–15 %
Molecular mass (KD)	167	40
Subunits	4	1
Substrate specificity	*L*-tryptophan	Broad-spectrum
Cofactor	Heme	Heme
G atoms of heme/mole	2	1
Other requirements	O_2	O^-_2
Induction by Glucocorticoids	Yes	No
Activation by TRP	+++	+
Activation by heme	++	–
Free apoenzyme	Yes	No
Induction by Cytokines	No	Yes
Inhibition by 1-methyl TRP	No	Yes

TRP Tryptophan

IDO1 and IDO2 [20]. In fact, 1-MT upregulates IDO1 in human cancer cells [21]. Accordingly, its immunosuppressive effects could be attributed to actions other than IDO inhibition [20].

Under normal conditions, the combined tissue IDO activity in humans (and most probably also animals) is at best 15 % of that of hepatic TDO. However, under conditions of immune activation, IDO assumes a significantly greater role in TRP degradation (for references, see [1]). However, a word of caution is appropriate here against extrapolation of mechanisms of changes in conditions of heightened, to those of mild, immune activation regarding the role of IDO. For example, as will be discussed below, contrary to currently held views, IDO induction does not play a significant, if any, role in the 5-HT deficiency of major depressive disorder, nor in determining the TRP status in pregnancy. This is, however, not to say that mild immune activation in these conditions does not exert other effects, which may have important clinical consequences.

Assessment of Tryptophan Oxidation in the Kynurenine Pathway

In small experimental animals, TRP oxidation is assessed directly by various means including direct assay of TDO activity in liver and that of IDO elsewhere. Direct assay of TDO in human liver biopsies is not ethically justified, but, as TDO controls >95 % of TRP oxidation and given its response to glucocorticoids and other effectors, reliance on changes in plasma TRP disposition and changes in KYN and its metabolites is justified to an acceptable extent under normal and other conditions, excluding strong immune activation, of which IDO, rather than TDO, induction is the predominant feature. By contrast, although IDO can be measured in human peripheral blood cells, surprisingly its activity in rarely reported by direct assay. This may be due to its very low activity under basal conditions, detection of which may necessitate using sensitive laboratory procedures. Any measurable level above the limit of detection in peripheral blood cells after significant immune activation should, however, demonstrate its activity. Instead, investigators of IDO rely overwhelmingly on the plasma [KYN]/[TRP] ratio or the interferon-γ (IFN-γ) marker neopterin to express TRP oxidation by IDO. In using this ratio, a number of factors should be considered. Firstly, because of the significant renal handling of KYN [22], elevation of its plasma levels occurs when extent of TRP oxidation exceeds renal handling (e.g., after robust induction of TDO or IDO or loading with TRP or KYN). Accordingly, the absence of an increase in plasma [KYN] does not rule out IDO/TDO induction or simple increased flux of TRP down the pathway. Measurement of plasma KYN metabolites could be more informative in these situations (see below). Secondly, the [KYN]/[total TRP] ratio reflects not only IDO, but also TDO, activity. Whereas using this ratio to express IDO activity is valid in conditions of heightened immune activation (e.g., HIV infection or after treatment of hepatitis C viral infection with IFN-α), this may not be the case in conditions of mild immune activation (e.g., depression or pregnancy). In fact, IDO does not play

a role in these 2 conditions. Thirdly, an increase in this ratio due to a lower [total TRP] (in the absence of an increase in KYN) does not automatically prove TDO or IDO induction. This induction requires demonstration of simultaneous and proportionate decreases in [free TRP]. In fact, using the [KYN]/[Free TRP] ratio instead may be more informative [23]. The importance of measuring free TRP is further emphasized by the role of NEFA in inflammation and immune function [7]. As well as by using free TRP, expression of TRP oxidation could be more meaningful if total KYNs, rather than KYN only, are used in new expressions [23]. These are the percentage total TRP oxidation (TTOX) and total TRP oxidation relative to free TRP (TTOXF). The former is 100×[total KYNs]/[total TRP], whereas the latter is 100×[total KYNs]/[free TRP]. These new expressions may be particularly useful in situations wherein TDO or IDO induction is modest or when formation of KYN metabolites is enhanced by simple flux of TRP down the pathway without TDO or IDO induction [23]. I urge the TRP research community to consider the above comments and the criteria outlined in Table 2.4 for assessing TRP oxidation, and to explore the utility of these 2 new expressions in future studies, especially as rapid high-performance liquid-chromatographic (HPLC) and mass spectrometric procedures are available to quantify TRP and various KYN metabolites simultaneously.

Table 2.4 Criteria for assessment of tryptophan oxidation in the kynurenine pathway

Parameter	Criteria
Decreased flux of TRP	Decreased plasma [free TRP] due to inhibition of lipolysis
	Decreased plasma % free TRP
	Decreased plasma [KYN(s)]
	Normal or decreased [KYN(s)]/[free TRP] ratios
Increased flux of TRP	High plasma free TRP due to displacement
	Normal or decreased plasma total TRP
	Increased plasma % free TRP
	Increased plasma [KYN(s)]
	Normal or increased plasma [KYN(s)]/[free TRP] ratios
	Increased plasma [KYN(s)]/[total TRP] ratios
TDO inhibition	Increased plasma free and total [TRP]
	Normal plasma % free TRP
	Decreased plasma [KYN(s)]
	Decreased plasma [KYN(s)]/[free or total TRP] ratios
TDO/IDO induction	Decreased plasma-free and total [TRP]
	Normal plasma % free TRP
	Increased plasma [KYN(s)]
	Increased plasma [KYN(s)]/[free or total TRP] ratios
IDO induction also	Increased IDO activity in peripheral blood cells, or
	Neopterin elevation (by ~100 %?)

TRP Tryptophan
KYN Kynurenine

The Hepatic Kynurenine Pathway in Diseases

The HKP appears to play a central role in normal pregnancy and at least 4 clinical conditions, as discussed below.

Alcoholism

The HKP has been studied in alcoholism mainly in relation to the brain 5-HT status. As the rate-limiting enzyme, TDO has received the greatest attention, with changes in man and experimental animals depending on whether alcohol (ethanol) is consumed acutely or chronically and if studies are performed during the chronic phase or acute or medium-term withdrawal [2].

Briefly, in fed rats, acute ethanol intake enhances TDO activity by a substrate (TRP)-type mechanism involving lipolysis-dependent release of albumin-bound TRP, whereas in starved rats, activation is heme-mediated [2]. In fasting normal human volunteers, TDO activation (judged by proportionate decreases in both free and total plasma [TRP] without altered TRP binding to albumin) is also likely to be heme-mediated, because of the absence of TRP or cortisol elevation. The resultant decrease in plasma TRP and its availability to the brain may explain alcohol-induced depression and loss of control with potential aggressive behavior in susceptible individuals [24]. As will be discussed below, inhibition of 5-HT synthesis is a major feature of major depressive disorder (MDD), and may also be involved in alcohol appetitive behavior, as the alcohol-preferring C57BL/6 J mouse strain exhibits a 5-HT deficiency mediated by a higher TDO activity, compared with the non-preferring CBA strain [25]. By contrast, chronic alcohol intake inhibits TDO activity in rats by an allosteric mechanism mediated by NAD(P)H as a result of ethanol metabolism [2]. Activities of HKP enzymes beyond TDO are not influenced by chronic alcohol intake [26]. In humans, evidence for TDO inhibition during chronic alcohol consumption includes decreased urinary excretion of KYN, 3-HK, and XA, increased cerebrospinal fluid [TRP], and decreased serum [KYN] at admission and upon relapse at 3 months after detoxification [2].

Subsequent withdrawal in rats causes a glucocorticoid induction of TDO by corticosterone released following cessation of alcohol intake [2]. Shortly before the appearance of the alcohol withdrawal syndrome in men, a large increase in plasma-free [TRP] and an elevation of cortisol are observed [27]. This suggests that human TDO may be induced during alcohol withdrawal. With TDO induction or increased TRP availability during withdrawal, increased production of KYN metabolites could be expected. In fact, serum [KYN] is higher following alcohol withdrawal, but, as stated above, is again decreased in patients relapsing 3 months after detoxification [28]. Elevation of circulating KYN can increase its entry into the brain and thus lead to increased cerebral synthesis of the NMDA receptor antagonist KYNA and agonist QUIN, thereby creating an environment leading to anxiety and other

clinical features of the alcohol-withdrawal syndrome [29]. Immune activation does not appear to play a role in TRP disposition during short- or medium-term alcohol withdrawal [30].

Pellagra

Alcohol misuse could induce or aggravate pellagra by a variety of mechanisms including the ethanol effects on the HKP [29]. However, in the absence of alcohol, pellagra is a disease of many causes, including malnutrition, conditions of gastrointestinal dysfunction, and certain drugs. These share a common final mechanism, that of decreased availability of the KYN metabolite NA (niacin, vitamin B_3) [29]. Nutritional aspects of the HKP are discussed in detail elsewhere in this publication. The liver occupies a central position in relation to NAD+ synthesis, because it contains all the necessary enzymes. Deficiency of protein and of some members of the B vitamin family, notably B_6, causes significant disturbances in the HKP, and these are compounded by the presence of large amounts of leucine in the traditional TRP-poor staples associated with pellagra, namely maize and sorghum. The mechanisms of the pellagragenic action of leucine involve activation of TDO and inhibition of KYNU [29]. Defective niacin synthesis in pellagra induced by drugs involves inhibition of TDO synthesis at the transcriptional or translational level (e.g., by antibiotics and immunosuppressants) or inhibition of KYNU activity by agents that bind to and thus inactivate the PLP cofactor (e.g., isoniazid and estrogens).

Major Depressive Disorder

As stated above, brain [TRP] and peripheral factors controlling it determine the level of activity of the rate-limiting enzyme of brain 5-HT synthesis, TRP hydroxylase, in particular the TPH2 isoform, which is some 50–75 % unsaturated with its TRP substrate [31]. 5-HT deficiency constitutes a fundamental aspect of MDD, but its biochemical mechanism(s) has not received critical appraisal until recently [31]. The deficiency has been known for many decades, and the role of the HKP is based on a number of observations. First, cortisol, which induces TDO synthesis, is elevated in ~50 % of MDD patients. Second, plasma total [TRP] is decreased in MDD by 20–29 % and the [total TRP]/[CAA] ratio by 16–36 %, with the decrease in the latter not involving [CAA] elevation [31]. A decrease in [free TRP] has also been observed in some studies, but, because free TRP is a labile parameter [6], the absence of a decrease may be explained by catecholamine-induced lipolysis-dependent displacement from albumin-binding sites superimposed on accelerated hepatic degradation [31]. It is also possible that elevation of catecholamines, which occurs in MDD, may cause a TRP-mediated TDO activation in subjects without a cortisol elevation. It should also be noted that cortisol and TRP cause an additive

enhancement of TDO activity [11, 31]. Lastly, a large number of antidepressant drugs of different chemical classes and pharmacological profiles, and also the mood stabilizers carbamazepine and lithium, share the single property of direct TDO inhibition both in vitro and after oral administration [31]. Some adjuncts to antidepressants, e.g., low-dose aspirin (salicylate) and estrogens, also inhibit TDO activity. The poor antidepressant efficacy of TRP is related to its accelerated hepatic degradation by TDO, and efficacy can be restored or enhanced by combination with TDO inhibitors, including existing antidepressants. Development of new and stronger TDO inhibitors not possessing the side effects of existing antidepressants may be a worthy goal [31].

Current evidence suggests that IDO does not play a role in the 5-HT deficiency in MDD. This is because of absence of evidence for peripheral or central IDO induction, or elevation of KYN, IFN-γ, or most cytokines (for references, see [31]). However, the modest elevation of the IFN-γ marker neopterin of ~40 % in MDD reported in 2 out of 4 studies [31] suggests a mild degree of immune activation, but is insufficient to deplete plasma [TRP], in contrast with the huge neopterin elevations of 300–600 % in HIV viral infection, which are associated with only a 25 % decrease in plasma total [TRP] [32, 33]. Modulation of the immune responses in MDD is therefore likely to occur as a result of the flux of TRP down the HKP following TDO induction, but this requires experimental scrutiny.

Porphyria

As stated above, TDO utilization of the small cytosolic regulatory-heme pool that controls heme synthesis by feedback repression of synthesis of the rate-limiting enzyme, 5-ALAS, may have important implications for the hepatic porphyrias and their therapy. Certain drugs and chemicals induce hepatic porphyria in experimental animals by undermining the feedback control mechanism, by causing loss of heme by direct destruction, inhibiting its synthesis, or increasing its utilization. Many drugs (e.g., barbiturates, chloroquine, chlorpropamide, ethanol, griseofulvin, α-methyldopa, and sulphonamides), estrogens, and other chemicals can exacerbate porphyria by inducing an acute attack and a laboratory test for detecting such exacerbators was developed based on heme utilization by rat liver TDO [34]. In this test, the decrease in the TDO heme saturation induced by the porphyrogen 3,5-diethoxycarbonyl-1,4-dihydrocollidine, as a result of destruction of heme and inhibition of its synthesis, is further potentiated by exacerbators, but not by non-exacerbators, of porphyria.

Activity of 5-ALAS is normal in hepatic porphyrias, but is enhanced during acute attacks. The genetic basis of the 6 types of hepatic porphyrias involves deficiencies in 6 enzymes beyond 5-ALAS (from 5-aminolaevulinate dehydratase to ferrochelatase) [35]. An acute porphyric attack is triggered by: (i) deficiency or loss of heme caused by the above mechanisms as in experimental porphyria; (ii) lipophilic drugs and chemicals interacting with nuclear receptors activating the

drug-response elements of the 5-ALAS enhancer; or (iii) deficiency of glucose and gluconeogenic substrates, thereby derepressing 5-ALAS synthesis [35]. Induction of 5-ALAS during acute attacks results in accumulation of 5-ALA and subsequent intermediates of the pathway, proximal to the genetically defective point, in blood and tissues, causing the various bodily and neurological features of the disease. Disturbances in TRP metabolism could also contribute to symptoms of these attacks (see below). The main treatment of an acute attack is intravenous heme arginate and oral glucose [35, 36], both of which suppress 5-ALAS synthesis. Two mechanisms have been proposed: repression by heme, downregulating transcription of 5-ALAS1 (the housekeeping liver enzyme) and its mitochondrial import post-translationally [36], and downregulation by glucose of the peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α) [37]. A third mechanism encompassing both heme and glucose and involving heme utilization by TDO has earlier been proposed [38], though not examined clinically. Starvation (which can induce an acute porphyric attack) increases heme utilization by TDO by a corticosterone- (or cortisol-) mediated increase in TDO synthesis utilizing heme to saturate a half of the newly synthesized apoenzyme. This results in a doubling of 5-ALAS activity. The greater induction of 5-ALAS by the porphyrogen 2-allyl-2-isopropylacetamide, which induces a loss of TDO heme, can be reversed by cortisol administration, due to restoring heme utilization by (newly synthesized) TDO [38]. The above effects of starvation are reversed by glucose, thus increasing the availability of the regulatory-heme pool for 5-ALAS repression. Glucose also inhibits the 5-ALAS induction by this porphyrogen, but cortisol reverses this inhibition [38]. It may therefore be suggested that the heme utilized by TDO may be a common factor in 5-ALAS repression by glucose or heme in humans. TDO inhibitors could be potential therapies of the hepatic porphyrias.

Disturbed TRP metabolism in the hepatic porphyrias may explain some of the clinical features of the acute attacks. The relative heme deficiency in the hepatic porphyrias (exemplified by impaired metabolism of drugs by the major hepatic hemoprotein cytochrome P450) decreases TDO saturation with heme, resulting in TDO inhibition and consequent elevation of plasma TRP and hence brain 5-HT. Demonstration of these changes in an experimental porphyria model led to the suggestion [39] that some of the attack symptoms are mediated by 5-HT, notably the gastrointestinal disturbances. Acute hepatic porphyrias appear to be associated with a functional vitamin B_6 deficiency, suggested by the increase in urinary excretion of KYN, 3-HK, KYNA, and XA following an oral TRP load [40], possibly through PLP depletion following 5-ALAS induction. However, neither porphyric symptoms nor these TRP metabolic abnormalities are reversible by B_6 supplementation [40]. This remains an unresolved issue, and it was suggested [40] that, in acute hepatic porphyria, the huge elevation of urinary KYN may involve impaired KYN hydroxylation.

As is the case in the above experimental hepatic porphyria model [39], evidence for decreased TDO activity during acute attacks is suggested by the observed 38 % higher plasma [total TRP] in porphyric patients than in controls [41] and the increased urinary excretion of the 5-HT metabolite 5-HIAA [41, 42]. The increased

excretion of 5-HIAA argues against the presence of a functional B_6 deficiency, which could otherwise impair 5-HTP decarboxylation (see above). Urinary levels of the 2 KYN metabolites KYNA and XA, however, show only a trend towards lower values [40–42], but are dramatically increased only after an acute TRP load [40]. The increased levels of plasma [TRP] [41] and urinary [5-HIAA] [41, 42] are abolished by intravenous heme administration, suggesting that TDO activity is restored and/or enhanced following heme repletion. The absence of changes in 24 h-urinary KYNA and XA following heme administration [41, 42] may be due to a potential short-lived activation of TDO by heme. A 24 h time-course study with urine collection at frequent (6 h) time intervals may demonstrate changes. Plasma TRP and KYN metabolite analysis over a 24 h after heme administration may also be more informative. In these 2 latter studies, heme was administered as heme arginate [41] or heme–albumin complex in equimolar amounts [42]. As plasma-free and total [TRP] were not measured [42], it is not clear if the decrease in urinary [5-HIAA] after heme-albumin was caused by decreased TRP availability for 5-HT synthesis by heme activation of TDO and/or increased TRP binding to albumin by the exogenously administered albumin. In isolated rat hepatocytes, addition of albumin decreases the flux of TRP down the HKP [43]. Thus, whereas TRP metabolism along the HKP is disturbed in hepatic porphyria, detailed studies are required to assess more fully the biochemical abnormalities in TRP disposition and their potential roles in the clinical features.

Pregnancy

As an essential amino acid, TRP plays a vital role in pregnancy. TRP availability in the material circulation is enhanced throughout pregnancy in rats [44] by TDO inhibition during the first 15 days (mediated by progesterone and estrogens) and subsequently by the release of albumin-bound TRP by a combination of albumin depletion (due to hemodilution) and displacement by a large NEFA elevation (due to enhanced lipolysis). Albumin depletion and NEFA elevation also occur in human pregnancy [7]. As stated above, if displacement is strong and sustained, the increase in free [TRP] is accompanied with a decrease in total [TRP], due to increased tissue uptake and the rapid equilibration between the free and bound fractions. This situation occurs in human pregnancy [7]. However, only total [TRP] was measured in most studies of pregnancy, with only two studies measuring and demonstrating the increase in free [TRP] (see [7] for additional references). Failure to measure free TRP led to the erroneous conclusion that pregnancy is associated with TRP depletion, upon which the TRP depletion concept of Munn et al. [45] concerning defense against fetal rejection is based. This concept cannot be reconciled with many observations, and a positive "TRP utilization" concept involving increased maternal TRP availability and flux down the HKP has been proposed as an alternative [7]. The increase in maternal TRP availability provides several advantages favoring fetal growth and development, namely provision of: (i) TRP for fetal protein synthesis

and growth; (ii) 5-HT for signaling pathways; (iii) KYNA for neuroprotection; (iv) QUIN for NAD^+ synthesis; and (v) other KYNs for suppression of T cell responses. T cell responses and their suppression are considered elsewhere in this publication.

Conclusions

The HKP controls TRP metabolism and hence availability of both TRP and its various metabolites for functions throughout the body. It is hoped that this chapter has provided a better understanding of mechanisms influencing TRP metabolism and disposition and clarified the role of the HKP in health and disease.

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Chapter 3
Nutritional Aspects of Tryptophan Metabolism

Katsumi Shibata

Abstract Niacin is biosynthesized from L-tryptophan (Trp). There is a substantial amount of Trp in the body which can be efficiently converted to niacin especially during critical situations. Therefore, it is very important to know the factors affecting the conversion ratio of Trp to niacin. In animal experiments, the conversion ratios decreased with increasing dietary protein levels. Feeding of diets containing unsaturated fatty acids increased the conversion ratio, while feeding of diets with saturated fatty acids did not. In the effects of carbohydrate, the conversion ratio was higher in diets containing starch than in diets containing sucrose. Vitamin B_1, vitamin B_2, or vitamin B_6 deficiency affects the metabolism of Trp to niacin. Mineral deficiency also affects the metabolism of Trp to niacin. With regard to hormones, thyroxin increased the Trp to niacin conversion ratio, while estrone, progesterone, and adrenaline decreased. Certain chemicals can also impact niacin synthesis; alloxane and streptozotocin decreased the conversion ratio, while pyrazinamide, phthalates, valproic acid, and clofibrate increased the ratio. Moderate food restriction greatly decreased the conversion. In human experiments, the urinary excretion ratio of anthranilic acid formation from Trp was calculated to be approximately 0.06 % against Trp intake, kynurenic acid 0.13 %, xanthurenic acid 0.11 %, 3-hydroxyanthranilic acid 0.13 %, and quinolinic acid 0.34 % in basal metabolic level. Supplementing healthy women with up to 5.0 g/day of Trp had no adverse effects. Urinary excretion of 3-hydroxykynurenine is a good surrogate biomarker for excess Trp ingestion.

Keywords Tryptophan • Niacin • Urine • Nicotinamide • N^1-methylnicotinamide • N^1-methyl-2-pyridone-5-carboxamide • N^1-methyl-4-pyridone-3-carboxamide • Quinolinic acid

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List of Abbreviations

ACMS	2-Amino-3-carboxymuconate-6-semialdehyde
ACMSD	2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase
AnA	Anthranilic acid
3-HA	3-Hydroxyanthranilic acid
3-HK	3-Hydroxykynurenine
IDO	Indoleamine 2,3-dioxygenase
KA	Kynurenic acid
KYN	Kynurenine
MNA	N^1-methylnicotinamide
Nam	Nicotinamide
NiA	Nicotinic acid
2-Py	N^1-methyl-2-pyridone-5-carboxamide
4-Py	N^1-methyl-4-pyridone-3-carboxamide
PiA	Picolinic acid
QA	Quinolinic acid
TDO	Tryptophan 2,3-dioxygenase
Trp	L-Tryptophan
XA	Xanthurenic acid

Introduction

The vitamin niacin is biosynthesized from the essential amino acid the L-tryptophan (Trp) (Fig. 3.1), and one defines it as the conversion ratio of Trp to niacin. Over 20 % of enzymes need pyridine nucleotide coenzymes. When the metabolic rate is accelerated, the need of pyridine nucleotide coenzymes concomitantly increase. Such a case, the conversion ratio of Trp to niacin would be increased. Therefore, it is very important to know what affects the conversion ratio of TRP to niacin.

It has been generally accepted in the nutrition field that in the normal state, 60 mg of Trp has equal activity to 1 mg of nicotinamide (Nam) in humans based on several human studies [1–4]. During pregnancy, there is an observed increase in the TRP to niacin conversion ratio [5].

Analyses

Metabolites

TRP [6] and its metabolites, including kynurenine (KYN) [7], kynurenic acid (KA) [8], anthranilic acid (AnA) [9], 3-hydroxykynurenine (3-HK) [9], xanthurenic acid (XA) [10], 3-hydroxyanthranilic acid (3-HA) [10], 2-oxoadipic acid (2-OAA) [11],

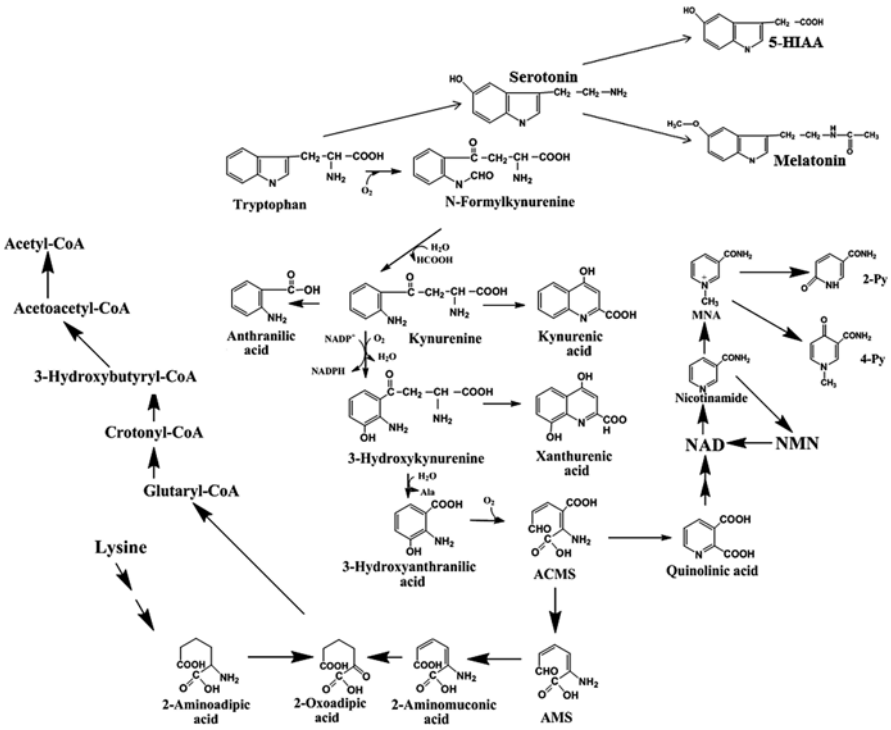

Fig. 3.1 Major metabolic pathway of Trp

quinolinic aid (QA) [12], N^1-methylnicotinamide (MNA) [13], Nam [14], *N1-methyl-2*-pyridone-5-carboxamide (2-Py) [14], and N^1-methyl-4-pyridone-3-carboxamide (4-Py) [14], were measured as described.

Enzymes

Trp 2,3-dioxygenase (TDO) [EC 1.13.11.11] [15], kynureninase [EC 3.7.1.3] [15], kynurenine aminotransferase [EC 2.6.1.7] [15], 3-hydroxyanthranilic acid dioxygenase [EC 1.13.11.6] [15], 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD) [EC 4.1.1.45] [16], and quinolinic acid phosphoribosyltransferase (QPRT) [EC 2.4.2.19] [17] were measured as described.

Conversion Ratio

The conversion ratio was calculated from the following equation:

$$\text{Conversion ratio} = \left(\text{urinary excretion of Nam} + \text{MNA} + 2 - \text{Py} + 4 - \text{Py}, \text{mol / day}\right) / \left(\text{Trp intake}, \text{mol / day}\right) \times 100$$

Rat Experiment

Effects of Dietary Protein Levels

The conversion ratio decreased with increasing dietary protein levels (Table 3.1). The activity of ACMSD increased with increasing dietary protein levels [18], while those of the other enzymes did not. The increased ACMSD activity means that ACMS, which is synthesized from Trp, is metabolized into acetyl-CoA but not into niacin. Therefore, the low conversion ratio in the group of high-protein diet might be attributed to the increase in the activity of liver ACMSD.

It is known that niacin itself is not necessary in rats when Trp is given in adequate amounts, because rats can biosynthesize niacin from Trp [19]. The rats fed the 20 % and 40 % casein diets did not require niacin for optimum growth, but the rats fed the 70 % casein diet needed it [20]. This phenomenon was attributed to the supposition that liver ACMSD activity increased according with the dietary casein levels. The conversion ratio in rats fed on the 70 % casein diet became extremely low, and then the rats needed niacin.

Effects of Dietary Constant Protein Levels but Differing Trp Level

As it had been known that the amounts of Nam increase with increasing intake of Trp, our data [18, 20] conflict common sense for the conversion. The sum total urinary excretion of Nam and its metabolites did not increase as the level of dietary casein increased [21]. While the sum total urinary excretion of Nam and its catabolites

Table 3.1 Effects of dietary protein levels on the conversion ratio of Trp to niacin and the activity of liver ACMSD

	20 % casein diet	40 % casein diet	70 % casein diet
Conversion ratio of Trp to niacin (%)	1.87 ± 0.27^{c}	1.01 ± 0.03^{b}	0.402 ± 0.03^{a}
ACMSD (μmol/h/g of liver)	1.67 ± 0.03^{a}	2.97 ± 0.33^{a}	11.67 ± 0.67^{b}

Statistical significance was determined by analysis of variance followed by Tukey's multiple comparison tests. Values are mean ± SE for five rats. Mean values in a row with uncommon superscript letter differs, $p < 0.05$

in the groups fed the 10 % casein-30 % gelatin, 20 % casein-20 % gelatin, and 40 % casein diets were about 5, 7, and 11 μmol/day, respectively, values were significantly different from each other [22]. Therefore, it was found that only when the total protein intake was constant the sum total urinary excretion increases with increasing intake of Trp.

Effects of Composition of Amino Acids

The conversion ratio in the group fed with a preformed niacin-free, 9 % casein-sucrose diet was 3.5 %. The ratio was decreased to 0.2 % by the addition of 2 % glycine, 0.078 % L-threonine, and 0.2 % L-cystine and restored to 3.2 % by the further addition of 0.1 % Trp [23]. The same phenomena were observed in other types of Trp-limiting diets [24]. These results clearly prove that the conversion ratio was lowest when the rats were fed with the Trp-limiting diets. Therefore, we think that the pellagragenic factor of corn is simply due to a low content of Trp, but the adverse effect is due to a low conversion ratio of Trp to niacin.

The conversion ratio was significantly lower in the groups fed with the 3 % L-leucine-, L-valine-, or L-isoleucine-added diet than in the group fed with the control diet [25]. Namely, the inhibition of the conversion was observed not only by the addition of L-leucine but also by the addition of L-valine or L-isoleucine. The ratio in the group fed with an amino acid diet-simulating rice protein was around 2.5 %. The ratio was decreased by the addition of the limiting amino acids, except for Trp, and increased by the addition of all the limiting amino acids, including Trp [26]. The composition of amino acids proved to greatly affect the conversion ratio.

Effects of Dietary Fat

Feeding of diets containing unsaturated fatty acids increased the conversion ratio, while diets containing saturated fatty acids did not affect it (Table 3.2). The increased conversion ratio in the group fed with unsaturated fatty acids might be attributed to the decreased activity of ACMSD because it is known that feeding of diets containing unsaturated fatty acids inhibits the ACMSD activity but that feeding diets containing saturated fatty acids does not [27].

Table 3.2 Effects of dietary fat level and the kind of dietary fat on the conversion ratio of Trp to niacin

	20 % casein + 0 % fat diet	20 % casein + 20 % corn oil diet	20 % casein + 20 % soybean diet	20 % casein + 20 % lard diet
Conversion ratio of Trp to niacin (%)	2.27 ± 0.20^{a}	4.40 ± 0.57^{b}	4.47 ± 0.58^{b}	2.66 ± 0.38^{a}

Statistical significance was determined by analysis of variance followed by Tukey's multiple comparison tests. Values are mean ± SE for five rats. Mean values in a row with uncommon superscript letter differs, $p < 0.05$

Table 3.3 Effect of dietary carbohydrate on the conversion ratio of tryptophan to niacin

	Starch diet	Sucrose diet
Conversion ratio of Trp to niacin(%)	1.83 ± 0.13	0.80 ± 0.12*

Statistical significance was determined by Student's *t*-test. Values are mean ± SE for five rats
*$p < 0.05$

Effects of Dietary Carbohydrates

The conversion ratio was higher in diets containing starch than in diets containing sucrose (Table 3.3) [18]. This phenomenon also might be due to the change of the activity of ACMSD.

Effects of Dietary Vitamins

Vitamin B_1-Free Diet

We clarified the following two phenomena: (1) severe food restriction gave an increase of the conversion ratio compared to free access control group [28, 29] and (2) the conversion ratio also increased by vitamin B_1 deficiency compared to free access control group [30]. So, another study was set up to clarify whether or not a true reason about an increase in the conversion ratio is vitamin B_1 deficiency or severe food restriction [31].

Vitamin B_2-Free Diet

In riboflavin-deficient rats, the activities of 2-Py-forming MNA oxidase and 4-Py-forming MNA oxidase were not detected in vitro [32]. These results show that the two MNA oxidases are FAD-dependent enzymes. On the contrary, the Nam methyltransferase activity was higher in riboflavin-deficient rats than in control rats. Therefore, the resulting excretion ratio of (2-Py + 4-Py/MNA) was greatly lower in the riboflavin-deficient rats than in the control rats.

Vitamin B_6-Free Diet

The urinary excretion of KA decreased while that of XA increased drastically in the vitamin B_6-free diet. The conversion ratio was lower in the vitamin B_6-free diet than in the vitamin B_6-containing diet [33].

Effects of Hormones

Thyroxine

It has been reported that thyroxine increased the activity of kynurenine aminotransferase and reduced the activity of kynurenine 3-monooxygenase, with a resulting increase in the reaction of kynurenine to KA [34]. Traditionally, these changes were taken to mean that thyroxine suppressed the conversion ratio; however, the effect of this hormone on the conversion ratio has not been actually demonstrated. We, therefore, investigated the effect on the conversion of feeding to rats, a preformed niacin-free, 20 % casein diet containing 0.002 % thyroxine [26]. The ratio was statistically higher in hyperthyroid rats than in the control rats. Furthermore, the present data suggests that ACMSD played a critical role in this conversion.

Sex Hormones

It is known that deaths attributable to pellagra, which is considered to be a disease caused by the disturbance of Trp metabolism, have been approximately twofold higher in women than in men [35]. Administration of ovarian hormones significantly decreased the conversion ratio [30, 36]. Feeding a diet containing testosterone had no effect on any parameter [30].

Adrenaline

The administration of glycemia-affecting chemicals such as alloxane [15], streptozotocin [37], and 6-aminonicotinamide [38] decreases the conversion ratio. Adrenaline is also known to increase the glucose level. The conversion ratio was reduced to half by the intraperitoneal injection of adrenalin [39].

Prednisolone

The urinary excretion of Nam, MNA, 2-Py, and 4-Py was each higher in the prednisolone group than in the physiological saline group after 1 day; however, each excretion was lower in the prednisolone group than in the saline group after 3, 4, 5, and 6 days [40]. On around the 7th day, the urinary excretion in the prednisolone group was restored to the initial values. The initial rapid increase of prednisolone decrease in the urinary excretion of Nam and its metabolites by an injection of prednisolone is considered to be attributable to a respective elevation of the liver TDO level and the liver ACMSD level.

Chemicals Substances

Table 3.4 summarizes the effects of some chemical substances on the metabolism of Trp to niacin.

Food Restriction

The conversion ratio was lower in both restricted groups than in the ad libitum-fed control group during the experimental period (control group, 1.37 ± 0.24 %; 80 %-restricted group, 0.20 ± 0.04 %; 65 %-restricted group, 0.15 ± 0.02 %. control vs. restricted groups, $p < 0.01$) [29]. Food restriction, even at mild levels, suppressed the conversion ratio compared to the ad libitum-fed control group.

Table 3.4 Effects of some chemical substances on the metabolism of Trp to niacin

Chemicals	Effects
Alloxane [15]	A marked increase was observed in the activities of TDO, ACMSD, and Nam methyltransferase upon the injection of alloxane; on the other hand, the activities of kynureninase and NAD^+ synthetase were decreased by the injection of alloxane. These changes mean that the conversion ratio is lower in the alloxane diabetic rat than in normal rat
Streptozotocin [37]	Marked increases in the activities of ACMSD and Nam methyltransferase were observed in the streptozotocin-induced diabetic rats, but increased TDO activity was not observed
Pyrazinamide [41, 42]	The administration of pyrazinamide and pyrazinoic acid, antituberculosis drugs, significantly increased the metabolites, 3-HA and beyond, especially QA, Nam, MNA, 2-Py, and 4-Py, and therefore significantly increased the conversion ratio. However, no difference in the upper metabolites of the Trp to Nam pathway such as AnA, KA, and XA was apparent by the addition of pyrazinamide and pyrazinoic acid
Phthalates [7, 43]	Of the phthalate esters with different side chains tested, di(2-ethylhexyl) phthalate (DEHP) and its metabolite, mono(2-ethylhexyl)phthalate (MEHP), most strongly enhanced the production of QA and degradation products of Nam, while 3-HA was unchanged. This pattern of metabolic change led us to assume that these esters lowered ACMSD protein or its activity. Although DEHP could not be tested because of its low solubility, MEHP reversibly inhibited ACMSD from rat liver and mouse kidney, and also the recombinant human enzyme. Correlation between inhibition of ACMSD by phthalate esters with different side chains and urinary excretion of QA supports the notion that phthalate esters perturb Trp metabolism by inhibiting ACMSD
Valproic acid [44]	Valproic acid is a short-chained, branched fatty acid that is widely used in humans as an anticonvulsant and mood stabilizer. The conversion ratio was increased by feeding a diet containing valproic acid. Of the intermediates formed during the conversion of Trp to Nam, the Trp to 3-HA step was not affected by the administration of valproic acid, while such metabolites beyond QA as Nam and its catabolites were significantly increased
Clofibrate [45]	Clofibrate enhanced the conversion ratio without any side effects and supports again the claim that the activity of ACMSD exerts a critical influence on the conversion ratio

Loading

Tryptophan

Young rats were fed on a 20 % casein diet with 0, 0.5, 1.0, 2.0, or 5.0 % added Trp for 30 days [46]. The apparent toxicity and growth retardation was observed in the 5.0 % Trp-added group. Metabolites of the Trp-Nam pathway such as AnA, KA, XA, 3-HA, and QA in urine increased in a dose-dependent manner. Of these metabolites, urine KA progressively increased, and urine AnA dramatically increased in the 2.0 and 5.0 % Trp-added groups. The urine ratio of AnA/KA is a useful index to monitor an excessive Trp intake.

Quinolinic Acid

Dietary QA had no adverse effects up to 35 mg/100 g of diet. Dietary QA is approximately 1/9 as active as niacin in growing rats [47].

Picolinic Acid

Picolinic acid (PiA) is an endogenous metabolite of Trp that has been reported to possess a wide range of physiological actions. Toxicity of PiA was higher compared with analogs such as nicotinic acid and QA. Feeding an ordinary diet containing 0.05 % and 0.1 % PiA did not elicit decreased intake of food or loss in body weight. PiA did not affect the in vitro liver activities of QPRT and ACMSD, a Zn-dependent enzyme. PiA administration did not affect Trp metabolites such as AnA, KA, and XA. However, QA and subsequent metabolites such as Nam and its catabolites were increased by administration of a diet containing 0.05 % PiA but not by a 0.1 % PiA diet. These results suggest that the in vivo activity of ACMSD is controlled by Zn level [48].

Human Experiments

Basal Metabolic Fate

Fate of dietary Trp in humans was investigated [49]. The urinary excretion amounts of Trp, kynurenine, AnA, KA, 3-HK, XA, 3-HA, and QA were about 40, 20, 4, 1, 10, 4, 3, 5, and 20 μmol/day, respectively. In this experiment, Trp was ingested at levels of 674 mg/day or 3300 μmol/day. The percentage of AnA formation from Trp was calculated to be approximately 0.06 %, KA 0.13 %, XA 0.11 %, 3-HA 0.13 %, and QA 0.34 %.

Tryptophan Loading

Healthy Japanese women were randomly assigned to receive placebo (0 g/day) or 1.0, 2.0, 3.0, 4.0, or 5.0 g/day of Trp for 21 days each with a 5-week washout period between trials [50]. Food intake, body weight, general biomarkers in blood and urine, and amino acid composition in blood and urine were not affected by any dose of Trp. Administration of up to 5.0 g/day Trp had no effect on a profile of mood states category measurement. The urinary excretion of NAm and its catabolites increased in proportion to the ingested amounts of Trp, indicating that participants could normally metabolize this amino acid. The urinary excretion of Trp metabolites, including kynurenine, AnA, KA, 3-HK, 3-HK, and QA, all of which are intermediates of the Trp–kynurenine–QA pathway, was in proportion to Trp loading. The response of 3-HK was the most characteristic of these Trp metabolites. This finding suggests that the urinary excretion of 3-HK is a good surrogate biomarker for excess Trp ingestion.

Nicotinamide Loading

To determine if *de novo* Nam synthesis from Trp is influenced by Nam intake itself, young women consumed controlled diets containing 0, 10.9, 37.8, or 68.6 mg/day respectively, and urinary excretion of Trp metabolites were measured [51]. None of the intermediates, including AnA, KA, XA, 3-HA, and QA, changed at all. That is, exogenous Nam did not affect *de novo* Nam synthesis.

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Chapter 4
Kynurenines and Brain Development

Trevor W. Stone, Caroline M. Forrest, and L. Gail Darlington

Abstract The mechanisms by which environmental factors such as stress, infection, or inflammation could affect the early brain development of the embryo are largely unknown. One metabolic pathway that could be important is the kynurenine pathway for the oxidative metabolism of tryptophan, since it is activated by each of these factors and it generates an agonist (quinolinic acid) and an antagonist (kynurenic acid) at glutamate receptors sensitive to *N*-methyl-D-aspartate. These receptors are known to be important in neurogenesis, neuron precursor migration, neurite formation, axon guidance, and synapse formation. This review summarizes the effects of inhibiting the pathway, during pregnancy in rats, on brain development in the neonates and adult offspring. The widespread changes in protein expression and localization with changes in neuronal excitability and plasticity reinforce the view that kynurenines play a significant role in early brain development and may be the substrates on which subsequent cues act to enhance or restrict behavioral and cognitive function.

Keywords Development • Neonates • Plasticity • Excitability • Kynurenic acid • Quinolinic acid

List of Abbreviations

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CNS	Central nervous system
DISC1	Disrupted in schizophrenia-1
E14, E16, E18	Embryonic day 14, 16, 18

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GABA	Gamma-amino-butyric acid
3HAA	3-Hydroxyanthranilic acid
3HK	3-Hydroxykynurenine
IDO	Indoleamine-2,3-dioxygenase
KMO	Kynurenine-3-monoxygenase
LTP	Long-term potentiation
NMDA	*N*-Methyl-D-aspartate
P21, P60	Postnatal day 21, 60
Ro61-8048	3,4-Dimethoxy-*N*-[4-(3-nitrophenyl)thiazol-2-yl]benzene-sulfonamide
Shh	Sonic hedgehog
TDO	Tryptophan-2,3-dioxygenase
Unc5H3	Uncoordinated 5H3
VAMP-1	Vesicle-associated membrane protein-1
VGAT	Vesicular GABA transporter
VGLUT	Vesicular glutamate transporter

Glutamate Receptors and Brain Development

The embryonic brain begins to develop—later than many peripheral tissues—from the early blastocyst. In rodents much brain development occurs in the second half of gestation and continues well beyond parturition and even beyond weaning, which occurs at postnatal day 21 (P21) in most rat strains. The human brain develops much more slowly than this, beginning within the first trimester of pregnancy but continuing into adolescence. Many authorities place a time point of approximately 2 years postnatally for the completion of the greater part of human brain development, including neurogenesis and the establishment of the major projection pathways and interactions, with refinements of these and the continuing development of neuroplasticity into the early second decade.

During these early developmental periods, glutamate receptors, especially those sensitive to *N*-methyl-D-aspartate (NMDA), are involved in neuronal generation and integration. After the initial formation of new neurons, migration to their eventual "correct" locations in the brain is influenced substantially by the activation of NMDA receptors. That activation is partly regulated by the level of excitability and electrical activity in the growing brain. Although much of the migratory activity takes place in the embryo, some continues into adulthood, albeit in relatively restricted regions such as the subgranular zone of the hippocampal dentate gyrus. If NMDA receptors are blocked in the prenatal or neonatal brain, there is a marked loss of neurons and disruption of network formation which can lead to significant changes of behavior [1]. At the cellular level, NMDA receptors affect synaptic transmission, dendrite growth, and spine formation [2–4].

The Kynurenine Pathway and Glutamate Receptors

The kynurenine pathway will be described and presented in several different ways in this volume, but Fig. 4.1 provides an overall view of its main components, originating in the essential amino acid tryptophan and proceeding via kynurenine and quinolinic acid to nicotinamide and the enzyme cofactor nicotinamide adenine dinucleotide [5, 6]. The kynurenine pathway has emerged as a major candidate for regulating the physiological and pathological activity of NMDA receptors since it includes both a selective agonist and an antagonist. The agonist properties of quinolinic acid were first observed in its ability to excite neocortical neurons in vivo [7],

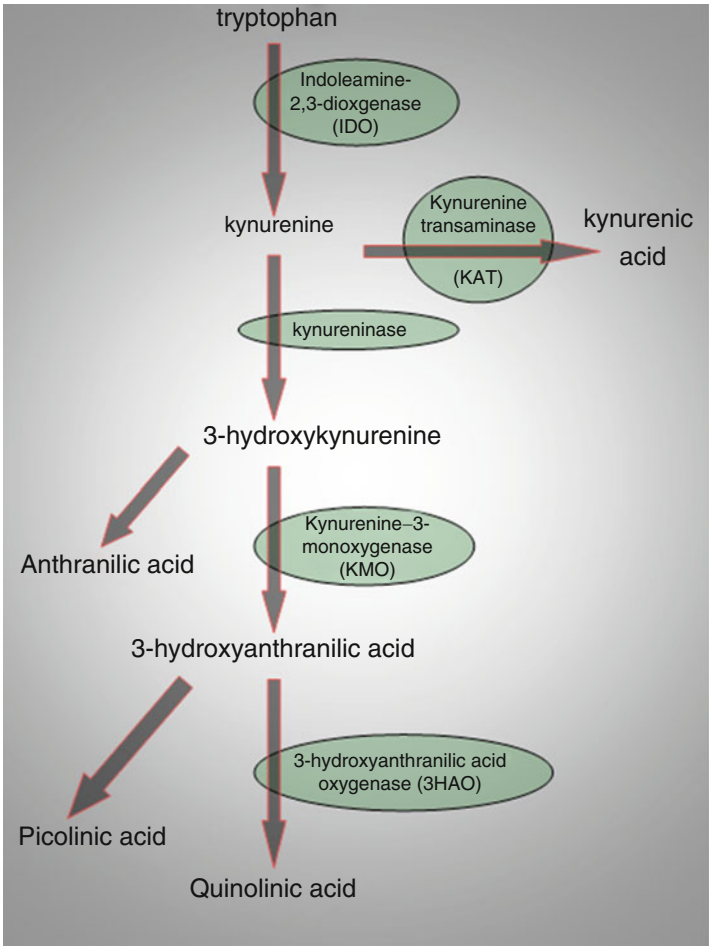

Fig. 4.1 A simple schematic illustrating the major compounds and enzymes involved in the kynurenine pathway of tryptophan metabolism

and its effects were blocked by the recently identified selective NMDA antagonists 2-amino-5-phosphonopentanoic acid and the heptanoate analog 2-amino-7-phosphonoheptanoic acid [7, 8]. As possible actions of other components of the kynurenine pathway were then explored, it was realized that kynurenic acid was an antagonist at NMDA receptors, although with significant antagonist actions at kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [9].

The major enzymes linking these compounds are also indicated in Fig. 4.1, as it is these that form the most crucial target for potential interference by extraneous factors. Most important are the initial enzymes tryptophan-2,3-dioxygenase (TDO), found primarily in the liver but also now known to occur in several tissues including the brain, and indoleamine-2,3-dioxygenase (IDO) which occurs primarily in cells of the immune system. The two enzymes (including their respective subtypes IDO1, IDO2, TDO1, and TDO2) are structurally and functionally dissimilar, although both include, as a major plank in their activity profiles, the ability to oxidize tryptophan to kynurenine. Both enzymes are of fundamental relevance in trying to appreciate the external control of tryptophan metabolism. IDO, for example, is induced, and its level of activity correspondingly increased substantially by pro-inflammatory cytokines including tumor necrosis factor-α, interleukin-1β, and, most potently, interferons. A second enzyme further along the pathway, kynurenine-3-monoxygenase (KMO), which oxidizes kynurenine to 3-hydroxykynurenine (3HK), is also induced and activated by cytokines and interferons, with the net effect that inflammation generates a very substantial increase in pathway activity. An important consequence of this activation is that any inflammatory stimuli, including autoimmune events and infections, will greatly enhance activity along the kynurenine pathway with increased levels of both the NMDA agonist quinolinic acid and the antagonist kynurenic acid. If embryos were exposed to either immune activation or maternal stress, the altered concentrations of quinolinic acid or kynurenic acid could modulate the activation of NMDA receptors (and potentially kainate and AMPA receptors) to modify neurogenesis, migration, and contact formation as noted above.

The activity of TDO is little affected by inflammatory mediators but does respond to blood levels of corticosteroids. Since these adrenal hormones are produced in increased amounts during times of stress and activation of the hypothalamic–pituitary–adrenal axis, any stressful situation will also provide increased activation of the kynurenine pathway, providing direct links between exposure to stress and the modification of brain development.

Clearly there is a strong possibility that such external, environmental influences on the early formation of the brain could lead to psychiatric or neurological disorders in the offspring or might increase the susceptibility of the postnatal brain to other factors contributing the initiation of those disorders.

To test this concept, an experimental program was established to examine the effects of inhibiting the kynurenine pathway during gestation in rats, at a time when brain development in the embryos was proceeding rapidly. The protocol adopted was to inhibit the kynurenine pathway by blocking the key enzyme KMO using 3,4-dimethoxy-*N*-[4-(3-nitrophenyl)-thiazol-2-yl]-benzenesulfonamide (Ro61-8048). This compound has been used by several groups since its first synthesis [10]. In almost

Table 4.1 Summary of proteins whose expression was changed after prenatal exposure of the embryos to Ro61-8048 [16–19]

	Changes of protein expression after prenatal Ro61-8048	
Tissues examined	Proteins increased	Proteins decreased
P21 hemisphere	PSD-95, GluN2A, GluN2B, DCX, PCNA, NFkB	RhoB, Shh
P60 hippocampus	DCX	GluN2A, Shh
P60 neocortex	GluN2A, Unc5H3, DISC1	DCX, Shh, COX-2
P60 cerebellum	–	DCX, DISC1, Unc5H1, Unc5H3, Shh

Abbreviations: *PSD-95* postsynaptic density protein-95, *GluN* glutamate NMDA receptor subunits, *DCX* doublecortin, *PCNA* proliferating cell nuclear antigen, *NFkB* nuclear factor kappa-B transcription factor, *Shh* sonic hedgehog, *DISC1* disrupted in schizophrenia-1, *Unc5H* uncoordinated movement 5H, *COX-2* cyclooxygenase-2

Proteins that were not affected in any region included synaptophysin, synaptotagmin, vesicle-associated membrane protein-1 (VAMP-1), RhoA, GluN1, 5-hydroxytryptamine-2c receptor (5HT-2c), (sex-determining region-2)-Box2 (Sox-2), tyrosine hydroxylase, α-synuclein

all previous studies, Ro61-8048 has increased the concentration of kynurenic acid in the tissues of interest by up to 100 times their resting levels [10–13]. Those previous studies have included work on stroke-induced pathway activation and activation by parasitic infections. Perhaps most dramatically, the inhibition of KMO and the resulting generation of kynurenic acid were sufficient to prevent the death of animals infected with strains of plasmodium parasites capable of inducing the normally fatal condition of cerebral malaria in mice [14], with a similar protective activity against the brain pathology produced by trypanosomes which induce sleeping sickness [15]. Although not previously tested during pregnancy, a similar, up to 100-fold increase in kynurenate levels in the blood and brain of the dam and in the embryonic brain was observed within 5 h of administering Ro61-8048 to pregnant rats [16].

Kynurenine Pathway Inhibition and Brain Development

Since the primary objective was to assess the effects of disrupted kynurenine metabolism in utero upon the development and functional status of the postnatal and adult brain, most analyses were performed on animals culled at the day of weaning (postnatal day 21, P21) or at young adulthood on day P60. The results are summarized in Table 4.1.

An analysis of protein expression revealed that early inhibition of KMO resulted in significant changes in protein expression in all regions of the CNS examined, although the degree and direction of change could be different in the several brain regions tested (Table 4.1). Nevertheless, from the spectrum of results obtained, it is possible to conclude that the state of the kynurenine pathway during early brain development does influence brain development in such a way as to modulate the protein profile of compounds known to play significant roles in neuronal structure and function.

Glutamate Receptor Changes

Particularly intriguing is the change in expression of the NMDA receptor subunits. Substantial changes were noted in the levels of two NMDA receptor subunits GluN2A and GluN2B, with a decreased expression of GluN2A and increased GluN2B. A balance between these subunits is known to change throughout development, and the magnitude and direction of that ratio are thought to be crucial in regulating neuronal function and early brain development [20, 21]. Normally, there is a shift between NMDA receptors dominated by GluN2B-containing subunits in the early stages of neuronal formation and their replacement by mainly GluN2A-containing receptors in later stages of development [22, 23]. A lower expression of GluN2A subunits increases the efficacy of newly formed synapses. This in turn could result partly from NMDA receptor-induced changes in dendrite formation and structure, as discussed below, since the two subunits GluN2A and GluN2B exert distinct influences on these parameters [24, 25].

The NMDA subunit balance will also influence the occurrence of, or susceptibility to, excitotoxicity and neurodegeneration [26]. Both GluN2A and GluN2B subunits modulate excitotoxicity [27, 28], although GluN2A subunit-containing receptors can be neuroprotective, implying from our results that the neocortex may become relatively resistant to degeneration, although the hippocampus may be more vulnerable. GluN2A subunits contribute to early stages of learning [29] although this may vary between brain regions [30]. Fear memory, anxiety, and depressant behaviors are also affected by the loss of GluN2A subunits [31, 32].

Interestingly, the Ro61-8048-induced changes in NMDA receptor expression are paralleled by changes in other aspects of glutamate-releasing neurons. In common with most classical neurotransmitters, glutamate is normally stored in synaptic vesicles, taken up by one of the vesicular glutamate transporters VGLUT1 or VGLUT2. The occurrence and localization of these proteins have been widely acknowledged as a useful indicator of excitatory glutamatergic transmission. The gestational exposure of rats to Ro61-8048 resulted in an increased number of synaptic terminals staining for VGLUTs, using an antibody that was nonselective for VLGUT1 and VGLUT2 [17]. This change was not accompanied by any alteration in the density of VAMP-1-positive terminals, indicating that the VGLUT changes were not a secondary consequence of a generalized loss of synapses. This conclusion was supported by the absence of any change in the vesicular GABA transporter VGAT.

As glutamate receptor formation, localization and function are partly dependent on cytoskeletal proteins, it is of interest that kynurenine pathway inhibition also changes expression of the small GTPases known as Rho proteins. Rho enzymes in the dendritic trees of neurons in the Xenopus brain are involved in the effects of glutamate on dendritic structure, consistent with similar roles described elsewhere, and they are also likely to play a role in other neuronal responses to NMDA [33] including excitotoxicity and neuronal development [34, 35]. Both RhoA and RhoB exist in the hippocampus, with a distinct cellular distribution and changed expression in response to the induction of long-term potentiation [36–38]. RhoA may inhibit

synaptic plasticity while RhoB facilitates it, accounting for a possible role in high-frequency stimulation-induced long-term potentiation (LTP). This effect requires the activation of NMDA receptors, but changes in RhoGTPase activation are sufficient to modify the induction of LTP [37].

The most likely link between the kynurenine pathway and these various receptor changes is via the substantial, 100-fold increase in kynurenic acid concentrations produced by Ro61-8048 [16], since the blockade of NMDA receptor is known to result in increased neuronal apoptosis. The kynurenine-induced changes in subunit composition are also likely to contribute to behavioral changes in postnatal animals, as described by other groups. Abnormalities of learning, for example, are associated with altered expression of NMDA receptor subunits [39].

Neurodevelopmental Proteins

Doublecortin

Prenatal exposure to Ro61-8048 reduced levels of doublecortin in the dentate gyrus when examined microscopically, with fewer granule cells present with extended dendritic processes. Doublecortin is a microtubule-associated protein known to have key roles in early neuronal migration, especially of inhibitory interneurons [40] and is associated with recently generated neurons, for which it is used as an indicator. Doublecortin (referring to "double cortex," a severe malformation of the brain) is associated with mental retardation and epilepsy and has been linked with neuronal development in the neocortex and hippocampus [41]. Its expression declines in early brain development. It is expressed primarily in newly formed neurons, while a deficiency in doublecortin expression lowers neuron formation and hinders recovery after brain trauma.

The increase in hippocampal doublecortin expression levels produced by Ro61-8048 at day P21 and in the hippocampus at P60 probably indicates increased formation of new neurons, although this did not correlate with the absence of any apparent difference in stained neurons counted directly using immunostaining.

Sonic Hedgehog

The sonic hedgehog (Shh) protein is involved in the development, differentiation, and maturation of recently generated cells [42–45]. In contrast to doublecortin, which exists primarily in immature neurons, Shh is expressed largely in fully differentiated cells [46]. Shh is a major factor in the early development, morphological organization, and orientation of many tissues and in the production and regulation of stem cells. It may mediate excitation-induced neurogenesis [47] and the proliferation of neural progenitor cells that occurs following cerebral injury or ischemia [46, 48]. It is probably the main factor secreted by Purkinje cells which promotes

cerebellar granule cell proliferation [49, 50]. In several regions of the CNS where adult neurogenesis is unusual (such as neocortex), it can be reinitiated by Shh [51].

Certainly, Shh is one of the few proteins whose expression is changed by Ro61-8048 in most regions of the brain examined, suggesting that it may be particularly vulnerable to early blockade of NMDA receptors and, therefore, the endogenous levels of kynurenic acid in the embryonic brain. It must be emphasized, however, that the loss of protein was not reflected by the counting of immunostained cells, which indicated an increase in Shh-positive neurons. The resolution of this difference remains unclear, but may suggest that there is an increased rate of production of new neurons after Ro61-8048 treatment but that those cells grow and mature more slowly than normal. Alternatively, since Shh is a secreted protein, there may be a defect in Shh production despite the increase in cell numbers.

There is good evidence to suggest that Shh might be especially important in the generation and stabilization of dopaminergic neurons, since it facilitates the emergence of a dopaminergic phenotype from uncommitted stem cells and promotes the differentiation of a dopaminergic phenotype in neurons of the ventral midbrain [52]. It also plays a highly significant role in the polarization of cells in their immediate postmitotic stages and affects the organization and orientation of the various organs and tissues [43, 44, 47, 49, 51]. It does so partly by controlling cell proliferation and movement by chemotaxis [48].

In view of the changed electrophysiological plasticity in the adult brain, it is relevant that Shh continues to occur in regions such as the hippocampus in fully differentiated neurons [46].

Uncoordinated-5H3

In addition to the major proteins discussed above, expression of several others was affected by Ro61-8048 treatment in some regions of the CNS. The dependence receptor uncoordinated 5H3 (Unc5H3) was increased in the neocortex, but expression was lowered in the cerebellum, an observation that could be of some importance in view of the crucial nature of this protein's contribution to cerebellar development: a loss of Unc5H3 results in severe abnormalities of cerebellar structure and motor control [53].

Synaptic Transmission and Plasticity

While an analysis of protein expression provides valuable information on the extent to which CNS organization may be compromised by Ro61-8048 exposure, it does not yield information on the true functional status of the brain. Plasticity is an especially interesting phenomenon in the treated animals in view of the changes in NMDA receptor subunits. Both the GluN2A and GluN2B subunit-containing NMDA are involved in LTP although the details of their sites and mechanisms of action remain

uncertain [18, 54]. The former carry a greater involvement in LTP than do GluN2B, a distinction that would correlate with behavioral studies showing a similar difference in their participation in learned fear responses.

Measurements of synaptic function in hippocampal slices indicated an increase in neuronal excitability which might be partly attributed to increased expression of receptors containing GluN2 subunits. Responses to paired electrical stimuli showed no differences in control tissue and that exposed to Ro61-8048, suggesting no change in presynaptic function between the two groups. Exposure to Ro61-8048 resulted in increased levels of LTP although its counterpart, LTD, was unaffected.

It is also possible that the increased number of VGLUT terminals contributes to the reduced paired-pulse inhibition [17, 55]. Although paired-pulse inhibition and facilitation are primarily determined, presynaptically the final level of paired-pulse interaction is partly influenced by the number of excitatory and inhibitory terminals on the neurons being recorded. The larger number of excitatory terminals defined here, therefore, could be responsible for the reduced paired-pulse inhibition. It has also been noted by others that the expression of VGLUT is a factor in the relative and overall efficacy of excitatory and inhibitory neurotransmission [56, 57].

The increased neuronal excitability and LTP might be related to a reduced synaptic inhibition which might in turn result from the altered expression of doublecortin [58]. This protein influences hippocampal lamination and dendritic structure but plays a role in regulating synaptic inhibition since it co-localizes with GABA and the GABAergic neuron markers glutamate decarboxylase and parvalbumin [40]. There is good evidence that neurons containing doublecortin are usually inhibitory neurons for which doublecortin is required in migration to their appropriate sites within the CNS [59]. The increased expression of GluN2B is also likely to regulate inhibitory neuron function indirectly [60] as reflected in the changes of inhibitory transmission following a blockade of NMDA receptors. This procedure, intriguingly, also influences the hippocampal location of inhibitory interneurons [61].

Spine Density and Neuronal Morphology

One aspect of neuronal development which is crucial to the appropriate organization and function of the CNS is the generation and functional maturation of the dendritic tree [62]. Most of the excitatory connections within the CNS and many of the inhibitory ones are made onto dendrites, and their associated spiny projections and changes in any of the morphological parameters of the dendrites are often linked with alterations in the efficiency of neurotransmission and various aspects of behavior including learning and memory processes. Dendritic changes have even been linked with a range of neuropsychiatric conditions [63].

It is known that dendritic structure is partly dependent on the presence and activation of NMDA receptors [64], raising the possibility that the increased expression of the GluN2B subunit in embryos 5 h after the administration of Ro61-8048 and also at P21 [16, 17, 55] could have influenced dendritic growth in the developing CNS.

Indeed, this subunit does affect the density of spines on some neuronal populations [20]. This possibility is especially intriguing since several groups have linked GluN2B with both spine density and neural plasticity [28, 64].

The prenatal administration of Ro61-8048 proved to have significant effects on dendritic structure. In the hippocampus, changes were noted in both the apical and basal dendritic trees, with a decrease in total and secondary basal dendrite length, fewer spines, and lower complexity. Some of these changes were consistent with involvement of the kynurenic acid–glycine-binding site on the NMDA receptor [5, 65, 66]. The involvement of both apical and basal dendrites is important as these regions have distinct plasticity thresholds and transmitter sensitivities which may well account for some of the electrophysiological modifications described after Ro61-8048 [55]. In contrast, there were no significant changes of structure of specific dendritic areas of the neocortex, but a Sholl analysis [67] revealed change in dendritic complexity. This parameter reflects the number of dendritic intersections within given distances from the cell soma. Layer 3 of the neocortex showed particularly clear increases in complexity and, as noted above, might reflect a different balance between excitatory and inhibitory inputs.

Given the presence of these structural changes, it is again relevant that doublecortin expression was altered, as noted above, since the expression of this protein is intimately related to the expression of actin, a cytoskeletal protein which plays a major role in determining dendritic length and branch complexity [59].

Glutamate Receptor Changes and CNS Disorders

A major rationale for studies such as these is, as noted earlier, the possibility that altered brain development may precipitate or predispose to CNS disorders. One major disorder of the CNS, for which the kynurenine pathway and its effects on NMDA receptor subunits may be a fundamental cause, is schizophrenia. The currently favored view seems to be that this psychiatric condition is based on reduced activation (hypofunction) of NMDA receptors [68, 69]. Blocking NMDA receptors in neonatal animals can produce alterations in the brain such as abnormal neuronal numbers and GluNR1 subunit expression and reduce preterminal markers such as synaptophysin [70–72]. As the brain develops into adulthood, it becomes possible to discern schizophrenia-like behaviors including suppressed pre-pulse inhibition, a hallmark of several psychiatric conditions including schizophrenia.

Given the potential involvement of the kynurenine pathway, especially kynurenic acid, in schizophrenia [73] with evidence for raised concentrations of kynurenic acid in the brain of patients with schizophrenia and in animal models [74–77], the protein analysis performed after Ro61-8048 treatment included disrupted in schizophrenia-1 (DISC1) [17, 19, 55]. The expression of this protein was increased in the neocortex, although it was decreased in the cerebellum. Chronically increased levels of kynurenic acid (produced here by Ro61-8048), therefore, might produce some of their schizophrenia-like effects by changing the functional concentrations

of the DISC1 protein. Stress in prenatal and neonatal life can also induce behavioral features of depression and anxiety [78, 79] that could complicate any attempt to interpret the results in terms of schizophrenia alone.

The effects of Ro61-8048 on brain protein expression and synaptic function do support the concept that abnormal levels of kynurenines, especially kynurenic acid, can affect brain development. Do the effects produced also lead to alterations in behavior relevant to schizophrenia? Using simple avoidance tasks, no significant effects on rodent behavior were produced by prenatal Ro61-8048, but it has been suggested that simple learning tasks can be performed via neural projections that do not involve the hippocampus, whereas some of the most marked protein and synaptic plasticity changes were seen in this region. On the other hand, when kynurenic acid levels were increased by the administration of its precursor, kynurenine, or decreased by inhibition of kynurenine aminotransferase, behavioral effects were reported. The most obvious changes were in pre-pulse inhibition, stimulus discrimination, and attentional set-shifting paradigms [80–88]. Several of these tasks do reflect aspects of the subtle behavioral changes seen in patients with schizophrenia and, overall, support a role for the reportedly increased kynurenic acid concentrations in patients in the behavioral symptomatology.

A Synthesis

The work summarized in this chapter strongly suggests that there is basal, constitutive activity in the kynurenine pathway which is active in the developing CNS of normal embryos and inhibition of which can affect NMDA receptor structure, as well as the expression of major neurodevelopmental proteins and, ultimately, neuronal plasticity. The changes which remain at P60 after prenatal inhibition of the pathway are not the same as those detected after 5 h or at P21 suggesting that the kynurenine pathway may have a series of temporally changing influences on brain development. The occurrence of these various changes may cause psychiatric or neurological disorders or predispose individuals to develop them. Indeed, an important consideration is that, since the kynurenine pathway is induced and activated by pro-inflammatory cytokines and steroid stress hormones, it could be responsible for transmitting peripheral or environmental influences in such a way that embryonic development is compromised [89–91]. Even dietary intake of compounds such as the brassinins may affect brain development in this way, since these and related compounds inhibit enzymes along its route [92], mimicking the effects of the experimental drug Ro61-8048 used here. This conclusion has important implications for the converse possibility that external factors promoting or suppressing basal kynurenine pathway activity may interfere with brain development and, as a result, brain structure and function in the adult. Maternal exposure of humans to infection and stress during pregnancy has been linked to the emergence of disorders such as schizophrenia in postnatal life. The kynurenine pathway could be a major contributor to that association.

The fundamental underlying mechanism for many of the effects described here is likely to be the massively increased concentration of kynurenic acid. However, although the weight of evidence overwhelmingly supports the view that the major action of this compound in the brain is a blockade of NMDA receptors, other actions may contribute to its overall effects. Similarly, other kynurenine metabolites may have actions that enhance or oppose those of kynurenic acid. For example, several kynurenine metabolites, including quinolinic acid and its precursors 3HK and 3-hydroxyanthranilic acid (3HAA), exhibit redox activity that may modulate the injurious effects of neurotoxins. Kynurenic acid itself has proved particularly interesting in that it has been claimed to block nicotinic cholinoceptors [93] and also to be an agonist at the aryl hydrocarbon receptor and the orphan G protein-coupled receptor GPR35 [64]. The possible effects at nicotinic receptors are disputed, however, and several groups have not been able to confirm this activity of kynurenic acid [94, 95] or its quantitative relevance [96]. Overall, the current major focus of interest has remained securely around its blockade of glutamate by kynurenic acid, especially acting at NMDA receptors. Whatever its detailed mechanism or mechanisms of action, this compound appears to play a significant role in the early development and maturation of the mammalian brain.

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Chapter 5
Role of Kynurenine Pathway in Aging

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Abstract Aging is a degenerative process that affects all living organisms and is characterized by physiological and functional changes that drives to numerous aging-related disorders contributing to death. Cellular changes during the aging process involve oxidative stress, inflammation, mitochondrial dysfunction, and cognitive and immune response decline. In this context, changes in tryptophan metabolism are also found. Tryptophan is an essential amino acid required for the synthesis of proteins, and it is mainly metabolized via the kynurenine pathway generating several metabolites with neuroactive and/or redox properties. Due to kynurenine pathway, metabolites have been related with aging and some aging-related diseases; we review here the alteration in kynurenine pathway components during aging and study the relationship between these metabolites and other factors present during the aging process, with the goal of exploring their therapeutic value, as targets or biomarkers of different age diseases or aging states.

Keywords Aging • Kynurenine pathway • Kynurenic acid • Oxidative stress

List of Abbreviations

ACMSD	2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase
AMO	Anthranilate 3-monooxygenase
CNS	Central nervous system
CSF	Cerebrospinal fluid
3-HANA	3-Hydroxyanthranilic acid
3-HDO	3-Hydroxyanthranilate 3,4-dioxygenase
3-HK	3-Hydroxykynurenine

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IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
KYNA	Kynurenic acid
KATs	Kynurenine aminotransferases
KMO	Kynurenine 3-monooxygenase
KP	Kynurenine pathway
L-KYN	L-Kynurenine
NAD^+	Nicotinamide adenine dinucleotide
NMDAr	N-methyl-D-aspartate receptor
PIC	Picolinic acid
QUIN	Quinolinic acid
QPRT	Quinolinic acid phosphoribosyltransferase
ROS	Reactive oxygen species
Trp	Tryptophan
TDO	Tryptophan 2,3-dioxygenase
XA	Xanthurenic acid

Introduction

Tryptophan

In humans, tryptophan (Trp) is an essential amino acid that can only be taken up through diet, and it enters in cells by active transport. Around <1 % of Trp is used for protein synthesis, and it is also a precursor of biomolecules with specialized functions mainly through two major metabolic pathways: (1) the methoxyindole pathway, which leads to the formation of serotonin (implicated in regulating mood, appetite, and reproduction) and, subsequently, melatonin (implicated in circadian rhythm) [1], and (2) the kynurenine pathway (KP), which metabolizes about 95 % of Trp and produces metabolites with neurochemical and redox properties leading to the NAD^+ as a final product. The cellular environment can affect the regulation of Trp metabolism leading to changes in the physiological and behavioral process that can be related with aging and aging-related diseases. In this context, the study of the KP is key to designing strategies with potential to prevent and treat aging-related diseases, improve health, and extend lifespan.

Kynurenine Pathway

In mammals, Trp is mainly metabolized via the kynurenine pathway (KP) (Fig. 5.1), where tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) catalyze the first step, which consists in the oxidative opening of the indole ring.

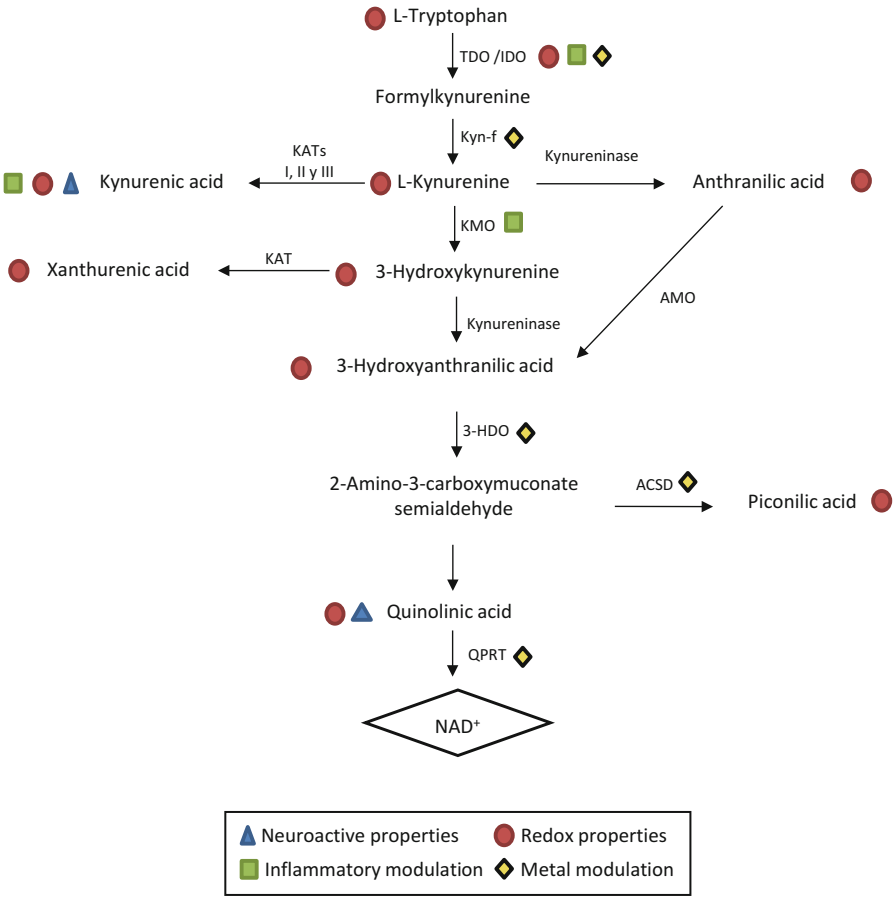

Fig. 5.1 The kynurenine pathway. The *symbols* show the modulation and properties found until now for each metabolite or enzyme. Tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase (IDO), kynurenine formamidase (Kyn-f), kynurenine aminotransferases I, II, and III (KATs I, II, and III), kynunerinase (Kynu), kynurenine 3-monooxygenase (KMO), anthranilate 3-monooxigenase (AMO), 3-hydroxyanthranilate 3,4-dioxygenase (3-HDO), 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD), quinolinic acid phosphoribosyltransferase (QPRT)

These enzymes are expressed in different tissues, and their expression is induced upon exposure to different stimuli. TDO is expressed mainly in the liver and its expression is induced by Trp itself or by stress hormones. In contrast, IDO is found in most mammalian cells, including macrophages and cells of the central nervous system (CNS). IDO is induced by the pro-inflammatory cytokine interferon (IFN)-γ and other immune stimulants, including lipopolysaccharide [2, 3]. *N*-Formylkynurenine is the product of TDO and IDO and is rapidly degraded to L-kynurenine (L-KYN) by kynurenine formamidase.

L-KYN is a key metabolite in KP because it can be a substrate for different enzymes: (1) kynurenine aminotransferases (KATs) to produce kynurenic acid (KYNA), the only endogenous antagonist of *N*-methyl-D-aspartate receptor (NMDAr); (2) kynureninase to produce anthranilic acid, which then is taken by anthranilate 3-monooxygenase (AMO) to produce 3-hydroxyanthranilic acid (3-HANA); and (3) kynurenine 3-monooxygenase (KMO) to produce 3-hydroxykynurenine (3-HK). 3-HK can be metabolized to xanthurenic acid (XA) through KATs or can be also metabolized to 3-HANA by kynureninase. 3-HANA is subsequently converted to 2-amino-3-carboxymuconate semialdehyde by 3-hydroxyanthranilate 3,4-dioxygenase (3-HDO). The intermediate product is taken by 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD) to form picolinic acid (PIC) or also can suffer nonenzymatic cyclization (spontaneous) to produce quinolinic acid (QUIN). QUIN is known as excitotoxin due to its NMDAr agonism. Finally, QUIN is metabolized through quinolinic acid phosphoribosyltransferase (QPRT) to NAD^+.

These KP metabolites possess different properties, and their imbalance can have impact in aging or aging-related diseases. The most studied metabolites are KYNA, which higher levels are related with cognitive deficit attributable to its capability to block α7 nicotine acetylcholine receptor; higher levels of QUIN are related with excitotoxicity and oxidative stress, while 3-HK and 3-HANA are considered pro-oxidant agents and can induce cell death. 3-HK has also been involved in several neurodegenerative disorders [4].

Aging and Oxidative Stress

Human aging is a multifactorial process involving genetic, environmental, epigenetic, and lifestyle factors and is characterized by progressive deterioration in physiological functions and metabolic processes. One consequence of aging is an increased incidence of aging-related diseases, such as cancer and neurodegenerative disorders. Recent reports have shown that the activity of antioxidant enzymes decreases and oxidative markers are elevated with aging in various organs [5, 6], particularly in the brain, making some cerebral areas more susceptible to the oxidative damage [7]. The brain is highly vulnerable to reactive oxygen species (ROS) effects, as it contains high concentrations of polyunsaturated fatty acids, which are the target of lipid peroxidation; also the brain does not possess an efficient antioxidant system as compared to other organs. The damage to other systems, such as glutamatergic, cholinergic, and catecholaminergic pathways, increases the organs' susceptibility to oxidative damage.

Additionally, the increase in ROS formation is accompanied by a loss of thiol groups, Trp degradation, and increased lipid peroxidation during aging [8]. There are a lot of damaged macromolecules, failure of protein repair, and maintenance systems which all are indicators of aging at a cellular level. Under certain condi-

tions, imbalance of ROS levels or disturbances in ROS signaling drives to pathological states like cardiovascular diseases, inflammation, cancer, and dementias [9].

On the other hand, aging also is characterized by inflammation. In fact, levels of inflammatory mediators typically increase with the age even in the absence of acute infection or another physiologic stress [10]. In this line, IFN-γ and TNF-α production and higher oxide nitric levels have been observed in senescence-accelerated mice [11]. Another consequence of aging is a decline in immune response, affecting the rate at which naive B and T cells are produced as well as the composition and quality of the mature lymphocyte pool [12]. In this context, T cell populations show poor response, which is one of the main changes that happen in immunosenescence, as has been measured in aged subjects. The changes in oxidative markers as well as the inflammatory parameters and the decline in immune function found during aging can be related with the KP metabolite alterations, due to the fact that these kynurenines possess redox and neuroactive properties and can be modulated by the inflammatory response. Until now, the main question is whether the general cellular status in aging is modified by the levels of kynurenines or if the KP metabolites are directly implicated in the cellular changes observed during the aging process. The exact mechanisms which modify these factors with aging have not been fully understood; however, we can assume that all of them are closely related in some way.

Relationship Between Aging and the Kynurenine Pathway

As was mentioned before, KP metabolites possess redox properties, and KP enzymes are also modulated by the environment (free radicals, antioxidants, and pro-inflammatory cytokines). Alteration in the levels of KP metabolites in aging suggests a complicated stage that provokes cells more vulnerable to damage, which is closely related with cognitive impairment, inflammation, oxidative stress, immune response decline, and events that can lead to aging-related diseases. This implies that Trp metabolites could be potent regulators of aging and aging-related diseases, offering new targets for lifespan intervention.

Different studies in various organisms have implicated Trp metabolism as a powerful regulator of aging and aging-related diseases (Table 5.1). For example, considering that IDO is involved in immune response and also suppresses T cell activity, Pertovaara et al. [20] measured the KYN/Trp ratio (Trp degradation rate, IDO activity) in a nonagenarian population finding that it was higher in older people than healthy controls and also was able to predict mortality in these individuals. They postulate that IDO activity reflects the fact that T cell response declines with age, although Trp levels did not change in both groups. In line with this, the authors also noted that both neopterin levels (induced by IFN-γ via IDO) as well as the KYN/Trp ratio increase in older people [11]. Similarly, the ratio of L-KYN/Trp was also increased in aged rats (28–32 months) compared with mature rats (4–6 months) [12].

Table 5.1 Changes of KP components found during the aging in diverse species and tissues

Metabolite or enzyme	Species	Tissue or sample	Modulation in aging	Reference
Trp	Female Wistar rat	Brain Liver Kidney	↓ ↓ ↓	[13]
	Male Wistar rats	Lenses	↑ 7–10 days ↓ from 45 days and older	[14]
	Sprague-Dawley rats	Brain	↑ from prenatal to birth ↓ from birth to P4	[15]
TDO	Female Wistar rat	Brain Liver Kidney	↓ ↓ ↓	[13]
	Sprague-Dawley rats	Liver	↓	[16]
	Human	Serum	↑	[17]
	Male Mill Hill hooded rats	Liver	↓ 6–18 months ↑ 24 months	[18]
IDO	Female Wistar rat	Brain Liver Kidney	↑ ↓ ↓	[13]
	Sprague-Dawley rats	Small intestine	↓	[16]
	Primiparous Sprague-Dawley rats	Brain	= pre- and postnatal	[19]
	Humans	Blood	↑	[20]
KYN	Female Wistar rat	Brain Liver Kidney	↑ ↓ 12 months = 24 months ↓ 12 months ↑ 24 months	[13]
	Humans	Lenses	↓	[21]
	Male Wistar rats	Lenses	↑ from 7 to 60 days ↓ 6 months and older	[14]
	Human	CSF	↑	[22]
	Sprague-Dawley rats	Brain	↓ from prenatal to P4	[15]
AA	Primiparous Sprague-Dawley rats	Brain	↓ from prenatal to birth ↑ one postnatal week ↓ thereafter	[19]
KAT	Female Wistar rat	Brain Liver Kidney	↑ = ↑	[13]
	Sprague-Dawley rats	Liver Kidney	= ↑	[16]
	Sprague-Dawley rat	Brain	↑	[23]

(continued)

Table 5.1 (continued)

Metabolite or enzyme	Species	Tissue or sample	Modulation in aging	Reference
KYNA	Female Wistar rat	Brain Liver Kidney	↑ = ↑	[13]
	Humans	CSF Serum	↑ =	[22, 24]
	Merino/Border Leicester cross sheep	CSF	↑	[25]
	Brown Norway rats	Retina	↑ from E20 to P0 ↓ from second week postnatal to 12 months	[26]
	White Leghorn chickens	Retina	↓ from E12 to birth and thereafter	[26]
	Male Sprague-Dawley rats	Brain (regions)	↑	[27, 28]
	Primiparous Sprague-Dawley rats	Brain	↓ from E15 to E20 ↑ E21 ↓ from birth to P7 ↑ after P7	[15, 19]
	Baboons (*Papio* sp.)	Cortex	↓	[15]
	Syrian hamsters	Plasma	=	[29]
	Male Sprague-Dawley rats	Liver	=	[28]
KMO	Sprague-Dawley rats	Liver Kidney	↓ ↓	[16]
Kynu	Sprague-Dawley rats	Liver Kidney	↓ ↓ at 12 months ↑ at 18 months	[16]
3-HDO	Sprague-Dawley rats	Liver Kidney	↑ vs. 1 week ↑ vs. 1 week	[16]
QUIN	Female Wistar rat	Brain Liver Kidney	↑ ↑ ↓ 12 months = at 24 months	[13]
	Male Wistar rats	Cortex brain	↑ 3 days to 3months	[30]
	Primiparous Sprague-Dawley rats	Brain	↑ at birth vs. prenatal ↓ postnatal period	[19]
ACSMD	Sprague-Dawley rats	Liver	↑ vs. 1 week No changes between 3, 12, and 18 months	[16]
		Kidney	↑ vs. 1 week ↓ at 18 months vs. 12 months	

(continued)

Table 5.1 (continued)

Metabolite or enzyme	Species	Tissue or sample	Modulation in aging	Reference
PIC	Female Wistar rat	Brain Liver Kidney	↑ ↑ ↓ 12 months = at 24 months	[13]
	Humans	CSF	↑	[31]
QPRT	Female Wistar rat	Brain Liver Kidney	↓ ↓ ↑	[13]

Abbreviations: *Trp* Tryptophan, *TDO* tryptophan 2,3-dioxygenase, *IDO* indoleamine 2,3-dioxigenase, *KYN* L-kynurenine, *AA* anthranilic acid, *KAT* kynurenine aminotransferase, *KYNA* kynurenic acid, *KMO* kynurenine 3-monooxygenase, *Kynu* kynunerinase, *3-HDO* 3-hydroxyanthranilate 3,4-dioxygenase, *QUIN* quinolinic acid, *ACMSD* 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase, *PIC* picolinic acid, *QPRT* quinolinic acid phosphoribosyltransferase

In other studies, it was found that depletion or inactivation of TDO in *C. elegans* or in flies resulted in increased lifespan [32, 33]. Moreover, it was observed that through the regulation of α-synuclein, amyloid-β, and polyglutamine proteins, the Trp levels are increased, suggesting that TDO2 could be a metabolic regulator of age pathologies and lifespan independent of kynurenines [32]. Other interesting findings observed in *C. elegans* were their gut granules containing anthranilic acid glucosyl ester which seems to serve as a marker for organism death; however, it is still under study whether there is relation with aging [34].

Braidy et al. [13] examined the KP metabolites and enzymes in the brain, liver, and kidney of female Wistar rats during aging. They found that Trp levels and TDO activity decreased in all tissues with age. In contrast, brain IDO activity increased with age. In this context, Mazarei et al. [35] studied IDO1 expression patterns during mouse brain development (9 days postnatal, 3 months, and 12 months) in the striatum, cortex, hippocampus, and cerebellum and found that IDO1 was significantly elevated in the striatum at all the time points compared with the other regions tested; the cortex was higher compared to the hippocampus, and there was no expression in the cerebellum. IDO2 exhibited a similar expression pattern as IDO1, being enriched in the striatum with no expression detectable in the cerebellum at 3 months of age. TDO2 expression was exclusive to the cerebellum. According to this, there is evidence that shows that depletion of TDO2 extends lifespan in *C. elegans* [32]. Additionally, this study also showed that KYNA, AA, XA, 3-HK, PIC, QUIN, and 5-hydroxyindoleacetic acid (5-HA) levels decreased in the striatum at 12 months compared with 3-month-old mice, while 3-HANA and serotonin increased in the striatum of older mice. In the cerebellum, AA, 3-HK, 3-HANA, PIC, serotonin, and 5-HA levels decreased, while KYN, XA, and NAD^+ levels were

increased in 12-month-old mice compared with the cerebellum from 3-month-old animals.

Nevertheless, Braidy et al. [13] also found that KYN, KYNA, PIC, and QUIN levels increased with age in the rat brain, which can be related with the increase in IDO expression and activity as well as the inflammatory activity and cytokine activation during aging. In another study, PIC levels were determined in the CSF of patients with or without CNS disease. A linear correlation was found between CSF PIC concentration and the age of patients in both the disease and control populations [31]. These findings are consistent with previous studies that demonstrated an age-associated elevation in ACMSD activity in rat kidney [16]. Therefore, the increase in ACMSD activity could have the effect of reducing the biosynthesis of nicotinic acid and NAD^+ in aging which could also cause impairment of cell function, leading to cell death.

Comai et al. [16] showed the correlation between some KP enzymes activities and age in the liver, kidney, and small intestine of male Sprague-Dawley rats. First, TDO enzyme activity decreased significantly with age progression in liver homogenates as well as KMO and kynureninase activity decreased both rat liver and kidneys. However, kynurenine-oxoglutarate transaminase, 3-HDO, and ACMSD activities increased with age in rat kidney [16].

Braidy et al. [13] also found that QUIN levels increased during aging in the liver and brain of female Wistar rats which can be correlated with the decrease in QPRT activity in the same samples, suggesting a decrease in NAD^+ levels. Similar findings were reported by Moroni et al. [30] who noted an increase in QUIN levels in rat brain cortex with age.

Age-dependent changes of KYNA were previously studied in male rats of different ages by Moroni et al. [27]. The brain concentration of KYNA was extremely low during the first week of life, increased at 3 months and furthermore at 18 months of age. This effect in KYNA levels can be related with a previous study done by Baran and Schwarcz [23], in which KAT activity increased in all brain areas (but not in the liver) in rats between 3 days and 3 months postnatal. KAT activity increased in the parietal cortex 34-fold during the observation period, whereas enzyme activity in the cerebellum and substantia nigra increased only three- to five-fold over the same time interval. Between 3 and 24 months of age, the changes in KAT were particularly pronounced in the cortex and in the striatum, where enzyme activity increased three-fold during the period studied. KYNA production was significantly enhanced in the cortex and hippocampus of old animals [28]. In accordance with these results, Gramsbergen et al. [28] showed increased KAT levels between 3 and 24 months of age in different rat brain regions, mainly in the cortex and in the striatum, where enzyme activity increased three-fold during the period studied. It could be possible that the increased KYNA levels in aging confer to the brain resistance to neurotoxic damage since striatal injection of QUIN in old rats showed reduced susceptibility to toxicity of QUIN [36].

In humans, Heyes et al. [22] observed higher CSF levels of KYNA and KYN in older control subjects. Similarly, KYNA levels were evaluated in the CSF and serum in a group of human subjects without detectable neurological disease with a range of age between 25 and 74 years. Subjects under 50 years of age showed lower KYNA levels in CSF compared with subjects over 50 years (around 2.86 fmol/μL and 4.09 fmol/μL, respectively), while no differences on KYNA levels were found in the serum. The increase in KYNA levels was not related with KAT-I or KAT-II activities. In the same samples, KYNA levels were correlated with IgG and β_2-microgobulin levels (a marker of activation of immune cells) [24]. Elevated KYNA metabolism may be involved in the hypofunction of the glutamatergic and/or nicotinic cholinergic neurotransmission in the CNS of aging humans. Experimental studies show that reductions in hippocampal KYNA constitute an effective strategy for cognitive improvement [37]. This approach might be especially useful in the treatment of cognitive impairment associated with increased brain KYNA levels as in aging.

Miaztal et al. [38] infused QUIN intracerebrally in rats for 2 weeks observing deficits in memory which resembles the cognitive impairment observed in aging. This observation was accompanied by neuronal loss from the forebrain. These findings are in accordance with results found by Moroni and colleagues who observed increased kynurenines levels in the CSF and brain in older animals suggesting that the metabolites are involved in the aging process [27, 30]. Different glycine-site antagonists of NMDAr (e.g., KYNA) have also been administered in rats and can result in both impaired working memory [39] and improved recognition memory [40].

Conclusion

In the aging process, KP metabolism shows characteristic patterns of changes throughout the lifespan, which has the capacity to impact cellular functions either by their own properties or by the NAD^+ availability. Modulation in KP metabolism could be considered a target to alter or minimize age-associated cellular degenerative changes. Furthermore, better understanding of the involvement of KP metabolism in aging may help design additional strategies with the potential to improve the aging process. Other possibilities would be the use of KP metabolites as biomarkers at different levels, in peripheral fluids, and at central levels, in order to prevent or detect some diseases or imbalances between them. It would be necessary to begin in different populations and different diseases in order to count with level ranges.

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Chapter 6
Role of Tryptophan Metabolism in Mood, Behavior, and Cognition

Johanna M. Gostner, Kathrin Becker, Barbara Sperner-Unterweger, Florian Überall, Dietmar Fuchs, and Barbara Strasser

Abstract During Th1-type immune response, tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) becomes activated and accelerates the breakdown of tryptophan, as expressed by a higher kynurenine to tryptophan ratio. Lowered tryptophan concentrations were detected in patients suffering from immunopathologies like virus infections, autoimmune syndromes, and certain types of cancer, and in some of these clinical conditions, an association between enhanced tryptophan breakdown and mood disturbances was observed. Tryptophan is required for the biosynthesis of 5-hydroxytryptamine (serotonin), and the availability of tryptophan in the blood is linked to its concentration in the brain, as tryptophan can cross the blood-brain barrier. In patients at risk for cardiovascular disease, higher concentrations of neopterin are associated with lower concentrations of vitamins C and E and other antioxidants. Data may indicate that chronic immune activation leads to an enhanced degradation of oxidation-sensitive biomolecules. Likewise, additional antioxidant vitamin supplementation might be able to counteract the inflammation process. However, this concept is mainly derived from in vitro data, whereas in vivo findings remain scarce. In vitro, it was also documented that several antioxidant compounds including vitamins C and E and stilbene resveratrol but also food preservatives and colorants are able to slow down Th1-type immune activation leading to a suppression of IDO activity. Similar effects were observed for extracts of beverages known to be rich in antioxidants like wine, beer, cacao, and coffee. The

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suppressive effects of antioxidant molecules and extracts on tryptophan breakdown could relate to their mood-enhancing properties.

Keywords Antioxidants • Immune activation • Indoleamine 2,3-dioxygenase • Inflammation • Interferon-γ • Neopterin • Reactive oxygen species • Serotonin • Tryptophan • Vitamins

List of Abbreviations

GCH	GTP cyclohydrolase I
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
iNOS	Inducible nitric oxide synthase
Kyn/Trp	Kynurenine to tryptophan ratio
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor-κB
ROS	Reactive oxygen species
TDO	Tryptophan 2,3-dioxygenase
TNF	Tumor necrosis factor

Introduction

Tryptophan is one of the essential amino acids in humans, which means that the human organism is incapable of synthesizing it by itself. Therefore, nutrition is most relevant for tryptophan supply. Alternatively, it can be recruited only via degradation of proteins. Because tryptophan is also the least abundant amino acid in food, the availability of tryptophan is an important regulator of protein biosynthesis. Additionally, tryptophan is the precursor of two important biochemical pathways, which in a first step involve either enzyme tryptophan 5-monoxygenase (tryptophan 5-hydroxylase) that initializes the synthesis of neurotransmitter 5-hydroxytryptamine (serotonin) or tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenases (IDO1 and IDO2). The latter enzymes introduce tryptophan into the so-called tryptophan-kynurenine pathway, which is named after its first stable intermediate kynurenine.

Further downstream metabolites are kynurenic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, picolinic and quinolinic acid, and nicotinamide adenine dinucleotide (NAD) [1]. It has to be mentioned that substrate specificity of IDO is less than that of TDO, which solely accepts tryptophan as a substrate, whereas IDO also degrades other indoleamine derivatives like serotonin, albeit with lesser efficacy.

TDO is mainly expressed in hepatocytes, but TDO activity can rarely be detected outside the liver. Enzyme activity is regulated by the tryptophan level in the blood and upregulated by corticosteroids. The conversion of tryptophan to kynurenine leads to a rather constant kynurenine to tryptophan (Kyn/Trp) ratio in the blood, which in human is around 27 ± 8.1 μmol/mmol (tryptophan 73 ± 15 μmol/L and kynurenine 1.9 ± 0.58 μmol/L), women presenting with approximately 15 % higher levels than men [2]. In the normal situation, expression and activity of IDO are low, but can be increased severalfold during inflammatory conditions by pro-inflammatory mediators like lipopolysaccharides (LPS) or specific cytokines, whereby Th1-type cytokine interferon-γ (IFN-γ) is the strongest inducer [3–5]. This explains why increased tryptophan breakdown, as indicated by increased Kyn/Trp concentrations, is commonly observed in patients under treatment with interferons or other stimulatory cytokines like interleukin-2 [6], but also in patients suffering from viral infections like HIV-1, autoimmune syndromes such as systemic lupus erythematosus [7], malignant cancers like malignant melanoma [8], or adult T-cell leukemia [9]. In such clinical conditions, significant correlation between Kyn/Trp ratio and concentrations of immune activation markers like serum soluble cytokine receptors sTNF-R75 and sIL-2R or of the macrophage product neopterin further substantiates the conclusion that the increased tryptophan breakdown rate is due to cytokine-induced IDO activity rather than TDO [10].

The alterations of Kyn/Trp ratio are usually more significant than the absolute kynurenine concentrations. Still significant association between concentrations of kynurenine and immune activation marker neopterin was observed in patients with HIV-1 infection, but it was not significant in patients with colorectal cancer [11], or adult T-cell leukemia [9], or in healthy pregnant women [12]. Thus, even when increased Kyn/Trp ratio shows that the conversion of tryptophan to kynurenine is accelerated, kynurenine does not accumulate much because most likely in the liver the compound is rapidly converted to downstream products of the kynurenine pathway as mentioned above [1] and these concentrations increase [13]. However, for example, in the absence of sufficient supply with B vitamins, the conversion of kynurenine by downstream enzymes kynurenine monooxygenase and kynurenine aminotransferases is decreased, and kynurenine concentrations might accumulate [14].

Tryptophan Breakdown and Immunity

During Th1-type immune response, IDO becomes activated mainly by IFN-γ and manifests in increased Kyn/Trp concentrations (Fig. 6.1). Tryptophan breakdown and deprivation are part of the antiproliferative strategy of the immune system to halt growth of pathogens and of malignant cells. Restrictions of essential nutrient availability as well as metabolic reprogramming are universal strategies to mediate growth inhibition. For example, iron and zinc pools in circulation can be reduced by increasing renal excretion or storage at other body sites. Also, the supply of lipids becomes disturbed in inflammatory conditions [15].

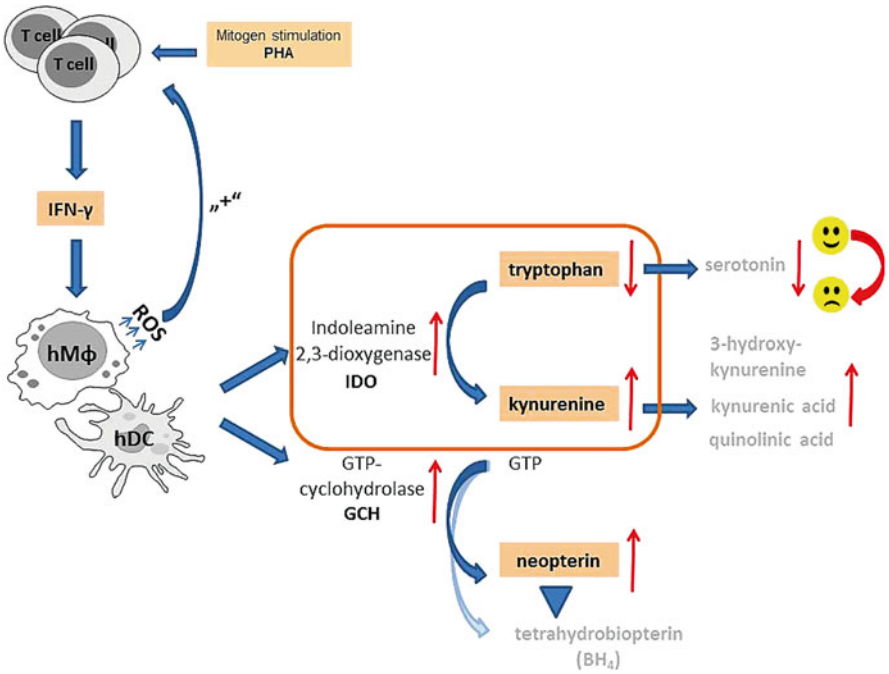

Fig. 6.1 Upon stimulation of type 1 T-helper cells (T cell) by, e.g., mitogen phytohemagglutinin (PHA), cytokine interferon-γ (IFN-γ) is released that activates ["+"] antimicrobial strategies and immunoregulatory cascades in target cells like macrophages (hMφ) and dendritic cells (DC). Among them are the production of cytotoxic reactive oxygen species (ROS) and the induction of tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) and GTP cyclohydrolase (GCH), which usually leads to the formation of tetrahydrobiopterin (BH_4), the necessary cofactor of inducible nitric oxide synthase (iNOS), but in human monocyte-derived cells like hMφ and hDC gives rise to the production of neopterin at the expense of BH_4. Diminished blood tryptophan levels due to IDO activity will slow down production of serotonin in the brain and lower mood. The possible direct effect of IDO degrading serotonin further strengthens the influence of IDO activation on neuropsychiatric performance of patients suffering from inflammatory conditions

Already in 1984, it has been clearly shown by E.R. Pfefferkorn that the growth of *Toxoplasma gondii* was inhibited upon treatment with IFN-γ [16], and the induction of tryptophan degradation was responsible for this effect: as a "note added in proof," the involvement of IDO in this biochemical alteration was suggested. Moreover, this study showed that the growth of *T. gondii* could be reestablished, when tryptophan was added back to culture, and it could be excluded that the accumulation of toxic tryptophan breakdown products was important for growth inhibition. In parallel, tryptophan breakdown was found to be accelerated in patients with cancer under treatment with IFN-γ [6], and in many clinical conditions, which go along with activated T-cell/macrophage responses, e.g., infections, autoimmune syndromes, and cancer, an enhanced Kyn/Trp ratio was observed in serum, plasma, and other body fluids such as cerebrospinal fluid [10].

Activated IDO by pro-inflammatory cytokines like IFN-γ represents the background for lowered tryptophan concentrations in patients suffering from certain types of cancer [10]; although a few tumor cell lines were observed to spontaneously express IDO, TDO, or both and degrade tryptophan, the IDO activity is enhanced severalfold in the presence of IFN-γ [17, 18]. Frequently, a higher tryptophan breakdown rate is associated with a reduced residual lifespan in patients, which seems to indicate a diminished functional immune response. In fact, patients with poor prognosis are characterized by several signs of an activated immune system, including elevated neopterin concentrations and tryptophan breakdown rates. Thus, it turns out that the antiproliferative effects of tryptophan deprivation also interfere with the development and proliferation of T cell and their responsiveness. Moreover, the accumulation of immunotoxic tryptophan breakdown products like quinolinic acid and 3HAA seems to be of major relevance for the induction of T-cell apoptosis [19] and generation of regulatory T cells (Treg) [20].

Tryptophan and Mood in Inflammatory Conditions

Signs of depression are common in patients suffering from a wide range of inflammatory conditions including infections, autoimmune pathologies like systemic lupus erythematosus, cardiovascular disease, and cancer, but also in elderly people that are characterized by lower levels of tryptophan. In general, the development of neuropsychiatric disturbances seems to represent a sign of poor outcome. In some of these clinical conditions, an association between enhanced tryptophan breakdown and increase of neuropsychiatric symptoms was observed [21–26].

Also, the treatment with cytokines like IFN-γ of patients with hepatitis C virus infection or malignant melanoma is associated with an increased risk for developing mood changes. Under treatment with IFNs, the enhanced tryptophan breakdown rate, which is already common in untreated patients, is further enhanced [6]. Likewise, in patients with malignant melanoma, an association was observed between depression development and the decline of tryptophan during follow-up of therapy [27]. In the same way, studies showed an association between lower blood tryptophan levels in cancer patients and higher degree of fatigue and quality of life scores, in, e.g., patients with colorectal cancer [11, 25]. In older individuals, several significant relationships were observed between tryptophan metabolic alterations in the blood and signs of mood alterations [28–30]. However, in most of these studies, either the absolute tryptophan concentrations or Kyn/Trp ratio revealed significant relationships, but the alterations of the absolute kynurenine levels were not significant. It appears that the kynurenine produced out of tryptophan becomes rapidly converted to its downstream products in the pathway like quinolinic acid, quinolinic acid concentrations themselves correlating rather well with neopterin levels [13]. Interestingly, in suicide attempters with major depression, plasma kynurenine levels were higher than in those without [31].

Also studies performed in animal models were able to show an association between tryptophan breakdown and the development of depressive behavior as, e.g., in the forced swim test or tail suspension test with mice, when tryptophan breakdown was induced upon exposure to *Bacillus Calmette-Guerin* [32]. In conclusion, pro-inflammatory cytokines like IFN-γ that are released during Th1-type immune activation and inflammation restrict tryptophan availability, and thus, also serotonin production is hampered, and the risk of low mood and depression can be increased. From the existing literature, it appears that IDO activity is a key player in the pathogenesis of depressive mood that are associated with inflammation and immune activations, although also biochemistry downstream of kynurenine may play a role as it is claimed in individuals with depression but excluding inflammation [33]. Neuroactive kynurenine downstream metabolites such as kynurenic acid, quinolinic acid, and 3-hydroxykynurenine are involved in several important regulatory processes in the brain, and besides their involvement in the development of depression and other psychiatric disorders, dysregulated production of these metabolites is associated also with several neurologic disorders and neurodegeneration. The levels of brain kynurenine pathway metabolites are at least partially dependent on peripheral concentrations of tryptophan, kynurenine, and 3-hydroxyanthtranilic acid, as these compounds can cross the blood-brain barrier. Additionally, other immunocompetent cells within the brain such as microglia are able to activate the tryptophan-kynurenine pathway themselves in response to immune activation signals.

One might hypothesize that the association between inflammatory conditions and an accelerated tryptophan breakdown could also be related with partnering behavior of individuals, when partners are selected depending on their mood level [34]. Many studies revealed that individuals with humor and positive mood are more likely to be chosen as partners than those with opposite attitudes, suggesting that a partner with positive thoughts, optimistic views, and fun is more likely free of infectious or inflammatory conditions and thus having a longer residual lifespan. Such partners may present with positive mood due to higher tryptophan levels which indicate less inflammation activity and lower risk of malignant or infectious diseases, i.e., higher probability of togetherness for a longer period of time, because such individuals are less likely to suffer from inflammatory conditions. However, potential associations of tryptophan levels, personality profiles, and partner selection have still to be analyzed.

Anti-inflammatory and Antioxidant Compounds Slow Down Tryptophan Breakdown

From in vitro and in vivo, it is well established that pro-inflammatory cytokines like IFN-γ, LPS, and TNF-α induce IDO and thus tryptophan breakdown in human macrophages and dendritic cells and also in other cells like fibroblasts or tumor cell lines [17, 18]. In monocyte-derived macrophages, IFN-γ was noted to be the strongest inducer [3, 5]; whereas, in dendritic cells and astrocytes, IFN-α, IFN-β, and

IFN-γ were found to be of equal potency [35, 36]. In contrast, immunosuppressants like cyclosporine A and rapamycin [37], anti-inflammatory compounds like acetylsalicylic acid and salicylic acid [38], and also statins [39] slow down Th1-type immune response, and thus, tryptophan breakdown ceases. Because IFN-γ is also the strongest inducer for the production of reactive oxygen species (ROS) in human macrophages [40] together with neopterin, the induction of the cells during Th1-type immune response is usually accompanied by a high output of ROS, and when antioxidant pools become wiped out, oxidative stress will develop. Moreover, the formation of ROS further enhances the formation of pro-inflammatory cytokines in an autocrine manner via translocation of the nuclear factor-κB (NF-κB), the central signal transduction element in the expression of various pro-inflammatory cytokines like TNF-α [41], and also specific enzymes like the inducible nitric oxide synthase (iNOS) [42].

Antioxidant compounds are therefore able to counteract the pro-inflammatory cytokine cascade at least in the in vitro setting and are considered to be of help in clinical situations that are associated with excess ROS production as it is the case in inflammatory conditions and chronic immune activation. In vitro studies have also shown that antioxidant compounds like vitamins C and E, the stilbene resveratrol, and also food preservatives like sodium sulfite or benzoate and colorants like curcumin and beet root juice suppress the production of IFN-γ [43, 44], and in turn, the activity of IDO is diminished (Fig. 6.2). As a consequence, tryptophan levels rise. Similar effects were observed for extracts of beverages known to be rich in antioxidants like wine, beer, or cacao and coffee. Thereby, the antioxidative effects of flavonoid compounds contained in such beverages are considered to be of major relevance for the immunosuppressive properties [45]. Thus, the increased use of dietary supplements including preservatives and colorants may contribute to a suppression of Th1-type immune response. In freshly isolated human peripheral blood mononuclear cells in vitro, it was shown that antioxidant compounds like vitamins C and E but also food preservatives and colorants exert significant suppressive effects on the Th1 immune activation cascade [46, 47]. The effects observed may be based on the interaction of antioxidant compounds with pro-inflammatory cascades involving important signal transduction elements such as NF-κB. Likewise, in dendritic cells, the antioxidant vitamins C and E have been demonstrated to promote production of Th2-type cytokines IL-4 and IL-10 in a dendritic cell coculture system, and it was shown that the T cells expressed FoxP3 and, besides being anergic, might have acquired Treg properties [48].

In vivo, for example, in patients at risk for cardiovascular disease, higher concentrations of vitamins C and E and other antioxidant biomolecules like lycopene, lutein, zeaxanthin, and carotenes were found to be associated with lower neopterin concentrations [49]. One might conclude that insufficient supply with such vitamins could feed the inflammation process and additional antioxidant vitamins might be able to counteract it. Alternatively, data may relate to the fact that chronic immune activation leads to an enhanced degradation of oxidation-labile biomolecules such as antioxidant vitamins. On the other hand, recent studies indicated that antioxidant treatments often treat only symptoms but not disease causes, and moreover, the

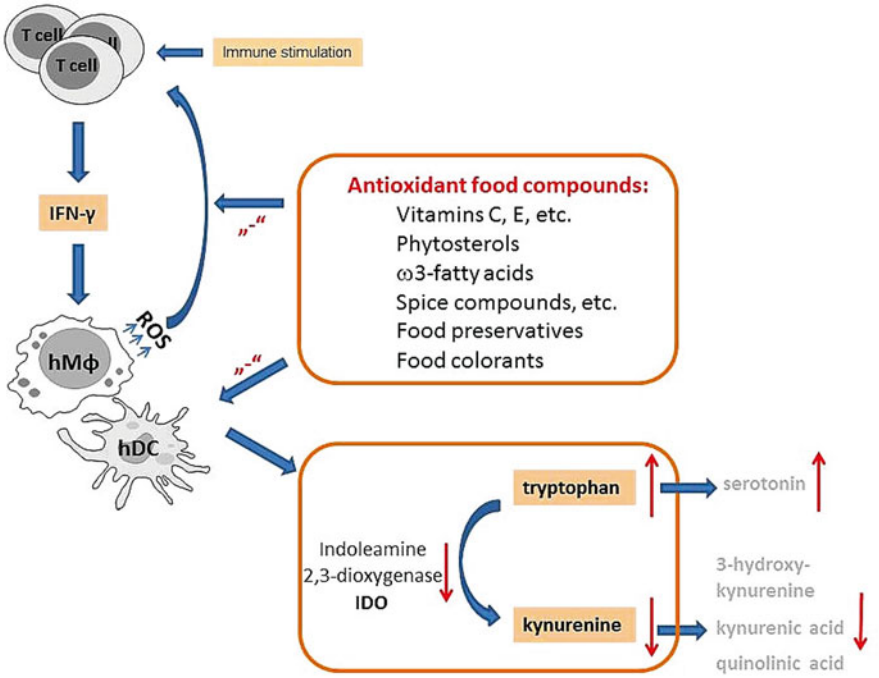

Fig. 6.2 Antioxidant compounds are able to neutralize reactive oxygen species (ROS) and slow down ("–") Th1-type immune activation cascades. This is especially true if compounds are added in high concentrations as food supplements (= extra vitamins) and also in the form of food preservatives and colorants. Such supplements suppress IDO activity, which on the one hand counteracts immune defense and thus may increase malignant growth and on the other hand will enhance serotonin availability and improve mood

excessive use of antioxidants may lead to adverse outcomes, e.g., development of allergies in children or even increased mortality [46].

When the immune activation process is suppressed by such food compounds, not only the neopterin levels decline, but also tryptophan breakdown ceases. As a consequence, tryptophan levels rise, and its transport into the brain may increase allowing a higher rate of serotonin production. Thus, typical mood food compounds such as antioxidant vitamins and flavonoids and also omega-3 fatty acids are contained in high concentrations in chocolate, berries, and nuts [50–53], but also in beverages like tea, wine, beer, cacao, and coffee [43, 44, 54] which possess the ability to improve tryptophan availability as a source for serotonin production. Consequently, mood might be enhanced after the intake of such beverages. This sequence of events that can be induced by the antioxidants contained in beverages could represent a major aspect why such nutrients possess their mood-enhancing ability and why such beverages became popular for recreation throughout centuries. From the PREDIMED study, it was concluded that moderate consumption of wine may reduce the incidence of depression, while heavy drinkers seem to be at higher risk [55].

For wine and beer, it was demonstrated in vitro that similar effects can be obtained with their alcohol-free analogs. So the alcohol (alone) is not what makes one feel good and maybe even dependent on such beverages; the contained antioxidants appear to be important as well or may be even more important than the alcohol. Ethanol became important to increase the suitability for storage of beverages, and ethanol will contribute to the enhancement of mood solely by increasing the solubility and resorption of compounds in the gut. The potential influence of antioxidant contents in alcoholic beverages to the drinking behavior may also shed some new light on the development of depression during and after alcohol withdrawal, when the decline of antioxidants will abrogate the suppression of immune responses and IDO activity [56]. As a consequence, tryptophan breakdown will increase, and tryptophan and serotonin availability may suffer. Thus, supplementation with tryptophan might help alcoholics under withdrawal to slow down depressive symptoms similar to selective serotonin reuptake inhibitors (SSRI).

Interestingly, smoking, as another widespread lifestyle behavior (although all victims are aware of the eventual negative side effects), can influence tryptophan availability because carbon monoxide (CO) is a potent inhibitor of Th1-type immune response and thus suppresses production of IFN-γ. Enzyme heme oxygenase 1 exerts its anti-inflammatory effects via the release of CO [57], and also in vivo exposure to low to moderate concentrations of CO was found to increase survival expectations in patients with sepsis [58]. Accordingly, among patients with cardiovascular disease, smokers were found to present with lower neopterin levels [59], and also lower tryptophan breakdown rates were observed [60]. So the resulting higher tryptophan levels in smokers may be associated with higher serotonin availability and improve their mood and make them feel better.

Antioxidant Compounds and Obesity

Depressive mood appears to modulate nutrition behavior, and it is thought that the reverse is also true, i.e., that low mood is associated with carbohydrate graving and that carbohydrate intake increases mood by several means. Enhanced serotonin levels appear to represent an important aspect, because the consumption of carbohydrate causes an increase of tryptophan in the plasma and the brain and of serotonin in the brain [61]. Thus, the availability of tryptophan in the blood represents an important aspect for the serotonin supply as does the ratio of tryptophan to branched chained amino acids for the transport of tryptophan into the brain. The higher intake of calories due to carbohydrate craving in situations of low mood may contribute to overeating and weight gain.

After smoking cessation, there is an increased risk for weight gain [62]. Although cardiovascular risks decrease after quitting smoking, body weight often increases in the early period after smoking cessation [63]. In light of the effects of smoking and CO on Th1-type immunity and IDO activity, smoking cessation seems to result in an increase of IDO activity, and as a consequence tryptophan and serotonin

availability decreases, which leads to a higher intake of carbohydrates and fat. Because of the intake of such calorie-rich foods, victims will regain weight. However, this represents not the only the mechanistic aspect; several other biochemical pathways relevant for nutrition behavior are affected by antioxidant compounds. This is also particularly true for the adipokine leptin, an important regulator of food intake [64]. In the normal situation, higher leptin levels cause a reduced food intake as a kind of feedback mechanism initialized by fat tissue, in the sense of a saturation signal. The influence of antioxidant compounds, e.g., food preservatives and colorants, was investigated in the in vitro model of the mouse embryonic fibroblast cell line NIH 3 T3, and the compounds were found to significantly suppress the leptin production rate in an inflammatory environment [65]. Thus, the increased intake of food supplemented with antioxidant compounds including antioxidant vitamins could play an important role in the obesity epidemic in the Western world. Also spices contain powerful antioxidant compounds like curcumin, piperine, and capsaicin which were found to exert similar anti-inflammatory effects on NF-κB expression and on pro-inflammatory cytokines [66]. Spices are in use to improve taste of food and these compounds make you feel good. This effect may be again due to their influence on the tryptophan biochemistry, and they are also prone to enhance the amount of food and thus calories to be taken in [67].

Naturally, a lifestyle with less movement and exercise is also an important denominator in the obesity epidemic. The most secure way to ensure human health is to provide individuals the required dosage of nutrients and exercise in a most exact way, never too little and never too much, first expressed by Plato 428–347 BC. Accordingly, overweight and obese individuals are often referred to weight reduction programs which include intake of lower calories per day and to perform exercise [26, 68, 69]. However, the adherence of victims to these programs is often limited, and they may quit before reaching their goals. In a recent study, we observed that low-calorie weight loss diet lowered not only leptin levels, but also levels of essential amino acid tryptophan decreased significantly [70]. The disturbed metabolism of tryptophan might affect biosynthesis of serotonin and could thereby increase the susceptibility for mood disturbances and carbohydrate craving, increasing the cessation probability of weight reduction programs. Supplementation with tryptophan while dieting [71] could be helpful in improving mood status and preventing uncontrolled weight gain or neuropsychiatric symptoms.

Adverse Effects of Antioxidant Compounds

There is accumulating evidence that large-scale supplementation with antioxidant compounds may increase infectious risks when they may counteract the killing strategies of immunocompetent cells. Moreover, in vitro results indicate that the anti-inflammatory property of compounds could shift the Th1–Th2-type immune balance toward Th2-type immunity. Thus, in particular the increasing use of antioxidant food supplements including preservatives and colorants may relate to the

increase of allergies in the Western world [46]. Moreover, increased Th2-type immune response was shown to include the risk of weight gain [67] and chronic disease development like diabetes mellitus.

Conclusion

There are manifold influences of antioxidant compounds on the expression and inhibition of biochemical and immunobiological pathways that are widely accepted. Usually, the consequences are considered only in a mono-directed way, when antioxidants are considered promising to combat inflammatory diseases. However, as ever there is nothing beneficial without negative side effects. Their influence on tryptophan biochemistry by slowing down Th1-type immune activation and production of pro-inflammatory cytokines like IFN-γ seems to be of major relevance for influencing mood. A role of tryptophan breakdown in the precipitation of mood disturbances is well acknowledged since the last two decades, when reduced tryptophan availability during inflammatory conditions was found to be associated with mood lowering and precipitating depression. In line with this, treatment with SSRI is well established and helpful at least in half of the patients. Certainly, there are more than just tryptophan metabolic disturbances, which play a role in the pathogenesis of depressive mood and depression, and the dopaminergic and adrenergic/noradrenergic pathway is of major importance. Interestingly enough, also this pathway is hampered by inflammation when ROS and other mechanisms seem to interfere with supply of 5,6,7,8-tetrahydrobiopterin, the necessary cofactor of important monooxygenases in this pathway [72, 73]. Unfortunately, animal models used to mimic what is going on in humans in such diseases are not well representative, because humans and mice differ regarding the potential influence of nitric oxide (NO) to inhibit IDO activity and because human macrophages produce less NO as compared with cells of other species like mice and rats, and thus, IDO plays a greater role in humans than in these animal species [74]. Alternatively, NO biochemistry is more important in murine macrophages than in human cells. As a consequence, any animal model used for studies of tryptophan metabolism is less than perfect to investigate the roles of tryptophan metabolism in human diseases.

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Part II
Systemic Diseases

Chapter 7
Role of the Kynurenine Pathway in Immune-Mediated Inflammation

Adam P. Cribbs and Richard O. Williams

Abstract Indoleamine 2,3-dioxygenase (IDO) plays a key role in immune homeostasis via depletion of tryptophan and accumulation of kynurenines and is recognized as an important factor contributing to suppression of antitumor immune responses. However, the possibility of harnessing the IDO pathway for the therapy of autoimmune disease represents an intriguing possibility, and in this review, we highlight recent research on the involvement of IDO in immune-mediated inflammatory diseases, with a focus on rheumatoid arthritis. Inhibition of IDO was shown to exacerbate experimental arthritis and increase numbers of pathogenic Th1 and Th17 cells in the joints and draining lymph nodes. Analysis of the kinetics of expression of kynurenine pathway enzymes in animal models also pointed to a potential role for tryptophan metabolites in disease resolution and administration of L-kynurenine or [3,4-dimethoxycinnamonyl]-anthranilic acid (a synthetic derivative of 3-hydroxyanthranilic acid) reduced the severity of disease. A more recent study has identified an association between defective regulatory T cells in rheumatoid arthritis with reduced capacity to activate the kynurenine pathway. These findings suggest that strategies to activate IDO in a targeted manner may be effective in the therapy of autoimmune disease.

Keywords Rheumatoid arthritis • Collagen-induced arthritis • Indoleamine 2,3-dioxygenase • Inflammation • Autoimmunity

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List of Abbreviations

1-MT	1-methyl tryptophan
APCs	Antigen-presenting cells
AHR	Aryl hydrocarbon receptor
CIA	Collagen-induced arthritis
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DMARDs	Disease-modifying anti-rheumatic drugs
EAE	Experimental autoimmune encephalomyelitis
GPR35	G-protein-coupled receptor 35
GWAS	Genome-wide association studies
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL-1	Interleukin 1
IRF1	Interferon-regulatory factor
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NK	Natural killer
NOD	Nonobese diabetic
NFAT	Nuclear factor of activated T cells
pDCs	Plasmacytoid DCs
Tregs	Regulatory T cells
RA	Rheumatoid arthritis
STAT1	Signal transducer and activator of transcription 1
TCR	T cell receptor
Th	T helper
TGF	Transforming growth factor
TDO	Tryptophan 2,3-dioxygenase
TTS	Tryptophanyl-tRNA-synthetase
TNF	Tumor necrosis factor
T1D	Type 1 diabetes

Introduction

The degradation of the essential amino acid L-tryptophan by indoleamine 2,3-dioxygenase (IDO) is the first and rate-limiting step in the kynurenine pathway. IDO is encoded by an evolutionarily ancient gene that precedes the development of the adaptive immune system. In 1984, Pfefferkorn observed that the pro-inflammatory cytokine interferon-γ (IFN-γ) induced tryptophan degradation and prevented the growth of *Toxoplasma gondii* [1, 2]. In the intervening years, much research has focused on investigating the link between tryptophan

degradation and innate immune responses. However, Munn and colleagues, who discovered that the activity of IDO could prevent the rejection of allogenic fetuses in pregnant mice, revealed that IDO-induced tryptophan depletion limited the supply of tryptophan for proliferating T cells, which in turn prevented a T cell response against the developing fetus [3]. This evidence suggested that the kynurenine pathway, as well as having a role in the innate immune system, also plays a major role in the adaptive immune system. In this chapter, we will focus on the role of the kynurenine pathway in inflammatory diseases, using rheumatoid arthritis (RA) as an example to highlight the potential of manipulating this pathway therapeutically.

The Regulation of IDO Expression During Inflammation

In mammals, three genes encode enzymes that catalyze oxidative degradation of tryptophan: IDO1, IDO2, and tryptophan 2,3-dioxygenase (TDO). Each enzyme catalyzes the same reaction, the cleavage of the 2,3 double bond in the indole ring [4]. TDO expression is mainly confined to the liver where it is involved in the homeostatic regulation of tryptophan and is not induced in response to immune stimulation. IDO1 and IDO2 are expressed in antigen-presenting cells (APCs) of the immune system and respond to a variety of inflammatory signals.

IDO1 and IDO2 share significant identity at the protein level but are not related structurally to TDO. The IDO genes are well conserved and contain several inflammatory response sequences [5], such as multiple response elements for interferon type I (IFN-α/β) and interferon type II (IFN-γ) signaling [6–9]. A number of inflammatory signals can induce IDO expression. For example, signal transducer and activator of transcription 1 (STAT1) and IFN-regulatory factor (IRF1) function in response to IFN-γ signaling to induce IDO, with a failure to induce IDO occurring in mice lacking either IFN-γ or IFR1 [10, 11]. Lipopolysaccharide (LPS), interleukin 1 (IL-1), and tumor necrosis factor (TNF) act synergistically with IFN-γ to enhance IDO expression in vitro [12]. However, other cytokines have been shown to act negatively. For example, TGF-β signaling can abrogate the effect of IFN-γ-induced IDO by reducing mRNA stability [13]. Similarly, administration of IL-10 both in vivo and in vitro leads to a decrease in the expression of IDO in DCs and splenic DCs [14].

Interestingly, an unexpected finding that IDO can be induced in DCs in response to ligation of CD80/86 by soluble recombinant cytotoxic T-lymphocyte-associated protein 4 fusion protein (CTLA-4-Ig) demonstrated that other pathways in addition to cytokines could regulate IDO expression [15]. IDO induction by ligation of CD80/86 has been demonstrated in numerous diseases in both humans and mice [16–18]. This observation also suggested the possibility that CTLA-4$^+$ regulatory T cells (Tregs) may also regulate the induction of IDO in DCs. Indeed, this has been supported by observations that both human and mouse CD4$^+$CD25$^+$ Tregs induce IDO both in vivo and in vitro in a CTLA-4 dependent mechanism [16, 19, 20].

Tryptophan Depletion as a Mechanism to Regulate the Immune System

Tryptophan is an essential amino acid found in low abundance that is involved in protein synthesis. Local depletion of tryptophan by IDO is one proposed mechanism of immunomodulatory action for this pathway. T cell proliferation is critical for an effective immune response, and it has been shown that inhibition of T cell proliferation by tryptophan depletion represents a critical component of the immunosuppressive function of IDO, which is mediated through IDO expressing macrophages and DCs [21, 22]. Munn et al. have shown that a consequence of tryptophan depletion is the activation of the kinase GCN2, in T cells that act as a mediator for several tryptophan depletion effects, such as reduced proliferation and increased anergy [23]. GCN2 is a stress response kinase that is activated by elevations in uncharged tRNA in response to amino acid starvation [24]. Activation of GCN2 results in cell-cycle arrest, differentiation, and apoptosis [23, 25]. T cells that do not upregulate GCN2 proliferate normally in response to IDO positive DCs and are not rendered anergic [23]. However, whether tryptophan degradation represents the major mechanism for the immunosuppressive action of IDO activity remains questionable, since results that support this have been obtained using in vitro assays and may not be reproduced in vivo. Moreover, tryptophan levels in tissue are expected to be replaced faster than the local degradation rate [26]. Therefore the hypothesis that tryptophan degradation is the primary explanation for the immunosuppressive effect of IDO needs to be validated in vivo.

The Regulation of the Immune System Through Tryptophan Catabolites

The breakdown of tryptophan by IDO leads to the formation of L-formylkynurenine, which is further degraded to L-kynurenine by formamidase. L-kynurenine acts as a substrate for a number of other enzymes that result in further breakdown into kynurenic acid, anthranilic acid, and 3-hydroxykynurenine. Further breakdown of 3-hydroxykynurenine by kynureninase produces 3-hydroxyanthranilic acid (Fig. 7.1). Each of these different tryptophan catabolites can differentially regulate the immune system, with most studies focusing on their anti-proliferative and apoptotic effects on T cells. L-kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid have all been shown to suppress $CD4^+$ and $CD8^+$ T cell proliferation and induce cell death [27, 28]. Moreover, tryptophan catabolites can preferentially induce apoptosis of Th1 cell clones but not Th2 cell clones, raising the possibility that tryptophan catabolites can alter the Th1/2 cell balance and potentially prevent a Th1-mediated response [28]. In addition to T cells, kynurenines also suppress natural killer (NK) cell function and can induce B cell death [27, 29], highlighting that the kynurenine pathway is involved in the suppression of a number of diverse

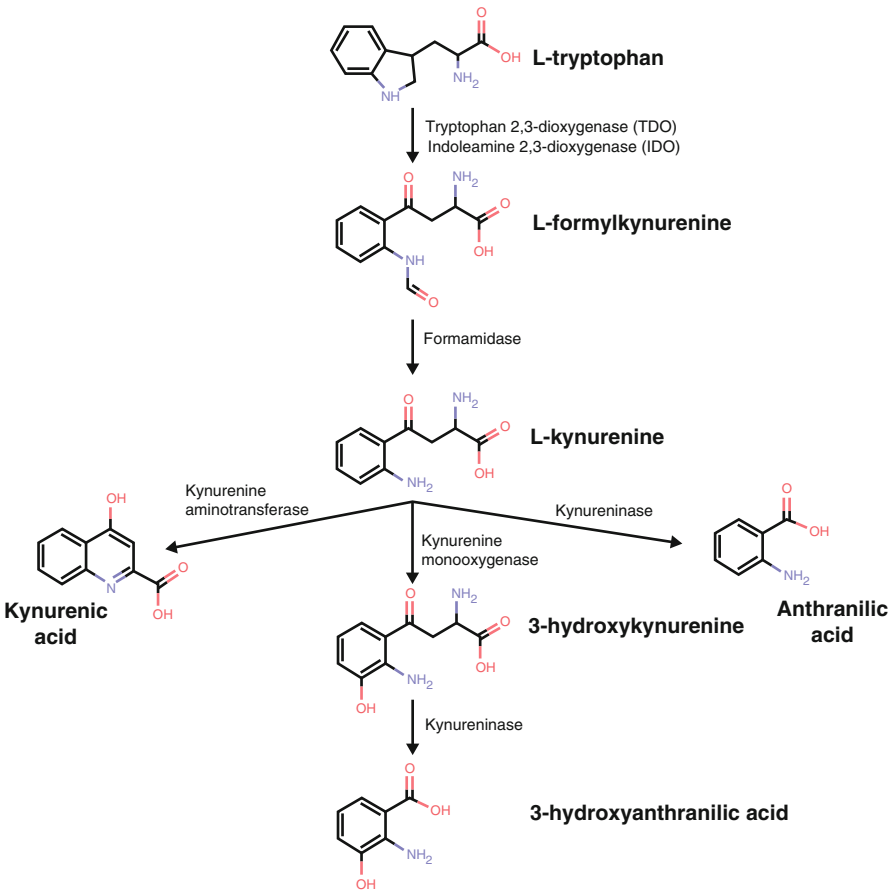

Fig. 7.1 The kynurenine pathway showing the catabolism of tryptophan

inflammatory processes. A direct effect on APCs may also represent another mechanism for tryptophan catabolite-mediated immune suppression. DCs appear to be nonresponsive to kynurenines; however, macrophage-derived APCs are susceptible to apoptosis following culture with 3-hydroxyanthranilic acid [30].

In addition to direct apoptotic effects of kynurenine and its metabolites, tryptophan catabolites may also be instrumental in skewing the immune system away towards anti-inflammatory responses. For example, IDO$^+$ plasmacytoid DCs (pDCs) promote the de novo differentiation of Tregs from naïve T cells [19]. This differentiation also occurs when naïve T cells are cultured in low tryptophan/high kynurenine conditions resulting in a downregulation of the TCRζ chain and generation of a Treg phenotype [31]. In addition to the generation of Tregs, IDO$^+$ DCs also promote the expansion of nTregs [32]. In this study, it was shown that IDO contributes to LPS and TNFα + poly(I:C) stimulated DC maturation, since inhibition of IDO resulted in a failure to induce mature DCs.

Kynurenine Targets of the Immune System

Kynurenine has been identified as the endogenous ligand for the aryl hydrocarbon receptor (AHR) [33]. Ligation of the AHR results in the promotion of Tregs and the suppression of pathogenic T cells. For example, co-cultures of AHR deficient DCs and naïve T cells led to reduced differentiation of Tregs [34]. In contrast, the addition of L-kynurenine promotes the differentiation of Tregs and suppresses Th17 differentiation [34]. Kynurenine metabolites may also mediate their inflammatory responses through other pathways. Kynurenic acid is the ligand of the orphan G-protein-coupled receptor 35 (GPR35), which is expressed on a number of inflammatory cells, and the interaction of kynurenic acid with GPR35 inhibits LPS-driven TNFα production in monocytes [35]. Another kynurenine metabolite, 3-hydroxyanthranillic acid, was found to reduce Th1 and Th17 cells in experimental autoimmune encephalomyelitis (EAE) [36], inhibit the generation of nitric oxide synthase in macrophages, and promote the production of TGF-β [37]; however, its primary molecular target is currently unknown.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints that affects approximately 1% of the world's population and remains a major cause of morbidity and mortality. The disease usually manifests itself as swelling of the joints, pain, and stiffness, with ankylosis developing in many cases [37]. A key observation made many years ago was that specific alleles of the class II major histocompatibility complex (MHC) confer susceptibility to RA [38]. In particular, susceptibility to RA was found to be associated with certain HLA-DRB1 alleles [39]. Subsequently, genome-wide association studies (GWAS) have revealed a number of other non-HLA susceptibility alleles that have also been implicated in disease, such as PTPN22, PADI4, CD40, and CTLA-4 [40], many of which are associated with immune responses. The involvement of inflammatory cells as drivers of RA pathogenesis is further emphasized by the presence of highly activated immune cells, particularly $CD4^+$ T cells within the joints of RA patients.

The Role of the Kynurenine Pathway in Animal Models of RA

Animal models of RA have been extensively used to investigate the efficacy of preclinical therapeutics, understand the biology underpinning RA, and investigate the genetic susceptibility involved in the development of RA. Many models of arthritis exist; however, the collagen-induced arthritis (CIA) model has

been widely investigated, since it shares many pathological features of RA, such as synovial hyperplasia, mononuclear cell infiltration, and cartilage degradation [41, 42]. Similar to RA, the genetic susceptibility in CIA is strongly linked with the MHC class II genes, in particular the susceptibility is highest in mouse strains that bear MHC types I-A^q and I-A^r [43]. Analysis of the levels of IDO mRNA expression by quantitative q-PCR in the spleen, lymph nodes, and paws of CIA mice has shown that there is significant increase in the expression of IDO following arthritis onset in the lymph nodes, but not in the spleen or paws [44]. Further analysis of the expression of IDO in these lymph nodes revealed that it was mainly confined to dendritic cells [45]. To understand the role played by the kynurenine pathway in arthritis, the levels of tryptophan and its metabolites were measured through the course of CIA [46]. The tryptophan concentration in lymph nodes decreased progressively during the development and resolution of arthritis, while the concentrations of kynurenine increased, indicating increased IDO activity [45]. Measurement of the downstream kynurenine metabolites revealed increased presence of anthranilic acid and 3-hydroxyanthranilic acid only during the resolution stage of arthritis [45]. This raises the possibility that the downstream kynurenine metabolites may play a role in disease resolution, since it is known that 3-hydroxyanthranilic acid plays a role in inhibiting T cell responses [47].

To investigate the role of IDO in CIA in more detail, the progression of CIA was monitored in mice treated with the IDO inhibitor, 1-methyl tryptophan (1-MT). Administration of 1-MT following disease onset increased the severity of CIA, in terms of increased paw thickness and enhanced humoral and cellular responses [44, 48]. A more comprehensive investigation into the role of IDO in CIA has been performed using IDO knockout (*Indo*$^{-/-}$) mice [49]. One of the most important findings to emerge from this work was an earlier onset of arthritis in the *Indo*$^{-/-}$ mice compared to wild-type mice [44]. *Indo*$^{-/-}$ mice showed increased clinical severity, which was accompanied by a significant increase in bone erosion and cellular infiltration. Analysis of the cytokine production in *Indo*$^{-/-}$ mice revealed higher production of IL-17 and IFN-γ in the lymph nodes and FACS analysis showed increased frequency of Th1 and Th17 cells in the paws of the *Indo*$^{-/-}$ mice [44]. These findings highlight the importance of IDO expression in CIA and confirm the important role that IDO plays in regulating the pathogenic Th1/Th17 responses.

In comparison to CIA, studies in the K/BxN animal model, which is an antibody-mediated model of arthritis, have shown that administration of 1-MT results in the amelioration of arthritis due to reduced level of B cell activity [50]. Further analysis revealed that the effect of 1-MT was to inhibit differentiation of autoreactive B cells into autoantibody-secreting cells, without influencing the activation or survival of these cells [51]. These results suggest that the concept of IDO having a purely immunosuppressive function needs re-evaluation, since it may have differential effects depending upon which cells play a significant part in each model.

The Role of the Kynurenine Pathway in RA

Patients with RA display significantly decreased levels of circulating tryptophan and increased levels of kynurenine when compared to healthy individuals [52–54]. In addition, decreased levels of 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and xanthurenic acid are detected in RA patients, while levels of kynurenic acid are normal when compared to healthy individuals [52]. Increased kynurenine may be associated with disease progression or increased as a result of treatment. For example, increased tryptophan correlates with increased disease progression [54]. However, another study found that RA patients had lower levels of kynurenine in both the synovial fluid and peripheral blood [55]. The discrepancy between these studies may be explained by differences in the patient demographics, in particular, exposure to disease-modifying anti-rheumatic drugs (DMARDs). To investigate differences in the kynurenine pathway early in disease, we have used a cohort of DMARD-naïve RA patients. Levels of tryptophan and kynurenine in serum were determined using HPLC-UV and we found no significant difference in the expression of tryptophan; however, a significant reduction in circulating levels of kynurenine was observed when compared to healthy controls (Fig. 7.2). These results suggest that low levels of kynurenine may contribute to the pathogenicity of RA in the early stages of disease.

The Molecular Mechanisms Underpinning Differences in the Kynurenine Expression in RA

Further insight into the function of the kynurenine pathway in RA may give a better understanding of the pathogenesis of RA. Understanding the precise molecular mechanisms underpinning the differences in kynurenine expression described above

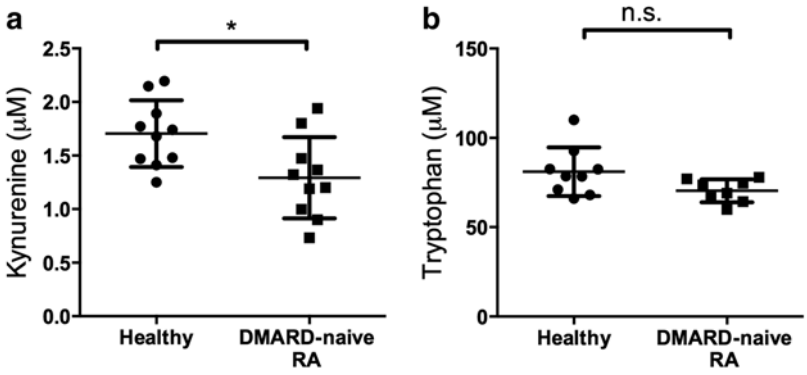

Fig. 7.2 Kynurenine and tryptophan levels in the human serum of DMARD-naïve RA patients. A reduction in the serum kynurenine levels was observed between DMARD-naïve RA patients and healthy individuals. n.s. = not significant, $*P > 0.05$ as measured using an Students *t* test

may ultimately lead to new therapeutic targets. Evidence has begun to emerge to suggest that synovial joint but not peripheral blood isolated T cells from RA patients are less responsive to IDO-mediated suppression by IDO^+ DCs [56]. Synovial T cells had increased expression of tryptophanyl-tRNA-synthetase (TTS), a cytoplasmic enzyme that mediates the association of tryptophan to tRNA that acts as a reservoir of tryptophan for protein synthesis. This led to enhanced intracellular storage of tryptophan and a resistance to IDO-mediated deprivation of tryptophan [56]. The resistance of T cells to IDO-mediated deprivation of tryptophan could be overcome by using blocking antibodies against IFN-γ or TNFα, which act to reduce the enhancement of TTS, suggesting that resistance to IDO activation represents a mechanism by which autoreactive T cells are maintained in the synovial joint.

Work from our own laboratory suggests that there is an intrinsic defect in the ability to induce expression of IDO in RA patients. We were the first to suggest this following investigation centered on examining dysfunctional Tregs from the peripheral blood of DMARD-naïve RA patients. Tregs act as potent inducers of the IDO pathway [19, 57], mediated in part through the ligation of CD80/86 by CTLA-4 on APCs [15]. We found that DMARD-naïve Tregs were unable to induce IDO expression in APCs following co-culture, which was associated with a reduction in both total and surface CTLA-4 expression [58]. The mechanism underpinning this reduction was through methylation of a single CpG within the *Ctla4* promoter. Methylation of this site prevented the binding of the transcription factor and activator of CTLA-4, nuclear factor of activated T cells (NFAT), which in turn led to a reduction in the transcriptional activity of *Ctla4* gene (Fig. 7.3). Overall, we identified that defective Treg function leads to an inability to induce IDO expression in APCs, leading to reduced local and systemic kynurenine levels and an inability to suppress effector T cells responses [58, 59].

The Kynurenine Pathway and Other Inflammatory Diseases

As discussed previously, inhibition of IDO promotes disease progression in animal models of RA. This finding is consistent with studies in other murine models of inflammatory diseases. For example, treatment with 1-MT, the competitive inhibitor of IDO, accelerated disease in EAE and type 1 diabetes (T1D) [60, 61]. Grohmann and colleagues have shown that nonobese diabetic (NOD) mice exhibit a defect in IDO-mediated T cell suppressive function mediated though excessive production of peroxynitrite by DCs, which could be reversed with CTLA-4 fusion protein-mediated IDO induction [62, 63]. Similar to findings in RA, this suggests that defects in the ability to induce IDO in DCs of the NOD mice underlie the susceptibility to T1D and signaling though the CTLA-4/CD80/86 pathway can restore IDO-mediated suppressive function. However, it is unknown whether the inability to induce IDO is a primary cause of disease or mediated through secondary upstream effects. Moreover, these findings have not been explored in the context of human T1D so the relevance to human disease is currently unknown. In parallel with synovial

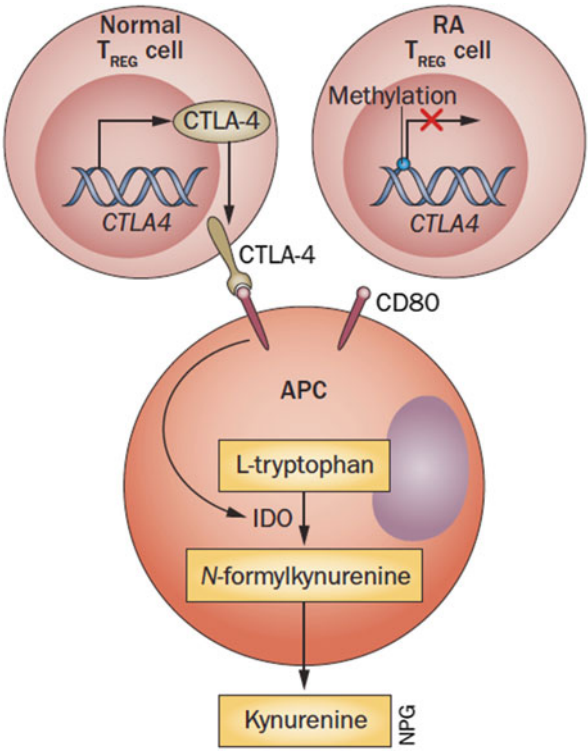

Fig. 7.3 Defective activation of the kynurenine pathway in DMARD-naïve patients with rheumatoid arthritis. The molecular mechanism underpinning reduced kynurenine expression in patients with RA is mediated through methylation of an NFAT binding site within the *Ctla4* promoter of Tregs. This results in a reduction in CTLA-4 protein expression. In co-culture with APCs, the reduction of CTLA-4 on RA Tregs fails to induce IDO expression, leading to reduced activation of the kynurenine pathway. Reprinted with permission from Macmillan Publishers Ltd: Nature reviews rheumatology [59]

T cells from RA patients that are resistant to IDO-mediated suppression, increased IDO resistance is also a feature of T1D. T cells from NOD mice display increased resistance to Treg-mediated suppression [64], which is also seen in the synovial T cells of patients with RA [65].

In EAE, amelioration of disease following stem cell transfer is mediated, in part, through the induction of IDO^+ $CD11c^+$ DCs, an effect that is abrogated following administration of the IDO inhibitor 1-MT [66]. Thus, inhibition of IDO increased disease progression and prevented disease resolution. Additionally, in both human disease and in animal models of cystic fibrosis, deficiencies in the ability to activate IDO contribute to a failure to eradicate pathogenic fungi from the lungs. Defective IDO expression was linked to an imbalance in Treg/Th17 responses, which could be corrected with the addition of tryptophan metabolites [67]. Therefore, although IDO does not contribute to the pathophysiology of cystic fibrosis, it plays a significant role in the ability to prevent the infection associated with the disease.

Targeting the Kynurenine Pathway Therapeutically for Inflammatory Disease

Evidence showing that the kynurenine pathway exerts immunosuppressive effects on the immune system has led to the idea that therapeutic manipulation of this pathway might be beneficial for treating inflammatory diseases. In order to determine whether kynurenine itself could be useful as a potential therapy, the progression of CIA was evaluated in CIA mice following treatment with L-kynurenine or vehicle alone following onset of arthritis. L-kynurenine significantly reduced the clinical and histological scores of disease [44], suggesting that kynurenine administration may have the potential to modify the progression of RA. However, it still needs to be established whether the therapeutic effects of L-kynurenine administration are mediated through kynurenine or one of its metabolites. Tranilast, a clinically approved 3-hydroxyanthranillic acid derivative used to treat allergy, has also been shown to reduce clinical and histological disease in CIA [68]. Therefore, suggesting that at least some of the therapeutic effects of L-kynurenine administration may be mediated through 3-hydroxyanthranillic acid. In addition to strategies designed to increase the systemic circulating levels of kynurenines, it has also been suggested that strategies designed to increase kynurenine levels at sites of inflammation may be beneficial for inflammatory diseases such as RA [69]. Xue et al. [70] have shown that in vitro administration of the DNA methyltransferase inhibitor zebularine given in combination with IFN-γ to human THP-1 cells results in demethylation of the IDO1 promoter and an increase in the activation of the IDO pathway.

Conclusion

Overall, it is clear that the kynurenine pathway plays a role in the pathogenesis of many inflammatory diseases. The contribution of the kynurenine pathway to the prevention of inflammation in murine models of disease is becoming clearer. Moreover, it has been demonstrated that administration of kynurenine and its downstream metabolites in murine models may have therapeutic potential for treating inflammatory conditions. However, more work is needed to ascertain whether therapeutic manipulation of this pathway in humans can provide real clinical benefits. However, the consequences of upregulating IDO activity systematically are likely to be detrimental; therefore, strategies for upregulating the pathway in a tissue specific manner require further elaboration. In this regard, the studies of Xue et al. [70] are potentially informative as they demonstrate a synergistic effect between zebularine and IFN-γ, a cytokine expressed specifically at sites of inflammation. Given the burden of autoimmune disease on society, understanding and exploiting the IDO pathway remains an important objective for the future.

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Chapter 8
Role of Kynurenine Pathway in Allergy

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Abstract Tryptophan catabolism, including kynurenine pathway, is known to be involved in immunoregulation and its metabolites play a role in immune modulation. In the context of allergic diseases, it has been proposed that tryptophan degradation pathway, rather than tryptophan deprivation itself, plays an important role in tolerance induction during allergen-specific immunotherapy, which is, by far, the only causal treatment of allergic diseases. In addition, it has recently been demonstrated that kynurenine and, perhaps, its metabolites can act through the aryl hydrocarbon receptor, a unique cellular chemical sensor, and regulate immune functions. The present review intends to highlight the recent development on the involvement of kynurenine pathway in the regulation of allergic diseases.

Keywords Kynurenine • Allergy • Allergic diseases • Mast cells • Allergen-specific immunotherapy • Immune tolerance • Aryl hydrocarbon receptor

List of Abbreviations

1-MT	1-Methyl-L-tryptophan
AA	Anthranilic acid
AD	Atopic dermatitis
AhR	Aryl hydrocarbon receptor
AR	Allergic rhinitis
BMMCs	Bone marrow-derived mast cells
CRSsNP	Chronic rhinosinusitis without nasal polyps

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CRSwNP	Chronic rhinosinusitis with nasal polyps
DCs	Dendritic cells
FcεRI	High-affinity receptor for IgE
ICS	Inhaled corticosteroids
IDO	Indoleamine 2,3-dioxygenase
KA	Kynurenic acid
KYN	Kynurenine
QA	Quinolinic acid
SIT	Allergen-specific immunotherapy
TDO	Tryptophan 2,3-dioxygenase
TPH-1	Tryptophan hydroxylase-1
TRP	Tryptophan
XA	Xanthurenic acid

Introduction

Tryptophan (TRP), an essential amino acid, plays an important role in the protein synthesis and serves as a precursor of many biologically active substances, such as kynurenine (KYN). The degradation of TRP takes place in the liver, kidney, brain, and peripheral tissues via three biochemical pathways: KYN pathway, tryptamine pathway, and serotonin pathway (Fig. 8.1). The KYN pathway is the major route for TRP metabolism [1]. Of the dietary TRP that is not used in protein synthesis, approximately 99 % is metabolized by the KYN pathway, where TRP is catabolized by rate-limiting enzymes, TRP 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO), to N-formyl-KYN, which is then converted to KYN [2]. Under normal physiological conditions, the level of KYN is low but is upregulated in response to infection and Th1-mediated inflammation [3] through IFN-γ-induced expression of IDO. Increase in KYN mediated by IDO/TDO activation is also found in various disease states, such as autoimmune disorders and malignant diseases [4].

While the role of TRP and its metabolites in immune regulation has been extensively investigated, their role in allergy has been less well defined. In recent years, a clear association has been made between TRP metabolism and immune or inflammatory responses in a vast array of disease states. These studies have focused mainly on the KYN pathway of TRP degradation occurring in the immune system, rather than in the serotonin pathway, and the importance of this pathway in allergic inflammation has recently been suggested; but, despite recent increasing interest in TRP metabolism in the field of allergy, particularly as it pertains to the tolerogenic properties of IDO or KYN metabolites in controlling allergic reactions, the biological significance of immune-related TRP breakdown remains still unclear. This review considers the current understanding of the role of the TRP metabolites, particularly those in the KYN pathway, in orchestrating and regulating the expression of allergic diseases.

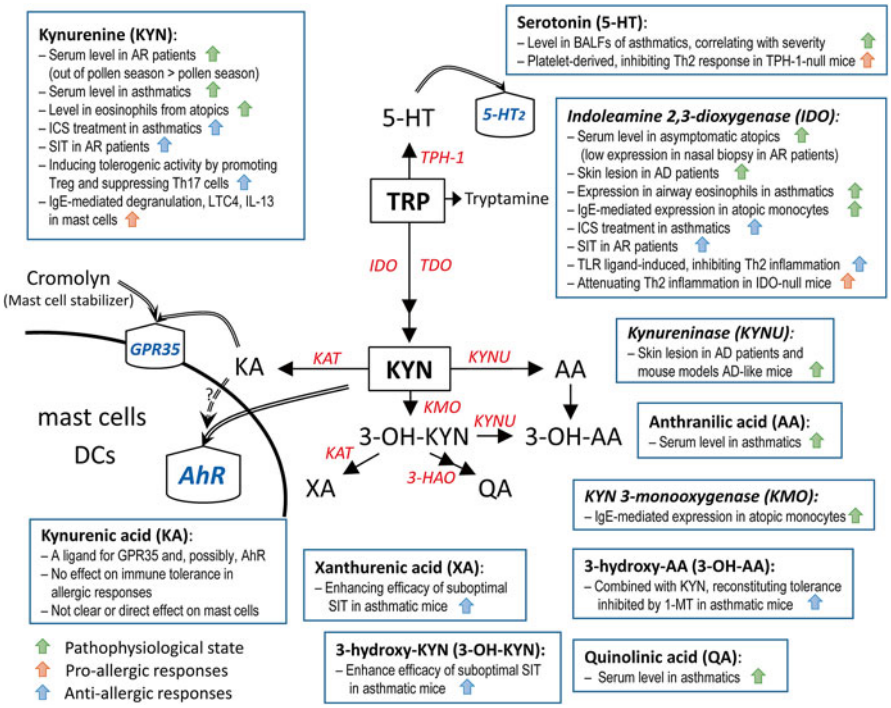

Fig. 8.1 A schematic diagram depicting the tryptophan metabolic pathway and its metabolites that are suggested to be involved in the regulation of allergic diseases. Pathophysiological states of related enzymes and metabolites in allergic diseases and their functional impacts are summarized. *Abbreviations*: *AR* allergic rhinitis; *AD* atopic dermatitis; *ICS* inhaled corticosteroids; *SIT* allergen-specific immunotherapy; *DCs* dendritic cells; *AhR* aryl hydrocarbon receptor; *1-MT* 1-methyl-L-tryptophan; *TPH-1* tryptophan hydroxylase-1; *5-HT* 5-hydroxytryptamine

Metabolites and Enzymes Along the KYN Pathway in Allergic Diseases

There are limited numbers of studies examining the level of KYN and its metabolites or activity of IDO in patients with allergic diseases. In seasonal allergic rhinitis (AR), higher plasma KYN/TRP ratio in asymptomatic atopic individuals was found during seasonal allergen exposure compared to those in symptomatic patients with AR and normal subjects [5]. In addition, serum KYN concentration was significantly higher in patients with AR than in normal subjects and was also higher out of the pollen season than during the season [6]. These results suggest that enhanced IDO activity may be involved in the maintenance of clinical unresponsiveness. Furthermore, it has been reported that patients with AR seem to have constitutively elevated serum TRP concentrations [7], suggesting the activity of

IDO is lower in symptomatic patients than in nonatopics or asymptomatic atopics. As a corollary, studies have shown that the expression of IDO was not increased in the nasal mucosa of patients with AR [8]. Therefore, the potential relationship between modified tryptophan metabolism and clinical responsiveness in AR need to be confirmed in further studies. Nonetheless, IDO seems to associate with chronic rhinosinusitis (CRS) [9].

CRS is known to be a refractory, versatile, and multifactorial immunological disease of nose and paranasal sinuses. CRS is characterized by increased local eosinophilia and a Th2 polarization with high levels of IL-5 and IgE or a Th1 polarization with high levels of IFN-γ and TGF-β, depending on patients with (CRSwNP) or without nasal polyps (CRSsNP), respectively. Luukkainen et al. found that the maxillary sinus mucosa from patients with CRSwNP, but not CRSsNP, showed a higher level of IDO expression in leukocytes but not in the epithelium when compared with normal subjects [10]. The findings of IDO expression in sinonasal biopsies were independent of AR, aspirin intolerance, asthma, smoking, use of intranasal or oral corticosteroids or antihistamines, previous operations, recurrence of polyps, sex, and age [10]. Thus, in the upper airways, IDO expression seems to associate with CRSwNP, but not with AR.

Regarding asthma, only limited studies have been performed in humans. It has been suggested that KYN/TRP ratio is decreased in sputum of asthmatics [11] and in cultured dendritic cells (DCs) from dust mite-sensitive patients with asthma compared to normal subjects [12]. It has also recently been reported in a prospective study that KYN concentration in exhaled breath condensates in stable mild asthmatics was lower at baseline level than that in normal subjects [13]. On the other hand, systemic levels of TRP and its metabolites, KYN, anthranilic acid (AA), and quinolinic acid (QA) were markedly higher in patients with allergic asthma and were associated with eosinophilic inflammation and symptom scores during experimental rhinovirus infection [13]. Thus, IDO expression appears to be associated with asthma.

But, in murine models of asthma, there have been inconsistent results with regard to the KYN's function in either promoting or suppressing asthmatic responses. One earlier study showed that KYN elevation in the lung induced by a TLR9 ligand, synthetic immunostimulatory sequence-containing oligodeoxynucleotide (CpG-ODN) was able to inhibit Th2-driven pulmonary inflammation, which could be reversed by the addition of 1-methyl-tryptophan (1-MT), a pharmacological inhibitor of IDO [14], suggesting an immunosuppressive or tolerogenic effect of the KYN pathway. In contrast, utilizing IDO-deficient mouse models, it has been reported that IDO deficiency did not impair the induction of immune tolerance in Th2-driven pulmonary inflammation and that IDO-deficient mice displayed blunted Th2-driven airway inflammation and airway hyperresponsiveness [15], indicating a pro-inflammatory effect of IDO. The reasons for these discrepant results are currently unclear. Different models and stimuli used could be the reasons for observing differential outcomes. It has been cautioned that genetically modified mice might have developmental defects associated with the IDO deficiency from birth. Further investigations into these issues are needed to clarify these issues. In the case of atopic

dermatitis (AD), the expression of IDO and kynureninase has been shown to be upregulated in the skin lesions as compared to the uninvolved skin of patients with AD [16]; also, their upregulated expression was noted in human-cultured epidermal keratinocytes and in the skin lesion of NC/Nga mice, which are considered to be an AD-like animal model [16].

Kynurenine Pathway in Monocytes, Eosinophils, and Mast Cells

Functional IDO has been detected in multiple cell types involved in allergic inflammation, including DCs [17], monocytes/macrophages [18], endothelial cells [19], fibroblasts [20], epithelial cells [21], and granulocytes [22]; but, IDO expression in mast cells or basophils has not been reported. In allergic individuals, the first contact with allergen is suggested to lead to IL-4- and IL-13-dependent production of allergen-specific IgE, with subsequent binding of these antibodies to the high-affinity receptor for IgE (FcεRI) on the surface of mast cells and basophils. Allergen reexposure results in cross-linkage of membrane-bound IgE and subsequent mediator release that induces typical immediate-type hypersensitivity reactions. FcεRI is constitutively expressed on mast cells and basophils but can also be detected on additional immune regulatory cell types, including antigen-presenting cells, of atopic individuals. In human monocytes, it has been reported that the enzymes along the KYN pathway, including IDO, were highly expressed in FcεRI-activated monocytes derived from atopics than those from nonatopics, and its expression is increased after cross-linkage of the receptors by IgE and anti-IgE [18]. These enhanced expression of IDO in monocytes from atopics lead to production of higher amount of KYN than those from nonatopics.

Eosinophil accumulation is a prominent feature of allergic inflammatory responses, such as those occurring in the lung of the allergic asthmatics [23]. Human eosinophils derived from atopic donors constitutively express IDO [22], and when treated with IL-5 in the presence of IFN-γ, they produce considerable amount of KYN. CD28 cross-linking also results in measurable KYN in culture supernatants and is inhibited by a neutralizing anti-IFN-γ antibody. Moreover, extensive infiltration of IDO-positive cells has been observed in the tissues from patients with asthma, where eosinophils are the prominent cell type expressing IDO.

Mast cells are known to be a critical cell type in the regulation of allergic responses and can be activated by a multitude of stimuli resulting in the release of inflammatory mediators and cytokines, contributing to various pathophysiological events in acute and chronic inflammation [24]. Considering the strategic location of mast cells at the site of tissue mucosa where exposure of TRP and its metabolites may occur, it is likely that KYN pathway metabolites, derived endogenously or from other cell types in the inflammatory microenvironment, may exert there in controlling the mast cell responses. Furthermore, it is still remain unsolved as to

how KYN metabolites act as an immune-regulating mediator. One plausible mechanism may involve KYN serving as a ligand for aryl hydrocarbon receptor (AhR) [25]. AhR is constitutively expressed in mouse and human mast cells [26–28]. The initial report showed that KYN and kynurenic acid (KA) could affect degranulation and intracellular calcium signaling in murine mast cell lines, although the dependence of AhR and the underlying mechanisms were not investigated. We have recently demonstrated that KYN enhanced IgE-mediated mast cell responses, including degranulation, leukotriene C4 (LTC4) release, and IL-13 production in mouse bone marrow-derived cultured mast cells (BMMCs) through the activation of PLCγ, Akt, and MAPK p38 and the increase of intracellular calcium [29]. KYN also enhanced cutaneous anaphylaxis in vivo in a mouse model of passive cutaneous anaphylaxis (PCA). In addition, KYN had similar enhancing effects on human peripheral blood-derived cultured mat cells. It was noted that the effective concentration of KYN showing the enhancing effect on mast cells was around 50 μM, which could be a physiologically relevant concentration in the microenvironment at the site where inflammation occurs [25].

Metabolites in KYN Pathway Act via Aryl Hydrocarbon Receptor (AhR)

KYN's effects on mast cells are dependent on AhR [29]. In our recent studies, the enhancing effects of KYN on IgE-mediated mast cell responses were not observed in AhR-deficient BMMCs and could be inhibited by an AhR antagonist. KYN's enhancing effects on human-cultured mast cells could be inhibited by an AhR antagonist as well. On the other hand, KA has been proposed as a potential endogenous AhR ligand in mouse and human hepatocytes [30], although its binding activity for human AhR is 100-fold higher than that for mouse AhR. However, both KA and QA did not show any additive and synergistic effects directly in mast cells when treated with KYN [29]. Thus, KA seems not to be associated with allergic responses in mast cells. Interestingly, in the study by Maaetoft-Udsen et al., KA was suggested to be an AhR ligand and affected mast cell responses [26], but its stimulatory effect appeared to be AhR independent or through a secondary activating mechanism. Indeed, KA has been reported to be a ligand for GPR35 [31], a G-protein-coupled receptor expressed in a variety of tissues, including mast cells, which was significantly upregulated when mast cells were exposed to IgE antibodies [32]. Further, cromolyn disodium and the second-generation nedocromil sodium, known as mast cell stabilizers, have been recently reported as ligands for GPR35 [33]. But, the underlying mechanism through which mast cell degranulation is inhibited remains unclear. This suggests the possible existence of a sequential event originating from the AhR-KYN axis and its subsequent activation of the GPR35-KA axis in regulating mast cell functions. Further detailed studies are clearly needed.

Role of KYN Pathway on Allergen-Specific Immunotherapy and Induction of Immune Tolerance in Allergic Diseases

The concept of allergen-specific immunotherapy (SIT) was first introduced 100 years ago [34]. SIT is the only available causative treatment of allergic diseases that induces a number of allergen-specific immunological changes. IDO seems to play a role in the induction of SIT. It has been reported that tolerance induction against allergens is partially mediated by activation of the KYN pathway during allergen-specific sublingual immunotherapy [35, 36]. TRP administered during SIT does not inhibit the effect of SIT, and pharmacological inhibition of IDO with 1-MT does not impair allergic inflammation during the challenge [37], suggesting the likely involvement of the KYN pathway metabolites rather than TRP deprivation itself in the induction of tolerance.

There is increasing evidence that IDO could be an important modulator of immune tolerance in a variety of immune responses. At the cellular level, IDO expression in DCs is important in inducing T-cell tolerance, as well as through direct effects on T cells or through effects of IDO on the DCs themselves. The mechanism by which IDO expression in DCs induces T-cell tolerance is hypothesized to be due to either the induction of T-cell anergy [38], the induction of T-cell apoptosis [39], the deviation of the immune response (Th1/Th2) [3], or the induction of regulatory T cells/suppression of Th17 cells [40].

A recent report, in the setting of allergen-specific immunotherapy, suggested that KYN pathway metabolites were able to downregulate allergic responses by potentiating tolerance induction in a mouse model of asthma [37]. KYN, 3-hydroxy-KYN and xanthurenic acid (XA), but not KA, QA and 3-hydroxy-AA, enhanced the efficacy of suboptimal immunotherapy with allergen. Thus, certain KYN pathway metabolites particularly KYN might potentiate immune tolerance during allergic responses, with anti-allergic properties. Therefore, it is likely that KYN might contribute to potentiate both pro-allergic (enhancing mast cell functions) and anti-allergic responses (promoting immune tolerance) depending on the timing of exposure to KYN and the types of immune cells during the progression of allergic diseases.

Serotonin Pathway in Allergy

Only 1 % of ingested TRP is converted into serotonin as mentioned above [2], whereas the majority of TRP is subject to degradation via the KYN pathway. However, it has been suggested that the KYN- and serotonin-metabolic pathway compete for their mutual precursor, TRP [41]. It has recently been reported that serotonin pathway in platelets also contribute to allergic inflammation [42]. It has long been recognized that serotonin may play an important role in the pathogenesis of allergic asthma. Elevated plasma serotonin level was found in symptomatic patients with asthma, correlating

with clinical severity and pulmonary function [43]. Furthermore, pharmacologic blockade of its receptors was shown to attenuate the development of allergic airway inflammation and remodeling in mice [44]. TRP hydroxylase (THP)-1 is a critical enzyme for the biosynthesis of serotonin outside of the central nervous system. Dürk et al. have reported that platelets, rather than mast cells, were the main source of serotonin released during an allergic inflammation by utilizing THP-1-deficient and mast cell-deficient mice and pharmacological approaches [42]. These results are consistent with several earlier clinical observations reporting platelet activation accompanying allergic asthmatic responses [45, 46].

Conclusion

Recent studies have made a significant progress in our understanding of the immunoregulatory properties of TRP metabolites, particularly in the context of allergic diseases. Recent advancement in elucidating the AhR-KYN and GPR35-KA axis in immune regulation provides a novel and promising regulatory mechanism supporting the importance of KYN's metabolic pathway in the pathophysiology of allergy and other diseases as well. However, the knowledge accumulated thus far has revealed a more diverse and complex network of regulation and function than we had previously recognized. Future research might benefit greatly from increasing attention on the metabolic regulations of these two receptor-ligand axis and their functional consequences, as well as of the functional link between KYN and serotonin pathways.

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Conflicts of Interest The authors declare that they have no conflicts of interest.

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Chapter 9
Role of Kynurenine Pathway in HIV/AIDS

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Abstract Two of the most fundamental requirements for the host are the ability to survive periods of starvation and the capacity to mount an effective response against pathogenic invaders while tolerating mucosa-associated commensal microbes. The kynurenine (Kyn) pathway (KP) is at the crossroad of these two fundamental requirements and therefore plays an important role in HIV infection. The combination of HIV infection and tryptophan catalytic bacteria causes CD4 Th17/Th22 dysfunction in the gut mucosa leading to microbial translocation that creates a systemic KP activation cycle. This self-sustaining feedback loop has deleterious effects on disease progression and on neurocognitive impairment in HIV-infected patients while fuelling a systemic state of immune activation.

Keywords IDO • HIV • Microbiota • Kynurenine • Antiretroviral therapy • Regulatory T cells • Th17 cells

List of Abbreviations

AhR	Aryl hydrocarbon receptor
AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
CSF	Cerebrospinal fluid
CRP	C-reactive protein

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HAND	HIV-associated neurocognitive disorder
HIV	Human immunodeficiency virus
IAld	Indole-3-aldehyde
IDO	Indoleamine 2,3-dioxygenase
I-FABP	Intestinal fatty acid-binding protein
IFN	Interferon
IL	Interleukin
KP	Kynurenine pathway
Kyn	Kynurenine
NK	Natural killer cells
sCD14	Soluble CD14
Treg	Regulatory T cells
Trp	Tryptophan

Introduction

The hallmark of HIV infection is the progressive loss of CD4 T cells in the context of chronic immune activation [1]. The persistence of immune activation even after several years of antiretroviral therapy (ART) is associated with an increased risk of AIDS- and non-AIDS-defining illnesses including cardiovascular, liver and kidney diseases, cancers and alteration of neurocognition. By hijacking the immune system, HIV is capable of drastically altering the gastrointestinal environment leading to significant changes to the gut microbiota and mucosal permeability resulting in microbial translocation from the gut into the peripheral blood. Such changes made locally in the gut have far-reaching consequences as they contribute to create a state of systemic immune activation [2]. We and others have identified several factors associated with profound T-cell dysfunction linked to the markers of immune activation [3–5]. Among these factors, the enhanced expression of inhibitory molecules like indoleamine 2,3-dioxygenase (IDO) plays the most deleterious role on immune dysfunction observed in HIV infection [4–6]. Recently, the IDO-dependent tryptophan/kynurenine pathway (KP) has been recognized as a key factor contributing to HIV immune dysfunction leading to a distinctive damage to gut mucosa and brain tissue integrity.

Kynurenine Pathway: A Crossroad Between Nutrition, Microbes and Host

IDO-1 is an immunomodulatory enzyme present in dendritic cells (DCs) and macrophages, which breaks down tryptophan (Trp) into a series of catabolites, including kynurenine (Kyn). IDO is induced by IFN-γ in response to inflammatory signals [7]

and its catabolites regulate immune homeostasis by acting as aryl hydrocarbon receptor (AhR) ligands allowing the generation of regulatory T cells (Treg) [8–10]. AhR, a ligand-activated transcription factor, was also found to feedback with IDO and Kyn to maintain a state of immune tolerance between commensal microbiota and the host [11, 12].

During the last decade, a wealth of information indicates that enhanced immunosuppressive Kyn production by IDO plays a harmful role in cancers, different neurological conditions and chronic viral infections including HIV [3, 6, 13, 14]. It is well established that monocyte-derived DCs specifically expressing IDO promote Treg expansion [15, 16] and contribute to the control of a hyper-inflammatory state. Increased plasma Kyn levels and Kyn/Trp ratios represent a negative predictor of clinical outcome and have been found in patients with different inflammatory conditions and in sepsis [17]. The KP is a double-edged sword as it is both initially helpful and harmful long term. During acute infection the KP is part of an endotoxin tolerance defence pathway to prevent an exaggerated immune response; however, during chronic infection the KP participates in the induction of an immune tolerance/exhaustion status that prevents tissue damage but surrenders the immune system to the microbial infection [12].

KP During HIV Disease Progression and Impact of ART

The link with increased KP and HIV infection was first established by Huengsberg et al. reporting on the elevation of Kyn/Trp ratio when compared to control individuals in a group of HIV-infected patients [18]. A strong association between the Kyn/Trp ratio, CD4 T-cell counts and the stage of the disease was observed. The median Kyn/Trp ratio for asymptomatic and AIDS patients was two- and threefold higher, respectively, than controls. The authors concluded that elevated Kyn/Trp ratio led to accumulation of quinolinic acid that in turn contributed to AIDS dementia. Following this, Zangerle et al. assessed the contribution of ART on improving the KP. After 6 months of therapy, the Kyn/Trp ratio decreased by more than twofold [14]. Interestingly, Kyn/Trp ratio positively correlated with neopterin, a macrophage-derived pro-inflammation marker, plasma viral load and CD4 T-cell counts.

Byakwaga et al. corroborated these findings in their own Ugandan study showing a decrease in Kyn/Trp over time spent on ART [19]. Our group also reported that long-term successfully ART-treated patients had similar Kyn/Trp ratio to their aged-matched controls [3]. Collectively, these study findings indicate that Kyn/Trp ratios are consistently linked to CD4 T-cell counts, level of T-cell activation and viral load making it a tempting new marker for HIV disease progression.

KP as a New Independent Marker for HIV Disease Progression

Predictor of Mortality

In the Byakwaga et al. study, patients were advanced in their infection with low CD4 T-cell counts, high viral loads and elevated Kyn/Trp ratios before initiating ART. After adjustment for baseline CD4 T-cell counts, viral load, body mass index and age, higher Kyn/Trp ratio independently predicted a lower CD4 T-cell recovery and increased mortality.

The same group of investigators also reported on the predictive value of Kyn/Trp ratio on mortality in US patients receiving ART [20]. They showed that Kyn/Trp ratio was an independent predictor for mortality along with other soluble factors of inflammation such as IL-6, C-reactive protein (CRP) and D-dimer [20].

Predictors of Non-AIDS Events Related to Inflammation

Despite the major influence of ART on patient survival, complications remain more frequent in HIV-infected patients than age-matched uninfected adults. Markers of immune activation and inflammation have been previously identified as predictors for non-AIDS events independently of other cardiovascular risk factors [21]. The predictive power of KP on non-AIDS events was recently demonstrated by Tenorio et al. who conducted a case-control study of HIV-infected patients successfully treated with ART [22]. After controlling for confounders, Kyn/Trp ratio along with IL-6 and D-dimers were the only markers associated with non-AIDS events when measured 1 year after ART initiation.

Serrano-Villar et al. identified the contribution of CD4/CD8 ratio on morbidity and mortality in a cohort of ART-treated patients and were able to show that this association was driven by the heightened CD8 T-cell count, even for patients having a satisfactory CD4 T-cell recovery exceeding 500 cells/mm^3 [23]. As expected from previous studies, the CD4/CD8 ratio was inversely associated with levels of markers of innate immune activation like IL-6, CRP, sCD14 and Kyn/Trp ratio [24–26]. Interestingly, in the patients with CD4 T cell counts more than 500 cells/mm^3, such associations with CD4/CD8 ratio where lost for all the innate markers with the exception of Kyn/Trp ratio. Furthermore, Kyn/Trp ratio had the strongest association for predicting non-AIDS events when compared to IL-6, sCD14 and I-FABP, a marker of gut epithelial integrity. Kyn/Trp ratio was the only innate immune marker, which remained associated with gut integrity markers in patients with CD4 T cell counts more than 500 cells/mm^3 [23].

Collectively, these studies represent a paradigm shift, as lymphoid markers of immune activation are no more predictors for both mortality and morbidity, while myeloid cell inflammation markers have become predictors for patients receiving long-term ART.

Players Involved in the KP Cycle During HIV Infection

HIV Proteins Nef and Tat and IFN-γ

Because quinolinic acid acts as a neurotoxic product of the KP and has been shown to be elevated in the cerebrospinal fluid (CSF) of patients with AIDS dementia, Smith et al. first reported on the direct effects of HIV viral proteins Tat and Nef on the induction of human macrophages. They showed that both Tat and Nef were able to induce IDO and quinolinic acid production by macrophages. However, it was unclear whether IDO was induced directly by Tat protein or indirectly following IFN-γ production [27]. To untangle the direct contribution of Tat on IDO induction, Planes et al. used IFN-γ and IDO inhibitors as well as in vitro co-culture assays to demonstrate the direct effect of Tat on IDO induction [16]. These results indicated that Tat acts as a viral pathogenic factor by inducing IDO in DCs, independently of IFN-γ activation during infection. In treated infections, where very low levels of viral replication occur, low levels of Tat can still contribute to IDO induction in macrophages and DCs [28]. Such finding indicates that HIV persistence as a viral reservoir in treated infection can still contribute to a KP-driven immune activation [29].

Gut Mucosal Shift of the Th17/Th22/Treg Balance by IDO

In 2010, Favre et al. linked the KP with the decrease in a functional subset of CD4 T cells secreting IL-17 (Th17) and the increase of Tregs in both blood and in rectal mucosal tissues in HIV-infected patients [6]. By using in vitro activation assays in the presence of varying concentrations of Trp catabolites, the investigators were able to modulate the balance of Th17 and Tregs. The loss of mucosal protector Th17 population was associated with induction of IDO by myeloid DCs and with increased plasma concentration of microbial products. They were also able to show that this deleterious effect on Th17/Treg imbalance was mediated by Kyn, an IDO downstream Trp catabolite. Collectively, these data support a model by which persistent activation of IDO diminished the host capacity to maintain protective Th17 cells and favour the generation of immunosuppressive Tregs [30]. The consequence of

this imbalance is a progressive loss of the mucosal epithelial barrier leading to an increased microbial translocation [31]. The systemic circulation of microbial products in turn fuels myeloid cell activation creating a vicious cycle where KP induction further induces immunosuppression and a chronic state of inflammation [32].

The KP Induction and Composition of Gut Microbiota

Vujkovic-Cvijin et al. showed that HIV-infected viremic patients had microbiota communities distinctly enriched in *Proteobacteria*, which includes known pathological microbes [33]. In addition, viremic patients displayed a decrease in *Bacteroides* and *Alistipes*, also depleted in inflammatory bowel disease [34]. Importantly, the particular changes on microbiota composition from these patients were found to be linked to a decrease in Th17 cells in gut biopsies as well as an increase in IDO enzymatic activity (Kyn/Trp) [33]. Most importantly, bacteria enriched in viremic patients possess enzymatic homologues of IDO, able to locally produce Kyn from Trp. Such bacterial communities are able to outcompete their counterparts local Kyn production through IDO and, once established, capable of producing Kyn, which further fuels their growth. A partial gut mucosal repair and enriched lactobacilli in the gut microbial composition were reported by Perez-Santiago et al. following ART initiation [35].

Zelante et al. further explored microbial interplay with the KP using *Lactobacilli* in a mouse model [36]. Specifically, *L. reuteri* are able to catabolize Trp into indole-3-aldehyde (IAld) and are in turn able to stimulate natural killer (NK) cells via AhR to produce IL-22 which controls the gut microbiota, ensuring a diverse ecosystem. However, in this model when IDO activity is elevated due to the excessive migration of IDO-expressing DCs to gut mucosa, Trp is preferentially catabolized into immunosuppressive Kyn. Higher levels of Kyn and the subsequent expansion of Tregs create a tolerogenic/immunosuppressive environment where normal commensals like *Candida albicans* can become numerous and pathogenic leading to candidiasis. Interestingly, the same study showed that oral administration of IAld to mice experiencing mucosal candidiasis were able to restore IL-22 production by NK cells and controlled the candidiasis. This distinctive use of Trp by *Lactobacilli* may in part account for its association with improved clinical outcomes by limiting Kyn production and may represent an important strategy for treatment. Accumulating evidence in animal and human research also strengthens the concept of the importance of the KP in the microbiota-gut-brain axis [37].

KP and Neurological Impairment in HIV Infection

HIV directly infects myeloid-derived cells in the central nervous system resulting in a cognitive impairment and in absence of ART can lead to dementia [38]. These two conditions are designated by the term "HIV-associated neurocognitive disorder" (HAND). Ageing patients receiving ART have higher burdens of neurological disorders compared to HIV-uninfected controls [39]. Research has shown that the KP has been implicated in the pathogenesis of HAND. Lower Trp and elevated Kyn levels have both been associated with a reduction of serotonin and serotonin transporter expression, as well as an accumulation of Trp metabolites. In fact, Kyn and quinolinic acid, found in the CSF of HIV-infected patients, correlate with the severity of HAND [40–42]. Local synthesis of Kyn can occur in the brain as blood-brain barrier endothelial cells can generate Kyn after Tat or IFN-γ activation [43, 44]. Furthermore, high circulating levels of IDO in patients are associated with severity of depression and can be reversed with ART [45]. The mechanisms by which the KP directly participates in HAND have been mainly based on animal models and have been recently reviewed [38].

Conclusion

The KP plays an important role in regulating composition of microbiota, local gut mucosal immune tolerance as well as systemic control of immune activation in the host (Fig. 9.1). The treatments using IDO inhibitors are under development as anticancer therapies and some are assessed in clinical trials [46–50]. The key role of the KP in HIV infection on immune dysfunction and neurocognition paves the way for strategies to modulate this immune-metabolic pathway likely in combination with other immune checkpoint inhibitors. Collaborative efforts between oncology, infectious diseases and immunology will soon establish the clinical benefit of modulation of the KP [51].

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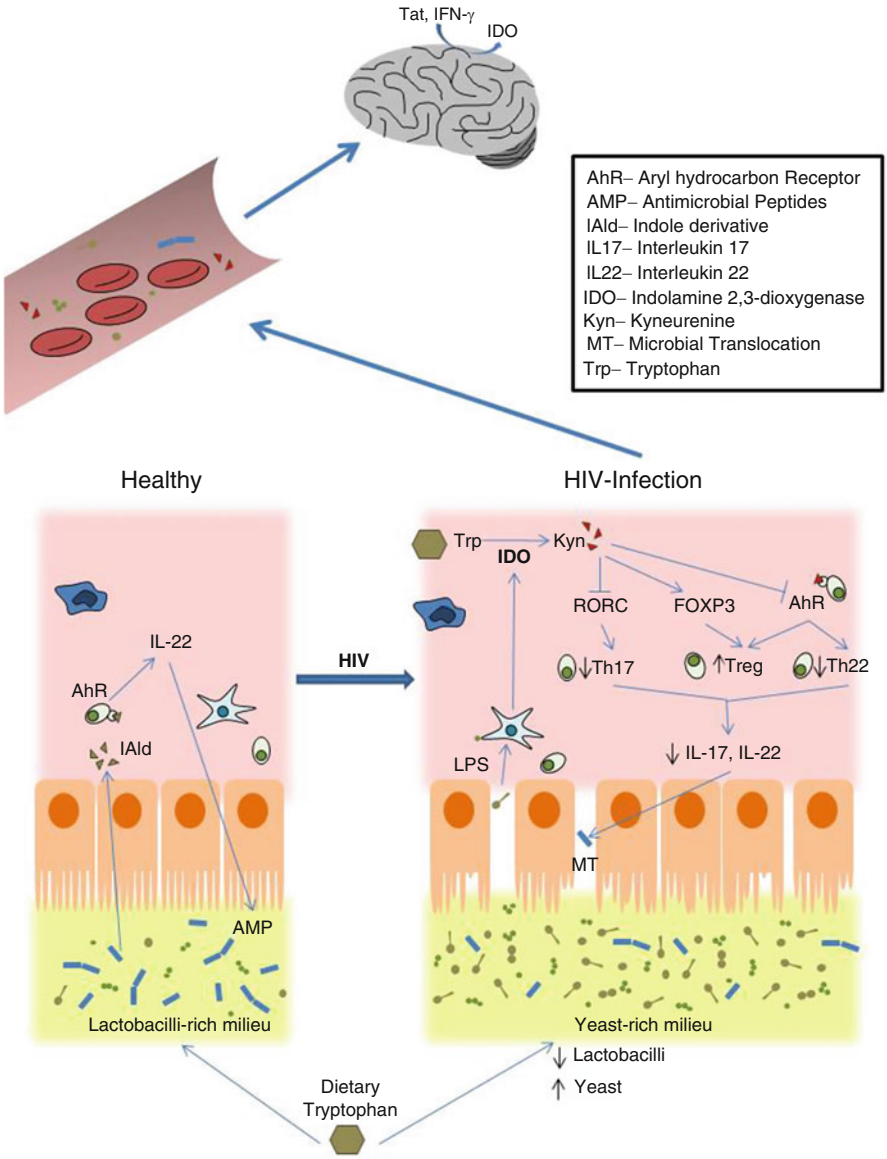

Fig. 9.1 In a healthy state, the microbiota interacts with the immune system to create immune balance and microbial control. After HIV infection, the microbiota shifts in composition which creates microbial translocation and IDO activation. IDO leads to an imbalance of immune cells as microbial and KP products enter the blood stream and eventually the brain causing serious downstream events

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Chapter 10
Role of Kynurenine Pathway in Cardiovascular Diseases

Harald Mangge, Eva Reininghaus, and Dietmar Fuchs

Abstract Atherosclerosis (AS) is a major pathologic sequel of obesity. It causes cardiovascular disease (CVD), the most common contributor to death in the Western world. A systemic chronic low-grade immune-mediated inflammation (scLGI) is substantially involved in AS and CVD. Pro-inflammatory cytokines released during cellular immunity play a major role, with the Th1-type cytokine interferon-gamma (IFN-γ) being a key mediator. Among other effects, IFN-γ activates the enzyme indoleamine 2,3-dioxygenase (IDO) in monocyte-derived macrophages, dendritic, and other cells, which ultimately decreases serum levels of the essential amino acid tryptophan (TRP). Hence, patients with CVD show increased serum kynurenine to tryptophan concentrations (KYN/TRP), a result of an increased TRP degradation. Importantly, a strong increased KYN/TRP is associated with a higher likelihood of fatal cardiovascular outcomes. Moreover, an increased production of the pro-inflammatory adipokine leptin, in combination with IFN-γ and interleukin-6 (IL-6), represents a central link between obesity, AS, and CVD. Leptin has also been shown to be involved in a Th1-weighted T cell polarization.

Tryptophan is not only an important source for protein production but also counts for the generation of the neurotransmitter 5-hydroxytryptamine (serotonin) by the tetrahydrobiopterin-dependent TRP 5-hydroxylase. As with duration of scLGI, the availability of free serum TRP decreases, and brain serotonergic functions will be affected. The accumulation of neurotoxic KYN metabolites such as quinolinic acid, via NMDA glutamatergic stimulation, was also associated with the development of depression. Notably, depression had been brought into connection with CVD

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endpoints. Nevertheless, a secondary loop connecting excess adipose tissue with cardiovascular morbidity and mortality via scLGI appears to be more reliable in this context than a direct causal interaction.

Accelerated catabolism of TRP is also involved in the pathogenesis of the anemia of scLGI. The pro-inflammatory cytokine IFN-γ suppresses growth and differentiation of erythroid progenitor cells, and the depletion of TRP limits protein synthesis. Hence, also hemoglobin production is decreased which reduces the oxygen supply. This constellation contributes to a worsening of ischemic vascular disease. Moreover, the influence of KYN and kynurenic acid on the complex processes involved in the destabilization of vascular atherosclerotic remains to be more elucidated.

In summary, within this chapter we show the impact of TRP breakdown pathways, and the related complex mechanisms on the prognosis and individual course of CVD. The analysis of TRP, KYN concentrations, and calculation of the KYN/TRYP ratio may contribute to a better understanding of the interplay between immune-mediated inflammation, metabolic syndrome, mood disturbances, anemia, and destabilization of atherosclerotic plaques.

Keywords Cardiovascular disease • Chronic immune–inflammatory activation • Tryptophan breakdown

List of Abbreviations

AS	Atherosclerosis
CVD	Cardiovascular diseases
GTP-CH1	Guanosine triphosphate cyclohydrolase-1
HDL-C	High-density lipoprotein cholesterol
IDO	Indoleamine 2,3-dioxygenase
iNOS	Inducible nitric oxide synthase
IFN-γ	Interferon-γ
KYN	Kynurenine
LDL-C	Low-density lipoprotein cholesterol
MetS	Metabolic syndrome
MI	Myocardial infarction
NO	Nitric oxide
ROS	Reactive oxygen species
T5H	Tryptophan 5-hydroxylase
TDO	Tryptophan 2,3-dioxygenase
TNF-α	Tumor necrosis factor-α
TRP	Tryptophan

Introduction

L-tryptophan (TRP) is an essential amino acid. The following TRP metabolites are involved in important biologic processes: (1) 5-hydroxytryptamine, serotonin, formed by tryptophan 5-hydroxylase (T5H) after decarboxylation; (2) kynurenine, formed by tryptophan 2,3-dioxygenase (TDO); and (3) indoleamine 2,3-dioxygenase (IDO) (Fig. 10.1).

Tryptophan 2,3-dioxygenase and IDO initiate the first step in the catabolism of TRP. This is called the "kynurenine pathway" leading to nicotinic acid, the vitamin niacin, and nicotinamide adenine dinucleotides, as end products [1]. Interferon-γ (IFN-γ) is a potent in vitro and in vivo stimulator for IDO. Tryptophan 2,3-dioxygenase breakdown was detected in antigen-presenting cells like monocyte-derived macrophages, dendritic cells, fibroblasts, and other cells [2–5] (Fig. 10.2) as well as in humans after injection of IFN-γ [6].

An in vivo, cytokine-induced breakdown of TRP occurs usually in context of a cellular Th1-weighted immune response. A decrease of the serum TRP concentration is accompanied by increased kynurenine (KYN) or other TRP catabolites [6–8]. Thus, KYN levels are well related to TRP concentrations. The KYN/TRP ratio is a reasonable indicator of the TRP breakdown and provides a better approach than the analysis of the absolute TRP or KYN concentrations. Immune activation is essential to refer TRP breakdown to the activation of IDO and not to TDO [9, 10]. Hence, IDO is active when KYN/TRP and IFN-γ are positively correlated. Neopterin is another diagnostic marker to monitor a Th1-type immune activation [11].

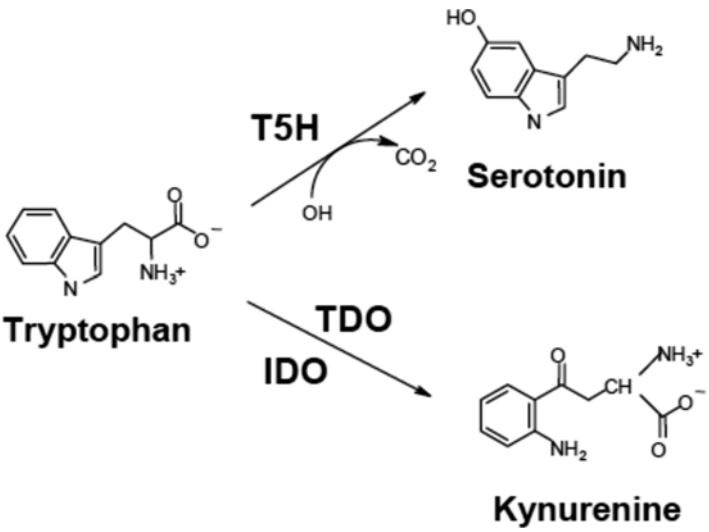

Fig. 10.1 Tryptophan metabolism. Tryptophan 5-hydoxylase (*T5H*) catabolizes the synthesis of 5-hydroxytryptamin (*serotonin*); tryptophan 2,3-dioxygenase (*TDO*) and indoleamine 2,3-dioxygenase (*IDO*) initiate the formation of kynurenine

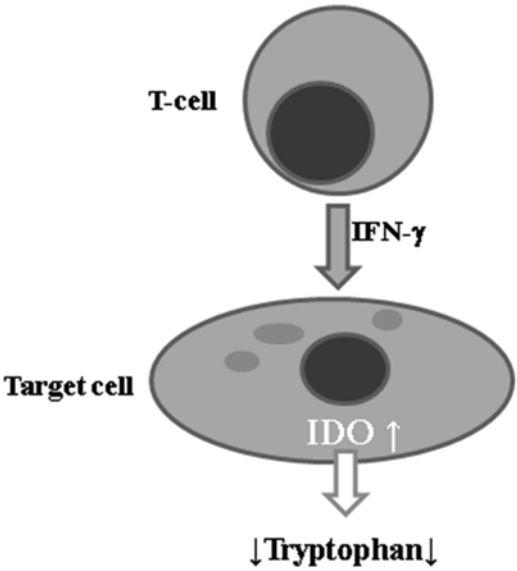

Fig. 10.2 The Th1 cytokine interferon-γ (*IFN-γ*) is released during the innate and adaptive immune response. It induces the enzyme indoleamine 2,3-dioxygenase (*IDO*) in macrophages and dendritic and other cells

Interferon-γ levels have been positively correlated to acute atherosclerotic complications [12], and KYN/TRP levels at admission predicted the later outcome in patients with ischemic stroke [13]. We showed recently that the TRP-KYN metabolism was essentially deregulated in obese persons in dependence of age and parameters of the metabolic syndrome (MetS) [14].

Tryptophan Metabolism Is Involved in Hypertension and Stroke

Blood pressure control is essential for the clinical course of cardiovascular diseases (CVD). Pharmacological inhibition of IDO increased blood pressure in mice but not in mice deficient in either IDO or IFN-γ which is required for IDO induction [15]. Both TRP and KYN have been shown to dilate preconstricted porcine coronary arteries [15]. The dilating effect of TRP required the presence of active IDO and an intact endothelium, and the effect of KYN was endothelium independent. Kynurenine-induced arterial relaxation was mediated by activation of the adenylate and soluble guanylate cyclase pathways. Summarized, IDO-mediated TRP metabolites seem to be involved in the regulation of the vascular tone [15]. Nevertheless, it was shown that an increased IDO activity (measured by KYN/TRP ratio in patients suffering from sepsis) caused a potent inhibition of the inducible nitric oxide synthase (iNOS) [16] indicating a complex relationship between the vasodilators nitric oxide (NO) and IDO which appear to reciprocally inhibit each other. These interactions may contribute to the pathological process of microvascular reactivity in myocardial infarction (MI), sepsis, and stroke. Indeed, activated IDO and higher

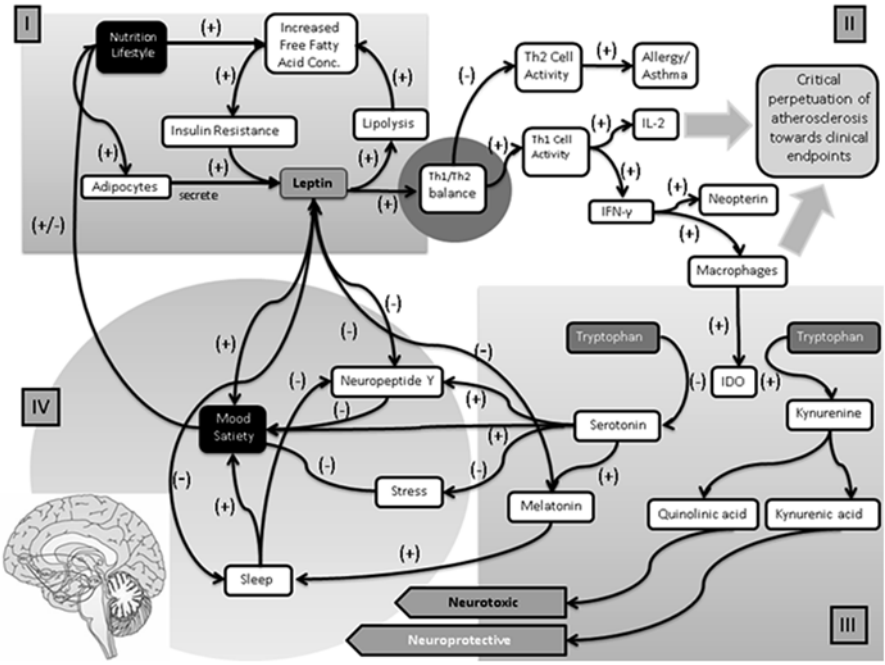

Fig. 10.3 The complex involvement of TRP metabolism in serotonin availability, mood alterations, T-cell-mediated inflammation, and clinical end points of CVD

concentrations of several KYN metabolites have been observed poststroke, where they have been associated with increased mortality and the development of poststroke cognitive impairment [17]. It sounds contradictory that despite IDO and KYN act as arterial relaxing factors [18], they are associated with increased poststroke mortality and cognitive impairment. Possibly, both IDO and KYN are produced due to inflammation as a kind of feedback mechanism to counteract the vasoconstriction mediated by pro-inflammatory events.

Multiple studies found evidence for TRP depletion as well as IDO activation in mood disorders [19–21]. Consistently, we recently reported that patients with bipolar disorder have elevated KYN and KYN/TRP ratios than mentally healthy control participants [22]. Moreover, concentrations of KYN and KYN/TRP were significantly increased in overweight compared to normal-weight bipolar patients [22]. Other studies demonstrated activated T cells, presumably of the Th1 subtype, over the whole course of manic-depressive illness [23]. Notably, the catabolic KYN pathway contributes to the neuroprotective–neurodegenerative/neurotoxic changes via production of kynurenic acid and quinolinic acid [24]. Activation of the KYN pathway also promotes generation of reactive oxygen species (ROS) and lipid peroxidation essentially involved in the oxidative stress as a consequence of chronic inflammation [25]. These molecular changes, involved in both atherosclerosis (AS) and mood disorders, could underlie the bidirectional association between mood disorders and CVD, as well as other inflammation-mediated medical conditions (Fig. 10.3) [26].

Tryptophan Metabolism and Melatonin: Specific Links to Cardiovascular Disease

Derived from L-tryptophan, melatonin is produced by the pineal gland during the biological ("internal") night driven by the master circadian pacemaker in the hypothalamus and released in dim light or darkness [27]. Impaired melatonin synthesis has been shown to be associated with age-related conditions including CVD [28–30]. A disrupted maturation of the photoneuroendocrine system caused by a genetic absence or mutation of genes involved in melatonin synthesis (including TRP hydroxylase) has been shown to cause an imbalance in the cross talk among serotonin, progesterone, catecholamines, and intracellular calcium [31]. Hence, an impaired TRP catabolism with successive melatonin deficiency may favor CVD by abnormal hormone levels (e.g., blood pressure increase through water retention by aldosterone) [32]. In addition, evidence exists that melatonin has anti-inflammatory, antioxidant, antihypertensive, and possibly antilipidemic properties [33]. For example, treatment with melatonin ameliorated the symptoms of MetS and decreased blood levels of low-density lipoprotein cholesterol (LDL-C) [34]. Further, melatonin has been shown to significantly suppress the formation of cholesterol and reduce LDL-C in isolated human mononuclear leucocytes [35]. In vitro studies have given evidence for antioxidant actions of melatonin on LDL-C oxidation [36]. In keeping with this, Dominguez-Rodriguez et al. [37] reported a relationship between nocturnally elevated oxidized serum LDL-C and reduced circulating melatonin levels in patients with acute MI. Hence, treatment with melatonin reduces blood pressure in rodents [38] and humans [34, 39]. It may be possible that decreased melatonin secretion secondary to increased TRP breakdown could represent an additional pathophysiological pathway toward CVD in patients with chronic inflammation of various causes, including obesity and associated MetS.

Tryptophan Metabolism, Anemia, Chronic Inflammation, and Cardiovascular Disease

The pro-inflammatory cytokines IFN-γ and tumor necrosis factor-α (TNF-α) suppress the growth and differentiation of erythroid progenitor cells [40]. Patients with anemia of chronic inflammation had decreased TRP plasma levels positively correlated with decreased hemoglobin [41]. Hence, IFN-γ-induced TRP deprivation may be involved in the cytokine-induced suppression of hematopoiesis seen in patients with chronic systemic inflammation. With respect to chronic inflammation in patients with coronary artery disease, anemia is probably a "player" rather than a "bystander" because it worsens the oxygenation of the myocardial tissue already reduced by coronary obstruction [42]. Furthermore, the tachycardia of anemia increases the myocardial oxygen demand. Summarized, chronic inflammation and anemia, based on an insufficient TRP supply due to IDO activation, may have a robust effect on mortality in CVD patients.

Tryptophan Metabolism, Aging, Immune Function, and Cardiovascular Risk

Systemic chronic low-grade immune-mediated inflammation in aging induces alterations in two enzymatic pathways, the IDO and the guanosine-triphosphate-cyclohydrolase-1 (GTP-CH1) pathways, both involved in the biosynthesis of monoamines [43] and potential neuropsychiatric symptoms. KYN/TRP was found significantly higher in nonagenarians compared with controls [44]. In a similar way, elevated neopterin levels have been described in nonagenarians and have been associated with a shorter residual life span [45]. Further, IDO activity predicted subsequent mortality in nonagenarians [44]. As aging of the immune system has been previously associated with a decline in T-cell function [46], one may consider increased IDO activity as an important mechanism involved in the decline of T-cell responses in immunosenescence [44]. Thus, accelerated TRP catabolism could be a modifiable step on this causal pathway. As T cells play an important role in the process of progression of CVD toward MI, a suppressed T-cell function is expected to be beneficial rather than detrimental to the incidence of coronary events, conceptually supported by the low MI rates in subjects >85 years. On the other hand, stroke frequency increases in subjects >85 years. IDO may have also a detrimental role in early AS, particularly in young female adults [47]. In these subjects, IDO activity correlated significantly with several risk factors for AS, i.e., with LDL cholesterol, body mass index, and inversely with high-density lipoprotein cholesterol (HDL-C) and triglyceride. Thus, IDO may be involved in the immune regulation of AS in a gender- and age-dependent way.

Therapeutic Considerations

To achieve a normalization of the TRP metabolism would be an important goal for the treatment of related symptoms in patients suffering from CVD. For instance, administration of the IDO-inhibitor 1-methyl tryptophan [48] may represent a pharmacological approach to be considered for future investigation. However, IDO is well known for its immunosuppressive properties, and inhibiting it may be detrimental, as it may stimulate pro-inflammatory mechanisms. Likewise, an elevated KYN/TRP ratio might represent a natural response of the human immune system to counteract inflammation. Anti-inflammatory drugs such as aspirin and statins may affect the IDO-mediated pathways. Specifically, atorvastatin suppresses IFN-γ-induced neopterin formation and TRP depletion in human peripheral blood mononuclear cells and in monocytic cell lines [49]. Moreover, immunosuppressants like rapamycin [50] counteract IDO induction during the pro-inflammatory response in vitro. The same is true for several natural compounds with anti-inflammatory properties and immunosuppressant capacity in vitro, e.g., resveratrol [51]. Finally, melatonin seems to have cardioprotective properties via its direct free radical scavenger and its indirect antioxidant activity [52].

Concluding Remarks

Herein we showed that the Th1-type cytokine IFN-γ causes increased IDO activity which ultimately decreases serum levels of the essential amino acid TRP. As a chronic low-grade systemic inflammation with activation of T-cell subsets (Th1, less Th2) [53] is usually present in patients with CVD, these subjects have an increased IDO activation leading to an increased TRP breakdown with an increased KYN/TRP ratio. Importantly, elevated KYN and KYN/TRP indicate a higher likelihood of fatal cardiovascular outcome.

In prolonged states of chronic low-grade systemic inflammation, availability of free serum TRP is persistently diminished, and thus, serotonergic functions are affected. Accumulation of neuroactive KYN metabolites such as quinolinic acid is neurotoxic and may contribute to mood dysregulation, also implicated in increased likelihood of MI.

Accelerated catabolism of TRP is also essentially involved in the pathogenesis of the anemia of chronic low-grade systemic inflammation. Interferon-γ suppresses erythroid progenitor cells, and the depletion of TRP decreases hemoglobin production. Thus, anemia caused by chronic low-grade systemic inflammation and increased TRP breakdown add to the detrimental effects of preexistent reduced vascular reserves.

Taken together, analysis of TRP, KYN concentrations, and calculation of the KYN/TRP ratio are important predictors of an unfavorable outcome in patients with CVD. It will be important to investigate if these parameters can provide a basis for more successful and precise biologically grounded therapeutic protocols to further reduce cardiovascular morbidity and mortality.

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Chapter 11
Role of Kynurenine Pathway in the Respiratory System

René Lutter

Abstract The tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase 1 (IDO1) is prominently expressed and active in the respiratory system during influenza infection and in several granulomatous diseases, such as sarcoidosis and tuberculosis. Manipulation of IDO1 activity in experimental models had profound effects on the course of influenza infection and on that of infection with the causative pathogen of tuberculosis, *M. tuberculosis*, and thus IDO1 qualifies as a prime target for therapeutic intervention. Based on the literature and unpublished work, working concepts for IDO1 activity in respiratory infections and granulomatous disease are proposed.

Keywords IDO • Sarcoidosis • Tuberculosis • Granulomatous disease • Mycobacterium • Influenza • Lung infection • Lung disease

List of Abbreviations

CGD	Chronic granulomatous disease
EBC	Exhaled breath condensate
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
NO	Nitric oxide
TDO	Tryptophan 2,3-dioxygenase
TtS	Tryptophanyl tRNA synthase

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Introduction

Tryptophan is an essential amino acid for many organisms including humans. This implies that tryptophan is crucial for survival but is not synthesized by the organism and thus has to be acquired via uptake. In humans, tryptophan is critical for the synthesis of proteins and that of several important bioactive substances like serotonin, melatonin, tryptamine, and vitamin B3. One of the major metabolic pathways of tryptophan is mediated by the constitutively expressed liver enzyme tryptophan 2,3-dioxygenase (TDO) and yields, via *N*-formylkynurenine, kynurenine, a precursor of vitamin B3. Together this indicates that regulation of the tryptophan metabolism must meet several requirements, and thus interference may lead to serious limitations.

In the late 1960s, the inducible indoleamine 2,3-dioxygenase (IDO) was discovered which too generates *N*-formylkynurenine by oxidizing tryptophan and thus potentially interferes with tryptophan metabolism. At the time, IDO was considered an antioxidant. It was not until the studies by Mellor and Munn [1] and independently those by Däubener and MacKenzie [2, 3] that the role of tryptophan depletion was placed center stage. Mellor and Munn [1] showed that tryptophan depletion by IDO1 activity halted activation of T cells and led to apoptosis of T cells. And further, by the generation of kynurenine, IDO1 activity promoted (premature) apoptosis of T cells. Däubener and MacKenzie [2, 3] showed the antimicrobial role of IDO-mediated tryptophan depletion, preventing bacterial growth and viral replication. Since early 2000, the volume of research into IDO has steadily increased, substantiating its immunomodulatory role [4], on subpopulations of T cells [5] and inflammatory cells such as neutrophils [6], and has also led to the discovery of a gene analogue, IDO2, which led to renaming of IDO to IDO1 [7]. IDO2 displays enzymatic characteristics and an expression profile distinct from IDO1 and may be involved particularly in regulating tolerance [8]. In this chapter, we discuss the role of IDO1 in the respiratory system and in particular that in relation to respiratory viral infections and in granulomatous disease.

IDO1 Activity and Respiratory Viral Infections

Yoshida and colleagues [9, 10] were first to show that IDO1 was induced in the airway mucosa during an influenza infection, peaking at day 11 and slowly decreasing over a 3 week period. Further they showed that this was primarily dependent on interferon-γ (IFN-γ), which is a key antiviral mediator [11]. Jacoby and Choi [12] showed that the epithelial cells exposed to influenza were expressing IDO1, although this likely is by uninfected epithelial cells only [13]. Interestingly, influenza-induced IDO1 activity resulted in a systemically enhanced kynurenine/kynurenine + tryptophan ratio, [14] and increased IDO1 activity was even found in other organs besides the lungs and trachea [9, 10]. We have developed conditional double transgenic

mice that can be induced to express human IDO1 specifically in the airway epithelium by supplementation of doxycycline to drinking water (van der Sluijs et al., submitted). With these mice we found a similar systemic decrease of tryptophan as upon influenza infection and an even more marked decline in the lungs. Together this indicates that IDO1 activity in structural cells like epithelial cells during an influenza infection may have a profound impact on the bioavailability of tryptophan in the lungs and likely other organs. This links to another important issue in that cells that express IDO1 are bound to deplete themselves of tryptophan, which would halt synthesis of proteins and that of bioactive compounds and ultimately lead to their death. This is countered by the IFN-γ-mediated enhanced expression of tryptophanyl tRNA synthase (TtS) and that of alternatively spliced forms of TtS, which have a higher affinity for tryptophan [15]. Aminoacyl tRNA synthases are required for protein synthesis, and TtS is the only aminoacyl tRNA synthase in eukaryotic cells of which the expression can be regulated. This will allow IDO1-expressing cells to increase the bioavailability of tryptophan for their protein synthesis, which may prevent their death. In contrast, cells that do not express more TtS and its alternatively spliced forms may not survive in an environment depleted of tryptophan by IDO1.

What now is the consequence of IDO1 activity in the lungs during an influenza infection? Based on the previous section, IDO1 activity is expected to display opposing antiviral effects by attenuating antiviral T cell responses and by inhibiting viral replication. As anticipated, IDO1 activity indeed attenuated antiviral $CD8^+$ responses, and it was also found to modulate T cell memory responses and attenuate Th17 cells during a recall response [16–18]. As part of a study into the impact of mucosal IDO1 activity by epithelial cells on the course of an influenza infection, we exposed conditional human IDO1-expressing mice (FVB × C57Bl/6 background) to influenza (A/PR/8/34). When IDO1 was expressed and tryptophan was depleted at the start of an influenza infection, at peak symptoms (day 8), IDO1-expressing mice had 100-fold higher titers of influenza and also succumbed at higher numbers and faster to lethal infections with influenza. Studies with another founder of these conditional human IDO1-expressing mice, i.e., with a lower IDO1 expression, yielded similar but attenuated effects, such as ten-fold higher viral titers. In parallel, numbers of $CD8^+$ and $CD4^+$ T cells, irrespective of their specificity, and macrophages and granulocytes were reduced in these IDO1-expressing mice. The enhanced susceptibility to influenza, however, was due to apoptosis-mediated reduction of $CD8^+$ cell numbers as was confirmed by showing that antibody-mediated depletion of $CD8^+$ cells leveled the effect of IDO1. So this indicates that IDO1 expression by structural cells can, in an apparent non-cognate manner, also attenuate T cell responses, which may relate to failure to upregulate TtS activity. Interestingly, titers of influenza-specific antibodies were not affected in these IDO1-expressing mice, indicating that either this process happened outside the area affected by IDO1 activity or is insensitive to IDO1 activity. And finally, despite the potent in vitro inhibition of influenza replication by IDO1-expressing H292 epithelial cells, as shown previously for other viruses, the enhanced viral loads in these IDO-expressing mice indicated that IDO1 activity does not or not fully halt viral replication in vivo.

IDO1 activity also promotes apoptosis of inflammatory cells such as neutrophils [6] and so potentially IDO1 may dampen inflammatory responses. Previously, we have reported that IDO1 activity had reduced neutrophil numbers 14 days after influenza exposure [14]. And also, in the above-described experiments with the conditional IDO1 mice, we found that IDO1 activity significantly reduced neutrophil numbers. In that same study, we also compared influenza infection in BALB/c wild-type and IDO1 knockout mice [kindly provided by Profs. M. Moser (Université Libre de Bruxelles, Gosselies, Belgium) and A. Mellor (Georgia Regents University, Augusta, Georgia, USA)] and found that IDO1 reduced macrophage numbers and trend wise that of neutrophils, paralleled by an increase in body weight. In addition to the induction of inflammatory cell apoptosis, IDO1 also enhanced IL-10 production in response to *S. pneumonia* [14], which prevents degranulation of neutrophils and thus neutrophil-induced damage [19]. Together this shows that IDO1 indeed may dampen inflammatory responses, which is in line with its relative prolonged expression after influenza has been cleared, 12–14 days after initial infection. The downside of these anti-inflammatory effects of IDO1 may be that also the antibacterial capacity is attenuated by IDO1 activity and may explain the manifestation of bacterial superinfection following influenza infection [14].

Differential Expression and Activity of IDO1

The Th1 mediator IFN-γ induces IDO1, whereas the Th2 mediators IL-4 and IL-13 dampen IDO1 expression. This may explain why an influenza infection in Th2-prone BALB/c mice induces a delayed and also lower IDO1 expression than, e.g., in Th1-prone C57Bl/6 mice (our unpublished results). As mentioned above, IDO1 attenuated the inflammatory cell numbers in BALB/c mice, which was reflected by a prolonged pathology in IDO1 knockout mice, whereas there were no differential effects on T cells. This shows that the timing and extent of IDO1 expression may vary depending on the genetic background, and this has to be taken into consideration for its immunomodulatory activities. In view of this, our recent study on the role of IDO1 in rhinovirus-induced asthma exacerbations is of interest [20]. Although rhinovirus16 failed to induce IDO activity in healthy controls, which likely is due to its low virulence and thus failure to induce a marked IFN-γ response, pulmonary IDO1 activity was significantly lower in asthmatics than controls and may underlie the enhanced inflammatory response in response to a viral infection in asthma patients.

IDO1 is a heme-containing enzyme, and therefore nitric oxide (NO) that can bind to the heme-moiety is a potent inhibitor of IDO1. Interestingly, the inducible nitric oxide synthase (iNOS) that produces NO is also induced by IFN-γ, which is suggestive of a negative feedback loop. These findings indicate that besides the timing and extent of IDO1 expression also inhibitory mechanisms like that of NO and possibly the mechanisms that repair NO-induced damage such as heme-oxygenase-1 are crucial determinants in assessing the immunomodulatory effect of IDO1.

A Working Concept

Taken together and as depicted in Fig. 11.1, high IDO1 activity early during an influenza infection or low IDO1 activity late during an influenza infection both lead to more severe pathology, but by different mechanisms: i.e., by a reduced antiviral $CD8^+$ response and by a prolonged inflammatory cell response, respectively (van der Sluijs et al., manuscript submitted). This implies that appropriately controlled IDO expression may limit pathology and thus that both reducing and enhancing IDO1 activity may be relevant therapeutic options. As IDO1 is also induced by other pro-inflammatory stimuli such as bacterial lipopolysaccharide (LPS) and muramyl peptide [21], it is likely that the proposed mode of actions of IDO1 also apply to bacterial infections. Less is known, however, on the impact of high IDO expression on local synthesis of proteins and bioactive compounds, which given the relevance of tryptophan metabolism clearly warrants further research. Contrary to expectations, we have shown previously that IDO1 activity may promote pro-inflammatory mediator production by airway epithelial cells [14], which was reversed by adding tryptophan. Although the underlying mechanism is unknown as yet, I assume that this is due to a stress response previously described as superinduction [22], which may aggravate inflammation at high IDO1 activity.

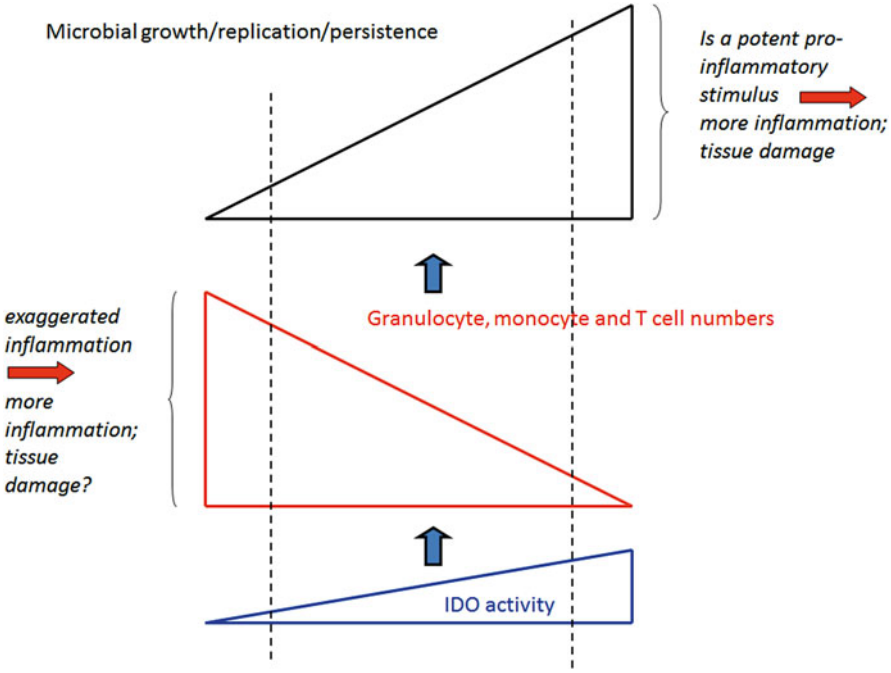

Fig. 11.1 Consequences of high or low IDO activity during a respiratory infection

IDO1 Activity and Granuloma

Popov et al. [23] have shown that cells in granulomas of various granulomatous diseases express IDO1. So far only few studies have, however, addressed the role of IDO1 activity in granulomas and thus in granulomatous diseases. Given its immunomodulatory and antimicrobial functions, IDO1 could play quite a prominent role in granuloma function.

We have confirmed and extended the finding for three granulomatous diseases with pulmonary manifestation (tuberculosis, sarcoidosis, and chronic granulomatous disease (CGD) [24]) using antihuman IDO1, kindly provided by Prof. Benoit van den Eynde (Ludwig Institute for Cancer Research, Brussels, Belgium). Common to all three diseases, IDO1 was expressed by cells surrounding cells in the center of granuloma, which were not expressing IDO1 and often were apoptotic. In some cases, apparently with relatively high IDO1 expression, we also found apoptotic cells outside the ring of IDO1-expressing cells. This suggests that these cells were not exposed to IFN-γ, as they did not express IDO1, and likely were recruited into the center of granuloma, or were near the granuloma, where they became apoptotic because of tryptophan depletion and/or cytotoxic tryptophan metabolites. Our pilot data for sarcoidosis even indicated that IDO1 expression is higher at stage IV of the disease, in line with an earlier report on enhanced systemic levels of kynurenine metabolites in more severe stages of sarcoidosis [25]. Together this suggests that granulomatous IDO1 actively degrades tryptophan and may inactivate recruited cells and cells in the neighborhood of granuloma and so could attenuate immune and inflammatory responses.

In a recent study, we found that tryptophan and its metabolites in EBC and serum do not correspond, suggesting that EBC indeed samples the airways and is not overwhelmed by systemic levels of tryptophan and its metabolites [20]. Given that systemic kynurenine correlated with sarcoidosis staging, and in a more recent study systemic kynurenine appeared a prognostic factor in tuberculosis [26], we extended the use of exhaled breath condensate (EBC) to granulomatous diseases. We collected and analyzed exhaled EBC for the analyses of tryptophan and various kynurenine metabolites by ultra-performance liquid chromatography linked to tandem mass spectrometry. Indeed we found that these metabolites were markedly enhanced in CGD and to a lesser extent for sarcoidosis (not shown), although we have not yet been able to determine whether this correlates with pulmonary pathology. Potentially, this offers a means to assess granulomatous IDO activity in the lungs and thus confirm pulmonary manifestation.

The role of IDO1 in granulomatous diseases has been studied in CGD and tuberculosis only, but only for tuberculosis this was focused on granulomatous IDO1. Romani et al. reported that IDO1 activity in CGD was inhibited as a consequence of the failure to generate superoxide by the defective NADPH oxidase [27]. This led to unrestrained reactivity of specific T cells, defective regulatory T cell functions, enhanced production of the pro-inflammatory IL-17, and acute inflammation. So the absence of IDO1 activity was proposed to lead to hyperinflammation, a prominent

comorbidity in CGD. Subsequent studies, however, have unambiguously shown that IDO1 activity is not inhibited in CGD, also supported by our analyses of EBC from CGD patients. Indeed IDO1 can also degrade tryptophan via oxygen and an electron and not strictly by superoxide [28]. As argued above for influenza infections, I propose that a high IDO activity in CGD may also lead to hyperinflammation. No further studies have been reported.

Blumenthal et al. [29] showed that IDO1 activity was induced upon infection with *M. tuberculosis* in C57Bl/6 mice, but that the course of the infection was not influenced in mice where IDO1 activity was inhibited with methyl-tryptophan. In our studies to assess the role of IDO1 in murine tuberculosis, we used BALB/c mice that display a relatively low IDO1 activity. As it takes time to develop an adaptive immune response consisting of IFN-γ-producing mycobacterial specific-T cells, we anticipated a delayed onset of IDO1 expression. Indeed, 2 weeks after intranasal infection with *M. tuberculosis* (Erdman strain), no IDO1 was expressed yet. Six weeks after infection, IDO1 mRNA was detected in lungs and IDO1 protein was expressed in affected areas in the lung. In line with this delayed onset and in comparison to wild-type BALB/c mice, BALB/c IDO1 knockout mice showed no difference at 2 weeks after infection. At 6 weeks after infection, however, the mycobacterial load was significantly reduced in IDO1 knockout mice, both in the lung and liver. Levels of IL-1β and IL-17 and granulocyte numbers were lower in IDO1 knockout mice, whereas levels of IFN-γ and IL-10 and numbers of macrophages and $CD4^+$ and $CD8^+$ cells were similar to IDO1 knockout mice. It makes sense to express these inflammatory parameters relative to the mycobacterial load as the bacteria drive the inflammatory response. When expressed per mycobacterial load, cell numbers and levels of inflammatory mediators were relatively enhanced in IDO1 knockout mice. Together this suggests that IDO1 inhibits immune and inflammatory responses as a consequence of which the mycobacterial load is reduced less. To test this concept further, we subjected our conditional IDO1-expressing mice to *M. tuberculosis* infection. As expected, pulmonary macrophage, granulocyte and T cell numbers, and even *M. tuberculosis*-specific T cells were reduced markedly in IDO1-expressing mice. In parallel, we found that *M. tuberculosis* disseminated more successfully to the liver and even into the bone marrow. So this confirms that local IDO1 activity can have a profound effect on the course of a *M. tuberculosis* infection, by promoting apoptosis of immune and inflammatory cells. In apparent contradiction, mycobacterial loads in lungs from IDO1-expressing mice remained similar to that in control littermates. As the inflamed area was significantly larger in IDO1-expressing mice, we hypothesize that also in the lungs there is more dissemination, but the high IDO1 activity inhibits the increase of *M. tuberculosis*. These findings differ markedly from those of Blumenthal et al. [29] in C57Bl/6 mice. The reason for this discrepancy is unclear, but as we have found that IDO1 activity affected the course of a *M. tuberculosis* infection in two independent models we assume that our findings are genuine. A possible explanation is that C57Bl/6 mice used by Blumenthal et al. [29], in contrast to the BALB/c mice used in our studies, have an enhanced NO production by iNOS, which inactivates IDO1.

IDO1 and Intracellular *M. tuberculosis*

Tryptophan is also an essential amino acid to microorganisms, and thus IDO1 activity can directly attenuate bacterial growth [2] and viral replication [3]. This is of particular importance when microorganisms are contained within cells that express IDO1. However, while for pathogens that are natural tryptophan auxotrophs (e.g., Chlamydia), this strategy is predicted to be successful; the genome of *M. tuberculosis* harbors a tryptophan biosynthetic gene clusters *trpE-C-B-A* (*rv1609/11/12/13*) and trpD (*rv2192c*) that help the bacterium to circumvent such host-mediated antimicrobial defense mechanisms. In a recent study, it was shown that an *M. tuberculosis* mutant strain that had one of the *trpE* gene of tryptophan biosynthesis operon replaced by an antibiotic marker became very sensitive to IDO1-mediated killing imposed by host macrophages [30]. Similar effects were also reproduced in wild-type *M. tuberculosis* strains by the use of a small molecule inhibitor that interfered with the bacterium's tryptophan biosynthesis [30]. From these reported findings, we conclude that the tryptophan metabolism of *M. tuberculosis* is an important pathway for its survival and therefore the impact of IDO1 and possibly TtS and its alternatively spliced forms should be investigated further. For example, it is not known whether IDO1-mediated tryptophan depletion sets off expression of other genes in *M. tuberculosis,* which may prevent clearance of *M. tuberculosis* from granulomas. And, possibly manipulation of the IDO1 pathway may provide an alternative to control tuberculosis.

A Synthesis

Based on the pilot findings described above and the literature, we propose that the functioning of granulomas is controlled by granulomatous IDO1 activity (Fig. 11.2) and that of TtS. IDO1 activity promotes death of recruited cells that are reactive to antigen and pathogens contained within granulomas, which prevents clearance of the antigen and pathogens and thus resolution of granulomas. These recruited cells have not been exposed to IFN-γ and therefore do not express the alternatively spliced form and enhanced levels of TtS, which would have made these cells cope with low tryptophan concentrations. A similar incapacity to deal with low tryptophan concentrations applies to immune and inflammatory cells in the vicinity of granulomatous IDO1 activity, which may underlie the development of comorbidities such as hyperinflammation, associated other inflammatory diseases, and increased risk of malignancies. Enhancing TtS activity in these cells should save their immune and inflammatory functions and thus may lead to resolution of granuloma and reduce comorbidities. In infectious granuloma, granulomatous IDO1 activity can kill the infectious agent. However, in case of *M. tuberculosis*-infected macrophages, *M. tuberculosis* can survive and reducing granulomatous IDO1 activity will lead to dissemination of the infection. TtS in the infected macrophage

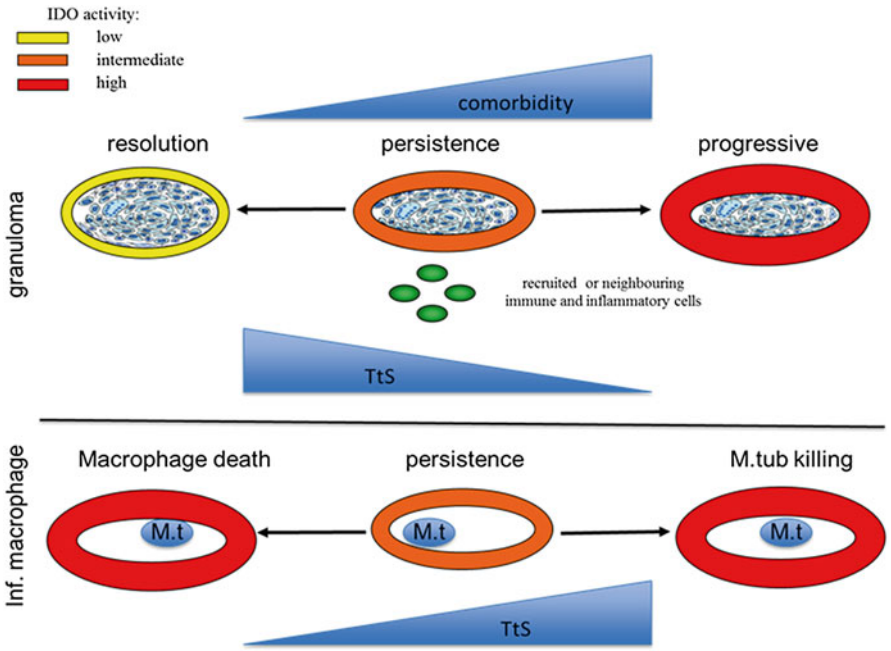

Fig. 11.2 Concept presentation of role of IDO and TtS in all granulomas (*top*) and *M. tuberculosis-containing* macrophages (*bottom*)

prevents death of the macrophage and thus promotes survival of *M. tuberculosis*. By modulating TtS activity in infected macrophages with high IDO1 activity, either macrophage death can be induced or possibly *M. tuberculosis* can be outcompeted for tryptophan.

Concluding Remarks

IDO1 activity is a key modulator of immune and inflammatory responses by inducing apoptosis or halting activation of involved cells and possibly by driving inflammatory mediator responses. Although IDO1 activity can halt viral replication and bacterial growth in vitro, we have failed to find evidence for these antimicrobial activities in vivo. However, as has been suggested by Prof. Däubener, the antimicrobial and immune modulatory activities of IDO1 may be affected by different tryptophan concentrations and so we may have missed it. The timing and extent of IDO1 activity determine its immunomodulatory effects, where too high or too low IDO1 activity appears to lead to pathology. Careful clinical and basic studies are required to further delineate the role of IDO1 activity in infectious and granulomatous diseases, in which local sampling such as that achieved with EBC for assessing kynurenine and its metabolites may prove valuable.

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Chapter 12
Role of Kynurenine Pathway in Gastrointestinal Diseases

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Abstract In the course of obtaining nutrients from the environment to sustain life, the gastrointestinal (GI) tract encounters a myriad diversity of foreign antigens, microbiota, and environmental toxins. To maintain homeostasis in this setting, the GI mucosal immune system employs a multitude of techniques to distinguish the helpful from harmful. Recent studies reveal amino acid metabolism pathways as an integral mechanism to maintaining homeostasis at the mucosal interface. Tryptophan metabolism along the kynurenine pathway in particular is now recognized as an important feature of both GI inflammation and malignancy. Indoleamine 2,3 dioxygenase (IDO1), the most prevalent of the tryptophan catabolizing enzymes, is expressed widely across tissues and cell types within the GI tract. This chapter will address the current knowledge of kynurenine pathway functions in the diseased GI tract and highlight the potential benefits of exploiting this pathway for therapeutic purposes.

Keywords Indoleamine dioxygenase • Kynurenine • Tryptophan • Gastrointestinal disease • Gut • Inflammation • Metabolome • Metabolomics • Cancer • Crohn • Ulcerative colitis • Biomarker

List of Abbreviations

1-MT	1-Methyl-DL-tryptophan
AhR	Aryl hydrocarbon receptor
AOM	Azoxymethane
APCs	Antigen presenting cells
CD	Crohn's disease
DCs	Dendritic cells
DRE	Dioxin-responsive element

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DSS	Dextran sodium sulfate
GI	Gastrointestinal
IBD	Inflammatory bowel disease
IDO1	Indoleamine 2,3 dioxygenase 1
IDO2	Indoleamine 2,3 dioxygenase 2
IFN-γ	Interferon-γ
KYN	Kynurenine
MAMPs	Microbial-associated molecular patterns
NAD^+	Nicotinamide adenine dinucleotide
PRRs	Pattern recognition receptors
QA	Quinolinic acid
TDO	Tryptophan 2,3 dioxygenase
TNBS	Trinitrobenzene sulfonic acid
TLR	Toll-like receptor
Tregs	Regulatory T cells
TRP	Tryptophan
WT	Wild type

Introduction

As the internal interface between an organism and its external environment, the gastrointestinal (GI) tract fulfills the important function of a selective physical, chemical, and immunological barrier. These barriers are formed through the interplay of many cell types (e.g., epithelia, stroma, immune cells, microbiota), all of which must be considered in a discussion of GI disease. As an immunological barrier, the tissue must be able to differentiate between a multitude of self-antigens and those of potentially dangerous pathogens, while maintaining an appropriate balance of symbiotic and commensal microorganisms. Disruption of this balance can lead to a number of GI maladies, including inflammatory bowel disease (IBD) and neoplasia. As such, mammals have evolved intricate mechanisms of immune regulation to maintain homeostasis within the GI tract. Metabolism, and particularly amino acid catabolism, is a frequent and important feature of both inflammatory responses and tumor progression in the gut.

One protein that has emerged as an important component of GI immune modulation is the tryptophan metabolizing enzyme indoleamine 2,3 dioxygenase 1 (IDO1). While not the only known enzyme to perform the first and rate-limiting step in tryptophan catabolism along the kynurenine pathway, IDO1 is by far the most characterized enzyme in the gut [1]. Catabolism of tryptophan by IDO1 results in a number of biologically active metabolites, collectively known as kynurenines [2]. IDO1 is expressed by professional antigen presenting cells (APCs) and is inducible in intestinal epithelial cells by a variety of proinflammatory stimuli [3, 4]. Hence, IDO1 expression is highly upregulated in human IBD and in animal models of coli-

tis, as well as in malignancies [5–8]. The induction of the tryptophan metabolizing enzyme IDO1 in several disease contexts, along with the biological activity of its metabolic products, indicates tryptophan catabolism is poised to modulate GI disease pathology [9]. The established and emerging roles of IDO1 and kynurenines in immune tolerance, inflammatory diseases, gastric and colorectal cancer, and host–microbial interactions will be discussed in detail herein.

IDO1 in Colitis and Inflammatory Bowel Disease

A low expression of IDO1 is observed in the GI tract in the normal state, mostly in the cells of lamina propria. However, its expression is highly upregulated in both the intestinal epithelium and the lamina propria APCs in human IBD and mouse models of colitis [1].

Inhibition or upregulation of IDO1 affects several forms of colitis. Pharmacologic inhibition or genetic deletion of IDO1 exacerbates inflammation in trinitrobenzene sulfonic acid (TNBS) and dextran sodium sulfate (DSS) induced models of colitis in mice [10–12]. Conversely, we have shown that inducing IDO1 by a Toll-like receptor (TLR) 9 agonist limits the severity of DSS induced colitis in mice [13]. Similarly, Jasperson et al. showed in a model of graft versus host disease that $IDO1^{-/-}$ mice had more severe colitis and mortality than wild-type (WT) mice which expressed IDO1 in the colonic epithelium [14]. This group also showed that induction of IDO1 by TLR7 and TLR8 agonists in APCs ameliorated colitis in the same model of graft versus host disease [15]. El-Zaatari et al. showed that $IDO1^{-/-}$ mice developed a severe form of *Clostridium difficile* colitis characterized by increased accumulation of neutrophils, increased production of interferon-γ (IFN-γ) by neutrophils, and severe immunopathology when compared to $IDO1^{+/+}$ mice [16]. Taken together, these findings indicated that IDO1 acts as a natural brake to various forms of gut inflammation.

Several mechanisms and cell types may mediate the tolerogenic effects of IDO1 activity and kynurenines in the gut. Matteoli et al. demonstrated that inhibiting IDO1 in $CD103^{+}$ dendritic cells (DCs) exacerbates DSS colitis by promoting the development of Th1 and Th17 T cells, producers of proinflammatory cytokines IFN-γ and IL-17 [10]. Conversely, IDO1 inhibition decreased development of anti-inflammatory T regulatory cells (Tregs). Tregs have been shown to be capable of suppressing Th17 cell response in the presence of IL-10 in vivo [17]. Although Matteoli's study did not focus on this specifically, it could be postulated that skewing toward Th17 inflammation in the setting of IDO1 inhibition was mediated in part by the reduction of Tregs. Neutrophils also appear to be affected by IDO1 and kynurenines. El-Zaatari et al. showed that kynurenine directly induces apoptosis in neutrophils [16], thus identifying a novel function of IDO1 in limiting cecal inflammation associated with *C. difficile*. Again, it could be extrapolated from this study that kynurenines, through IDO1 expression in DCs or the epithelium itself,

could perform a similar role in limiting the inflammation found in human IBD and mouse models of colitis.

We and others have shown that IDO1 expression is upregulated in the colonic epithelium in DSS induced colitis, and inhibition of IDO1 worsened DSS induced colitis, highlighting that IDO1 and kynurenines can limit inflammation in T cell-independent fashion [10, 13]. We have recently shown that kynurenine and quinolinic acid (QA) can induce proliferation in HCT116 cells [18]. The effect of IDO1 activity and kynurenines on the normal, non-malignant epithelium is not yet elucidated, but may have relevance for pharmacologic agents targeting the IDO1-kynurenine pathway for its anti-inflammatory properties.

Gastrointestinal Cancer

The expression of IDO1 and similar enzymes which metabolize tryptophan along the kynurenine pathway [indoleamine 2,3 dioxygenase 2 (IDO2) and tryptophan 2,3 dioxygenase (TDO)] has been reported in several tumors. In GI tumors, IDO1 is the principle enzyme to be upregulated [1] with existing data pointing to it having a significant role in the development and/or progression of GI tumors. Human observational studies examining IDO1 in colon cancer [19–21] have identified that high IDO1 expression correlated with reduced survival. This suggests that IDO1 is involved in colon cancer pathogenesis, and inhibition of IDO1 could be beneficial in colon cancer.

Inhibiting IDO1 Reduces Tumor Burden

A few studies have examined the functional mechanisms of IDO1 in the pathogenesis of colon cancer. Our group recently identified a novel role for IDO1 expression in the neoplastic epithelium using the azoxymethane (AOM)/DSS model of colitis-associated cancer [18]. We showed that pharmacologic inhibition or genetic deletion of IDO1 resulted in a reduced tumor burden and a lower proliferation index in the neoplastic epithelium when compared to control mice. These observations were also made in Rag1/IDO1 double-knockout mice compared with $Rag1^{-/-}$ mice (which lack mature adaptive immunity) [18]. Examining a rat model of pre-neoplastic colon lesions, Ogawa et al. showed that AOM administration alone upregulates IDO1 expression. Though the study was also evaluating green tea catechins, they found that pharmacological inhibition of IDO1 by 1-methyl-DL-TRYPTOPHAN (1-MT) prevented the development of pre-neoplastic lesions and suggested that IDO1 was associated with early phases of colon carcinogenesis [22]. Together these observations provide new insight and a novel role for IDO1 in promoting tumor growth and proliferation independent of impact on the adaptive immune system.

Tryptophan Metabolites as Mediators of Tumor Progression

In other cancers, both tryptophan depletion and tryptophan metabolites have been shown to promote tumor progression via immunomodulatory mechanisms [23–26]. High expression of IDO1 by tumor cells depletes local tryptophan concentrations leading to T-effector cell anergy and apoptosis. Tryptophan metabolites also directly suppress T cell function and induce apoptosis. Though our studies only found a trend toward this immune effect in the AOM/DSS model of colitis-associated cancer, it is likely that tumor progression in other models of colon cancer may also involve these mechanisms [18].

Based on the finding in mice lacking mature adaptive immunity, we sought to further examine the possibility that IDO1 activity may be promoting growth of colon neoplasia in a cell autonomous fashion. Using in vitro techniques, we identified a link between kynurenine metabolites and active β-catenin signaling. Inhibiting IDO1 expression using 1-MT and siRNA suppressed the proliferation in HT29 and HCT116 colorectal cancer cell lines in vitro [18]. The addition of kynurenine and QA reversed the effects of IDO1 inhibition in a concentration-dependent fashion by inducing proliferation in cancer cells and stabilizing the expression of β-catenin and canonical Wnt signaling. These findings clarify that IDO1 expression in the neoplastic epithelium directly promotes tumor growth in a kynurenine pathway-dependent manner. Figure 12.1 illustrates our current understanding of how IDO1 and kynurenine promote colon cancer progression by both supporting tumor immune tolerance and stimulating tumor cell growth.

Several possibilities exist to explain the mechanisms by which IDO1 and kynurenines mediate both immune-dependent and immune-independent promotion of tumor progression. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor which binds the nuclear xenobiotic- or dioxin-responsive element (DRE). Opitz et al. showed that kynurenine, a natural AhR ligand, promotes clonogenic survival and malignant progression in human brain tumors through activation of AhR [27]. In this study, kynurenines came from TDO, a tryptophan catabolizing enzyme like IDO1, but with a more limited expression pattern (brain and liver). It is thus possible, though not yet demonstrated, that the IDO1-AhR pathway is active in colon cancers. The nicotinamide adenine dinucleotide (NAD^+) stress response pathway may represent another mechanism by which kynurenines facilitate growth of colon neoplasia. NAD^+ plays a key role in maintaining cellular integrity in response to stressors such as hypoxia and high proliferative activity. Cancer cells rapidly consume NAD^+ and thus require alternative synthesis pathways (other than via nicotinamides). The tryptophan–kynurenine pathway generates QA which is one alternative substrate for NAD^+ synthesis. QA, as a precursor to NAD^+, has recently been identified as an important factor in conferring resistance of gliomas to oxidative stress [28]. Notably, we identified both kynurenine and QA as able to normalize colon cancer cell line proliferation in the setting of IDO1 inhibition [18].

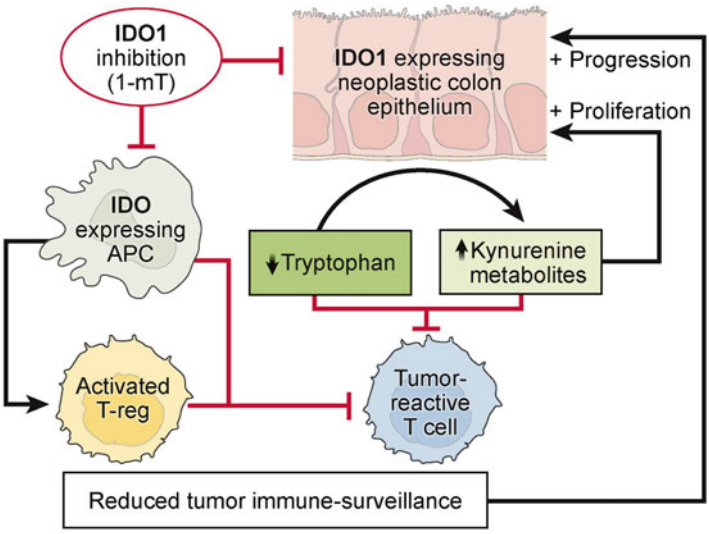

Fig. 12.1 Dual functions of IDO1 and kynurenines in colon cancer. IDO1 is expressed by the neoplastic epithelium and APCs of the tumor draining lymph nodes or tumor microenvironment. A decreased tryptophan/kynurenine metabolite ratio suppresses tumor-reactive effector T cells and promotes regulatory T cell activation to limit tumor immune surveillance and thus facilitate tumor progression. Concurrently, increased local levels of kynurenine metabolites directly stimulate proliferation in the neoplastic epithelium through activated nuclear β-catenin. Inhibition of IDO1 activity (1-MT) reduces tumor progression and proliferation. Reproduced with permission from Thaker et al. 2013 [18]

Gastric Carcinoma

A recent abstract by El-Zaatari et al. shows that in *Helicobacter felis* infection, myeloid expression of IDO1 was elevated in WT mice. This IDO1 expression correlated with increased kynurenine levels and an increase of bioactive IL-1β through the activation of the caspase 1 pathway [29]. Several investigators are currently working on elucidating the involvement of IDO1 in gastric carcinoma, and more such interesting studies are expected in the near future.

Tryptophan and Kynurenine Levels as a Biomarker in IBD and Colon Cancer

Beeken et al. was the first group to historically show that Crohn's disease (CD) patients had lower serum tryptophan levels than normal subjects [30]. Recently, we showed that elevated expression of IDO1 in the gut correlated with low serum tryptophan levels and a high kynurenine/tryptophan (KYN/TRP) ratio in patients with active CD compared to those in remission and normal subjects [31]. We also showed

that the KYN/TRP ratio reached normal levels as the inflammation subsided in CD patients [31]. Two small cohort studies also reported elevated kynurenine and kynurenic acid in patients with CD and ulcerative colitis [32, 33].

An increased KYN/TRP ratio has also been reported in patients with colon cancer when compared to normal populations [34]. Huang et al. further showed a correlation between reduced serum tryptophan and deterioration of quality of life in patients with metastatic colorectal cancer [35]. Finally, Walczak et al. showed that mucosal aspirates in colon carcinoma patient had elevated kynurenic acid than control subjects [36]. Thus, measuring kynurenine and tryptophan levels in the serum of patients with IBD and colon cancer can be exploited as a potential biomarker in these GI disorders.

Kynurenines at the Host–Microbial Interface

Outnumbering host cells by ~10-fold, the human intestinal microbiota is now recognized as a significant contributor to both health and disease [37, 38]. Host cells interact with resident microbial populations by the direct binding of microbial-associated molecular patterns (MAMPs) to host pattern recognition receptors (PRRs), as well as through uptake of secreted metabolites [39, 40]. These mechanisms of cross talk work in concert to fine-tune the host mucosal immunity to maintain a proper balance between tolerance of symbiotic microorganisms and protection against potential pathogens [41]. Through the study of tryptophan catabolizing enzymes such as IDO1, kynurenines are emerging as key players in a number of such host–microbial interactions.

Although it has been shown to perform signaling functions independent of its enzymatic activity, IDO1 promotes a tolerant microenvironment primarily through its enzymatic function [42–44]. Local tryptophan depletion and kynurenine activation of the AhR are necessary for the peripheral generation of Tregs [45, 46]. Interestingly, inoculation of mice with *Clostridium* clusters IV and XIVa leads to IDO1 upregulation and an increase in Tregs, and imparted resistance to colitis [47]. A similar finding was reported in mice treated with *Lactobacillus salivarius* Ls33 peptidoglycan and subjected to TNBS induced colitis [48]. The IDO1-AhR pathway is also necessary to regulate systemic inflammation associated with lipopolysaccharide challenge, and was shown to protect against immunopathology associated with *S. enterica* Typhimurium and group B *Streptococcus* infection, indicating a role for kynurenines in disease tolerance [49]. The role of kynurenines in immune tolerance was further illustrated in mice lacking IDO1, which showed increased *C. difficile*-associated immunopathology even with a lower bacterial burden [16]. The enhanced pathology was attributed to an increased neutrophil density and overproduction of IFN-γ [16].

Host cells are not the sole source of kynurenines in the gut. In the absence of IDO1 in mice, a kynurenine of microbial origin engages the AhR and induces a state of IL-22-dependent immune tolerance [40]. This immune tolerance was not univer-

sal, however, as the host was protected against gastric colonization by *Candida albicans*, indicating a role for kynurenines in maintaining a pro-health microbiota [40]. Not surprisingly, the loss of IDO1 was also shown to enhance the innate immune response to commensal microorganisms [50]. Interestingly, this inappropriately enhanced innate immune response to the resident microbiota provided protection against *Citrobacter rodentium* colonization [50]. Taken together, these studies indicate that kynurenines are crucial in a number of host–microbial interactions involved in maintaining an immunologically tolerant environment within the gut epithelia, regulating the resident microbial populations, and protection from pathogen colonization.

Concluding Remarks and Future Directions

As summarized in Table 12.1, the robust impact of kynurenines on GI health and disease is being brought to light. As IDO1 is a potential target in the treatment of a number of GI diseases, IDO1 inhibitors, including 1-MT [51, 52] and INCB023843 [34], are currently under investigation and are showing promising results in clinical trials. The proposed effect of inhibiting IDO1 is to break immune tolerance against tumor-associated antigens [53], but the impact of these inhibitors on the normal GI tissues must be considered. In summary, kynurenines are emerging as important regulators of GI health, and the elucidation of the roles these molecules fulfil in numerous GI diseases will yield novel therapeutic approaches.

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Table 12.1 Impact of tryptophan catabolism along the kynurenine pathway on gastrointestinal physiology

Function	Molecular interactions	Associated pathology	References
Inflammatory response	TNFα; IFN-γ; TLRs; IL-17; IL-1β; IL-6	Infectious colitis; IBD; Dysbiosis; Cancer; Immunopathology	[10, 11, 13, 16, 29]
Promotion of tolerance	IL-10; IL-22; TGF-β; IgA; AhR	Immunopathology; Gut homeostasis; Cancer; Infectious colitis; IBD	[10, 45, 47, 48]
Epithelial physiology	β-Catenin; AhR; NAD^+; IL-27	Epithelial regeneration; IBD; Cancer	[18, 19, 27, 28, 54]

While not an inclusive list, this table summarizes key molecular players which interact directly or indirectly with tryptophan metabolizing enzymes and their metabolites. Interactions have been demonstrated in the GI tract and extrapolated from other organ systems

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Chapter 13
Role of Kynurenine Pathway in Insulin Resistance: Toward Kynurenine Hypothesis of Insulin Resistance and Diabetes

Gregory F. Oxenkrug

Abstract Diabetogenic effect of kynurenine (KYN) derivative of tryptophan (TRP), xanthurenic acid (XA), has been suggested last century. Recent interest to this hypothesis was stimulated by data revealing that TRP–KYN pathway can be upregulated by both chronic low-grade inflammation and stress, the two conditions involved in the pathogenesis of insulin resistance (IR), diabetes type 2 (T2D), and, probably, type 1 (T1D). Pro-inflammatory factors (e.g., interferons) and stress hormones (e.g., cortisol) activate enzymes catalyzing first two steps of TRP–KYN pathway: indoleamine- or tryptophan-2,3-dioxygenases and kynurenine 3-monooxygenase (KMO), resulting in increased formation of KYN and 3-hydroxyKYN (3-HK), respectively. In addition to overproduction of 3-HK, inflammation/stress-induced increased demand for pyridoxal 5′-phosphate (P5P) leads to functional deficiency of P5P. 3-HK is a substrate for two competing pathways dependent on P5P: formation of 3-hydroxyanthranilic acid (3-HAA) catalyzed by kynureninase (KYNase) and formation of xanthurenic acid (XA) catalyzed by 3-HK-transaminase (3-HKT). Since KYNase is more sensitive to P5P deficiency than 3-HKT, inflammation/stress-induced P5P deficiency inhibits KYNase and shifts downstream metabolism of overproduced 3-HK from formation of 3-HAA toward excessive formation of XA. Recent data revealed additional mechanisms of diabetogenic effects of XA, 3-HK, and their derivatives. This chapter suggests that dysregulation of KYN metabolism is one of the mechanisms mediating the impact of chronic low-grade inflammation and/or stress on pathogenesis of IR, T2D, and T1D. Up- and downstream 3-HK derivatives might be used as biomarkers predicting inflammation and/or stress-induced progression from IR to T2D. TRP–KYN pathway might be a new target for prevention and treatment of IR and diabetes.

Keywords Xanthurenic acid • Insulin resistance • Diabetes • Inflammation • Stress

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List of Abbreviations

3-HAA	3-Hydroxyanthranilic acid
3-HK	3-Hydroxykynurenine
3-HKT	3-HK transaminase
HCV	Hepatitis C virus
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IR	Insulin resistance
KAT	Kynurenine aminotransferase
KMO	Kynurenine 3-aminooxygenase
KYN	Kynurenine
KYNA	Kynurenic acid
KYNase	Kynureninase
NAD	Nicotinamide adenine dinucleotide
P5P	Pyridoxal 5′-phosphate
TDO	Tryptophan 2,3-dioxygenase
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TRP	Tryptophan
XA	Xanthurenic acid

Introduction

Diabetogenic effect of kynurenine (KYN) derivative of tryptophan (TRP), xanthurenic acid (XA), has been described by Japanese researchers [1], and role of kynurenines in diabetes mellitus was suggested in 1985 [2]. Recent interest to this hypothesis was stimulated by data on upregulation of TRP–KYN metabolism by chronic low-grade inflammation and stress (for review [3, 4]), the two conditions involved in the pathogenesis of insulin resistance (IR), diabetes type 2 (T2D), and, probably, type 1 diabetes [5–9].

The current chapter focuses on inflammation and stress-induced dysregulation of kynurenine (KYN) pathway of tryptophan (TRP) metabolism and diabetogenic effects of some of derivatives of KYN.

Inflammation/Stress and Tryptophan–Kynurenine Metabolism

Up- and Downstream 3-Hydroxykynurenine Metabolic Pathways

KYN pathway is the major nonprotein route of TRP metabolism leading to production of NAD^+. Initial step of TRP–KYN pathway, formation of KYN from TRP (via N-formyl-KYN), is catalyzed by tryptophan- or indoleamine-2,3-dioxygenases

(TDO or IDO, respectively). KYN conversion into 3-hydroxykynurenine (3-HK) is catalyzed by flavin adenine dinucleotide-dependent KYN-3-monooxygenase (KMO) [10].

Downstream metabolism of 3-HK depends on two pyridoxal 5′-phosphate (P5P)-dependent enzymes, kynureninase (KYNase), catalyzing conversion of 3-HK into 3-hydroxyanthranilic acid (3-HAA) along the NAD^+ biosynthetic pathway, and 3-HK-transaminase (3-HKT), catalyzing conversion of 3-HK into xanthurenic acid (XA) [11]. Additionally, P5P is involved in conversion of KYN into kynurenic acid (KYNA) catabolized by P5P-sensitive KYN aminotransferase (KAT) [10].

Inflammation/Stress Activation of Upstream 3-HK Metabolism

Inflammatory factors (e.g., interferons) activate IDO while stress factors (e.g., cortisol) activate hepatic and brain located TDO [10]. Both inflammatory and stress factors activate not only IDO/TDO but KMO as well [12, 13]. It is noteworthy that IDO and TDO could not be activated concurrently [14]. We did not find reports of studies assessing the possibility of concurrent activation of KMO by inflammation and stress. The end result of inflammation/stress on the initial steps of TRP–KYN pathway is increased formation of 3-HK.

Overproduction of 3-HK might not be sufficient to increase formation of XA. Thus, mouse lung infection, resulted in a 100-fold induction of IDO, was accompanied by a 16- and 3-fold increase of KYN and 3-HK levels, respectively, without increase of XA [15]. Therefore, overproduction of 3-HK is the necessary but not sufficient condition for increased formation of XA from 3-HK. P5P is a cofactor for key enzymes of downstream 3-HK metabolism.

Inflammation/Stress-Induced Functional P5P Deficiency Dysregulation of Downstream 3-HK Metabolism

P5P, the active form of vitamin B6, is a cofactor for over 200 enzymes activated in response to inflammation and stress. Increased demand for P5P leads to functional deficiency of P5P. Low levels of plasma P5P are associated with a variety of inflammatory conditions independent of dietary intake or excessive catabolism of the vitamin B6, or congenital defects in B6 metabolism, and may represent a passive consequence of increased utilization of P5P [16] (Figs. 13.1 and 13.2).

Because KYNase is more sensitive to P5P deficiency than 3-HKT [17], P5P deficiency leads to inhibition of KYNase and, consequently, to shifting of excessively produced 3-HK from formation of 3-HAA toward production of XA [18–20].

Stress-induced P5P deficiency affects up- and downstream 3-HK metabolism in a dose-dependent manner: moderate deficiency yielded increased 3-HK levels while more severe P5P deficiency resulted in an additional increase in XA and KYN [21].

Vitamin B6 depletion drastically increased while vitamin B6 supplementation normalized urinary 3-HK and XA excretion after TRP load in cardiac and obese

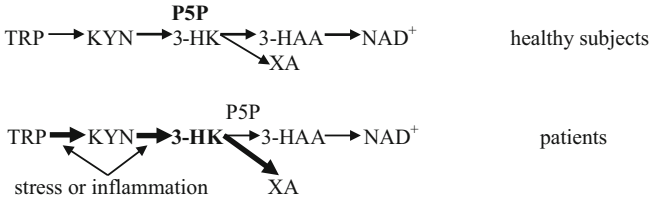

Fig. 13.1 Inflammation/stress and P5P deficiency: effect on TRP–KYN metabolism. *Abbreviations*: *TRP* tryptophan, *KYN* kynurenine, *3-HK* 3-hydroxyKYN, *P5P* pyridoxal 5'-phosphate, *3-HAA* 3-hydroxyanthranilic acid, *NAD*+ nicotinamide adenine dinucleotide, *XA* xanthurenic acid

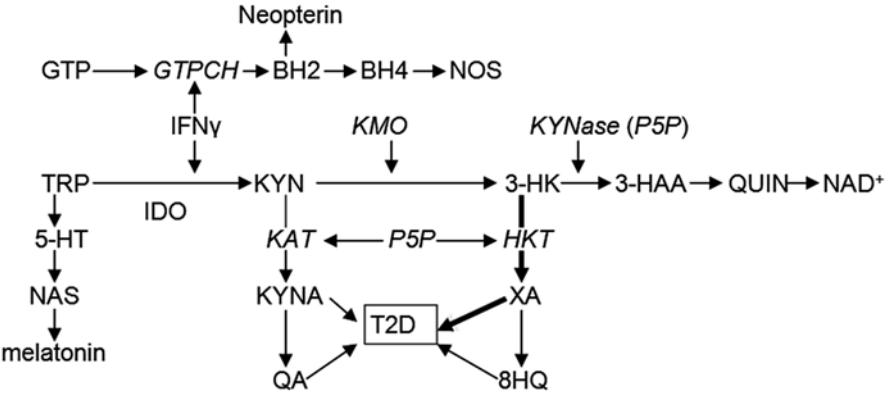

Fig. 13.2 TRP–KYN metabolic pathways and T2D. *Abbreviations*: *TRP* tryptophan, *IFN-γ* interferon-γ, *IDO* indoleamine 2,3-dioxygenase, *KYN* kynurenine, *KMO* KYN-3-monooxygenase, *3-HK* 3-hydroxyKYN, *P5P* pyridoxal 5′-phosphate, *3-HAA* 3-hydroxyanthranilic acid, *QUIN* quinolinic acid, *NAD*+ nicotinamide adenine dinucleotide, *KYNA* kynurenic acid, *XA* xanthurenic acid, *QA* quinaldic acid, *8-HQ* 8-hydroxyquinaldic acid, *GTP* guanosine triphosphate, *GTPCH* GTP cyclohydrolase I, *BH2* 7,8-dihydroneopterin, *BH4* tetrahydrobiopterin, *T2D* type 2 diabetes, *5-HT* serotonin, *NAS* N-acetylserotonin

patients and rats [22–24]. Therefore, the above mentioned literature suggests that inflammation and stress, two conditions prominently associated with pathogenesis of diabetes, stimulate production of XA because of (a) an excessive formation of KYN and 3-HK due to activation of IDO/TDO and KMO and (b) shifting of 3-HK metabolism from formation of 3-HAA toward production of XA due to inflammation/stress-associated decreased availability of P5P as a cofactor for the KYNase.

Diabetogenic Effects of Kynurenine Derivatives

Experimental Studies

Diabetogenic Effect of Xanthurenic Acid XA was isolated as a yellow crystal of substance from the urine of rats fed with fibrin [25]. Pyridoxine administration resulted in disappearance of XA from urine of rats fed with pyridoxine deficient but TRP rich diet. It was concluded that XA is an abnormal metabolite of TRP when the body is on a pyridoxine deficient diet [26]. XA was found in the urine of humans, rabbits, dog, and guinea pig [27, 28].

XA induced experimental diabetes in rats [1]. Elevated urinary excretion of XA was found in alloxan- and streptozotocin-induced diabetic rats [22, 29, 30], while decreased activity of P5P-dependent KYNase was observed in alloxan-induced diabetic rabbits [18]. A metabolomic study revealed a strong association between alloxan and XA in rats fed with high caloric diet [31].

Much faster disappearance of TRP from the bloodstream was observed in streptozotocin-diabetic rats after TRP load [32]. Surplus of dietary TRP induced IR in pigs [33].

Clinical Studies

Increased urine XA excretion, accompanied by reduction of 3-HAA after TRP load, was reported in prediabetic [34] and diabetic patients [1]. Increased expression of IDO, decreased plasma TRP concentrations, and increased KYN and 3-HK were reported in patients with T1D and T2D in comparison with healthy controls patients [35]. XA was identified by a thin-layer chromatogram in epidermis of diabetes patients [36].

Low plasma concentrations of P5P have been reported in conditions associated with increased fasting glucose and glycated hemoglobin [37].

Insulin Resistance and Kynurenine Metabolism

Upregulated TRP–KYN metabolism and increased concentrations of 3-HK and XA were reported in neurodegenerative disorders [38] and depression [39] conditions associated with high incidence of IR [40]. Hepatitis C virus (HCV) patients have fourfold higher incidence of IR than healthy controls or chronic hepatitis B patients

[41]. Although HCV per se is diabetogenic [42], presence of HCV alone does not affect IR [43]. Upregulated IDO expression in liver [44], dendritic cells [45], and increased serum KYN/TRP ratio [44] were reported in HCV patients. We found correlations between plasma levels of KYN and homeostasis model of insulin resistance assessment (HOMA-IR) scores and between KYN and HOMA-beta (pancreatic beta cell function in HCV patients). Serum KYNA concentrations correlated with KYN but not with TRP concentrations [46]. Our study of only upstream 3-HK metabolites suggests the possibility of involvement of downstream 3-HK metabolites in pathogenesis of IR and diabetes.

Plasma concentrations of neopterin, an indirect marker of IFN-γ-induced upregulation of KYN metabolism [47], positively correlated with IR in Caucasian [48–51] and Puerto Rican populations [52], and negatively correlated with P5P levels, suggesting association of inflammation (elevated neopterin concentrations) with P5P deficiency [52, 53].

Possible Mechanisms of Diabetogenic Effects of XA

XA might contribute to development of diabetes via (a) induction of pathological apoptosis of pancreatic beta cells through XA-induced activation of caspase 3 [54]; (b) binding of XA with Zn++ in beta cells with formation of complexes exerting toxic effect on pancreatic beta cells [55]; (c) inhibition of pro-insulin synthesis in pancreatic beta cells [56]; (d) inhibition of glucose [56] and leucine-induced insulin release from pancreas [57]; and (e) binding of XA to histidine and tyrosine of the insulin molecule with formation of complexes that are indistinguishable from insulin as antigens but having 49 % lower activity than pure insulin [30].

XA-induced inhibition of KYNase might sustain the elevated levels of 3-HK and KYN at the expense of NAD^+ production [58] while XA-induced inhibition of pyridoxal kinase, the enzyme catalyzing the formation of P5P from vitamin B6, might perpetuate P5P deficiency [59]. P5P deficiency-induced downregulation of KYNase leads to decreased formation of NAD+ resulting in inhibition of synthesis and secretion of insulin and the death of pancreatic beta cells [22].

Pro-inflammatory cytokine, IFN-γ, is one of the most powerful transcriptional inducers of IDO [60] and KMO [12]. Changes in KYN metabolism are likely to mediate IFN-γ-induced apoptotic death of pancreatic beta cells [61, 62]. IFN-γ effect was not related to nitric oxide generation, suggesting that IFN-γ-induced death of pancreatic cells were mediated by pro-oxidative KYN metabolites (e.g., quinolinic acid) [63]. These studies, however, did not assess the possible involvement of XA in the apoptotic effect of IFN-γ on pancreatic islets.

Involvement of TRP–KYN metabolism in pathogenesis of diabetes might be further supported by anti-diabetic effect of berberine [64] and minocycline [65], known to inhibit KYN formation from TRP [66–68].

Conclusion

Kynurenine hypothesis of diabetes synthesizes evidences of diabetogenic effects of XA and of effects of chronic low-grade inflammation and/or stress on TRP–KYN metabolism to suggest that dysregulation of TRP–KYN metabolism is one of mechanisms mediating the impact of chronic low-grade inflammation/stress on pathogenesis of T1D and progression from IR to T2D. It proposes that dysregulation of KYN metabolism consists of: (a) upregulation of TRP conversion into KYN and KYN conversion into 3-HK due to inflammation or stress-induced activation of IDO/TDO and KMO and (b) downregulation of utilization of 3-HK as a substrate for formation of 3-HAA due to inflammation/stress-associated functional deficiency of P5P. Combination of increased formation of 3-HK with decreased availability of 3-HK as a substrate for 3-HAA results in upregulation of utilization of 3-HK for formation of diabetogenic 3-HK derivative, XA.

Kynurenine hypothesis does not propose that dysregulation of TRP–KYN metabolism is the cause of diabetes development. Alternatively, there is a possibility that diabetes might trigger dysregulation of TRP–KYN metabolism. Thus, insulin dose-dependently decreases urine excretion of XA in rats after TRP load. However, dysregulation of TRP–KYN metabolism exacerbates the development of T1D and contributes to progression from IR to T2D. Therefore, indices of TRP–KYN metabolism might be used as novel biomarkers of risk for diabetes in addition to traditional clinical risk factors. KYN pathway of TRP metabolism might be a new target for prevention and treatment of diabetes.

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Conflict of Interest and Disclosure Author reports no conflict of interest.

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Chapter 14
Role of Kynurenine Pathway in Infections

Silvia Kathrin Eller and Walter Däubener

Abstract Tryptophan is an essential amino acid, not only for mammals but also for a broad range of pathogens. The majority of the dietary tryptophan is degraded via the kynurenine pathway to kynurenine and other downstream tryptophan metabolites, together termed "kynurenines". The induction of the kynurenine pathway in the context of infections is currently the focus of many human and mouse studies. It has been shown that infections with viruses, parasites and bacteria result in a reduction in tryptophan levels and enhanced levels of kynurenines in the plasma/serum and cerebrospinal fluid. These changes influence the survival of pathogens and the activity of the immune system, respectively. On the one hand, low levels of tryptophan mediate antimicrobial effects through tryptophan starvation but also inhibit an adequate immune defence. On the other hand kynurenines exert toxic effects on both pathogens and the cellular immune system. The interplay of all these antimicrobial and immunoregulatory effects of kynurenines is the focus of this chapter.

Keywords Kynurenines • Infection • Interferon-gamma • Pathogen • Bacterium • Virus • Parasite • Indoleamine 2,3-dioxygenase

List of Abbreviations

1-MT	1-Methyl tryptophan
3-HK	3-Hydroxykynurenine
3-HAA	3-Hydroxyanthranilic acid
CSF	Cerebrospinal fluid
DCs	Dendritic cells

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IC_{50}	Half maximal inhibitory concentrations
IDO	Indoleamine 2,3-dioxygenase
IDO-2	Indoleamine 2,3-dioxygenase-2
IFN-γ	Interferon-γ
KYN	Kynurenine
Mtb	*Mycobacterium tuberculosis*
NO	Nitric oxide
PA	Picolinic acid
QUIN	Quinolinic acid
TDO	Tryptophan 2,3-dioxygenase
TRP	Tryptophan

The Kynurenine Pathway in Infections

The kynurenine (KYN) derivates 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA), picolinic acid (PA) and quinolinic acid (QUIN), referred to as the "kynurenines", can readily enter the bloodstream and are able to serve as messengers that affect blood pressure [1], neuronal function [2] and also the immune system [3]. This chapter will focus on the role of the KYN pathway in response to infections.

The enzymes indoleamine 2,3-dioxygenase (IDO), indoleamine 2,3-dioxygenase-2 (IDO-2) and tryptophan 2,3-dioxygenase (TDO) catalyse the degradation of the essential amino acid tryptophan along the KYN pathway. Despite large variations in their expression pattern in various organisms, all enzymes promote the oxidative cleavage of tryptophan (TRP) to N-formylkynurenine that is further converted to KYN. The resulting KYN can be converted locally to the downstream derivates mentioned above.

Although IDO, IDO-2 and TDO provide the same basis for the KYN pathway by producing KYN, it has been well established that IDO has a key position in the field of immune regulation. Despite a low constitutive expression in mammalian cells, IDO expression is markedly induced in confined cell types during infection or inflammation. It has been shown that IDO is highly expressed in professional antigen-presenting cells, such as dendritic cells (DCs) in response to LPS as well as to cytokines, especially interferon-gamma (IFN-γ). Since IFN-γ itself alters the activity of other enzymes along the KYN pathway, it is also considered a major regulator of the production of kynurenines [4]. Furthermore, KYN itself signals via an autocrine signalling loop involving the aryl-hydrocarbon receptor, resulting in an enhanced tryptophan degradation as shown in human cancer cells [5] (❶ in Fig. 14.1).

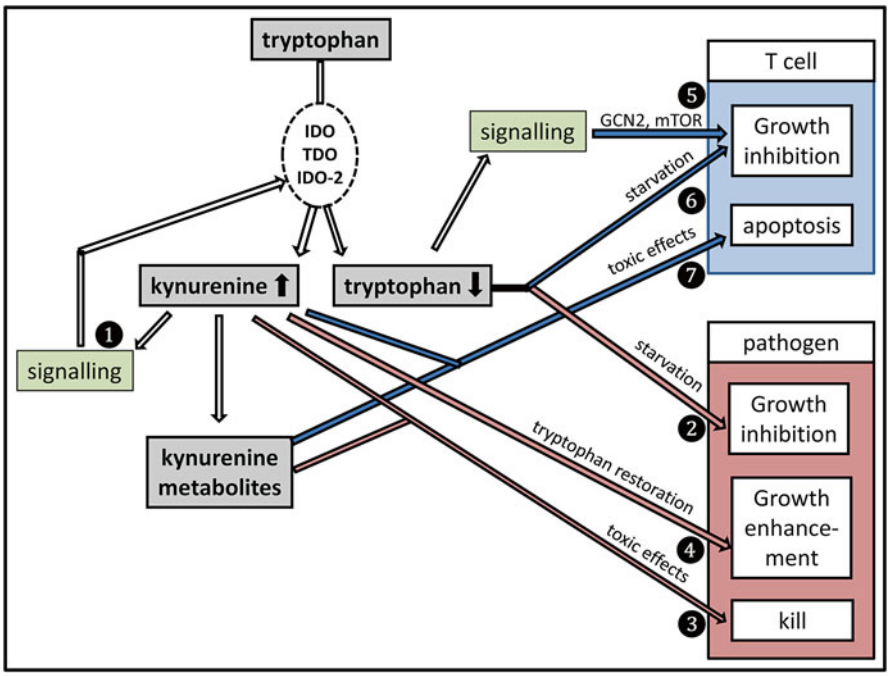

Fig. 14.1 Influence of tryptophan-pathway products on T cells and pathogens in infectious diseases. The mechanisms of the antimicrobial and immunoregulatory effects are mentioned in the text and depicted by encircled numbers

Upregulation of Kynurenine Pathway During Infectious Diseases in Humans

Studies investigating different pathological conditions in humans revealed a major role of the IDO-dependent KYN pathway in pathogenesis. The upregulation of the KYN pathway was not only observed in neurological disorders, autoimmune diseases and malignancies but also during infectious diseases. Probably the best studied disease in this context is the infection with the human immunodeficiency virus (HIV).

Kynurenines in Viral Diseases

It was shown by several groups that HIV patients exhibit lower serum TRP levels as compared to healthy controls (e.g. 44.6 vs. 52.6 μM) accompanied by increased levels of KYN (e.g. 4.1 vs. 2.7 μM) and IFN-γ (259 vs. 23.5 U/L) [6]. This acceleration of TRP degradation and the resulting production of KYN were detected not

only in serum but also in cerebrospinal fluid (CSF) or brain tissue of HIV-infected patients [6]. Interestingly, one study even revealed that the elevated KYN/TRP ratio predicted the onset of AIDS in HIV-infected patients, discriminating between asymptomatic and symptomatic stages of the disease [7]. However, not only KYN but also QUIN levels were elevated in sera of HIV patients (e.g. 848 vs. 303.3 nM), correlating with the progression of disease as demonstrated by the fact that QUIN levels were significantly reduced during the highly active antiretroviral therapy HAART [8]. Altered levels of kynurenines were also found in humans suffering from other chronic viral infections. For example, it was observed that IDO is expressed in the liver of hepatitis B virus- or hepatitis C virus-infected patients, which was concomitant with increased KYN/TRP ratios and advanced liver inflammation and fibrosis [9].

Kynurenines in Parasitic Diseases

Beside viral infections, infections with parasites also lead to an IDO-mediated production of kynurenines. A study of patients with cerebral malaria caused by an infection with *Plasmodium falciparum* revealed an increase in QUIN, KYNA and PA levels in CSF. Again, elevated levels of QUIN and PA were associated with a higher rate of convulsions and a fatal outcome, although the levels of these metabolites were detected only in a nanomolar range (QUIN = 90 nM; PA = 180 nM) [10]. In contrast, TRP and KYN levels and their ratio did not significantly differ in *Toxoplasma gondii* (*T. gondii)* sero-positive and sero-negative pregnant patients [11]. However, since these patients were chronically infected with Toxoplasma, this study cannot provide information about the involvement of the KYN pathway during the acute phase of infection.

Kynurenines in Bacterial Diseases

The KYN pathway is also markedly induced during major bacterial infections such as tuberculosis or bacterial sepsis in human beings [12, 13]. All analyses revealed that the magnitude of IDO induction, as determined by the KYN/TRP ratio in the sera, correlated with the severity of the diseases. Therefore, the induction of IDO and the accompanying changes in the KYN/TRP ratio were proposed as diagnostic marker, e.g. for the therapy of tuberculosis [12, 13].

In summary, the induction of the KYN pathway seems to be a hallmark for an IFN-γ dominated immune response during major infectious diseases in humans. Nevertheless, the functional consequence of TRP starvation and/or production of kynurenines on the replication and survival of microorganisms remain unclear.

Direct Antimicrobial Effects Mediated by Kynurenines

Within the last decades, several in vitro studies have been performed investigating the effect of IDO-mediated TRP degradation and KYN production on the growth of different pathogens. Most of these studies concentrated on IFN-γ induced IDO activity in different human cell types such as fibroblasts or epithelial cells.

IDO activity in these cells could effectively inhibit the growth of bacteria (e.g. Chlamydia [14] or enterococci [15]), viruses (e.g. Cytomegalovirus [16] or Herpes simplex virus type 2 [17]) and parasites (e.g. *Neospora caninum* [18]). Most interestingly, these antimicrobial effects were only observed towards TRP auxotroph microbes, and the growth inhibition was abrogated by the supplementation of TRP or by the addition of the IDO-specific inhibitor 1-methyl tryptophan (1-MT). Therefore, the antimicrobial effect was strictly assigned to the IDO-mediated lack of the essential amino acid TRP (❷ in Fig. 14.1).

Of note, emerging data have also demonstrated a direct antimicrobial activity of kynurenines (❸ in Fig. 14.1). In an antimicrobial assay, 3-HK and PA could significantly reduce the growth of methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, *Escherichia coli* and multidrug-resistant *Pseudomonas aeruginosa* (MDRP) [19]. However, the lowest half maximal inhibitory concentration (IC_{50}) of all metabolites that was effective was 14 μg/mL (113.7 μM) for PA, leaving the question as to whether these experiments can be considered physiological, since these high micromolar concentrations of kynurenines have never been measured in patients (Table 14.1). The exact concentration of kynurenines reached locally in infected human tissue or cells has not yet been determined. Another study underlining the impact of kynurenines was the recently described growth inhibition of *Listeria monocytogenes* (*L. monocytogenes*) in the presence of KYN and 3-HK [20]. Both metabolites were clearly bactericidal for *L. monocytogenes* with an IC_{50} of about 12 μM. This concentration was in approximation of levels detected by the same group in supernatants of IDO-expressing human DC in vitro. In contrast to *L. monocytogenes* growth inhibition, KYN and 3-HK had a weaker effect on *Streptococcus pyogenes* growth and no influence on *Listeria innocua* survival [20]. The observed differences in the sensitivity of diverse bacteria to various kynurenines make it tempting to conclude that KYN-pathway metabolites act highly pathogen-specific.

Support for this pathogen specificity comes from the fact that not all pathogens are TRP auxotroph and that some of them are able to synthesize TRP themselves (❹ in Fig. 14.1). Interestingly, the intracellular bacterium *Chlamydia psittaci* (*C. psittaci*) has the potential to synthesize its own supply of TRP from host-generated KYN by usage of kynureninase and a number of tryptophan-pathway genes [21]. Thus, *C. psittaci* has the ability to recruit host-derived defence molecules for its own use. Such capability represents a special feature even among other Chlamydia strains.

Another interesting observation is the fact that the bacterium *Pseudomonas aeruginosa* (*P. aeruginosa*), isolated from cystic fibrosis patients, produces high levels

Table 14.1 Concentrations of kynurenine metabolites that have been detected in plasma/sera or cerebrospinal fluid (CSF) in humans and concentrations of kynurenine metabolites effective in in vitro and in vivo studies

	Metabolite concentration			In vitro/in vivo effectivity [IC_{50}]	
	Source	Healthy control	Pathologic situation	Pathogen/Cell	Concentration
TRP	Plasma/Serum	67.3 ± 18.5 µM [6]	28.4–143.3 µM [6]	*S. aureus*, Enterococci[a] T cell inhibition[a]	≤1.0–1.5 µM [36] ≤0.098 µM [36]
	CSF	1.9 ± 0.6 µM [6]	1.3 nM–5.0 µM [6]		
KYN	Plasma/Serum	2.4 ± 0.8 µM [6]	1.9–70.4 µM [6]	*L. monocytogenes*[a] T cell inhibition[a]	12 µM [20] 553 µM [37]
	CSF	38.4 ± 15.7 nM [6]	0.002–2.6 µM [6]		
KYNA	Plasma/Serum	36.9 ± 16.2 nM [6]	24.3–57.8 nM [6]	n.a.	n.a.
	CSF	1.8 ± 1.0 nM [6]	0.002–0.1 µM [6]		
3-HK	Plasma/Serum	0.38 ± 0.02 µM [6]	0.38 µM [6]	MRSA, MDRP *S. epidermidis, E. coli*[a] *L. monocytogenes*[a] *T. cruzi*[a] *T. cruzi*[b] T cell inhibition[a]	≥107 µM [19] 12 µM [20] 20 µM [24] ≥1 mg/kg/day [24, 25] 187 µM [37]
	CSF	n.d. [45]	n.a.		
Quin	Plasma/Serum	0.37 ± 0.1 µM [6]	0.07–16.8 µM [6]	MRSA, MDRP *S. epidermidis, E. coli*[a]	≥842.5 µM [19]
	CSF	23.2 ± 9.3 nM [6]	0.03–3.8 µM [6]		
AA	Plasma/Serum	19.0 ± 3.5 nM [6]	139 nM [6]	MRSA, MDRP *S. epidermidis, E. coli*[a]	≥700 µM [19]
	CSF	16.3 ± 4.2 nM [6]	n.a.		
3-HAA	Plasma/Serum	16.0 ± 11.2 nM [6]	1.04–24.5 nM [6]	*T. cruzi*[a] T cell inhibition[a]	20 µM [24] 96 µM [37]
	CSF	n.d. [6]	n.a.		
PA	Plasma/Serum	2.4 ± 0.4 µM [6]	1.4 µM [6]	MRSA, MDRP *S. epidermidis, E. coli*[a]	≥113.7 µM [19]
	CSF	0.5 ± 0.1 µM [6]	0.2–0.4 µM [6]		

[a]in vitro
[b]in vivo
n.d. not detectable
n.a. not available

of KYN on its own. Following contact with neutrophils, *kynA*, the first gene involved in the KYN pathway, has been found to be transcriptionally activated, which in turn allowed an effective production of KYN in *P. aeruginosa*. This KYN inhibited reactive oxygen species (ROS) in activated neutrophils by scavenging hydrogen peroxide and, to a lesser extent, superoxide [22]. Thus, bacteria-derived KYN was able to promote the survival of its own species. Since this virulence factor could be a widespread strategy throughout all bacteria that possess a functional *kynA* gene or comparable genes, further studies on this subject will be of great interest.

Upregulation and Function of Kynurenines During Infectious Diseases in Mice

To further analyse the possible role of the KYN pathway in the defence against infections in vivo, animal models have to be employed. However, the capacity of specific cell types to express IDO in in vitro experiments differs between human or murine cell lines [23]. Using mice as an in vivo model system, it has been shown in several studies that the KYN pathway is indeed induced upon infections. BALB/c mice that had been infected with the parasite *Trypanosoma cruzi* (*T. cruzi*) expressed IDO protein in spleen, skeletal muscle and heart, while it was absent in uninfected mice [24]. As expected, KYN was produced in these tissues and enriched in the circulation over time. The induction of the KYN pathway seemed to be beneficial for the host as a blockade of IDO production by the administration of 1-MT resulted in an impaired resistance to the infection, exacerbated infection-associated pathology and parasite load. More detailed in vitro analyses of this antiparasitic effect revealed that 3-HK and 3-HAA (20 μM) promoted the inhibition of intracellular *T. cruzi* replication and, additionally, that 3-HK was toxic against the trypomastigote stage found in the bloodstream. Finally, the therapeutic treatment of *T. cruzi*-infected mice with 3-HK (1–500 mg/kg/day) controlled the parasite load, reduced cardiac lesions and improved the survival of the mice [24, 25]. All these data underline an antiparasitic action of KYN pathway metabolites in mice in vivo during the course of an infection with *T. cruzi.*

Other in vivo studies also revealed an inhibitory role of IDO activity on the growth of bacteria. The infection with *Francisella novicida* (*F. novicida*) resulted in a strong organ-specific upregulation of IDO in the lungs of mice [26]. In this study, it was shown that the bacterial genes involved in TRP biosynthesis were required for *F. novicida* survival, since the ablation of these genes resulted in an attenuation of bacterial growth. This growth inhibition of TRP biosynthesis mutants could be partially rescued in the lungs of $IDO^{-/-}$ mice, indicating that the observed growth inhibition was due to TRP starvation [26].

Similar results have been obtained in a murine infection model utilizing *Mycobacterium tuberculosis* (Mtb). CD4 T cells made up an important defence against Mtb infections by activating infected macrophages. As a consequence, IDO

expression within the macrophages resulted in the depletion of the cytoplasmic TRP pool, initiating mycobacterial TRP biosynthesis for bacterial survival. The loss or inhibition of TRP biosynthesis renders Mtb hypersusceptible to IFN-γ-mediated killing within macrophages in vitro and in vivo [27]. Therefore, the KYN pathway is, at least in part, involved in the defence against Mtb.

Further studies in mice revealed also a strong IDO induction in the early phase of a *T. gondii* infection. Especially in lung tissue, high IDO expression was found which corresponded to the complete degradation of the local TRP pool and resulted in a substantial production of KYN [28]. The consideration of all these data prompted the assumption that the IDO-mediated TRP degradation acts as an effective defence mechanism in the early stage following an infection with toxoplasma in mice. The observation that $IDO^{-/-}$ animals exhibited no difference in their condition after *T. gondii* infection, neither in their survival rate nor in an accelerated weight loss, was unexpected [29]. Moreover, although depletion of lung tryptophan did not occur in these $IDO^{-/-}$ animals, the parasite load was not enhanced [29]. This leads to the speculation that in mice, IDO rather benefits the parasite than being a main antiparasitic regulator in vivo. Further studies with other pathogens will be needed to obtain better insights into the complex mechanisms that occur during infections in vivo. However, there is a multitude of studies showing that IDO is not the main antimicrobial effector molecule in mice, whereas nitric oxide (NO) produced by the inducible nitric oxide synthase [30], immunity-related GTPases [31] and guanyle binding proteins like mGBP2 [32] are well-known antimicrobial effectors in the murine system.

Role of the Kynurenine Pathway in the Regulation of the Immune System

The immunoregulatory function of IDO was first described by Mellor and Munn in 1998 [33]. This group published that the administration of 1-MT would cause rejection of semiallogeneic foetuses in mice, arguing for a regulatory function of IDO during pregnancy. Indeed, several mechanisms by which TRP degradation and KYN production modify immune cells have been identified in the meantime. Firstly, the starvation of the amino acid TRP itself activates the general control non-derepressible-2 kinase (GCN2 kinase) and inhibits the master metabolic regulator mTOR leading to an arrest of protein synthesis and therefore preventing T cell proliferation [34, 35] (❺ in Fig. 14.1). Secondly, T cells are sensitive towards TRP starvation and certain kynurenines in vitro [36] (❻ in Fig. 14.1). In line with this KYN, 3-HK and 3-HAA exert a cytotoxic action on activated $CD3^{+}$T cells [37, 38] (❼ in Fig. 14.1). Additionally, these kynurenines also dispatch B cells and natural killer cells, whereas DCs are not affected, having far reaching consequences for immunoregulated processes as they appear during infections. Thirdly, the generation of regulatory T cells (Treg) is induced upon exposure of naïve T cells to

IDO-expressing DCs [39]. In this context, small numbers of IDO^+ DCs are able to mediate a remarkable immunosuppressive potency, also referred to as "infectious tolerance". Considering this powerful impact of the KYN pathway on the immune system, it is hardly surprising that pathogens have acquired strategies that exploit the function. Infectious pathogens provoke tissue inflammation that is attended by a strong cytokine release and induction of IDO activity in immune cells such as macrophages and NK cells [40]. For example, Leishmania parasites induce IDO expression in human myeloid-derived DCs that suppress the allogeneic lymphocyte proliferation and thereby may circumvent immune clearance within the infection microenvironment [41]. Such parasitic exploitation of the IDO-mediated immunoregulatory effect is also observed during murine *Leishmania major* (*L. major*) infections in vivo. The administration of 1-MT reduced parasite burdens within infected mice, revealing a constitutive requirement for IDO to maintain pathogenesis [42]. In line with this observation are new findings that Tregs present in *L. major* infection sites proliferated specifically in response to *L. major*-infected DCs, that they were restricted to sites of infection and that their survival was strictly dependent on parasite persistence [43]. All these data suggest that host Tregs are programmed by the parasites to suppress host cell immunity.

Other murine infection models using murine leukaemia virus LP-BM5 or the encephalomyocarditis virus also revealed a tolerogenic role of the KYN pathway. $IDO^{-/-}$ mice that have been infected with one of those viruses presented an upregulated level of type I interferons and a reduced virus replication [44, 45]. The same effect was observed after the administration of the IDO inhibitor 1-MT. Additionally, the authors could show that the induction of a protective tolerance during infection was due to the presence of kynurenines. When infected $IDO^{-/-}$ mice were treated with a mixture of kynurenines (20 mg/kg/d KYN, 3-HK and 3-HAA), the effect of the IDO knockdown was eliminated. This suggests that kynurenines regulate the production of type I interferons by decreasing the number of macrophages, which are actively involved in all phases of inflammation as effector and regulatory cells [46].

Concluding Remarks

In summary, the dual regulatory role of IDO and the KYN pathway in the pathophysiology of infectious diseases is complex. Both the type of pathogen and the stage of disease determine whether the production of kynurenines has a detrimental or tolerogenic effect on the pathogen. IDO is a natural immune-regulatory substance that is exploited by infectious pathogens to maintain survival within hosts. Therapeutic interventions such as the induction of the KYN pathway, the control of systemic TRP concentrations, the regulation of TRP synthesising genes or the administration of kynurenines might be beneficial for patients with acute or chronic infections.

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Part III
CNS Disorders

Chapter 15
The Role of the Kynurenine Pathway in Neurodegenerative Diseases

Zsófia Majláth, József Toldi, and László Vécsei

Abstract The kynurenine pathway, the main metabolic route of tryptophan degradation, produces several neuroactive molecules. Alterations in the kynurenine pathway have been described in a number of neurological disorders; a feature of special importance is the elevation of neurotoxic metabolites, which may promote glutamate-mediated excitotoxic neuronal damage. The delicate balance between the neurotoxic and neuroprotective compounds participating in the kynurenine pathway has been suggested to play an important role in the regulation of glutamatergic neurotransmission and in inflammatory processes. Synthetic kynurenic acid derivatives and kynurenine-monooxygenase inhibitors have proved in several preclinical studies to have beneficial effects. The kynurenine pathway offers a promising target for future drug development with the aim of achieving neuroprotection.

Keywords Neurodegenerative disorders • Neuroprotection • Kynurenine pathway • Parkinson's disease • Huntington's disease • Amyotrophic lateral sclerosis • Multiple sclerosis

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List of Abbreviations

3-HANA	3-Hydroxyanthranilic acid
3-OH-KYN	3-Hydroxykynurenine
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CNS	Central nervous system
CSF	Cerebrospinal fluid
EAE	Experimental autoimmune encephalomyelitis
Glu	Glutamate
HD	Huntington's disease
IDO	Indoleamine-2,3-dioxygenase
KAT	Kynurenine aminotransferase
KMO	Kynurenine monooxygenase
KP	Kynurenine pathway
KYN	Kynurenine
KYNA	Kynurenic acid
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple sclerosis
NMDA	*N*-methyl-D-aspartate
PD	Parkinson's disease
QUIN	Quinolinic acid
TDO	Tryptophan-2,3-dioxygenase
Trp	Tryptophan

Introduction

Although neurodegenerative disorders are demonstrating an increasing prevalence, our understanding of their pathobiochemical background is still limited. These devastating diseases have a serious impact on the quality of life of the patients affected, and their pharmacological management is often not satisfactory. Therapeutic approaches are mainly symptomatic, and drugs capable of modifying disease progression or offering neuroprotection are still awaited. The exact neurochemical and molecular processes in the pathomechanisms of neurodegenerative disorders have not yet been clarified, though several common features have been described. Mitochondrial disturbances, neuroinflammation, and oxidative stress have been implicated in the pathological course of neurodegeneration [1–3]. Glutamate (Glu) is the main excitatory neurotransmitter in the central nervous system (CNS), but overactivation of the Glu receptors may lead to neuronal damage; this process, excitotoxicity, has also been linked to the pathomechanisms of several acute and chronic neurological diseases [4]. An energy impairment as a result of a mitochondrial

disturbance may cause partial membrane depolarization, leading to relief from the voltage-dependent magnesium (Mg^{2+}) blockade of the *N*-methyl-D-aspartate (NMDA) receptors. In this case, even a physiological Glu concentration may induce excitotoxic neuronal damage [5].

The kynurenine pathway (KP) is the main route of tryptophan (Trp) degradation leading to the production of nicotinamide adenine dinucleotide [6]. The KP produces several neuroactive metabolites, some of which are capable of influencing the Glu receptors. The first and rate-limiting step of the pathway is catalyzed by the enzyme indoleamine-2,3-dioxygenase (IDO), which can be induced by inflammatory cytokines. This step can also be catalyzed by tryptophan-2,3-dioxygenase (TDO), which is mainly present in the liver. The metabolic route has two main branches, dividing at the key intermediate, L-kynurenine (KYN). KYN is either metabolized by the enzyme kynurenine-aminotransferase (KAT) to kynurenic acid (KYNA) or through a cascade of enzymatic steps that produces several toxic compounds, such as 3-hydroxykynurenine (3-OH-KYN), 3-hydroxyanthranilic acid (3-HANA), and quinolinic acid (QUIN) [6, 7]. The two branches of the KP are separated in different cell types in the CNS: KATs are localized mainly in the astrocytes, which are therefore primarily responsible for KYNA synthesis, while microglial cells harbor mainly kynurenine-monooxygenase (KMO), the enzyme responsible for 3-OH-KYN synthesis (Fig. 15.1) [8–10].

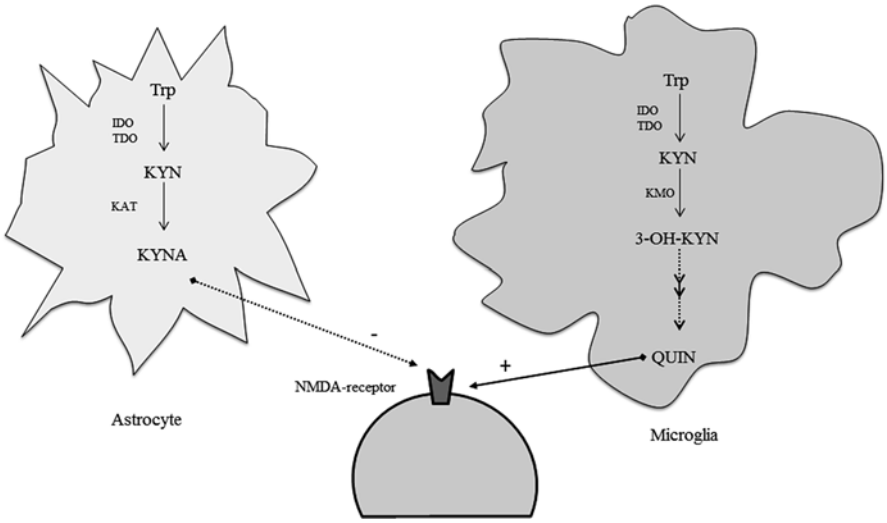

Fig. 15.1 The kynurenine pathway. The KP is the main route of Trp metabolism, which produces the only known endogenous neuroprotective compound, KYNA. The pathway is responsible for the synthesis of several neurotoxic molecules as well: the free radical generator 3-OH-KYN and the NMDA agonist QUIN. The synthesis of the different neuroactive metabolites can be attributed to different cell types in the CNS. The astrocytes do not contain KMO, and produce therefore only KYNA, while microglia release mainly the neurotoxic metabolites of the KP. *Abbreviations*: *3-OH-KYN* 3-hydroxykynurenine, *CNS* central nervous system, *KMO* kynurenine-3-monooxygenase, *KP* kynurenine pathway, *KYNA* kynurenic acid, *QUIN* quinolinic acid, *Trp* tryptophan

KYNA is the only known endogenous neuroprotective compound; its beneficial effects have been proved in multiple preclinical studies [11]. It is a broad-spectrum endogenous antagonist of Glu receptors, with the highest affinity for the strychnine-insensitive glycine site of the NMDA receptor [12–14]. On the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, KYNA exhibits a concentration-dependent dual effect: in nanomolar concentrations it facilitates these receptors, while in the micromolar concentration range it antagonizes them [15–17]. It is also a noncompetitive inhibitor of the α7-nicotinic acetylcholine receptor and a ligand of the previously orphan G protein-coupled receptor GPR35 [18, 19]. In response to KYNA, α7-nicotinic acetylcholine receptors and GPR35 receptors both reduce extracellular Glu release and therefore excitatory neurotransmission [20, 21]. 3-OH-KYN and 3-HANA are potent free radical generators, while QUIN is a neurotoxic compound exerting its effect mainly by NMDA agonism [22, 23].

Increasing evidence suggests that alterations in the kynurenine metabolism might play a role in the neurodegenerative process and are also implicated in immunological processes [24].

Parkinson's Disease

A mitochondrial impairment has been described in Parkinson's disease (PD), and mitochondrial toxins are often used in animal models of the disease. The mitochondrial complex I inhibitor 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) results in dopaminergic neurotoxicity and induces characteristic changes in the KP. MPTP, and another toxin, 6-hydroxydopamine, both decrease KAT-I immunoreactivity in the substantia nigra neurons [25, 26]. Another study confirmed that MPTP inhibits KAT-II activity too, thereby resulting in a decrease in KYNA concentration [27]. Alterations of the KP have been described not only in preclinical but also in human studies. A decreased Trp level and an elevated KYN/Trp ratio have been found in both the plasma and cerebrospinal fluid (CSF) of PD patients, while in the blood diminished KAT-I-II activity and lower KYNA levels have been observed [28, 29]. An enhanced CSF/plasma Trp ratio has also been demonstrated [30]. Widner et al. suggested that an increased KYN/Trp ratio reflects immune activation, this being confirmed by elevated neopterin levels [29]. Human postmortem investigations revealed lower KYNA and KYN and higher 3-OH-KYN levels in several brain regions of PD patients as compared with controls [31]. 3-OH-KYN has recently been identified by metabolomic analysis as a biomarker in the CSF of PD patients [32]. An elevated level of 3-OH-KYN can contribute to the degree of oxidative stress. A decrease in the NMDA-antagonist KYNA may lead to the vulnerability of neurons to excitotoxic processes [33]. KYNA is not only involved in glutamatergic neurotransmission, but additionally has a bidirectional modulating effect on dopamine [34, 35]. All these alterations point to a shift in the KP toward the synthesis of neurotoxic metabolites, which may reflect neuroinflammation and contribute to excitotoxic neuronal damage [36]. KP metabolites may also connect

dopaminergic and glutamatergic neurotransmission in PD and have been suggested as a possible link between PD, depression, and inflammatory processes [37, 38].

Huntington's Disease

Alterations in the KP metabolism have been clearly demonstrated in Huntington's disease (HD). Intrastriatal administration of QUIN produces characteristic behavioral and histological changes resembling HD, and QUIN has therefore been widely used as an animal model of HD [39–41]. QUIN-induced excitotoxicity has been demonstrated to increase the immunoreactivity of huntingtin in mice [42]. In a yeast model of HD, KMO inhibition reduced huntingtin toxicity, while KAT deletion enhanced it [43]. Similar results have been described in a Drosophila model of HD, where KMO inhibition resulted in elevated KYNA levels and diminished neurodegeneration [44, 45].

Human studies confirmed the KP changes. Several brain regions of HD patients exhibited an elevated QUIN and 3-OH-KYN levels [46, 47]. In HD, early microglia activation is present, and these neurotoxic compounds are both synthesized in these cell types. In contrast, KYNA levels are reduced in the striatum and cortex of HD brains, accompanied by a decreased KAT activity [48, 49]. There is also evidence of elevated IDO activity, as measured by an increased KYN/Trp ratio in HD patients, which is in accordance with elevated levels of inflammatory markers [50]. Changes in KP metabolites have been reported to correlate with the disease severity [51].

Alzheimer's Disease

Alterations in the KP have been implicated in the pathogenesis of Alzheimer's disease (AD) by an increasing body of evidence [52–54]. The first study describing changes in the KP metabolism, in 1999, detected decreased levels of 3-OH-KYN and L-KYN and significantly increased levels of KYNA and KAT-I activity in several brain regions of pathologically confirmed AD patients [52]. Later, enhanced QUIN and IDO immunoreactivity was observed in the hippocampus of AD patients, indicating the role of QUIN in the neurodegenerative process [55]. In accordance with this, QUIN was found to be co-localized with hyperphosphorylated tau in the AD cortex, and it has also been described to induce tau phosphorylation in in vitro studies [56]. A recent study confirmed these alterations in triple transgenic AD mice and the human AD brain and revealed elevated TDO immunoreactivity [57]. Alterations in the KP have additionally been detected in the periphery. Reduced levels of KYNA have been demonstrated in the serum, red blood cells, and CSF of AD patients, while the KAT-I or KAT-II activity was not altered in the serum [58, 59]. The contribution of inflammatory processes to the pathogenesis of AD was confirmed by the observation of an increased serum KYN/Trp ratio, reflecting

elevated IDO activity, which correlated with several immune markers [53]. An increased level of Trp degradation proved to be associated with cognitive decline [53]. A recent study indicated that the KYNA concentration did not correlate with the cognitive decline, but did correlate significantly with the tau level and inflammatory markers [60]. Another recent in vitro investigation provided evidence that KYNA treatment was able to reduce amyloid-induced inflammatory cytokine production in the microglia [61]. In a recent study, 3-OH-KYN has been suggested as a possible biomarker for AD, following the finding that 3-OH-KYN elevation was specific for AD patients, ant it was postulated that increased 3-OH-KYN availability may result in an enhanced level of QUIN production in the brain [62]. The converging evidence supports the role of KP alterations in the pathomechanism of AD, providing a link between amyloid production, neuroinflammation, and oxidative stress.

Multiple Sclerosis

Activation of the KP has been also implicated in multiple sclerosis (MS), but the exact role of this in the autoimmune process has not yet been fully clarified. In experimental autoimmune encephalitis (EAE), an animal model of MS, IDO activation suppresses, while IDO inhibition exacerbates the autoimmune process [63–66]. Accordingly, in relapsing-remitting patients, interferon-β therapy induced IDO activity, resulting in increased serum KYN and KYN/Trp levels [67]. The balance of neurotoxic and neuroprotective compounds seems to be altered as an increased QUIN content has been observed in the spinal cord of animals with EAE [68]. MS patients displayed elevated CSF KYNA levels during an acute relapse, while in the chronic remission phase the concentration of KYNA was lower [69, 70]. These data indicate an upregulation of the KP in MS, with the preferential formation of QUIN, which may contribute to the neurodegenerative process. Further, IDO activation has an important impact on the regulation of T-cells and immune activation. The exact roles of IDO and KP alterations in the pathomechanism of MS await comprehensive investigations.

Amyotrophic Lateral Sclerosis

KP alterations have been implicated in the pathomechanism of amyotrophic lateral sclerosis (ALS) by a number of studies. Chen et al. provided an extensive description of the Trp metabolism in the disease, with increased levels of Trp, KYN, and QUIN in the serum and CSF and enhanced IDO activity in the CSF of ALS patients. Additionally, the presence of activated microglia in the spinal cord and motor cortex of ALS patients provided evidence of an inflammatory process, the QUIN and IDO

immunoreactivity in the same regions also proving to be enhanced [71]. The levels of the neuroprotective KYNA are higher in the CSF and lower in the serum of patients with a severe clinical status [72]. These findings indicate an inflammatory process in ALS, which leads to upregulation of the KP, and the formation of neurotoxic metabolites such as QUIN. An excessive amount of QUIN may contribute to damage of the motor neurons by inducing Glu excitotoxicity through NMDA agonism. This concept was confirmed by a recent in vitro study on NSC-34 cells, where QUIN resulted in severe neuronal damage, which could be prevented by KYNA or picolinic acid [73]. Currently, the only drug available for the pharmacological management of ALS is riluzole, which also has a complex anti-glutamatergic effect. These data suggest that the NMDA antagonist KYNA might well be a valuable candidate for drug development.

Future Therapeutic Options by Modulating the KP

A growing body of evidence indicates that changes in the KP may play an important role in the pathomechanism of neurodegenerative disorders. As KYNA has proved to be neuroprotective in several preclinical studies, influencing the KP provides a rationale for possible therapeutic options. However, the systemic administration of KYNA is not feasible, due to its poor ability to cross the blood–brain barrier [74]. There are several ways to achieve an elevated KYNA level in the brain: the administration of either a prodrug or synthetic KYNA derivatives, or acting on the enzymatic machinery of the KP with enzyme inhibitors. One option would be the peripheral administration of KYN, the precursor of KYNA, and also neurotoxic KP metabolites, and it may be assumed that it would give rise to the formation of QUIN too. Interestingly, evidence from animal experiments indicated that, in the event of excitotoxic damage in the brain, the production of neurotoxic kynurenines is less pronounced, indicating a shift toward KYNA synthesis [75]. KYN administration together with probenecid, an organic acid transport inhibitor, results in an elevated KYNA level in the brain [76]. Moreover, this treatment regime afforded neuroprotection and prevented the histochemical changes in toxin models of PD and AD [77, 78]. Synthetic KYNA derivatives are also able to provide neuroprotection in an MPTP-induced PD model and a HD animal model [79, 80].

KMO is the main candidate for enzyme inhibition with the aim of achieving an enzymatic shift in the KP, because it results in an enhanced formation of KYNA, while the synthesis of neurotoxic kynurenines is diminished. The beneficial neuroprotective effects of KMO inhibitors have been demonstrated in animal models of AD and HD, where neuronal damage and characteristic behavioral alterations were both prevented [45]. Interestingly, KMO inhibitors administered together with levodopa reduced dyskinesia, whereas they did not influence the antiparkinsonian effect of levodopa [81, 82].

Conclusions

The KP involves several neuroactive metabolites, which have been linked to several key features of the neurodegenerative process: Glu excitotoxicity, oxidative stress, and neuroinflammation. An increasing body of evidence suggests that alterations in the delicate balance of the KP may play an important part in the pathomechanism of several neurological disorders. Synthetic KYNA analogs or KP enzyme inhibitors may be promising candidates for the development of novel pharmacological tools to achieve neuroprotection.

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Chapter 16
Role of the Kynurenine Pathway in Epilepsy

Monika Sharma, Chaitali Anand, and Diane C. Chugani

Abstract A role for the kynurenine pathway (KP) of tryptophan (TRP) metabolism in epilepsy was first discovered when injections of the KP metabolite quinolinic acid (QA) in brain produced seizures in mice (Lapin IP. J Neural Transm. 42(1):37–43;1978). Subsequent studies have reported altered KP metabolites in animal models of epilepsy and in human epilepsy. Epilepsy is a complex group of disorders involving abnormal firing of neurons linked to an imbalance of excitatory and inhibitory mechanisms in the brain. KP metabolites act at several neurotransmitter receptors and can produce both excitatory and inhibitory effects. Thus, the role of the KP mechanisms in epilepsy, a condition characterized by an imbalance in excitation and inhibition, is important to consider. Furthermore, the KP is induced by inflammatory cytokines and is part of the inflammatory response increasingly recognized to play a role in epilepsy and epileptogenesis (Vezzani A. Epilepsy Curr. 14(1 Suppl):3–7;2014). In this chapter, we discuss evidence for the role of the KP in the balance of excitation and inhibition and the inflammatory process in animal models, as well as evidence for a role of kynurenines in human epilepsy. Finally, therapeutic targets for epilepsy in the KP are discussed.

Keywords Epilepsy • Kynurenine • Tryptophan • Inflammation • α-[^{11}C] methyl-L-tryptophan

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List of Abbreviations

3-HK	3-Hydroxykynurenine
ACMSD	α-Amino-β-carboxymuconate-ε-semialdehyde decarboxylase
AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMT	α-[^{11}C]methyl-L-tryptophan
CSF	Cerebrospinal fluid
IDO	Indoleamine 2,3-dioxygenase (IDO)
KATs	Kynurenine aminotransferases
KMO	Kynurenine monoxygenase
KP	Kynurenine pathway
KYNA	Kynurenic acid
NMDA	*N*-methyl-D-aspartate
NORSE	New-onset refractory status epilepticus
PET	Positron emission tomography
PTZ	Pentylenetetrazole
QA	Quinolinic acid
TRP	Tryptophan
TSC	Tuberous sclerosis complex

Role of Kynurenine Metabolites on Inhibition and Excitation

The first evidence that the kynurenine pathway (KP) may be involved in epilepsy came from a study that showed that an intracerebroventricular injection of the KP metabolite quinolinic acid (QA) produced seizures in mice [1]. QA was subsequently shown to be excitatory by acting as a selective agonist at the *N*-methyl-D-aspartate (NMDA) glutamate receptor [2]. Several other mechanisms have been found to be associated with neurotoxic properties of QA, including induction of lipid peroxidation [3,4] and production of reactive oxygen species [4,5]. Furthermore, the intravenous injection of QA in rats was shown to cause EEG changes but no seizures when the blood–brain barrier was intact [6], while tonic–clonic seizures were observed following administration in rats in which the blood–brain barrier was disrupted by irradiation. Increased QA and KP enzymes have been reported in epilepsy-prone E1 mice [7,8]. Seizures associated with measles virus infection are related to activation of microglia and astrocytes in weanling Balb/C mice after inoculation with hamster neurotropic measles virus and were associated with increased levels of the neurotoxic kynurenine metabolites 3-hydroxykynurenine (3-HK) and QA in the hippocampus [9]. The authors hypothesized that elevations of 3-HK and QA cause subsequent seizures and neurodegeneration in this model. Thus, QA administered to brain induces seizures, and increased levels of QA have been measured in several animal models of epilepsy.

Conversely, the KP metabolite kynurenic acid (KYNA) is an antagonist at the NMDA receptor, as well as an antagonist at the kainate and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) glutamate receptor sites [10]. KYNA acts as a competitive antagonist at the glycine site on NMDA receptors [11]. Furthermore, KYNA is an antagonist at the α7-nicotinic acetylcholine receptor [12]. Actions at the α7-nicotinic acid receptor have been shown to regulate extracellular concentrations of both the excitatory neurotransmitter glutamate and the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in rat cortices [13,14]. Finally, KYNA has been reported to interact with GPR35 [15] and the aryl hydrocarbon receptor [16,17]. KYNA was shown to modulate calcium channels through the GPR35, a G protein-coupled receptor that is highly expressed in immune cells, the gastrointestinal tract, and brain, and to induce internalization of the receptor [15,18]. KYNA is an agonist at the aryl hydrocarbon receptor, and agonists such as dioxin induce expression of indoleamine 2,3dioxygenase enzymes IDO1 and IDO2, the first enzymatic step in the KP, in dendritic cells [19]. Thus, KYNA may impact excitation and inhibition through multiple mechanisms and may be involved in positive feedback regulation of KP pathway.

Several studies have shown that KYNA inhibits the progression of kindled seizures. Pretreatment with intracerebroventricular KYNA prior to administration of electrical kindling stimulus in rats significantly reduced the rate of kindling [20]. Kindled seizures were associated with significant alterations in the levels of KYNA and TRP. Levels of KYNA and ratio of KYNA/TRP were increased in the amygdala and hippocampus of kindled animals whereas TRP was decreased in the prefrontal cortex and hippocampus [21]. Increased release of KYNA was found in the extracellular fluid in hippocampus in kindled animals and was associated with progression of kindling development to the stage of generalized tonic or clonic convulsions [22]. In another study, KYNA was increased in nucleus accumbens following kindling but not in hippocampus, striatum, cerebral cortex, olfactory bulb, thalamus, tectum, pons/medulla, cerebellum, or plasma [23]. Pentylenetetrazole (PTZ)-induced seizures were inhibited by KYNA, and KYNA protected the animals from the death induced by repeated PTZ injections [24]. The same group showed elevation of KYNA in the pilocarpine, bicuculline, and kainic acid seizure models [25]. Tissue concentrations of KYNA were decreased in caudate, entorhinal cortex, piriform cortex, putamen, amygdala, and hippocampus after PTZ-induced seizures [26]. KYNA converted in situ from kynurenine blocked the epileptiform discharges induced in Mg2+-free medium, and application of kynurenine prevented spontaneous activity in hippocampal slices [27]. Similarly, pretreatment of hippocampal slices from young animals with kynurenine decreased neural hyperecxitability and prevented the neuroexcitatory effect of PTZ [28]. Finally, administration of kynurenine hydroxylase and kynureninase inhibitors employed to increase brain levels of KYNA protected the rats from electroshock-induced seizures and DBA/2 mice from audiogenic seizures [29]. While QA induces seizures, there is abundant evidence using many animal models that KYNA blocks seizure induction and progression.

Activation of KP Pathway with Inflammation in Astrocytes and Microglia Differs

Increases in reactive astrocytes and microglia have been reported in brain tissue surgically resected for treatment of medically intractable epilepsy and in animal models of epilepsy [30]. The relative proportions of the two types of cells play an important role in the levels of different kynurenine metabolites. Astrocytes express kynurenine aminotransferases (KATs) and synthesize KYNA but do not express kynurenine 3-monoxygenase (KMO), while microglial cells express KMO and synthesize the neurotoxic KP metabolites 3-HK and QA [31]. When present alone, astrocytes predominantly produce KYNA, but in the presence of macrophages and/or microglia, production of large amounts of kynurenine in astrocytes can be metabolized to QA by neighboring microglia or infiltrating monocytic cells [32]. Inflammation results from a number of the environmental risks for epilepsy such as traumatic brain injury, infection, and stroke, and seizures themselves can lead to inflammatory responses [33]. The inflammatory process leads to a complex signaling cascade involving the production of proinflammatory cytokines in multiple cell types in the brain [34]. The indoleamine 2,3-dioxagenase enzymes (IDO, IDO1, and IDO2) are the first and rate limiting step of the KP, and proinflammatory cytokines IL-1β, TNF-α, and IFN-γ are inducers of IDO. Furthermore, IFN-γ also induces the expression of KMO [35]. The induction of the KP with inflammatory signals in epilepsy may be protective or neurotoxic depending on the relative contributions from astrocytes and microglia.

Evidence for Alter Kynurenine Pathway in Human Epilepsy

Based on the role of the KP in animal models of epilepsy, it was postulated that kynurenine pathway metabolites might play a role in human epileptogenesis. Young et al. measured KYN levels in the cerebrospinal fluid (CSF) from patients having complex partial seizures and found that the mean values for CSF kynurenine were lower for the patients with the complex partial seizures, compared to the controls [36]. Feldblum et al. reported lower activity of quinolinic-phosphoribosyl transferase, an enzyme which metabolizes QA, in brain tissue involved in the epileptic discharge from patients undergoing epilepsy surgery compared to postmortem control brain tissue [37]. However, Heyes et al. found that QA was not increased in the seizure focus as compared with nonfocus brain regions from adults undergoing temporal lobectomy [38]. More recently, a stop codon mutation (p.Trp26Stop) in the α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD) gene, which encodes part of the KP at the branch to picolinate or QA, was identified as the disease-segregating mutation in a family with cortical myoclonic tremor and epilepsy [39].

Imaging of tryptophan metabolism with positron emission tomography (PET) with the tryptophan analogue α-[^{11}C]methyl-L-tryptophan (AMT) has provided evidence that there is activation of the KP in the epileptic focus in several types of epilepsy. Increased uptake of AMT in a subset of brain lesions called tubers and surrounding tubers associated with epileptic discharges was reported in patients with tuberous sclerosis complex (TSC) and medically refractory epilepsy [40]. AMT, initially developed as a PET ligand to measure serotonin synthesis in the brain, more accurately reflects KP metabolism based upon analysis of tissue resected for seizure control [40]. Immunocytochemical studies showed expression of IDO, not only in microglia/macrophages and astrocytes, but also in balloon cells and in the neuropil in surgical specimens from TSC patients [41]. Increased uptake of AMT in TSC patients has been replicated by two groups [42,43], although there are differences in the reported sensitivity of the technique [42]. Increased tryptophan metabolism measured with AMT-PET has also been reported in epilepsy patients with malformations of cortical development [44–46], temporal lobe epilepsy [47], brain tumors [48], periventricular nodular heterotopia [49], dysembryoplastic neuroepithelial tumors [50], and new-onset refractory status epilepticus (NORSE) [51]. Application of AMT-PET has provided an in vivo measure to identify activation of the KP in patients with epilepsy of multiple etiologies.

Therapeutics Aimed at the KP

A number of different approaches have been suggested for the development of therapeutic agents based upon the KP. One approach is to use KYNA analogues as antagonists at glutamate receptors. Another is to inhibit the enzymes leading to QA synthesis. Several pharmacological approaches to treating epilepsy with agents aimed at the KP in animal models have been reported. Chiarugi et al. studied *m*-nitrobenzoylalanine, an inhibitor of kynurenine hydroxylase, and *o-methoxybenzoylalanine*, an inhibitor of kynureninase, and found that both agents increased the concentration of KYNA in hippocampal extracellular space in rats [52]. In addition, they showed that these agents protected against audiogenic seizures in DBA/2 mice. Nemeth et al. administered kynurenine with probenecid to rats with PTZ-induced seizures and showed marked inhibition of electrophysiological and behavioral seizure activity [24]. Schwarcz and colleagues have administered 4-chloro-kynurenine, which is converted by astrocytes to the NMDA glycine-site antagonist 7-chloro-kynurenic acid, to rats with kainate-induced seizures [53] and in chronic limbic epilepsy [54]. 4-chloro-kynurenine administration in kainate treated rats delayed seizure onset, reduced total time in seizures, and prevented lesions in piriform cortex in the CA1 region of the hippocampus. Administration of 4-chloro-kynurenine resulted in decreased amplitude and number of population spikes in response to electrical stimulation in the chronic limbic epilepsy rats, but showed no effect on the evoked response in control animals.

The same group reported enhanced formation of 7-chloro-kynurenic acid from administered 4-chloro-kynurenine in the pilocarpine seizure model [55]. They hypothesized that enhanced conversion of the drug was related to astrocytosis induced by pilocarpine treatment. These pharmacological agents may lead to new targets for the treatment of human epilepsy.

Summary and Future Directions

Study of alterations in TRP metabolism by the KP in epilepsy both in animal models and in human epilepsy has a long history. However, until the relatively recent studies showing increased tryptophan metabolism in epilepsy patients using in vivo imaging with AMT-PET, this information had not impacted clinical assessment or treatment of patients with epilepsy. AMT-PET is now used in planning for epilepsy surgery in clinical studies. However, this imaging tool may also guide future pharmacological trials for the selection of candidates for drugs aimed at the KP as well as a biomarker for monitoring of drug response.

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Chapter 17
Role of the Kynurenine Pathway in Stroke

Heidi Ormstad and Robert Verkerk

Abstract Stroke is the second and fifth leading cause of death for people aged >60 and 15–59 years, respectively. Many stroke survivors suffer from chronic health problems that necessitate a long-term process of recovery and rehabilitation. There is increasing evidence that inflammation plays an important role in acute ischemic stroke (AIS), indicating the presence of important interactions between the nervous and immune systems. Furthermore, there are currently strong indications for a close relationship between the immune system and indoleamine-2,3-dioxygenase (IDO)-induced tryptophan catabolism [the kynurenine (KYN) pathway]. Although KYN pathway metabolites can produce excitatory and oxidative neurotoxicity, they can also protect neurons from inflammatory damage and attenuate excitatory neurotoxicity via *N*-methyl-D-aspartate receptor antagonism. Thus, activation of IDO in the central nervous system might be a double-edged sword. Recent studies indicate that the KYN pathway is activated immediately after a stroke, that this is related to the stroke-induced inflammatory response, and also that this IDO-induced tryptophan catabolism is correlated with a worse outcome. Since activation of the KYN pathway may disturb brain serotonin (5-hydroxytryptamine) and glutamate neurotransmission, it is reasonable to assume that inflammation-induced IDO activity in AIS is involved in several sequelae following stroke, such as cognitive impairment, depression, and fatigue. Many AIS survivors suffer from post-stroke fatigue and post-stroke depression, indicating the importance of increasing the base of knowledge about the mechanisms underlying these sequelae. In this chapter, we present and discuss findings that support the notion that the AIS-induced immune response and IDO activation are related to post-stroke fatigue but not to post-stroke depression.

Keywords Stroke • Inflammation • Cytokines • Kynurenine pathway • Kynurenine metabolites • Serotonin • Glutamate • Post-stroke depression • Post-stroke fatigue

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List of Abbreviations

3-HK	3-Hydroxykynurenine
AIS	Acute ischemic stroke
CRP	C-reactive protein
CSF	Cerebrospinal fluid
hsCRP	High-sensitivity C-reactive protein
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
KA	Kynurenic acid
KATs	Kynurenine aminotransferases
KYN	Kynurenine
NIHSS	National Institutes of Health Stroke Scale
NMDA	*N*-methyl-D-aspartate
PSD	Post-stroke depression
PSF	Post-stroke fatigue
QA	Quinolinic acid
SAR	Superoxide anion radical
TDO	Tryptophan 2,3-dioxygenase
TNF-α	Tumor necrosis factor-α
TRP	Tryptophan

Tryptophan and the Kynurenine Pathway

Tryptophan (TRP) is an essential and rare amino acid. Its metabolism is under tight metabolic control, such that the organism adapts its rate of TRP catabolism to its availability. A shortage of TRP activates mechanisms designed to preserve it, both at the whole-organism and intracellular levels. TRP homeostasis is achieved mostly via the oxidative or kynurenine (KYN) pathway, a concise outline of which is shown in Fig. 17.1. This pathway is by far (quantitatively) the major metabolic pathway of TRP; the only exception is the brain, in which almost half of the TRP is used for the synthesis of serotonin. Thus, TRP deficiency may impair other important metabolic pathways, such as that responsible for serotonin synthesis.

Enzymes

The enzymes tryptophan 2,3-dioxygenase (TDO; historically also known as tryptophan pyrrolase, EC 1.13.11.11) and indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.17) catalyze the conversion of TRP to *N*-formyl-kynurenine, which is further rapidly converted to KYN in a rate-limiting step by the enzyme kynurenine

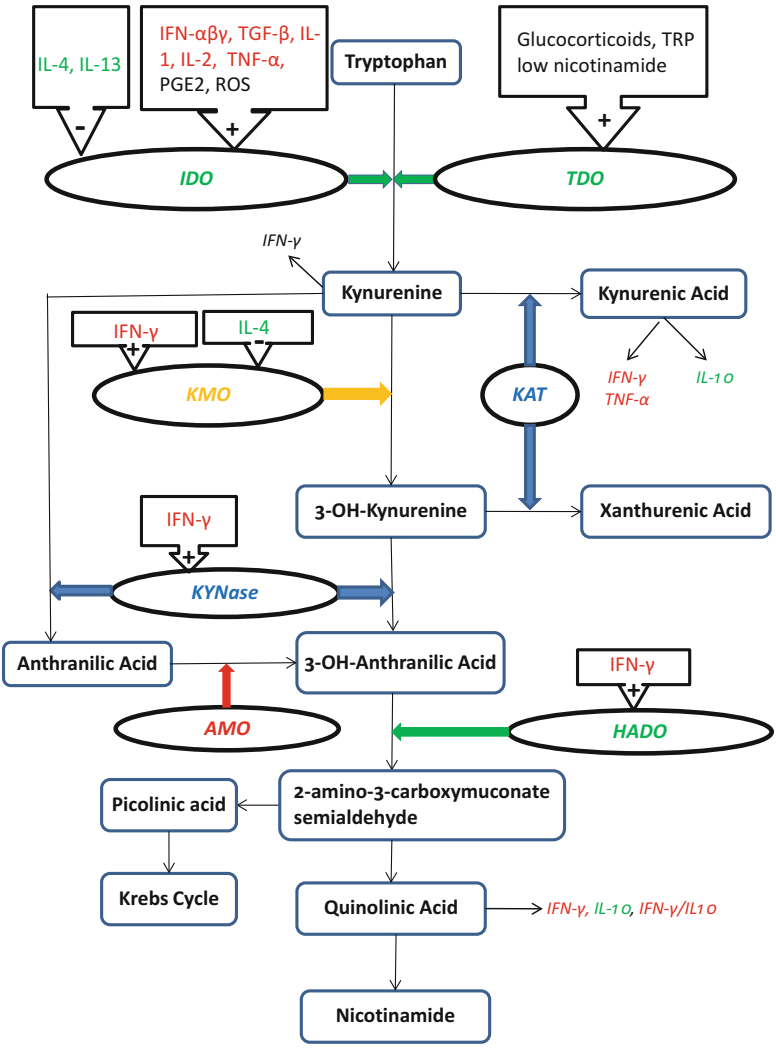

Fig. 17.1 Overview of the KYN pathway showing that the expression of many enzymes is influenced by cytokines, while metabolites modulate the immune system by elevating (*thin solid arrows*) or lowering (*thin dashed arrows*). The *color* of the *thick arrows* indicates the specific cofactor dependence of the enzyme: B6 dependent, FAD dependent, THB dependent, and iron dependent

formamidase (EC 3.5.1.9). A recent genomic analysis has revealed the existence of an additional isoenzyme in this pathway, called IDO2 [1].

TDO is an important housekeeping enzyme for TRP. It leads TRP to the Krebs cycle to produce energy in the case of excess TRP or stress or to nicotinamide synthesis in the case of TRP deficiency. Although TDO is located in the liver, its mRNA has been detected in the brain of schizophrenia patients [2].

IDO is distributed ubiquitously, but it is found mainly in the intestines and lung, and is membrane-bound. Rather than being a house-keeping enzyme, it serves specific purposes in the vicinity of the membranes of the cells in which it is expressed. The major flow of the catabolic cascade is to the Krebs cycle via picolinic acid (see Fig. 17.1 for a schematic of the enzymes and their inducers and cofactors), which may be important for maintaining the metabolic flow and the concentration of metabolites. Iron-dependent enzymes may be inhibited by nitric oxide or, hypothetically, by carbon monoxide. Vitamin B6 deficiency, an independent risk factor for vascular disease, may inhibit several enzymes.

KYN and 3-hydroxykynurenine (3-HK) are converted to kynurenic acid (KA) and xanthurenic acid by the action of kynurenine aminotransferases (KATs). There are known to be at least four KATs present in brain (arbitrarily named KATs I–IV), all of which are dependent on vitamin B6. They are identical to other known transaminases [3]. Deviations in their activity may have major consequences for other metabolic pathways. Another deviation from the main route leads to the production of nicotinamide via the well-known neurotoxin quinolinic acid (QA).

Receptors

QA and KA can bind to receptors to modulate cellular mechanisms, although not exclusively in brain, certainly of utmost importance there. The effect of QA is partly mediated through binding the glutamate-binding *N*-methyl-D-aspartate (NMDA) receptor [4]. KA acts as an endogenous antagonist to the same receptor and is therefore thought to be neuroprotective by inhibiting excessive glutamate excitation and antagonizing the neurotoxic QA [5]. Imbalance of QA and KA levels may therefore result in excitotoxicity. QA also reduces γ-aminobutyric acid levels in the brain [6]. KA also binds to other important receptors, such as alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid [7], kainate, G protein-coupled receptor GPR35 [8], and α7-nicotinic receptors [9].

Kynurenine Pathway and the Immune System

There is a tight relationship between IDO-induced KYN pathway and immune system activity. Activation of IDO in the cell membrane creates a microenvironment devoid of TRP, whereby lymphocytes become anergic, thus protecting the cell against the immune system [10]. Evidently, the combination of TRP depletion and the cytotoxic properties of some KYNs is responsible for immune tolerance by causing a shift from T-helper (Th)1 to Th2 and regulator T cells. There are also indications of KYN-induced natural killer cell apoptosis and dysfunction [11]. The fact that KYNs influence the immune system and that elements of that immune system induce or inhibit several enzymes of the KYN pathway suggests a reciprocal balancing.

Cytokines are small proteins (~5–20 kDa) that are important in cell signaling and can be produced by a broad range of cells, including immune cells, endothelial cells,

fibroblasts, and various stromal cells. The cytokines modulate the balance between humoral and cell-based immune responses, as well as regulate the maturation, growth, and responsiveness of particular cell populations, by acting via specific receptors. They act in a complex network, often involving cascades and feedback loops, and the overall response is dependent upon their synergistic or antagonistic action. One way of categorizing cytokines is to divide them into functional classes. For example, some cytokines are primarily lymphocyte growth factors, others polarize the immune response to the antigen, and yet others play a role in infection and/or inflammation [12]. The concept that some cytokines function primarily to induce inflammation (pro-inflammatory cytokines) while others suppress inflammation by suppressing the activity of pro-inflammatory cytokines (anti-inflammatory cytokines) is fundamental to both cytokine biology and clinical medicine [13].

IDO expression is induced by lipopolysaccharide and pro-inflammatory cytokines [14–16], and is probably inhibited by anti-inflammatory cytokines [17–20]. Prostaglandin E2 and interferon-γ can also induce IDO expression [16], and glucocorticoids may promote superinduction of IDO above that achieved with interferon-γ [21, 22]. During immune activation, hepatic TDO may be suppressed while IDO is induced, rendering the latter the more important enzyme for TRP catabolism [23].

Many other enzymes downstream of IDO and TDO are regulated by cytokines; however, KATs are not influenced by cytokines. Hence, the inflammation-induced activation of IDO causes a metabolic flux with a preferential route toward nicotinamide adenine dinucleotide/Krebs cycle via QA, to the detriment of KA [24]. The KYN/KA ratio has thus been used as a surrogate neurotoxic index. In our study, which is described below, we indeed found a strong correlation between QA/KA and KYN/KA in both patients and controls.

Kynurenine Pathway and Oxidative Stress

The KYNs are involved in oxidative stress; in particular, QA produces reactive oxygen species [25, 26] and causes lipid peroxidation [27], while 3-hydroxyanthranilic acid and 3-HK generate free radicals [28]. IDO is also induced by oxidative stress [29]; it is the only enzyme—apart from superoxide dismutase—that uses the superoxide anion radical (SAR) as a co-substrate, enabling it to reduce oxidative stress [29]. Considerable amounts of SAR may be generated during the acute phase of stroke and during reperfusion.

Stroke

According to the World Health Organization, stroke is the second and fifth leading cause of death for people aged >60 and 15–59 years, respectively. Worldwide, over 15 million individuals suffer a stroke each year resulting in death in nearly 6 million of them (http://www.world-stroke.org/advocacy/world-stroke-campaign). Stroke is characterized by the sudden loss of cerebral blood circulation, resulting in a

corresponding loss of neurological function. Two main subtypes exist: (1) acute ischemic stroke (AIS), which is caused by thrombosis or embolism and constitutes over 80 % of all stroke events and (2) hemorrhagic stroke, which is caused by rupture of an artery. AIS is a heterogeneous and multifactorial disorder that involves a complex interplay between environmental and genetic factors. Much is now known about the mechanisms underlying stroke (e.g., cardioembolism, artery-to-artery embolism, and hypoperfusion), and several well-known conventional vascular risk factors (e.g., increasing age, hypertension, smoking, and diabetes) and underlying etiologies (e.g., atherothrombosis, atrial fibrillation, and small-vessel disease) have been established [30].

AIS results in a complex interplay between multiple processes, including excitotoxicity, acidotoxicity, ionic imbalance, peri-infarct depolarization, oxidative and nitrative stress, inflammation, and apoptosis [31], resulting in neural death within minutes in the so-called ischemic core. This short phase is not accessible to therapeutic interventions and is followed by several physiological disturbances that together are known as the ischemic cascade (although "cascade" is a misnomer since the events do not always follow the same or linear order). The ischemic cascade may last for hours or even days after the initial insult [32]. Moreover, reperfusion is accompanied by oxidative and nitrative stress, causing further injury. In this post-stroke period, the extent of the peri-infarct zone influences the prognosis and outcome and may thus putatively be modified by therapeutic interventions [33]. Some elements of the ischemic cascade, notably oxidative stress and inflammation, have a profound influence on the cerebral KYN pathway, and its metabolites may have deleterious effects.

The Immune System in Stroke

There is increasing evidence that inflammation plays an important role in AIS, indicating important interactions between the nervous and immune systems [34, 35]. Cerebral ischemia induces a strong inflammatory reaction that involves several cell types. Numerous studies have focused on the inflammatory reaction after the ischemic episode, identifying the roles of important inflammatory signaling molecules, mainly cytokines [36–38]. It can be assumed that this inflammatory state influences the KYN pathway and vice versa.

Cytokines are up-regulated in the brain after stroke and are expressed not only in immunological cells but also in glial cells and neurons [39]. The pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 are secreted in the ischemic region by activated immune cells, driving the inflammatory process and accelerating additional inflammatory processes by inducing the expression of inflammatory molecules. These molecules recruit more circulating leukocytes, which infiltrate the ischemic region and lead to further loss of neuronal cells and brain tissue, thereby possibly enlarging the cerebral infarct area [40, 41]. Whether post-ischemic inflammatory responses are deleterious or beneficial to brain recovery

is presently a matter of debate [37, 42]. The findings of studies examining the relationship between the inflammatory response following AIS and infarct volume [43–51] and stroke subtype [51] have been inconsistent; the roles of the cytokines involved thus remain unclear. Our research group performed a study in which the serum levels of 13 cytokines were evaluated in blood samples taken from 45 acute stroke patients (within 72 h of stroke onset) and 40 healthy controls [52]. Serum concentrations of IL-1Ra, IL-6, IL-8, IL-9, IL-10, IL-12, IL-18, and GRO-α [chemokine (C-X-C) ligand 1] were significantly higher in the stroke patients than in the controls. These results lend further support to the existence of an early pro-inflammatory response and an early activation of endogenous immunosuppressive mechanisms [52].

Kynurenine Pathway in Human Stroke

Saito et al. were the first to implicate the KYN pathway in cerebral ischemia, demonstrating an increase in the levels of QA several days following transient cerebral ischemia in gerbils [53]. The plasma concentration of QA was found to be significantly correlated with both the plasma concentration of QA in the cerebrospinal fluid (CSF) and in the cerebral cortex. The role of KYN pathway activation in acute stroke has been explored in four clinical studies, whereby serum levels of KYN metabolites were measured in stroke patients [54–57]; the main findings of these studies are presented in Table 17.1 and are briefly discussed below.

Darlington et al. compared blood components in samples taken from 50 AIS patients at the point of diagnosis and 1, 2, 3, 4, 7, and 14 days post-stroke, with samples from 35 healthy control subjects [54]. The serum concentration of TRP was lower, while the KYN/TRP ratio (reflecting IDO activity) was higher in patients than in controls at day 1, 7 and 14. KYN was higher in the patients only at the first day. The KYN/TRP ratio was correlated with serum neopterin (a marker for inflammation). KA was not elevated in the patient group as a whole, but it was significantly higher in those patients who died within 21 days of the initial stroke event. Based on these results, Darlington et al. suggested that increased TRP catabolism is initiated before or immediately after a stroke, and that this is related to the inflammatory response and oxidative stress, with a major change in 3-hydroxyanthranilic acid levels.

Brouns et al. evaluated the KYN pathway in 149 AIS patients in whom blood was sampled at admission and at 24 h, 72 h, and 7 days after stroke onset (there was no control group) [55]. The KYN/TRP ratio was correlated with infarct volume and the National Institutes of Health Stroke Scale (NIHSS) score, and patients with poor outcome (i.e., a high modified Rankin scale score at 3 months) had significantly elevated KYN/TRP ratios. The KYN/TRP ratio was also correlated with markers for inflammation [C-reactive protein (CRP), erythrocyte sedimentation rate, and neutrophil/lymphocyte ratio]. Brouns et al. demonstrated that KYN-pathway activity in AIS is correlated with stroke severity and long-term stroke outcome, and that TRP oxidation is related to the stroke-induced inflammatory response [55].

Table 17.1 The findings of four studies examining the role of activation of the KYN pathway in acute stroke patients by measuring KYN metabolites in serum samples

Metabolite/ratio	Paper	TRP	KYN	KYN/TRP (IDO activity)	KA	KYN/KA	QA	QA/KA	TRP index (TYR/CAA)
Sign. higher/lower level in blood samples from stroke patients, compared to healthy controls	Darlington et al.	Lower	Higher	Higher	Higher in patients who died in 21 d	–	–	–	–
	Brouns et al.	–	–	–	–	–	–	–	–
	Mo et al.	Lower	nsd	Higher	Lower	Lower	–	–	–
	Ormstad et al.	Lower	nsf	nsf	nsf	nsf	nsf	nsf	Lower
Sign. correlated with clinical parameter (infarct volume, NIHSS or BI-20)	Darlington et al.	–	–	–	–	–	–	–	–
	Brouns et al.	–	–	Pos. corr. with infarct volume and NIHSS	–	–	–	–	–
	Mo et al.	–	–	Pos. corr. with NIHSS at adm and 3w PS	–	–	–	–	–
	Ormstad et al.	Pos. corr. with BI-20	nsf	Neg. corr. with BI-20	nsf	nsf	nsf	nsf	nsf

Sign. correlated with immunological component(s)	Darlington et al.	–	–	Pos. corr. with neopterin	–	–	–	–	–
	Brouns et al.	–	–	Pos. corr. with CRP, ESR and N/LR	–	–	–	–	–
	Mo et al.	–	–	Pos. corr. with hsCRP	–	–	–	–	–
	Ormstad et al.	nsf	Neg. corr. with IL-1b	nsf	Pos. corr. with IL-10	Neg. corr. with IL-10	nsf	Pos. corr. with IL-6	Pos. corr. with IL-10

The metabolites presented were chosen based on the study performed by our own research group [57]
nsf none significant findings, – not analyzed/reported, *NIHSS* National Institutes of Health Stroke Scale, *BI-20* the baseline Barthel Index 20 scale, *PS* post-stroke, *Adm* admission, *w* weeks, *TRPindex* 100×TRP/(TYR+VAL+PHE+ILE+LEU), *ESR* erythrocyte sedimentation rate, *N/LR* neutrophil/lymphocyte ratio

A recent study tested for KYN pathway metabolites in blood samples taken from 81 AIS patients within 24 h after stroke onset and 35 normal controls [56]. The stroke patients had significantly lower levels of TRP and KA, but a higher KYN/KA ratio (reflecting neurotoxic potential). Furthermore, the levels of high-sensitivity CRP (hsCRP) and KYN/TRP were much higher in the stroke group, and the KYN/TRP ratio was positively correlated with hsCRP in the patients. Finally, hsCRP and IDO levels were positively associated with the NIHSS score at both admission and at 3 weeks post-stroke. These results were considered indicative of an inflammatory response characterized by up-regulated IDO activation in ischemic stroke.

Our research group recently measured serum levels of TRP, 5-hydroxytryptamine (5-HT), KYN, KA, and QA in 45 acute AIS patients (blood drawn within 72 h of stroke onset) and 40 control subjects [57]. In addition, competing amino acids (CAAs)—tyrosine (TYR), valine (VAL), phenylalanine (PHE), isoleucine (ILE), and leucine (LEU)—and significant ratios thereof were measured. The serum TRP and TRPindex [100×TRP/(TYR+VAL+PHE+ ILE+LEU)] were significantly lower in the stroke patients than in the normal controls, and KYN in the stroke group was negatively correlated with IL-1β, while QA/KA (reflecting neurotoxic potential) was positively correlated with IL-6. KA and TRPindex were positively correlated with IL-10, while KYN/KA (reflecting neurotoxic potential) was negatively correlated with IL-10. TRPindex was negatively correlated with glucose. None of the correlations found in the stroke group were present in the control group. These findings indicate an increase in TRP oxidation and reduced capability for 5-HT synthesis in the brain following AIS. Furthermore, it appears that the pro-inflammatory response in AIS may be responsible for the reduced capacity for excitotoxic overactivation of the NMDA glutamate receptors involved in necrotic brain damage after ischemic stroke. Conversely, the anti-inflammatory IL-10 appears to facilitate higher TRP bioavailability for 5-HT synthesis in the brain and possesses neuroprotective potential. We suggest that these mechanisms are involved in several sequelae following AIS, such as cognitive impairment, post-stroke depression (PSD), and post-stroke fatigue (PSF).

The four above-mentioned studies have produced largely consistent findings. First, the three studies in which control groups were used all revealed that serum TRP was lower in the stroke group [54, 56, 57]. Second, both Darlington et al. [54] and Mo et al. [56] found that the KYN/TRP ratio was significantly elevated in stroke patients [54, 56]. Although we also noted a higher ratio in the stroke group, the difference was not statistically significant [57]. It is interesting to note that Darlington et al. found the KYN/TRP ratio in patients who had survived for 21 days after stroke onset to be significantly elevated relative to controls only at days 1 and 7 and not during the intervening period. This is consistent with our findings, since the blood samples in our study were taken within 72 h of stroke onset. Darlington et al. also found significantly higher KYN concentrations in patients compared with controls in blood samples taken 24 h after stroke onset, although the subsequently measured values did not differ significantly from those of the controls. Mo et al. and our research group found no significant difference in KYN levels between the patient and control groups. Again, the fact that blood was drawn later in our study compared to Darlington et al. could explain the different findings. Moreover, as far as we can

tell, Darlington did not analyze serum KYN separately in the group of patients who survived for 21 days after the stroke. The patients in our study probably had a better outcome than those of Darlington et al.; none of the patients in our study died within 21 days after stroke onset, which again may explain some of the difference. Mo et al. found KA to be significantly lower and the KYN/KA ratio to be significantly higher in the stroke group than in the control group, whereas neither Darlington et al. nor our group found any significant difference in these parameters. However, Darlington et al. found that KA was significantly higher in those patients who died within 21 days of stroke onset compared to those who survived past that point. With respect to the association between KYN pathway activity and clinical outcome, Broun et al., Mo et al., and our research group found a significant correlation between KYN/TRP ratio and a worse outcome. Finally, it was noted in all four studies that TRP oxidation appears to be related to the stroke-induced inflammatory response.

Possible Relationship Between Kynurenine Pathway Activity and the Sequelae of Stroke: Cognitive Impairment, Post-Stroke Depression, and Post-Stroke Fatigue

Since KYN pathway metabolites can produce excitatory and oxidative neurotoxicity, but also protect neurons from inflammatory damage and attenuate excitatory neurotoxicity via NMDA receptor antagonism, overactivation of IDO in the central nervous system might be a double-edged sword [58]. It is therefore relevant to determine whether KYN pathway activity is associated with clinical cognitive outcomes after stroke.

It has been demonstrated that TRP depletion impairs learning and memory [59, 60], and is associated with depression [61–63], supposedly as a result of a decrease in 5-HT turnover in the brain. However, it might also be that the worse outcome may be related to the burden of neurotoxic or neuroactive metabolites such as QA, KYN, and KA. In fact, the production of these neuroactive metabolites has previously been associated with cognitive impairment. For instance, an elevated KYN/TRP ratio has been demonstrated in Alzheimer's disease [64], and an association between the KA/KYN ratio and Mini-Mental State Examination scores has been found [65]. A possible protective effect of KA on the production of KYN and other metabolites has been suggested [65]. A relationship between cognitive impairment and activation of the KYN pathway post-stroke has been demonstrated [66].

Many stroke survivors suffer from chronic health problems that necessitate a long-term process of recovery and rehabilitation. Both PSF and PSD are common but often overlooked symptoms that may develop, both of which have a high negative impact on the quality of life, daily activities, and rehabilitation of patients.

PSF represents a complex interaction between biological, psychosocial, and behavioral phenomena, which makes it challenging to define [67]. A recent systematic review of nine longitudinal studies assessing fatigue following stroke found that the frequency of PSF ranged from 35 % to 92 % [68]. Despite the considerable

clinical relevance, few studies have investigated the pathophysiology of PSF. However, it has been hypothesized that PSF is attributable to the proinflammatory activation that occurs in AIS [69].

A recent systematic review found a pooled prevalence of depression observed at any time point of 29 %, with prevalence rates of 28 %, 31 %, 33 %, and 25 % at <1 month, 1–6 months, 6–12 months, and >1 year post-stroke, respectively [70]. Although there has been widespread research on PSD over the past few decades, the etiology of this condition remains unclear. The most frequently cited mechanism is the pro-inflammatory cytokine hypothesis of PSD, in which an increased production of pro-inflammatory cytokines in response to stroke is considered to cause a widespread activation of IDO and a subsequent depletion of 5-HT [71, 72].

In follow-up studies of our aforementioned study performed in 2011 [52], we investigated the association between PSF and PSD, and stroke type, infarct volume, and laterality, and the levels of various cytokines and other blood components [69, 73]. PSF and PSD were measured using the Fatigue Severity Scale (FSS) and Beck Depression Inventory, respectively, at 6, 12, and 18 months after stroke onset. Our results clearly indicated a role for the post-stroke pro-inflammatory response in the appearance of PSF. The finding that IL-1β seems to be a predictor of PSF [69] may be explained by the concept of cytokine-induced sickness behavior, whereby sickness behavior is induced by physiological concentrations of pro-inflammatory cytokines acting in the brain after infection, the symptoms being loss of appetite, sleepiness, withdrawal from normal social activities, fever, aching joints, and fatigue [74]. The most plausible mechanism by which pro-inflammatory cytokines could induce mental fatigue is disturbance of glutamate signaling, which is crucial for information gathering and processing within the brain. The proinflammatory cytokines TNF-α, IL-1β, and IL-6 may influence the pathophysiology of mental fatigue via their ability to attenuate the astroglial clearance of extracellular glutamate, their disintegration of the blood–brain barrier, or their effects on astroglial metabolism and the neuronal metabolic supply, thereby attenuating glutamate transmission [75].

We did not find an association between cytokines and PSD, despite numerous reports of an association between PSD and PSF [73]. In this context, the aforementioned model of Dantzer et al. [74] for sickness behavior should be considered. Based on this model, depressive disorders develop from cytokine-induced sickness behavior only in vulnerable patients [76]. A review of clinical studies in which patients were treated continuously with recombinant cytokines found that depression is a late phenomenon that develops over a background of early appearing sickness behavior [77]. Furthermore, incorporating this feature in animal models of inflammation-associated depression has verified that alterations of brain serotoninergic neurotransmission do not play a major role in its pathogenesis. As discussed by the authors, this is in contrast to the activation of IDO, potentially generating neurotoxic metabolites. Since glutamate signaling is crucial for information intake and processing within the brain, and plays an essential role in the brain metabolism of glutamate, alterations in glutamate transmission may play a role in fatigue, as mentioned above. Since QA can act as agonists to the glutamatergic receptor, increased IDO activity may lead to alterations in brain serotonin neurotransmission and disturbed glutamatergic activity.

We have also investigated the mechanisms involved in PSD and PSF by studying the relationship between KYN pathway activity in the acute ischemic phase and subsequent PSF and PSD in the stroke sample referred to above [78]. TRP index was significantly lower in patients with an FSS score of ≥4 than in those with an FSS score of <4 at 12 months. Furthermore, the serum level of KA was significantly higher in patients with an FSS of score ≥4 than in those with an FSS score of <4 at 18 months. These findings indicate that stroke patients with PSF have a lower bioavailability of TRP for 5-HT synthesis in the brain in the acute stroke phase. However, they also appear to have greater neuroprotective potential during that phase. In contrast to PSF, no predictors of PSD were found. Together these findings suggest that the immune response and IDO activation that follow AIS can predict PSF but not PSD. A hypothetical scheme of the potential role of cytokines and KYN pathway activity in PSF and PSD is shown in (Fig. 17.2). In our latest paper [78],

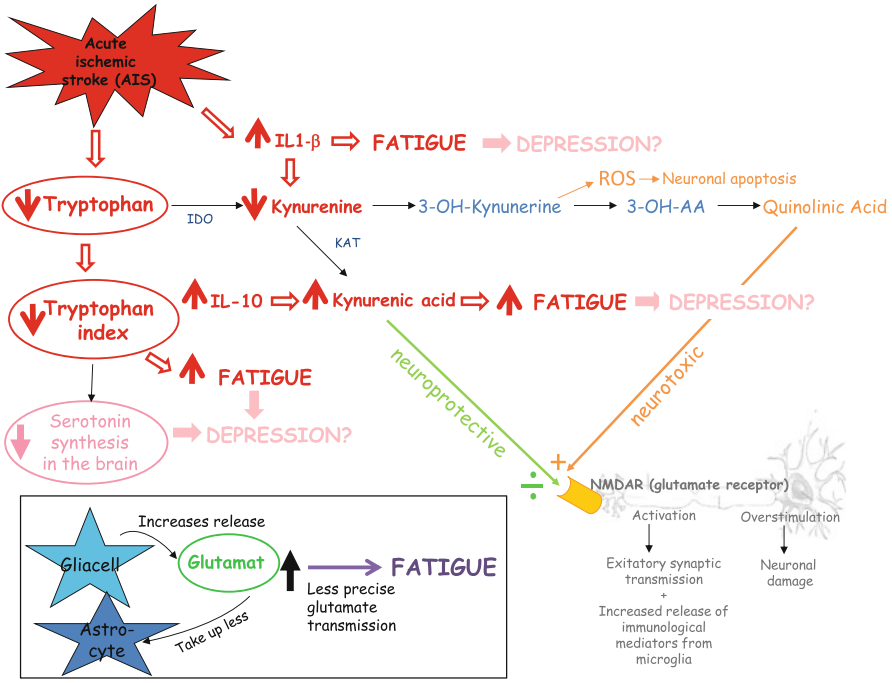

Fig. 17.2 A hypothetical scheme of the potential role of cytokines and KYN pathway activity in post-stroke fatigue (PSF) and post-stroke depression (PSD). The *open red arrows* indicate significant associations found in studies performed by our own research group and not necessarily involving known or explainable mechanisms. The *bold red arrows* indicate higher or lower levels of the various biological components, as measured in our studies. *Pink arrows* illustrate hypothetical consequences or mechanisms. The *inset* illustrates a possible mechanism underlying PSF as consequence of disturbed glutamate signaling. Since glutamate signaling is crucial for information intake and processing within the brain, and plays an essential role in the brain metabolism of glutamate, alterations in glutamate transmission may play a role in PSF. Since QA can act as agonists to the glutamatergic receptor, increased IDO activity may thus lead not only to alterations in brain serotonin neurotransmission but also to disturbed glutamatergic activity

we discuss the possibility that PSD differs from clinical depression and that the development of clinical depression post-stroke may be countered if the experience of PSF is taken into account (i.e., the patient accepts and uses appropriate coping mechanisms).

Future Research

Since IDO induces immune tolerance and may prevent ischemic tissue from being damaged by inflammatory factors, elevated IDO activity is potentially beneficial to AIS patients. The immunosuppressive effects of IDO are probably mediated by both TRP starvation and the actions of downstream KYN metabolites. However, as discussed above, since the metabolites that are capable of initiating immunological tolerance are potentially toxic to neurons, the overactivation of IDO in the central nervous system might be a double-edged sword. Further studies of KYN pathway activity in stroke are therefore warranted. Studies are also required to determine whether knowledge of the levels of specific metabolites or enzymes such as KAT and KYN 3-monooxygenase may be useful when determining pharmaceutical interventions for stroke patients related to the post-stroke neuropsychological and neurobehavioral sequelae.

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Chapter 18
The Role of Kynurenine Pathway in Pain and Migraine

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Abstract Migraine is a highly prevalent, disabling primary headache disorder which has a high socioeconomic impact. Its pathomechanism involves peripheral and central sensitization processes and activation of the trigeminovascular system. Alterations in the glutamatergic neurotransmission and the activation of excitatory receptors leading to neuronal hyperexcitability have been implicated in the pathomechanism of different pain syndromes, including migraine and neuropathic pain. The pharmacological management of these disorders is often a challenge, and the identification of possible new druggable targets is therefore at the focus of research interest. The kynurenine pathway involves several neuroactive metabolites which can influence glutamatergic neurotransmission and may therefore be promising novel candidates for drug development.

Keywords Migraine • Kynurenine pathway • Kynurenic acid • Hyperexcitability • Glutamate • Neuropathic pain • Nociception

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List of Abbreviations

3-HK	3-Hydroxykynurenine
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CSD	Cortical spreading depression
Glu	Glutamate
KAT	Kynurenine aminotransferase
KMO	Kynurenine monooxygenase
KP	Kynurenine pathway
KYN	L-Kynurenine
KYNA	Kynurenic acid
Mg^{2+}	Magnesium
NMDA	*N*-methyl-D-aspartate
nNOS	n-Nitric oxide synthase
QUIN	Quinolinic acid
Trp	Tryptophan

Introduction

Chronic pain syndromes have a high socioeconomic impact and a deteriorating effect on the patient's quality of life. Migraine, a highly prevalent, disabling primary headache disorder, has an overall prevalence of around 16 % in the adult population with a female predominance. Neuropathic pain, another common pain syndrome, involves a variety of etiological factors, but the main hallmark is an abnormal pain sensation with sensory disturbances without nociceptive stimuli. Chronic pain with neuropathic characteristics has a prevalence of 6–10 % in the general population. Migraine and neuropathic pain share common pathomechanistic features: glutamate (Glu)-mediated hyperexcitability and a sensitization process. Glu is the main excitatory neurotransmitter in the human brain, but the excessive stimulation of Glu receptors may result in neuronal damage, in the process of excitotoxicity. *N-methyl-d*-aspartate (NMDA) receptors are of outstanding importance in this process, and NMDA antagonism has long been suggested as a potential therapeutic option. However, complete NMDA antagonism is not feasible because of severe cognitive side effects, and more selective drugs are needed which can influence glutamatergic neurotransmission to restore the physiological balance.

The Kynurenine Pathway

The kynurenine pathway (KP) is responsible for more than 90 % of the peripheral degradation of the essential amino acid tryptophan (Trp) in the human brain (Fig. 18.1) [1]. The first, rate-limiting step of the KP is the enzymatic conversion of Trp into L-kynurenine (KYN) by indoleamine 2,3-dioxygenase. The pathway then divides into two branches, KYN being converted either by kynurenine-aminotransferase (KAT) to kynurenic acid (KYNA) or by kynurenine monooxygenase (KMO) to 3-hydroxykynurenine (3-HK). 3-HK is further metabolized in a sequence of enzymatic steps, this cascade giving rise to formation of the neurotoxic quinolinic acid (QUIN). The enzymatic machinery of the two arms of the KP is physically divided in the different cell types: KAT is mainly harbored in the astrocytes, while KMO is predominantly present in microglia [2, 3].

KYNA is a wide-spectrum endogenous antagonist of ionotropic Glu receptors and can therefore prevent excitotoxic neuronal death. KYNA is a noncompetitive antagonist of NMDA receptors by binding to the strychnine-insensitive glycine-binding site or with lower affinity to the ligand-binding site [4, 5]. The neuroprotective action of KYNA is mainly based on NMDA antagonism. Interestingly, on the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, KYNA displays a concentration-dependent dual effect: in nanomolar concentrations it may facilitate these Glu receptors, while in micromolar concentrations it behaves as an antagonist of them [6, 7]. This concentration-dependent effect may indicate a regulatory role in the brain. KYNA is also a ligand for the α-nicotinic-acetylcholine receptors and thereby participates in the regulation of presynaptic Glu release [8, 9]. All these are suggestive that KYNA is an important modulatory agent in glutamatergic neurotransmission [10, 11]. As concerns the neurotoxic kynurenines, QUIN exerts its harmful action mainly by NMDA agonism, but it also contributes to mitochondrial dysfunction, oxidative stress, and lipid peroxidation [12].

Migraine, Hyperexcitability, and Kynurenines

Despite extensive research, the exact pathomechanism of migraine has still not been fully elucidated. Although there are open questions, Glu-mediated hyperexcitability, peripheral and central sensitization, neurogenic inflammation, and activation of the trigeminovascular system seem to be key features [13]. Neuronal hyperexcitability has been confirmed in multiple investigations including various electrophysiological and neuroimaging studies [14–16]. Glu has been linked to the process by neurochemical measurements, indicating an upregulation of the glutamatergic metabolism [17–19]. Magnesium (Mg^{2+}) levels have been demonstrated to be decreased in the plasma, erythrocytes, and saliva of migraine patients interictally [18, 20–22]. The predominance of excitatory amino acids and low Mg^{2+} levels may further enhance NMDA receptor activation, thereby promoting hyperexcitability.

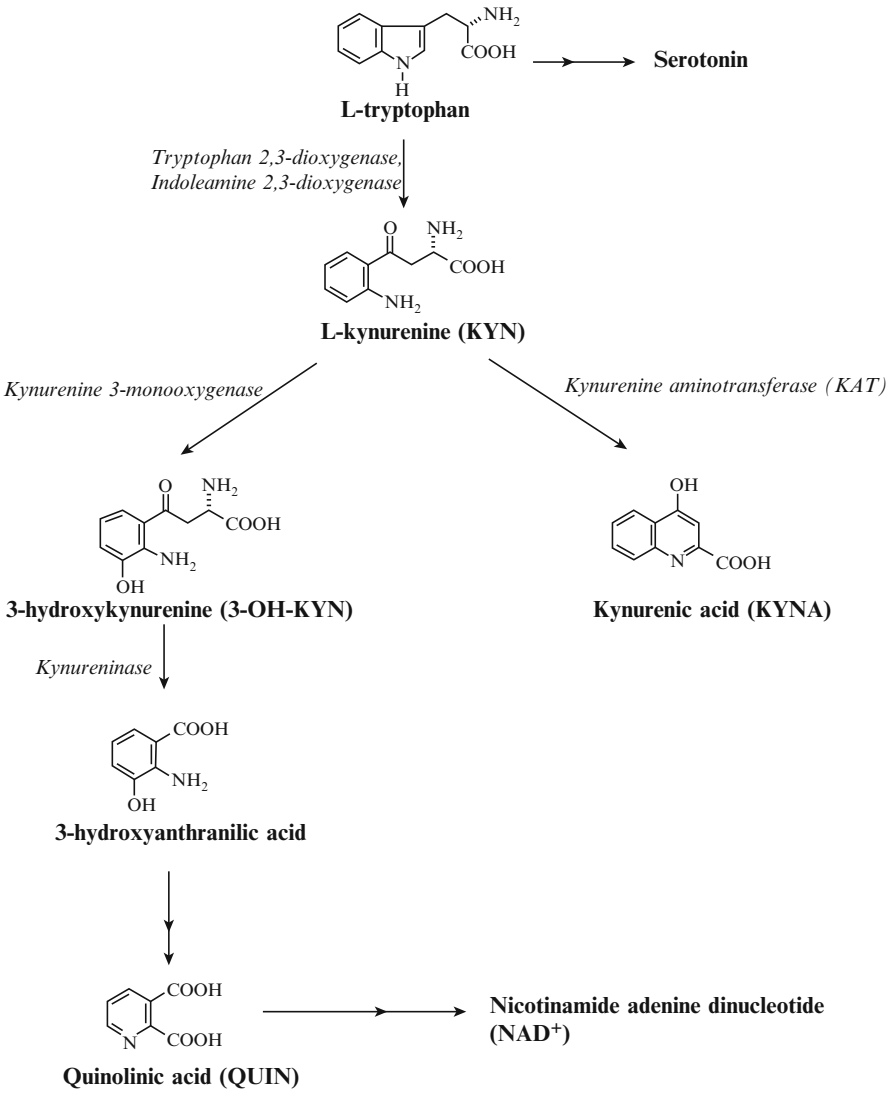

Fig. 18.1 The kynurenine pathway
This figure displays the enzymatic machinery of the KP, the main route of Trp metabolism in the human brain, which is responsible for the formation of the essential coenzyme NAD. The metabolic route divides at KYN, which can be converted either to the neuroprotective KYNA or in a metabolic cascade it yields several neurotoxic compounds such as 3-OH-KYN and QUIN.
Abbreviations: *3-OH-KYN* 3-hydroxykynurenine, *KP* kynurenine pathway, *KYN* L-kynurenine, *KYNA* kynurenic acid, *NAD* nicotinamide adenine dinucleotide, *QUIN* quinolinic acid

Evidence has emerged of the involvement of Glu in trigeminovascular nociception, and NMDA inhibition has been reported to inhibit trigeminovascular nociception [23–26]. Since the KP produces both an NMDA agonist and an NMDA antagonist, kynurenines were early implicated in migraine [27].

The coadministration of KYN with probenecid, an organic acid transport inhibitor, results in an elevated KYNA level in the brain [28]. This treatment in a nitroglycerine-induced or electrical stimulation migraine model resulted in a reduction of the c-Fos immunoreactivity in the rat trigeminal ganglion [29, 30]. Further, KYN with probenecid or a novel KYNA derivative prevented the nitroglycerine-induced expression of n-nitric oxide synthase (nNOS) [31]. The KYNA analogue effectively blocked calmodulin-dependent protein kinase II alpha and calcitonin gene-related peptide expression in the same animal model [32]. Cortical spreading depression (CSD) was successfully abolished by KYNA in in vitro models [33, 34]. KYN or KYNA suppressed CSD in a rat model; interestingly, this effect was influenced by the presence of ovarian hormones [35, 36]. Besides CSD suppression, KYNA decreased the permeability of the blood–brain barrier [36]. KYNA is also capable of influencing the brainstem nuclei. Injection of KYNA into the periaqueductal gray matter potentiated the antinociceptive effect of morphine [37]. In another animal experiment, a novel KYNA-amide (*N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride) diminished nitroglycerol-induced neuronal activation and sensitization in the cervical part of the trigemino-cervical complex [38].

All of the above findings clearly demonstrated that KYNA and its derivatives can influence the key structures and processes of migraine and achieve an antinociceptive effect.

Kynurenines in Other Pain Conditions

The antinociceptive effect of local KYNA treatment has been confirmed in a carrageenan-induced rat model of inflammatory pain [39]. KYN+ probenecid treatment prevented allodynia in an animal model of neuropathic pain by increasing the KYNA concentration [40]. In another study involving an inflammatory trigeminal pain model, probenecid treatment alone reduced the characteristic behavioral response and c-Fos and nNOS immunoreactivity, the effect being suggested to be mediated at least partly by KYNA [41]. Sciatic nerve stimulation induces the strong activation of noradrenergic locus coeruleus neurons, this being prevented by KYNA pretreatment [42]. The antinociceptive effect has been suggested to be mediated by NMDA receptor antagonism. One major concern with NMDA antagonist therapies is the occurrence of motor or cognitive side effects, but in these experiments no motor side effects developed. In a recent study of inflammatory pain, activation of the GPR35 receptor by KYNA led to an antinociceptive effect [43]. This effect of KYNA was suggested to be mediated by the inhibition of Ca^{2+} channels and Glu release [40, 44, 45]. Further extensive investigations are needed to elucidate the therapeutic potential of kynurenines in neuropathic pain conditions.

Conclusions

Glu hyperexcitability plays an important role in the pathomechanisms of migraine and neuropathic pain; Glu receptors are therefore an important therapeutic target. The KP includes several neuroactive metabolites which exert effects on NMDA receptors. Preclinical studies have yielded evidence that treatment with KYNA or its analogs can prevent CSD, allodynia, and trigeminovascular activation and achieve an antinociceptive effect. Further comprehensive investigations are warranted to clarify the therapeutic potential of these molecules as drug candidates.

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Chapter 19
The Role of Kynurenine Pathway Metabolites in Neuropsychiatric Disorders

Brian M. Campbell, Ana Pocivavsek, Francesca M. Notarangelo, and Anna I. Parachikova

Abstract In recent years, the role of kynurenine and its neuroactive metabolites as they relate to human disease has been intensely studied. Though much work has focused on kynurenine metabolism in neurodegenerative diseases, it is becoming clear that disruption of this pathway can have profound behavioral effects that are also relevant for neuropsychiatric indications. The following chapter will review both preclinical and clinical studies where kynurenine and its most abundant metabolic products were evaluated in relation to a range of highly prevalent neuropsychiatric disorders including schizophrenia, depression, and ADHD. Each section will summarize the kynurenine disruptions thought to be associated with a particular disorder, discuss the functional consequences of these effects, and speculate on means to improve unmet medical needs through manipulation of the kynurenine pathway.

Keywords Kynurenines • Schizophrenia • Depression • Attention deficit hyperactivity disorder • Psychiatric disorders

List of Abbreviations

1-MT	1-Methyl tryptophan
3-HANA	3-Hydroxyanthranilic acid
α7nAChR	α7 Nicotinic acetylcholine receptors
AA	Anthranillic acid

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BCG	Bacillus Calmette-Guerin
CNS	Central nervous system
CSF	Cerebrospinal fluid
FST	Forced swim test
IDO	Indoleamine 2,3-dioxygenase
3-HK	3-Hydroxykynurenine
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
KATs	Kynurenine aminotransferases
KMO	Kynurenine monooxygenase
KOKP	Kynurenine pathway Knockout
KYN	L-Kynurenine
KYNA	Kynurenic acid
LPS	Magnesium
MDD	Major depressive disorde
NMDA	*N*-methyl-D-aspartate
QUIN	Quinolinic acid
SNP	Single-nucleotide polymorphism
TDO	Tryptophan 2,3-dioxygenase
TTrp	tryptophan

Introduction

The kynurenine pathway (KP) metabolites serve multiple functions in mammalian physiology. In the past decade, there has been a tremendous surge in new information regarding these neuroactive metabolites, including their impact on brain physiology as well as putative roles in a variety of neuropsychiatric disorders. The focal points of this chapter are the KP metabolites, the genes, and the enzymes that influence their regulation and physiological processes, related to neuropsychiatric disorders, that induce activation of the pathway.

Kynurenine metabolism, as it relates to central nervous system (CNS) function, has been extensively studied in recent years [1, 2]. Kynurenine is a product of tryptophan metabolism mediated by two rate limiting enzymes, indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). The KP is further bifurcated into at least two branches by the enzymes kynurenine 3-monooxygenase (KMO) and kynurenine aminotransferases (KATs I-IV). In the brain, these branches, expressed in microglia and astrocytes, respectively, have often been considered as "neurotoxic" and "neuroprotective" or "excitatory" and "inhibitory", based on the neuroactive properties of the metabolites [3]. The microglial branch of the pathway, initiated by KMO, converts kynurenine to 3-hydroxykynurenine (3-HK) which leads to the formation of 3-hydroxyanthranilic acid (3-HANA) and quinolinic acid (QUIN) [4, 5]. QUIN acts as an excitotoxic agonist at the *N-methyl*-d-*aspartate* (NMDA) receptor and along with the other metabolites of the microglial pathway, namely 3-HK, QUIN can contribute to oxidative stress. The astrocyte branch metab-

olizes kynurenine to the end-product kynurenic acid (KYNA), a neuroinhibitory product that antagonizes both NMDA receptors [6] and α7 nicotinic acetylcholine receptors (α7nAChR) [7]. Recent studies also suggest that KYNA may bind G protein-coupled receptor GPR35 [8] and the aryl hydrocarbon receptor [9].

The two branches of the KP compete for the bioavailability of kynurenine and consequently its degradation into either KYNA or QUIN. Kynurenine is readily transported into the brain by the large neutral amino acid transporter [10]. Therefore, peripheral sources of kynurenine can impact brain biochemistry. Conversely, many of the metabolites of the pathway, including KYNA and QUIN, do not readily cross the blood brain barrier and thus local synthesis of these metabolites determines CNS concentrations [11].

In this chapter, we focus on the dysregulation of the KP in schizophrenia, bipolar depression, major depressive disorder (MDD), and attention-deficit and hyperactivity disorder (ADHD). The goals of the chapter are to: (i) review the evidence of dysregulation; (ii) examine the relationship to symptomology and disease pathology; (iii) discuss the potential cause of dysregulation; and iv) propose possible points for therapeutic intervention.

Kynurenine Pathway in Schizophrenia and Bipolar Disorder

Schizophrenia is a psychiatric disorder characterized by positive symptoms, such as delusions and hallucinations, and negative symptoms including lack of emotional arousal, mental activity, and social drive. The pathophysiology of the disorder involves dopaminergic and glutamatergic dysfunctions [12]. Bipolar disorder is characterized by episodes of mania and depression interspaced by symptom-free intervals (euthymia). Common features of both disorders are cognitive dysfunctions, including deficits in explicit memory, working memory, attention, and executive function [13, 14].

Mounting evidence indicates that abnormalities in KP metabolism exist in the brain of individuals with schizophrenia and bipolar disorder. Consistent findings suggest increased levels of KYNA in the prefrontal cortex [15, 16] as well as in cerebrospinal fluid (CSF) [17–19] of patients with schizophrenia compared to healthy controls. Importantly, elevations in endogenous KYNA levels were observed in CSF of drug naïve, first episode schizophrenia patients, as well as patients undergoing treatment with antipsychotics [17–19], implying that increased KYNA is a function of the disorder rather than a consequence of treatment. Further supporting the notion that activation of the KP is not related to long-term antipsychotic use, chronic treatment of rats with haloperidol or risperidone did not alter KYNA levels [15, 16]. Elevations in kynurenine, the direct bioprecursor to KYNA, were observed in the prefrontal and anterior cingulate cortex [15, 20], as well as CSF [19] of individuals with schizophrenia. In contrast, no differences in 3-HK or QUIN levels were detected [15, 21]. Though most studies report an increase in astrocyte-mediated kynurenine metabolism in schizophrenia, at least one study

reported an increase in the microglial branch metabolite 3-HANA in postmortem brains of patients with schizophrenia [22]. This elevation could be explained by decreased activity of 3-hydroxyanthranilic acid dioxygenase, the enzyme that catalyzes its degradation [16].

Patients with bipolar disorder show similar changes in KP metabolism compared to patients with schizophrenia. Specifically, elevated levels of both KYNA [23, 24] and kynurenine [20, 22] were reported in CSF and postmortem tissue. However, no changes specific to the microglial branch of the pathway, including 3-HANA levels, were found [22]. Increased concentration of KYNA in the CSF of euthymic patients was associated with lifetime psychotic features and recent occurrence of mania [24]. Significant increases in kynurenine were also reported in bipolar patients with psychosis [22]. Similar to data in schizophrenic patients, these finding suggest that bipolar depression patients are burdened with an excess production of kynurenine that is metabolized primarily in the astrocyte branch of the KP to KYNA.

The specific activity of KAT II, the primary enzyme responsible for KYNA synthesis in the CNS, was not reportedly increased in the brains of individuals with schizophrenia, suggesting that the increase in KYNA is likely related to availability of its precursor kynurenine through mass action. Elevated kynurenine levels may derive from an increased conversion of tryptophan by IDO and TDO. Supporting this notion, elevations in TDO mRNA and protein were reported in postmortem brain of schizophrenia patients as well as in cases of bipolar disorder [20, 25].

Increased kynurenine availability may also be derived by a decreased conversion to 3-HK via the enzyme KMO [26]. Interestingly, significant decreases in KMO activity were detected in the prefrontal cortex [16], and reduced activity of the enzyme was associated with decreased mRNA expression in the frontal eye field in postmortem schizophrenia brain tissue [27]. The *Kmo* gene is located on chromosome 1q42, a chromosomal region that has been implicated in schizophrenia [28]. The first report of a polymorphism in the *Kmo* gene was reported in a Japanese cohort of patients with schizophrenia [29], but not replicated in an independent sample. The same single-nucleotide polymorphism (SNP) was associated with deficits in cognitive endophenotypes, predictive pursuit eye-tracking, and visuospatial working memory in both healthy volunteers and patients with schizophrenia [27] suggesting a relationship to specific symptom clusters rather than the disease per se. Alternatively, a nonsynonymous SNP has been associated with increased KYNA levels in CSF [30]. However, no association has been found between *Kmo* SNPs per se and clinical diagnosis of schizophrenia [27, 30].

Changes in KMO have also been reported in bipolar disorder. Specifically, while no differences have been reported between bipolar disorder patients and healthy controls, expression of KMO in the prefrontal cortex was decreased in patients with psychotic features compared with nonpsychotic patients [31]. In different group of patients, a SNP in *Kmo* was more common among patients with psychotic features and associated with higher CSF KYNA [31].

The relevance of elevated brain KYNA in the pathophysiology of schizophrenia and psychotic features of bipolar disorder is strongly supported by preclinical studies in rodents. In animal studies, increases in KYNA are paralleled by

decreases in extracellular levels of dopamine, glutamate, and GABA in various brain areas implicated in behavioral symptomology associated with schizophrenia, including the hippocampus, striatum, and prefrontal cortex [32–35]. Positive symptoms, a common feature of both disorders, are defined by hyperactivity in mesolimbic dopamine pathways and exogenous application of KYNA, like application of classical NMDA receptor antagonists PCP and MK-801, increases firing of midbrain dopaminergic neurons in rodents [36, 37]. Additionally, elevated KYNA is associated with cognitive dysfunction, a core domain of the psychopathology of both schizophrenia and bipolar disorder. These deficits appear to be related to glutamatergic and nicotinergic abnormalities, involving NMDA and α7nACh receptors distributed throughout the brain. Preclinical studies in rodents reveal that cognitive processes are modulated by fluctuations in the brain content of KYNA. Specifically, elevations in endogenous KYNA impair [32, 38–42], whereas reductions in KYNA improve [32, 43], cognitive functions. Furthermore, the connection between KYNA and schizophrenia may have a developmental dimension as several of the risk factors associated with schizophrenia, including perinatal stress and infections, result in the activation of IDO, a cytokine-responsive enzyme that catalyzes an increase in the formation of kynurenine, the direct bioprecursor of KYNA. Recent preclinical studies have therefore focused on elevating KYNA during pre- and early postnatal developmental periods. This concept has direct ramifications for the pathophysiology in schizophrenia, and in line with the prodromal events supporting the neurodevelopmental hypothesis of schizophrenia, results in long-lasting cognitive deficits in adult animals [44–46].

The KYNA hypothesis of neuropsychiatric dysfunction is aligned with evidence suggesting that a reduction in KYNA function in the brain may be beneficial for patients with schizophrenia and bipolar disorder. Targeting the enzyme KAT II, which preferentially controls a pool of KYNA in the brain that can be rapidly mobilized, may be a promising target for pharmacological intervention. Reductions in KYNA, achieved by specific inhibition of KATII, increased extracellular levels of both glutamate and dopamine and improved learning, attention, and working memory in rodents and primates [32, 47, 48]. Pharmacological inhibition of KAT II may be a particularly effective approach to combat cognitive dysfunction in individuals with psychiatric disorders.

Kynurenine Metabolism in Depression

Depression is the most prevalent neuropsychological disorder worldwide. It is estimated that ~20 % of people will experience a major depressive episode throughout the course of their lifetime [49]. Understanding the etiology of major depressive disorder (MDD) is complicated by social and demographic factors as well as the contribution of polygenetic and epigenetic factors. Emerging evidence shows that dysregulation of the immune system, chronic inflammation, and altered tryptophan

metabolism, including kynurenine metabolism, are contributing factors in at least some populations of MDD patients [3, 50].

Evidence for a role of kynurenine dysregulation in MDD is based primarily on the observation that proinflammatory mediators, including a variety of cytokines which impact kynurenine metabolism (e.g., interferon (IFN)-α,β,γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, 6) may be causally linked to depressive symptoms. The most direct evidence stems from studies in which immune stimulating agents such as IFN-α or endotoxin (lipopolysaccharide (LPS) *from E. coli*) induce depressive symptoms in patients and/or healthy subjects. For example, up to 50 % of hepatitis C patients who received IFN- α therapy developed depressive symptoms that were maintained throughout the course of treatment, but subsided within a short period after completion [51, 52]. In patients treated with IFN-α, tryptophan metabolism favored an increase in the ratio of kynurenine/tryptophan (K/T) in serum [53], a measure indicative of increased IDO and/or TDO enzyme activity. Importantly, increased K/T ratios were correlated with greater symptoms of depression and anxiety [52, 54]. Though not all patients reached the criteria to be considered depressed, those that did also showed the greatest disruption in tryptophan metabolism and highest K/T ratios. In healthy volunteers, stimulation of the immune system with low doses of endotoxin increased proinflammatory cytokine levels such as TNF-α and IL-6 and increased KP activation (K/T ratios) over a time course consistent with depressive symptoms [55]. In other studies, higher plasma K/T ratios correlated with anhedonia scores in adolescents with MDD [56], and elevated K/T, kynurenine levels, and 3-HANA/kynurenine ratios were associated with severity of depressive symptoms [57].

Though studies evaluating a genetic link in MDD patients to disrupted kynurenine metabolism are sparse, a few suggest that such a connection may exist in certain patient populations. Increased IDO activity, as measured by serum K/T, was demonstrated in MDD patients with IFN-γ (+874) T/A genotypes. Women with the higher IFN-γ producing T allele had elevated levels of kynurenine production as well as a higher incidence of depression than the AA genotype [58, 59]. More recently, an IFN-γ CA repeat polymorphism was identified that conferred lower tryptophan levels and higher kynurenine production [60], though the relationship between symptoms of depression and kynurenine metabolism has yet to be evaluated in these patients. More directly, a polymorphism in the promoter region of the IDO gene correlated with increased depression in hepatitis C patients treated with IFN-α [61]. Finally, in the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trial, two common SNPs in the IDO1 gene were associated with treatment outcome for either citalopram or overall antidepressant treatment [62]. Overall, these data support the concept that patients with increased kynurenine metabolism may have an elevated risk for depression.

Increased kynurenine metabolism, or elevated K/T levels in serum, is a relatively common finding in MDD studies; however, fewer reports have evaluated kynurenine metabolism in the CNS in depression. Where it was studied, QUIN was elevated in the anterior cingulate cortex in postmortem tissue of depressed patients, but only in severely depressed individuals [63]. In addition, studies have now demonstrated

that, along with increased plasma kynurenine [64], QUIN was elevated in the cerebrospinal fluid (CSF) of suicide attempters [65]. Intriguingly, the correlation between activation of the QUIN branch of the kynurenine pathway in suicide attempters was confirmed in non-MDD patients as well. In a subsequent study, where patients were followed for several months, QUIN levels were reduced in the CSF relative to the suicide attempt, though still elevated over time [66]. Furthermore, these same patients had reduced KYNA levels indicating a further imbalance in kynurenine metabolism favoring the KMO, or microglial, branch of the pathway relative to the KAT II arm. These data suggest that in addition to elevated IDO activity peripherally, selective metabolism of kynurenine along the "neurotoxic" (e.g., QUIN) branch may occur in the brains of severely depressed patients and those at high risk for suicide.

Preclinical studies strongly support the link between immune stimulation, induction of kynurenine metabolism, and development of depressive-like symptoms [67, 68]. Acute immune stimulation with LPS induced expression of IDO and KMO, without affecting KAT II expression, in the CNS in rodents [69–71]. Concurrent with these effects, animals responded poorly in forced swim (FST) and tail suspension (TST) tests, assays commonly used to measure depressive-like behavior in rodents. Blockade of IDO with 1-MT prevented the induction of IDO, attenuated the increase in K/T in the brain and periphery, and alleviated behavioral impairments. Interestingly, IDO inhibition did not affect elevated IFN-γ, TNF-α, and IL-1β suggesting that induction of proinflammatory cytokines occurred upstream or in parallel to KP activation. In another study, mice treated with LPS developed an anhedonic phenotype measured by sucrose or saccharine preference which was also blocked by IDO inhibition [72]. When infused directly into the brain, LPS increased K/T ratios in both the CNS and periphery [73]. Inhibition of IDO with 1-MT prevented elevation of K/T as well as reduced immobility in FST, suggesting that increased kynurenine production contributed to the depression-like phenotype caused by a direct inflammation challenge in the CNS. Similar effects were reported with the Toll-like receptor (TLR) 3 ligand, PolyI:C, which increased IDO expression and kynurenine metabolism in the brain while also producing a depressive-like phenotype [74].

In addition to acute experiments with LPS, chronic inflammation studies further support a link between kynurenine metabolism and depressive-like phenotypes in rodent models. Bacillus Calmette-Guerin (BCG), an attenuated strain of *Mycobacterium bovis*, produces a chronic low-grade lung infection in mice associated with a depressive-like phenotype and increased kynurenine metabolism [75, 76]. BCG treatment induces an acute sickness period in mice lasting up to 5 days followed by a more prolonged depression-like phase that was sustained for weeks [75]. Kynurenine levels were also increased for up to 3 weeks within the brain [76]. Dissection of the mechanism by which BCG regulated kynurenine metabolism and caused a depressive-like phenotype demonstrated that brain IDO, IFN-γ, and TNF-α mRNA were up-regulated in concordance with depressive-like behavior. The depressive phenotype and kynurenine dysregulation produced by BCG inoculation was absent in IDO and IFN-γ KO mice [77, 78]. However, proinflammatory cytokines

(e.g., IL-1β) remained elevated in IDO KO mice, and to a lesser extent IFN-γ KO mice, suggesting that the impact on depression-like behaviors of cytokine induction subsequent to BCG treatment occurred through a downstream effect mediated by kynurenine metabolism.

Both clinical and preclinical data support a link between inflammation, kynurenine metabolism, and symptoms of depression. Induction of IDO and KMO in the brain and/or peripheral tissues by proinflammatory cytokines which cause disruption of normal physiological metabolism of tryptophan and/or kynurenine appears to be an important link in the cascade of events leading to certain forms of depression and may be associated with a higher risk of suicide within these patients. Though the precise mechanism by which kynurenine dysregulation could contribute to development of depression is not well understood, the most prevalent theory suggests that an imbalance in QUIN production relative to KYNA results in excessive stimulation of NMDA receptors. Where tested in animal models, blockade of IDO has been beneficial in reversing or preventing the development of depressive phenotypes, though additional studies with better pharmacological and/or genetic tools will be needed to determine whether more selective inhibition of the QUIN branch yield similar effects. Because limited tools are available for testing the clinical benefit of manipulating the KP, it will be important for current (e.g., IDO inhibitors for the treatment of cancer) and future studies to evaluate the psychological status of patients in a systematic way to better understand the therapeutic potential of this system in MDD.

Kynurenine Metabolism in Attention Deficit Hyperactivity Disorder

Attention Deficit Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder with an estimated global prevalence in pediatric populations of 5–7 % [79, 80]. Clinically, ADHD is characterized by inattention, impulsivity, and hyperactivity (DSM-V criteria). Children with ADHD exhibit a high degree of impulsivity and hyperactivity, which tend to wane early in life, as well as inattention which persists throughout adulthood [81, 82]. Imaging studies show that ADHD is associated with deficits in the structure, functioning, and connectivity of fronto-striatal, parieto-temporal, and fronto-cerebellar networks [83]. Importantly, ADHD patients exhibit hypoactivation of the prefrontal cortex, parietal cortex, and basal ganglia during attentional tasks [84, 85]. Longitudinal data suggests that clinical outcome focused on attentional parameters may be underscored by delayed cortical maturation [86, 87].

Evidence for altered kynurenine metabolism in the pathophysiology of ADHD is limited. The first proposed link emerged in 1981 where an imbalance in tryptophan metabolism was hypothesized in ADHD [88]. Since then only a handful of studies have further evaluated the role of tryptophan and kynurenine metabolism in ADHD subjects. In these studies, tryptophan was elevated and a trend was noted for decreased 3-HK in serum from ADHD children, though no strong associations were

uncovered between kynurenine metabolites and ADHD symptoms [89]. However, it was speculated that if similar effects would be found in the CNS then decreases in 3-HK could reflect a reduction of normal synaptic pruning in the brain during development, consistent with delayed maturation of the cerebral cortex. Oades and colleagues further demonstrated that biochemical alterations in inflammatory signaling including IFN-γ, a cytokine known to impact kynurenine metabolism, correlated with clinical parameters of attention, impulsivity, and hyperactivity [89]. The authors found associations between increased serum tryptophan and decreased 3-HK to inattention, though the effects were marginal. Lastly, shorter pregnancy and lower birth weight of ADHD patients were linked to symptom severity, increased 3-HK and IFN-γ levels [90], while SNPs associated with KAT I and KAT II may be among several genetic risk factors that link low birth weight and ADHD symptoms [91]. Taken together, these data suggest that patients with ADHD may possess deficiencies in their ability to convert tryptophan to kynurenine or to further metabolize kynurenine into additional neuroactive products such as 3-HK. However, it's important to note that these effects, if present, are subtle and may contribute as a risk factor rather than having a strong etiological impact. Therefore, the exact nature of any potential effect (e.g., decreased vs increased 3-HK) needs to be more clearly determined.

Though few studies have evaluated kynurenine metabolites in ADHD patients, associations can be hypothesized between kynurenine metabolism and ADHD neuropathologies and/or clinical manifestations. As discussed above, preclinical studies indicate that KYNA may negatively modulate dopamine, acetylcholine, and GABA release as well as inversely regulate glutamate levels in the CNS [33, 35, 92, 93], thus influencing key neurotransmitter systems associated with cognitive function. Additionally, blockade of KAT II improved performance in sustained attention, working memory, and spatial memory tasks in rodents and primates, highlighting the impact of KYNA on cognitive processes.

In conclusion, current evidence to support a link between kynurenine dysregulation and ADHD remains limited. Few strong associations have been found, though where this has been studied, the data seem to predict an imbalance in metabolism that favors a higher ratio of KYNA to 3-HK/QUIN, or perhaps an overall impairment in tryptophan conversion to kynurenine. From a treatment perspective, preclinical data suggests that reduction of KYNA could have therapeutic value in treating deficits in cognitive symptom domains which remains a large unmet need in ADHD.

Summary

Dysregulation of kynurenine metabolism is increasingly seen as an important factor in the pathophysiology and/or symptom domain clusters for a variety of neuropsychiatric disorders. As described above, disruption or augmentation of kynurenine production, and imbalances in the primary metabolites, may have a contributing role in these disorders. Elevated production of KYNA inhibits neurotransmitter

systems (e.g., glutamate, dopamine) important for normal cognitive performance, specifically in domains of attention, working memory, and executive function. Alternatively, excess production in the QUIN branch appears more closely related to mood state, possibly by increasing neuronal vulnerability in conjunction with inflammation and/or stress. The position of this pathway at the interface between immune and neuronal signaling provides a unique opportunity to study how differential regulation of an enzyme pathway may result in behavioral endophenotypes associated with a broad range of human diseases. Successful development of clinical agents able to test the role of kynurenine metabolism might open the venue for novel therapeutic interventions in a wide range human CNS disease.

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Part IV
Oncology

Chapter 20
Role of the Kynurenine Pathway in Stem Cell Biology

Simon P. Jones, Gilles J. Guillemin, and Bruce J. Brew

Abstract The KYN pathway (KP) is the main catabolic pathway of the essential amino acid TRP. The KP has been identified as playing critical roles in the regulation of the immune response in a variety of experimental settings. It is also known to be involved in several neuroinflammatory diseases including Huntington's disease, amyotrophic lateral sclerosis and Alzheimer's disease. This chapter considers the current understanding of the role of the KP in stem cell biology. Both these fundamental areas of cell biology have independently been the focus of a burgeoning research interest in recent years. However, little is known about how these two important fields of research interact. Several of the inflammatory and infectious diseases in which the KP has been implicated include those for which stem cell therapies are being actively explored at a clinical level. Therefore, it is highly pertinent to consider the evidence that the KP influences stem cell biology and impacts on the functional behaviour of progenitor cells.

Keywords Kynurenine pathway • Indoleamine 2,3-dioxygenase • Embryonic stem cell • Haematopoietic stem cell • Mesenchymal stem cell • Neural stem cell

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List of Abbreviations

1-MT	1-Methyl tryptophan
3-HAA	3-Hydroxyanthranilic acid
AhR	Aryl hydrocarbon receptor
CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
ESC	Embryonic stem cell
FASD	Fetal alcohol spectrum disorders
hESC	Human embryonic stem cells
HSC	Haematopoietic stem cell
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
KMO	Kynurenine 3-monooxygenase
KP	Kynurenine pathway
KYNA	Kynurenic acid
MSC	Mesenchymal stem cell
NAD+	Nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
NSC	Neural stem cell
PGE2	Prostaglandin E2
PIC	Picolinic acid
QUIN	Quinolinic acid
TDO	Tryptophan 2,3-dioxygenase
TGF-β1	Transforming growth factor-β1
TRP	Tryptophan

Introduction

Stem Cells

A stem cell is defined by two fundamental properties: a capacity to self-renew and the ability to differentiate into mature cells. A hierarchy of stem cell potential exists, with the pluripotent embryonic stem cells (ESCs) at the apex (Fig. 20.1). ESCs are derived from the inner cell mass of the developing blastocyst and can give rise to mature cells of all three germ layers. Due to a range of moral, bioethical and technical issues, there are numerous hurdles to the clinical application of ESCs. Consequently, the last three to four decades have witnessed an increased interest in the use of adult stem cells. Adult stem cells can be isolated postnatally from a host of different organs and tissues. They typically have a more limited differentiation potential—often restricted to mature cells of one germ layer. Of the diverse range of adult stem cells, the haematopoietic stem cell (HSC) represents perhaps the best-studied multipotent cell and can give rise to all cells of the blood.

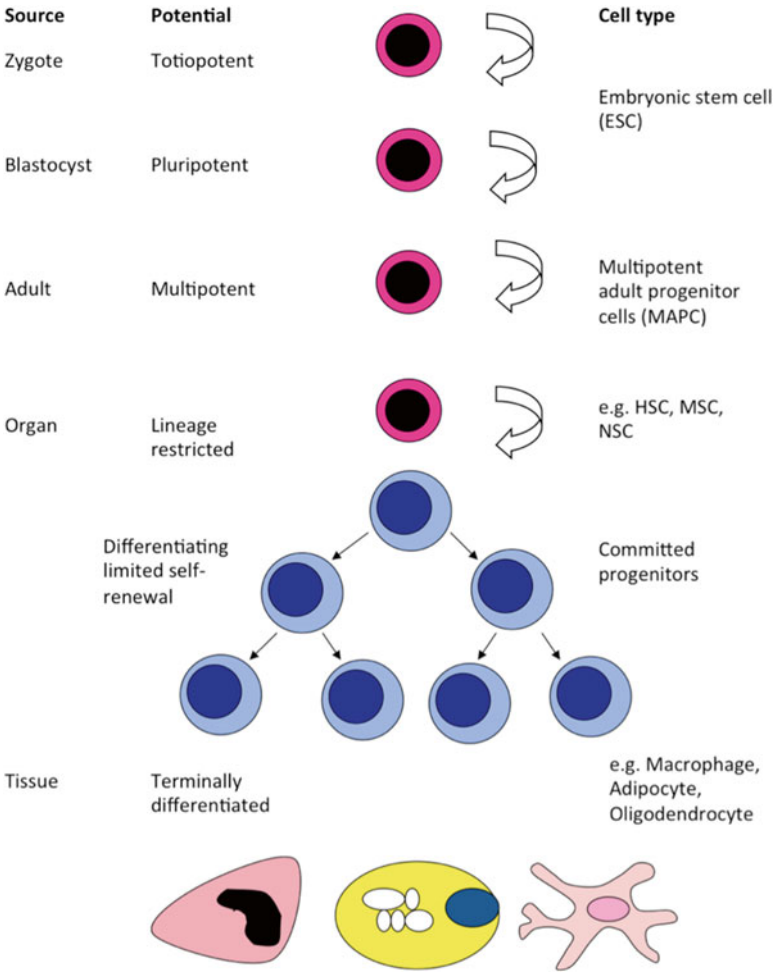

Fig. 20.1 The hierarchy of stem cells

Another bone marrow resident stem cell is the mesenchymal stem cell (MSC). MSCs can differentiate into cells of mesodermal origin, typically osteoblasts, chondrocytes and adipocytes. Neural stem cells (NSCs) represent a relatively recently identified organ-specific adult stem cell that can differentiate into neurons, astrocytes and oligodendrocytes (Fig. 20.1).

The KYN Pathway

Tryptophan (TRP) is one of the 9 essential amino acids that the human body is unable to synthesise and, thus, has to be provided through diet. Once absorbed by the body, TRP travels around the peripheral circulation. TRP is the only amino acid

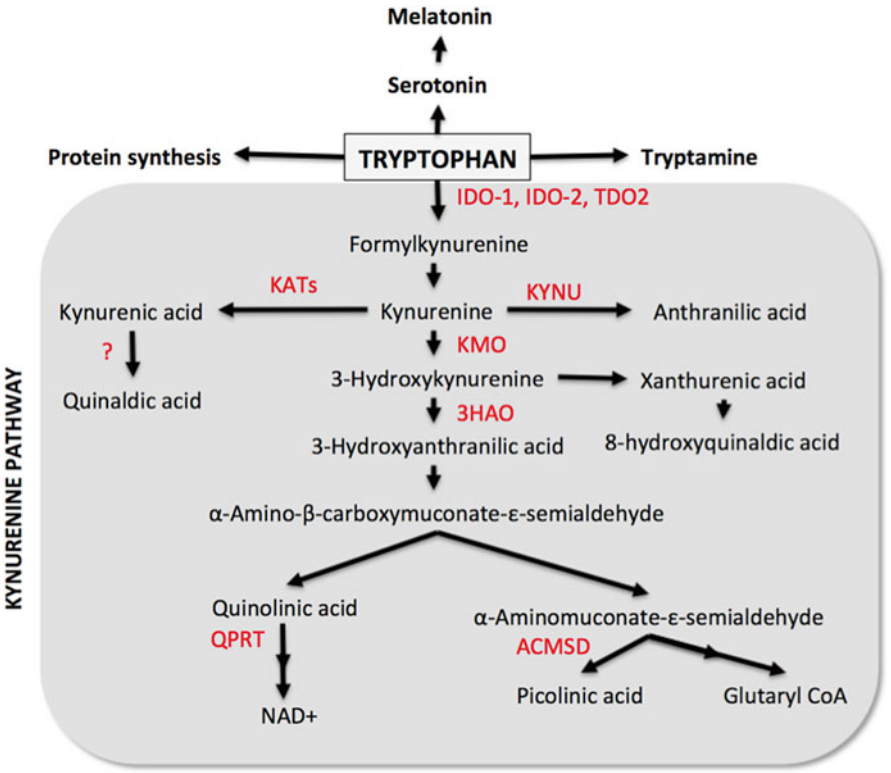

Fig. 20.2 Overview of the KYN pathway of TRP metabolism. Key enzymes are indicated in *red*. Abbreviations: *IDO*, indoleamine 2,3-dioxygenase; *TDO2*, TRP 2,3-dioxygenase; *KYNU*, kynureninase; *KATs*, KYN aminotransferases; *KMO*, KYN 3-monooxygenase; *3HAO*, 3-hydroxyanthranilic acid oxygenase; *ACMSD*, alpha-amino-beta-carboxymuconate-epsilon-semialdehyde decarboxylase; *QPRT*, quinolinic acid phosphoribosyltransferase

that binds to albumin in the plasma with approximately 10 % of the total plasma TRP in the free form and 90 % transported bound to albumin, with these two states existing in equilibrium [1]. However, TRP can only be transported across the blood–brain barrier in its free form by the competitive and non-specific L-type amino acid transporter. Once in the central nervous system (CNS), TRP acts as a precursor to several metabolic pathways including general protein synthesis, serotonin/melatonin synthesis and kynurenine (KYN) production [1] (Fig. 20.2).

In both the peripheral and central systems, the kynurenine pathway (KP) represents the major route for the catabolism of L-TRP, resulting in the production of the essential cofactor pyridine nucleotide nicotinamide adenine dinucleotide (NAD^+) and other neuroactive intermediates (Fig. 20.2). TRP is oxidised by cleavage of the indole ring, initiated either by tryptophan 2,3-dioxygenase (TDO2), indoleamine 2,3-dioxygenase 1 (IDO-1) or indoleamine 2,3-dioxygenase 2 (IDO-2) [2–5]. TDO2 is primarily expressed in the liver [6, 7] but is also present in the CNS [8]. TDO2 is

induced by its substrate TRP and by corticosteroids [7]. TDO2 can be inhibited by indoleamines and nicotinamide analogues as well as some antidepressant drugs [9]. IDO-1 is the predominant enzyme extrahepatically and can be found in most of the cell types, including macrophages, microglia, neurons and astrocytes but not in oligodendrocytes [10–12]. IDO-1 is upregulated by several inflammatory molecules including lipopolysaccharides, amyloid peptides and HIV proteins [13–15] but to date its most potent activator is interferon-γ (IFN-γ) [5, 16]. IFN-γ induces both the gene expression and enzymatic activity of IDO-1 [17, 18]. Whilst IDO-2 possesses similar structural and enzymatic activities to IDO-1, IDO-2 displays a different expression pattern and signalling pathway [4, 19]. In the CNS, the KP enzymes are variably expressed in most of the cell types [11], including astrocytes [20], neurons [21], infiltrating macrophages and microglia [22], oligodendrocytes [12] and endothelial cells [23]. Infiltrating macrophages, activated microglia and neurons express the full range of KP enzymes, whereas astrocytes and probably oligodendrocytes lack the crucial enzymes: kynurenine 3-monooxygenase (KMO) and IDO-1, respectively [12, 20].

In terms of KP metabolites, KYN is the first stable intermediate formed. Subsequently, several other neuroactive intermediates are generated including the free-radical generator, 3-hydroxyanthranilic acid (3-HAA) [24]; the excitotoxin and N-methyl-D-aspartate (NMDA) receptor agonist, quinolinic acid (QUIN) [25]; the NMDA antagonist, kynurenic acid (KYNA) [26] and the neuroprotectant, picolinic acid (PIC) [27]. Amongst all the KP metabolites, QUIN is likely to be one of the most important in terms of biological activity [28].

In recent years, IDO-1 has been the focus of attention because of its potent immunosuppressive effects on T lymphocytes, resulting partly from TRP depletion and partly from direct effects of TRP catabolites [29–32]. Some of the KYNs, such as QUIN and 3-HAA, can selectively target immune cells undergoing activation, consequently suppressing T-cell proliferation [33, 34]. More recently, KYN has also been involved in immuno-regulation through its ligand function for the aryl hydrocarbon receptor (AhR) [35]. KP metabolites can also act in concert to produce an additive effect [31]. IDO-1 upregulation and accelerated and sustained degradation of TRP represent a key indicator of inflammation. Indeed, inflammation and resulting immune activation lead to the activation of the KP and the concomitant increased production of the excitotoxin QUIN [36]. To date, QUIN has been associated with the pathogenesis of a wide range of inflammatory diseases and disorders [37–40].

Evidence of KP Involvement in Stem Cell Biology

Embryonic Stem Cells

Currently, there is very little known about the role of the KP in ESC biology. As one might expect for such a critical metabolic pathway, the rate-limiting and key enzymes of the KP are expressed in human ESCs [41]. In a quest for biomarkers of

developmental toxicity, Cezar et al. identified that small molecules of the KP, in particular TDO2, were upregulated in ESCs when treated with the antiepileptic drug valproate [41]. The authors propose that the KP plays a role in the pathogenesis of neurodevelopmental disorders. They hypothesised that activation of the KP reduces the bioavailability of TRP leading to a reduction in serotonin synthesis and subsequent neurodevelopmental defects. Aside from this study, there has been limited exploration of the KP in ESC biology.

Haematopoietic Stem Cells

HSCs represent a population of progenitor cells that, relative to other adult stem cells, have been well characterised as the precursor of the cells of their respective tissue system. The role of HSCs and their niche in haematopoiesis, transplantation biology and treatment of malignancies have been the focus of much interest in recent years. Cells of the haematopoietic lineage were amongst the first studied to reveal a link between the KP and immune regulation. The expression of IDO has been well characterised in professional antigen-presenting cells [29, 42]. In these studies, the activation of the KP in dendritic cells and macrophages was a potent mechanism for the regulation of T-cell proliferation. In fact, IDO production from donor monocytes following haematopoietic stem cell transplantation is thought to be responsible for the depressed T-cell function often seen in these patients [43]. Work on TRP catabolism in lineage-committed haematopoietic cells has recently been extended to investigate, for the first time, the activation of the KP in their progenitor forebears: HSC [44]. In this study, the authors found that both acute and chronic stimulation with IFN-γ caused an increase in the production of KYN in $CD34^+$ HSC cultures. They hypothesised that the IFN-γ-mediated activation of the KP might inhibit haematopoiesis. However, they did not observe any functional suppressive effect of IDO on erythropoiesis in their experiments. This is possibly due to the fact that TRP concentrations were not significantly depleted to starve HSC. Kurz et al. suggested that other bone marrow resident progenitor cells could therefore account for the inhibition of erythropoiesis seen in conditions such as anaemia [44].

Mesenchymal Stem Cells

As a purported population of adult stem cells, links between TRP metabolism and MSC biology have been more widely investigated. MSCs are multipotent progenitors initially identified in the bone marrow [45] where they are thought to have a physiological role in maintaining the haematopoietic stem cell niche [46]. Recently, MSCs have also been isolated and expanded from a wide range of postnatal and fetal tissues [47–49] with some investigators suggesting they are present in nearly

all adult tissues [50]. The defining feature of MSCs is their ability to differentiate in various tissues of mesodermal origin, typically osteoblasts, chondrocytes and adipocytes [51]. Interestingly, it seems MSCs are not restricted to a mesodermal fate. They have also been shown to differentiate into endothelial cells [52] and neural cells [53]. This plasticity of MSC, along with their ability to migrate to sites of inflammation, has attracted much attention in the last decade for their potential use in transplant and regeneration studies [54]. Another aspect of MSC biology that makes them of particular interest is the finding that they display potent immunomodulatory functions. MSCs from a variety of species inhibit the response of T cells to antigenic, mitogenic and polyclonal stimuli [55–57]. This effect is targeted mainly at the level of T-cell proliferation and has been further characterised as an arrest of the cell cycle in the G_1 phase [58]. This has been shown to be a pro-survival affect with MSC rescuing T cells from apoptosis [59]. The immunosuppressive effects of MSCs are not limited to T cells. B-cell proliferation and differentiation [60], dendritic cell maturation and antigen-presenting function [61] as well as natural killer cell proliferation and cytotoxicity [62] are all targets of MSC-mediated suppression. There is also growing evidence that MSC exert similar effects on the innate immune system (for a review of this subject, see [63]).

The mechanisms by which MSCs produce these compelling immunomodulatory functions remain unresolved. Whilst cell-to-cell contact seems to be required to 'license' MSCs to become suppressive, the inhibitory effect is ultimately mediated by a soluble factor(s) [59]. Several candidate molecules have been proposed including transforming growth factor-β1 (TGF-β1) [56], hemeoxygenase-1 (HO-1) [64], prostaglandin E_2 (PGE_2) [65] and nitric oxide (NO) [66]. Interestingly, in light of this chapter, activation of the KP has also been investigated as a potential mechanism for this immunosuppressive effect. Meisel et al. showed that IDO could be induced in MSC by exposure to IFN-γ in a dose-dependent manner. This finding was confirmed in cocultures of MSC and mixed lymphocyte reactions (MLRs) where significant IDO activity was detected as compared to MSC or MLR cultures alone, suggesting MSCs were the primary source of the IDO activity. Importantly, in the MSC/MLR cocultures, the addition of TRP significantly restored T-cell proliferation. The authors did not investigate the effect of IDO inhibitors in this system [67]. In support of these findings, a more comprehensive study of the MSC immunomodulatory function found that the separate addition of two different competitive inhibitors of IDO, 1-methyl tryptophan (1-MT) and norharmane, reduced the suppressive effect of MSC on T-cell proliferation [68]. A third study has also confirmed a role for IDO in the MSC-mediated suppression of allogeneic T-cell proliferation. The treatment of MSC/MLR cocultures with 1-MT significantly, but not completely, restored allo-driven proliferation of lymphocytes [69]. As an interesting aside, it has been documented that ESCs share similar immunosuppressive functions with MSC [70]. However, it remains to be seen whether the KP plays a role in this inhibitory effect. In a recent study, using murine ESC and MSC, the authors found that IDO production did not have a role in stem cell-mediated immunosuppression in their system [71].

A role for the KP in the immunosuppressive effects of MSC has been supported by in vivo studies. Using experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis, Matysiak et al. showed that mice transplanted with MSC experienced significantly lower clinical scores and greater improvement than control mice with EAE [72]. The authors reported a higher than twofold increase in the expression of IDO in the spleens of EAE mice treated with MSC, and on closer analysis, it was found that $CD11c^+$ dendritic cells were the population predominantly expressing IDO. The use of 1-MT to treat MSC-transplanted EAE mice restored clinical scores to similar levels as those of control mice with EAE and no transplanted MSC. Therefore blocking IDO activity led to a loss of the immunosuppressive function of MSC in vivo and exacerbation of inflammatory disease [72].

In addition to their effects on T cells, MSCs have been shown to inhibit NK cell proliferation and cytotoxicity, which is at least partially mediated by IDO [73]. Furthermore, in this study, the addition of the PGE_2 inhibitor, NS-398, to MSC and NK cell cocultures along with 1-MT (inhibitor of IDO) almost fully recovered IL-2-stimulated NK cell proliferation. This suggests a mechanistic synergy between IDO and PGE_2 in the immunosuppressive effect of MSC. The authors speculate that the synthesis of IDO by MSC is induced both directly—by exposure to pro-inflammatory cytokines such as IFN-γ—and indirectly by autocrine stimulation of cells by PGE_2. This hypothesis is supported by earlier work using dendritic cells where PGE_2 exposure induced the de novo expression of IDO mRNA [74].

Despite the evidence outlined above, the notion that the KP regulates the MSC-mediated suppression of immune cells remains contentious. Gieseke and co-workers used MSC cultured from the bone marrow of a boy with a frame shift mutation in the IFN-γ receptor 1 (R1). These $MSC^{IFN\gamma R1-}$ failed to respond to IFN-γ in vitro but displayed the same ability to inhibit PBMC proliferation as wild-type MSC. Additionally IDO expression was completely absent and non-inducible in $MSC^{IFN\gamma R1-}$ yet they were still able to inhibit the proliferation of allogeneic PBMC [75]. These findings clearly contest the notion that IFN-γ stimulated IDO expression is required for MSC to exert their inhibitory effects on immune cells. However, they do not rule out the fact that other factors are able to upregulate IDO. More recently, Lanz et al. demonstrated that murine MSCs suppress the activation of myelin-specific T cells independently of IDO [76]. The pharmacological inhibition of IDO, using 1-MT, failed to rescue the immunosuppression of MOG-activated splenocytes. This is based on the reasoning that 1-MT is a potent inhibitor of IDO. Work from our laboratory and others has shown that 1-MT, along with other pharmacological inhibitors, at best only offers partial inhibition of IDO activity. Moreover, the authors showed that in an EAE model, IDO gene ablation ($IDO1^{-/-}$ MSC) did not attenuate the therapeutic effects of MSC. Both wild-type MSC and $IDO1^{-/-}$ MSC reduced EAE clinical scores by a similar magnitude, potentially via an inhibition of pro-inflammatory cytokines, notably IL-17 [76].

Interestingly, there seems to be significant species differences between human and murine KP expression in what may be expected to be an evolutionarily conserved pathway. Our group has found that, in contrast to human MSC, mouse MSCs do not experience significant IFN-γ-inducible changes in the expression of several

KP enzymes at the RNA level [77]. Such findings may go some way to explain the conflicting evidence outlined above. In the same paper, Croitoru-Lamoury et al. showed that the inhibitory effects of KP activation are not limited to immune cells. In fact, IFN-γ inhibits the proliferation of both mouse and human MSC through activation of the KP. The addition of excess TRP to cultures of MSC stimulated with IFN-γ blocked the anti-proliferative activity of the cytokine on MSC. Moreover, blocking IDO action by addition of 1-MT rescued cell viability of cytokine-stimulated MSC. However, in long-term cultures of 50 days, addition of IDO inhibitors norharmane and 1-MT did not recover the significant decrease in proliferation of IFN-γ-treated MSC. The effect of IFN-γ-mediated activation of the KP on the proliferation of MSC was extended to investigate its effects on MSC differentiation. Using gene expression of recognised osteogenic and adipogenic differentiation markers (osteopontin, integrin-binding sialoprotein II and adipsin, adipoQ, Fabp4, respectively), it was demonstrated that treatment with IFN-γ inhibited the gene expression of all these markers. Importantly, the addition of norharmane to differentiation cultures partially recovered the gene expression of osteopontin, adipsin and adipoQ. Similar findings were extended to the neural differentiation of MSC [77]. Taken together, this evidence points to a role for the KP in modulating essential stem cell functions: proliferation and differentiation potential.

Neural Stem Cells

NSCs have been shown to express high levels of IDO mRNA and protein when activated by IFN-γ. This feature of NSC was utilised in a cell-based assay to examine the effects of a number of naturally occurring anti-inflammatory phytochemicals on IDO expression. The authors suggested that the suppressive effect of some of these compounds on IDO may account for their observed antitumour and neuroprotective properties [78]. Our group has previously established that much of the KP 'machinery' is expressed in NSC [77]. Using murine NSC, transcripts encoding all the major KP enzymes were detected under basal conditions. Ongoing experiments in our laboratory are examining the expression and function of the KP in neural progenitor cells. We hope to ascertain whether modulation of the KP can affect the survival, proliferation and differentiation of neural progenitor cells and ultimately their functional ability to remyelinate damaged neurons. This has huge potential to offer novel therapeutic opportunities for the treatment of inflammatory and neurodegenerative disorders.

The KP has been implicated in the pathophysiology of depression and its associated deficits in neurogenesis. Zunsdain et al. found that human hippocampal progenitor cells constitutively express IDO and TDO and that treatment with IL-1β significantly upregulated IDO. IL-1β also induced a decrease in expression of the 3 major KYN aminotransferase (KAT) enzyme isoforms, which catalyse the conversion of KYN into the neuroprotectant kynurenic acid (KYNA). Conversely, transcripts for two enzymes that act along the neurotoxic branches of the KP—KMO

and kynureninase (KYNU)—were increased. Thus the authors postulated that IL-1β inhibits neurogenesis in human hippocampal progenitor cells by increasing neurotoxicity and suppressing neuroprotection, although they provided no direct evidence. Treatment with the KMO inhibitor Ro 61-8048 partially reversed the detrimental effects of IL-1β on neurogenesis confirming the observed effects were mediated via the KP [79]. Interestingly, the authors showed that IL-1β had contrasting effects on the differentiation and proliferation of neural progenitor cells: IL-1β inhibits neural differentiation whilst promoting proliferation of undifferentiated cells. It was postulated that this difference was facilitated through activation of the neurotoxic branch and inhibition of the neuroprotective branch of the KP during differentiation. In contrast, IL-1β activated both the neuroprotective and neurotoxic branches in proliferating cells. A major limitation of this study was the use of a progenitor cell line under in vitro experimental conditions.

However, in vivo support for a role of the KP in neural stem cell differentiation comes from a report exploring the role of the KP in neurogenesis and anxiety-related behaviour using $TDO^{-/-}$ mice [80]. $TDO^{-/-}$ mice had significantly elevated plasma levels of TRP; however, concentrations of KYN and KYNA remained at physiological levels. IDO was suggested as the most likely candidate to compensate for the lack of TDO in maintaining downstream KP metabolite concentrations. In terms of the phenotype of neural progenitor cells, this study reported that there was a marked increase in the proliferation of NSC in the subventricular zone of $TDO^{-/-}$ mice brains. This suggests the KP plays a role in the regulation of adult neurogenesis and possibly higher brain functions. Evidence to support the latter came from observations that $TDO^{-/-}$ mice displayed anxiety-related behaviour in two classical behaviour tests.

An investigation into the effect of alcohol exposure on neural development in the fetus treated human embryonic stem cells (hESCs), neural progenitors and neurons with ethanol in vitro and analysed the metabolome of hESCs during these stages of neurogenesis [81]. Interestingly, ethanol induced the significant alteration of TRP metabolism in hESC and NSC. A number of KP metabolites were elevated upon alcohol exposure including 3-hydroxy-L-KYN, 5-hydroxy-L-KYN, L-KYN and indole-3-acetaldehyde. This may provide potential biomarkers to help with the diagnosis of fetal alcohol spectrum disorders (FASD) and also suggests aberrant TRP metabolism plays a mechanistic role in FASD.

Therapeutic Applications

This chapter has hopefully established that there already exists a considerable body of evidence to suggest that the KP plays a significant role in the development and function of stem cells. The clinical application of this knowledge is in its infancy but there is promising evidence that targeting stem cells through the manipulation of the KP could provide clinical benefit. Several synthetic TRP catabolites (tranilast, teriflunomide and laquinimod) are in phase II and phase III clinical trials for the prevention of a number of autoimmune disorders including multiple sclerosis [82].

Although it is thought the mechanistic action of these compounds is mediated largely through their immunosuppressive effect on T cells and natural killer cells, their influence on endogenous progenitor cells has yet to be investigated. It is noteworthy that in a recent double-blind, placebo-controlled phase III study investigating the efficacy of teriflunomide in multiple sclerosis patients, there was a significantly reduced risk of disability progression in the treatment groups [83]. This suggests a regenerative action above and beyond a solely anti-inflammatory effect.

Two studies outlined in this review identify KP metabolites in stem cells as potential biomarkers for neurodevelopmental disorders [41, 81]. More than their putative role as markers of disease, it is highly likely that the KP plays a mechanistic role in a number of cognitive and motor deficits associated with neurodegeneration. There is recent compelling evidence in both *Drosophila* [84] and mice [85] that the genetic and pharmacological inhibition of KMO reverses neurodegeneration in models of Alzheimer's disease and Huntington's disease. This effect was mediated by a concomitant decrease in the neurotoxin 3-HK and increase in KYNA. The authors advocate the prompt exploration of KMO inhibitors at the clinical level. Interestingly, the mode of action of some antidepressant drugs has been linked to reducing KMO levels and stimulating KAT activity, which leads to the production of KYNA from KYN [86]. Ensuring the correct balance of neurotoxic and neuroprotective KP metabolites in future stem cell therapies must be of prime importance. This notion is consistent with clinical data demonstrating a decrease in KYNA levels in depressed patients [87].

Conclusion

In this chapter, we have tried to outline the known links between the KP and stem cell biology. In ESC and HSC, there is relatively little known about the role of TRP metabolism in their function. However, in the case of MSC and NSC, there exists a rapidly growing body of evidence demonstrating a crucial link between the KP and the role of these progenitor cells in healthy and diseased tissue. It is clear that the KP has huge potential for the treatment of a wide range of inflammatory and degenerative diseases. The precise mechanistic role that progenitor cells play in this remains to be further elucidated. There are many questions and hypotheses to be tested; a better understanding of how KP metabolites impact on stem cell populations may help to overcome some of the current barriers to their efficacious use in animal models and enhance future therapeutic transplantation. This chapter heralds an exciting time for the exploration of TRP metabolism as a modulator of stem cell behaviour.

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Chapter 21
Role of Kynurenine Pathway in Cancer Biology

Christopher G. Mowat

Abstract The kynurenine pathway for tryptophan catabolism is responsible for the production of the essential cofactor NAD^+, but many of the pathway catabolites play roles in many different disease states. The involvement of the kynurenine pathway enzymes and catabolites in cancer occurs via both immune and nonimmune mechanisms. In this chapter, the consequences of the immune response to developing tumors will be summarized, and the role played by indoleamine 2,3-dioxygenase in enabling tumor immune escape via tryptophan depletion will be outlined. In addition, the role played by other enzymes, such as tryptophan 2,3-dioxygenase—which modulates the immune response by producing kynurenine—is described. Further to this, the involvement of downstream enzymes and catabolites of the pathway in tumor development is discussed.

Keywords Kynurenine • Indoleamine 2,3-dioxygenase • Tryptophan 2,3-dioxygenase • Immune escape • Interferon-γ • Aryl hydrocarbon receptor • Tryptophan catabolites

List of Abbreviations

KP	Kynurenine pathway
IDO	Indoleamine 2,3-dioxygenase
IDO2	Indoleamine 2,3-dioxygenase-2
TDO	Tryptophan 2,3-dioxygenase
NMDA	*N*-methyl-D-aspartate
TRP	L-tryptophan
KYN	Kynurenine
KYNA	Kynurenic acid
3-HK	3-Hydroxykynurenine

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AA	Anthranilic acid
3-HA	3-Hydroxyanthranilic acid
PIC	Picolinic acid
QUIN	Quinolinic acid
NAD^+	Nicotinamide adenine dinucleotide
IFN	Interferon
IL	Interleukin
TNF	Tumor necrosis factor
APC	Antigen-presenting cell
DC	Dendritic cell
AHR	Aryl hydrocarbon receptor
ACMSD	Aminocarboxymuconate semialdehyde decarboxylase
1-MT	1-Methyl-tryptophan
LPS	Lipopolysaccharide

The Kynurenine Pathway for Tryptophan Catabolism

Tryptophan (TRP) is an essential amino acid whose catabolism is primarily effected and controlled by the enzymes of the kynurenine pathway (KP). This route accounts for around 95 % of L-TRP turnover [1] and ultimately results in the production of the essential cofactor NAD^+. The enzymes and metabolites of the KP are summarized in Fig. 21.1, and the balance between these metabolites is of crucial importance due to their differing physiological roles. Several of these metabolites are neuroactive and play essential roles in the regulation of NMDA (*N*-methyl-D-aspartate) receptor function and in associated free radical generation. It is the excitotoxicity, and resulting excessive free radical production, mediated by the NMDA receptor that is involved in the pathology of neurodegenerative conditions such as (but not limited to) Alzheimer's, Parkinson's, and Huntington's diseases, and there is evidence to suggest that this is due to imbalances in levels of KP metabolites [2]. There is also evidence that dysregulation of the KP is involved in several other diseases, such as multiple sclerosis, pain syndromes, and immune conditions.

It is the role played by KP enzymes and metabolites in cancer, related to immunomodulation and possible other nonimmune factors, that is the focus of this chapter.

Tumor Development and the Immune Response

It has long been considered that there is a functional relationship between inflammation and development of tumors, and the theory of cancer immunosurveillance was developed throughout the first half of the 20th century, being described by

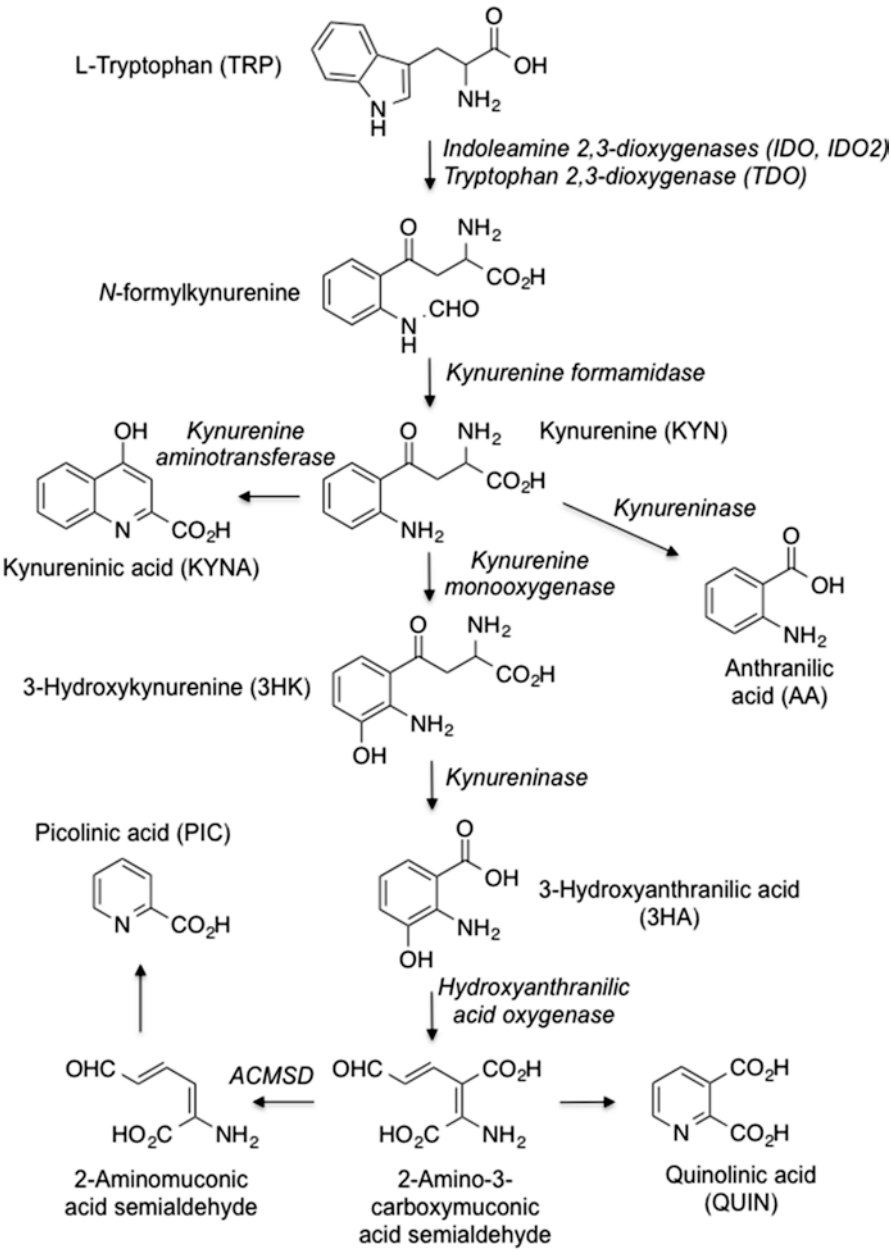

Fig. 21.1 The kynurenine pathway for tryptophan catabolism. Enzymes responsible for each step are shown in italics. *ACMSD*, aminocarboxymuconate semialdehyde decarboxylase. The pathway continues from quinolinic acid toward eventual synthesis of NAD^+

Burnet in 1957 [3]. While at times controversial, the operation of immunosurveillance is supported by studies on immunodeficient mice [4]. The concept of immunosurveillance involves the immune system of the host protecting against tumor formation, with the immune function of lymphocytes being stimulated by recognition of stress ligands or antigens expressed on transformed (malignant) cells, thus leading to their elimination [5].

The antitumor response is initiated when the presence of a growing tumor alerts the cells of the innate immune system by causing local tissue disruption. This in turn can cause the production of pro-inflammatory molecules that, alongside chemokines produced by tumor cells [6], further encourage the innate immune system to respond to the source of the challenge [7, 8]. This response may occur via various mechanisms, all of which have the consequence that interferon-γ (IFN-γ) is produced, a critical event for progression of the antitumor response. The presence of IFN-γ leads to a positive feedback system, resulting in more IFN-γ being released and the activation of several IFN-γ-dependent processes [9–19] that result in a proportion of the tumor being killed. These dead tumor cells subsequently provide a source of tumor antigens that enrolls the adaptive immune system into a tumor-specific response. This involves the activation of immature dendritic cells (DCs) at the site of the tumor, and these acquire tumor antigens prior to migrating to the draining lymph node [20]. At this point, naïve tumor-specific $CD4^+$ T cells are activated, and these enable the development of $CD8^+$ cytotoxic T lymphocytes [21–24]. This development of tumor-specific adaptive immunity thus allows $CD8^+$ T cells to kill antigen-positive tumor cells directly while also engaging mechanisms of cell cycle inhibition, apoptosis, and tumoricidal macrophage activity. This model of immunosurveillance, whereby both the innate and adaptive limbs of the immune system act to reject a developing tumor, forms only one part of the larger theory of immunoediting.

Immunoediting is perhaps best described as being comprised of three phases, the first of these being the "elimination" phase described above. If this phase is successful in eradicating the tumor, then the immunoediting is complete; otherwise, the process moves on to the "equilibrium" phase. This is described by Dunn et al. [4] as an essentially Darwinian phenomenon; the surviving tumor cells and the host immune system reach a dynamic equilibrium that serves to contain the unstable and mutating cells to the tumor bed, with the continual destruction and emergence of variant cells of increasing ability to resist the immune response. In this way, the immune system serves to sculpt the population of new tumor cells over a period of time that can (for some tumor types) amount to years, resulting in tumors that are able to resist the host immune system.

The third phase of immunoediting has been described as the "escape" phase. In this scenario, those tumor cells that have been "selected for" during the equilibrium phase are able to grow progressively, eventually becoming clinically detectable. The molecular basis of tumor escape has been shown to operate via immunosuppressive cytokines or T cells (regulatory T cells) [25, 26] and/or as a result of changes at the tumor itself. These changes may affect tumor recognition by the cells of the immune system (e.g., decrease in antigen expression or development of IFN-γ insensitivity) or provide mechanisms for avoiding immune destruction.

The result of all of this is an increased understanding of the role of the immune system in cancer development. While the properties of malignant tumors (survival, growth, invasion, metastasis) are often described in terms of the "hallmarks of cancer," it is now clear that the ability of a tumor to progress despite the presence of a normally functional immune system should be included among these descriptors.

Understanding the mechanisms of immune escape is critical because it is these that distinguish preclinical dormant lesions from clinically relevant disease. Potentially developing methods to overcome these mechanisms could allow cancer to be treated like an acute infection. The following sections will examine the role(s) played by specific KP enzymes and catabolites.

Tryptophan Depletion by IDO and Immunosuppression

The first step in the KP for TRP catabolism is the dioxygenation of L-Trp to form L-kynurenine (KYN). From Fig. 21.1, it can be seen that this may be catalyzed by one of three enzymes encoded in the human genome: indoleamine 2,3-dioxygenase (IDO), tryptophan 2,3-dioxygenase (TDO), and indoleamine 2,3-dioxygenase-2 (IDO2). The identity of the enzyme responsible for this reaction is determined by their respective expression patterns; TDO is primarily a hepatic enzyme that is has also been detected in the skin, brain, and early concepti [27–30], and the distribution of IDO2 in humans is, as yet, unclear [31] but IDO2 mRNA and/or protein has been detected in various tumor types [32, 33]. IDO, however, is expressed in most tissues, but especially in the gut, lung, epididymis, and thymus. The immunological role of TRP catabolism, induced by inflammatory agents, was thought to be in inhibiting growth of pathogens and cancer cells by starving them of TRP [34]. However, the discovery that IDO activity prevented rejection of allogeneic fetuses in pregnant mice by suppressing T-cell activity changed the focus of research into the role of TRP catabolism in immunity [35]. In this seminal work, the authors suggested that localized depletion of TRP caused by IDO activity limited the amount of free TRP available to proliferating T cells, thus preventing their response against the developing fetus. This finding led to other work supporting the idea that IDO has a broadly immunosuppressive role in viral, bacterial, and parasitic infections [36–38], but since the 1950s, there has been an awareness of the relationship between elevated TRP catabolism and cancer. First recognized in bladder cancer patients [39, 40], this was followed during the 1960s by similar findings for other types of cancer [41–44]. This upregulation is now attributed to IDO activity, is still being discovered in various tumor types including thyroid and colorectal cancers, and indeed can provide a diagnostic marker in some cases [45–47].

Production of IDO is induced by the presence of viruses, lipopolysaccharides (LPS), and several pro-inflammatory cytokines including interleukins (IL-1β and IL6), tumor necrosis factor (TNF)-α, and interferons (IFN-α and IFN-γ) [48–50]. Of these, it is the role of IFN-γ that is perhaps most discussed/best understood, and it is known to be a major inducer of IDO in several tumor types, including nasopharyngeal, cervical, colorectal, hepatocellular, ovarian, endometrial, and thyroid [51–54].

In this respect, there is interest in the mechanism of IFN-γ induction of IDO. This process relies on phosphorylation and nuclear translocation of the transcription factor STAT1 (as well as other transcription factors). The recent discovery that this mechanism is inhibited both by (−)-epigallocatechin gallate in colorectal cancer cells [55] and by sodium butyrate (via increased acetylation and decreased phosphorylation of STAT1) raises the possibility that it may be possible to modulate IDO expression as a means of alleviating IDO-mediated immune tolerance [56].

This induction of IDO expression by IFN-γ is particularly important in antigen-presenting cells (APCs), and these are found to create an environment that contributes to immune tolerance of tumors. Munn et al. showed that (in tumor-draining lymph nodes in mice) plasmacytoid dendritic cells (pDCs) expressing IDO [57] potently suppressed the response of T cells to antigens on these same pDCs, while also suppressing the response to antigens on other APCs. The precise mechanism of IDO-mediated T-cell suppression is thought to involve synergy between the effects of TRP depletion and the production of downstream KP catabolites [58, 59], both direct consequences of IDO activity.

The concept of local TRP depletion by IDO as a mechanism for immune modulation is now supported by a large and growing body of evidence, and some of the immunosuppressive effects induced by TRP depletion are reversible upon addition of the amino acid. Work by Munn et al. [60] identified GCN2, a stress-response kinase, as a downstream mediator of several key effects of TRP depletion by IDO. GCN2 is activated as a result of accumulation of uncharged transfer RNA (tRNA) as a consequence of insufficient levels of amino acid (in this case L-Trp) [61]. This GCN2 activation causes initiation of the integrated stress response, which can cause arrest of the cell cycle, differentiation, adaptation, or apoptosis [62], and it has been demonstrated that expression of GCN2 is necessary in T cells in order for them to be susceptible to anergy induced by the presence of IDO-producing dendritic cells [60]. The role of IDO activation and TRP depletion in tumor immune escape is simplified and summarized in Fig. 21.2.

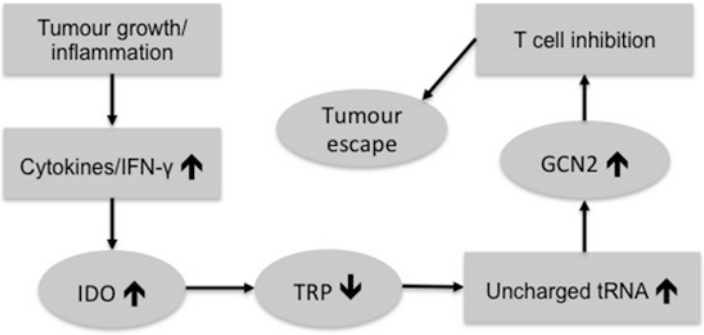

Fig. 21.2 The role of IDO in tumor immune escape. Inflammation and tissue disruption at the site of the tumor cause releases of several cytokines, including IFN-γ which strongly induces IDO production. This causes tryptophan depletion, leading to a stress response and activation of GCN2, which in turn inhibits T-cell activity and the immune response to the tumor

IDO2: A Relative Newcomer Related to IDO

The GCN2-dependent stress response induced by TRP depletion is also caused by the action of a recently discovered second enzyme, IDO2, which is closely related to IDO [63, 64] but appears to be less widely expressed in the body. In mice, it has been demonstrated that IDO2 is present in the liver, epididymis, and kidney, but as yet it is unclear whether it is also present in the brain. Similarly, a lack of studies examining both protein and mRNA expression in humans means that it is too soon to confidently assess the distribution of IDO2 in humans. However, as mentioned earlier, IDO2 has been detected in several tumors and cancer cell lines, along with mouse dendritic cell lines [32, 33, 64], with upregulation of its mRNA in response to IFN-γ. However, another study has shown that this phenomenon was not observed in HeLa cells treated with IFN-γ, even though IDO mRNA was expressed strongly [65]. This apparent inconsistency is perhaps due to the complexity of the transcription of the IDO2-encoding gene [66] and underlines the need for protein expression levels to be measured. In addition to this uncertainty, it has proven difficult to carry out reproducible and consistent biochemical characterization of IDO2 with respect to its ability to catabolize TRP. It has been suggested that IDO2 may be inactive in humans or that the conditions under which it is optimally active have yet to be determined [67], but the weight of evidence implies that IDO2 participates in similar cellular immunoregulation processes via TRP catabolism as IDO does.

Tryptophan Catabolites and the Involvement of TDO

While the specific response to TRP depletion goes some way to explaining IDO-induced immunosuppression, the question of whether TRP levels are maintained at such a necessarily low level in vivo (<0.5–1 μM) raised the hypothesis that other IDO-dependent (or indeed IDO-independent) mechanisms exist.

In this respect, numerous reports have been made on the effects of downstream KP catabolites. KYN, kynurenic acid (KYNA), 3-hydroxykynurenine (3-HK), and 3-hydroxyanthranilic acid (3-HA) have all been shown to suppress T-cell function and can also cause T-cell apoptosis [68], while potential receptors have been identified for individual catabolites [69]. Of most interest, perhaps, is the mounting evidence that the aryl hydrocarbon receptor (AHR) plays a role as a target for kynurenine binding [70–72]. The AHR is a member of the basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) family of transcription factors and is activated by xenobiotic ligands such as benzo[*a*]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [73]. When activated, the AHR is responsible for the regulation of a number of genes, and in particular, it has been shown that the gene encoding the cytochrome P450 CYP1A1 is induced by AHR with an EC_{50} for KYN of 12.3 μM [70]. The

specificity of the interaction of AHR with KYN has been confirmed by radioligand binding assays with mouse liver extract, as well as by specific inhibition or genetic knockdown of the receptor. The role of the AHR in carcinogenesis has been the subject of much recent work, and evidence is growing to indicate a tumor-promoting role for the receptor, with its activation promoting clonogenicity and invasiveness of cancer cells. Genetic knockdown of the AHR in several tumor types results in decreased proliferation and invasion of cancer cells, while in vivo studies in mice that overexpress the constitutively active AHR show enhanced development of stomach and liver cancers [74]. While the precise mechanism of AHR-mediated tumor immunity remains unclear, it has been proposed to involve suppression of $CD4^+$ and $CD8^+$ T-cell activity [70]. The demonstration that these effects are dependent upon KYN concentration, and that the presence of KYN is dependent upon TDO activity, raises the importance of TDO activity in cancer development and the possibility that the TDO–KYN–AHR signaling pathway may be a means of modulating inflammation and immunity and thus may represent a therapeutic target. Figure 21.3 shows a simplified scheme for the effect of TDO activity on tumor survival.

This idea is further supported by the finding that TDO, normally primarily a hepatic enzyme responsible for regulating systemic TRP levels, is shown to occur in several different tumor types [75]. In this setting, the enzyme acts to prevent rejection of tumors in mice, and administration of the specific TDO inhibitor LM10 restores the ability of these mice to reject TDO-expressing tumors.

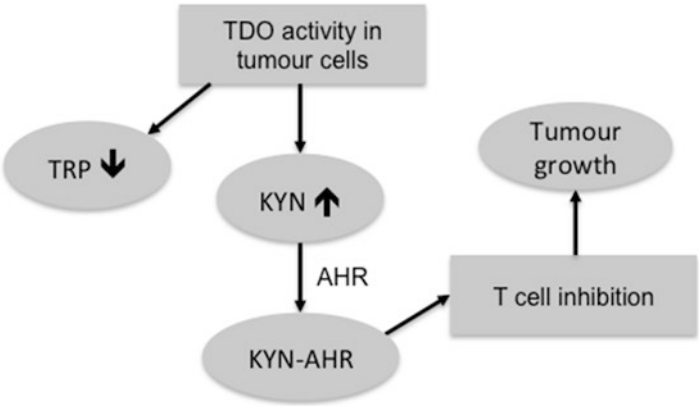

Fig. 21.3 Kynurenine produced by TDO in tumor cells acts as an endogenous ligand of the aryl hydrocarbon receptor. This leads to activation of several genes with the effect that T-cell activity is inhibited and progression of the tumor is enabled

The Lower Kynurenine Pathway Enzymes and Their Catabolites

Research literature into the role of the KP in cancer is overwhelmingly focused on the role of IDO in TRP depletion and immune tolerance. Recently, key papers have highlighted the immunomodulatory role played by the TDO–KYN–AHR pathway resulting from TRP catabolism in TDO-expressing tumor cells. In this respect, the involvement in cancer development of other downstream TRP catabolites and the enzymes responsible for their production (Fig. 21.1) remains relatively unexplored. It has been demonstrated that T-cell proliferation is inhibited by micromolar concentrations of KYN, picolinic acid (PIC), 3-HK, and 3-HA, while both 3-HA and quinolinic acid (QUIN) induce apoptosis in antigen-specific $CD4^+$ T-helper cells in vitro and in vivo [59, 76]. PIC has also been shown to have antitumor activity in mice [77], perhaps due to IFN-γ-mediated macrophage activation and nitric oxide production [78]. In this context, the later enzymes of the KP are responsible for determining whether catabolites are converted to QUIN (for eventual NAD^+ synthesis) or to PIC [79, 80]. In particular, the level of ACMSD (aminocarboxymuconate semialdehyde decarboxylase, Fig. 21.1) activity is inversely proportionate to the amount of NAD^+ synthesized in the brain, and inhibition of ACMSD leads to higher levels of QUIN and its metabolites at the expense of the production of PIC with its demonstrated antitumor properties [81]. The result of all of this work is that there appears to be a complex relationship between the expression levels and activities of the various KP enzymes that leads to shifting balance between the various catabolites. It should also be noted that this complicates the use of detection of specific TRP catabolites as potential biomarkers in patients [65, 70]. Given that many of these molecules mediate multiple effects in tumor development, more work is needed to assess their relative importance and therefore the potential for regulation of KP enzymes in cancer treatment.

Kynurenine Pathway Inhibitors and Therapeutic Potential

The previous sections of this chapter have outlined potential targets for cancer chemotherapy and immunotherapy, and several inhibitors of these have been used in order to delineate and demonstrate their roles in tumor development. The sheer complexity of the interconnected reactions of the KP, their differential importance in tissues and cell types, and the task of finding specific inhibitors of the pathway enzymes mean that while there are great opportunities for developing future therapeutic agents, there remain great challenges.

In this respect, perhaps the best-studied candidate molecule is 1-methyltryptophan (1-MT), and this has been shown to compromise immunosuppression and cause tumor regression [82, 83]. However, some of the evidence surrounding the inhibitory characteristics of 1-MT (both the L- and D-stereoisomers) has served

to further illustrate the complexity of the issue [31, 32, 84]. Nevertheless, the recent discovery that D-1MT acts in a mouse model to alleviate IDO-dependent inhibition of the immunoregulatory kinases mTOR and PKC-Θ goes some way to helping unravel this conundrum [85].

Concluding Remarks

What is clear from this chapter is that the KP is of great importance in tumor development and progression and thus represents a crucial target for development of future cancer immunotherapy and chemotherapy methods. The existence of synergism and antagonism between the various components renders this problematic, but the continual and incremental gains in our understanding can provide a level of confidence in the future success of such an approach to treatment of this most ruinous of diseases.

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Chapter 22
Role of Kynurenine Pathway in Neuro-oncology

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Abstract Despite decades of research, both primary and metastatic brain tumors remain intractable clinical problems. While the kynurenine (KYN) pathway of tryptophan metabolism has been well explored in other cancer types, there are few studies of human brain tumors. The rate-limiting enzymes of the conversion of tryptophan to kynurenine (both forms of indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO2)) have been studied predominantly in gliomas with in vitro methods, in vivo animal studies, and ex vivo tissue studies of surgically resected specimens. In these studies, IDO1 has been shown regulated by interferon-γ (as in other cancer types) and TDO2 by the glucocorticoid receptor. IDO and TDO2 have also been positively correlated with tumor grade and negatively correlated with patient survival in glioma. One seminal study also identified KYN as an activator of the aryl hydrocarbon receptor (not previously shown in any other cancer types). Therefore, the KYN pathway may represent new opportunities for treatment strategies for brain tumors.

Keywords Glioma • Glioblastoma • Meningioma • Metastatic brain tumor • Tryptophan • Indoleamine 2,3-dioxygenase • Tryptophan 2,3-dioxygenase

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List of Abbreviations

3-HK	3-Hydroxykynurenine
AMT	α-[^{11}C]-methyl-L-tryptophan
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
AhR	Aryl hydrocarbon receptor
CNS	Central nervous system
FKBP52	FK506-binding protein 52kDa
GBM	Glioblastoma
GR	Glucocorticoid receptor
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
KYNA	Kynurenic acid
KYN	Kynurenine
KMO	Kynurenine 3-monooxygenase
MRI	Magnetic resonance imaging
MEN	Meningioma
MBT	Metastatic brain tumor
PET	Positron emission tomography
QUIN	Quinolinic acid
Tregs	Regulatory T cells
TRP	Tryptophan
TDO2	Tryptophan 2,3-dioxygenase

Introduction

Despite the wealth of data regarding the kynurenine (KYN) pathway of tryptophan (TRP) metabolism in the context of cancer, very few studies have addressed the role this pathway may play in central nervous system (CNS) tumors. The most common primary brain tumor types are glioma (originating from glial cells in the brain) and meningioma (MEN, originating from the arachnoid cap cells in the meninges), while the most common extracranial cancers that generate metastatic brain tumors are lung, breast, and melanoma [1–3]. Regardless of the tumor type (primary or metastatic), few treatment options are available once surgical resection and radiotherapy have been exhausted. Even with the best standard of care, tumor recurrence and disease progression lead to dismally short survival times, particularly for metastatic brain tumors (MBT, ~6–9 months) [1, 4] and the highest grade of gliomas, glioblastoma (GBM, ~15 months) [5, 6]. Exploration of the KYN pathway in CNS tumors has been predominantly in gliomas.

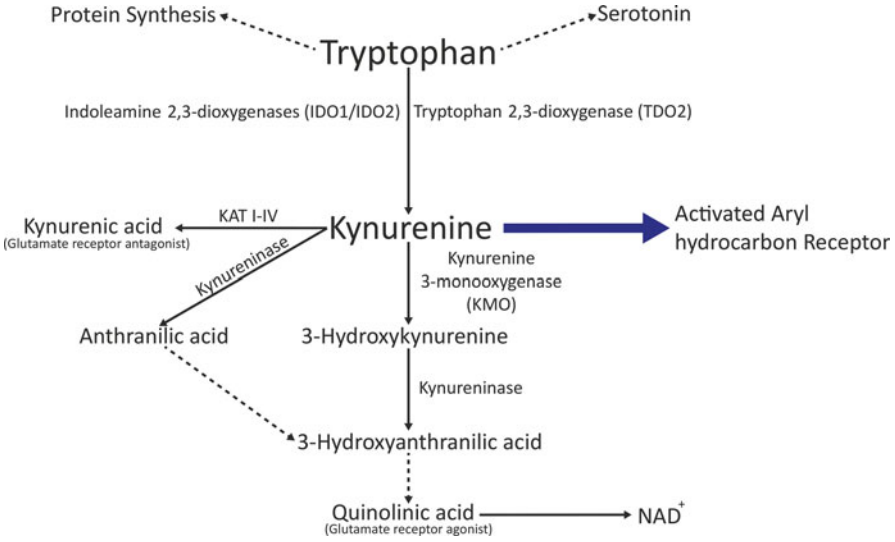

Fig. 22.1 Overview of the KYN pathway. Tryptophan has three predominant metabolic pathways in the body: incorporation into proteins, serotonin production, or the kynurenine pathway. Some KYN metabolites are neurotoxic (QUIN, which activates glutamate receptors), while KYNA is neuroprotective (due to antagonism of glutamate receptors). KYN was also recently shown to be an AhR receptor agonist in the context of human glioma

The general schema of the KYN pathway is shown in Fig. 22.1. The rate-limiting conversion of TRP to KYN may be mediated by either of two forms of indoleamine 2,3-dioxygenase (IDO) or by tryptophan-2,3-dioxygenase (TDO2). The KYN may be further metabolized to kynurenic acid (KYNA, can antagonize glutamate receptors), 3-hydroxykynurenine (3-HK), or anthranilic acid, and further downstream, quinolinic acid (QUIN, glutamate receptor agonist) [7]. However, a recent study has shown that in the context of glioma (and will likely be relevant not only to other brain tumor types but many other cancers), KYN directly activates the aryl hydrocarbon receptor (AhR, also known as the dioxin receptor) [8]. The IDO1, IDO2, and TDO2 enzymes of the KYN pathway have been the primary targets of research in neuro-oncology.

In Vitro and In Vivo Animal Studies

In a hybrid neuroblastoma cell line, 3-HK which is generated from KYN by KYN 3-monooxygenase (KMO), was found to be cytotoxic and induce oxidative stress. In these experiments, KYN itself was not toxic to the neuroblastoma cells [9].

Experiments on GBM cell lines have also been performed in which cells were treated with KYNA and cell proliferation was assessed. The two studies reported conflicting results, possibly due to different GBM cell lines and culture conditions. The first study utilized human U343MG GBM cells and found 1–10 μM KYNA increased proliferation in low serum medium [10]. The second study was conducted with human T98G GBM cells, and these authors found a decrease in proliferation using normal levels of serum in the medium, but at much higher concentrations of KYNA (0.5 mM and higher) [11]. Further studies are warranted before any conclusions can be drawn about a role for KYNA in GBM.

Most recent studies are focused on the enzymes IDO and TDO2. In U87MG cells, one of the standard in vitro models of human GBM, interferon-γ (IFN-γ) decreases TRP and increases KYN in the cell culture media and induces IDO expression and activity [12]. Similarly, in patient-derived primary cultures of MEN cells, IFN-γ induced IDO gene expression and increased protein levels with corresponding increases of KYN production [2]. Also in the U87MG cells as well as other GBM cell lines, TDO2 expression levels were found to correlate with KYN measured in cell culture media. Inhibition of TDO2 activity with a specific inhibitor or gene knockdown decreased both the KYN in the GBM cell assays [8]. Furthermore, these studies demonstrated clearly that the KYN produced was able to activate AhR [8], linking the KYN pathway with the xenobiotic-response pathway long known to play a role in the development of cancer induced by environmental toxins [13].

A recent study extended these findings of TDO2 in the U87MG cells by demonstrating that glucocorticoid receptor (GR) expression is negatively correlated with TDO2 expression and that the glucocorticoid agonist dexamethasone *decreases* TDO2 expression in GBM and other human neural cells in vitro and also in vivo in mice bearing intracranial GBM tumors generated from U87MG cells [14]. The authors also demonstrated that dexamethasone *increases* TDO2 expression in both mouse and human cells of liver origin [14], which was congruent with studies characterizing the human TDO2 promoter from alcohol-dependent patients [15]. This glucocorticoid response in GBM cells is likely mediated through the association of the GR with the FK506-binding protein FKBP52 [14] (also known as FKBP4 and Hsp56). Intriguingly, the immunosuppressive drug FK506 (used clinically to reduce organ rejection posttransplant) [16] increased TDO2 expression and KYN production in U87MG cells, further implicating that TDO2 plays a role in the generation of the immunosuppressive microenvironment that allows tumors such as GBM to prosper [14].

In parallel, studies of glioma in mouse knockouts lacking IDO have also clearly established that the IDO expressed *by the tumor* and not by the immune system is adequate to drive the accumulation of suppressive regulatory T cells (Tregs) [17]. These studies used mouse glioma GL261 cells [18], one of the classic GBM models used in laboratory research [19]. In both wild-type and IDO knockout mice, intracranial GL261 tumors had comparable profiles of tumor-infiltrated T cells and similar post-injection animal survival [17]. However, knocking down IDO in the GL261 cells prior to intracranial injection markedly reduced tumor volume, reduced sup-

pressive Tregs, and increased the post-injection overall survival period from around 40 days to 150 days in both wild-type and IDO knockout mice. Interestingly, the GL261 cell line was originally developed by the intracranial implantation of pellets containing methylcholanthrene, which at the time was known to be carcinogenic [18], but which is now known as an AhR agonist [20]. Furthermore, activation of AhR with the classic environmental toxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) upregulated both IDO1 and IDO2 gene expression and enzyme activity in mice and in dendritic cells in vitro, respectively [21]. Therefore, the prominence of the KYN pathway in this mouse tumor model is not surprising.

Human Studies

In early neuro-oncology studies, substantial TRP uptake was found in astrocytomas (one subtype of glioma) although the mechanism and consequences of excess TRP in tumor cells were not known [22]. Radiolabeled TRP was used to image gliomas qualitatively. TRP appeared to be concentrated in these tumors [23]. Another set of studies assessed KYN metabolism in astrocytomas and GBMs. In these experiments, exogenous KYN was added to ex vivo human tumor specimens and the secreted metabolites were measured. KYNA was produced dose-dependently in astrocytomas and GBMs (with higher levels in astrocytomas) [24]. A smaller study conducted only in astrocytomas found KYN-induced QUIN production also [25]. Similarly, pediatric patients with brain tumors were found to have moderate levels of KYN and QUIN in the cerebrospinal fluid, thought to be secreted by the tumor cells [26].

More recent studies have focused on the rate-limiting enzymes in the pathway, IDO and TDO2. One study found that 9 out of 10 GBMs tested were positive for IDO [27], while another study found that IDO level in gliomas could serve as a prognostic factor (with lower levels correlating with better patient outcome) [17]. These studies were also congruent with the determination that IDO protein levels were greater in high-grade gliomas compared to low-grade gliomas; that IDO levels were more pronounced in recurrent, progressive gliomas; and that patients with more robust IDO immunostaining had worse survival rates [28]. Another recent study showed that IFN-γ stimulation significantly increased the expression of IDO1, IDO2, KYNU, and KMO in cultured human glioma cells [29]. Compared to controls, plasma of GBM patients showed significantly lower concentrations of neuroactive KYN pathway metabolites TRP, KYNA, QUIN, and picolinic acid [29]. TDO2 may also play a much larger role in glioma pathology than previously understood. In studies of grade II, III, and IV glioma specimens, TDO2 protein levels positively correlated with KYN levels and also with Ki-67 proliferative index. Co-localization of TDO2 protein with AhR was also found in gliomas, with high expression levels of both leading to markedly decreased patient survival [8]. Also, as in the in vitro studies described above, GR expression was negatively correlated with TDO2 expression in GBM and also neuroblastoma [14].

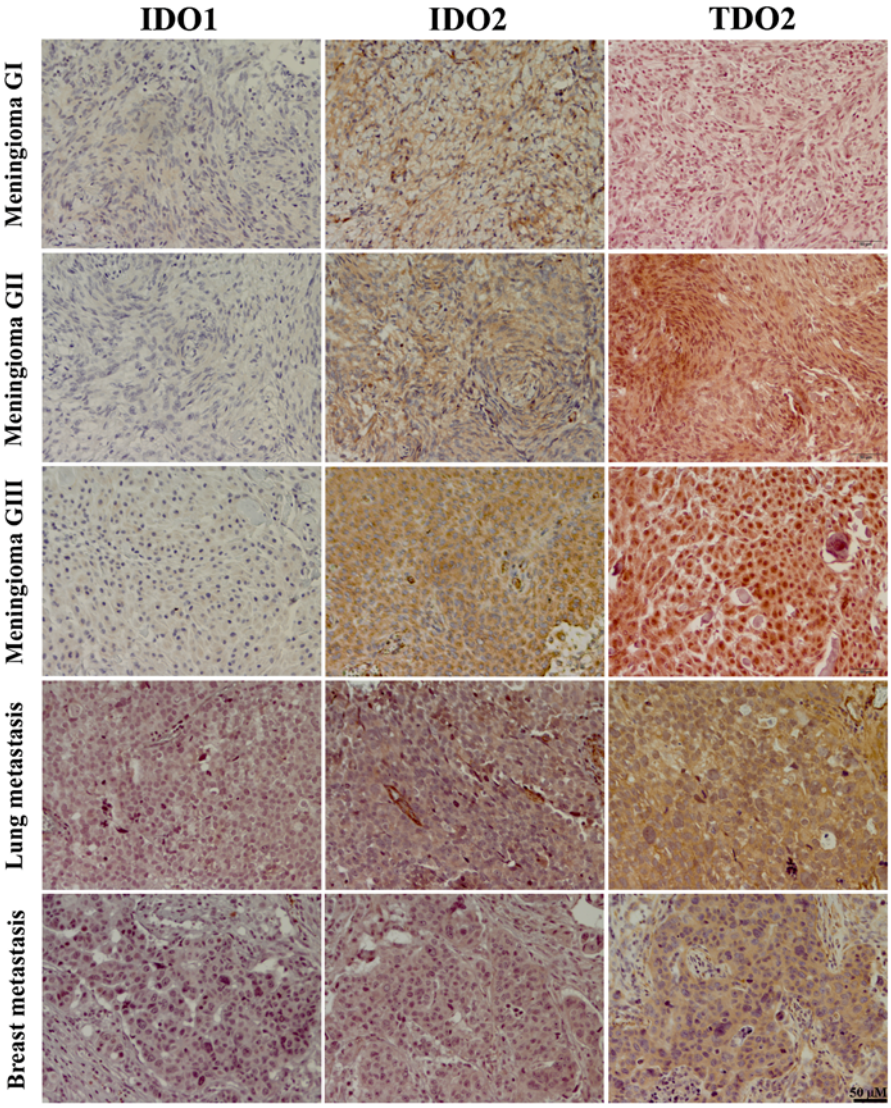

Fig. 22.2 Tryptophan-catabolizing enzymes in meningioma and metastatic brain tumors. Tissue sections were obtained from MEN grade I, II, and II specimens, as well as lung cancer brain metastases and breast cancer brain metastases. Standard immunostaining protocols were followed. Primary antibodies used were IDO1 (Novus Biologicals; catalog# NBP1-87702), IDO2 (Aviva Systems Biology; catalog#:OAAB08672), and TDO2 (Novus Biologicals; catalog# NBP2-13424). All sections were counter-stained with hematoxylin. Scale bar shown is 50 μM and applies to all panels

Although the majority of work described here has focused on glioma, the KYN pathway may have equal importance in MEN and MBTs derived from lung or breast cancer. As shown in Fig. 22.2, all three grades of MEN have pronounced detection of IDO2 and TDO2, with minimal IDO1. In MBTs, only the TDO2 staining is prominent, and much more so in the lung cancer brain metastases than the breast cancer MBT. While these are just qualitative examples of individual tumor specimens, collectively they underscore the need to assess all three enzymes in future studies.

In addition to the tumor tissue studies described above, activity of the KYN pathway has been assessed in neuro-oncological tumors in vivo noninvasively with the radiotracer α-[^{11}C]-methyl-L-tryptophan (AMT). As this positron emission tomography (PET) imaging modality is the focus of another chapter in this volume, it will be only briefly discussed here. AMT is actively taken up into tumor cells via the large amino acid transporter and is not incorporated into proteins. Accumulation of the radiotracer is measured by PET imaging and can be quantified [30]. In contrast to conventional magnetic resonance imaging (MRI), AMT-PET imaging can detect brain tumor recurrence and distinguish it from radiation treatment-induced injury [31], can identify tumor type and grade (glioma vs. MBT) [32], and can define significant prognostic factors in previously treated high-grade gliomas [33].

Concluding Remarks

In summary, while the KYN pathway clearly plays a profound role in the development and recurrence of brain tumors, study of the salient enzymes in the context of neuro-oncology is still in its infancy. Many studies were performed before publication of the discovery of IDO2 [34] or with IDO antibodies with ambiguous antigen epitopes. Studies of MEN and MBTs are severely lacking. Given the dearth of effective treatments for both primary brain tumors (glioma and MEN) and MBTs, further examinations of the KYN pathway will be an important endeavor. Treatment strategies for brain tumors targeting the enzymes IDO1, IDO2, and/or TDO2 will likely prove to be highly clinically valuable.

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Chapter 23
Role of Kynurenine Pathway in Hematological Malignancies

Sarah Parisi and Antonio Curti

Abstract Similarly to solid tumors, hematological malignancies create an immunosuppressive microenvironment, where both innate and adaptive immune responses are profoundly deregulated. Some recent reports have shed new light on the mechanisms underlying the induction of immunological tolerance by leukemic cells. Among these, tryptophan catabolism toward kynurenines by indoleamine 2,3-dioxygenase 1 (IDO1) has been recently described as a potent immunosuppressive pathway in several hematological tumors. The first evidence of a role of kynurenine pathway in regulating immune escape was described in acute myeloid leukemia (AML). Such preliminary finding has been recently extended to pediatric leukemias and Hodgkin and non-Hodgkin lymphomas. The tolerogenic activity of IDO1 has been reported both as a consequence of IDO1 expression by leukemia/lymphoma cells and by an increased IDO1 activity in the tumor microenvironment. The immunosuppressive role of IDO has been recently investigated for the induction of graft tolerance, including allogeneic stem cell transplantation (SCT), which represents a fundamental therapeutic strategy in the management of most hematological malignancies. In this context, some recent reports have indicated IDO1 as a critical regulator of graft-versus-host disease (GVHD). Aim of this chapter is to summarize the most significant and recent advances in the field.

Keywords Indoleamine 2,3-dioxygenase • Hematological malignancies • Immune system

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List of Abbreviations

IDO1	Indoleamine 2,3-dioxygenase 1
AML	Acute myeloid leukemia
SCT	Stem cell transplantation
GVHD	Graft-versus-host disease
HSCs	Hematopoietic stem cells
1-MT	1-Methyl tryptophan
COX-2	Cyclooxygenase-2
CR	Complete remission
IFN-γ	Interferon-γ
HGF	Hepatocyte growth factor
DC	Dendritic cells
APC	Antigen-presenting cell
CTLA-4	T-lymphocyte-associated antigen 4
pDCs	Plasmacytoid DCs
LDH	Lactate dehydrogenase
MM	Multiple myeloma
MSCs	Mesenchymal stem cells
GVL	Graft versus leukemia

Introduction

Over the last few years, a large body of evidence has been provided supporting the notion that, along with tumor cell-autonomous defects, cell-extrinsic microenvironmental factors have a crucial role in leukemia generation and maintenance [1, 2]. In particular, inflammatory networks within the leukemia milieu appear to play a crucial role in tumor initiation and progression, as well as in response to chemotherapy. Indeed, on the one hand, the hyper-activation of inflammatory networks has been indicated as a key contributor to tumor development. On the other hand, the release of abundant inflammatory mediators within the tumor microenvironment, i.e., during chemotherapy, has been shown to trigger pro-inflammatory networks and enhance adaptive immune responses, promoting the presentation of tumor-associated antigens and attenuating tolerogenic pathways. During the tumor development, tumor cells acquire some properties, which are defined through the interaction with the host environment (cell extrinsic). Such process remains poorly understood as well as its interplay with other aspects of malignant conversion, such as tumor cell proliferation and apoptosis. In that context, the immunological microenvironment seems to act as a fundamental background where cell-to-cell interactions and interplay may influence tumor growth, including leukemia [3]. In particular, the immune system-tumor interaction plays a dual role both by eliminating tumor cells and by facilitating tumor escape from immune control [4].

Indoleamine 2,3-dioxygenase (IDO) is a key enzyme in the tryptophan metabolism that catalyzes the initial rate-limiting step of tryptophan degradation along the kynurenine pathway [5]. Tryptophan starvation by IDO consumption inhibits T-cell activation [5, 6], while products of tryptophan catabolism, such as kynurenine derivatives and O_2 free radicals, regulate T-cell proliferation and survival [5, 7]. For these reasons, IDO has immunosuppressive activity. Recent works have demonstrated a crucial role for IDO in the induction of immune tolerance during infection, pregnancy, transplantation, autoimmunity, and neoplasia, including hematological malignancies [7–10]. As for tumors, the tryptophan catabolism has been indicated as a potent pathway which mediates the escape of the antitumor immune response by tumor cells.

Evidence for a Biological Role of Kynurenine Pathway in Leukemia Development

A wide variety of human solid tumors have been demonstrated to express an active IDO protein and transfecting IDO1 into tumor cells prevents their rejection by preimmunized hosts [9, 11]. Several reports demonstrate a clear correlation between IDO1 expression by cancer cells with reduced T-cell infiltration and poor prognosis [12, 13]. Indeed, for solid tumors, preliminary clinical trials are currently ongoing testing the safety, and clinical potential, of novel compounds acting as IDO inhibitors. As for hematological malignancies, the first evidence of a role of kynurenine pathway in regulating immune escape was referred to acute myeloid leukemia (AML).

IDO1 Expression by Leukemic Cells

AML cells, and not normal hematopoietic stem cells (HSCs), were demonstrated to constitutively express IDO, which, in turn, exerts its inhibitory effect on T-cell immunity by inducing the conversion of $CD4^+CD25^-$ into $CD4^+CD25^+$ T regulatory cells [10, 14–16]. Firstly, IDO expression was correlated with increased circulating $CD4^+CD25^+Foxp3^+$ T cells in AML patients at diagnosis. In vitro, IDO^+ AML cells increased the number of $CD4^+CD25^+$ T cells expressing surface CTLA-4 and Foxp3 mRNA, and this effect was completely abrogated by the IDO inhibitor, 1-methyl tryptophan (1-MT). Moreover, in mice, intrasplenic injection of IDO^+ leukemia/lymphoma cells induced the expansion of bona fide Treg cells by conversion of $CD4^+CD25^-$ T cells, and this effect was counteracted by 1-MT treatment. Such inhibitory effect was not reverted by differentiating human AML blasts into leukemic dendritic cells, which still express IDO and expand a fully functional population of Tregs [16]. To investigate the mechanism of IDO1 overexpression in AML

cells, especially upon inflammatory conditions, one recent report indicated a correlation between IDO1 expression and cyclooxygenase (COX)-2, the rate-limiting enzyme in the synthesis of prostaglandins. In particular, COX-2 inhibition by nimesulide, a preferential COX-2 inhibitor, resulted in reduced kynurenine production, thus suggesting COX-2 inhibitors as potential drugs to be used to circumvent IDO1-mediated immune tolerance in AML). At the clinical level, the expression of IDO by AML blasts has been correlated with poor clinical outcome in terms of overall survival. Clinically, IDO expression seems to act at different levels through a reduced response to chemotherapy and a higher frequency of relapse in patients who achieve complete remission (CR) [17]. More recently, childhood AML blasts have been tested for IDO1 expression and function [18]. Interestingly, no constitutive expression of IDO1 protein was detected, whereas about half of cases upregulated IDO1 upon exposure to interferon (IFN)-γ. To better elucidate the signaling pathway underlying IDO1 expression, the authors demonstrated a STAT3-dependent induction of IDO1 protein, which was strongly corroborated by the inhibitory effect of STAT3 on IDO1 expression. At the functional level, IDO1-expressing AML blasts were confirmed to have increased tolerogenic capacity by blunting T-cell proliferation and inducing a population of Tregs. Ex vivo, IDO-expressing AML patients showed higher serum level of IFN-γ as compared to IDO-nonexpressing patients. These data confirm the first demonstration of a role of tryptophan catabolism in AML by expanding the observation to the pediatric field. Taken together, these data indicate IDO expression as a novel mechanism of leukemia escape from immune control, and its inhibition may represent a novel antileukemia therapeutic strategy. Besides AML, one recent report indicates IDO1 expression in multiple myeloma cells, where it acts as immunosuppressive agents by promoting the differentiation of CD4+ T cells into bona fide Tregs. IDO 1 expression was correlated with the release of hepatocyte growth factor (HGF) and with the frequency of Tregs [19].

IDO1 Expression by Accessory Cells Within Leukemic/ Lymphoma Microenvironment

IDO is expressed by a wide variety of cell types in response to several stimuli. Within the immune system, dendritic cells (DCs) and other accessory cells, including mesenchymal stromal cells, have the capacity to modulate IDO expression in response to external stimuli, depending on the microenvironment [20–23]. In particular, IDO expression in DCs may result in depletion of tryptophan and increase of tryptophan metabolites within the interface between antigen-presenting cell (APC) and T cell. This alteration of the intercellular microenvironment during the activation of the immune response has been proposed as a critical mechanism of inhibition of T-cell responses. Indeed, pro-inflammatory signals, such as IFN-γ as well as signals from T cells, such as cytotoxic T-lymphocyte-associated antigen 4

(CTLA-4), expressed by Tregs, are known to induce IDO expression in DCs [23–25]. Plasmacytoid DCs (pDCs) express IDO in response to certain inflammatory stimuli and negatively affect the activation of T-cell-mediated immune response [26].

Then, it is not surprising that tryptophan catabolism within the kynurenine pathway may play a role in the tumor microenvironment, including the leukemic one, which is rich in accessory and stromal cells [27]. For example, mesenchymal stromal cells have been demonstrated to regulate immunity in tumor microenvironment via IDO expression [28]. As for hematological malignancies, IDO has been shown to be expressed in histiocytes, DCs, and some endothelial cells in Hodgkin lymphoma [29]. Interestingly, in this setting, IDO was not expressed in tumor cells. IDO-positive cells were more frequently observed in mixed cellularity type and were independently associated with worse clinical outcome. Moreover, upregulated expression of IDO in non-Hodgkin lymphoma tissues correlates with increased regulatory T-cell infiltration [30]. In this study, increased IDO1 expression was clinically associated with advanced stage, larger tumors, and unfavorable prognostic factors, such as high serum lactate dehydrogenase (LDH). In multiple myeloma (MM), inflammatory stimulation of mesenchymal stem cells (MSCs) specifically induces IDO, which in turn mediates a marked sensitivity of proximal MM cells to tryptophan depletion in the microenvironment [31]. These data suggest a role for tryptophan catabolism as one potential microenvironmental pathway in the regulation of MM cell proliferation and growth.

Evidence for a Biological Role of Kynurenine Pathway in Stem Cell Transplantation

The immunosuppressive role of IDO has been recently widely investigated for the induction of graft tolerance. In several transplant models, increased IDO activity in transplanted cells has been demonstrated to have antirejection properties both in vitro and in vivo [32–34]. Several authors reported the capacity of immunosuppressive agents, such as CTLA-4Ig and CD40Ig, to favor acceptance of transplanted organ by inducing immune tolerance [33]. As recently demonstrated, much of its effect is mediated through the upregulation of IDO expression in DCs, which are then capable to induce a peripheral tolerogenic pathway via the increase of Tregs [24, 33]. Moreover, in an MHC-mismatched renal allograft model, spontaneous acceptance of transplanted organ involves several mechanisms leading to Treg expansion via IDO expression in DCs [34].

Recently, IDO has been demonstrated to act as a critical regulator of graft-versus-host disease (GVHD) in a mouse model of allogeneic hematopoietic stem cell transplantation [35]. GVHD is the main cause of morbidity and mortality after allogeneic stem cell transplantation. In recent years, our knowledge of the pathophysiology of GVHD has increased, but the immunological mechanisms by which GVHD is

regulated both at local and systemic level are still incompletely understood. By using an $IDO^{-/-}$ KO mouse model, it has been demonstrated that the absence of IDO results in increased colon GVHD and reduced survival of transplanted $IDO^{-/-}$ mice as compared to wild-type mice. Interestingly, in $IDO^{-/-}$ mice, the major effect is described at the site of GVHD, mainly in the gut, with increased infiltration of proliferating $CD4^+$ and $CD8^+$ T cells, whereas little, if any, systemic effect was found, i.e., activation of T cells in lymphoid organs and/or induction of Tregs. The critical role for IDO expression in orchestrating T-cell function during GVHD at the site of inflammation represents a major advance in our knowledge on the environmental effects influencing GVHD tissue injury and offers the rationale for a novel approach to GVHD management. Interestingly, MSCs, which are currently under clinical investigation for the induction of immunological tolerance within allogeneic stem cell transplantation [36], have been shown to express a functionally active form of IDO after exposure to IFN-γ [37]. This observation offers the rationale for exploiting IDO-mediated tolerance as part of a cell-therapy approach to GVHD. The modulation of IDO in GVHD target organs may represent an interesting strategy to limit GVHD by acting at sites where host and donor cells interact, whereas other interventions, such as Treg infusional therapy, may be used to induce systemic T-cell tolerance within lymphoid organs. Indeed, Blazar and collaborators reported that, during GVHD, upon arrival in the colon, activated donor T cells produce a high amount of IFN-γ that upregulates IDO, especially on antigen-presenting cells (APCs). Therapeutically, such pathway may be inhibited by the administration of exogenous metabolites, including an agonist to toll-like receptor-7/8, which is primarily expressed on APCs and is capable of reducing injury in the colon via the upregulation of IDO [38]. Additionally, in a murine allogeneic BM transplantation model, the treatment of DCs with histone deacetylase (HDAC) inhibitors, which are commonly used as antitumor agents, resulted in increased IDO1 expression, thus preventing GVHD [39]. One major task in SCT transplantation is dissociating the GVHD, which is the main cause of transplant-related morbidity and mortality, from the graft-versus-leukemia (GVL) effect of donor T cells. It is well-known that the clinical success of allogeneic SCT is hampered by the GVHD effect. However, since GVHD and GVL are parts of the same process, our attempts to reduce GVHD have always been associated with a consequent reduction in GVL, thus resulting in poor clinical effect. It is, then, noteworthy that in a recent report, the induction of IDO1 synthesis by a subset of donor DCs (plasmacytoid DCs, pDCs) resulted in an altered balance between donor Tregs and inflammatory T cells. Indeed, after transplant, IDO1-expressing pDCs expanded in vivo and augmented the GVL activity while attenuating their GVHD effect. Under this viewpoint, the manipulation of the composition of donor DCs within allogeneic SCT may be considered a novel method to skew the balance of GVL/GVHD activity in the direction of GVL [40].

Concluding Remarks

Over the last years, great expectations have been raised by the hypothesis of killing leukemic cells by targeting relevant and crucial cell-intrinsic genetic alterations. The clinical translation of such approach by using targeted novel drugs has been proven limited and far from being curative, mainly due to the extraordinary genetic complexity of AML cell biology. If the deeper knowledge of the molecular mechanisms underlying leukemic transformation still represents a fundamental task for leukemia research, increasing interest is gaining the contribution that microenvironmental factors have in leukemia development and maintenance, as well as in disease progression and drug resistance. Similarly to solid tumors, hematological malignancies are capable of creating an immunosuppressive microenvironment, where both innate and adaptive immune responses are profoundly deregulated. Some recent reports have shed new light on the mechanisms underlying the induction of immunological tolerance by leukemic cells. Novel and important pathways of immunological escape by tumors, including leukemias and lymphomas, have been recently established. In particular, a better knowledge of the role of immunological checkpoint regulators, such as PD-1 and CTLA-4, in the induction of immunological tolerance against tumors, represents an important step forward in the definition of critical pathways for the manipulation of antitumor immune response. In addition, the role of tryptophan catabolism and kynurenine pathway has emerged as crucial, as briefly summarized in the present chapter. From a clinical point of view, promising albeit preliminary trials targeting the kynurenine pathway in the hematology field are currently ongoing. Moving from the pioneering experience in solid tumors, novel compounds are under active investigation and different hematological diseases have been chosen as potential targets for IDO1 inhibition.

In conclusion, harnessing the immune system against cancer, including leukemia, has been exploited for a very long time, as the immune system is clearly able to recognize and attack leukemic cells. The understanding of the factors responsible for the escape from immune destruction in AML, which become more prominent with disease progression, is necessary for the development of innovative immunotherapeutic treatment modalities for hematological malignancies. In particular, the combination of conventional therapies with compounds, such as IDO1 inhibitors, which are capable of disrupting the tolerogenic pathways within the immunosuppressive tumor microenvironment may represent an interesting approach, which nowadays is based on well-established biological pieces of evidence.

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Part V
Experimental Models and In Vivo Imaging

Chapter 24
Experimental Models to Study the Kynurenine Pathway

Anthony R. Guastella, Sharon K. Michelhaugh, and Sandeep Mittal

Abstract The kynurenine pathway is the major pathway that degrades tryptophan that has not been incorporated into proteins. The metabolites that make up the pathway have roles in a broad range of disease states spanning from neurodegenerative diseases, such as Huntington's disease, to infections to cancer. The studying of this pathway is crucial to further our understanding of the many diseases it is incorporated in and furthermore presents the opportunity to discover novel therapeutics. In this chapter, we hope to shed light on the transgenic animal models available (*Ido1, Ido2, Tdo2, Kmo,* and *KatII*) and the diseases they have currently been used to study, as well as give a glance at some of the inhibitors available.

Keywords Kynurenines pathway • Tryptophan • Transgenic • Tryptophan 2,3-dioxygnase • Indoleamine 2,3-dioxygenase • Kynurenines 3-monooxygenase • Kynurenines aminotransferase • Inhibitors

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List of Abbreviations

TRP	Tryptophan
KP	Kynurenine pathway
NAD^+	Nicotinamide adenine dinucleotide
IDO1	Indoleamine 2,3-dioxygenase
IDO2	Indoleamine 2,3-dioxygenase 2
TDO2	Tryptophan 2,3-dioxygenase
KYN	Kynurenine
KMO	Kynurenine 3-monooxygenase
KAT	Kynurenine aminotransferase
QUIN	Quinolinic acid
3HK	3-Hydroxykynurenine
NMDA	*N*-methyl-D-aspartate
KO	Knockout
IFN-γ	Interferon-γ
WT	Wild-type
α7nACh	α7 nicotinic acetylcholine
Unc5h3	Uncoordinated-5H3
CNS	Central nervous system
DG	Dentate gyrus
SVZ	Subventricular zone
GCL	Granular cell layer
HD	Huntington's disease
PD	Parkinson's disease
LD	L-3,4-dihydroxyphenylalanine
LID	Levodopa-induced dyskinesia
5-HT	Serotonin
TH	Tryptophan hydroxylase
BBB	Blood brain barrier
LLC	Lewis lung carcinoma
AHR	Aryl hydrocarbon receptor
DRE	Dioxin-responsive elements
Htt	Huntingtin protein

Kynurenine Pathway Overview

Tryptophan (TRP) has numerous metabolic fates within the body and is most commonly associated with the production of the neurotransmitter serotonin. However, an alternate route of metabolism known as the kynurenine pathway (KP) is the principal method for TRP degradation; it accounts for ~99 % of

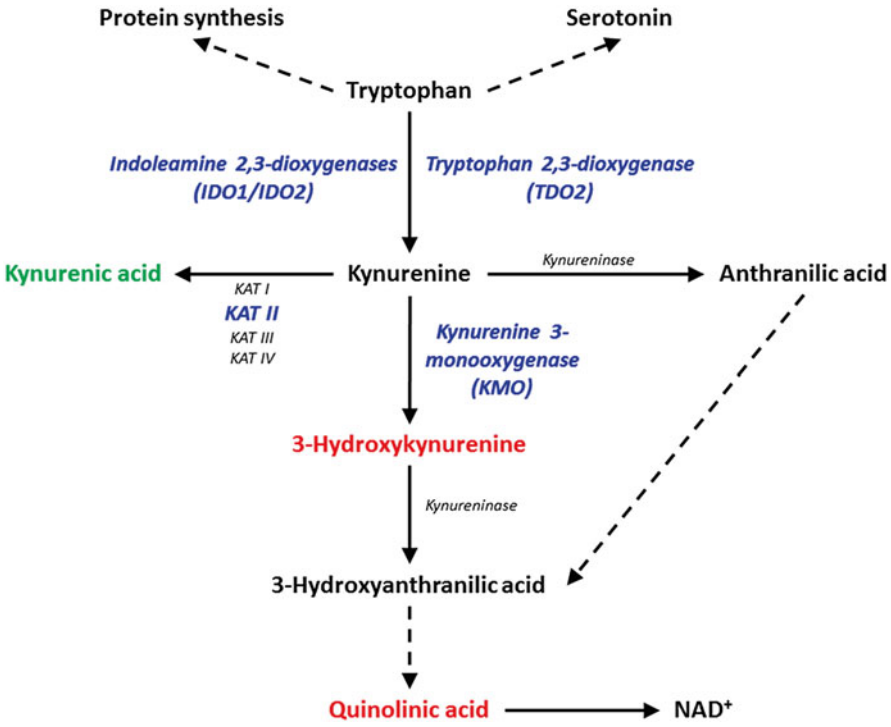

Fig. 24.1 The three metabolic pathways for tryptophan: protein synthesis, serotonin production, or the kynurenine pathway (KP). Metabolites along the KP show both neurotoxic (in *red*), 3HK and QUIN, as well as neuroprotective activity (in *green*), KYNA. To study the effect of the various kynurenines, knockout mice have been generated for the highlighted enzymes

tryptophan metabolism and is a source of the coenzyme nicotinamide adenine dinucleotide (NAD^+) [1]. The KP has gained much attention for its metabolites and their link to numerous systemic and nervous system diseases including various neurodegenerative diseases, arthritis, and cancer [2]. Three enzymes catalyze the first, as well as rate-limiting, step in this pathway (Fig. 24.1): indoleamine 2,3-dioxygenase 1 (IDO1), indoleamine 2,3-dioxygenase 2 (IDO2), and tryptophan 2,3-dioxygenase (TDO2). Upon conversion of TRP to kynurenine (KYN), the pathway reaches a branch point between neuroprotective and neurotoxic arms. Kynureninase, kynurenine 3-monooxygenase (KMO), and kynurenine aminotransferases (KATs) I-IV produce quinolinic acid (QUIN), 3-hydroxykynurenine (3HK), and kynurenic acid (KYNA), respectively. 3HK is a free radical generator and QUIN is an *N*-methyl-D-aspartate (NMDA) receptor agonist, and both metabolites are neurotoxic. Conversely KYNA is an NMDA receptor antagonist and is considered neuroprotective [3].

Experimental Models

The KP appears to be well conserved among mammals, having been experimentally identified in rabbits, guinea pigs, mice, and rats [4]. Lower organisms, such as fruit flies *(Drosophila melanogaster)* [3, 5], worms *(Caenorhabditis elegans)* [6], yeast *(Saccharomyces cerevisiae)* [7], and mosquitoes *(Aedes aegypti)* [8], also show KP similar to those of humans and have been used in a variety of studies [9]. Along the pathway there are many points in which loss of enzymatic function would lead to altered metabolite production. Beginning with the initial rate-limiting step, knockouts (KO) of IDO1, IDO2, and TDO2 are crucial for examining not only the overall effect that the KP has on a system but also to study the individual enzyme effects. IDO1 is expressed in most tissue, regulates cell growth and division [10], and has a physiological role in pregnancy [11]. It is often recognized as a defense molecule during infections, as its expression is upregulated by cytokines, such as interferon-gamma (IFN-γ) [2]. *Ido1*$^{-/-}$ mice (Jackson Laboratories, Bay Harbor, ME) therefore are invaluable tools for research focused on immune modulation. Recently it was discovered that another enzyme, IDO2 (then called INDOL1), also contributes to the enzymatic degradation of TRP. Although the isoforms are on adjacent chromosomes in mammals and are structurally similar, IDO2 appears to be nonredundant with IDO1, as seen in the characterization of *Ido2*$^{-/-}$ mice [12]. Much is yet to be discovered for IDO2 and its role in both normal physiology and diseased states. Conversely, TDO2 has been studied for quite some time and is often credited to being first purified from rabbit liver in the 1937 publication by Kotake et al. Under normal physiological conditions, TRP degradation takes place predominantly in the liver, mediated by TDO2 [1]. *Tdo2* is highly expressed in adult terminally differentiated hepatocytes, and *Tdo2*$^{-/-}$ mice have a substantial increase in systemic serum TRP levels compared to control [13, 14]. This finding suggests that TDO2 is the predominant enzyme for systemic TRP degradation. However, this may be too bold a statement as *Tdo2*$^{-/-}$ mice showed sustained plasma levels of KYN and KYNA, thereby implying compensatory activity, which is most likely resulting from IDO1 or IDO2 activity [13]. Therefore, it is more probable that IDO1, IDO2, and TDO2 work collectively in order to convert TRP to KYN. Upon conversion to KYN, there are several enzymes that can further metabolize KYN. KMO is one such enzyme and is directly responsible for the production of 3HK, which eventually leads to both QUIN and NAD^+ production (Fig. 24.1). KMO is responsible for production of the neurotoxic metabolites in the KP and is therefore a promising target for inhibition and KO. The recently generated *Kmo*$^{-/-}$ mouse model showed drastically reduced levels of 3HK in liver, brain, and serum, as well as lowered levels of QUIN in all three tissues compared to control mice. Interestingly, despite normal levels of enzymatic activity of the various KP enzymes, the production of KYN, KYNA, and anthranilic acid was all significantly increased in liver, brain, and serum levels compared to wild type (WT) [15]. This data suggests that even in the presence of other metabolically active enzymes, KYN is primarily catalyzed by KMO. Nonetheless, there are four other proteins that have been characterized in mammalian brains to interact with KYN: KATI, KATII, KATIII, and KATIV [16]. KAT enzymes play a large role in

the KP, as well as neurological function, as they are the primary source of KYNA biosynthesis. Alterations in the level of KYNA are implicated in numerous neurological diseases, as KYNA is an endogenous ligand for both NDMA and α7 nicotinic acetylcholine (α7nACh) receptors [17]. Of the four KATs, KATII has been found to have the most impact on KYNA levels, producing >70 % of the total KYNA [18]. The *Kat-2* mouse homolog *mKat-2* was targeted and deleted, producing *mKat-2*$^{-/-}$ mice that expressed a transient depression of KYNA levels for the first month of life, that return to normal thereafter [19]. Along with these KO mouse models, modulation of the KP is possible through the use of inhibitors for the numerous enzymes (Table 24.1). Together, these knockout mouse models and inhibitors offer approaches to investigate diseases in which KP metabolites are involved and afford possible avenues to generate and test novel therapeutics.

Neurological Aspects

Altered levels of the neuroactive kynurenines, 3HK, QUIN, and KYNA, have implications in many neurological disorders. Here we will concisely discuss certain studies and the model systems and/or inhibitors used to examine the disease.

Neurogenesis

Recently a correlation between the KP and early, embryonic neurogenesis was determined [20]. Briefly, pregnant rats were given an intraperitoneal injection of Ro61-8048, a KMO inhibitor, on days E14, E16, and E18. The resulting offspring were sacrificed postnatal day 60. Brain tissue was collected and analyzed for various proteins indicative of neurogenesis in the cerebellum, midbrain, and neocortex by means of western immunoblotting. The staining showed that within the neocortex the GluN2A subunit was increased and uncoordinated-5H3 (Unc5H3) was decreased. Within the cerebellum and midbrain, although to a lesser extent, there were increased Unc5H3 levels. Decline in both sonic hedgehog and doublecortin expression reinforce the notion that an NDMA antagonist, such as KYNA, would lead to a loss of neurons during central nervous system (CNS) neurogenesis. All of the data supports the premise that the KP plays a role in the embryonic brain development.

Previously, a relationship between modulation of tryptophan metabolism and adult neurogenesis was observed [13]. *Tdo2*$^{-/-}$ mice showed substantially elevated TRP levels in both blood plasma and the brain. Using BrdU and nestin, neuronal progenitor proliferation was accessed in 13-week-old *Tdo2*$^{-/-}$ mice, specifically in the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ). Both areas showed marked increase of double-stained cells, suggesting increased neurogenesis. Accelerated neurogenesis was also detected within the granular cell layer (GCL) of the olfactory bulb. These findings show that TDO2 plays an integral role in neurogenesis, and when combined with the embryonic study results, it is evident that the KP plays a role throughout the life of an individual.

Table 24.1 Selected inhibitors of the kynurenine pathway

Compound	Inhibitor kinetics	Kinetics determination	Ref
IDO1 inhibitors			
Norharman	$K_i = 120$ μM	In vivo	[50]
1-methyl-D-tryptophan (the observed K_i represents the intermediate DL isoform) 1-methyl-L-tryptophan	$K_i = 35$ μM $K_i = 19$ μM	In vitro (B16F10 and BT10 mouse tumor models) and in vivo	[51]
INCB024360	$K_i = 71.8$ nM	In vitro	[52]
NLG919	$K_i = 7.2$ nM	In vivo (B16F10 mouse tumor model) and in vitro	
Methyl-thiohydantoin-tryptophan	$K_i = 11.6$ μM	In vitro	[53]
Annulin A Annulin B Annulin C	$K_i = 690$ nM $K_i = 120$ nM $K_i = 140$ nM	In vitro	[54]
TDO2 inhibitors			
LM10 [(E)-6-Fluoro-3-[2-(1H-tetrazol-5-yl) vinyl]-1H-indole]	$K_i = 5.6$ μM	In vitro	[46]
680C91 [(E)-6-fluoro-3-[2-(3-pyridyl)vinyl]-lH-indole)]	$K_i = 42$ nM	In vivo (WT rats)	[55]
709 W92 [(E)-6-fluoro-3-[2-(4-pyridyl)vinyl]-lH-indole)]	$K_i = 40$ nM	In vivo (WT rats)	[55]
KMO inhibitors			
Nicotinylalanine	$IC_{50} = 0.9$ μM	In vitro	[56]
4-Aryl-2-hydroxy-4-oxobut-2-enioc acid derivatives	$IC_{50} = 0.32–1.9$ μM	In vitro	[57]
Benzoylalanine family	$IC_{50} = 0.1–8$ μM	In vitro	[57]
Phenylcinnamic acids	$IC_{50} = 0.5–2.9$ μM	In vitro	[57]
Ro-61-8084	$IC_{50} = 37$ nM	In vitro	[58]
JM6	$IC_{50} = 4$ μM	In vitro	[25]
UPF 648	$IC_{50} = 40$ nM	In vitro	[59]
KATII inhibitors			
L-cysteine sulphinate	$IC_{50} = 2$ nM	Computerized linear regression analysis of quantal log dose-probit function	[60]
L-glutamate	$IC_{50} = 73$ nM	Computerized linear regression analysis of quantal log dose-probit function	[60]
ESBA [(S)-4-(ethylsulfonyl) benzoylalanine	$IC_{50} = 6.1$ μM	In vitro	[61]
PF-04859989 (7)	$IC_{50} = 23$ nM	In vitro	[34]
BFF-122	$IC_{50} = 1$ μM	In vitro	[62]

Huntington's Disease

One hypothesis for the neurodegenerative process of Huntington's disease (HD) states that activation of NDMA receptors causes an increase in the intracellular Ca^{2+} levels to the point of toxicity, leading to neuronal death. Current ideology suggests that it is not an increase in the receptor agonist levels but rather a decrease in the levels of endogenous neuroprotectants [21]. The "kynurenergic hypothesis of HD" proposes that an increased QUIN level, compared to that of KYNA, contributes to HD pathology. This hypothesis is substantiated through numerous studies measuring the levels of 3HK, QUIN, and KYNA in patients with stage 1 HD and HD transgenic mice [22], *mKat-2*$^{-/-}$ mice [23], and R6/2 HD mouse model [24, 25]. In patients and the HD transgenic mice, an increased 3HK:KYNA ratio was detected [22]. Within the *mKat-2*$^{-/-}$ mouse, a greater susceptibility towards QUIN was documented when there were lower levels of KYNA. However, this vulnerability was reversed using pharmacological enhancement of KYNA production through the KMO inhibitor, UPF 648 [23]. In one instance, R6/2 mice were injected with JM6, a prodrug version of the Ro-61-8048 KMO inhibitor, and were found to have increased life span and diminished synaptic loss [25]. In another experiment, R6/2 and age matched WT mice were injected with either tritiated KYN or tritiated TRP. Brain tissue was collected for ex vivo evaluation of ^{3}H-KYN from ^{3}H-TRP injected mice and tritiated kynurenines from the ^{3}H-KYN injected mice. R6/2 mice showed equivalent levels of ^{3}H-KYN to the controls; however, genotypic differences were observed with the formation of ^{3}H-3HK from ^{3}H-KYN. R6/2 mice attained a 200 % synthesis rate of 3HK relative to that of the control. Enzymatic analysis of mutant mice revealed that the increased levels of 3HK were a result of both increased synthesis (increased KMO activity) and decreased degradation (decreased kynureninase activity) [24]. *Drosophila melanogaster* were used to further explore this distorted 3HK:KYNA ratio in HD [3]. One advantage to this system is that *Drosophila* does not naturally produce QUIN, and consequently modulation of KYNA and 3HK can be accomplished through the use of inhibitors. KMO inhibitors UPF 648, JM6, and Ro61-8048, as well as flies deficient for TDO (v^{36f}/v^{36f}) and KMO (cn^{3}/cn^{3}), were used. This study shows that the inhibition of KMO, genetically and chemically, improves neurodegeneration in HD flies. Other studies using WT *Drosophila* have shown that impairing conversion of TRP to KYN increased life span of flies. WT flies lived on average 27 days, which was increased to 43.8, 47.6 and 45.5 days for two mutants with deficient TDO2 and one with reduced transmembrane transport of TRP, respectively [5]. These results align with a prior study that performed a genomic screening in yeast for loss of function, aiming to identify gene deletions that would cause the suppression of mutant huntingtin (Htt) fragment-induced toxicity, specifically that of the mutant Htt103Q fragment. From the screening, 28 suppressors were identified, and the most potent was Bna4, a gene that encodes for KMO. Cells lacking this gene showed no 3HK or QUIN production [7]. Combined, these studies suggest that KMO inhibition plays a role in ameliorating the 3HK toxicity experienced with HD and offers a possible novel avenue for HD therapeutics.

Parkinson's Disease

Parkinson's disease (PD) is the most common α-synucleinopathy and is characterized by the presence of α-synuclein inclusions known as Lewy bodies [26]. Recent studies using *Tdo2*$^{-/-}$ *C. elegans* showed an increase in lifespan, suppression of α-synuclein toxicity without affecting its expression, as well as suppression of toxicity associated with β-amyloid and polyglutamine [6]. Furthermore, it was verified through multiple KP enzyme KOs that this lowered α-synuclein toxicity was in fact due to the *Tdo2*$^{-/-}$ and no other KP enzyme or metabolite. These findings propose a new role for TDO2 and potentially the KP in general regulation of proteotoxicity and offer a novel approach to treating diseases caused by protein aggregation. Nonetheless, the current gold standard of care for treatment of PD is prescribing L-3,4-dihydroxyphenylalanine [Levodopa] (LD). Although the drug exhibits overall treatment efficacy, overtime patients run the risk of acquiring what is known as LD-induced dyskinesia (LID), which is associated with the upregulation of NMDA receptors [27, 28]. KYNA is a known antagonist of NMDA and α7nACh receptors and shows preferential binding to these receptors at micromolar concentrations. To increase the levels of KYNA produced from the KP, KMO inhibitors can be used to push the pathway down the neuroprotective branch. Coadministering Ro61-8048 with LD for 1 month in parkinsonian monkeys lead to reduced development of LID without compromising the effectiveness of the LD treatment [29]. The results from these studies indicate how broadly applicable the KP is to disease states and show that even for the same disease, there are multiple approaches to modulation of the KP.

Schizophrenia

The "hypo-glutamatergic" hypothesis for schizophrenia assumes the disorder results from the decrease in activation of glutamate receptors. KYNA is a known antagonist for NMDA and α7nACh receptors and has been shown to exist at elevated levels in those with schizophrenia [30]. A possible explanation for this observed KYNA:QUIN imbalance is the enzymatic activity of KMO and KATs, responsible for QUIN and KYNA production, respectively. KAT enzymes display a higher capacity for L-KYN, K_M values in the low millimolar range [31], and directly affect the endogenous levels of KYNA in the hippocampus of mice [17]. KMO has a $K_M \approx 20$ μM [32] yet is more easily saturated by L-KYN than the KATs, contributing to its potential as a rate-limiting step. Enzymatic activity and mRNA expression for KMO was measured in the frontal eye field in postmortem tissue of schizophrenic patients ($n = 32$) and control patients ($n = 32$). Both gene expression levels and enzymatic activity showed significant reduction for KMO [33]. Decreased expression and activity, in conjunction with KMO's high rate of saturation, leads to a shifting of KYN present towards KAT, thus increasing the KYNA as seen [30]. Future therapeutics for schizophrenia will come in the form of KAT inhibitors, with the most

promising compound being cyclic hydroxamic acid (PF-04859989). This is an irreversible inhibitor, is brain penetrant, and is able to reduce brain KYNA by 50 % at a dose of 10 mg/kg [34].

Depression

Evidence supports that of the TRP not utilized for protein synthesis, only 1 % is metabolized into serotonin (5-HT) [35]. It is hypothesized that antidepressants function by inhibiting TDO2 in order to increase the brain levels of TRP available for tryptophan hydroxylase (TRPH) to catalyze. This theory has been substantiated through the studying of *Tdo2*$^{-/-}$ mice who display increased levels of both TRP and 5-HT in the hippocampus and midbrain [13]. These findings further can be interpreted to postulate that TDO2 is the rate-limiting step, and not TRPH, for 5-HT synthesis. Current research using stressed rats and treatment with extracts of *Hypericum perforatum,* commonly referred to as St. John's Wort (SJW), corroborates the notion that hepatic TDO2 expression is inversely proportional to the brain levels of 5-HT [36]. TDO2 inhibition is not the only mode to modify the KP as it relates to depression. Newly published data demonstrates that endurance-style exercise induces the two skeletal muscle transcription factors PGC-1α1 and PPARα/δ. Together these factors excite KAT enzymes in skeletal muscle thus shifting peripheral KYN to KYNA, which is unable to cross the blood brain barrier (BBB) [37]. These results further validate the long established claim that exercise is beneficial for managing depression.

Cancer and Immunology

The KP has immunological association as seen by its role in various immune-compromised situations such as rheumatoid arthritis [38], bacterial/viral infections [39–41], and cancer. Cancer and its interaction with the immune system has been a focus of research for many years; however, limited translational progress has been made. The primary cause for this is the tumor's ability to use multiple immunosuppressive pathways to evade the body's immune system, one of which is the KP. IDO expression is induced through IFN-γ, as seen through IFN-γ gene transfer to *IDO1*$^{-/-}$ and Lewis lung carcinoma (LLC) tumor-bearing WT mice causing upregulation of IDO1 in several mouse organs [42]. This increase in IDO1 creates a localized depletion of TRP, a "TRP sink," which inhibits proliferation of T lymphocytes in the G1 phase when TRP concentration is below 1 μM [43]. Further studies show that IDO1 expressing tumor cells cause a lack of T cells at the tumor site, as well as silencing the immune response in preimmunized mice. This effect was diminished through the inhibition of IDO1 with 1-MT, suggesting that in order to improve the efficacy of cancer vaccines, there should be a concomitant treatment with an IDO1 inhibitor [44].

A recent study analyzed patient samples from multiple grades of astrocytoma and detected a correlation between the expression of IDO1, tumor grade, and prognosis; IDO1 expression was directly proportional to tumor grade, whereas it was inversely proportional to the prognosis. Furthermore, the group injected GL261 mouse glioma cells intracranially into *Ido1*$^{-/-}$ mice and found that the KO mice had prolonged survival and diminished Treg levels compared to that of WT mice [45]. When taken together, the studies done on IDO1 and cancer show that this enzyme has a major role in the disease and that it is a viable target for future therapeutics. Nevertheless, IDO1 is not the only KP enzyme that produces immunosuppression. A recent study injected P815 tumor cells, known to overexpress TDO2, into naïve syngeneic DBA/2 mice and treated with LM10, a novel TDO2 inhibitor. Systemic treatment with LM10 prevented growth of the P815 tumor cells in the mice, and no signs of toxicity were detected in the mice [46]. Additionally, immunodeficient *RAG2* KO mice were injected with P815 cells and treated with LM10. Tumor growth was unaffected in this system, demonstrating that the effect of LM10 is dependent upon the immune system [46]. Along with the immune modulation, TDO2 also affects the production of KYN in glioma cells. KYN has been found to be an endogenous aryl hydrocarbon receptor (AHR) ligand [47]. AHR is a transcription factor that forms a heterodimer with the AHR nuclear translocator and interacts with the dioxin-responsive element (DRE, also termed xenobiotic responsive element) in the regulatory region of AHR target genes, leading to tumorigenesis and inflammation [48]. Knockout for *AHR,* or the use of an AHR antagonist, inhibited KYN-induced AHR activation and AHR-regulated gene expression, showing that KYN is a specific agonist for AHR [47]. Furthermore, AHR has been revealed to partake in an autocrine AHR-IL-6–STAT3 signaling loop for IDO1, creating an autonomous mechanism for IDO1 expression [49]. These results strengthen the concept that the KP is a very complex and collective pathway, with elaborate interactions of the enzymes and metabolites. As more studies are performed, it is becoming more evident that monotherapies will not suffice in treating the KP and that combination therapies will prove to be most efficacious.

Conclusion

The KP has links with many diseases and there are many factors that alter its natural state. IDO1 is regulated by immunological responses and has a newly found autocrine loop that causes autonomous expression. There is still much to be learned of IDO2 and the role it plays in the human body and its possible interactions with IDO1. *Tdo2*$^{-/-}$ mice have shown that the initial metabolism of tryptophan is not associated with only one of the rate-limiting enzymes, but rather it is a combination of the three that produce KYN. Future studies aimed at resolving this complex collaboration may hold the key in developing novel therapeutics with optimized combinations of IDO1, IDO2, and TDO2 inhibitors. There appears to be much promise towards generating therapies for neurological disorders with the newly developed

Kmo$^{-/-}$ mouse model system, forcing the pathway towards the neuroprotective arm. Taken together, the tools presented in this chapter afford many options in targeting aspects of the pathway and should prove to be instrumental in the ongoing research.

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Chapter 25
Animal Models to Study the Role of Kynurenine Pathway in Mood, Behavior, and Cognition

Nathalie Castanon

Abstract Mood disorders and cognitive dysfunctions are often associated with a number of serious conditions/diseases, including aging and obesity. These disorders not only impair the quality of life of affected subjects but emerge as potent risk factors for related health outcomes. Understanding their etiology represents therefore a major public health challenge. In the present chapter, we provide converging evidence showing that inflammatory processes, and more importantly related activation of the kynurenine pathway, are involved in the development of neuropsychiatric comorbidities. Specifically, we give an overview of how experimental studies performed in relevant animal models of immune activation that can be used to better understand the role of kynurenine pathway in mood, behavior, and cognition bring strong support to this notion. We report experimental data demonstrating the link between inflammation-induced brain activation of indoleamine 2,3-dioxygenase (IDO), the enzyme that metabolizes tryptophan along the kynurenine pathway, and mood and cognitive alterations. Beyond highlighting the role of IDO in that context, we also show how animal models can contribute to identify the mechanisms by which kynurenine pathway activation promotes behavioral alterations. Lastly, we briefly present recent experimental data pointing to a broadly role of this pathway in a medical condition associated with inflammation, namely obesity. Altogether, these findings may prove valuable for introducing new therapeutic strategies targeting the kynurenine pathway to treat behavioral alterations.

Keywords Animal models • Kynurenine • Tryptophan • Indoleamine 2,3-dioxygenase • Depression • Anxiety • Cognition • Cytokines • Inflammation • Obesity

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List of Abbreviations

IL-1β	Interleukin-1β
TNF-α	Tumor necrosis factor-α
IFN	Interferons
LPS	Lipopolysaccharide
BCG	Bacillus Calmette-Guerin
IDO	Indoleamine 2,3-dioxygenase
3-HK	3-Hydroxykynurenine
QUIN	Quinolinic acid
KA	Kynurenic acid
BDNF	Brain-derived neurotrophic factor

Introduction

Neuropsychiatric disorders, notably mood disorders and cognitive dysfunctions, represent a major public health concern most notably as they are often associated with a number of serious conditions/diseases, including aging, obesity, type 2 diabetes, or cardiovascular diseases [1–3]. Such comorbid associations are particularly preoccupying as neuropsychiatric disorders not only impair the quality of life of affected patients, but they emerge as potent risk factors for aggravation of associated diseases [4, 5].

While different mechanisms are likely to be involved in the development of neuropsychiatric comorbidity, there is increasing evidence for a role of inflammatory processes and related activation of the kynurenine pathway (KP) [6, 7]. Indeed, most of the diseases associated with neuropsychiatric comorbidities share chronic inflammation as a common denominator [8, 9]. Besides, inflammatory processes are notorious for influencing pathways involved in the regulation of mood and cognition, including neurotransmitter metabolism, neuroendocrine function, and neural plasticity [6, 10]. Mounting research has also begun to shed increasing light on activation of the KP, not only as potential underlying mechanism for neuropsychiatric disorders but most importantly as potential mediator between inflammatory processes and development of neuropsychiatric comorbidity [7, 11]. Actually, tryptophan (TRP) metabolism along the KP accounts for most of the TRP that is not used for protein synthesis and includes compounds active in both the immune and nervous systems [12]. Several interesting reviews, mainly based on clinical findings, already described the role of inflammation-induced activation of the KP in the pathophysiology of neuropsychiatric disorders [7, 8, 13]. The objective of the present chapter is rather to provide an overview of how experimental studies bring strong support to this notion. At the light of the recent experimental results obtained in the field, we describe how relevant animal models can help deciphering the

mechanisms underlying the role of KP in mood and cognitive alterations associated with several inflammatory conditions.

Animal Models of Neuropsychiatric Disorders

Using animal models to study complex clinical pathological conditions, particularly those affecting brain and mental functions (e.g., mood alterations), often appears limiting and even controversial. However, these models are absolutely required to test particular hypotheses in complex in vivo settings that cannot be recapitulated by purified cells or ex vivo experiments. Animal models are often the more suitable way to deeply study the causes, underlying mechanisms and progression of diseases, and to test potential therapeutic interventions. Moreover, the development of consistent and reliable models focusing on different core symptoms of the human diseases rather than on the entire disorders per se has provided very useful tools to study their respective pathophysiology [14]. Interestingly, we present herein data showing that applying such experimental approach to study behavioral consequences of inflammation allowed identifying what has been certainly the least easily predicted result, namely the discovery of the profound action of the principle molecular actors of inflammation, namely the cytokines, on brain functions and their involvement in neuropsychiatric disorders [6].

Inflammation, Sickness Behavior, and Neuropsychiatric Symptoms

Inflammation is an active defense reaction resulting from the activation of the innate immune system. Although it serves a protective function in controlling infection and promoting tissue repair, it can also cause tissue damage and needs therefore to be tightly controlled. Cytokines are relatively large hydrophilic polypeptides that include interleukins (IL), interferons (IFN), tumor necrosis factors (TNF), chemokines, and growth factors. Although most cytokines have little or no function in healthy tissues, they are rapidly induced by activated innate immune cells in response to tissue injury or infection. They are also able to act systemically on distant organs, including the brain. Although cytokines do not readily cross the blood–brain barrier, several non-exclusive humoral, neural, and cellular pathways allow peripheral immune messages to be transmitted from the periphery to the brain (Fig. 25.1). Activation of immune-to-brain communication ultimately induces production of brain cytokines by activated endothelial and glial cells, particularly microglia. Upon detection of homeostatic disturbances, microglia are transiently activated and rapidly engaged in brain adaptive immune responses mainly due to their ability to produce cytokines, express their receptors, and amplify their signals

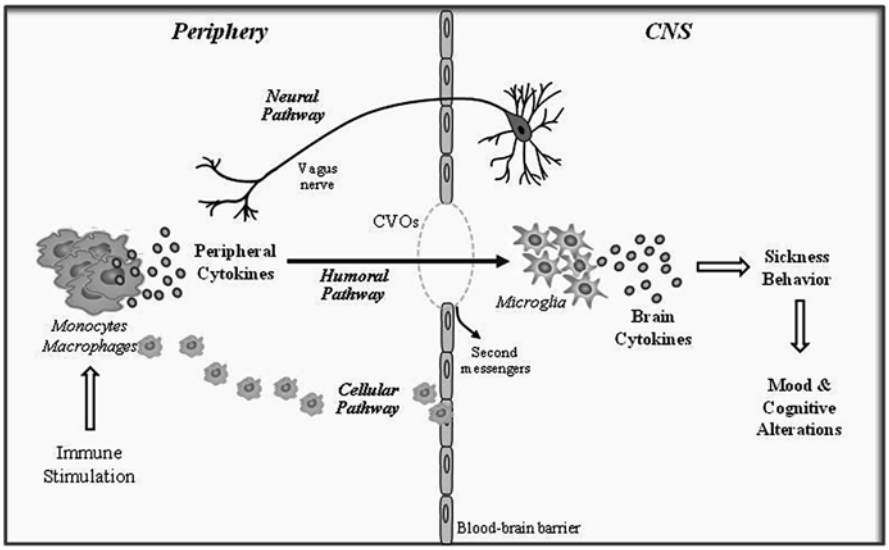

Fig. 25.1 Immune-to-brain communication.
In response to a systemic immune stimulation, activated monocytes and macrophages release inflammatory cytokines, which can reach the brain by several nonexclusive pathways. In the *neural pathway*, the vagus nerve that is activated by peripherally produced cytokines relays the information to the brain through activation of the area postrema or the nucleus of the tractus solitaries. In the *humoral pathway*, circulating cytokines reach the brain at the level of the circumventricular organs (CVOs) that are devoid of functional blood–brain barrier. Within the brain parenchyma, activated endothelial cells then release second messengers able to act on specific brain targets. Lastly, a direct entry into the brain parenchyma of peripherally activated monocytes can be detected in response to chemokines released by microglia (*cellular pathway*). All these pathways ultimately activated microglia that release brain cytokines responsible for the development of sickness behavior and mood and cognitive alterations when microglial activation endures

[15]. While microglia normally exert a protective action on the brain, their unregulated and chronic activation may in contrast become deleterious.

By altering neurotransmitter metabolism and function, neuroendocrine activity, neural plasticity, and/or brain circuitry, transient brain cytokine activation during an infection ultimately coordinates a large number of behavioral changes (including weakness, listlessness, malaise, anorexia, and fatigue) that have been referred to as sickness behavior. Necessary for infection recovery, sickness behavior usually resolves within few days, once microbial pathogens have been cleared and the innate immune system is no longer activated. However, in cases of unregulated activation of the systemic immune system and/or brain microglia, sickness behavior may evolve into clinically relevant neuropsychiatric symptoms, including depression, anxiety, and cognitive dysfunctions [6]. During the last decades, these findings have prompted a surge of interest for the circumstances precipitating the development of these symptoms. Clinical data have shown that if inflammation-related sick-

ness behavior and neuropsychiatric symptoms share some common components, they also differ by their respective duration and intensity [8, 13]. These findings suggest therefore that distinct mechanisms likely underlie the different behavioral consequences of inflammation. Interestingly, strong support for this assumption came from studies performed in immune-challenged animals, which are classically used to modulate inflammation-induced behavioral alterations.

Modeling Inflammation-Induced Behavioral Alterations in Rodents

Most animal studies aimed at identifying mechanisms of the cytokine-induced sickness behavior are based on acute immune activation by cytokine inducers (bacterial lipopolysaccharide: LPS, viral mimetic poly I:C), or recombinant inflammatory cytokines [16–18]. First attempts to experimentally assess the neurobiological bases of cytokine-induced emotional and cognitive alterations have concentrated on the same approaches [19–21]. The results of these studies could be biased by the profound lethargy and motor impairment that occur in the first hours following immune stimulation [16, 20]. There are however ways to circumvent this potential problem. By using an experimental design based on the clinical evidence showing that neuropsychiatric symptoms develop later than sickness in cytokine-treated patients [22], we differentiated in LPS-treated mice the initial phase of decreased motor activity that progressively returns to normal with delayed depressive-like behaviors that remain up to 24 h after treatment [23–25]. Similar dissociation between LPS-induced sickness behavior and increased anxiety-like behavior [26], or impaired spatial memory [27], was also reported using the same experimental design. Importantly, we showed at the neuroanatomical level a functional dissociation between the brain structures that respectively underlie LPS-induced sickness behavior and depressive-like behavior, strongly suggesting therefore that different mechanisms are likely involved [23].

It could be argued that a model of acute immune activation such as a LPS challenge is not the most relevant to the clinical situation and therefore may be less suitable to study the neurobiological bases of cytokine-induced neuropsychiatric symptoms than models of chronic immune activation. Actually, we showed that mice inoculated with *Bacillus Calmette-Guerin* (BCG), which chronically activates the immune system [28], also display a transient episode of sickness behavior (lasting 1 week) followed by a longer occurrence of depressive-like behavior (up to several weeks) [29–31]. Although each model has its own limitations and strengths, used together in a complementary fashion, they turned out to be very useful to identify the mechanisms underlying cytokine-induced emotional and cognitive alterations and, by doing so, to specifically demonstrate the role of the KP.

Role of Kynurenine Pathway in Inflammation-Induced Neuropsychiatric Symptoms: Clinical Evidence

Converging clinical findings support a main role for cytokines in mood disorders and cognitive decline through processes related to neuroinflammation, neurodegeneration, and structural remodeling [8, 13]. Interestingly, development of neuropsychiatric symptoms in medically ill patients chronically treated with IFN-α [13], elderly subjects [32], or patients with Alzheimer's disease [33] is associated with reduced circulating TRP levels and concomitant increase of one of its main metabolite, kynurenine (KYN). Similarly, neuropsychiatric symptoms correlate in some instances with increase in serum KYN/TRP ratio [34, 35]. These clinical findings suggested a possible link between cytokine-induced activation of the indoleamine 2,3-dioxygenase (IDO), that is the first and rate-limiting enzyme that degrades TRP along the KP, and neuropsychiatric symptoms.

As part of the immune response to infection, increased IDO activity occurring in activated monocytes, macrophages, and brain microglia is usually beneficial to the host [36]. However, sustained brain IDO activation can also be deleterious because of its negative impact on monoaminergic neurotransmission, particularly serotonin (5-HT) neurotransmission, and neuronal survival (Fig. 25.2). Since TRP is the biosynthetic precursor for the synthesis of 5-HT, increased degradation of TRP by IDO has been postulated to reduce 5-HT production. Concurrently, increased brain KYN

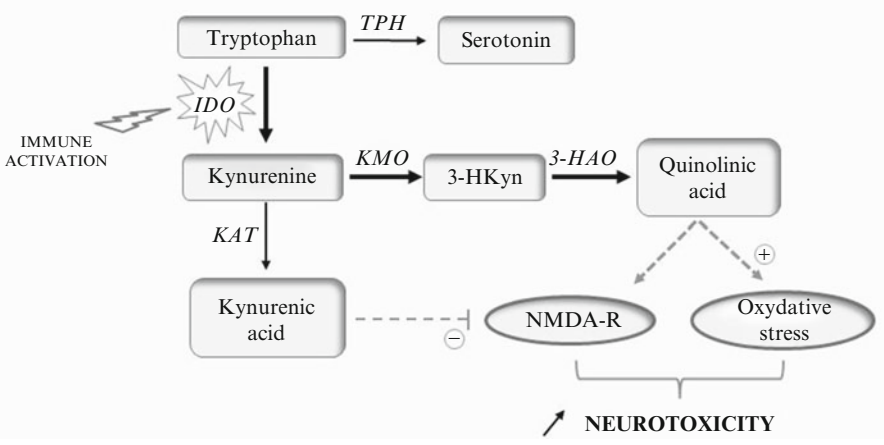

Fig. 25.2 The kynurenine pathway.
In conditions of immune activation, activated indoleamine 2,3-dioxygenase (IDO) catabolizes tryptophan through the kynurenine pathway. Kynurenine is then metabolized in different neuroactive glutamatergic metabolites, mainly 3-hydroxykynurenine (3-HKyn) and quinolinic acid. Elevated levels of quinolinic acid are neurotoxic by activating glutamatergic NMDA receptors and promoting oxidative stress. Conversely, kynurenic acid at high concentrations antagonizes NMDA receptors. 3-HKyn does not appear to interact with a specific receptor; rather, its neural-damaging properties are via free radical generation. *KAT*, kynurenine aminotransferase; *KMO*, kynurenine monooxygenase; *3-HAO*, 3-hyrdoxyanthranilic acid oxygenase; *TPH*, tryptophan hydroxylase

levels resulting from IDO activation can be further metabolized to produce several neuroactive glutamatergic compounds, including 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN), which play a key role in neuronal death and neurodegenerative diseases by stimulating *N*-methyl-D-aspartate (NMDA) receptors and promoting oxidative stress [12]. On the other hand, KYN can also be metabolized in kynurenic acid (KA) that rather displays neuroprotective properties. However, these apparently antagonistic pathways are compartmentalized in the brain, with microglia preferentially producing QUIN, whereas astrocytes produce KA. Immune activation therefore tips the scale in favor of neurotoxicity. Increased brain or cerebrospinal fluid concentrations of KYN and its neurotoxic metabolites have been reported in patients with major depression [7], schizophrenia [37], or neurodegenerative diseases [12]. Moreover, increased concentrations of these neurotoxic metabolites have been related with the stretch of brain damages, and with mood and cognitive impairments [12], suggesting that IDO activation may lead to both functional and structural alterations in the brain.

Role of Kynurenine Pathway in Cytokine-Induced Emotional and Cognitive Alterations: Contribution of Animal Models

In line with clinical findings, animal models of immune activation in which it is possible to experimentally dissociate inflammation-induced sickness behavior from emotional and cognitive alterations have provided very useful tools not only to highlight the causal relationship between IDO activation and these alterations, but also to thoroughly study the mechanisms of brain IDO activation by cytokines and its impact on brain functions.

We and others demonstrated in mice that an immune challenge with LPS or BCG increases peripheral, but also brain IDO activity [28, 38, 39], and that cytokine-induced IDO activation parallels development of depressive-like and anxiety-like behaviors [23–26, 29–31, 40, 41]. Thereafter, similar association between brain IDO activation and development of cognitive and emotional alterations was also reported in other models of immune activation [18, 42–44]. More importantly, pharmacological or genetic inhibition of IDO activity, obtained by acting either directly on the enzyme or indirectly on its inducing cytokines (mainly IFN-γ and TNF-α), prevents induction of depressive-like behaviors, anxiety-like behaviors, and/or cognitive impairments by systemic immune challenges [25, 26, 30, 31, 42, 44, 45]. On the contrary, inhibiting IDO activity has no impact on development of sickness behavior (Fig. 25.3). In addition, aged mice [24, 46] or mice exhibiting constitutive microglial over-activation [47] display sustained cytokine production after an immune challenge, together with protracted brain IDO expression and depressive-like behavior [24, 40, 48]. Taken together, these studies strongly pointed to activation of brain IDO, rather than activation of peripheral KP, as main mediator of cytokine-induced emotional alterations, although a potential contribution of

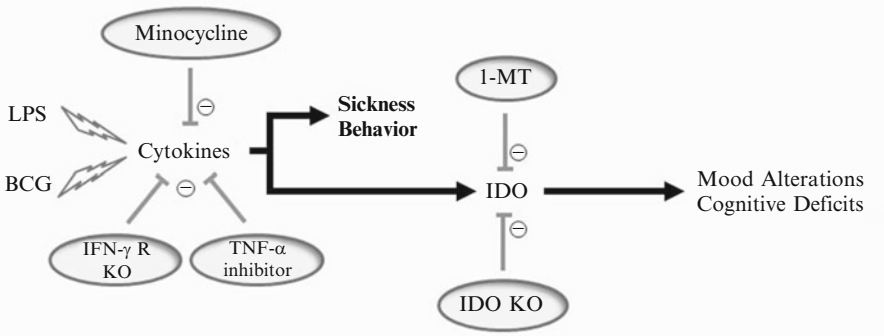

Fig. 25.3 Demonstration of the role of IDO in cytokine-induced mood and cognitive alterations. Blockade of LPS- or BCG-induced cytokines in mice abrogates both sickness behavior and mood and cognitive alterations, whereas blockade of IDO only inhibits mood and cognitive alterations. *LPS*, Lipopolysaccharide; *BCG*, Bacillus Calmette-Guerin; *TNF-α,* Tumor necrosis factor-α; *IFN-γ,* Interferon; *IDO,* Indoleamine 2,3-dioxygenase; *1-MT*, 1-methyl tryptophan

peripherally produced KYN, which can cross the blood–brain barrier, cannot obviously be totally excluded based on these data alone. However, further studies report that centrally induced inflammation, which only activates brain KP, is sufficient to elicit depressive-like behaviors [41, 49–51]. Interestingly, other studies shed light on the hippocampus, which is well known to control mood and cognition [52, 53], as important brain area for cytokine and IDO activation [23, 38, 45, 47, 50, 54], although they are broadly stimulated within the brain in response to immune challenges [38, 55]. Taken together, these results clearly point to a pivotal role of IDO activation, particularly in the hippocampus, in mediating cytokine-induced mood and cognitive alterations.

Beyond highlighting the role of IDO, animal models can also contribute to identify the pathways by which cytokine-induced brain IDO activation promotes behavioral alterations. Emotional alterations linked to hippocampus IDO activation by an immune challenge are associated with reduced hippocampal expression of the brain-derived neurotrophic factor (BDNF) [18]. This neurotrophin contributes to mood regulation and memory function, including in conditions of immune activation [56], by supporting synaptic plasticity and neuronal excitability [57, 58]. Of note, brain IDO activation did not result in these studies in a detectable reduction of 5-HT concentrations [18, 25], although increased hippocampal 5-HT/TRP ratio has been reported in another study following systemic blockade of IDO [44]. On the other hand, we recently showed that obesity in mice exacerbated LPS-induced increase of brain KYN levels and depressive-like behavior, but did not change the effect of LPS on brain TRP levels [59]. These results suggest therefore that exacerbated depressive-like behavior in LPS-treated obese mice may be due to the neurotoxic actions of KYN metabolites rather than to reduced brain TRP availability for 5-HT synthesis. Consistent with this assumption, peripheral KYN administration dose-dependently induces in mice depressive-like behaviors, anxiety-like behaviors, and cognitive impairment [25, 26, 60–62]. Of note, NMDA receptor blockade

abrogates cytokine-induced depressive-like behavior [63]. Moreover, mice deficient for IDO are protected against NMDA receptor-mediated excitotoxicity [64]. Altogether, although these results do not exclude the possible role of impaired 5-HT synthesis in inflammation-induced neuropsychiatric symptoms, they clearly support a key role for the neuroactive metabolites of KYN.

Role of Kynurenine Pathway in Emotional and Cognitive Alterations Associated With Obesity

In light of the present knowledge on the mechanisms leading to development of neuropsychiatric symptoms in conditions of elevated systemic and brain inflammation, an important question arises as to whether the same mechanisms may take place in conditions of chronic low-grade inflammation, such as obesity. While clinical data support a role for inflammation in obesity-related neuropsychiatric alterations [65], the literature is still sparse regarding the specific underlying mechanisms. We present here recent experimental data that may help to start addressing this issue.

Neuropsychiatric comorbidities, including mood and anxiety disorders, and mild cognitive impairment, are frequent in obesity and emerge as significant risk factor for aggravation of related health outcomes [1, 65]. In addition to peripheral organ dysfunction, basal systemic low-grade inflammation (i.e., increased plasma levels of cytokines, activation of macrophages, and inflammatory signaling pathways in multiple organs) is also a fundamental characteristic of obesity, which is now considered not only as a metabolic disorder but also as an inflammatory condition [66]. Interestingly, studies performed in rodent models of obesity, in particular those resulting from exposure to high-fat and/or high-sucrose diets or spontaneous mutations (e.g., *ob/ob* and *db/db* mice, respectively, deficient for leptin or its receptor), show that inflammation also exist within the brain. This neuroinflammation is not only found in areas involved in physiological homeostasis such as the hypothalamus [67, 68] but also in the hippocampus [59, 69–71] or the cortex [72]. More importantly, neuroinflammation in these structures is associated with increased emotional behaviors and cognitive impairments [59, 69, 70, 72]. These models of obesity are therefore especially suited for investigating the mechanisms linking obesity-associated inflammation to behavioral alterations, including potential involvement of the KP.

Blocking hippocampal IL-1β expression in obese *db/db* mice prevents their cognitive impairment by normalizing dendritic spine density and synaptic dysfunction in the hippocampus [73]. Moreover, cognitive impairment and emotional alterations reported in both diet-induced obese mice and *db/db* mice are linked to increased inflammation and reduced BDNF levels in the cortex [72] and the hippocampus [70]. Reciprocally, anti-inflammatory interventions in these mice normalize hippocampal levels of BDNF and prevent hippocampus-mediated cognitive impairments [74]. Given the present knowledge on the consequences of dysregulated hippocampal cytokines and neurotrophins expression, and impaired synaptic function on

mood, learning, and memory [6, 57, 58], these results point to a link between increased neuroinflammation, impaired neurogenesis/synaptic plasticity, and behavioral alterations in animal models of obesity. Consistent with the role of cytokine-induced brain activation of the KP in these processes, we recently reported in *db/db* mice a direct relationship between brain IDO activation induced by LPS and development of depressive-like behavior [75]. Similarly, we showed that diet-induced obesity exacerbates both hippocampal cytokine and IDO induction by LPS and related behavioral changes [59]. It can be argued that metabolic factors altered in obesity, including insulin or leptin, may also contribute to behavioral alterations by acting within the brain. Nevertheless, several studies including ours suggest that these factors per se are not sufficient to explain behavioral alterations occurring in that context [59, 69, 70]. In addition, mounting clinical reports support a role of KP in obesity. Severely obese individuals with high prevalence of neuropsychiatric comorbidity [1, 65] display increased plasma KYN levels [76, 77]. Of note, activation of the KP has been reported to be a key component in the initiation and propagation of obesity and associated medical complications, including metabolic syndrome and cardiovascular diseases [78]. Similarly, it could also contribute to the development of neuropsychiatric symptoms in this context (Fig. 25.4), in particular through the neurotoxic effects of KYN metabolites since obese patients display

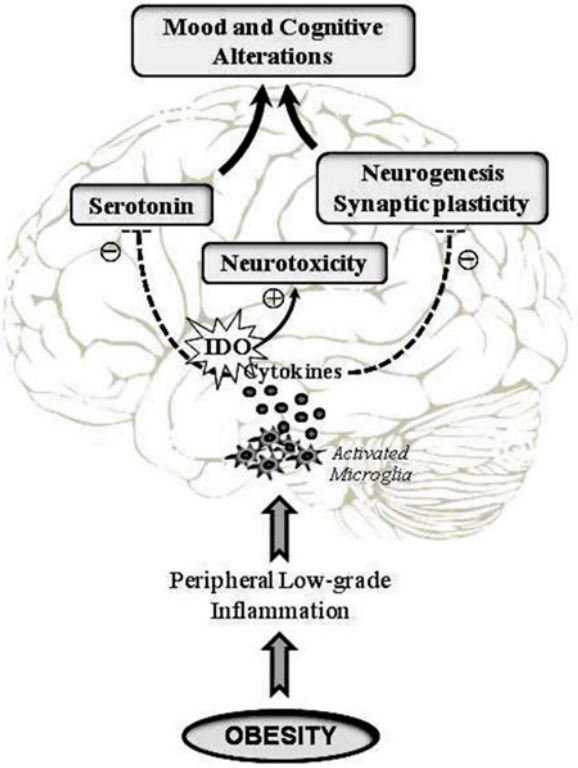

Fig. 25.4 Proposed role of kynurenine pathway activation in mood and cognitive alterations associated with obesity. **Obesity is characterized by peripheral low-grade inflammation with well-known impact on brain inflammation. By sustaining chronic activation of microglia, brain production of inflammatory cytokines, and indoleamine 2,3-dioxygenase (IDO), obesity may promote neurotoxicity. Increased neurotoxicity, associated with impaired 5-HT neurotransmission, neurogenesis, and synaptic plasticity, may together contribute to induce mood and cognitive alterations as reported in rodent models of obesity**

higher hippocampal atrophy than non-obese subjects [79]. Although more studies are needed to confirm the behavioral consequences of KP activation in obesity, the available data already point to a broadly role of this pathway in several medical conditions associated with inflammation.

Conclusion

Altogether, evidence provided in this chapter shows that brain dysfunctions related with neuroinflammation play a pathogenic role in the development of neuropsychiatric disorders and that degradation of TRP along the KP is certainly a pivotal contributing factor to this phenomenon. Several issues are still at stake, from the role of these mechanisms in nonimmune-mediated neuropsychiatric disorders to the respective importance of peripheral versus central production of KYN metabolites, although it is clear that the ultimate target is the brain. The different animal models presented in this chapter should likely help to address these issues. The experimental approaches used to model inflammatory-related behavioral alterations in rodents can indeed easily be adapted to broaden out the study of the pathogenic role of KP on behavior. Meanwhile, available animal models, which provide increasing knowledge on the respective role of the different protagonists of the KP in altering mood, behavior, and cognition, might help to define novel therapeutic strategies aiming at improving the health and quality of life of people suffering from neuropsychiatric disorders. Given the increasing prevalence of such disorders in modern societies and their role as risk factor for many other diseases, strategies to prevent their development are urgently required.

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Chapter 26
An Overview of PET Studies of the Cerebral Uptake of Amino Acids

Paul Cumming and Per Hartvig-Honoré

Abstract Molecular imaging with positron emission tomography (PET) has revolutionized the study of brain physiology. The facilitated diffusion of large neutral *L*-amino acids (LNAA) across the blood–brain barrier (BBB) is mediated by the LAT1 carrier in the capillary endothelium. A number of substrates for this carrier have been labeled with fluorine-18 and tested as PET oncology tracers; compounds such as [^{18}F]fluoro-ethyltyrosine enter the brain, but are not metabolized. Overexpression of LAT1 and other transporter systems in some tumors results in elevated LNAA uptake relative to the physiological background. Dynamic PET with the endogenous LNAA [^{11}C]tyrosine PET reveals not just reversible BBB transport but also irreversible trapping in brain protein, which can be quantified using linear graphic analysis. In theory, [^{11}C]tyrosine PET might also reveal a signal related to the synthesis of dopamine in nigrostriatal terminals, but in practice, the neurotransmitter pathway cannot be visualized against the background of protein synthesis. However, the trapping of [^{18}F]-fluorodopa (FDOPA) and other aromatic *L*-amino acid decarboxylase (AAADC) substrates reveals the integrity of the nigrostriatal dopamine pathway, without interference from a background of physiological protein synthesis. The serotonin precursor [^{11}C]-5-hydroxytryptophan seems to be decarboxylated preferentially by AAADC expressed in serotonin fibers; in theory, specificity for visualizing the serotonin pathway is to be obtained with α-[^{11}C] methyltryptophan ([^{11}C]AMT), which is not a substrate for protein synthesis. However, the rate of conversion of this tracer to α-[^{11}C]methyserotonin in serotonin neurons of living brain is low, such that its quantitation in PET studies is challenging. The issue of conversion is also central to prospects for molecular imaging of the kynurenine pathway in PET studies with [^{11}C]AMT.

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List of Abbreviations

5-HT	Serotonin
AAADC	Aromatic amino acid decarboxylase
AMT	α-[11C]methy-L-tryptophan
BBB	Blood–brain barrier
COMT	Catechol-O-methyltransferase
FDG	Fluorodeoxyglucose
FDOPA	6-[18F]fluoro-L-DOPA
FET	O-2-([18F]fluoroethyl)-L-tyrosine
FmT	6-[18F]-L-meta-tyrosine
HTP	β-[11C]-5-hydroxytryptophan
LAT1	L-type amino acid transporter
LNAA	Large neutral L-amino acids
MAO	Monoamine oxidase
OMFD	O-methyl-FDOPA
PET	Positron emission tomography
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase

Introduction

Access of solutes in the blood to the brain is restricted by the blood–brain barrier (BBB), which consists of a physical barrier to diffusion, imposed by the tight junctions between cells of the capillary epithelium, and also an active barrier mediated by ATP-cassette proteins such as the P-glycoprotein, which extrude broad classes of molecules from brain. Specific solutes and nutrients such as glucose and amino acids would normally be excluded from brain, but for specific facilitated diffusion carriers, also located in brain capillaries. Degradation of brain proteins provides some recycling of amino acids for protein synthesis in situ, but most of the brain's requirement for amino acids is met by influx from circulation. The transport of branched chain and aromatic amino acids across the BBB is mediated by a heterodimeric protein known as the large neutral L-type amino acid transporter (LAT1). Among its substrates are valine, leucine and isoleucine, phenylalanine, tyrosine, DOPA, tryptophan, and methionine, as well as a number of synthetic and exogenous amino acids. In classical studies of [^{14}C]-labeled amino acid transport, endogenous amino acids had Michaelis–Menten affinities in the range 100–800 μM and maximal transport rates also extending over a 10-fold range [1]. The substrate

affinities relative to the prevailing concentrations in blood result in considerable competition between substrates for transport, which explains the vulnerability of brain protein synthesis to perturbation of amino acid levels, as occurs in phenylketonuria [2], or hepatic encephalopathy. Likewise, competition at the BBB can limit availability in brain of amino acid precursors for the synthesis of neurotransmitters such as dopamine and serotonin (5-HT) and also for the kynurenine pathway. Molecular imaging of these pathways is critically dependent upon the rates of enzymatic conversion of the amino acid precursors in brain; this concept is the central theme of the present chapter.

Tumor Imaging with Large Neutral Amino Acids

The LAT1 is a revolving door; it is permissive to the bidirectional passage of its substrates across membranes and serves to establish equilibrium of substrate concentrations on either side of the BBB. Once entering brain, amino acids in the interstitial space are transported into living cells by LAT1 and by a separate, sodium-dependent process. Only then can they be incorporated into amino-acyl-RNA, the immediate precursors for protein synthesis. Tyrosine and tryptophan are also precursors for the synthesis of, respectively, catecholamine and indoleamine neurotransmitters. Consequently, positron emission tomography (PET) studies with these amino acids might convey information about both trapping processes in brain. However, molecular imaging studies by PET convey (at most) the blood concentration of the tracer as a function of circulation time (measured by serial blood sampling), and the total radioactivity concentration in brain as a function of time (measured by dynamic PET). The task of kinetic modeling is to obtain from these data a quantitation of one or more physiological processes. One of very first available PET tracers was the amino acid [^{11}C]methionine [3], which soon found use in PET studies of BBB transport and protein synthesis in brain tumors [4]. Taking [^{11}C]methionine-PET as an example, the processes describing its cerebral uptake and utilization are described in formal terms by a set of transfer coefficients and rate constants (Fig. 26.1). In general, this model is expressed as a two-tissue compartment model defined by unidirectional blood–brain clearance (K_1; ml g^{-1} min^{-1}), facilitated diffusion from brain back to circulation (k_2; min^{-1}), and the net rate constant for "irreversible" trapping in brain (k_3; min^{-1}), which here represents the composite of all processes leading to trapping of [^{11}C]methionine as protein. As will be discussed below, these kinetic steps are frequently collapsed into a single macro-parameter describing the net blood–brain transfer (K*; ml g^{-1} min^{-1}) of the PET tracer, defined as $K_1k_3/(k_2+k_3)$.

Any *L*-amino acid permeable to the BBB might serve for tumor imaging by PET. In a recent review, the most popular tracers were the endogenous amino acid [^{11}C]methionine, which is incorporated into protein in tumor and healthy brain tissue, and the exogenous tracers O-2-([^{18}F]fluoroethyl)-L-tyrosine (FET), 6-[^{18}F]fluoro-L-DOPA (FDOPA), and α-[^{11}C]methy-L-tryptophan (AMT), which are not

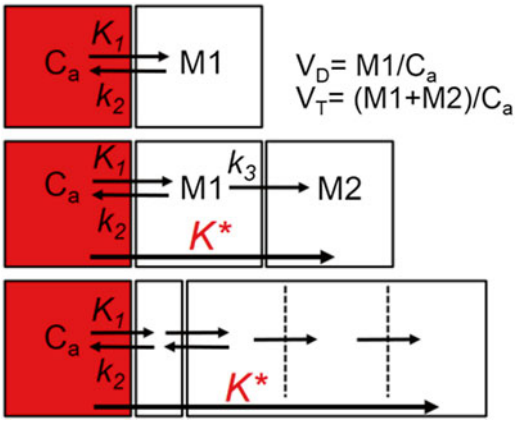

Fig. 26.1 The general compartmental model for PET tracers. The upper portion shows the cases for an inert tracer which simply partitions between blood and brain, obtained an equilibrium distribution volume (V_D; ml g^{-1}) defined by the ratio of the unidirection blood–brain clearance (K_1; ml g^{-1} min^{-1}) and the rate constant for diffusion of free tracer back into circulation (k_2; min^{-1}) The middle portion illustrates the case of a two-tissue compartment model for a tracer such as [^{11}C]tyrosine, which is irreversibly trapped in brain (k_3; min^{-1}). In this case, the total distribution volume (V_T; ml g^{-1}) entails both compartments, and linear graphic analysis gives the net influx, i.e., the macroparameter K^* (ml g^{-1} min^{-1}). The lower portion of the figure depicts a more physiological accurate model for the case of a metabolized tracer such as α-[^{11}C]methyltryptophan, which first enters the interstitial space (the small compartment), and then the cellular compartment, where it can undergo a series of biochemical transformations; the net influx is still represented by K^*

substrates for protein synthesis [5], but accumulate preferentially in tumors due to overexpression of LAT1 and other transporters. It remains to be established which of the many possible amino acid tracers is optimal for tumor staging or detection by PET. However, the main emphasis of this chapter concerns the amino acid tracers which are metabolized in brain in a manner specific for neurotransmitter or kynurenine synthesis.

PET Studies of Dopamine Synthesis: Tyrosine Hydroxylase Substrates

The classical rate limiting step for catecholamine synthesis is the enzyme tyrosine hydroxylase (TH), which is generally saturated with its substrate tyrosine. As such, [^{11}C]tyrosine presents itself as a potential tracer for dopamine synthesis in nigrostriatal terminals [6]. However, rat studies with [^{3}H]tyrosine indicate that its incorporation into brain protein is normally 10-fold greater than its entry into striatal dopamine synthesis [7]; under conditions of pharmacological blockade of presynaptic autoreceptors, the TH activity in vivo increased several fold, predicting that [^{11}C]tyrosine PET might under this challenge condition detect striatal catecholamine

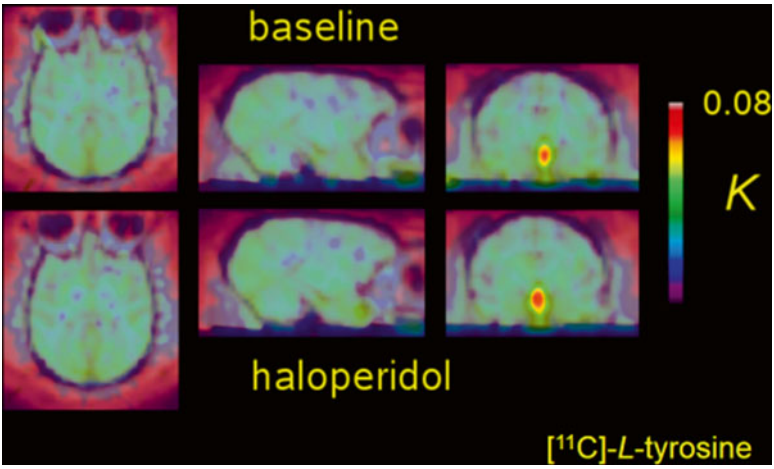

Fig. 26.2 PET examinations of [^{11}C]tyrosine uptake in brain of a monkey. The parametric maps show the net influx of the tracer into brain relative to the metabolite-corrected arterial input during 1 h. The result (K; ml g^{-1} min^{-1}) corresponds to the incorporation of the amino acid into brain protein. This is globally uniform, with a hotspot in the median eminence and pituitary, revealing a locus of neuropeptide synthesis. Contrary to prediction, pretreatment of the animal with an antagonist of dopamine D2/3 receptors (haloperidol; 0.3 mg/kg) did not discernibly stimulate catecholamine synthesis in the striatum. The PET results are projected upon a gray-scale MR image of monkey brain. Figure previously published in [73]

synthesis against the background of protein synthesis. We undertook a pilot study of this concept in monkey; linear graphical analysis of net influx [^{11}C]tyrosine relative to the metabolite-corrected arterial input curve was uniformly high in the untreated baseline condition and was unchanged following treatment with haloperidol, an antipsychotic known to stimulate TH (Fig. 26.2). The α-methyl-amino acids enter the brain via LAT1, but are not substrates for protein synthesis, predicting that α-[^{11}C]methyltyrosine might impart specific signal in living striatum. However, tests with α-[^{14}C]methyltyrosine did not reveal evidence for biotransformation and trapping in striatum of living rat [8]. Given these negative results, there seems to be no way to measure catecholamine synthesis rates in living brain by PET.

PET Studies of Dopamine Synthesis: Aromatic Amino Acid Decarboxylase Substrates

During the past 30 years, FDOPA, β-[^{11}C]DOPA, and some other substrates of aromatic amino acid decarboxylase (AAADC) have emerged as invaluable agents for PET investigations of the nigrostriatal dopamine innervation in living brain. Dynamic PET recordings with this class of tracer reveal initial influx that is spatially homogeneous, being mediated by LAT1 everywhere in the BBB.

However, the radioactivity concentration in striatum soon exceeds that in other brain regions, reflecting metabolism by AAADC, yielding (in the case of FDOPA) [^{18}F]fluorodopamine, which is trapped in synaptic vesicles. Once entering striatum, FDOPA is decarboxylated by AAADC, yielding [^{18}F]fluorodopamine, which is retained for a time within dopamine vesicles, but is ultimately decomposed by the successive actions of monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT). The resultant acidic metabolites, [^{18}F]DOPAC and [^{18}F]HVA [9], are free to diffuse out of brain. The specific radiolabeling of dopamine terminals attains a maximum at about 1 h after FDOPA (or β-[^{11}C]DOPA) injection [10] and is followed by slow washout. Thus, a prolonged FDOPA-PET recording reveals three distinct phases: (1) the initial tracer uptake across the BBB, (2) the formation and trapping of [^{18}F]fluorodopamine in dopamine vesicles, and (3) the late washout phase dominated by diffusion of [^{18}F]fluorodopamine acid metabolites from brain. Physiological quantitation of FDOPA scans requires explicit pharmacokinetic models, as in Fig. 26.1; the several FDOPA-PET models entail trade-offs between real physiological complexity, and the simplicity and robustness of the method [11]. Despite this complexity, the ratio between striatum and cerebellum radioactivity at 1 h is a robust indicator of the integrity of the nigrostriatal dopamine pathway. The diverse clinical findings in neurology and psychiatry PET studies with AAADC tracers have been reviewed [12, 13].

Endogenous L-DOPA is normally synthesized in living brain by TH, such that the actual brain L-DOPA concentration is generally unknown. However, the AAADC enzyme can never be saturated by L-DOPA in living brain, given the 100 μM affinity. It follows that PET studies with AAADC substrates cannot generally reveal the *rate* of catecholamine synthesis, but they can provide an index of the *capacity* for catecholamine synthesis from L-DOPA in circulation. Since FDOPA is also a substrate for AAADC throughout the body, subjects for brain PET studies are generally pretreated with the peripherally acting AAADC inhibitor carbidopa. In this condition, plasma FDOPA is mainly metabolized by liver COMT. Within 30 min of FDOPA injection, the inert metabolite *O*-methyl-FDOPA (OMFD) is the major radioactive species in plasma of humans [14] and rats [15]. The several approaches for the quantitation of cerebral FDOPA kinetics must somehow correct for the plasma-derived OMFD, which presents a homogeneous background radioactivity somewhat obscuring the signal related to dopamine synthesis, in analogy to the background of protein synthesis presented by [^{11}C]tyrosine, above.

Although FDOPA and L-DOPA are not kinetically distinguished by AAADC in vitro [16], FDOPA is a better substrate for COMT than is natural L-DOPA [17, 18]. The tracer β-[^{11}C]DOPA is thus preferable for PET studies of AAADC, since it yields much less of the interfering plasma metabolite [19]. Furthermore, the shorter half-life of [^{11}C] (20 min) relative to [^{18}F] (110 min) might permit pharmacological challenge studies, in which a subject is scanned twice in succession on the same scanning day. Unfortunately, the complex radioenzymatic synthesis of β-[^{11}C]DOPA seems to have discouraged its widespread use. On the other hand, the [^{18}F]-precursor or FDOPA itself can be shipped hundreds of kilometers from a cyclotron/radiochemistry facility. The alternate AAADC tracer 6-[^{18}F]-L-*meta*-tyrosine

(FmT) is not a substrate for COMT and therefore does not yield a troublesome brain-penetrating metabolite. FmT rapidly enters brain via LAT1 and gives rise to a more intense labeling of striatum than is typical of FDOPA [20]. Influx plots of FmT or FDOPA were equally sensitive to the nigrostriatal degeneration in monkeys with MPTP-induced parkinsonism, although only FDOPA revealed clear evidence of the late washout phase related to breakdown of dopamine [21]. Perplexingly, the decarboxylated product of FmT, 6-[^{18}F]-*meta*-tyramine, is not sequestered in synaptic vesicles [22], but is instead rapidly metabolized by MAO, yielding the acidic metabolite [^{18}F]fluoro-hydroxyphenylacetic acid [23]. In general, acidic metabolites should diffuse from brain, so it is unclear how the specific FmT signal is retained so effectively within living striatum.

FDOPA/β-[^{11}C]DOPA kinetic modeling is based upon the earlier experience with fluorodeoxyglucose (FDG), the tracer for PET studies of glucose consumption [24]. Whereas the model for analysis FDG-PET safely assumes irreversible trapping of the tracer in brain, as in Fig. 26.1, the FDOPA model is considerably more complex. Indeed, the kinetic modeling of FDOPA is a "worst case" scenario, due to the entry of OMFD in brain, and due also to formation of diffusible brain metabolites and progressive violation of the assumption of irreversible trapping, i.e., washout. In practice, it is usually necessary employ a simplification of the over-specified physiological model. In the simplest kinetic analysis of PET data with AAADC substrates, the radioactivity curve in some brain region devoid of AAADC is used as a surrogate for the free concentration of precursor. Graphical reference tissue analysis yields the fractional rate constant of tracer decarboxylation in striatum, known as k_3^r (min^{-1}). In β-[^{11}C]DOPA studies using this approach, substantial blockade of AAADC was shown in striatum of monkeys treated with a centrally acting AAADC inhibitor [25], as distinct from the potentiation of uptake by a peripherally acting inhibitor. Thus, benserazide treatment increased tracer influx without altering the magnitude of β-[^{11}C]DOPA-k_3^r in monkey striatum, although concomitant COMT inhibition potentiated a central effect of the AAADC inhibitor [26]. The k_3^r of β-[^{11}C]DOPA exceeded that of 6-fluoro-β-[^{11}C]DOPA, but treatment with a COMT inhibitor increased the apparent rate constant for the latter tracer [27]; given that FDOPA is a better COMT substrate than is L-DOPA (noted above), this finding indicates that OMFD in the reference region propagates to underestimation of k_3^r, due to overestimation of the precursor pool. In human β-[^{11}C]DOPA studies, influx to white matter was inversely proportional the sum of the plasma concentrations of the LNAAs, which gives a formal demonstration of the competition for BBB transport [28]. As such, a protein meal is best avoided in the hours preceding the PET scan. In clinical FDOPA studies of patients with Parkinson's disease, k_3^r was reduced by one half in putamen [29], indicative of the extent of nigrostriatal degeneration resulting in motor symptoms.

Treatment with the TH cofactor tetrahydrobiopterin and tyrosine increased the β-[^{11}C]DOPA k_3^r in monkey striatum, this by an unknown mechanism [30]. Treatment of monkeys with L-DOPA also increased the magnitude of β-[^{11}C]DOPA k_3^r by 20 % in monkey striatum [31], an effect also seen in health controls [32],

patients with advanced Parkinson's disease [33], and for FDOPA in healthy humans, but not in Parkinson's disease patients [34]. The basis of this "feed-forward" stimulation of AAADC by its main substrate or by TH stimulation is unclear, but an inverse relationship reported in the former study between baseline k_3^r and the magnitude of stimulation implicates autoreceptor tonus as a regulatory mechanism. This concept was developed further in β-[^{11}C]DOPA studies with the autoreceptor agonist (−)OSU6162, which likewise showed effects on k_3^r which depended on the baseline AAADC activity [35]. Similarly, apomorphine challenge decreased [^{3}H] DOPA decarboxylation in rat striatum [36] and decreased the β-[^{11}C]DOPA k_3^r in monkey striatum in a tonus-dependent manner [37]. Conversely, blockade of dopamine autoreceptors with haloperidol stimulates the uptake and utilization of FDOPA in striatum of healthy humans [38]; this principle was the basis of the test of [^{11}C] tyrosine uptake after activation of TH (Fig. 26.2).

As distinct from reference tissue analyses of AAADC substrates, compartmental models require serial arterial blood sampling. In the case of FDOPA, fractions of untransformed FDOPA and the metabolites OMFD are measured in plasma extracts from serial blood samples collected during the PET scan. These fractions can be separated from blood plasma using powdered alumina [14], but this solid phase method has been largely supplanted by HPLC methods [39]. The end result is a pair of arterial inputs, one for the declining fraction of untransformed FDOPA and one for the increasing amounts of OMFD. When the dual arterial inputs are available, FDOPA uptake in brain can then be analyzed by compartmental analysis, and also by a convenient linear graphical analysis (K^*, above), in direct analogy to the method for calculating net flux of FDG to brain [40–42]. Graphic analysis is based on linear regression of the plot of tracer distribution volume ($C_t(t)/C_p(t)$) versus the normalized arterial input ($\mathrm{Int}C_p(t)/C_p(t)$, as shown in Fig. 26.3. In the case of FDOPA, the troublesome plasma metabolite OMFD also enters brain and soon becomes the predominant radioactive compound in most brain regions [13, 43]. To isolate an index of the dopamine synthesis capacity, it is first necessary to remove from consideration the global brain radioactivity due to OMFD. Linear graphic analysis after frame-by-frame subtraction of the entire radioactivity in the reference region approximates the FDOPA K^* [44], as defined in Fig. 26.2. Using this analysis method with FDOPA [45] and β-[^{11}C]DOPA [46], increased capacity for dopamine synthesis was seen in brain of patients with schizophrenia; this has become one of the best-established molecular imaging findings in schizophrenia. While the reference tissue subtraction removes interference from OMFD, it results in bias in the magnitude of net influx due to over-subtraction of the FDOPA precursor. Compartmental analysis of FDOPA recordings more properly accounts for the presence in brain of OMFD, and can be used to estimate the magnitude of k_3, in this context corresponding to the relative activity of AAADC [47, 48]. This compartmental analysis may be the most rigorous approach, but is seldom undertaken due to its technical complexity.

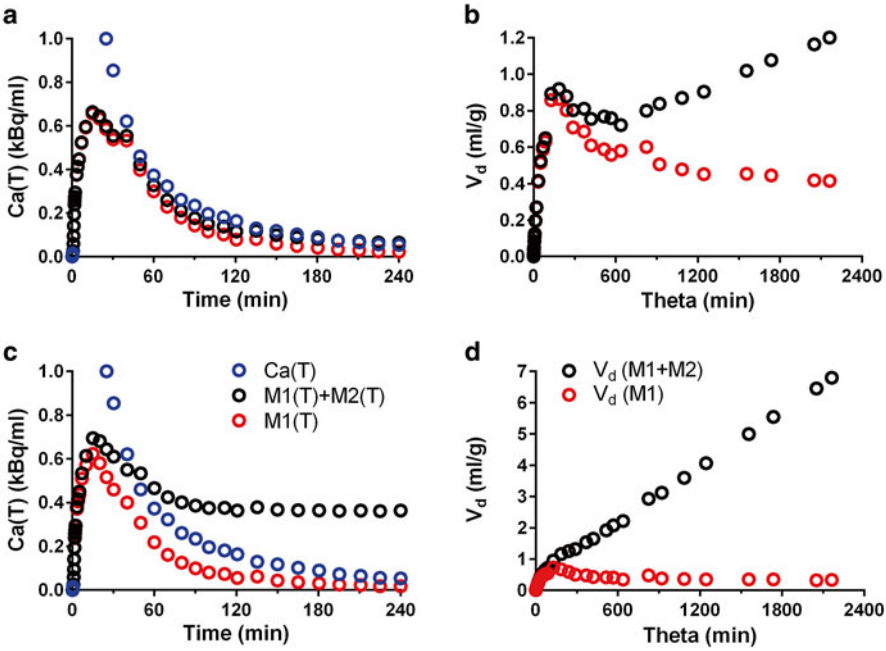

Fig. 26.3 Arterial [^{11}C]AMT input (C_a(T)) and calculated brain curves and influx plots for [^{11}C]AMT (M1) and the sum of [^{11}C]AMT together with α-[^{14}C]methylserotonin (M1+M2) calculated with canonical values of K_1 (0.015 ml g^{-1} min^{-1}), k_2 (0.04 min^{-1}), and (**a**) low k_3 (0.001 min^{-1}) and (**c**) high k_3 (0.01 min^{-1}). Comparison of the two figures indicates the substantial conversion expected for the case of high k_3. Replotting these data shows the expected distribution volume as a function of the normalized arterial input (theta) during 240 min for (**b**) low k_3 (K^* = 0.00039 ml g^{-1} min^{-1}) and (**d**) high k_3 (K^* = 0.0032 ml g^{-1} min^{-1}). The off-scale initial peak of the input curve (C_a(max); 15 kBq/ml) occurred at 1.5 min. Note that M1 and M2 are of approximately equal magnitude at (**a**) 180 min for low k_3 and (**c**) 60 min for high k_3, corresponding to 50 % conversion in vivo. Figure presented earlier in [73]

PET Studies of Serotonin Synthesis

Brain 5-HT synthesis is mediated by the concerted and sequential actions of two enzymes: tryptophan hydroxylase (TPH) and AAADC, the same enzyme catalyzing the decarboxylation of FDOPA and β-[^{11}C]DOPA. Molecular imaging of 5-HT synthesis is motivated by the important neurological and psychiatric conditions in which 5-HT is implicated. However, PET studies with β-[^{11}C]tryptophan are unfit to reveal brain 5-HT synthesis due to the entry of tracer into the brain protein pool [49], which masks the much lesser flux of tracer into the 5-HT synthesis pathway. Furthermore, lesion studies indicated that trapping of β-[^{11}C]-5-hydroxytryptophan (HTP) in rhesus monkey striatum was largely mediated by AAADC in dopamine fibers, rather than 5-HT terminals. Two-tissue compartment modeling of this

tracer in human brain showed AAADC activity (k_3) ranging from 0.02 min^{-1} in cerebellum to 0.1 min^{-1} in caudate nucleus [50], indicating very substantial transformation in the course of the PET recording. Pretreatment with HTP or a TPH inhibitor both increased the influx of [^{11}C]HTP to human brain; this seems paradoxical, since the two treatments should have had opposite effects on the specific activity of the tracer in brain [51]. In contrast, HTP pretreatment competitively inhibited k_3^r in monkey striatum, but more sensitively with respect to [^{11}C]HTP than for β-[^{11}C]DOPA, suggesting a certain specificity of the former tracer for AAADC in 5-HT fibers [52]. This effect stands in contrast to the potentiation of FDOPA uptake by L-DOPA, noted above. Pretreatment of monkeys with the AAADC co-factor pyridoxine (vitamin B6) stimulated the decarboxylation of [^{11}C]HTP in monkey striatum [53], indicating lack of saturation in vivo; cofactor sensitivity may conceivably account for the failure of μPET with [^{11}C]HTP to reveal serotonin synthesis in brain of living rats [54]. In a semiquantitative clinical study, [^{11}C]HTP uptake was globally reduced in brain of patients with major depression [55], but k_3^r was unaffected in a subsequent report [56], a discrepancy apparently due to the low specific activity in the former study. However, a link between [^{11}C]HTP uptake and mood across the menstrual cycle was seen in women with premenstrual dysphoria [57].

The incomplete specificity of [^{11}C]HTP for 5-HT fibers lead to the proposal of AMT as a selective PET tracer for 5-HT synthesis. The AMT method is predicated upon entry of the tracer into the 5-HT pathway, in analogy to other metabolized tracers such as FDG and FDOPA. AMT is a substrate LAT1 in the BBB; upon entering brain, it can be converted to α-methy-L-serotonin by the successive actions of TPH and AAADC in 5-HT fibers [58]. The α-[^{14}C]methylserotonin so formed from [^{14}C]AMT can be released as a false neurotransmitter [59]. Since AMT is not a substrate for protein synthesis, and since α-methylserotonin is not a substrate for monoamine oxidase, the final enzymatic product is likely to accumulate specifically and irreversibly in 5-HT neurons. Indeed, there was a high correlation between autoradiographic trapping of [^{14}C]AMT at 24 h after injection and TPH protein levels within the rat raphé nuclei [60]. Furthermore, the autoradiographic method reveals progressive labeling of the rat raphé and pineal gland, with less distinct labeling in the ventral hippocampus, ventral striatum, and other regions with a relatively sparser 5-HT innervation [61]; linear graphic analysis of [^{14}C]AMT uptake during 150 min gives a net influx (K^*) which should be proportional to the local 5-HT synthesis rate. In this model, the microparameter k_3 defines the activity of TPH (Fig. 26.1). In point of fact, the Trp product OHAMT, like other LNAAs, is a substrate for LAT1, such that it is free to leave brain unless decarboxylated, or irreversibly bound to AAADC enzyme, in the manner of α-fluoromethyl-DOPA [62]. Thus, k_3 is more properly corrected for the fraction of the intermediate which fails to be irreversibly trapped. This fraction is unknown for OHAMT, but is close to 80 % for the case of FDOPA [63]; we suppose that the majority of OHAMT is likewise committed to the 5-HT pathway.

TPH activity is low in most brain regions. Therefore, it is important to define the minimum k_3 imparting a discernible TPH signal, given the constraint of 100 min PET recordings with [^{11}C]AMT. This consideration requires a review of the enzymology and neurochemistry of TPH (Table 26.1). Available data shows that the

Table 26.1 Biochemical properties of tryptophan hydroxylase (TPH), the Michaelis–Menten kinetic parameters (V_{max}, K_m) in several preparations, and the relative TPH activity (here, k_3) observed in living brain, along with concentrations of tryptophan (Trp) and reduced pterin or DMPH4

TPH assay			Reference
Rat brain, reduced pterin concentration		1.4 μM	[74]
Purified from porcine kidney Pig brainstem homogenate	K_m (Trp): 500 μM K_m (Trp): 20 μM	1 mM DMPH4	[75]
Rat brainstem, partial purification	K_m (Trp): 75 μM	K_m (DMPH4): 240 μM	[76]
Cat forebrain homogenate[a]	V_{max}: 1.5 nmol g^{-1} min^{-1} K_m (Trp): 37 μM k_3: 0.04 min^{-1}	K_m (DMPH4): 43 μM	[77]
Rat raphe homogenate[a]	V_{max}: 3.3 nmol g^{-1} min^{-1} K_m (Trp): 390 μM k_3: 0.008 min^{-1}	K_m (DMPH4): 280 μM	[78]
Rat brain, OHTrp accumulation after NSD 1015 treatment:[b] Cerebellum Cerebral cortex Midbrain	V: 1.3 pmol g^{-1} min^{-1} k_3: 0.00005 min^{-1} V: 15 pmol g^{-1} min^{-1} k_3: 0.0006 min^{-1} V: 56 pmol g^{-1} min^{-1} k_3: 0.002 min^{-1}		[79]
Young rat, OHTrp accumulation after NSD 1015 treatment:[b] Cerebral cortex Midbrain	V: 8.3 pmol g^{-1} min^{-1} k_3: 0.0065 min^{-1} V: 20 pmol g^{-1} min^{-1} k_3: 0.013 min^{-1}	[Trp] in whole brain: 25 μM	[80]
Rat, OHTrp accumulation after NSD 1015 treatment:[b] Cerebellum Cerebral cortex Dorsal raphe	V: 3.6 pmol g^{-1} min^{-1} k_3: 0.00014 min^{-1} V: 18 pmol g^{-1} min^{-1} k_3: 0.00072 min^{-1} V: 610 pmol g^{-1} min^{-1} k_3: 0.024 min^{-1}		[81]

[a] V_{max}, the maximal activity of TPH under saturating conditions in vitro, calculated relative to tissue wet weight, and k_3, calculated as V_{max}/K_m

[b] V, the activity of TPH measured ex vivo from the accumulation in brain of OHTrp during 30 min after treatment with the DOPA decarboxylase inhibitor NSD 1015, and k_3 calculated from V relative to the brain Trp concentration, taken to be 25 μM

TPH V_{max} in vitro is much higher than the enzyme velocity (V) calculated ex vivo from the accumulation of 5-hydroxytryptophan (OHTrp) after pharmacological blockade of AAADC. Also, the concentrations in brain of the TPH substrates Trp and tetrahydrobiopterin are lower than their Michaelis–Menten affinities in vitro, explaining why TPH activity in vivo (V) is lower than V_{max}. Finally, TPH activity is approximately 100-fold higher in the raphe nucleus than in cerebellum. HPLC analysis of micro-dissected rat dorsal raphe at 180 min after [^{14}C]AMT injection showed nearly 50 % conversion to α-[^{14}C]methylserotonin in [64], matching closely the expected conversion for k_3 = 0.001 min^{-1}, as presented below (Fig. 26.3a).

Others, however, failed to detect much α-[^{11}C]methylserotonin in extracts of monkey midbrain at 90 min and could not discern a linear phase in the graphic analysis of [^{11}C]AMT uptake [65]. Nonetheless, Diksic presented figures showing distinctly linear influx phases for [^{14}C]AMT to rat brain and [^{11}C]AMT to human brain and argued that the kinetic model does not necessarily depend upon biochemical transformation of AMT [66]. This argument was developed further by Chugani and Muzik [67], who concluded that K^* reveals the composite of α-[^{11}C]methylserotonin synthesis, together with a *much larger* pool of trapped but unmetabolized precursor(s), presumably destined for later enzymatic conversion. The physiological nature of this hypothetical compartment is unclear, but might be related to binding of tracer to the AAADC enzyme.

Conventional two-tissue compartment analysis of [^{11}C]AMT uptake in human [68] or nonhuman primate brain [69] relative to the arterial input measured during 60 min PET recordings gives estimates of the unidirectional blood–brain clearance (K_1; 0.015 ml g^{-1} min^{-1}), the fractional rate constant for diffusion of [^{11}C]AMT back to circulation (k_2; 0.04 min^{-1}), and the relative TPH activity (k_3; 0.01–0.02 min^{-1}), to be contrasted with the FDOPDA-k_3 of nearly 0.1 min^{-1} in striatum [47, 48]. However, Muzik et al. [70] noted that 60 min recordings were insufficient to support a two-tissue compartment model. Prolonged arterial inputs for [^{11}C]AMT are unattainable due to the 20 min physical half-life. Therefore, we used a 240 min FDOPA input from a human PET study (unpublished observations) as a surrogate input. We can then calculate the brain curves expected for [^{11}C]AMT (M1) and the sum of [^{11}C]AMT together with α-[^{14}C]methylserotonin (M1+M2), given canonical values of K_1 and k_2 (as above) and low k_3 (0.001 min^{-1}; Fig. 26.3a) or high k_3 (0.01 min^{-1}; Fig. 26.3c). For linear graphic analysis, these data are replotted as distribution volume (V_d) as a function of the normalized [^{11}C]AMT input (theta) during 240 min for low k_3 (Fig. 26.3b) and high k_3 (Fig. 26.3d). Both of these latter figures show an initial linear phase for tracer delivery to brain, i.e., K_1. After some time there is a breakpoint, and the plot enters a final linear phase, corresponding to K^*, from which 5-HT synthesis rate is calculated. For low k_3, this breakpoint occurs at 500 min of theta, i.e., 100 min of tracer circulation, but for high k_3, the final linear phase is already established at 125 min of theta, i.e., 40 min of tracer circulation. This simulation implies that linear graphic analysis of brief [^{11}C]AMT PET scans could serve for quantitation of K^* in regions of highest TPH, such as the dorsal raphe, but is likely inadequate for quantitation in regions with activity as low as that expected for cerebellum or cerebral cortex.

These factors may contribute to the low range of the magnitudes of K^* estimates reported in various regions of rat brain (<3-fold [70]) and from 60 min PET recordings of human brain (<2-fold [71, 72]), this despite the 100-fold range of TPH activity between cerebellum and raphé noted above, which predicts a 15-fold range of K^* by region. Forcing a linear fit to early data will naturally overestimate the true magnitude of K^*, due to incomplete separation or trapping from the BBB transport phase. Data from human cerebral cortex nonetheless show a final linear uptake phase beginning in human cortex with only 30 min of theta, giving K^* of 0.005 ml g^{-1} min^{-1} [71]. This K^* is consistent with a k_3 close to 0.01 min^{-1} in cortex (Fig. 26.3), which seems rather

high. The biochemical data in Table 26.1 require that [^{11}C]AMT-PET must be interpreted with caution, being most likely valid in regions of highest TPH activity, such as the dorsal raphe. This region emerged as a site of relatively increased 5-HT synthesis in a new PET study of human users of the psychostimulant MDMA (Ecstasy) [72], suggesting accumulation of Trp enzyme in the same region due to partial axotomy. However, as argued above, the present PET method may be unfit to quantify 5-HT synthesis in regions of low TPH activity. An unexplored possibility for better quantitation might be obtained with the untested PET tracer α-[^{18}F]fluorotryptophan, which might enter into the 5-HT and kynurenine pathways, while affording PET recordings lasting several hours, a more adequate time for developing specific trapping signal. As in all PET tracers for amino acid metabolism, the physiological interpretation of PET recordings must be always be informed by a priori knowledge of the extent of biochemical transformation of the tracer.

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Chapter 27
Molecular Imaging of Serotonin Synthesis in the Brain

Otto Muzik, Sandeep Mittal, and Csaba Juhász

Abstract Tracer kinetic analysis of ^{11}C-labeled alpha-methyl-tryptophan (AMT) allows quantitative assessment of serotonin synthesis using positron emission tomography (PET) imaging in human brain. The obtained K-complex represents an index of serotonin synthesis (termed serotonin synthesis capacity) that is well suited to study serotonergic mechanisms in the developing brain. Assessment of global and local developmental changes of serotonin synthesis capacity in brains of autistic children indicates impaired serotonin metabolism that affects neuronal differentiation of serotonergic neurons during critical developmental periods. Moreover, increased serotonin synthesis capacity in various supratentorial regions of children with alternating hemiplegia is observed during ictal and postictal periods, suggesting increased regional serotonergic activity associated with hemiplegic attacks in extended neural networks. Finally, children with Tourette syndrome show decreased serotonin synthesis capacity in the bilateral dorsolateral prefrontal cortex and increased serotonin synthesis capacity in the bilateral thalamus and caudate, suggesting abnormal tryptophan metabolism in the fronto-striato-thalamic circuits.

Keywords Positron emission tomography • Alpha-methyl-tryptophan • Tracer kinetic modeling • Serotonin synthesis rate • Autism • Tourette syndrome • Alternating hemiplegia • Migraine

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List of Abbreviations

5-HIAA	5-Hydroxyindoleacetic acid
5-RIAA	5-Hydroxyindole acetic acid
ADHD	Attention-deficit hyperactivity disorder
AMHT	Alpha-methyl-5-hydroxytryptophan
AMS	Alpha-methylserotonin
AMT	Alpha-[11C]methyl-L-tryptophan
OCD	Obsessive-compulsive disorder
PET	Positron emission tomography
TS	Tourette syndrome

Introduction

In vivo imaging using positron emission tomography (PET) tracers aimed at investigating serotonergic function has been an area of great interest, especially because there is evidence that abnormalities of serotonergic neurotransmission are associated with a number of neurological and psychiatric disorders including autism, migraine, sleep disorders, depression, movement disorders, and epilepsy (for review see [1]). Early attempts to study serotonergic function using radioactive-labeled tryptophan suffered from methodological problems involving the necessity of chemical separation of metabolites in the blood pool and removal of the part of the tracer incorporated into proteins [2–6]. Another problem with the use of labeled tryptophan as a tracer is the very fast removal of 5-hydroxyindoleacetic acid (5-HIAA) from the brain, which is the terminal metabolite of the neurotransmitter serotonin. To overcome these problems, an analogue of tryptophan, alpha-[^{11}C] methyl-L-tryptophan (AMT), has been developed as a tracer for serotonin synthesis with PET by [7]. This analogue has been shown to possess better metabolic characteristics than the natural substrate for kinetic modeling of tracer in tissue. After the administration of labeled or unlabeled AMT in rats, the synthesis of alpha-methylserotonin (AMS) in brain has been shown by highpressure liquid chromatography [7, 8]. [^{3}H]AMS synthesized in brain was localized in serotonergic neurons by combined autoradiography and tryptophan hydroxylase immunocytochemistry at the electron microscopic level [9] proving the specificity of the tracer for serotonergic nerve terminals. Furthermore, [^{3}H]AMS present in nerve terminals could be released by K^{+}-induced depolarization, suggesting that this tracer is stored with the releasable pool of serotonin [9]. Because AMS, unlike serotonin, is not a substrate for the degradative enzyme monoamine oxidase [8], AMS accumulates in serotonergic nerve terminals in proportion to the synthesis rate of serotonin. In addition, AMT, unlike tryptophan, is not incorporated into proteins, nor are metabolites released into the blood pool [7, 10]. These properties of AMT make it an excellent tracer substance for the measurement of serotonin synthesis rate in vivo.

Quantitative Assessment of Serotonin Synthesis

It was shown in animal studies [7, 11] that the kinetics of the tracer [^{11}C]AMT can be described by a three-compartment model using first-order rate constants (schematically represented in Fig. 27.1). The inflow rate constant K_1 and the outflow rate constant k_2 both describe the exchange of [^{11}C]AMT between vascular space and the cell cytoplasm (transport across the blood–brain barrier, the interstitial space, and the cell membrane). Once present in the cell cytoplasm, [^{11}C]AMT is converted to [^{11}C]AMS (C_m) by means of an irreversible, two-step enzymatic process characterized by the rate constant k_3. It is generally accepted that the rate-limiting step of this process is the hydroxylation of [^{11}C]AMT to alpha-[^{11}C]methyl-5-hydroxytryptophan ([^{11}C]AMHT) that is mediated by an enzyme only found in serotonergic neurons.

Using dynamic PET imaging, the time course of AMT tracer concentration (μCi/cc) can be determined quantitatively for various anatomical regions directly from the images, yielding the combined signal from free AMT tracer in cytoplasm ($C_f(t)$) and the metabolized portion representing AMHT ($C_m(t)$) denoted as $C_{PET}(t)$ (Fig. 27.2). If the arterial input function ($C_a(t)$) is known, a set of differential equations can be formulated that describes the relationship between dynamic changes in $C_{PET}(t)$ and $C_a(t)$ as a function of the rate constants K_1, k_2, and k_3.

The solution for the set of differential equations describing this three-compartment model was previously reported by numerous investigators (e.g., [12, 13]). Furthermore, it was shown that a combination of these rate constants (the term $[K_1k_3/(k_2 + k_3)]$ also called the K-complex) is directly proportional to the total amount of tracer entering the third "metabolic" compartment.

$$\text{Serotonin synthesis rate}\left[\mu\text{mol}/\text{g}/\text{min}\right] = \text{scale} - \text{factor}\left[\mu\text{mol}/\text{mL}\right] * \text{K} - \text{complex}\left[\text{mL}/\text{g}/\text{min}\right] \quad (27.1)$$

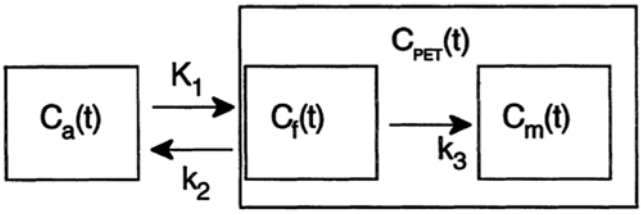

Fig. 27.1 Tracer kinetic model for alpha-[^{11}C]methyl-tryptophan ([^{11}C]AMT). The inflow rate constant K_1 and the outflow rate constant k_2 both describe the exchange of [^{11}C]AMT between vascular space (C_a) and the cell cytoplasm (C_f). Once present in the cytoplasm, [^{11}C]AMT is converted to alpha-[^{11}C]methylserotonin (C_m) by means of an irreversible, two-step enzymatic process that is characterized by the rate constant k_3. The PET tissue signal is denoted as C_{PET} and represents a combination of both the C_f and C_m signals. K_1: inflow rate constant; k_2: outflow rate constant; C_a: vascular space; C_f: cell cytoplasm; C_m: alpha-[^{11}C]methyl-serotonin; C_{PET}: PET tissue signal

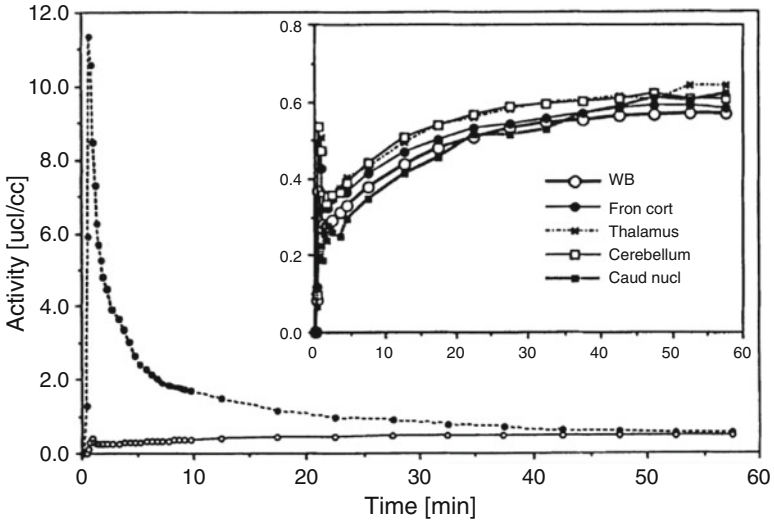

Fig. 27.2 Arterial plasma and whole brain tissue concentration—time course of [^{11}C]AMT (corrected for radioactive decay) in a young healthy control subject. The whole brain uptake was low with less than 2% of the injected dose present in the brain after 60 min. Inset: Individual regional time-activity curves of whole brain and selected brain regions shown on an expanded scale. Abbreviations: *WB*, whole brain; *Fron cort*, frontal cortex; *Caud nucl*, caudate nucleus

The scale factor in the above equation is equal to the quotient of blood plasma tryptophan concentration available to be transported into brain tissue and the "lumped constant." The lumped constant accounts for the difference between serotonin synthesis rate and [^{11}C]AMS synthesis rate. As this difference is considered to be invariant between subjects of a given species [7, 14, 15], the lumped constant represents a true constant. Reliable estimates of the individual rate constants K_1, k_2, and k_3 for [^{11}C]AMT using nonlinear least-squares fitting algorithms can only be obtained for large regions of the brain due to the low identifiability of the model, which is caused by the low count statistics originating from the low single-pass extraction fraction of the tracer (approximately 2 %) and the short physical half-life of [^{11}C] [16]. The graphical approach described by Patlak et al. [17] is an alternative method of analysis, which allows the determination of the K-complex ($= K_1k_3/(k_2 + k_3)$) from the linear portion of a graph that relates the integral of the tracer concentration in blood to the tracer concentration of tissue at a later time point (Fig. 27.3). Upon reaching dynamic equilibrium, this relationship becomes linear and can be fitted with a straight line on a pixel-by-pixel basis.

This approach assumes that the system to be analyzed consists of a homogenous tissue region with any number of compartments that communicate reversibly with the blood. In addition, there must be at least one compartment that the tracer enters in an irreversible manner. As discussed by Patlak et al. [17], at long enough times after administration of tracer ($t > t^*$), the amount of tracer in the reversible

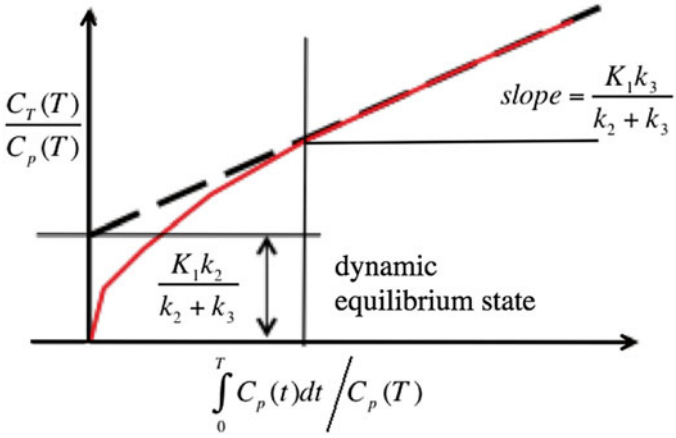

Fig. 27.3 Linearization of a two-tissue compartment model using the Patlak graphical analysis approach. The plot relating the integral of the blood input function (from time 0 to T) to the tissue activity at time T becomes linear once dynamic equilibrium is reached (i.e., when the amount of tracer extracted into tissue equals the amount of tracer metabolized). The slope of the fitted line is numerically equivalent to the K-complex ($K_1k_3/(k_2 + k_3)$), which is directly proportional to the tracer compound synthesis rate

compartments will be in effective steady state with plasma yielding a linear function. Although true dynamic equilibrium is reached only after ~80 min for [^{11}C] AMT, it was shown that applying the Patlak-plot method between 20 and 60 min postinjection is a good compromise between stability, bias, and contrast when creating parametric functional images of the K-complex, with a bias of 10–20% [16].

The above [Eq. (27.1)] is identical to that used by Sokoloff et al. [12] to estimate glucose metabolic rate using the tracer [^{14}C]2-deoxyglucose. Sokoloff exploited the property of 2-deoxyglucose which is irreversibly trapped in brain tissue (based on the rate of tracer accumulation in tissue) and allows quantitative assessment of metabolic rate of glucose. Similarly, AMS is not a substrate for the degradative enzyme monoamine oxidase [8]; thus, accumulation of AMS occurs in serotonergic terminals, possibly reflecting serotonin synthesis rate. However, although AMT is similar to 2-deoxyglucose with regard to an irreversible pool of tracer uptake in the third (metabolic) compartment, there are important differences between the two tracers, which affect interpretation of the K-complex.

Serotonin Synthesis Capacity

2-Deoxyglucose competes only with glucose for transport into the brain by means of the glucose transporter. The plasma concentration of glucose is approximately equal to the Km of the glucose transporter under normal circumstances (for review, see [18]).

In contrast, tryptophan accounts for only approximately 5% of the total large neutral amino acid pool [19, 20] with which the tracer [^{11}C]AMT competes at the blood–brain barrier for entry into the brain. Furthermore, the large neutral amino acid transporter is close to saturation under normal circumstances [20, 21]. Therefore, even large changes in plasma tryptophan concentration will have little effect on the transport of [^{11}C]AMT into the brain. Thus, even a 100% increase in plasma tryptophan results in only a 5% change in total large neutral amino acid pool with which [^{11}C]AMT must compete for brain entry. Thus, calculating the serotonin synthesis rate by multiplying the Kcomplex for [^{11}C]AMT by the plasma-free tryptophan level in analogy to the glucose model is erroneous. Shoaf et al. [22] showed that the values for AMT K-complex were highly reproducible in the same animal under the same test–retest conditions, whereas values for plasma tryptophan and free tryptophan were not. These data suggest that for AMT, the K-complex is a stable parameter characteristic for the individual and thus has meaning independent of the plasma tryptophan or plasma-free tryptophan, termed the serotonin synthesis capacity [23]. A representative set of images demonstrating both tracer accumulation between 30 and 55 min as well as serotonin synthesis capacity (mL/g/min) is shown in Fig. 27.4. Based on the studies and issues discussed above, we can conclude that AMT is a useful tracer for examination of serotonergic mechanisms, although this tracer cannot be used to measure the absolute serotonin synthesis rate. Furthermore, in certain pathologic states (e.g., in tumors and inflammatory conditions), increased AMT may represent increased metabolism by means of the kynurenine pathway, rather than increased serotonin synthesis (see further details in Chap. 28).

Developmental Course of Serotonin Synthesis in the Brain

Developmental changes in brain serotonin content and serotonin receptor binding have been demonstrated in nonhuman primates [24, 25]. There is a rise in serotonin content in cortex beginning before birth reaching a peak at 2 months of age, followed by a slow decline until 3 years of age [24]. Expression of serotonin receptors showed a similar developmental time course [25]. In humans, measures of the serotonin metabolite 5-hydroxyindole acetic acid (5-RIAA) in cerebrospinal fluid show higher values in children compared to adults [26, 27]. There is evidence that serotonin acts as a trophic or differentiation factor in addition to its role as a neurotransmitter, and that alteration of serotonin levels during brain development influence neuronal differentiation. For non-autistic children, Chugani et al. [28] showed that serotonin synthesis capacity was >200% of adult values until the age of 5 years and then declined toward adult values (Fig. 27.5). Furthermore, serotonin synthesis capacity values declined at an earlier age in girls than in boys [28]. The earlier decline in the developmental curve for whole brain serotonin synthesis capacity in girls as compared to boys is consistent with earlier onset of puberty in girls [29].

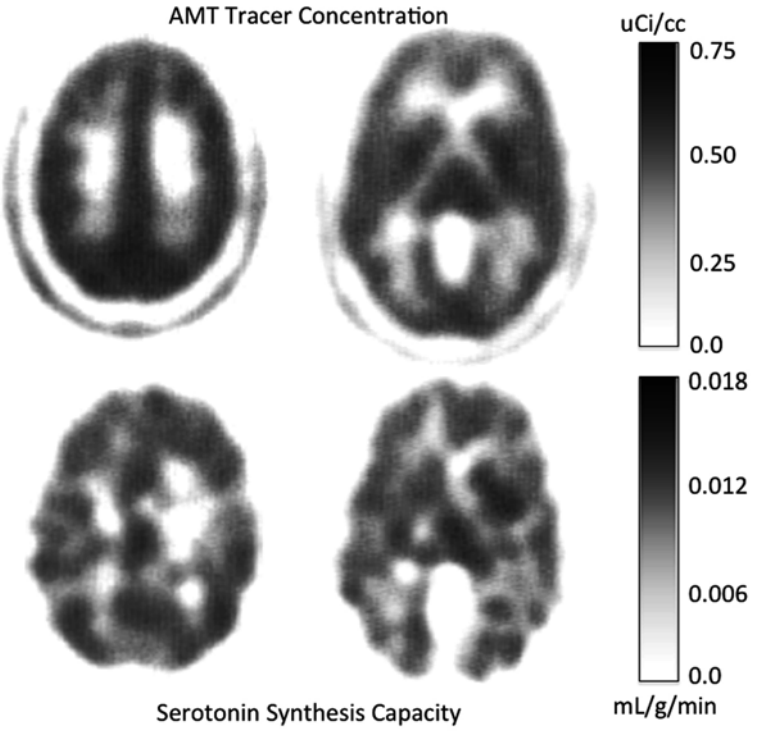

Fig. 27.4 alpha-[^{11}C]methyl-tryptophan distribution in healthy adult brain. The top row shows transaxial images at two levels generated by summing data obtained between 30 and 55 min after tracer injection and shows widespread distribution of the tracer throughout brain gray matter. The shaded bar indicates activity concentration in units of μCi/cc. The bottom row shows parametric images of serotonin synthesis capacity at corresponding brain levels. The shaded bar indicates activity concentration in units of mL/g/min

Impaired Serotonin Synthesis in Autism

There are numerous lines of evidence suggesting a role of serotonin in autism (for review see [30]). In contrast to the developmental pattern described for non-autistic children above, serotonin synthesis capacity increases gradually between the ages of 2 and 15 years to values 1.5 times those of normal adult values and show no gender difference in autistic children [28]. These data suggest that the developmental process with a period of high brain serotonin synthesis capacity during childhood is disrupted in autistic children (Fig. 27.6).

In addition to a global difference in whole brain serotonin synthesis capacity in autistic children compared to age matched non-autistic children, Chugani et al. [31] have determined focal asymmetries of [^{11}C]AMT uptake in the frontal cortex, thalamus, and the contralateral dentate nucleus of the cerebellum in autistic boys

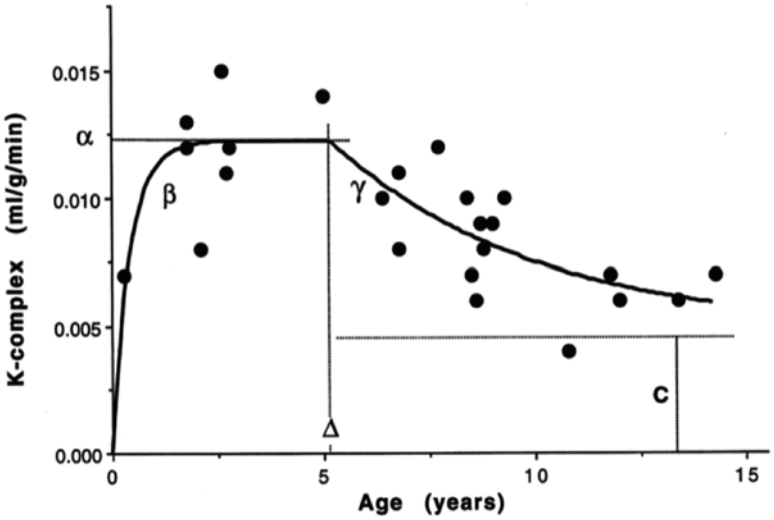

Fig. 27.5 Serotonin synthesis capacity in non-autistic children ($N = 24$). Global brain values for the K-complex (mL/g/min) for the siblings of autistic children and children with epilepsy were fitted according to a five-parameter developmental model. Parameter α (0.0122 ± 0.0008 [±SD]) represents the magnitude of the plateau phase, and parameter C (0.0047 ± 0.0064) describes adult values. The shape parameters β and γ represent the rate of increase ($\beta = 2.33 \pm 1.06$) and decline ($\gamma = 0.2066 \pm 0.3177$) of the developmental process with age. The parameter Δ (5.16 ± 2.03) indicates the age at which the plateau phase ends and the decline to adult values begins [28]

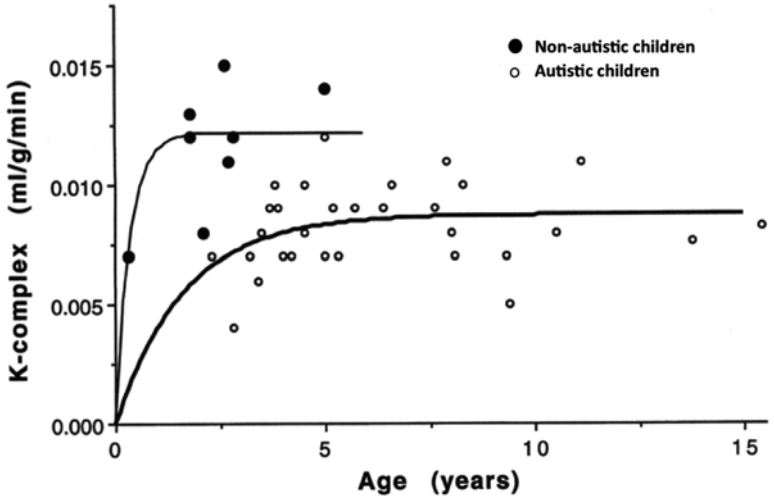

Fig. 27.6 Serotonin synthesis capacity in autistic children ($N = 30$, *open circles*) compared with non-autistic children for ages less than Δnon-autistic ($N = 8$, *closed circles*). Global brain values for the K-complex (mL/g/min) for the autistic children were fitted with an α (1-exp(−βt)) function, representing the increase to the plateau phase, because no decline phase was detected. The magnitude of the plateau α was significantly lower for the autistic than the non-autistic group ($p = 0.0036$) [28]

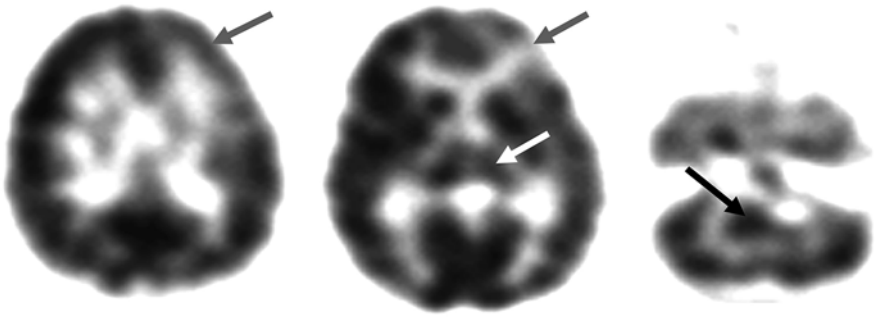

Fig. 27.7 Dentatothalamocortical asymmetries of AMT uptake in an autistic boy. Three axial cuts of an AMT-PET scan showing decreased left frontal cortical (*gray arrows*) and left thalamic (*white arrow*) AMT uptake along with increased AMT uptake in the contralateral right dentate nucleus

(Fig. 27.7), but not in non-autistic siblings. Specifically, decreased [^{11}C]AMT tracer accumulation was observed in the left frontal cortex and thalamus in 5 out of 7 boys studied, accompanied by elevated tracer accumulation in the right dentate nucleus. In the remaining 2 autistic boys, the pattern was mirrored, showing decreases in the right frontal cortex and thalamus and increases of [^{11}C]AMT tracer accumulation in the left dentate nucleus, but this reversed pattern was not related to handedness. These three brain regions showing abnormal serotonin synthesis capacity are synaptically connected via the dentatothalamocortical pathway. Purkinje cells in the cerebellar cortex (which are decreased in autistic brains) project to the dentate nucleus, afferents from which constitute the major output pathway of the cerebellum. Dentate neurons project to the ventral lateral nucleus of the contralateral thalamus in the dentatorubrothalamic tract, crossing to the contralateral side of the brain in the decussation of the superior cerebellar peduncle. In turn, neurons in the thalamic ventral lateral nucleus project to the frontal cortex, including prefrontal cortex, Broca's language area, motor cortex, and supplementary motor cortex.

Moreover, additional AMT-PET studies in a large group of autistic children (N = 117) clearly demonstrated a relationship between cortical asymmetry and two measures of hemispheric specialization, language function and handedness [32]. Using an objective measure of cortical asymmetry, 55% (64/117) of the children with autism showed abnormal cortical asymmetry. Among the autistic children whose [^{11}C]AMT-PET scans showed abnormal asymmetry, left sided decreases of [^{11}C] AMT tracer uptake were seen in 31 subjects, right sided decreases in 29, and bilateral decreases in 4 children. Cortical [^{11}C]AMT tracer uptake decreases were detected in frontal cortex in 90% (58/64) of asymmetric cases and included temporal lobe in 47% (30/64) and parietal lobe in 30% (19/64) of cases (Fig. 27.8). Cortical regions showing asymmetry included inferior prefrontal cortex (Broca's area [BA] 44, 45, and 47), inferior parietal lobule (BA40), superior temporal gyrus (BA22 and 21), and inferior occipital gyrus (BA18 and 19). These asymmetries in

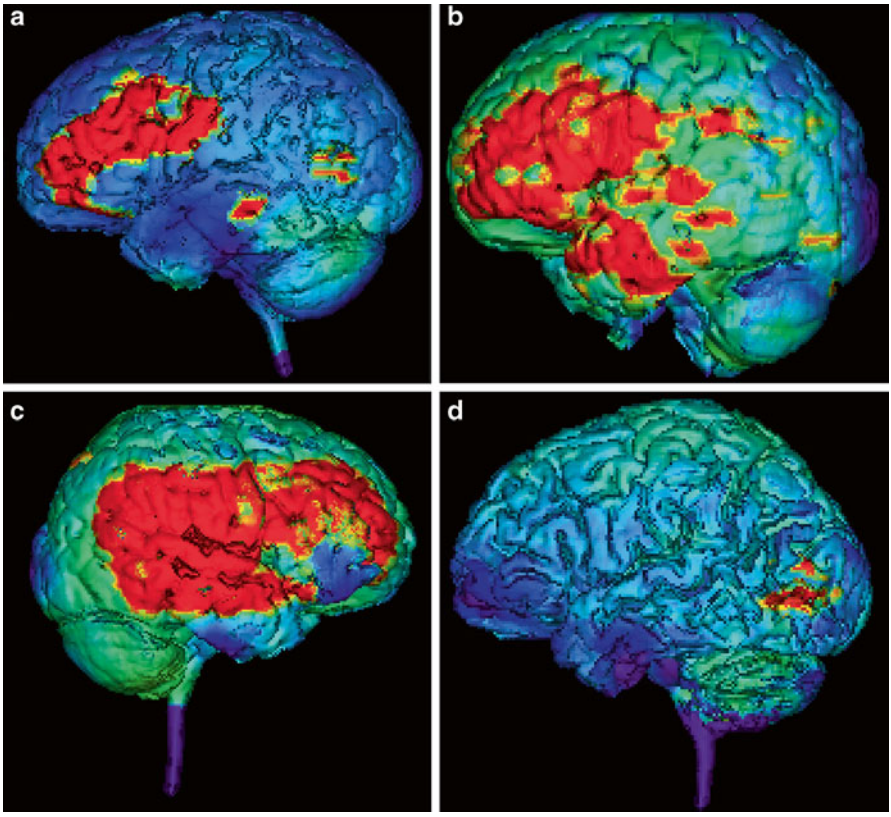

Fig. 27.8 Patterns of cortical serotonergic abnormalities in four autistic children. Representative 3D reconstructed MR image volumes with "marked" regions of significantly decreased cortical asymmetry (*red areas*) in [^{11}C]AMT tracer uptake. Serotonergic abnormalities in (**a**) the frontal cortex (*left hemisphere*), (**b**) frontal and temporal cortices (*left hemisphere*), (**c**) frontal, parietal, and temporal cortices (*right hemisphere*) have been the most common. Occipital cortical decreases (**d**) were less common and less extensive

serotonin synthesis capacity were related to differences in language function and handedness, indicating hemispheric specialization. Autistic children with decreased left cortical serotonin synthesis showed an increased incidence of severe language impairment compared to those with right side abnormalities and those without cortical asymmetry. Additionally, there was a trend for higher incidence of left and mixed handedness in the children with right cortical decreases in serotonin synthesis. These data suggest that the different patterns of cortical abnormality in serotonin synthesis capacity are related to dysfunction in hemispheric specialization in autistic children.

There are several possible explanations for the observed asymmetries of cortical serotonin synthesis capacity in autistic children. The first explanation is that at some

point in early development, cortical asymmetry of serotonin synthesis is normal and is part of the mechanism responsible for asymmetry of the cortical microcircuitry: the increased size and spacing of minicolumns on the left [33–35], the increased size of pyramidal cells on the left [36], the increased neuropil of the left, and the increased spacing between the functional macrocolumn units [37]. The presence of abnormal cortical asymmetry in serotonin synthesis observed in approximately one-half of the autistic children might then represent another manifestation of the abnormality in the developmental regulation of serotonin synthesis. Secondly, early damage to the dominant left hemisphere may lead to compensatory changes in the right hemisphere in order to support language based on the recognition that damage to the dominant left hemisphere leads to compensatory changes to the right hemisphere to support language [38–40]. Thus, the presence of smaller, more closely spaced minicolumns may be a type of "damage" requiring compensatory changes. This function might be shifted to the right as would happen with stroke, trauma, malformation, or hemispherectomy, in which the right hemisphere is a relatively suitable substrate for reorganization.

Alternating Hemiplegia of Childhood

Alternating hemiplegia of childhood is a rare neurologic disorder characterized by recurrent attacks of paralysis variably affecting the limbs. Ocular motor abnormalities, movement disorders, autonomic disturbances, and progressive mental impairment may also be seen. The onset of alternating hemiplegia of childhood is usually before the age of 18 months, and the duration of hemiplegic attacks lasts from minutes to days. Significant numbers of these children may also have seizures. Some observations have suggested involvement of the serotonergic system in alternating hemiplegia of childhood. For example, the clinical symptoms in alternating hemiplegia of childhood typically disappear during sleep, and it is well known that during transitions between wake and sleep there are many neurochemical changes involving serotonergic mechanisms in the brainstem. Moreover, alternating hemiplegia of childhood and migraine are both associated with recurrent and/or transient attacks, and there is abundant evidence implicating serotonin in the pathophysiology of migraine. Interictally, patients with alternating hemiplegia of childhood show bilaterally symmetric distribution of serotonin synthesis capacity, similar to a normal pattern obtained from a control group [41]. Increased serotonin synthesis capacity in various supratentorial regions of the brain is however observed during ictal and postictal periods, suggesting increased regional serotonergic activity associated with hemiplegic attacks in extended neural networks (Fig. 27.9).

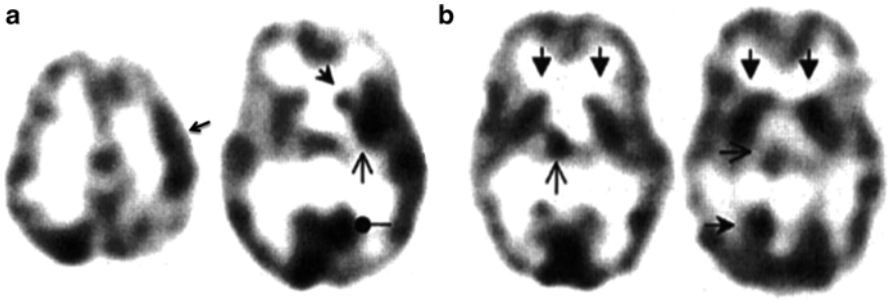

Fig. 27.9 Serotonin synthesis capacity in two representative children with alternating hemiplegia of childhood. The left side of images corresponds to the right side of the brain. (**a**) Ictal scans in a 4.5-year-old boy demonstrate increased serotonin synthesis capacity in the left frontoparietal (*arrow*) and lateral temporal cortex (*diamond arrow*), caudate nucleus (*stealth arrow*), putamen (*open arrow*), and hippocampal gyrus (*oval arrow*). (**b**) Ictal scan in a 14-year-old boy shows increased tracer uptake in the caudate nucleus and putamen (*arrows*) bilaterally, in the right thalamus (*open arrow*), and in the right hippocampal gyrus (*stealth arrow*) [41]

Increased Serotonin Synthesis Capacity in Migraine Patients

Although the etiology of migraine remains unknown, there is evidence that serotonin plays a role in its pathophysiology, and many of the pharmacological agents that are used to treat migraine interact with serotonin receptors. Moreover, drugs that release serotonin from tissue stores induce migraine attacks. Our findings indicating increased brain serotonin synthesis capacity in migraine patients are consistent with previous reports of systemic alteration of serotonin metabolism in patients without aura [28]. Our results also suggest that the mechanism of action of beta-adrenergic antagonists for migraine prophylaxis may involve regulation of serotonin synthesis.

Decreased Serotonin Synthesis Capacity in Tourette Syndrome

Tourette syndrome (TS) is a neuropsychiatric disorder characterized by motor and vocal tics and is commonly associated with neuropsychological deficits (e.g., visuomotor integration) and comorbid conditions such as obsessive-compulsive disorder (OCD) and attention-deficit hyperactivity disorder (ADHD). The pathophysiology of TS is postulated to involve a dysfunction of cortico-striatal-thalamic circuitry. Indeed, Behen et al. [42] demonstrated cortical and subcortical abnormalities of in vivo tryptophan metabolism in TS patients. Specifically, TS patients showed significantly decreased serotonin synthesis capacity in the bilateral dorsolateral prefrontal cortex (DLPF) and increased serotonin synthesis capacity in the bilateral

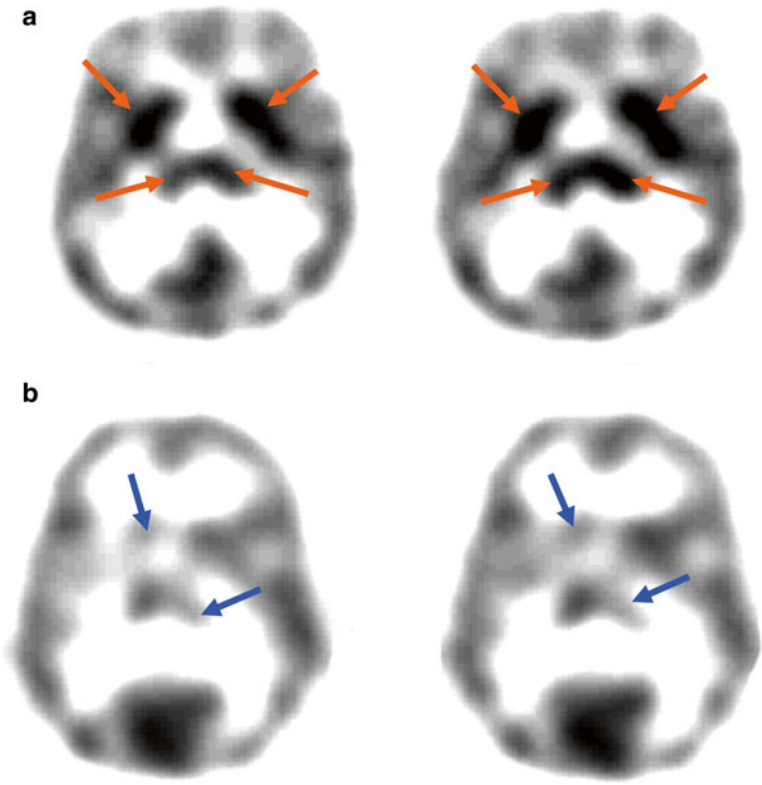

Fig. 27.10 Typical patterns of abnormal AMT uptake in Tourette syndrome: (**a**) Massive bilateral increase in the basal ganglia and thalamus (*orange arrows*). (**b**) Left>right uptake in the caudate nucleus (and basal ganglia more generally) and right>left in the thalamus (*blue arrows*), a pattern that was associated with ADHD in children with Tourette syndrome

thalamus and caudate (Fig. 27.10a). As a consequence, the serotonin synthesis capacity ratios between these subcortical and cortical regions were significantly higher in TS patients, suggesting abnormal tryptophan metabolism in the fronto-striato-thalamic circuit in TS. Finally, differences in regional [^{11}C]AMT uptake between behaviorally defined TS subgroups (ADHD, OCD) became evident. Analysis of [^{11}C]AMT tracer uptake pattern revealed decreased right DLPF for the ADHD group compared to the OCD group and opposite subcortical patterns for the ADHD (left > right basal ganglia (caudate and lentiform nuclei); right > left thalamus) and OCD (right > left basal ganglia; left > right thalamus) groups (Fig. 27.10b). These findings suggest a general impairment of cortical–subcortical serotonergic function in TS represented as an imbalance between direct and indirect striato-pallidal pathway tone that causes overactivity in neuronal circuits consistent with clinical symptoms of TS.

Summary and Conclusions

In summary, PET imaging using the [^{11}C]AMT tracer is a useful tool for quantitative examination of serotonergic mechanisms in the brain, although this method cannot be used to measure the absolute serotonin synthesis rate. Nevertheless, an index of serotonin synthesis rate can be derived (termed serotonin synthesis capacity) that allows quantitative assessment of abnormal developmental changes in serotonin synthesis rate in autistic children as well as detection of abnormal pattern of serotonin synthesis in neurodevelopmental disorders including Tourette syndrome and alternating hemiplegia. Finally, in certain pathologic states, increases in [^{11}C]AMT tracer uptake represent increased metabolism by means of the kynurenine pathway rather than increased serotonin synthesis.

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Chapter 28
Molecular Imaging of Tryptophan Metabolism in Tumors

Csaba Juhász and Sandeep Mittal

Abstract Molecular imaging of the kynurenine pathway can be achieved by positron emission tomography (PET) of radiolabeled tryptophan derivatives. While tryptophan is the substrate of protein synthesis, the PET tracer ^{11}C-alpha-methyl-L-tryptophan (AMT) is not incorporated into proteins; rather it is a substrate of indoleamine 2,3-dioxygenase (IDO), the initial and rate-limiting enzyme of the kynurenine pathway. Recent studies of AMT-PET have demonstrated high AMT uptake and accumulation in a variety of WHO grade II–IV gliomas, glioneuronal tumors, and meningiomas. Increased AMT uptake can also readily detect lung cancers and breast cancers. Tracer kinetic analysis of dynamic PET images can differentiate between tryptophan transport and metabolic rates, thus enhancing the clinical utility of AMT-PET. Studies of resected tumor specimens have shown high expression of LAT1, a key transporter of tryptophan, and also IDO and tryptophan 2,3-dioxygenase, in AMT-accumulating tumors. This chapter discusses the mechanisms of high AMT uptake on PET and provides a summary of potential clinical applications, including pretreatment tumor characterization, treatment planning, prognostication, as well as posttreatment tumor detection in various intra- and extracranial tumors.

Keywords Positron emission tomography • Molecular imaging • Amino acid radiotracer • ^{11}C-alpha-methyl-L-tryptophan • Gliomas • Lung cancer • Breast cancer • Indoleamine 2,3 dioxygenase

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List of Abbreviations

AHR	Aryl hydrocarbon receptor
AMT	^{11}C-alpha-methyl-L-tryptophan
DNET	Dysembryoplastic neuroepithelial tumor
FDOPA	^{18}F-fluoro-L-dihydroxy-phenylalanine
FEHTP	5-(2-^{18}F-fluoroethoxy)-L-tryptophan
FET	^{18}F-fluoroethyl-tyrosine
GTV	Gross tumor volume
HR-GTV	High-risk gross tumor volume
IDO	Indoleamine 2,3-dioxygenase
LAT1	L-type amino acid transporter 1
MET	L-[methyl-^{11}C]methionine
NSCLC	Non-small cell lung cancer
PET	Positron emission tomography
SUV	Standardized uptake value
TDO	Tryptophan 2,3-dioxygenase
TPH1	Tryptophan hydroxylase 1
VD	Volume of distribution

Introduction

Since tryptophan is the precursor of both serotonin as well as kynurenine and its downstream metabolites, positron emission tomography (PET) of radiolabeled tryptophan derivatives is a logical approach to image tryptophan metabolism via these pathways. Radiolabeled ^{11}C-DL-tryptophan indeed had been used in early PET studies to image tryptophan accumulation in cancer tissue [1, 2]. However, unmodified tryptophan cannot target the serotonin or kynurenine pathways specifically, because a significant portion of tryptophan may be incorporated into proteins. Instead, modified tryptophan molecules can be radiolabeled thus creating PET tracers that can target the serotonin and/or kynurenine pathways. For example, ^{11}C-labeled (20 min half life) 5-hydroxy-L-tryptophan was found to be useful to image and improve staging of neuroendocrine tumors that generate serotonin [3]. Recently, a longer half-life (110 min), ^{18}F-labeled derivative, 5-(2-^{18}F-fluoroethoxy)-L-tryptophan (FEHTP) has been tested for tumor imaging [4]. FEHTP was found to accumulate in both endocrine and non-endocrine tumors, and the authors provided preliminary data suggesting that this tracer may be useful to evaluate LAT1 transport activity (LAT1 is a member of transport system L for tryptophan and other large amino acids; see mechanism of uptake below). ^{18}F-labeled fluoropropyl tryptophan analogs have also been tested for tumor imaging [5], but none of these radiotracers are known to be metabolized via the serotonin or kynurenine pathways. Rather, these radioligands reflect tumoral tryptophan uptake and remain largely

unmetabolized, similar to other, more established amino acid PET tracers, such as O-(2-[^{18}F]fluroethyl)-L-tyrosine.

^{11}C-labeled alpha-methyl-L-tryptophan (AMT) was originally developed to measure brain serotonin synthesis [6–9]. After extensive validations in animal models and humans, AMT-PET has been used successfully to measure abnormalities of brain serotonin synthesis in a variety of neuropsychiatric disorders [10, 11]. Subsequently, AMT-PET was used for detecting epileptic foci, which can show increased AMT uptake even in the interictal state [12–15]. More recently, AMT was explored for PET imaging of human tumors due to its unique properties related to tryptophan metabolism via the kynurenine pathway, which is associated with tumoral immune resistance [16]. In the following section, we briefly summarize the putative mechanisms of tryptophan and AMT uptake in tumors followed by the summary of our experience with this radiotracer in molecular imaging of human brain tumors and extracranial cancers.

Mechanism of AMT Uptake and Trapping in Tumors

After intravenous injection of AMT, usually with a dose of 3.7 MBq/kg, AMT first has to cross the blood–tumor barrier in neuro-oncologic applications. If this barrier is intact, AMT can be transported to the tumor by various transport systems shared with other amino acids and their radiolabeled counterparts, such as L-[methyl-^{11}C] methionine [MET], ^{18}F-fluoroethyl-tyrosine [FET], and ^{18}F-fluoro-L-dihydroxyphenylalanine [FDOPA]. Among these transport systems, L-type amino acid transporter 1 (LAT1, a member of system L) plays a pivotal role in tumoral amino acid transport. LAT 1 can transport large neutral amino acids, and it is widely expressed in primary human cancers and cancer cell lines; it is also known to play an essential role in survival and growth of tumors [17, 18]. High LAT1 expression was found not only in gliomas but also in meningiomas and dysembryoplastic neuroepithelial tumors (DNETs) [19, 20]. Other transport systems (e.g., system A, xCT, glutamine, or the cationic amino acid transporters) have been also described and explored for tumor imaging [21–23]. For brain tumor imaging, system L remains the most suitable transport system because of its activity at the blood–brain barrier, which allows selected radiotracers (AMT, MET, or FET) to enter the tumor tissue even if this barrier is intact [24]. A recent study also demonstrated that radiolabeled amino acids with an alpha-methyl moiety, such as AMT, are more specific for tumor-derived LAT1 and, therefore, show more tumor-specific accumulation [25].

After transport to the tumor tissue from the bloodstream, different amino acid tracers have different metabolic fates; this can explain the different uptake kinetics and may lead to unique clinical usefulness of commonly utilized amino acid PET tracers (reviewed in [26]). For AMT, accumulation was first described in non-tumoral lesions such as in epileptic tubers [12]. Preliminary studies of resected epileptic tubers demonstrated high concentrations of quinolinic acid, a metabolite of tryptophan via the kynurenine pathway [27]. This finding suggested that increased

conversion and trapping of AMT in these lesions may be attributed, at least partly, to the upregulation of the inflammatory kynurenine pathway, which can be activated in such lesions [12, 27].

The interest in the kynurenine pathway in oncology was boosted by the seminal paper by Uyttenhove et al. in 2003, which demonstrated the high expression of indoleamine 2,3-dioxygenase (IDO, the initial and rate-limiting enzyme of the kynurenine pathway) in a number of malignancies [16]. The authors also showed that high IDO expression was associated with tumoral immune tolerance, which could be reversed by IDO enzyme inhibition with 1-methyl-tryptophan. Due to its poor substrate specificity [28, 29], IDO can convert not only tryptophan but also AMT to kynurenine metabolites. Thus, tumoral uptake and accumulation of AMT on PET may reflect IDO-derived conversion and trapping as a kynurenine metabolite, such as alpha-methyl-kynurenine. In tumor tissue, prolonged trapping of tryptophan (and AMT) metabolites may occur via the aryl hydrocarbon receptor (AHR, formerly known as dioxin receptor, a cell cycle regulator implicated in tumor progression), for which L-kynurenine has been identified as an endogenous ligand [30, 31]. Activation of AHR by kynurenine can lead to generation of FoxP3+ regulatory T cells [32], a mechanism through which tryptophan-derived kynurenine can promote an immune-suppressed tumoral microenvironment. Thus, in the presence of AHR, a prolonged increase of AMT-derived radioactivity in the tumor tissue may be an imaging marker of tumoral immune resistance. This ability of AMT-PET was supported by our studies showing high IDO protein expression in gliomas and glioneuronal tumors, detected by immunostaining in tumors demonstrating high AMT trapping on PET [33, 34]. In the first study of 15 patients with WHO grade I–IV gliomas and glioneuronal tumors, high IDO expression was found in tumor cells, as well as neuropil, endothelial cells, and perivascular tissue in some cases. Tumors with the most extensive IDO expression showed high AMT k_3 values, indicating high AMT metabolic rates in tumors with high IDO activity. In the second study of 11 temporal lobe DNETs associated with seizures, resected tumors showed moderate to strong IDO activity, with the strongest expression in tumor vessels. Interestingly, in two DNET patients with persistent seizures after complete tumor resection, increased AMT uptake extended to non-tumoral cortex (which was not surgically excised). This raised the possibility that high cortical AMT uptake in such cases may be associated with epileptogenicity, and affected cortex should be resected to optimize postsurgical seizure outcome [34].

Preliminary AMT-PET studies in WHO grade I meningiomas showed high tracer uptake, which was associated with strong IDO expression that could be blocked by a selective inhibitor of IDO1 but not IDO2 in meningioma cells lines [19]. In a subsequent study, differential protein expression of IDO1, IDO2, as well as tryptophan 2,3-dioxygenase (TDO) has been found in grade I-III meningiomas, with TDO showing the most robust expression [35]. TDO has also been found to contribute to tryptophan-to-kynurenine conversion in other brain tumors [30, 36]. Comparison with in vivo AMT kinetics in the meningioma study showed a strong correlation between TDO expression and tryptophan trapping rates; the latter also had a strong prognostic value for meningioma grade. These studies suggested that high AMT

uptake and accumulation on PET may be a useful imaging marker of IDO/TDO activity and related tumoral immune resistance in affected tumors. These results may have important therapeutic implications in clinical trials with emerging IDO inhibitors, also, potentially, with inhibitors of TDO. Clinical trials with IDO inhibitors are ongoing. For example, INCB024360 (Incyte Corp., Wilmington, DE) is a highly potent IDO1 inhibitor, which has progressed to a phase II clinical trial whose aim is to break tumoral immune resistance thus allowing chemotherapy to exert maximum effect [37]. In trials involving such enzyme inhibitors, molecular imaging with AMT may provide unique clinical information. First, high AMT accumulation may identify tumors with increased IDO/TDO activity, thus selecting tumors most amenable to enzyme inhibitor treatment. Second, AMT-PET may also be useful to monitor IDO activity noninvasively by showing changes in AMT trapping after administration of the enzyme inhibitor. The metabolic effects of novel IDO/TDO inhibitors can also be tested in animal tumor models before progressing to human trials. Therefore, a critical next step is to develop and validate tumor models with high baseline IDO and/or TDO expression and determine if they show in vivo tryptophan uptake similar to human tumors by using small animal micro-PET imaging with AMT as the radiotracer (Fig. 28.1). Translational research with such models will likely play a pivotal role in development and clinical introduction of novel IDO/TDO enzyme inhibitors as a new family of drugs to diminish tumoral immune tolerance.

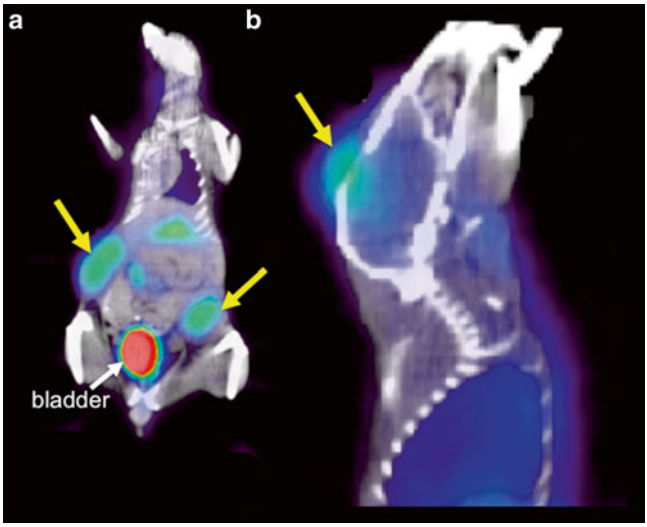

Fig. 28.1 AMT micro-PET/CT in human tumor xenografts implanted in immunodeficient mice. Human glioblastoma tissue growing in the flank bilaterally (**a**) and in the brain (expanding to extracranial tissue) (**b**) shows AMT accumulation (*arrows*). Maximum uptake values were reached 15–45 min after tracer injection, depending on tumor site, indicating prolonged tryptophan accumulation similar to gliomas in humans. AMT is excreted through the kidney to the bladder causing high bladder activity (*red area*)

Imaging Tryptophan Metabolism in Brain Tumors

The initial applications of AMT-PET for human tumor imaging involved various types of primary brain tumors [38]. AMT-PET was indeed found to be extremely sensitive to detect WHO grade II–IV gliomas (Fig. 28.2). These tumors almost invariably showed higher AMT uptake as compared to normal cortex irrespective of tumoral contrast enhancement on MRI [19, 34, 38, 39]. Using tracer kinetic analysis

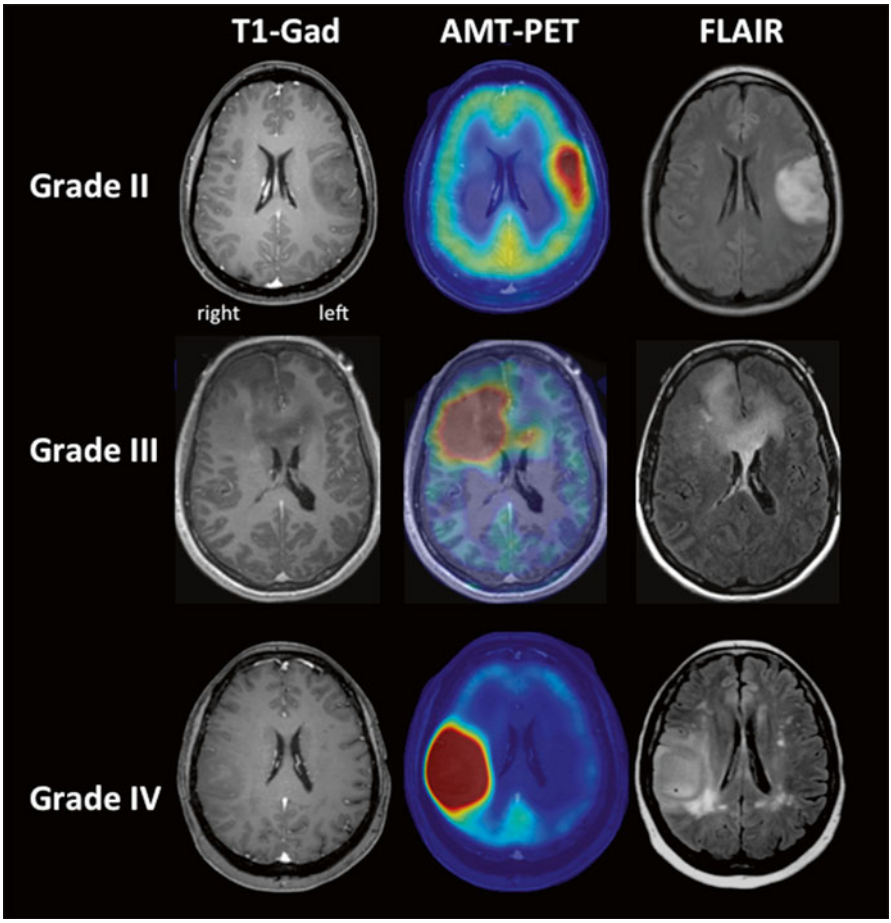

Fig. 28.2 AMT-PET and co-registered MR images in WHO grade II, III, and IV gliomas. All three tumors showed minimal contrast enhancement on gadolinium-enhanced T1-weighted MR images (T1-Gad). High AMT uptake, as compared to normal cortex, was seen in all three tumors, with the highest standardized uptake values (SUV) measured in the glioblastoma. Areas with high AMT uptake were smaller than FLAIR abnormalities, especially in the grade II and III gliomas, thus effectively differentiating active tumor tissue from vasogenic edema. Note that the patient with grade III glioma had bifrontal MRI abnormalities, which showed extensive high AMT uptake on the right side, while only a small AMT-intense nodule, indicating high-grade tumor, on the left

(such as the Patlak graphical approach with early imaging of the cardiac blood pool followed by venous blood sampling [38, 40]), the authors also explored the potential use of AMT uptake and different kinetic parameters for several clinical applications, as described below.

Differentiation of Newly Diagnosed Glioma Types

AMT-PET in untreated brain tumors was found to be useful to differentiate low-grade astrocytomas from oligodendrogliomas and DNETs [39]. These tumors cannot be distinguished reliably by conventional MR imaging and are difficult to differentiate with other amino acid PET tracers [41]. For AMT-PET, the best differentiating kinetic parameter was k_3, which characterizes metabolic trapping of AMT. Astrocytomas had higher k_3 tumor/cortex ratios than oligodendrogliomas (and DNETs) and could be differentiated with 100 % specificity and 75 % sensitivity when using a 1.30 cutoff threshold. Pretreatment differentiation of suspected low-grade glioma types may have high clinical value in timing of treatment (surgery) and for prognosis.

Differentiation of Malignant Gliomas from Brain Metastases

In a recent study of malignant brain tumors, we demonstrated that lesions with ring enhancement on MRI (common for both high-grade glioma and metastatic brain tumors) could be differentiated with up to 90 % accuracy based on tumor/cortex standardized uptake value (SUV) ratios (a measure that does not require blood sampling and kinetic analysis) [42]. This differentiation may have substantial clinical value in patients with newly diagnosed solitary ring-enhancing lesions.

Estimation of Glioma Proliferative Activity by PET

Among various AMT kinetic variables, AMT volume of distribution (VD, a measure of net tracer transport) proved to be an accurate predictor of the Ki-67 labeling index, a measure of tumor proliferative activity [43]. High VD values were associated with high proliferative activity measured in resected tumor tissue [43]. Tumor/cortex AMT VD ratios above 2.0 suggested at least 10 % Ki-67 labeling index, thus raising the suspicion for a high-grade glioma.

Detecting Glioma-Infiltrated Brain Tissue

Increased AMT uptake was observed not only in the glioma mass but also in tumor-infiltrated brain tissue (Fig. 28.3) [44]. In a study of 28 patients with WHO grade II–IV gliomas, AMT-PET-defined tumor volumes were greater than the MRI contrast-enhancing volume, but smaller than T2 abnormalities (which often delineated edema) [44]. AMT-positive regions were confirmed to be tumor-infiltrated brain by histopathology in a subgroup of cases. However, it remains unclear whether performance of AMT-PET is any different from that of other amino acid PET studies in this respect.

Detection of Tumor Recurrence and Prognosis After Initial Glioma Treatment

In a posttreatment study of 21 patients with MRI suspicion of glioma recurrence, the lesion to cortex ratio of AMT unidirectional uptake (K) differentiated recurrent gliomas from radiation necrosis with 90 % accuracy (Fig. 28.4) [45], which is similar to what has been reported in one study with FET [46] and higher than reported in MET-PET studies [47]. Furthermore, our recent data [48] also provided evidence

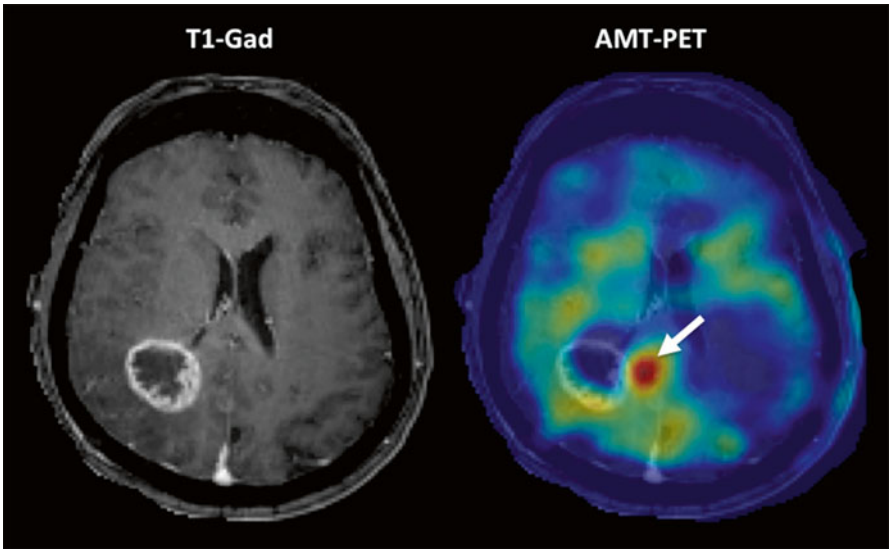

Fig. 28.3 Example of increased AMT uptake outside of the contrast-enhancing glioma mass in tumor-infiltrated brain tissue. This patient had a newly diagnosed ring-enhancing glioblastoma. Moderate AMT increase was seen in some of the contrast-enhancing tumor portions, while the highest AMT uptake (*arrow*) was adjacent, in the medial occipital area, suggesting that the most active glioma tissue was located outside the contrast-enhancing mass

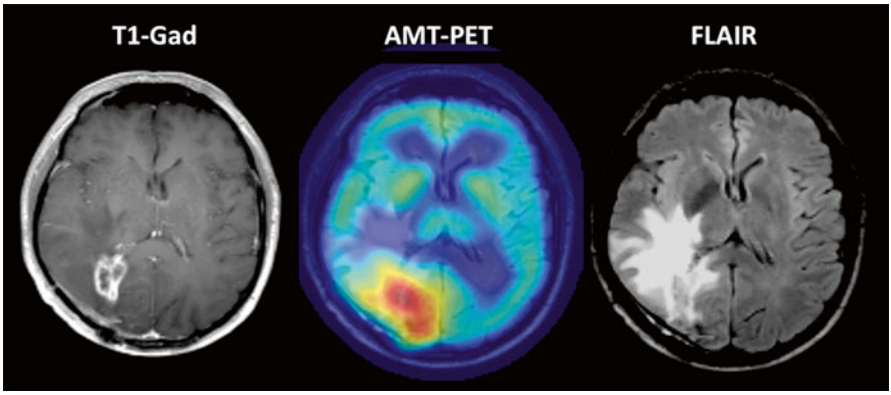

Fig. 28.4 Detection of posttreatment glioblastoma recurrence 5 months after initial treatment (resection followed by chemo-radiation). An area with enlarging contrast enhancement was suspicious for tumor recurrence on T1-weighted gadolinium enhanced (T1-Gad) images. AMT-PET showed high tryptophan uptake not only in the contrast-enhancing region but also in the adjacent medial area, which showed no enhancement and also no/minimal FLAIR abnormalities. The FLAIR image showed increased signal extending anteriorly, into low AMT uptake regions, consistent with edema. Kinetic analysis of dynamic PET images showed high AMT lesion/cortex K-ratio (1.62), consistent with glioma recurrence rather than pure radiation injury. This was supported by subsequent MRIs demonstrating progressive extension of the lesion with increasing mass effect

for the strong prognostic value of AMT uptake regarding survival in patients with previously treated high-grade gliomas. In this study of 39 patients with a suspected WHO grade II–IV recurrent glioma (after initial resective surgery and chemo-radiation), high AMT uptake in the suspicious lesion (based on conventional MRI) was associated with a short survival. Specifically, we found that patients with a lesional AMT SUV_{max} above 4.6 (Fig. 28.5) or lesion/cortex AMT K ratio above 1.63 had a median post-PET survival of 177 days, while those with values below these threshold values had a much longer median survival of 876 days. This strong predictive value of AMT uptake remained significant in multivariate analyses where other predictors, such as age, histologic grade, or presence of MRI contrast-enhancement, were also taken into account.

In recent studies, we have also found evidence for altered tryptophan metabolism in contralateral cortical and thalamic brain regions in patients with WHO grade III–IV gliomas after initial therapy [43, 49]. Interestingly, AMT uptake in the thalamus had a strong prognostic value for survival in these patients. Specifically, high thalamic SUVs and thalamic-cortical SUV ratios after initial treatment were associated with short survival, even when clinical predictors such as age, histologic grade, and time since radiation therapy were entered in the regression model. Altered tryptophan metabolism in non-tumoral brain may indicate abnormal serotonin synthesis, which may have implications in glioma-associated behavioral abnormalities such as depression.

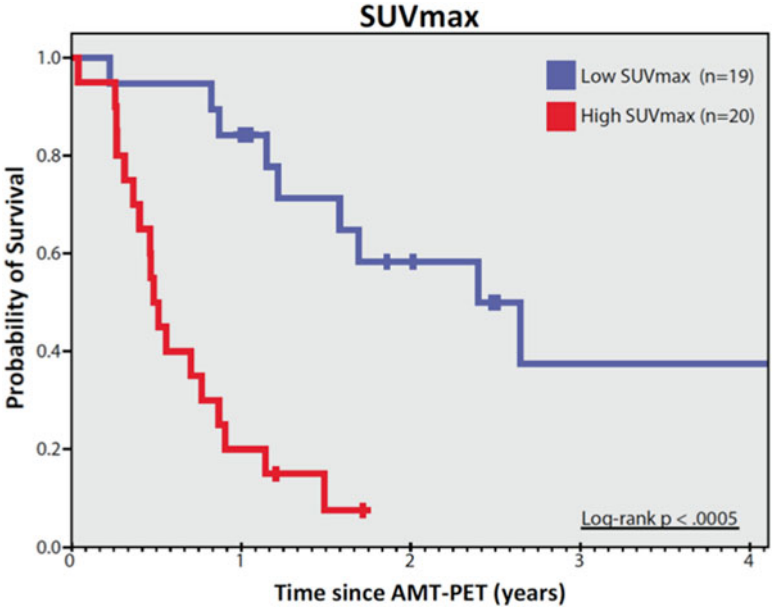

Fig. 28.5 Strong prognostic value of posttreatment AMT-PET for survival. Kaplan–Meier survival curves of patients with high vs. low values by maximum AMT standardized uptake values (SUVs, measured in the MRI-defined lesions suspicious for glioma recurrence) showed a major survival advantage of low AMT SUV. The optimal SUV cutoff threshold was 4.6 as determined by receiver operating characteristic (ROC) analysis based on 1-year survival

AMT-PET in Glioma Treatment Monitoring and Planning

In a longitudinal case study to observe the treatment progression on a patient with optic pathway glioma by AMT-PET, AMT uptake was high before and was considerably decreased after chemotherapy [50]. When the patient again developed vision difficulty, high AMT uptake reappeared while the MRI showed no obvious changes to identify the area of tumor for radiotherapy. This proof-of-concept case demonstrated that AMT-PET may be useful to monitor metabolic changes during chemotherapy of gliomas.

In a recent pilot study of 11 patients with high-grade glioma who underwent tumor resection followed by radiotherapy, we have also analyzed the potential value of AMT-PET for defining the optimal target volume for radiotherapy [51]. AMT-PET images were retrospectively fused to the simulation CT, and high-risk gross tumor volumes (HR-GTVs) generated by AMT-PET (GTV_{AMT}) were defined using a threshold previously established to distinguish tumor tissue from peritumoral edema (i.e., >36 % increase of AMT uptake, as compared to unaffected cortical uptake). HR-GTV for MRI was defined as the postoperative cavity plus any residual area of enhancement on post-contrast T1-weighted images. In patients with definitive radiographic progression, follow-up MRI demonstrating initial tumor

progression was fused with the pretreatment images and a progression volume was contoured. Although mean GTV_{MRI} (50.2 cm^3) and GTV_{AMT} (48.9 cm^3) were not significantly different, coverage of the initial recurrence volume by HR-GTV_{MRI} (mean, 52 %) was inferior to GTV_{AMT} (mean, 68 %; $p = 0.028$). There was also a trend towards better recurrence coverage with the combined $GTV_{MRI+AMT}$ than with GTV_{AMT} alone ($p = 0.068$). These data provided preliminary evidence that AMT-PET can achieve a superior coverage of the recurrence volume when compared to the GTV defined by clinical MRI [51].

Imaging Tryptophan Metabolism in Extracranial Cancers

After the initial results in AMT-PET imaging of human brain tumors, a logical extension of this imaging modality was the study of extracranial cancers, with a particular emphasis on common cancer types. The first oncologic application of AMT-PET in extracranial tumors involved lung cancer patients [52]. From a methodological point of view, an advantage of PET imaging of lung tumors (as opposed to brain tumors) is the easy noninvasive evaluation of arterial blood input function from the left ventricle of the heart; thus, kinetic analysis of blood radioactivity data did not require invasive blood sampling. From a mechanistic point of view, lung tumors along with several other cancer types are a good target for tryptophan imaging due to their overexpression of IDO (and, in some cases, also TDO) and the resulting increased tryptophan metabolism via the immunosuppressive kynurenine pathway [16, 53, 54]. In the first AMT-PET/CT study of 10 patients with lung tumors, we performed detailed kinetic analyses of AMT uptake in a total of 18 lung lesions [52]. All but three of these lesions were non-small cell lung cancers (NSCLC) on histopathology. Most of these NCSLCs showed a prolonged AMT accumulation (Fig. 28.6), with peak values reached about 20 min after tracer injection and maintained for up to 30 min afterwards. A few NSCLC lesions showed different tracer kinetics with a quick early accumulation followed by a washout phase after a few minutes. SUVs in NSCLCs ranged between 0.8 and 5.5. The reason and clinical significance of this metabolic heterogeneity across NSCLCs remains to be clarified. The two benign lung lesions (one hamartoma and one cyst) included in this study showed lower SUVs (values below 0.8). Further, kinetic analysis of the NSCLCs showed both high AMT net transport and unidirectional uptake rates, which could be consistent with the high activity of the LAT1 transporter and also high IDO activity, respectively, although we did not have tissue available to measure these proteins or enzyme activity in the specimens. Nevertheless, these initial findings showed the promise that AMT uptake and kinetics in these tumors could be useful molecular imaging markers of in vivo tryptophan transport and IDO activity.

The PET studies of tumoral tryptophan metabolism have been extended to breast cancers in a subsequent study [55]. In 9 women with stage II–IV breast cancer, we analyzed kinetic properties of AMT. In addition, tumoral expression of LAT1 and IDO was assessed by immunostaining. On dynamic PET imaging, tumoral AMT

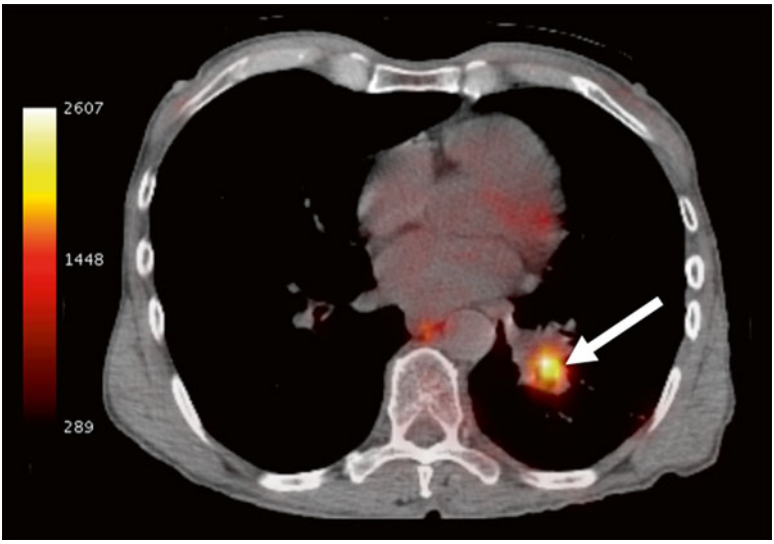

Fig. 28.6 AMT-PET/CT image showing increased AMT uptake (*arrow*) in a lung nodule, 20–50 min after tracer injection. Histology showed non-small cell lung cancer

uptake peaked at 5–20 min after tracer injection in 7 cases. In the remaining two patients, with advanced stage (IIIB and IV) invasive ductal breast carcinoma, a more prolonged AMT accumulation was observed, peaking at 20–30 min after tracer injection followed by a moderate decline (Fig. 28.7a–c). Similar to lung tumors, SUVs varied widely (2.6–9.8) and showed a strong positive correlation with VD values (i.e., net tracer transport) derived from kinetic analysis. An intriguing observation was that all six invasive ductal carcinomas showed higher SUVs (range: 4.75–9.8) than the three other rarer tumor types (two carcinosarcomas and one invasive micropapillary carcinoma: SUVs ranging 2.59–4.24). It remains to be determined if AMT uptake could differentiate various breast cancer types reliably. Immunohistochemistry studies of resected tumor tissues showed that tumor cells infiltrating the parenchyma surrounding the ducts were positive for both LAT1 and IDO; tumor cell staining intensity was moderate to strong in most specimens (Fig. 28.7d–f). In addition, we observed a similar strong immunostaining for a breast cancer brain metastasis included in the study. Increased IDO expression in these breast cancers was consistent with findings of a previous study [56]. In that study, expression of IDO did not correlate to histologic classification, or lymph nodes metastasis, but correlated to clinical stage. In our study, we have also found positive immunostaining for tryptophan hydroxylase 1 (TPH1), a tryptophan-metabolizing enzyme found to be associated with malignant phenotype in breast tumors [57]. Therefore, in breast cancers, a plausible (additional) mechanism of AMT uptake and trapping is via the serotonin pathway.

Accumulation of tumoral AMT followed by a washout in breast cancers may involve both unmetabolized AMT and metabolites, most likely in the form of

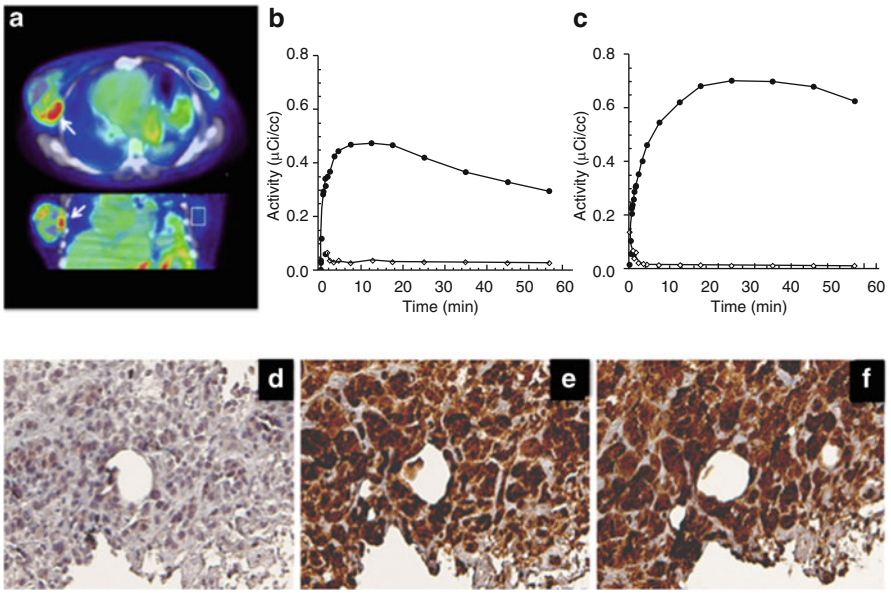

Fig. 28.7 AMT uptake and tracer kinetics in breast cancer co-expressing the LAT1 transporter and IDO. (**a**) AMT tracer accumulation in a breast tumor 5–20 min postinjection. (**b**) Corresponding time-activity curve (*black circles*) shows that tracer uptake peaked within the first 20 min followed by steady decline, in the same tumor. This time course was seen in most patients with breast cancer. (**c**) More prolonged accumulation with a peak at 20–30 min postinjection was seen in subjects with advanced stage breast cancer. The curves with open circles indicate the very low uptake in normal breast tissue (contralateral to the tumor). (**d–f**) Immunohistochemistry from resected breast cancer tissue: (**d**) No-primary control staining; (**e**) IDO showing strong staining intensity in close to 100 % of tumor cells; (**f**) LAT1 staining showing similar strong intensity in almost all cells in the same tumor (original magnification: 20×)

kynurenine metabolites and derivatives of the serotonin pathway. Increased kynurenine and decreased tryptophan levels were reported in the blood of breast cancer patients [58], suggesting kynurenine efflux and perhaps a tryptophan/kynurenine exchange from the site of tumoral tryptophan degradation. Such tryptophan influx/kynurenine efflux cycle could be executed by LAT1 [59, 60]. Therefore, high LAT1 expression observed in these tumors may result in increased tumoral tryptophan influx, but also, radioactive AMT products may be transported out of the tissue in the form of kynurenine metabolites by the same transport system. Overall, high LAT1 expression with simultaneous strong IDO activity would be consistent with a system that takes up available tryptophan rapidly, converts it to kynurenine metabolites, and then removes these in exchange to further tryptophan absorption. However, additional studies, involving animal tumor models, are needed to clarify the exact mechanisms of AMT uptake and metabolism in breast cancers as well as other tumors.

Conclusion

AMT-PET enhanced with tracer kinetic analysis is an excellent imaging tool to characterize tryptophan transport and metabolism in brain tumors and extracranial cancers. Tumoral accumulation of AMT, measured by PET, may be an imaging marker of tryptophan metabolism via the kynurenine pathway and could serve as an imaging tool to monitor therapeutic effects in clinical trials with emerging IDO (and TDO) enzyme inhibitors aiming at reversing tumoral immune tolerance. The clinical utility of this molecular imaging technique could gain widespread clinical applications in cancer imaging if similar radiotracers with longer half-life could be developed.

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Part VI
Therapeutic Approaches

Chapter 29
Inhibition of the Kynurenine Pathway of Tryptophan Metabolism

Raphaël Frédérick

Abstract This chapter summarizes the current state-of-the-art of inhibitors of the kynurenine pathway. After a brief presentation regarding the kynurenine pathway itself and the enzymes involved, the main series of inhibitors that were discovered are presented by targets. Among the enzymes investigated in the present study are the indoleamine 2,3-dioxygenases IDO1 and TDO, the kynurenine-aminotransferases (KATs), the kynureninase, the kynurenine 3-monooxygenase, and the 3-hydroxyanthranilic monooxygenase. All these enzymes are responsible for the formation of tryptophan metabolites, collectively called the kynurenines and thus constitute very interesting targets in various diseases.

Keywords Inhibitors • Medicinal chemistry • Kynurenine pathway • IDO • TDO • KAT • 3-HAO • Kynureninase • KMO

List of Abbreviations

1MT	1-Methyl-tryptophan
3-HAO	3-Hydroxyanthranilic monooxygenase
CNS	Central nervous system
HTS	High throughput screening
IDO1	Indoleamine 2,3-dioxygenase 1
KATs	Kynurenine aminotransferases
KMO	Kynurenine-3-monooxygenase (also called kynurenine 3-hydroxylase)
LE	Ligand efficiency
mM	Millimolar
MTH-Trp	*N*-methyl-thiohydantoine-tryptophane
NAD	Nicotinamide Adenine Dinucleotide

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nM	Nanomolar
PIM	4-Phenylimidazole
PLP	Pyridoxal-5′-phosphate
SAR	Structure–activity relationships
TDO	Tryptophan 2,3-dioxygenase
μM	Micromolar

Introduction

L-tryptophan is an essential amino acid. Besides its role in protein synthesis, L-tryptophan is metabolized, for less than 5 %, into serotonin via the so-called serotonin pathway [1] and, for more than 95 %, into kynurenine metabolites via the so-called kynurenine pathway [2] which involves a series of enzymatic reactions leading to important cofactors such as nicotinamide adenine dinucleotide (NAD^+) and other metabolites. Although this last degradation route has been known for decades, it is only during the last 15 years or so, that it has attracted attention for drug discovery, particularly in the field of immunomodulation [3] and central nervous system (CNS) diseases [4]. The aim of this chapter is to give the reader an overview of the inhibitors that were developed on the main target in the kynurenine pathway.

The Kynurenine Pathway: An Attractive Playground for the Development of Inhibitors

Indoleamine 2,3-dioxygenase (IDO1) and tryptophan 2,3-dioxygenase (TDO) both catalyze the first and rate-limiting step of L-tryptophan degradation along the kynurenine pathway (Fig. 29.1). IDO1 which is found in a number of tissues but not in the liver is a heme-containing enzyme converting L-tryptophan into *N*-formyl-L-Kynurenine. TDO, which shares less than 10 % sequence identity with IDO, is also a heme-containing enzyme but is mainly located in the liver. Recent studies have also reported its constitutive expression in neuronal cells. Once formed, *N*-formyl-L-Kynurenine is further degraded into L-Kynurenine by a formamidase. L-Kynurenine plays a central role in the kynurenine pathway. It is further degraded by three distinct pathways: the first is composed of kynurenine aminotransferases (KATs) which produce kynurenic acid, the second leads to 3-hydroxy-L-kynurenine via degradation by the kynurenine-3-monooxygenase (KMO; also called kynurenine 3-hydroxylase), and the third is responsible for the production of anthranilic acid by the action of a kynureninase. 3-Hydroxy-L-kynurenine can be further converted to xanthurenic acid by the action of KATs or to 3-hydroxy-anthranilic acid by degradation through a kynureninase. Nonspecific hydroxylation of anthranilic acid also leads to 3-hydroxyanthranilic acid. This later is then converted by a

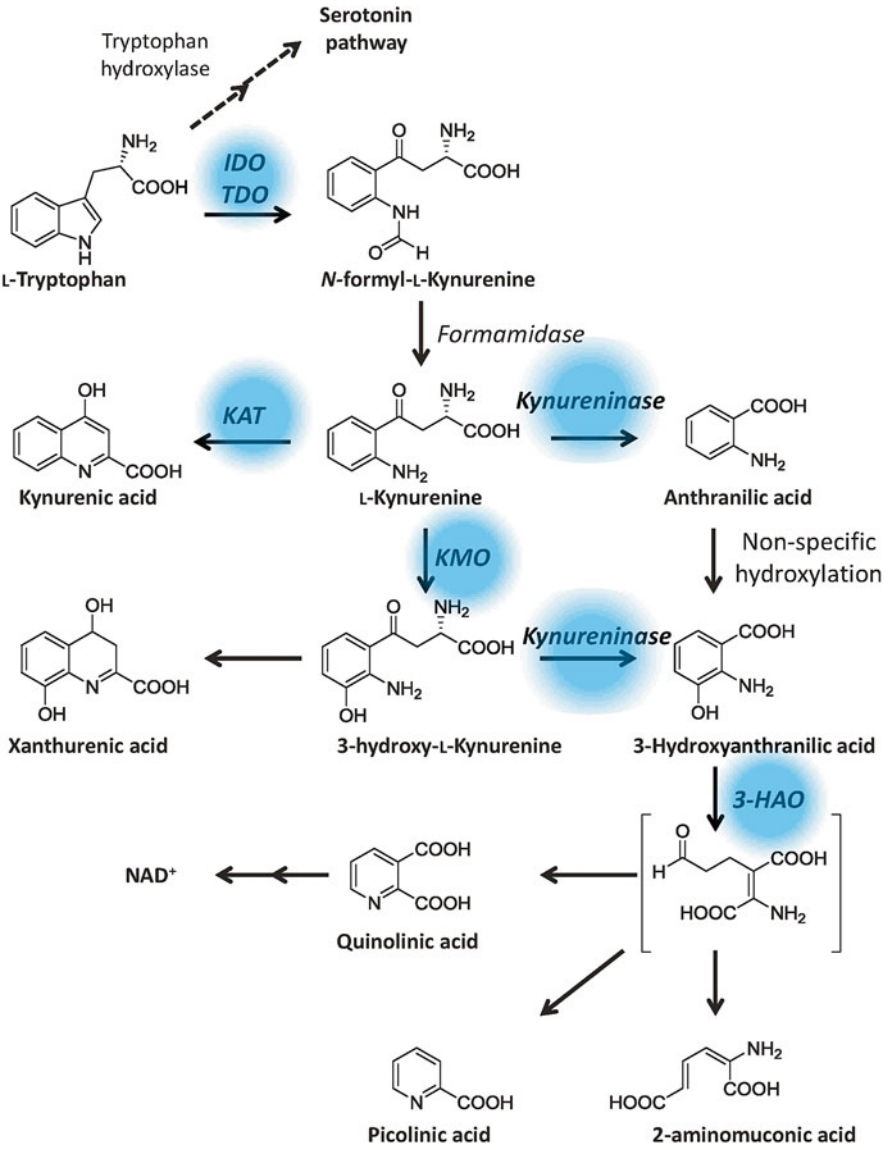

Fig. 29.1 Schematic diagram of the kynurenine metabolic pathway. The enzymes considered for therapeutic intervention are highlighted in *blue*

3-hydroxyanthranilate oxidase to 2-amino-3-carboxymuconate-semialdehyde, an unstable compound that is rapidly converted enzymatically into picolinic acid and chemically to quinolinic acid, the precursor of NAD^+ synthesis. The metabolites formed via this pathway are collectively called kynurenines. The pharmacology of these kynurenines being the main focus of the previous chapters of this book,

we will mainly focus on the inhibitors that were developed against the major enzymes of this pathway, namely IDO, TDO, KATs, kynureninase, KMO, and 3-hydroxyanthranilic monooxygenase.

Inhibitors of IDO and TDO

Among the various targets of the kynurenine pathway, IDO1 is probably the one that attracted much attention in the last years notably because of its involvement in the phenomenon of tumoral immune resistance. This has triggered considerable research efforts to discover novel potent IDO1 inhibitors [5]. Until recently, the best known IDO1 inhibitors (Fig. 29.2) displayed affinities in the micromolar (μM) range and comprised mainly indole-based compounds such as the usually recognized reference inhibitor 1-methyl-tryptophan (1MT) ($Ki = 34$ μM) [6]. Indeed, in 2008, Newlink Genetics entered the D-stereoisomer of 1MT (D-1-MT; NLG8189) into Phase I clinical trials although this stereoisomer was later shown not to be an IDO inhibitor, on the contrary to its L-stereoisomer (L-1MT) [7, 8]. In addition, an IDO paralogue, IDO2, has also been reported and implicated in tumor-driven immune suppression [9]; however, there has been an ongoing dispute over which enzyme is best inhibited by which 1MT enantiomer. Moreover, the physiological relevance of IDO2 remains unclear [10], and all the patented research so far has focused on IDO1.

Other indole-based derivatives also include β-carboline compounds such as the 3-butyl-b-carboline derivatives characterized with an IDO1 inhibition of 3.3 μM (Fig. 29.2) [11]. *N*-methyl-thiohydantoine-tryptophane (MTH-Trp, $Ki = 11.4$ μM) was also reported as an IDO1 inhibitor. New IDO inhibitors based on natural product brassinin were also published, the most potent compound having a *Ki* around 11 μM [12]. Finally, our group also reported a series of indol-2-yl ethanone IDO1 inhibitors following an in silico screening campaign with the 2-(pyridin-3-yl)-1-(6-(trifluoromethoxy)-1*H*-indol-2-yl)ethan-1-one being the best derivative in this series with an IC_{50} of 13 μM [13, 14].

In 2006, potent nanomolar inhibitors were isolated from marine invertebrate extracts, such as exiguamine A or Annulin B [15]. Based on these, Carr et al. identified tryptamine quinone as the core pharmacophore of exiguamine A and described a series of derivatives, the best showing a K_i of 0.2 μM [16]. Kumar et al. reported optimization of the naphthoquinone core of annulin B and designed derivatives, partly based on structural modeling, with IC_{50} values reaching 60 nM (Fig. 29.2) [17].

In 1989, 4-phenylimidazole (PIM) was identified as a modestly potent IDO1 inhibitor [18]. Despite the uncompetitive inhibition kinetics, the authors showed through spectroscopic studies that PIM binds into the active site of IDO1. The first crystal structure of human IDO1 complexed with this inhibitor was reported later and confirmed the binding of PIM into the active site pocket [19]. The two three-dimensional structures of IDO1, in complex with PIM and the cyanide ion, provided

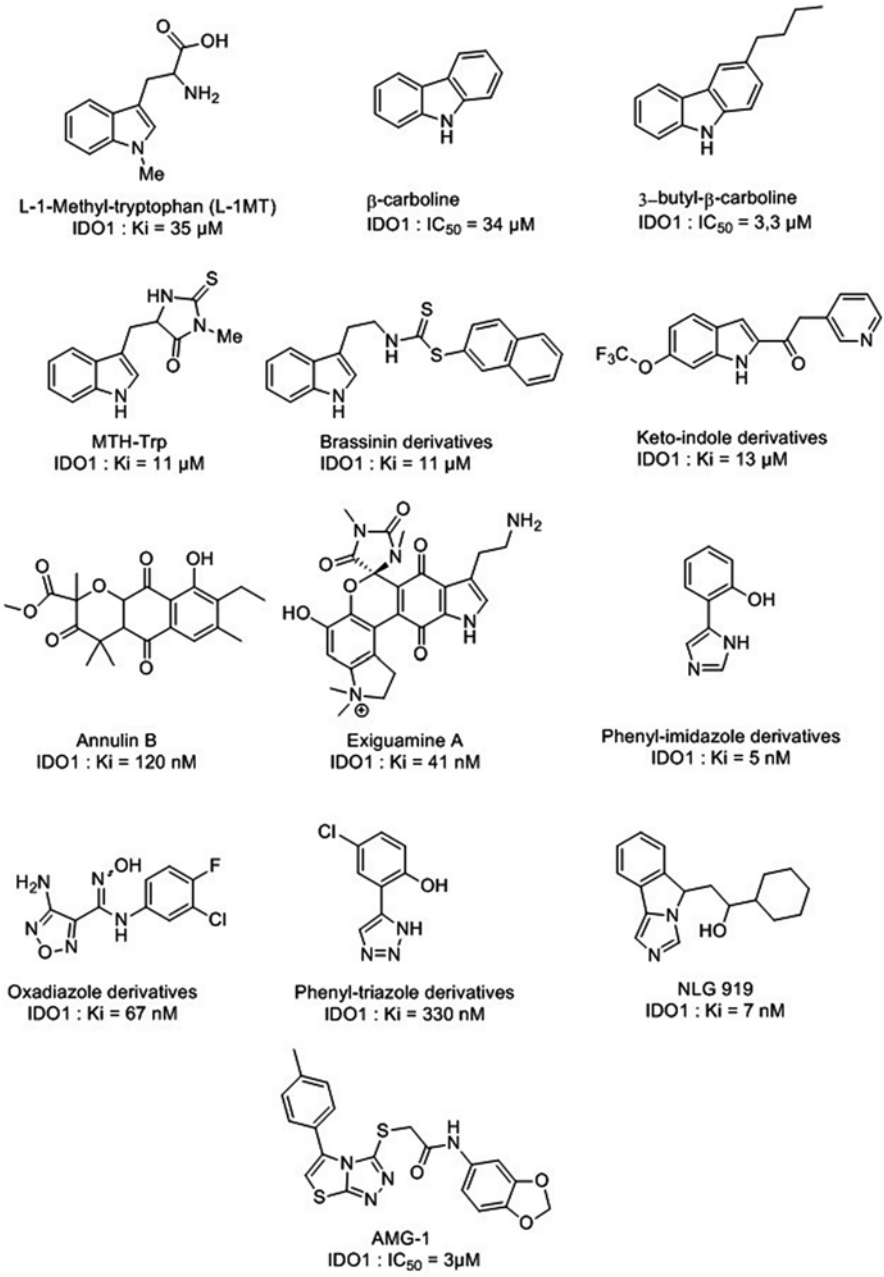

Fig. 29.2 Structure of reported IDO1 inhibitors. The inhibition values given may come from different experimental setup. They are not intended to be used for comparison between compounds but instead to give the reader an order of magnitude of the inhibitory potency

important data for the structure-based drug design of novel IDO1 inhibitors. Based on the co-crystal structure between PIM and IDO1, Newlink Genetics developed new IDO1 inhibitors based on the phenylimidazole structure [20]. The study notably revealed that introduction of a hydroxyl group in the 2-position or a thiol in the 2-, 3-, or 4-positions of the PIM structure improves the IDO1 inhibitory potency compared to the parent PIM. Later on, Newlink Genetics reported a patent application on IDO inhibitors based on a fused phenylimidazole scaffold from which the NLG919 compound (Fig. 29.2) being characterized with an *Ki* of 7 nM on IDO1 was selected to enter Phase I clinical trial (ClinicalTrials.gov Identifier: NCT02048709) [21].

In 2009, Incyte Corp. reported IDO1 inhibitors based on 4-amino-1,2,5-oxadiazole-3-carboximidamide lead structure obtained following a high throughput screening (HTS) of Incyte's corporate collection [22]. Direct binding of this compound to the IDO1 active site was confirmed by absorption spectroscopy and its good biological and physicochemical properties, that is, a reversible competitive IDO1 inhibition kinetic, some activity in a cellular assay, a good selectivity versus TDO, a low molecular weight, and a good ligand efficiency (LE), prompted for a detailed structure–activity study (SAR). The study revealed that meta-substituents were preferred on the phenyl ring and that the di-substitution in the 3- and 4-positions was also very well tolerated. Indeed compounds possessing a 3-Cl, 4-F and a 3-Br, 4-F di-substitution, respectively, were among the most potent IDO1 inhibitors in this series.

One of the compounds developed by the Incyte group (INCB24360), whose structure is undisclosed, was chosen to perform a dose escalation Phase I study to determine if it was safe, well-tolerated, and effective in patients with advanced malignancies (ClinicalTrials.gov Identifier: NCT02178722). The study is still ongoing and a Phase II study is ongoing to evaluate INCB24360 versus tamoxifen in biochemical recurrent only ovarian cancer patients following complete remission with first-line chemotherapy.

Finally, in 2011 AMG1 possessing a thiazolotriazole core and characterized with an IDO1 inhibition in the low micromolar range was discovered [23]. Although not being extremely potent, this compound was efficiently optimized to afford nanomolar compounds. One of these compounds as well as AMG1 have been recently co-crystallized with IDO thus affording additional structural information on IDO1 inhibition [24].

As noted earlier, a structurally different enzyme, but catalyzing the same biochemical reaction of Trp degradation, tryptophan 2,3-dioxygenase (TDO), has recently also been involved in the phenomenon of cancer immune escape [25, 26, 10]. Recently, starting from 680C91, a lead compound described in the literature, our group was involved in the development of new TDO inhibitors, with the best example (Fig. 29.3, LM10) retaining the same activity level but displaying significantly improved physicochemical and pharmacokinetic properties [27]. The most promising candidate LM10 was shown to reverse tumoral immune resistance in murine models. To date this series of compounds constitutes the only derivatives selectively inhibiting TDO and that are active in vivo.

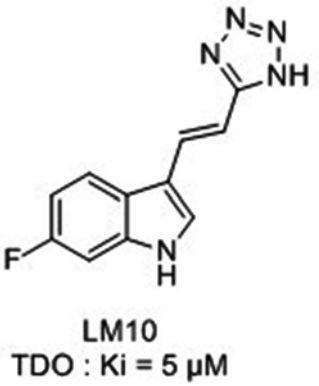

Fig. 29.3 Structure of LM10, a potent, bioavailable, and selective TDO inhibitor

Inhibitors of KATs

Four different subtypes of KATs (KAT1 to KAT4) have been identified, of which only KAT1 and KAT2 are thought to have a predominant role in humans. Indeed KAT2, a pyridoxal-5′-phosphate (PLP) dependent enzyme accounts for the majority of kynurenic acid in the rat and human brain and as such represents a key transformation in the kynurenine pathway [28].

The development of KAT inhibitors appears somehow limited in the literature with only few compounds reported to date. KAT1 selective compounds (Fig. 29.4a) are all indole-based derivatives with L-tryptophan [29], 3-indolepropionic acid [30], and the indole 3-lactic acid [30] as the main representative compounds. Apart from L-tryptophan that is characterized by an IC_{50} below 20 µM on KAT1, the other derivatives are only poorly active with IC_{50}'s in the high micromolar range. Regarding KAT2 selective derivatives (Fig. 29.4b), the compound (S)-4-ethylsulfonylbenzoylalanine (S-ESBA), developed in 2006 by the group of Pellicciari, was among the first reported KAT2 inhibitors with an IC_{50} of 6.1 µM and no inhibition versus KAT1 [31]. Later, the same group published an optimization of this series with compounds characterized by a 10 to 20-fold improvement in potency [32]. BFF-122 is another micromolar KAT2 inhibitor possessing a 2,3-dihydro-7H-[1, 4]oxazino[2,3,4-ij]quinolin-7-one core structure. Finally, recently the (S)-3-amino-1-hydroxy-3,4-dihydroquinolin-2(1H)-one (PF-04859989) which was shown to bind KAT2 irreversibly and tightly (IC_{50} = 23 nM) was discovered [33].

Inhibitors of Kynureninase

As it can be seen from Fig. 29.1, kynureninase catalyzes the degradation of both L-kynurenine and 3-hydroxy-L-kynurenine, respectively, into anthranilic and 3-hydroxy-anthranilic acids. A number of inhibitors have been developed that mimic the transition state for the kynureninase catalyzed reaction, including (4S)- and (4R)-dihydro-L-kynurenine and a series of S-aryl-L-cysteine S,S-dioxides (Fig. 29.5) [34].

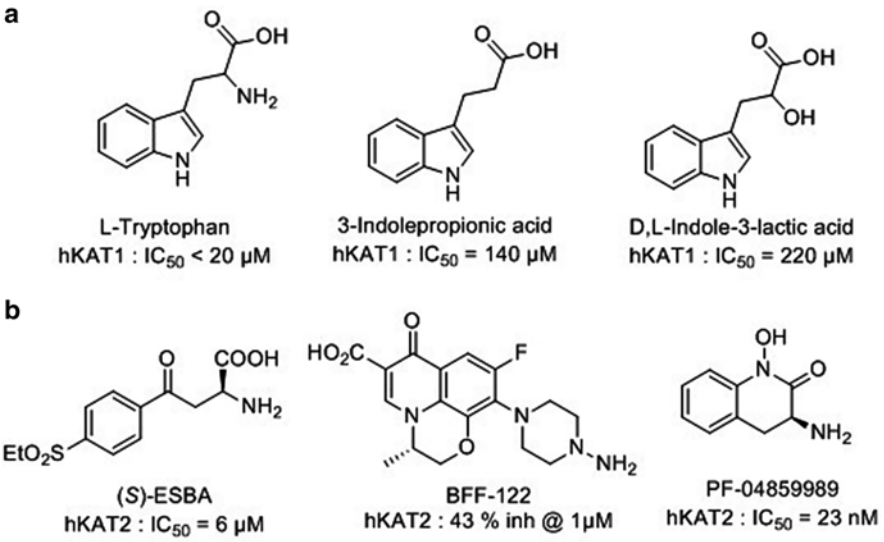

Fig. 29.4 Structure of reported (**a**) selective KAT1 and (**b**) selective KAT2 inhibitors. The inhibition values given may come from different experimental setup. They are not intended to be used for comparison between compounds but instead to give the reader an order of magnitude of the inhibitory potency

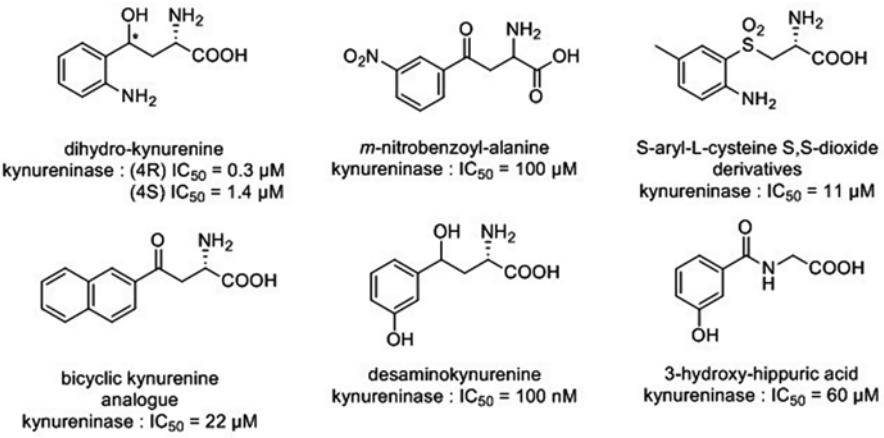

Fig. 29.5 Structure of reported kynureninase inhibitors. The inhibition values given may come from different experimental setup. They are not intended to be used for comparison between compounds but instead to give the reader an order of magnitude of the inhibitory potency

These compounds generally display kynureninase inhibition in the micromolar range. In 1994, the group of Pellicciari has shown that *m*-nitrobenzoyl-alanine was a moderately potent kynureninase inhibitor [35]. Indeed this compound was shown in the same study to be a far more potent inhibitor of the kynurenine-3-monooxygease (see below).

A series of naphtyl analogues of kynurenine with inhibition potencies in the low micromolar range were subsequently reported [36]. Further enhancement of inhibition was achieved via the synthesis of desamino-dihydrokynurenine compounds [37]. One of the best compounds in this series displays an inhibition of human kynureninase of 100 nM.

More recently, 3-hydroxy-hippuric acid was used in co-crystallization experiments and allowed determining the 3D-structure of kynureninase by X-ray diffraction [38]. The compound revealed to be only modestly potent on kynureninase with an inhibition potency around 60 μM.

Inhibitors of KMO

KMO catalyzes the hydroxylation of L-kynurenine at the third position. It is thus located at a critical step of the kynurenine pathway. A number of groups have thus searched for inhibitors of this enzyme, notably in the field of neurodegenerative diseases.

One of the very first KMO inhibitors was the nicotinylalanine derivative (Fig. 29.6) [39]. This compound is characterized with a modest KMO inhibition of 900 μM. Subsequent trials to optimize this compound led to the discovery of the

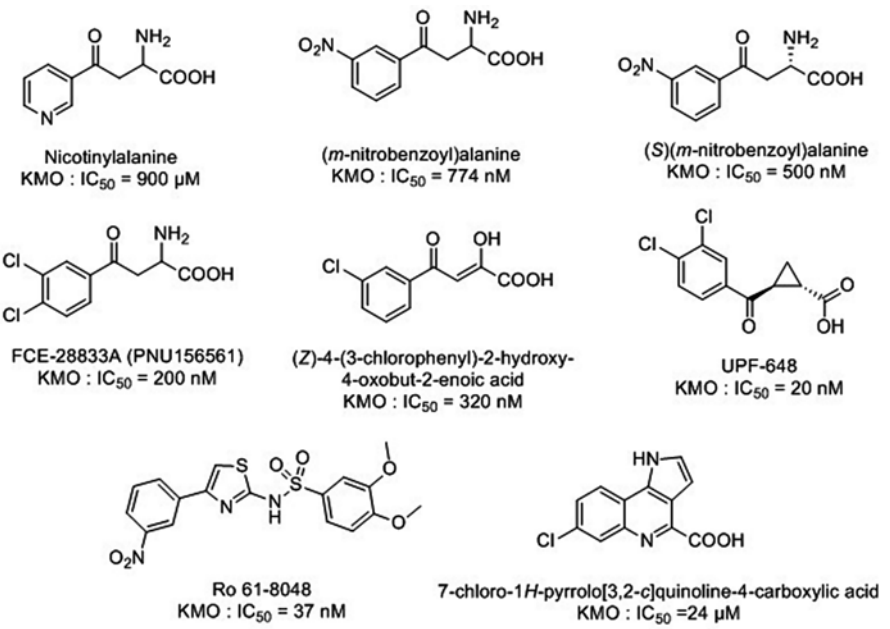

Fig. 29.6 Structure of reported KMO inhibitors. The inhibition values given may come from different experimental setup. They are not intended to be used for comparison between compounds but instead to give the reader an order of magnitude of the inhibitory potency

m-nitrobenzoyl-alanine compound that is endowed with an KMO inhibitory potency of 774 nM [40]. Separation of both enantiomers revealed that the S-(-)-isomer, possessing an KMO inhibition of 500nM, was responsible for the activity of this derivative [41]. Approximately at the same time, the compound FCE-28833A was discovered, also called PNU156561, where the *m*-nitrophenyl was replaced by an 3,4-dichlorophenyl group [42, 43]. This derivative is even more potent, being characterized with an IC_{50} of 200 nM. Subsequent structure–activity relationships in this series revealed that the carboxylic acid was essential for activity whereas the amino moiety could be replaced. This led to compounds such as the (Z)-4-(3-chlorophenyl)-2-hydroxy-4-oxobut-2-enoic acid possessing submicromolar inhibitory potency [42].

Further studies demonstrated that conformational rigification of the FCE-28833A derivative, notably by introduction of a cyclopropane moiety, led to an improvement of the inhibitory potency, the compound UPF-648 being one of the most potent KMO inhibitor yet reported with an IC_{50} of 20 nM [44]. Indeed this compound became very popular in industry and in academia and served as a valuable tool to detail the exact role of KMO in vivo. Other rigidifications of FCE-28833A led to the quinoline-based inhibitor with the 7-chloro-1H-pyrrolo[3,2-c] quinoline-4-carboxylic acid being the more potent ($IC_{50} = 24$ nM) [45].

Finally, Hoffmann-La Roche reported the discovery of new KMO inhibitors based on the *N*-(4-phenylthiazol-2-yl)benzenesulfonamide template [43]. These compounds were discovered based on a sulphonamide screening hit and the *m*-nitrobenzoyl-alanine derivatives that served as lead structures. Their study led to the discovery of the di-methoxy derivative (Ro 61-8048) that is characterized with an KMO inhibition of 37 nM. Interestingly, these compounds were not only potent in vitro but were also shown to inhibit KMO after oral administration, with ED_{50}'s in the range of 3–5 μmol/kg in gerbil brain. The compound Ro 61-8048 increases kynurenic acid concentration in extracellular hippocampal fluid of rats and appears to be more potent than FCE 28833.

Inhibitors of 3-HAO

3-Hydroxyanthranilic acid oxygenase (3-HAO) catalyzes the last step of the kynurenine pathway leading to kynurenines. The availability of 3-HAO inhibitors remains limited with only halogenated substrate-based derivatives (anthranilic acid derivative). Representative compounds include the 4-chloro- [46], the 4,5-dichloro-, and the 4,5-dibromo-3-hydroxyanthranilic acids that are endowed with 3-HAO inhibitory activity of 6, 0.3, and 0.3 nM, respectively (Fig. 29.7) [47]. More recently, the discovery of new 3-HAO inhibitors based on the 2-aminonicotinic acid 1-oxide template was reported [48]. These compounds, illustrated by the 2-amino-3-carboxy-6-methylpyridine 1-oxide, remain weakly potent on 3-HAO (IC_{50} of the best derivative in the low micromolar range), but seem to have the advantage of being more chemically stable compared to the parent hydroxyanthranilic compounds.

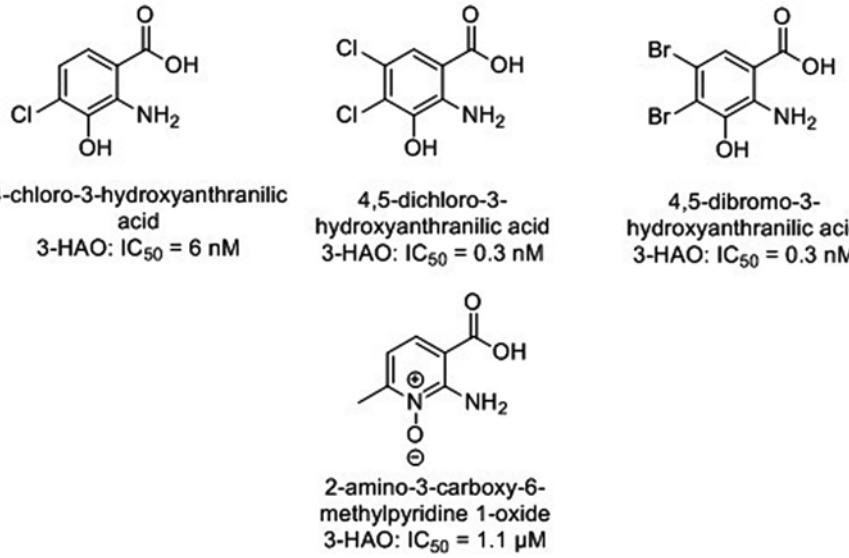

Fig. 29.7 Structure of reported 3-HAO inhibitors. The inhibition values given may come from different experimental setup. They are not intended to be used for comparison between compounds but instead to give the reader an order of magnitude of the inhibitory potency

Concluding Remarks

The kynurenine pathway of tryptophan metabolism is a very attractive area of research for drug discovery. In fact, kynurenines are involved in numerous physiological and pathological conditions so that the discovery of pharmacological tools targeting the enzymes contributing to their synthesis is the focus of intense researches. Recently, the discovery of new IDO1 and TDO inhibitors was for instance boost by the highlight of the implication of these enzymes in the phenomenon of tumoral immune resistance. As a result, some compounds targeting selectively IDO1 entered clinical trials. The discovery of small molecule inhibitors of the downstream enzymes of the kynurenine pathway has been mainly achieved in the context of CNS diseases. KATs and KMO have been the focus of most of the research efforts in this field with numerous compounds reported in the literature.

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Chapter 30
Clinical Trials Targeting the Kynurenine Pathway

Melanie Sheen and Hatem Soliman

Abstract The kynurenine pathway of tryptophan metabolism and it's first and rate-limiting enzymes, indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), play a key role in many cancers. Upregulation of IDO has been noted in several tumor types and enables tumors to evade the immune system. Overexpression of IDO appears to directly correlate with poor prognosis. In this chapter, we describe the experience using various IDO inhibitors (both clinically-approved and investigational drugs) for a variety of malignancies. The ongoing clinical trials of IDO inhibitors indicate that these drugs are well tolerated. Nonetheless, results using monotherapy have been somewhat limited. As such, future clinical trials using combination therapies and introduction of selective TDO inhibitors as well as identification of biomarkers are likely to improve outcomes and help with selection of patients.

Keywords Kynurenine pathway • Indoleamine 2,3-dioxygenase • Tryptophan 2,3-dioxygenase • Immune escape • Clinical trials • IDO inhibitors • TDO inhibitors

List of Abbreviations

ASCO	American Society for Clinical Oncology
D-1MT	Dextro-1-methyl-tryptophan
EGCG	Epigallocatechin-3-gallate
IDO	Indoleamine 2,3-dioxygenase
JAK-STAT1-IFNg	Janus-activated kinase-Signal Transducer and Activator of Transcription 1-interferon gamma

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L-1MT	Levo-1-methyl-tryptophan
mTOR	Mammalian target of rapamycin
TDO	Tryptophan 2,3 dioxygenase
TILs	Tumor infiltrating lymphocytes
Tregs	Regulatory T cells

Introduction

The process of tryptophan degradation in the intracellular environment first occurs by the enzymatic activity of the oxidoreductase enzymes tryptophan 2,3 dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO). In certain tissue types, such as dendritic cells and various malignant cells, depletion of tryptophan by IDO signals effector T cells to stop at G1, halting proliferation [1]. An increase in regulatory T cells (Tregs) is seen in many malignancies, and animal models have shown that by eliminating Tregs, the immune response is stimulated [2, 3]. Upregulation of IDO activity in antigen presenting cells results in conversion of naïve T cells into Tregs, leading to systemic anergy in the host [1, 4]. Many different tumor types show an increase in IDO activity on immunohistochemistry. An increase in IDO activity and a decreased concentration of tryptophan correlate with a poor prognosis in glioblastoma multiforme, pancreatic adenocarcinoma, melanoma, ovarian, and prostate cancer [2, 5–8]. Specifically, tumor draining lymph node sampling in pancreatic cancer showed increased IDO expression in all lymph nodes that were positive for metastatic disease, demonstrating that immune evasion by cancer cells allows for metastatic spread [5]. Inhibitors of IDO have been shown to be synergistic with chemotherapies in immunocompetent MMTV-neu breast mouse tumor models [9]. More recent data suggests that IDO activity can serve as a regulator of responses to checkpoint inhibitors such as CTLA-4 antibodies [8]. This data and other studies form the basis of studying IDO inhibitors in the treatment of various cancers.

Approved Drugs with IDO Inhibitor Activity

There are examples of FDA approved drugs for other indications that subsequently have been found to exhibit IDO inhibitor properties. In 2011, Balachandran et al. published the inhibitory properties of imatinib in mouse models of gastrointestinal stromal tumors [10]. The group found that imatinib not only inhibited the c-KIT pathway but also inhibited tumor expression of indoleamine 2,3-dioxygenase. Another example is the antiviral medication acyclovir. Muller et al. demonstrated that acyclovir inhibits indolamine-2,3-deoxygenase by suppressing free radical superoxide anion, which is a cofactor of IDO in the redox reaction that catabolizes tryptophan [11–13]. Acyclovir has been shown in vitro to suppress circulating Tregs in rat brains and glioblastoma tissue, but this preclinical data has yet to be validated in a clinical trial [14].

Certain herbal or alternative medicines have been shown to inhibit IDO activity which may in part contribute to their anti-proliferative effects in cancer. Curcumin is commonly used as an anti-inflammatory agent and is being studied in clinical trials for the treatment of certain malignancies such as colon cancer. Jeong et al. published data showing that curcumin is able to block induction of IDO in bone marrow derived dendritic cells through inhibition of the Janus-activated kinase-Signal Transducer and Activator of Transcription 1-interferon gamma (JAK-STAT1-IFNg) pathway [15]. The major constituent of green tea, epigallocatechin-3-gallate (EGCG), can also inhibit IDO induction similarly through the blockade of JAK-STAT1-IFNg signaling [16].

Ebselen is a powerful antioxidant currently under investigation for the prevention of cerebral vascular events and cisplatin induced hearing loss. Much like acyclovir, ebselen reduces hydrogen peroxide, eliminating free radicals thereby interfering with the redox activity of IDO [17]. Preclinical data also shows that ebselen reacts with cysteine residues of IDO. This reaction inhibits IDO by increasing nonproductive L-tryptophan binding, reducing overall tryptophan degradation [18]. Currently, ebselen is not specifically being clinically tested for its IDO properties but does show promise in other areas.

Tryptophan 2,3-dioxygenase, TDO, a hepatic enzyme in tryptophan metabolism, has also been implicated in immune evasion of tumor cells. Similar to IDO, this enzyme works by degrading tryptophan to kynurenine. However, TDO differs from IDO in its more constitutive expression pattern in the liver and its specificity for binding L-tryptophan [19]. TDO inihibitors have been used preclinically in combination with serotonin in neurologic and behavioral research in mice, but TDO alone has only recently been introduced in cancer immunotherapy [20, 21]. This is in part due to data suggesting that TDO can be pathologically overexpressed within certain tumors and may represent another target in the kynurenine pathway. While specific TDO compounds have yet to be tested in patients, IDO inhibitors with TDO inhibitory activity are being investigated.

Investigational IDO Inhibitors in Clinical Trials

1-Methyl-D-tryptophan (D-1MT)

1-Methyl-tryptophan is the first pharmaceutical initially studied to inhibit IDO. IDO inhibition works through two isomers, levo-1-methyl-tryptophan (L-1MT) and dextro-1-methyl-tryptophan (D-1MT). Both the racemic mixture and each stereoisomer have been studied in preclinical settings. The L-1MT isomer has been shown to be more effective at inhibiting tryptophan degradation in cell lines, whereas the D-1MT isomer has been shown to be more effective at reversing Treg suppression and inhibiting tumor growth from both murine and human dendritic cells [22, 23]. Preclinical models have shown 1-MT to both increase the effects of chemotherapy as well as support vaccination concurrently administered [6, 9, 24]. Since D-1MT (later named indoximod) demonstrated superior antitumor activity in preclinical

models, it was selected by the NCI as the lead compound for clinical development. The mechanism of action of indoximod was the subject of debate for quite some time as it didn't appear to have as potent an effect on IDO enzymatic activity in certain assays compared to the L stereoisomer [25, 26]. Initial studies suggested that indoximod acted through a splice variant of IDO known as IDO2, but the human version of IDO2 appears to have limited catabolic activity in vivo [27]. Subsequent work suggests that the effect of indoximod on the IDO pathway is downstream of the actual enzyme by acting as a tryptophan mimetic for amino acid sensing proteins that feed into mammalian target of rapamycin (mTOR) signaling in immune cells [28]. This in essence relieves immune cells from the effects of IDO activation in the tumor microenvironment.

The first in man phase I clinical trial of indoximod was activated in 2007 and continued accrual through to 2010 [29]. The trial ultimately enrolled 53 patients with the data on the entire cohort presented at the American Society of Clinical Oncology (ASCO) 2012 annual meeting. Forty-eight patients were treated, and a maximum tolerated dose was not achieved up to the highest dose level of 2000 mg BID. The best response noted was stable disease for over 6 months in five patients at 6 months with mixed responses in others [30]. The drug was well tolerated with the most common attributed adverse events being grade 1–2 fatigue, anemia, anorexia, and nausea.

A second phase 1/2 trial investigated the safety and efficacy of the combination of indoximod with a dendritic cell vaccine targeting p53 mutated patients [31]. The combination was very well tolerated with no significant toxicity attributed to the treatment. While there were no objective responses noted in the phase 1/2 cohort, it was noted that a higher than expected number of patients who were treated with subsequent chemotherapy responded. This chemosensitization effect of the vaccine was seen in a prior small cell lung cancer trial as well, so this aspect of the treatment is being explored further [32]. Another trial looking at indoximod combined with the prostate cancer dendritic cell vaccine, sipuleucel-T, is ongoing. Both of these trials aim to demonstrate that combining vaccines with indoximod will result in a boosted immune response that will ultimately lead to improved outcomes [33].

There are multiple completed and ongoing trials looking at the combination of chemotherapy with indoximod to see if the preclinical synergy observed is seen in patients with advanced tumors. Three of eight listed clinical trials involving indoximod combine the study medication with known, FDA approved chemotherapies. The first combination phase I trial paired docetaxel at 60 mg/m^2 and 75 mg/m^2 every 21 days with twice daily indoximod up to 1600 mg BID with maximum tolerated dose as the primary endpoint. The recommended phase II dose of docetaxel 75 mg/m^2 and indoximod 1200 mg BID was determined. Twenty-seven patients were treated and 22 were evaluable for response. The most common toxicities such as neutropenia, anemia, and fatigue were not higher than what would be expected with docetaxel alone. The objective response rate was 18 % with an additional 40 % of patients experiencing stable disease. Based on this data, a randomized phase 2 trial comparing docetaxel with docetaxel plus indoximod in metastatic breast cancer was initiated [34].

A third trial combining indoximod with chemotherapy (NCT02077881) is the most recently submitted ongoing study and combines the study drug with gemcitabine and nab-paclitaxel in patients with metastatic pancreatic cancer [35]. The study uses the recently FDA approved regimen gemcitabine 1000 mg/m^2 and nab-paclitaxel 125 mg/m^2 days 1, 8, and 15 of a 28 day cycle in conjunction with scaling doses of twice daily indoximod (600 mg, 1000 mg, and 1200 mg BID) to determine first maximum tolerated dose and second recommended phase II dosage.

As previously discussed, IDO may serve as a resistance mechanism against therapeutic CTLA-4 blockade. There is a clinical trial now underway combining indoximod with ipilimumab (NCT02073123) sponsored by NewLink Genetics [36]. The indoximod is dosed twice daily concurrently with standard ipilimumab scheduling. A trial in progress abstract was presented at ASCO 2014 during general poster presentation for melanoma and skin cancers [37].

A second compound developed by New Link Genetics, NLG919, appears to function more as a direct IDO enzyme inhibitor. Preclinical data showed NLG919 improved survival when combined with chemotherapy and also demonstrated synergy with indoximod in a B16 melanoma mouse model [24, 38]. The drug is currently in a phase Ia dose escalation trial and will complete accrual by the end of 2014 (NCT02048709) [39]. The trial starts with 50 mg of NLG919 twice daily on a 21 of 28 day cycle and escalates to 800 mg twice daily for 21 of 28 days per cycle [40].

INCB024360

INCB024360 is a small molecule currently in clinical trials by Incyte for the selective inhibition of IDO. It is a more potent IDO1 inhibitor than L-1MT with an IC50 concentration of 0.01 μM in cellular assays. INCB024360 was also shown to stimulate an increased cytokine production from dendritic and T cells cocultured with IDO producing HeLa cells [41]. INCB024360 increases tumor infiltrating lymphocytes (TILs) and decreases tumor and tumor draining lymph nodes kynurenine levels [42].

The phase I clinical trial for INCB024360 enrolled adult patients with advanced solid malignancies using a standard 3+3 design for dose escalation from 50 mg daily to 700 mg BID [43]. The trial enrolled 52 patients between July 2010 and July 2013, the majority of who had colorectal or melanoma (68 % combined). The initial data was presented at 2013 ASCO poster presentations. The trial found no significant dose-limiting toxicities at the maximum tested dose of 700 mg BID. Pharmacokinetics showed that doses ≥300 mg BID had maximal effect and approximately 90 % inhibition of IDO1. Therefore, the recommended phase II dosage for INCB024360 based on this clinical trial was 600 mg BID. The best observed activity was stable disease for >4 months in seven patients [44].

An advanced ovarian cancer preoperative immune response study is studying the ability of INCB024360 to favorably increase the antitumor response over a 2 week period [45]. The goal is enrollment of 12 patients with either stage III or IV epithelial

ovarian, fallopian tube, or primary peritoneal cancer. These patients are to be treated orally with the pharmaceutical for 2 weeks followed immediately by surgery on day 15. The primary outcome of the trial is to determine a CD8+ increase among participants. This trial began recruiting in December 2013 and is ongoing.

An additional single agent trial comparing INCB024360 with tamoxifen (NCT01685255) is a randomized, open label, phase II trial in patients with epithelial ovarian, fallopian, and primary peritoneal cancers [46]. This trial aims to enroll 110 patients with biochemical recurrence of CA-125 who have had complete remission after receiving only first line chemotherapy of paclitaxel, bevacizumab, or vaccine. The primary outcome of this trial is progression free survival by RECIST criteria. This trial opened in August 2012, and final data is projected to be collected in October 2014.

The fourth monotherapy clinical trial of INCB024360 (NCT01822691) is a phase II trial for treatment of patients with myelodysplastic syndromes [47]. The trial aims to treat 40 patients with INCB024360 over 17 weeks measuring the primary outcome of partial or complete response or hematologic improvement in any cell line based on International Working Group (IWG) 2006 criteria. This is the only currently active IDO inhibitor trial for hematologic malignancies.

There are two ongoing clinical trials combining INCB024360 with a vaccine. Both of these trials are in patients with epithelial ovarian, fallopian tube, or primary peritoneal cancers in remission. The remaining gynecologic malignancy vaccine trial, trial that is currently recruiting [48]. One ongoing study (NCT02166905) is a Phase I/II trial that pairs INCB024360 with the vaccine DEC-205/NY-ESO-1 fusion protein CDX-1401 with poly ICLC in women with NY-ESO-1 positive epithelial ovarian, fallopian, and primary peritoneal cancer. The phase I portion of this trial involves four arms: standard therapy, INCB024360 alone, INCB024360 with vaccine, and vaccine alone. The primary outcomes of this trial are safety and toxicity for phase I and progression free survival for phase II. The second INCB024360-vaccine trial (NCT01961115) combines INCB024360 with MELITA 12.1 peptide vaccine for patients with stage III or stage IV melanoma [49]. This trial includes patients with mucosal, ocular, and recurrent melanoma. This phase II open label trial pairs twice daily INCB024360 with vaccine injection weekly for 3 weeks followed by every 3 weeks for 9 weeks. Accrual is ongoing with an anticipated completion date of December 2014.

INCB024360 is also being explored in combination with immune checkpoint inhibitors. Both agents, ipilimumab and pembrolizumab, are approved in metastatic melanoma [50, 51]. One trial (NCT01604889) combines INCB024360 with ipilimumab, a CTLA-4 antagonist antibody [51]. The trial is a two arm phase I/II of patients receiving ipilimumab in combination with INCB024360 or with placebo. The first phase is a traditional 3 + 3 dose escalating study. As previously mentioned studies have shown, 700 mg of INCB024360 twice daily has been well tolerated as monotherapy. However, preliminary data from the phase I portion of this trial showed immune related adverse events at a dose of 300 mg BID. Seven patients were enrolled at this dose, but five had adverse events in the way of elevated liver enzymes. This dose level was discontinued due to toxicity concerns. Prior to the patients being taken off of the 300 mg BID dose, scans showed an immune related

partial response. Three patients did not require subsequent treatment for 3 months, while four of seven patients started new therapy after 6 months. The study was restarted with a lower dose of 25 mg BID and enrolled eight patients. This dosage showed similar results in terms of stable disease, but with fewer side effects or immune related adverse events. Three of the patients who received 25 mg BID had prolonged stable disease of 116 days, >173 days, and >187 days [52]. This clinical trial is currently enrolling for a 50 mg BID and a 75 mg daily cohort. The phase II blinded, placebo portion of this trial is awaiting the recommended phase 2 dose from the phase I portion. The pembrolizumab combination trial which opened in June 2014 includes selected solid tumors in its phase I portion, including NSCLC, transitional cell cancer of the bladder, renal cell cancer, triple negative breast cancer, adenocarcinoma of the endometrium, or squamous cell carcinoma of the head and neck. The phase II portion is enrolling advanced stage non-small cell lung cancer [50].

Conclusions

The ongoing clinical trials of IDO pathway inhibitors suggest that these agents overall are well tolerated. However, the clinical activity of these agents as monotherapy is limited when compared to the more dramatic effect of checkpoint inhibitors such as ipilimumab, nivolimumab, or pembrolizumab in solid tumors. This is likely due to the fact that IDO acts more as a fine-tuning modulator of the immune response in contrast to the on–off switch effect of checkpoint blockade. This obviously puts a priority on developing combination therapies that include IDO inhibitors in an attempt to enhance the immune activating properties of the other therapy. The lower toxicity of IDO inhibitors makes them attractive candidates for this type of approach. The number of different tumor types which could be studied is broad since IDO is overexpressed in many different tumor types. So prioritizing these clinical trials will likely be based on what interesting immunotherapy or cytotoxic agents are already in use for those different tumor sites. As other inhibitors of kynurenine pathways such as TDO become available, these trials will likely look at combinations of kynurenine inhibitors to maximize their therapeutic effect. What will be important is the development of good, practical biomarkers (imaging and blood-based) to help stratify and select patients for treatment. It is an exciting time in cancer immunotherapy, and inhibitors of the kynurenine pathways may play an important role in optimizing the clinical benefit of these treatments in the years to come.

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Chapter 31
The Next Decade in Tryptophan Metabolism Research

Chai K. Lim and Gilles J. Guillemin

Abstract Over the last three decades, tryptophan research has progressively moved from an "obscure" to a primary field of research. Interest in tryptophan metabolism and thus the number of publications has been growing concomitantly and almost exponentially. Looking at Pubmed today (November 2014), the overall number of publications about tryptophan, indoleamine 2,3 dioxygenase (IDO), and the kynurenine pathway has been rising significantly (Fig. 31.1). One of the first "boost" for tryptophan research was in the eighties with the identification of neuroactive activities of kynurenine pathway metabolites such as quinolinic acid [1, 2] and kynurenic acid [3] and especially their actions as agonist and antagonist of the N-methyl-D-aspartate receptor. Then, the field was subject to another major "hit" when IDO1 was identified as a key regulator of the immune response by the work of Munn and Mellor [4]. Of course, many more studies have demonstrated the pleiotropic roles played by tryptophan and its metabolites in both physiological and pathological conditions.

Keywords Tryptophan • Kynurenine pathway • Quinolinic acid • Biomarker • Therapeutic

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List of Abbreviations

AhR	Aryl hydrocarbon receptor
AMT	α-[^{11}C]methyl-L-tryptophan
IDO	Indoleamine 2,3 dioxygenase
IJTR	International Journal for Tryptophan Research

Introduction

Over the last three decades, tryptophan research has progressively moved from an "obscure" to a primary field of research. Interest in tryptophan metabolism and thus the number of publications has been growing concomitantly and almost exponentially. Looking at Pubmed today (November 2014), the overall number of publications about tryptophan, indoleamine 2,3 dioxygenase (IDO), and the kynurenine pathway has been rising significantly (Fig. 31.1). One of the first "boost" for tryptophan research was in the eighties with the identification of neuroactive activities of kynurenine pathway metabolites such as quinolinic acid [1, 2] and kynurenic acid

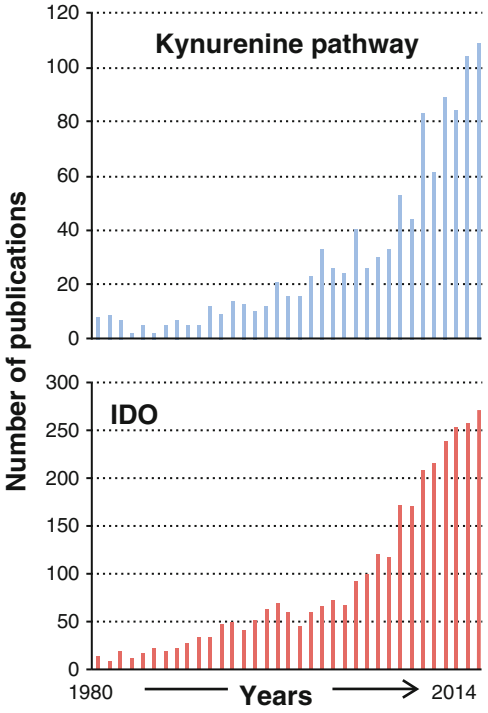

Fig. 31.1 Timeline of the number of publications between 1980 and 2014

[3] and especially their actions as agonist and antagonist of the N-methyl-D-aspartate receptor. Then, the field was subject to another major "hit" when IDO1 was identified as a key regulator of the immune response by the work of Munn and Mellor [4]. Of course, many more studies have demonstrated the pleiotropic roles played by tryptophan and its metabolites in both physiological and pathological conditions.

During the recent years, researchers have identified new roles for and uses of tryptophan metabolites. Some examples among these recent discoveries are: The ability of some of the kynurenine pathway metabolites to bind and regulate the aryl hydrocarbon receptor (AhR) is thought to be a crucial mechanism in the regulation of immune responses [5, 6]. A couple of publications have described that ability of some of the kynurenine pathway metabolites to modulate differentiation and/or proliferation of stem cells [7, 8]. Tryptophan and its metabolites are also now been considered as targets for both diagnostic, prognostic biomarkers but also for therapeutic strategies. Several recent publications and patents have described that tryptophan metabolism can be used as sensitive biomarker for several psychiatric disorders [9, 10] such as schizophrenia [11–13], bipolar disorder [14], depression [15, 16], suicidal behavior [17], and autism [18], but also for multiple sclerosis (PCT/IB2013/055902) or Alzheimer's disease (WO2006-105907). A new method measuring kynurenic acid in patient saliva in context of a brief stress challenge has been recently reported as a new and simple detection approach for psychiatric disorders [19]. Interestingly, the combination of the mathematic and kinetic models with expression data has been reported as a powerful diagnostic tool to predict alterations in tryptophan metabolism [20]. We can mention, for example, the use of new generation of tracers such as α-[^{11}C]methyl-L-tryptophan (AMT) PEt allows evaluation of brain serotonin synthesis and can also track up regulation of the immunosuppressive kynurenine pathway metabolites in tumor tissue. Increased AMT uptake is a hallmark of World Health Organization grade III–IV gliomas and other brain tumors [21–25]. Therapeutic using new generations of inhibitors targeting IDO1 and other enzymes such as KMO or KAT-II [26] has been a very popular new target over the recent years. Kynurenine pathway inhibitors have been studied in a large number of neurodegenerative diseases such as Alzheimer's disease [27, 28], dementia [29, 30], Parkinson's disease [31–33], amyotrophic lateral sclerosis [34], multiple sclerosis [35], Huntington's disease [36], and also psychiatric disorders [37–39]. Some of these inhibitors are already in clinical trial and in the next few years we will hopefully see them used in human therapy.

There are also a couple of more anecdotal points making tryptophan research attractive. The first one is that this field is fortunately to be still highly collaborative. As example the recent study published by Dr. Bessede's in *Nature* has involved 16 different research groups with scientists from eight different countries including France, Italy, Germany, Japan, Australia, The Netherlands, Canada, and USA [6]. Moreover the International Society for Tryptophan Research (ISTRY) has been concomitantly growing with the interest for the field, and the associated open access journal, *International Journal for Tryptophan Research* (IJTR), is about the get first impact factor soon. Figure 31.2 shows the number of views of IJTR papers per continent over the last 6 years and the obvious predominance of the United States.

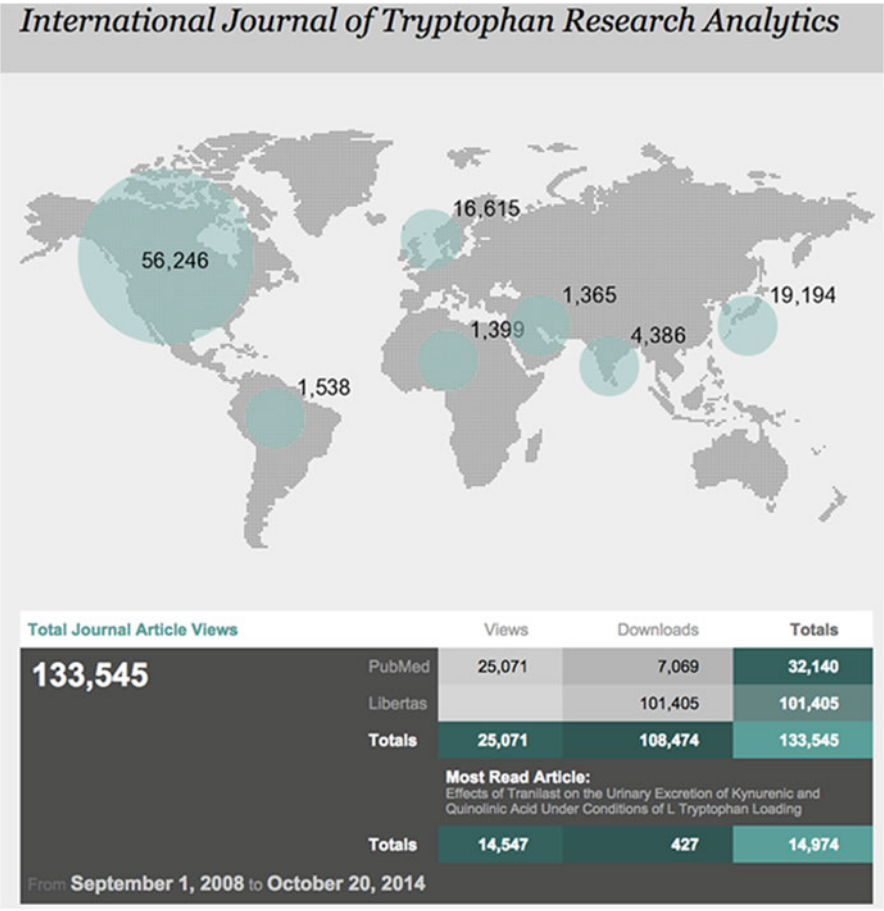

Fig. 31.2 World map outlining the number of views of IJTR papers per continent over the last 6 years

To conclude, tryptophan metabolism has progressively become a prime research topic and it is likely to stay as such. We believe that tryptophan and its metabolites are likely to have many more functions still to be identified and that we only see the tip of the iceberg.

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