

Lysine metabolism in mammalian brain: an update on the importance of recent discoveries

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Abstract The lysine catabolism pathway differs in adult mammalian brain from that in extracerebral tissues. The saccharopine pathway is the predominant lysine degradative pathway in extracerebral tissues, whereas the pipecolate pathway predominates in adult brain. The two pathways converge at the level of Δ^1 -piperideine-6-carboxylate (P6C), which is in equilibrium with its open-chain aldehyde form, namely, α -aminoadipate δ -semialdehyde (AAS). A unique feature of the pipecolate pathway is the formation of the cyclic ketimine intermediate Δ^1 -piperideine-2-carboxylate (P2C) and its reduced metabolite L-pipecolate. A cerebral ketimine reductase (KR) has recently been identified that catalyzes the reduction of P2C to L-pipecolate. The discovery that this KR, which is capable of reducing not only P2C but also other cyclic imines, is identical to a previously well-described thyroid hormone-binding protein [μ -crystallin (CRYM)], may hold the key to understanding the biological relevance of the pipecolate pathway and its importance in the brain. The finding that the KR activity of CRYM is strongly inhibited by the thyroid hormone 3,5,3'-triiodothyronine (T_3) has far-reaching biomedical and clinical implications. The interrelationship between tryptophan and lysine catabolic pathways is discussed in the context of shared degradative enzymes and also potential regulation by thyroid hormones. This review traces the discoveries of enzymes

involved in lysine metabolism in mammalian brain. However, there still remain unanswered questions as regards the importance of the pipecolate pathway in normal or diseased brain, including the nature of the first step in the pathway and the relationship of the pipecolate pathway to the tryptophan degradation pathway.

Keywords Ketimine reductase · Lysine degradation · Pipecolate pathway · Saccharopine pathway · Tryptophan metabolism · Thyroid hormones

Abbreviations

| | |
|--------------|---|
| AAD | α -Aminoadipate |
| AADT | α -Aminoadipate transaminase (aminotransferase) |
| AAS | α -Aminoadipate δ -semialdehyde |
| AASDH | α -Aminoadipate δ -semialdehyde dehydrogenase |
| α -KG | α -Ketoglutarate |
| AKA | α -Keto adipate |
| ALDH | Aldehyde dehydrogenase |
| ALS | Amyotrophic lateral sclerosis |
| CTBP | Cytosolic thyroid hormone-binding protein |
| CRYM | μ -Crystallin |
| DAAO | D-Amino acid oxidase |
| GAD | Glutamate decarboxylase |
| GTK | Glutamine transaminase K |
| GTL | Glutamine transaminase L |
| KAC | α -Keto- ϵ -aminocaproate |
| KAT | Kynurenine aminotransferase |
| KR | Ketimine reductase |
| Kyn | L-Kynurenine |
| KynA | Kynurenate |
| IL4I1 | Interleukin-4-induced protein 1 |
| LAAO | L-Amino acid oxidase |

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|----------------|--|
| LKR/SDH | Lysine- α -ketoglutarate reductase/ saccharopine dehydrogenase |
| P2C | Δ^1 -Piperideine-2-carboxylate |
| P6C | Δ^1 -Piperideine-6-carboxylate |
| PLP | Pyridoxal 5'-phosphate |
| POX | Pipecolate oxidase |
| Pyr2C | Δ^1 -Pyroline-2-carboxylate |
| Pyr5C | Δ^1 -Pyroline-5-carboxylate |
| T ₃ | 3,5,3'-Triiodothyronine |

Introduction

L-Lysine is an essential amino acid with the added importance that it is a limiting amino acid in the diet of many people around the world who rely on cereals as a staple in their diet (Papes et al. 2001). Our understanding of L-lysine catabolism in human tissues is therefore of paramount importance. Weissman and Schoenheimer (1941) demonstrated that L-lysine labeled with ^{15}N in the α position and deuterium in the side chain is incorporated into proteins in the rat without a detectable change in the ratio of ^{15}N to ^2H . This finding was regarded as proof of the prior suggestion that L-lysine is unique among the common amino acids in that it cannot *reversibly* share its α -amino nitrogen with other common amino acids. Weissman and Schoenheimer (1941) therefore suggested that a pathway unique among the common amino acids exists for lysine catabolism. We now know that lysine catabolism is indeed unusual in that it proceeds via two distinct major pathways, the saccharopine pathway and the pipecolate pathway, both of which later converge into a common degradative pathway. The findings of Weissman and Schoenheimer (1941) may be explained by the fact that conversion of the α -amino group of lysine to an α -keto function (pipecolate pathway) or conversion of the ϵ -amino group of lysine to an aldehyde (saccharopine pathway) results in products that rapidly cyclize, essentially rendering unfavorable the formation of lysine by a transamination reaction. In the adult brain the pipecolate pathway predominates, whereas in extracerebral tissues the pipecolate pathway is a minor pathway for lysine degradation (Chang 1976, 1978). However, in the developing fetal brain the saccharopine pathway is highly active and predominates. During development, the capacity of the pipecolate pathway increases, becoming the major catabolic pathway for lysine degradation in adult brain (Rao et al. 1992). This relationship suggests a specific neuronal developmental role for the pipecolate pathway and its intermediate metabolites.

The two lysine catabolic pathways differ in that the saccharopine pathway is predominantly mitochondrial,

whereas the pipecolate pathway is predominantly peroxisomal and cytosolic, as discussed in Sects. 3–7. Recently some of the key enzymes of the pipecolate pathway have been identified, yet questions still remain as to the relevance and importance of this pathway to the mammalian brain. A unique cyclic ketimine, which is not produced in the saccharopine pathway, is generated as an intermediate in the pipecolate pathway, namely, Δ^1 -piperideine-2-carboxylate (P2C). Perhaps P2C holds the key to elucidate the biological importance of the pipecolate pathway. This review traces the discoveries that highlight the importance of the pipecolate pathway in the brain and particularly the role of P2C in this pathway. The review also raises many unanswered questions that should provide the basis for future research, particularly in the areas designed to elucidate the neurochemical importance of lysine (and tryptophan) metabolism in normal and pathological states.

The saccharopine pathway: a major degradative pathway for lysine in extracerebral tissues and fetal brain but a minor pathway in adult mammalian brain

Higashino et al. (1965, 1967) were the first to identify saccharopine as a key intermediate in L-lysine degradation in mammalian tissues. These authors demonstrated that rat liver mitochondria *in vitro* convert L-lysine to saccharopine in the presence of α -ketoglutarate (α -KG), thus establishing the saccharopine pathway as a mitochondrial pathway (Fig. 1). The human enzyme that converts L-lysine to saccharopine in the presence of α -KG was investigated by Hutzler and Dancis (1968) and identified as an NADPH-dependent lysine- α -KG reductase (LKR). Studies by Dancis et al. (1969) on patients presenting with hyperlysinemia demonstrated that the accumulation of L-lysine is due to a deficiency of LKR. Although Higashino et al. (1971) found small amounts of free saccharopine in mouse liver, no detectable saccharopine was found in body fluids obtained from normal human volunteers (Carson et al. 1968) or in body fluids of rats that had been injected with ^{14}C -labeled L-lysine. Thus, a rapid *in vivo* turnover of saccharopine was hypothesized by Carson et al. (1968). Evidence in support of this hypothesis was obtained by Fellows and Lewis (1973) who demonstrated much higher specific activity of saccharopine dehydrogenase (SDH) than of LKR in liver from various mammalian species. However, saccharopine can accumulate under certain pathological conditions. Thus, Carson et al. (1968) described the presence of saccharopine in physiological fluids of patients presenting with saccharopinuria. Fellows and Carson (1974) showed that saccharopinuria is caused by an absence of SDH, which catalyzes the NAD^+ -dependent oxidation of saccharopine to AAS and glutamate. Fellows and Lewis

(1973) also demonstrated that a hepatic mammalian saccharopine oxidoreductase catalyzes the formation of L-lysine and α -KG from saccharopine in vitro, with highest specific activities in the livers of herbivores, and lowest levels in the livers of carnivores/omnivores. A cDNA clone that encodes a bifunctional mitochondrial LKR/SDH as well as a monofunctional SDH in mouse liver and kidney were isolated and characterized (Papes et al. 1999). Northern blot analyses indicated the existence of two mRNA species in mouse liver and kidney. The longer transcript, 3.4 kb in size, corresponds to the isolated cDNA and encodes the bifunctional mitochondrial enzyme. The shorter transcript, 2.4 kb in size, probably encodes a monofunctional dehydrogenase (Papes et al. 1999). A single gene encodes a bifunctional mitochondrial LKR/SDH protein in humans (Sacksteder et al. 2000).

Rao et al. (1992) measured LKR/SDH activity in developing rat liver and brain. The authors found that enzyme activity is highly dependent on the stage of development in the rat brain, with high levels of activity in developing brain and low activity in adult brain (Rao et al. 1992). The decrease in activity of LKR/SDH in the brain contrasts with the increase in activity of this enzyme in the liver, where it becomes the major contributor to L-lysine catabolism in the liver of adult rats. Rao et al. (1992) also showed that glucagon induces the enzyme in both adult liver and brain. Hutzler and Dancis (1968, 1975) had previously shown that LKR/SDH enzyme activity is low in adult rat brain, and, as mentioned above, Chang (1976, 1978) had shown that the pipercolate pathway is the dominant L-lysine degradative pathway in the adult rat brain. These findings for rat brain have recently been corroborated by Sauer et al. (2011) for adult mouse brain. These authors found no detectable LKR/SDH activity, coupled with low LKR/SDH mRNA expression, in adult mouse brain (Sauer et al. 2011). Thus, the prior contention by Papes et al. (2001) that cortical and cerebellar expression of LKR/SDH mRNA in the mouse are almost as high as that found in the liver requires clarification. Papes et al. (2001) appear to have neglected to take into account the developmental changes in expression as noted by Rao et al. (1992). It appears that Papes et al. (2001) compared embryonic liver LKR/SDH mRNA expression (which is significantly lower than in adult liver) with adult cerebral/cerebellar LKR/SDH mRNA expression. Moreover, the conclusion by Papes et al. (2001) that LKR/SDH mRNA is comparable in rat brain to that in liver appears to contradict their previous findings of low LKR/SDH gene expression in adult mouse brain (Papes et al. 1999) and also the work of Sauer et al. (2011) mentioned above. The authors did, however, show that ^{15}N label in the ϵ -amino position of L-lysine is transferred to glutamate in mouse cerebellar slices. The authors demonstrated that brain LKR/SDH

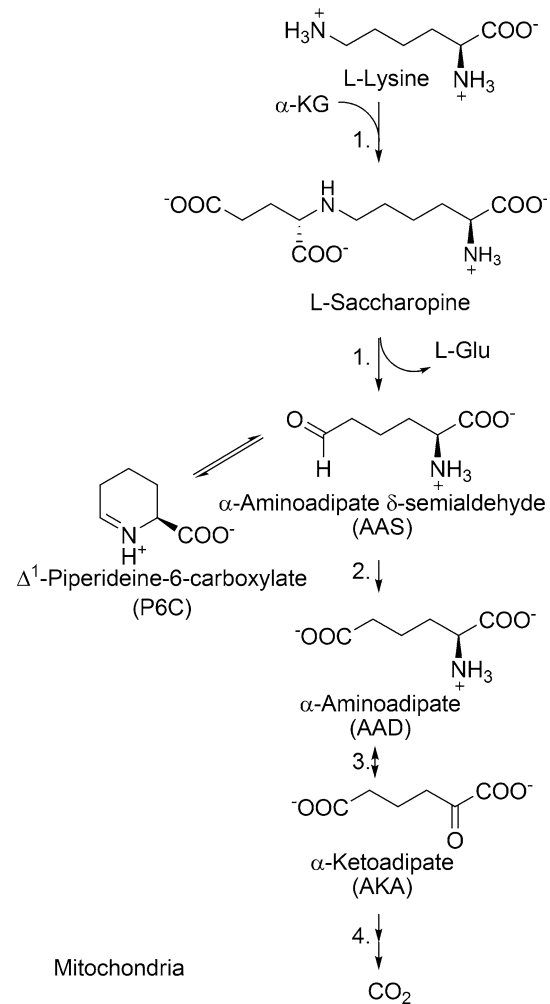


Fig. 1 The saccharopine pathway for the metabolism of L-lysine in mammals. In contrast to the pipercolate pathway (Fig. 2), which is predominantly cytosolic and peroxisomal, the saccharopine pathway occurs only in the mitochondria. The saccharopine pathway is the predominant pathway for L-lysine degradation in extracerebral tissues and developing brain. However, in the adult brain, the pipercolate pathway predominates. The two pathways converge at Δ^1 -piperideine-6-carboxylate/ α -amino adipate δ -semialdehyde (P6C/AAS). Enzymes: 1 the bifunctional enzyme L-lysine- α -ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH); 2 α -amino adipate semialdehyde dehydrogenase (AASDH); 3 α -amino adipate aminotransferase/kynurenine aminotransferase II (AADT/KAT II); 4 a series of mitochondrial enzymes common to L-lysine as well as tryptophan degradation (Fig. 4). Interconversion between AAS and P6C occurs spontaneously (non-enzymatically). Note that some of the enzyme steps shown here occur in other figures. The numbering of each enzyme-catalyzed step is maintained in the subsequent figures. Note also that some of the coenzymes and reactants/products in this pathway and other pathways (Figs. 2, 4) are omitted for simplicity

expression is predominantly neuronal and suggested that the associated enzyme activities may contribute to a de novo neuronal glutamate and GABA biosynthetic route in neurons independent from the astrocytic glutamine-glutamate cycle, with particular importance in fetal brain development. This suggestion is further discussed in Sect. 9

in relation to tryptophan metabolism in the brain. In summary, the literature supports the notion that the saccharopine pathway exists in the adult rodent brain, but that it is of secondary importance to the pipercolate pathway for the metabolism of L-lysine.

The net reaction of LKR/SDH enzyme activity is the reductive amination of α -KG to glutamate balanced by the oxidative deamination of the ϵ -amino group of L-lysine (Fig. 1). The product of the oxidative deamination is AAS, which is in equilibrium with its cyclic ketimine form, namely Δ^1 -piperideine-6-carboxylate (P6C) (Soda et al. 1968). The equilibrium greatly favors P6C. It is at the level of P6C that the saccharopine and pipercolate pathways converge, and thereafter follow the same common degradative pathway. The pathway for catabolism of AAS/P6C is discussed in Sect. 8.

The nature of the first step of the pipercolate pathway in the brain remains unsolved

L-Pipercolate (L-PA, Fig. 2) is a natural metabolite in mammalian tissue including brain. Reported levels for adult mouse brain vary from a low of 3.4 nmol/g (w/w) for cerebrum to a high of 7.2 nmol/g (w/w) (or about 4.3 and 9.0 μ M, respectively, assuming brain is 80 % water by weight) for the cerebellum (Kim and Giacobini 1985). Similar levels have been reported for adult rat brain (Nishio and Segawa 1983). Interestingly, L-pipercolate has been found to bind tightly to P2 fraction membranes of mouse brain. This binding is modulated by GABA (Gutierrez and Giacobini 1985). Given the neurochemical importance of L-pipercolate, it is somewhat surprising that its origin in brain is not well understood. One theoretical source of L-pipercolate is via the action of D-amino acid oxidases followed by reduction of the product (a cyclic ketimine).

D-Lysine has been known for over 45 years to be metabolized in rats to L-pipercolate (Grove and Henderson 1968). The peroxisomal enzyme D-amino acid oxidase (DAAO) was suspected of initiating the metabolism of D-lysine. Indeed, Scannone et al. (1964) had previously shown that D-lysine is a substrate of mammalian DAAO. The product of D-lysine oxidative deamination is α -keto- ϵ -aminocaproate (KAC), which spontaneously cyclizes to the ketimine P2C (Fig. 2). This ketimine is subsequently reduced to L-pipercolate by a cytosolic P2C reductase (Meister 1962). Interestingly, P2C has been shown to form a stable complex with DAAO, and P2C is a potential competitive inhibitor of this enzyme with a very high enzyme affinity (Nishina et al. 1991). In rat brain, the specific activity of DAAO is highest in cerebellum and brainstem, with very low specific activity in the cerebral cortex (Horiike et al. 1994). Chang (1978) studied the

metabolism in the rat brain of intraventricular administered 14 C labeled L- and D-lysine and demonstrated that the major metabolite formed from both labeled compounds is L- 14 C]pipercolate and that the two enantiomers are equally effective precursors of L- 14 C]pipercolate. The conversion of D- 14 C]lysine to L- 14 C]pipercolate was presumably initiated by the action of DAAO, despite the possibility of strong product inhibition, followed by an enantiomeric reduction of labeled P2C. In the same study, Chang (1978) noted that some labeled α -amino adipate (AAD) was also produced from both L- and D- 14 C]lysine in the rat brain. In earlier work, Chang (1976) noted that 14 C]pipercolate, but not 14 C]AAD, was present in the urine of rats intraperitoneally injected with L- 14 C]lysine. It was concluded that L-lysine metabolism in the brain occurs via a pathway involving pipercolate conversion to AAD (Chang 1976, 1978). As discussed above, this pathway (the pipercolate pathway) in the adult rat brain is distinct from the saccharopine pathway for lysine metabolism known to predominate in the liver and other tissues of the rat.

Given that the conversion of D-lysine to P2C in the brain is presumably catalyzed by a DAAO, how is L-lysine converted to P2C in that organ? Historically, Rothstein and Miller (1954) showed, using 15 N and 14 C as tracers and a "metabolite overloading" procedure, that L-lysine can be converted to L-pipercolate in the rat through loss of the α -amino group. But how is the α -amino group of lysine removed? The simplest explanation is that either an L-amino acid oxidase (LAAO) or an aminotransferase catalyzes the conversion of L-lysine to KAC. Because conversion of lysine to KAC is little understood, we provide here a detailed discussion of both possibilities. In both cases the product is KAC, which, as noted above, cyclizes to the ketimine P2C. Neuberger and Sanger (1943) suggested that acetylation of the ϵ -amino group may be the first step in the pathway leading to the oxidation of the α -amino group of L-lysine. In this regard, an acetyltransferase has been shown to be present in human tissues that acetylates thialysine (*S*-2-aminoethyl-L-cysteine) as well as L-lysine (to a lesser extent) at the N^ϵ position (Coleman et al. 2004). However, work by Hernandez and Chang (1980) demonstrated that the L-pipercolate formed in vitro in rat kidney and brain from N^ϵ -acetyl-L-lysine is derived from L-lysine. The study of Hernandez and Chang (1980) thus negates the possibility that N^ϵ -acetyl-L-lysine as an obligatory intermediate in the pipercolate pathway of L-lysine metabolism.

Murthy and Janardanasarma (1999) provided an evidence for the presence of an LAAO/lysine oxidase in mouse brain. The authors, using labeled 14 C-L-lysine as substrate, identified both P2C as well as its open-chain form (i.e., KAC) in mouse brain homogenates. However, the brain homogenate exhibited relatively low activity with lysine. Moreover, because a crude homogenate was used,

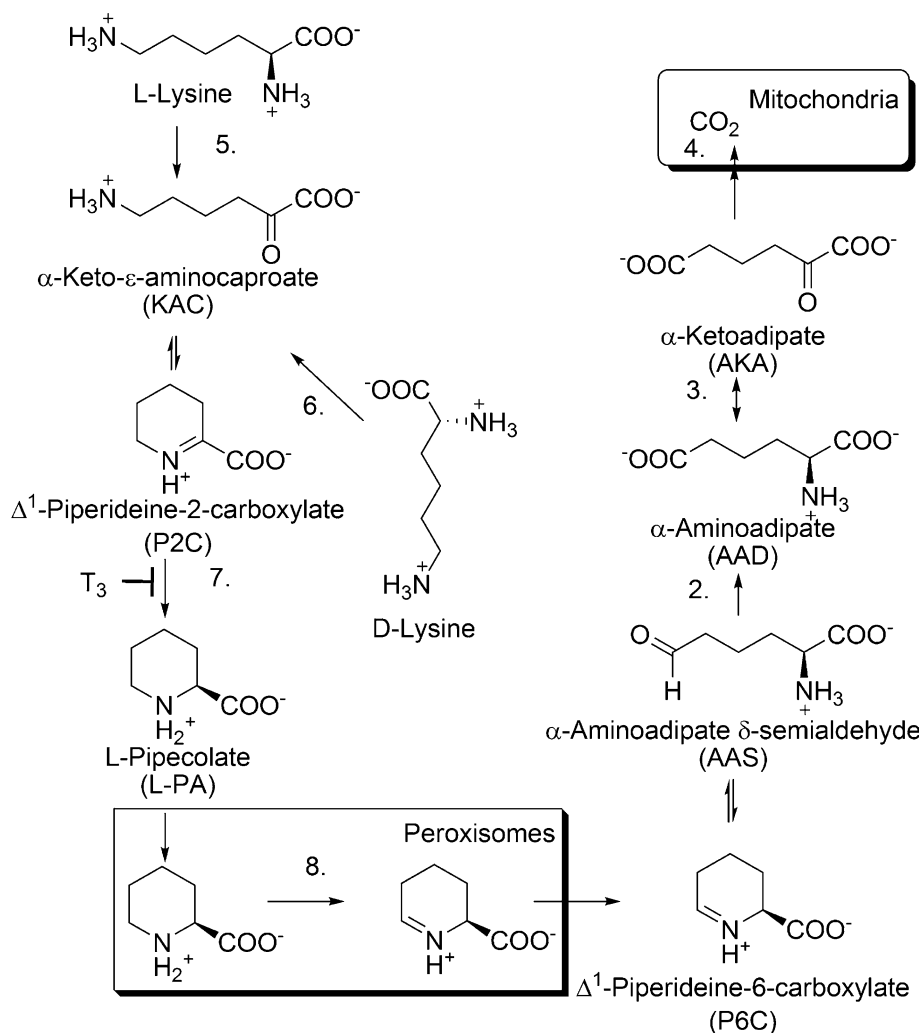


Fig. 2 The pipecolate pathway for the metabolism of L-lysine in mammals. This pathway is the main degradative pathway for L-lysine in adult mammalian brain. Enzymes 1 and 2 are cytosolic. The enzymes 2 (AASDH) and 3 (AADT/KAT II) are the same as those in the saccharopine pathway. These enzyme activities are present in both mitochondria and cytosol. Nevertheless, the pathway for the conversion of L-lysine to AKA can be considered predominantly cytosolic with a peroxisomal component (as discussed in the text). *Enzymes:* 5 an enzyme that catalyzes conversion of the α -amino group of L-lysine to a keto function, possibly kynurenine aminotransferase III/

glutamine transaminase L (KAT III/GTL); 6 D-amino acid oxidase (DAAO); 7 CRYM/ketimine reductase (CRYM/KR); 8 L-pipecolate oxidase (POX); 2–4 as per Fig. 1. Conversion between Δ^1 -piperideine-2-carboxylate (P2C) and α -keto- ϵ -aminocaproate (KAC) as well as between Δ^1 -piperideine-6-carboxylate (P6C) and α -amino adipate δ -semialdehyde (AAS) is spontaneous (non-enzymatic). Thyroid hormones may play a major part in regulating this pathway as CRYM/KR (step 7) has been shown to be strongly regulated by T_3 , the active form of thyroxine

the findings do not prove that an LAAO reaction is the major route for conversion of L-lysine to KAC in brain. Additionally, it is not clear what type of LAAO could catalyze such an oxidase reaction with L-lysine. A well-characterized mammalian (human) LAAO is an L-phenylalanine oxidase [interleukin-4-induced protein 1 (IL4I1)] (Chavan et al. 2002; Boulland et al. 2007). Two isoforms of the IL4I1 protein have been described. Isoform 1 is predominantly expressed in leukocytes, and isoform 2 is predominantly expressed in testis and brain (Wiemann et al. 2005). Isoform 2 may be identical to the enzyme described by Murthy and Janardanasarma. IL4I1 is lysosomal,

exhibits an acidic pH optimum, and “prefers” aromatic amino acids such as phenylalanine as substrates. Thus, L-lysine is a relatively poor substrate (Mason et al. 2004). Moreover, IL4I1 is expressed at relatively low levels in the brain (Wiemann et al. 2005). L-Hydroxy acid oxidases are known to catalyze the oxidation of certain L-amino acids to a limited extent. However, L-lysine seems not to be a substrate, and L-hydroxy acid oxidases are not significantly expressed in brain (Miles and Holmes 1975; Duley and Holmes 1976; Urban et al. 1988). In summary, the contribution of an LAAO to the formation of KAC in brain remains uncertain.

Could an aminotransferase be responsible for the conversion of lysine to KAC in the brain? In contrast to most amino acids that are commonly found in proteins, L-lysine has traditionally been regarded as inert to the action of mammalian aminotransferases (Higashino et al. 1967). However, Papes et al. (2001) showed that when mouse brain cortex or cerebellar slices were incubated with L-[¹⁵N]glutamate, label could be detected in the α -amino group of L-lysine, but not in the ϵ -amino group (Papes et al. 2001). The simplest explanation for this finding is that despite the perceived notion that transamination reactions involving lysine are not favorable, the α -amino nitrogen can be transferred from glutamate to KAC in the brain, possibly as a result of the very high levels of glutamate in brain (10–12 mM). This finding suggests that transamination between glutamate and KAC is reversible—i.e., that transamination of L-lysine can occur in mouse brain such that the α -amino group is transferred to α -KG (or another suitable α -keto acid acceptor), thereby generating KAC (the first intermediate of the pipercolate pathway). If this reasoning is correct, what is the identity of this aminotransferase? Alanine:glyoxylate aminotransferase II (AGT II) is an enzyme with a relatively broad substrate specificity that can catalyze in vitro transamination of the α -amino group of L-lysine (Ogawa et al. 1990). However, lysine is a poor substrate compared to L-alanine, and AGT II is unlikely to catalyze this reaction to any great extent in vivo. Moreover, Ogawa et al. (1990) stated that AGT II activity is present in the rat only in liver and kidney homogenates.

Here, we consider the possibility that three other aminotransferases of broad α -keto acid and amino acid specificities may catalyze transamination of lysine at the α position. Cooper and Meister (1972, 1981, 1985) identified two glutamine transaminases in rat tissues, which they named glutamine transaminase K (GTK) and glutamine transaminase L (GTL). In the rat, GTK [synonyms: kynurenine aminotransferase I (KAT I); cysteine conjugate β -lyase isozyme 1 (CCBL 1)] activity is most abundant in kidney, but appreciable activity is also present in liver and other tissue. In the rat, GTL [synonyms: kynurenine aminotransferase III (KAT III); cysteine conjugate β -lyase isozyme 2 (CCBL 2)] activity is most abundant in liver, but activity can be detected in other tissues. Interestingly, GTK occurs in both the cytosolic and mitochondrial compartments of rat tissues (Cooper and Meister 1981). The same gene codes for both forms, but alternative splicing dictates whether or not a 32-amino acid mitochondrial targeting sequence at the *N*-terminus is present in the expressed protein (Malherbe et al. 1995; Tamburin et al. 1999). Cooper and Meister (1981, 1985) noted that GTL activity also occurs in cytosolic and mitochondrial compartments. It is possible that since the genes encoding KAT I/GTK and KAT III/GTL are highly conserved (including the

N-terminus) (Yu et al. 2006), alternative gene splicing at the *N*-terminus may also dictate the relative distribution of KAT III/GTL between cytosol and mitochondria.

Both GTK and GTL exhibit very broad and somewhat overlapping amino acid and α -keto acid specificities. Thus, it is possible that one or both of these enzymes contribute to the formation of KAC from L-lysine in brain. In this regard, Pensa et al. (1989) purified an aminotransferase from bovine brain that catalyzes transamination of thialysine at the α position. [The standard assay reaction mixture employed by these authors contained 10 mM L-thialysine and 0.5 mM α -keto- γ -methiolbutyrate in 20 mM sodium pyrophosphate buffer (pH 9.2). Formation of the product (i.e., AECK) derived from cyclization of the α -keto acid product was continuously measured spectrophotometrically.] Curiously, despite the similarity in structure between thialysine and lysine, the authors did not determine whether the enzyme also catalyzes transamination of L-lysine. An enzyme that catalyzes transamination of thialysine was also purified from bovine liver (Costa et al. 1986). The authors suggested that the enzyme responsible for transamination of thialysine is a glutamine transaminase.

Cooper and Meister (1981, 1985) showed that the glutamine analog L-albizziin is a good substrate of rat liver GTL, but a relatively poor substrate of rat kidney GTK. It is interesting to note that thialysine transaminase from bovine liver is also able to use albizziin as a substrate (Costa et al. 1986). Taken together, we considered the possibility that transamination of L-lysine at the α position may be catalyzed by KAT III/GTL. Similar to GTL purified from rat liver (Cooper and Meister 1972), recombinant mouse KAT III/GTL (mKAT III/GTL) has recently been shown to exhibit broad substrate L-amino acid and α -keto acid specificity (Han et al. 2009). L-Lysine was shown to be a substrate of mKAT III/GTL. At a concentration of 10 mM, L-lysine was about 25–40 % as effective as a substrate as the “best” amino acid substrates (conditions: 10 mM L-amino acid substrate; 2 mM glyoxylate as co-substrate; borate buffer pH 9.0, 45 °C) (Han et al. 2009).

We have now tested recombinant human KAT I/GTK, recombinant human KAT II/ α -amino adipate aminotransferase (AADT), another aminotransferase of broad substrate specificity, and recombinant mKAT III/GTL for their ability to catalyze transamination between 1 mM L-lysine and 1 mM α -keto- γ -methiolbutyrate (a good α -keto acid substrate of all three enzymes) in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C (unpublished observations). These assay conditions are more physiological than those employed by Costa et al. (1986) and Pensa et al. (1989) for the measurement of thialysine transamination (see the previous paragraph). Both the disappearance of α -keto- γ -methiolbutyrate and appearance of methionine were followed by HPLC with CoulArray detection [e.g., (Lee

et al. 2009)]. Under these conditions, the ability of KAT I/GTK and KAT III/GTL to catalyze transamination of L-lysine was <1 % that exhibited with glutamine. The ability of KAT II/ α -aminoadipate aminotransferase to catalyze transamination between L-lysine and α -keto- γ -methiolbutyrate could not be detected. In a separate experiment (2-h endpoint determination; 37 °C), we investigated transamination between 10 mM L-lysine (or 10 mM L-glutamine) and 5 mM α -keto- γ -methiolbutyrate in 50 mM sodium pyrophosphate buffer (pH 9.2). As noted for the experiment carried out at pH 7.4, lysine was found to be an extremely poor substrate of KAT I/GTK at pH 9.2 (<0.1 % relative to that exhibited with glutamine) and activity with KAT II/ α -aminoadipate aminotransferase could not be detected. However, although lysine is a less effective substrate than is glutamine of KAT III/GTL, the amount of lysine transaminated at pH 9.2 is substantial (about 5 % relative to that exhibited with glutamine). The data are thus in accord with the findings of Han et al. (2009) that KAT III can catalyze transamination of lysine at high pH values (≥ 9.0) at a moderate rate.

A consideration of the chemical properties of P6C (a double bond positional isomer of P2C) suggests a possible explanation for the apparent low level of activity of KAT III/GTL toward lysine at pH 7.4, but relatively higher activity at pH 9.2. Theoretically, P6C could arise through a one-step transamination of the ϵ -amino group with a suitable α -keto acid acceptor in an analogous fashion to that of the well-characterized transamination of the δ -amino group of ornithine. Instead, nature has devised a two-step process to convert lysine to AAS/P6C. Why? In Sect. 8 we discuss how P6C can react with PLP to form a Knoevenagel adduct. Perhaps the two-step conversion of L-lysine to AAS/P6C via saccharopine is designed by nature to avoid the problem of syncatalytic inactivation of a PLP-dependent aminotransferase by a Knoevenagel condensation of PLP cofactor with product P6C. We suggest that P2C may also inhibit an enzyme that catalyzes transamination of lysine at neutral pH due to a condensation with PLP cofactor. This inhibition is expected to be less of a problem at pH 9.2, because a greater proportion of P2C will be in the enamine form (Lu and Lewin 1998). It is possible that the imine reductases have evolved in part to rapidly reduce the double bond of P2C, preventing product inhibition of the parent aminotransferase.

In conclusion, of several enzymes considered as a possible source of KAC in the brain, KAT III/GTL appears to be the most likely candidate. KAT III/GTL is present in mammalian brain (Han et al. 2009 and references cited therein), but it is not clear how effective this enzyme is in transaminating lysine at physiological pH values. It is even conceivable that neither an LAO nor an aminotransferase is mainly responsible for the generation of KAC, but rather

a dual enzyme system analogous to the LKR/SDH for the production of P6C from lysine operates to generate P2C from lysine. Evidently, much more work is required to define the mechanism by which L-lysine is converted to KAC/P2C in the brain.

The discovery of specific cytosolic P2C reductases

In studies on ketimine reductase (KR) from bovine cerebellum, Nardini et al. (1988a) determined that, among potential substrates tested, this enzyme exhibits maximal activity with P2C (and NADPH rather than NADH as cofactor) and that it is also a cytosolic enzyme. P2C reductase activity was later found to be present mainly in the cytosolic supernatant fraction of rat cortex (Chang and Charles 1995). As it seems likely that P2C reductase activity occurs mainly in the cytosol, it is possible that the synthesis of KAC from L-lysine also occurs in the cytosol.

Δ^1 -Piperidine-2-carboxylate formed from the spontaneous cyclization of KAC is reduced to L-pipecolate by specific reductases (Meister 1962; Meister and Buckley 1957; Aspen and Meister 1962a, b; Meister et al. 1957). Meister (1962), Meister and Buckley (1957) and Meister et al. (1957) partially purified an imine reductase from rat kidney and were the first to show that mammalian tissues contain reductase(s) that reduce the imine double bond of P2C and also that of its five-membered ring analog [i.e., Δ^1 -pyrroline-2-carboxylate (Pyr2C)], forming only the L-enantiomers of pipecolate and proline, respectively. The authors concluded that the same enzyme(s) catalyze(s) the reduction of both compounds, but are unable to catalyze the reduction of Δ^1 -pyrroline-5-carboxylate (Pyr5C) and P6C, which are isomers of P2C and Pyr2C, differing only in the position of the imine double bond. Pyr5C reductases that exist in various isoforms in mitochondria as well as in the cytosol were later characterized and shown to catalyze the reduction of both Pyr5C and P6C (Valle et al. 1973; Yeh et al. 1981). Note that enzymes that reduce Pyr5C/P6C and P2C/Pyr2C may be regarded as aldimine reductases and ketimine reductases (KRs), respectively.

Meister et al. (1957) showed that P2C/Pyr2C reductase is present in rat kidney, brain, cardiac muscle, spleen, and testis. With the exception of kidney, of all the organs investigated, rat brain exhibited the highest specific activity of P2C/Pyr2C reductase (Meister 1962; Meister et al. 1957). In later work, a P2C/Pyr2C reductase was partially purified from porcine kidney by Petrakis and Greenberg (1965) and these authors obtained a higher specific activity than that obtained previously from rat kidney by Meister et al. (1957). It is notable that the enzyme preparations obtained by both groups could use NADH as well as NADPH as cofactor. However, the results obtained by these authors do not

preclude the possibility that mammalian tissues contain multiple KRs that differ in their affinities for NADH versus NADPH. In this regard, Meister et al. (1957) showed that NADH and NADPH were equally effective as cofactor for the KR purified from rat kidney, whereas Petrakis and Greenberg (1965) showed that the KR purified from porcine kidney exhibited a marked preference for NADPH over NADH. A cytosolic KR from porcine kidney was partially purified by Nardini et al. (1988b) and unlike the preparation obtained by Petrakis and Greenberg (1965), but similar to the preparation obtained by Meister et al. (1957), the preparation obtained by Nardini et al. (1988b) utilized NADH and NADPH equally well as cofactor when P2C was employed as substrate.

Garweg et al. (1980) demonstrated the presence of P2C/Pyr2C reductase activity and L-pipecolate in several regions of mouse, dog, and monkey brain. Both the level of L-pipecolate and P2C/Pyr2C reductase-specific activity differed considerably among the different brain regions. The authors found highest specific activities in the frontal and temporal cortex, with the striatum having the maximal specific activity. The enzyme in mouse brain exhibited an optimal pH of 5.2 with P2C and 5.4 with Pyr2C as ketimine substrates. These pH optima were similar to those determined by Meister et al. (1957) previously for a partially purified rat kidney KR. Among the various brain regions investigated, L-pipecolate levels were previously reported to be highest in the cerebellum (Kasé et al. 1973; Nishio and Segawa 1983), yet Garweg et al. (1980) found minimal P2C reductase activity in that region. A possible explanation for this apparent discrepancy is that NADH (and not NADPH) was used as a cofactor by Garweg et al. (1980). The cytosolic KR purified from bovine cerebellum by Nardini et al. (1988a) was found to have a marked preference for NADPH over NADH as a cofactor when P2C was used as the ketimine substrate. Nardini et al. also found maximal activity in the cerebellum and cerebral cortex among the brain regions investigated. The bovine cerebellar KR exhibited a similar pH optimum to that previously reported for other P2C reductases of ~ 5 . The cerebellar enzyme was found to be a homodimer (monomer $M_r \sim 50,000$), whereas a KR purified from porcine kidney was found to have a native M_r of $\sim 70,000$ (Nardini et al. 1988b). However, no subunit composition data were presented for the porcine kidney enzyme. Both KRs purified by Nardini et al. were shown to reduce substrates other than P2C, namely several sulfur-containing cyclic ketimines. Perhaps not surprisingly, the sulfur-containing cyclic ketimine aminoethylcysteine ketimine (AECK), which is a thioether analog of P2C, was found to be a good substrate for both KRs.

In summary, the published findings strongly support the hypothesis that mammalian tissues contain at least two

distinct KRs that are capable of reducing P2C to L-pipecolate. Moreover, in the mammalian brain there also appears to be regional differences in the expression of these two enzymes.

μ -Crystallin: the first P2C reductase identified in mammalian genome databases

In 2011, we purified a cytosolic lamb forebrain KR to apparent homogeneity and showed that this enzyme is similar to the previously reported purified porcine kidney KR (Hallen et al. 2011). Notably, the purified enzyme was able to use the same substrates [AECK, cystathionine ketimine (CysK) and P2C], as those previously described by Nardini et al. (1988b) for the porcine kidney enzyme. Moreover, both enzymes were found to use NADH and NADPH equally well as cofactor. Finally, the purified lamb brain KR was shown to be a homodimer (M_r monomer, $\sim 36,000$). This value is consistent with the M_r reported by Nardini et al. (1988b) for the porcine kidney native enzyme ($\sim 70,000$). However, the size and substrate specificity of the lamb brain KR appear to be notably different from those of a KR previously purified from bovine cerebellum by Nardini et al. (1988a). As noted above, the bovine cerebellar KR was reported to be a homodimer (monomer $M_r \sim 50,000$).

Mass spectrometric proteomic analysis of the purified lamb brain KR showed that the enzyme is an orthologue of a protein annotated in human and other mammalian genome databanks as μ -crystallin (CRYM). This finding suggested that human CRYM has a previously unsuspected biological role as a KR. Direct confirmation that human CRYM is indeed a KR was obtained by showing that highly purified recombinantly expressed human CRYM possesses substantial KR activity (Hallen et al. 2011). As noted for KRs purified from rat liver, porcine kidney and bovine brain, the lamb brain KR exhibits a pH optimum of ~ 5 with P2C as substrate (Hallen et al. 2011).

It is interesting to note that CRYM is now known to be a multifunctional protein with at least three disparate properties. In addition to our findings that CRYM is a KR (Hallen et al. 2011), the protein was previously well characterized as a structural protein of diurnal marsupial lens (Segovia et al. 1997). [For a recent review and characterization of crystallins (including μ -crystallin) in kangaroo lens see Augusteyn (2011).] Moreover, CRYM also strongly binds the active form of thyroxine, namely 3,5,3'-triiodothyronine (T_3) in a redox-sensitive manner (Mori et al. 2002). The characterization of CRYM as a cytosolic thyroid hormone-binding protein (CTBP) is intriguing. The K_d for binding of T_3 in the presence of NADPH was reported to be 0.3 nM, which implies very strong binding

of T_3 to CRYM (Beslin et al. 1995). CTBP is considered the main cytosolic thyroid hormone-binding protein and it was originally purified and identified as CRYM on the basis of its extremely strong affinity for T_3 in the presence of NADPH (Vie et al. 1997). Thyroid hormones have long been known to exert a major influence on brain development, and neonatal hypothyroidism in humans is known to cause mental retardation and neurological damage (Bernal 1999; Delange 2000). What role CRYM/KR has in brain development remains an unanswered question.

Lennon et al. (1980) demonstrated, from data obtained by Scatchard analysis of thyroid hormones binding to charcoal-extracted cytosol in the presence of 50 μ M NADPH, that thyroid hormone binding is significantly lower in cerebellum compared to cerebrum at every stage of development. Suzuki et al. (1991) calculated the maximal binding capacity for cytosolic NADPH-dependent T_3 binding in various rat tissues and also showed that levels of thyroid hormone binding are noticeably lower in the cerebellum than in the cerebrum of adult rat brain at all stages of development. The differences noted in NADPH-dependent T_3 binding between cerebrum and cerebellum are reflected in the differences noted for mRNA CRYM/KR gene expression in adult mouse brain for the same regions, with very low expression in cerebellum compared to cerebrum (Tebbenkamp and Borchelt 2010). Among several tissues investigated in rats at various developmental stages, NADPH-dependent T_3 binding was only detected in kidney, liver, heart and spleen after birth, increasing over the next 6 weeks (Suzuki et al. 1991). In contrast, NADPH-dependent T_3 binding was detected in cerebrum and cerebellum 5 days before birth, increasing with a sharp transient spike at the time of birth, particularly in cerebrum. The NADPH-dependent T_3 binding in cerebrum decreased after birth, but began to increase again 2 weeks after birth. The level in cerebellum did not show this increase. This spike in NADPH-dependent T_3 binding at birth was not observed in other organs. In fetal rats, production of thyroid hormones is known to only occur 5–6 days before birth (Strait et al. 1990) with a surge at and after birth, reaching maximal levels 10 days after birth (Dubois and Dussault 1977). Prior to fetal production of thyroid hormones, the fetus is reliant on maternal thyroid hormones. The placenta, however, maintains a barrier to their supply and fetal thyroid hormone levels are known to be much lower than circulating maternal thyroid hormones prior to initiation of fetal thyroid hormone production (Calvo et al. 1992).

Studies on L-pipecolate levels in developing brain show a remarkable similarity to the observed changes in the concentrations of NADPH-dependent T_3 -binding protein. The highest levels were observed in mouse and rat brain during the perinatal period with levels 6–10 times higher

than those in adult brain, and a subsequent rapid decrease after birth (Nishio and Segawa 1983; Kim and Giacobini 1985). This spike in L-pipecolate levels almost exactly mirrors the change in NADPH-dependent T_3 -binding protein concentrations noted at birth. Synthesis of L-pipecolate from L-lysine has been shown to be accelerated in the perinatal period in chick and mouse brain homogenates and this may also correlate with the observed changes in L-pipecolate levels (Nomura et al. 1978). It is interesting to note that by day 10 in rats, brain L-pipecolate is markedly decreased from its maximum just after birth (Nishio and Segawa 1983; Kim and Giacobini 1985). As noted previously, apart from the unexplained transient spike in NADPH-dependent T_3 -binding protein at birth, there is a gradual increase of NADPH-dependent T_3 binding until adulthood, which also corresponds to the increases in thyroid hormone levels. It should be noted that the developmentally related expression of NADPH-dependent T_3 binding (and by inference CRYM/KR expression) in the rat brain roughly correlates with the development of the pipecolate pathway. These results suggest that CRYM/KR and thyroid hormone levels may be major determining factors in the development of the pipecolate pathway in the brain. The relationships of thyroid hormones and the pipecolate pathway are further discussed in Sect. 9 in relation to tryptophan metabolism.

A complicating issue as regards T_3 binding to its cognate receptor in the brain is the finding that there are at least two different CTBPs in cerebral tissues. As described above, CRYM/ketimine reductase (CRYM/KR) is a well-characterized CTBP. A distinguishing feature of human CRYM/KR is that it binds T_3 in the presence of NADPH, but not in the presence of $NADP^+$ (Vie et al. 1997). A 38-kDa protein (approximately the monomeric size of CRYM/KR) is present in rat liver cytosol and was found to bind T_3 far better than do other cytosolic proteins (Moreno et al. 2003). As with the human CTBP (CRYM/KR), the rat liver 38-kDa protein strongly binds T_3 in an NADPH-dependent fashion and may thus be identical to CRYM/KR. Interestingly, however, the protein also strongly binds 3,5-diiodothyronine (3,5- T_2), but in an NADPH-independent fashion. 3,5- T_2 is suspected of playing a non-genomic role in energy metabolism at the mitochondrial level independent of any protein synthesis (Goglia et al. 1994). Moreno et al. (2003) suggest that the NADPH/ $NADP^+$ ratio may regulate T_3 /3,5- T_2 intracellular translocation. The effects on CRYM/KR enzyme activity by 3,5- T_2 have yet to be evaluated.

Another CTBP has been isolated from rat brain that binds T_3 tightly (K_a 1.56 nM) (Lennon 1992). SDS-PAGE analysis of the purified protein suggested a monomeric M_r of \sim 58,000. The binding capacity of this CTBP toward T_3 increased two- to fourfold in the presence of NADPH with

only a slight change in K_a to 2.6 nM (Lennon 1992). NADP⁺ was found to bind to this protein, but only in the presence of the strong thiol reductant dithiothreitol. The protein purified by Lennon may be similar to the 58-kDa CTBP characterized and identified in *Xenopus laevis* liver as an aldehyde dehydrogenase (ALDH), which preferentially uses retinal as a substrate (Yamauchi and Tata 1994). However, T₃ was shown to only weakly inhibit (at micromolar concentrations) the mitochondrial and cytosolic forms of human liver ALDH [competitively with respect to aldehyde substrate (propional) and uncompetitively with respect to NAD⁺] (Zhou and Weiner 1997). T₃ was also shown to competitively inhibit the oxidation of octanal by recombinant mouse retinaldehyde dehydrogenase-3 at micromolar concentrations (Graham et al. 2006).

Motivated by the prior finding that T₃ binds very strongly to CRYM in the presence of NADPH, we evaluated T₃ as a potential inhibitor of KR and found that it is a strong reversible inhibitor when AECK (a sulfur-containing analog of P2C) is used as substrate at pH 5 (K_i , 278 nM) (Hallen et al. 2011). A much stronger inhibition using low nM concentrations was found at neutral pH. Owing to technical difficulties in using AECK as a substrate at neutral pH a true K_i could not be measured for T₃. At neutral pH AECK exists predominantly in a tautomeric form (enamine) that is not a substrate of CRYM/KR (Nardini et al. 1988b). Thus, it was necessary to use an enzyme concentration greater than inhibitor concentration, resulting in difficulties in calculating an exact K_i at neutral pH. Nevertheless, our results together with the low K_d value determined by Beslin et al. (1995) at neutral pH, imply that enzyme activity would be tightly regulated in vivo by nM or even sub-nM concentrations of T₃. Our kinetic data indicate that cyclic ketimines and T₃ compete with each other in the active site of CRYM (Hallen et al. 2011). Therefore, we hypothesize that there will be a strong reciprocal relationship between the in vivo concentration of cyclic ketimines (and NADPH) and the concentration of free (i.e., unbound) T₃. In this regard, it is interesting to note that CRYM had previously been implicated in regulating the bioavailability of T₃ by a mechanism that involves redox-dependent translocation of T₃ to the nucleus (Mori et al. 2002). Prior to our discovery that CRYM is a KR, this relationship was suggested to possibly be due to changes in the overall intracellular NADPH/NADP⁺ ratio (Beslin et al. 1995). However, while our findings are certainly consistent with this notion, they add an additional layer of complexity. Thus, the availability of KR substrates of CRYM may well be an even more important factor than the NADPH/NADP⁺ ratio in the regulation of T₃ translocation to the nucleus. Inasmuch as CRYM effectively catalyzes the NADPH-dependent reduction of P2C to pipecolate (Hallen et al. 2011), our findings emphasize the

important concept that T₃ bioavailability is likely strongly dependent on the pipecolate pathway activity. Thus, any biological process that results in increased availability of the cyclic ketimine substrate P2C also has the potential to decrease the binding of T₃ to CRYM and consequently to allow the translocation of free T₃ to the nucleus. Conversely, any biological process that results in decreased concentration of P2C will promote increased tight binding of T₃ to CRYM, reducing the translocation of free T₃ to the nucleus. This is a novel concept in which the T₃ bioavailability and its role as a transcription factor are regulated by a component of the pipecolate pathway (i.e., P2C reductase activity).

As the above discussion attests, the relationship between L-pipecolate levels and KR at the *whole brain* level is now well established. However, there are still some apparent inconsistencies that require an explanation. These include the observed high level of L-pipecolate in cerebellum yet low expression of CRYM/KR in that region, and the discovery by Nardini et al. (1988a) of a cerebellar KR with kinetic properties (and size) different from those reported by us for CRYM/KR. As we mentioned above, the findings suggest that the brain may contain at least two distinct P2C reductases/KRs, one of which is predominant in the forebrain (Hallen et al. 2011) and another that is prominent in the cerebellum (Nardini et al. 1988a). The forebrain enzyme is CRYM/KR that has been characterized in our laboratory. Although Nardini et al. (1988a) have partially characterized the cerebellar enzyme, its identity in mammalian genome data banks is not yet established. In future work, it will be important to determine not only the gene that codes for the cerebellar enzyme, but also to more fully characterize its kinetic and regulatory properties and to ascertain the relative importance of the two enzymes in the brain pipecolate pathway. It seems unlikely that the cerebellar enzyme will also be a thyroid hormone-binding protein. Thus, in future work it will be important to determine the mechanism involved in regulation of the cerebellar enzyme and why the brain requires two separate KRs.

At this point it is important to note that although our discussion has dealt mainly with the reduction of P2C, the KRs may also have additional important metabolic functions, particularly in relation to the sulfur-containing cyclic ketimines. The sulfur-containing ketimine substrates for CRYM/KR are thought to have neurotransmitter/neuro-modulator roles. For example, Duprè et al. (1993) showed that [³⁵S]lanthionine ketimine (LK) strongly binds to brain membranes with a resulting elevation of basal cAMP levels. [The structure of LK and some other sulfur-containing ketimines is shown in Fig. 3.] LK (and its ethyl ester derivative) has been shown by Hensley et al. (2010, 2011) to have neurotrophic properties, such as promoting neurite

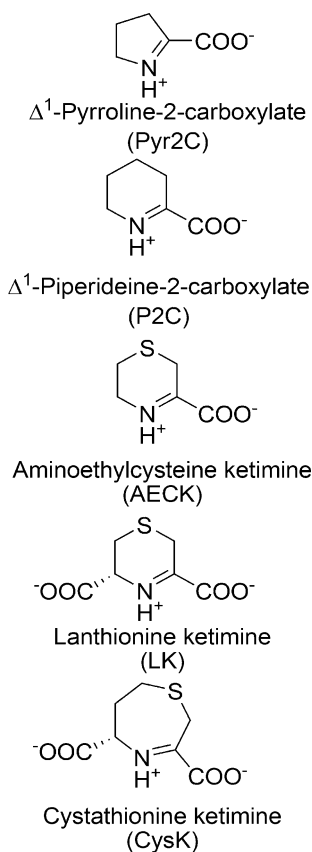


Fig. 3 Structures of several known CRYM/KR substrates and one putative substrate [Pyr2C]. The known substrates include P2C and the sulfur-containing ketimines, aminoethylcysteine ketimine (AECK), lanthionine ketimine (LK), and cystathionine ketimine (CysK). LK in particular has been shown to have neurotrophic properties (see the text). The five-membered ring analog of P2C, namely, Δ^1 -pyrroline-2-carboxylate (Pyr2C), has been reported to be a substrate for semi-purified P2C/Pyr2C reductases (Meister et al. 1957). However, although it seems likely to be a substrate, Pyr2C has not yet been tested as a substrate of highly purified CRYM/KR

outgrowth in duck dorsal root ganglia as well as in motor neuron-like cells. As yet, no such relationships have been studied or noted for P2C or Pyr2C. The discovery that CRYM is a P2C reductase, and its enzyme activity regulated by T_3 is consistent with the possibility that P2C and Pyr2C may also have neurotransmitter/neuromodulator roles. Thyroid hormones are known to affect neuronal differentiation and also to cause neurite outgrowth (Walter 1996). Further research is necessary to determine to what extent the inter-relationship between CRYM/KR, its cyclic ketimine substrates, as well as thyroid hormones, play in neuronal differentiation.

Northern blot analysis revealed that CRYM/KR mRNA in the mouse is most highly expressed in skin among the tissues evaluated, followed by brain and heart (Aoki et al. 2000). The expression of the CRYM transcript was found to follow the hair growth cycle with a significant increase

in expression during mid- and late anagen growth phases. The exact function and importance of CRYM as regards hair growth is unknown. Aoki et al. (2000) suggested the possible involvement of CRYM in the development of mouse hair follicles during the anagen phase. The discovery of an enzyme function for CRYM as a KR (Hallen et al. 2011) places the suggestion of Aoki et al. in a new light. We suggest that enzyme substrates (e.g., sulfur-containing cyclic ketimines such as AECK and LK, Fig. 3) may play a role in regulating cell growth and/or cell differentiation.

Steroid hormones have a marked effect on the expression of CRYM/KR. For example, androgens have been shown to induce CRYM expression in human prostate cancer (Malinowska et al. 2009). Estrogen has also been shown to increase CRYM expression in bovine mammary glands (Li et al. 2006). There are marked seasonal differences in the expression levels of CRYM/KR in the hypothalamus of the song sparrow (*Melospiza melodia*) with highest expression in spring and lowest in autumn (Mukai et al. 2009). A similar seasonal variation of CRYM/KR gene expression also has been noted in the ground squirrel *Ictidomys tridecemlineatus* (Schwartz et al. 2013).

It has long been thought that owing to the predominance of the pipecolate pathway in the brain, the pathway must play a specific role in neuronal development (Chang 1976, 1978). That role is still poorly understood. However, the findings that (1) CRYM/KR is a crucial enzyme in the pipecolate pathway, (2) the CRYM/KR activity is potentially regulated by T_3 , and (3) CRYM/KR substrates are potentially neuroactive and are highly informative. These findings are beginning to not only help in the elucidation of the biological role of the pipecolate pathway (and its associated enzyme activities), but also to emphasize the previously overlooked role of the pipecolate pathway in brain physiology and function.

Our discovery that T_3 regulates CRYM/KR activity (and enzyme substrate levels) raises the question as to whether at least some of the biological actions of T_3 are due to the interaction of T_3 with CRYM/KR. In our opinion, KR and their cyclic ketimine substrates (including sulfur-containing cyclic ketimines) are important topics for future neurochemical studies.

The significance of CRYM/KR in psychiatric and neurological disease

There is typically a delay of a few weeks before tricyclic antidepressants elicit a therapeutic response. This delay suggests that long-term neuroadaptive changes underlie the antidepressant actions rather than inhibition of neurotransmitter reuptake and signaling. This suggestion was the motivation for research performed on mice that were

treated chronically for 28 days with the tricyclic antidepressant amitriptyline at dosages, corrected for body weight, known to have a therapeutic effect in humans (Böhm et al. 2006). The nucleus accumbens is an area of the forebrain that has been suggested to be affected in schizophrenia (Chronister and DeFrance 1982; Gray et al. 1995) as well as in depression (Shirayama and Chaki 2006; Bewernick et al. 2010). Böhm et al. (2006) microdissected dopaminergic cells from the nucleus accumbens of control mice and of mice treated with amitriptyline, followed by RNA amplification and DNA microarray analysis. Among the 7,523 transcripts analyzed 64 were found to be significantly up-regulated and 31 significantly down-regulated. Interestingly, among those genes significantly up-regulated, CRYM/KR mRNA levels showed the greatest difference compared to control mice (24.3-fold increase) (Böhm et al. 2006). This finding suggests that amitriptyline may exert its effect, not only through its well-documented inhibition of the serotonin and norepinephrine transporters in the brain, but also possibly in part by stimulating the increased reduction of cyclic ketimines. This significant change in gene expression must be further evaluated in the context of the now known enzyme role of CRYM/KR.

D-Serine is an important co-agonist of the NMDA glutamate receptor. Endogenous levels of D-serine are regulated in part by DAAO (Snyder and Kim 2000). Hypostimulation of glutamate NMDA excitatory receptors is viewed as an important component of schizophrenia (Olney et al. 1999). Increased brain DAAO activity has been found in schizophrenia (Madeira et al. 2008) as well as decreased D-serine levels (Hashimoto et al. 2003). Beneficial effects of D-serine administration had previously been noted in schizophrenia (Tsai et al. 1998; Kantrowitz et al. 2010). The hypo-function of the NMDA receptor is commonly attributed to increased kynurenate (KynA) levels (as discussed in Sect. 9). However, we propose an additional contributory effect that is related to CRYM/KR of the pipecolate pathway in brain. As noted in Sect. 3, P2C is known to inhibit DAAO, but the sulfur-containing analog AECK is a far more potent DAAO inhibitor (Ricci et al. 1983). We suggest that levels of cyclic ketimine substrates of CRYM/KR (such as AECK and P2C) in the brain regulate DAAO activity and D-serine levels, and therefore also play a role in schizophrenia. The role of the pipecolate pathway in schizophrenia is further discussed in Sect. 9 in relation to tryptophan metabolism.

Gene expression profiling of schizophrenia pre-frontal cortex has revealed the CRYM/KR gene expression to be consistently decreased compared to that in controls (Middleton et al. 2002). In addition, CRYM/KR gene expression was shown to be down-regulated in pre-frontal cortices from a subset of schizophrenia patients (Akbarian et al. 2005). Moreover, low levels of the CRYM/KR protein have been

found in schizophrenia pre-frontal cortex (Martins-de-Souza et al. 2009). CRYM/KR protein expression has, however, been found to be up-regulated in the corpus callosum of schizophrenia patients (Sivagnanasundaram et al. 2007). Whether these differences are a primary cause or a secondary compensatory result of the pathology remains to be determined. Given the possible involvement of DAAO in schizophrenia and the potential role of cyclic ketimines such as AECK, in regulating activity of this enzyme, down-regulation of CRYM/KR gene/protein expression may represent a secondary compensatory mechanism to maintain glutamate NMDA receptor tone.

Dysregulation of CRYM/KR gene expression, with subsequent alterations in cyclic ketimine substrate levels, may be involved in the pathological or secondary compensatory mechanisms of a number of neurodegenerative and neuromuscular diseases. Abnormal expression of CRYM/KR has been reported in facioscapulohumeral muscular dystrophy (FSHD) with large increases of CRYM/KR protein detected in muscle biopsies from affected patients (Reed et al. 2007). No such increase was noted in biopsies of several other myopathies (Reed et al. 2007). In future work, the effects of resultant lower ketimine substrate levels should be evaluated as possibly being causal or contributory in the pathology. The CRYM/KR gene is also up-regulated in a murine model of familial amyotrophic lateral sclerosis (ALS) (Fukada et al. 2007). Fukada et al. (2007) suggested that this up-regulation may be related to the pathogenesis of familial ALS. In this regard, a member of the cyclic ketimine family, namely, LK, administered as its ethyl ester, has been shown to substantially alleviate ALS symptoms, and prevent weight loss in an ALS murine model (Hensley 2007). CRYM/KR gene expression has also been shown to be significantly up-regulated in a murine model of Alzheimer disease (George et al. 2010).

Finally, there are two known point mutations of human CRYM, both of which are associated with nonsyndromic deafness (Abe et al. 2003). The effects of these mutations on enzyme activity must now be evaluated. The inference is that levels of CRYM/KR substrates are important determinants in hearing as CRYM mRNA is highly expressed in human inner ear (Aoki et al. 2000).

The peroxisomal connection: oxidation of L-pipecolate

Patients with hyperlysinemia are known to excrete increased amounts of L-pipecolate in their urine relative to unaffected controls (Woody and Pupene 1970). The accumulation of L-lysine in these patients is now known to be due to a block in LKR/SDH (also known as AAS synthase) (Sacksteder et al. 2000). As noted above, the saccharopine

pathway is thought to be the main route for extracerebral L-lysine degradation in adult mammals. However, Woody and Pupene (1970) suggested that in patients with hyperlysinemia the increased level of L-pipecolate is indicative of an alternative pathway for lysine degradation i.e., via the L-pipecolate pathway. Zellweger syndrome is the result of a severe genetic disorder characterized by the failure to develop normal peroxisomes in affected individuals. The disease is characterized by impaired brain developmental and mental retardation (Versmold et al. 1977). Plasma L-pipecolate levels were found to be near normal in two newborn infants with Zellweger syndrome, but the level rose markedly within 2 months to pathological levels (Dancis and Hutzler 1986). The increase in plasma L-pipecolate in patients with Zellweger syndrome suggests that most, if not all, L-pipecolate oxidation occurs in peroxisomes in humans. Indeed, Wanders et al. (1988, 1989) showed that the oxidation of L-pipecolate is localized to peroxisomes in human liver and that this oxidation is deficient in liver from patients with Zellweger syndrome. These findings were subsequently confirmed by experiments showing that radiolabeled L-pipecolate is oxidized by a peroxisomal fraction of human liver and that this activity is deficient in patients with Zellweger syndrome (Mihalik et al. 1989). Rao and Chang (1990) also confirmed that L-pipecolate is oxidized in human liver peroxisomes and rigorously confirmed the identity of the product formed to be the cyclic ketimine P6C in equilibrium with its open-chain form AAS.

Early studies on the oxidation of L-pipecolate by *Pseudomonas putida* demonstrated that L-pipecolate is oxidized to P6C/AAS by an FAD-requiring dehydrogenase (Baginsky and Rodwell 1967). Subsequent studies showed that L-pipecolate oxidase (POX) is widespread in nature (Mihalik and Rhead 1989; 1991). Interestingly, however, differences occur in the subcellular location of the enzyme among various mammalian species. In rabbit, dog, guinea pig, pig, and sheep oxidation of L-pipecolate occurs in the mitochondrial fraction (Mihalik and Rhead 1991), whereas in rats oxidation of L-pipecolate occurs in both mitochondrial and peroxisomal fractions (Rao et al. 1993). In the rat brain, POX activity in the mitochondria is located in the matrix, whereas the activity in the peroxisomes is membrane-associated (Rao et al. 1993). POX activity in brain and liver was observed to occur early in rat fetal development, reaching maximum specific activity in the perinatal period immediately before birth, followed by a noticeable decline in specific activity with age (Rao et al. 1993). These changes are similar to those observed for CRYM/KR expression (Lennon et al. 1980) and also for L-pipecolate levels in brain (Kim and Giacobini 1985).

In contrast to the findings observed with tissues from other mammalian species (rabbit, dog, guinea pig, pig,

sheep, rat), POX in the kidneys of the Rhesus monkey is only present in the peroxisomal fraction (Mihalik and Rhead 1989). POX was subsequently purified to apparent homogeneity from Rhesus monkey liver and shown to be monomeric, with a M_r of $\sim 46,000$ and to possess a covalently bound FAD prosthetic group (Mihalik et al. 1991). Purified monkey liver POX was also shown to oxidize L-proline to a limited extent (Mihalik et al. 1991). Through peptide sequencing of the purified monkey liver POX, a reverse genetic approach was used to identify the cDNA for human POX (Dodt et al. 2000). The human POX possesses a C terminal AHL peroxisomal targeting sequence and is expressed in cultured human fibroblasts in a fashion consistent with its location in peroxisomes (Dodt et al. 2000). Previously, it was shown that D-pipecolate is an effective substrate of the peroxisomal enzyme DAAO (Zaar et al. 1986). Inasmuch as POX carries out an identical reaction to that catalyzed by DAAO (except that L-picolinate is the substrate rather than D-picolinate) it was considered that POX may be phylogenetically related to DAAO. However, comparative sequence analysis showed that human POX has no discernible relationship to DAAO (Dodt et al. 2000). Rather, human POX was found to be most similar to various monomeric sarcosine oxidases and dehydrogenases. The recombinantly expressed human enzyme was shown to oxidize both sarcosine (*N*-methyl glycine) and L-pipecolate equally effectively. [Both sarcosine and pipecolate may be regarded as *N*-substituted amino acids.] However, in patients with peroxisomal deficiency disorders, plasma pipecolate levels were found to be greatly elevated (95–126 μM ; control 1.8 μM), whereas plasma sarcosine levels were undetectable in both controls and patients. The authors suggested that POX mainly oxidizes L-pipecolate in vivo in humans (Dodt et al. 2000). As was found for the monkey POX, the human enzyme contains a covalently bound FAD prosthetic group. This covalent binding of the cofactor in human and Rhesus POX is in contrast to DAAO, where the cofactor is non-covalently bound (Dodt et al. 2000).

The markedly elevated levels of L-pipecolate in patients with Zellweger syndrome are thought to contribute to the neuropathological symptoms (Arneson et al. 1982). L-Pipecolate has been shown to reduce neuronal cell viability and cause neuronal apoptosis (Matsumoto et al. 2003). Matsumoto et al. (2003) suggested that L-pipecolate may cause encephalopathy by apoptosis rather than through depression of neurotransmission. The mechanism involved has yet to be elucidated. However, it may involve activation of POX and subsequent formation of reactive oxygen species (ROS) as a related enzyme, namely proline oxidase, has been implicated in contributing to apoptosis through generation of ROS (Liu et al. 2006). Significant apoptosis takes place during normal brain development (de

Graaf-Peters and Hadders-Algra 2006) and we suggest that formation of L-pipecolate and its subsequent oxidation by POX may be a contributing factor in this apoptosis.

The convergence of pathways and the mystery of B₆-dependent epilepsy solved

As noted previously in Sect. 2, the saccharopine and pipecolate pathways (Figs. 1, 2, respectively) for the metabolism of L-lysine both converge at the step in which AAS is oxidized to AAD. An NAD⁺-requiring oxidoreductase [namely, α -amino adipate semialdehyde dehydrogenase (AASDH)] responsible for the oxidation of AAS to AAD was first isolated from *P. putida* and characterized (Calvert and Rodwell 1966). Subsequently, an AASDH was shown to be present in *Streptomyces clavuligerus* and its highly conserved gene was successfully sequenced (Pérez-Llarena et al. 1998). Rao and Chang (1990) followed their successful isolation and characterization of AAS as the oxidation product of POX in tissues of several mammalian species with the characterization of a cytosolic AASDH enzyme in human liver (Chang et al. 1990). However, it was not until 16 years later that the human AASDH gene was identified.

The identification of the human AASDH gene came about as a result of a curious series of events. An inexplicable (at the time) severe intractable form of early onset epilepsy was first described in 1954 in which the patients exhibited resistance to conventional pharmaceutical interventions and yet showed an abrupt therapeutic response upon administration of pyridoxine (Hunt et al. 1954). [Pyridoxine is a B₆ vitamin that is converted in vivo to the most biologically active form of vitamin B₆, namely, pyridoxal 5'-phosphate (PLP)] The mechanism for the therapeutic effect of pyridoxine, however, remained unexplained for more than 50 years. Glutamate decarboxylase (GAD) is a PLP-dependent enzyme that converts the excitatory neurotransmitter glutamate to the inhibitory neurotransmitter GABA. Therefore, it was reasonable to suggest that a possible cause of seizures alleviated by vitamin B₆ is due to an endogenous compound that reacts with PLP and/or a deficiency of GAD (Gospe et al. 1994). However, no linkage was found between the two brain GAD isoforms and pyridoxine-dependent seizures (Battaglioli et al. 2000). Moreover, the gene defect associated with pyridoxine-dependent seizures was mapped to chromosome 5q31 (Cormier-Daire et al. 2000), a region not associated with GAD genes.

The first important clue as to the origin of pyridoxine-dependent seizures came from the observation that a patient with a defect in Pyr5C dehydrogenase (hyperprolinemia type II) presented with both a vitamin B₆

deficiency and seizures (Walker et al. 2000). It was suspected by Walker et al. (2000) that accumulation of Pyr5C resulting from this enzyme defect was in some way inactivating vitamin B₆. Farrant et al. (2001) confirmed that this is indeed the case and showed that Pyr5C reacts with PLP through a Knoevenagel condensation, thus effectively preventing it from being used as an enzyme cofactor. In effect, Pyr5C acts as an anti-vitamin by covalently removing PLP. Thus, a defect in Pyr5C dehydrogenase accounts for one form of epilepsy responsive to vitamin B₆ therapy. However, the majority of epileptic patients responsive to vitamin B₆ therapy do not have this enzyme defect. As emphasized previously in Sect. 2, AAS is in equilibrium with its cyclic form P6C, and P6C is an analog of Pyr5C. Thus, it was rationalized that a defect in the human homologue of the previously described microbial AASDH gene (Pérez-Llarena et al. 1998) may be responsible for the disorder. By using a highly conserved region of the microbial AASDH gene, the sequence in the human genome analogous to this microbial enzyme was identified as the gene coding for aldehyde dehydrogenase 7A1 (ALDH7A1) and this gene mapped to chromosome 5q31 previously identified through linkage analysis (Mills et al. 2006). Mutations in *ALDH7A1* were shown by Mills et al. (2006) and Plecko et al. (2007) to be present in several patients presenting with pyridoxine-responsive seizures. Interestingly, the AASDH encoded by the *ALDH7A1* gene was already well known as antiquitin, a highly conserved protein with 60 % identity to that, for example, of a protein present in the garden pea (Lee et al. 1994). The identity of human aldehyde dehydrogenase AASDH as antiquitin/ALDH7A1 was conclusively established by Mills et al. (2006). Thus, a defect in antiquitin/ALDH7A1/AASDH will result in accumulation of P6C. It was shown that P6C, in a similar manner to Pyr5C, also forms a Knoevenagel condensation product with PLP (Mills et al. 2006; Plecko et al. 2007). [It is not currently known whether P2C, which is a double bond positional isomer of P6C, can also react with PLP in a similar manner to that exhibited by P6C, but a condensation product between PLP and P2C seems highly likely. Thus, an important function of KRs may be to maintain levels of P2C at a low level to avoid compromising PLP-dependent enzymes.]

Folinic acid-responsive seizures also result from mutations in antiquitin/AASDH/ALDH7A1 (Hyland et al. 1995; Nicolai et al. 2006). However, the biochemical mechanism involved is not currently understood (Hyland et al. 1995; Nicolai et al. 2006). The implication is that the biochemical repercussions resulting from accumulated P6C may be more complex than can be explained solely on the basis of PLP removal. Moreover, even though increased urinary AAS is an important indicator of antiquitin/ALDH7A1/AASDH deficiency (pyridoxine-dependent epilepsy), this

compound is increased in the urine of patients with other disorders Mills et al. 2012). For example, sulfite was also shown in vitro with human liver homogenates to be a strong specific inhibitor of AASDH, with an accompanying increase in AAS (Mills et al. 2012). This finding may explain the marked accumulation of AAS in sulfite oxidase deficiency as well as in a deficiency of molybdenum, a sulfite oxidase cofactor (Mills et al. 2012).

At this point it is worth emphasizing the different subcellular localizations of the pipecolate and saccharopine pathways. The first step in the pipecolate pathway (Fig. 2), as discussed in Sect. 3, involves a conversion of L-lysine to KAC/P2C in the cytosol. As also discussed, this step is possibly catalyzed by GTL/KAT III, which is present in the cytosolic fraction of mammalian tissues. The second step of the pipecolate pathway, namely, the reduction of P2C by CRYM/KR also takes place in the cytosol. The next enzyme in the pipecolate pathway (POX, responsible for oxidation of pipecolate) occurs in the peroxisomes as discussed previously in Sect. 7. Another enzyme further downstream in the pathway (AASDH) is also present in the cytosolic compartment (Chang et al. 1990). It is thus reasonable to suggest that the pipecolate pathway is predominantly a cytosolic pathway with a peroxisomal component. The saccharopine pathway on the other hand is a well-established mitochondrial pathway. However, there is one caveat that requires discussion. Recently, AASDH was definitively established to be present in the cytosol and mitochondria of various mammalian tissues (Wong et al. 2010; Chan et al. 2011). This distribution results from two isoforms of the enzyme—a cytosolic isoform (ALDH7A1_v2) that lacks a mitochondrial targeting sequence, and a mitochondrial isoform that possesses an additional 28 amino acid mitochondrial signaling peptide at the N-terminus (ALDH7A1_v1) (Brocker et al. 2010). The mitochondrial ALDH7A1 was found, by Brocker et al. (2010), to be the predominant form in mouse kidney and liver in which the saccharopine pathway is the major lysine degradative pathway. Both cytosolic and mitochondrial ALDH7A1 isoforms are present in adult brain where the pipecolate pathway is predominant (Brocker et al. 2010). These findings suggest that even though the pipecolate and saccharopine pathways merge at the metabolite AAS, further catabolism of AAS may well be separated in different compartments depending on the pathway i.e., cytosolic/peroxisomal and mitochondrial. Fibroblasts normally metabolize L-lysine through the saccharopine pathway and not through the pipecolate pathway. Nevertheless, Struys and Jakobs (2010) demonstrated formation of L-pipecolate from L-lysine in AASDH-deficient fibroblasts. The authors presented an evidence that L-pipecolate is produced in these cells via saccharopine and AAS/P6C (Struys and Jakobs 2010). Struys and Jakobs (2010) suggested that the

enzyme responsible for this alternative pathway for the formation of L-pipecolate may be Pyr5C reductase.

AASDH is a member of the aldehyde dehydrogenase superfamily, and besides its role in the reduction of AAS it also is involved in the detoxification of other reactive aldehydes such as betaine aldehyde, converting it to the osmoprotectant glycine betaine (*N,N,N*-trimethylglycine) (Brocker et al. 2010). The enzyme can also act on longer chain aldehydes derived from the oxidation of lipids (Brocker et al. 2010). AASDH was first purified from Atlantic seabream (*Mylio macrocephalus*) and characterized as an aldehyde dehydrogenase based on its activity with acetaldehyde (Tang et al. 2002). The role of AASDH in lysine metabolism was unknown at that time. Reactive aldehydes are potentially toxic and can readily form adducts and condensation products with small molecules (e.g., amines) as well as with proteins and DNA (Marnett 2002; Brooks and Theruvathu 2005). In addition to its mitochondrial and cytosolic subcellular localization, AASDH is present in the nucleus (Brocker et al. 2010), where it is suggested to protect against aldehyde-induced DNA damage (Chan et al. 2011). In this regard, AASDH/ALDH7A1 is similar to aldehyde dehydrogenase 3A1 (ALDH3A1), which is also localized to both mitochondria and nucleus (Pappa et al. 2005). Interestingly, AASDH accumulates maximally in the nucleus during the G₁-S phase of replication, which is a time when oxidative stress is known to be most deleterious (Havens et al. 2006). However, its role in detoxification of aldehydes may not be its only function in the nucleus (Chan et al. 2011). We suggest that AASDH may also function in a similar manner to nuclear lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, both of which are known to be part of the histone 2B transcription activation complex (Dai et al. 2008).

In summary, mutations in AASDH can give rise to pyridoxine-dependent and folinic acid-dependent seizures. Moreover, AASDH is a multifunctional enzyme with roles in: (1) lysine degradation; (2) protection against oxidative stress by converting reactive aldehydes derived from lipid peroxidation (and other metabolic processes) to less reactive/toxic carboxylic acids; (3) conversion of betaine aldehyde to the osmoprotectant betaine; and (4) possible protection against DNA damage by reactive aldehydes and/or functioning to regulate DNA transcription.

Interconnections between lysine and tryptophan pathways: developmental and regulatory aspects

AAD formed by AASDH is subsequently transaminated to form α -ketoadipate (AKA). Historically, Broquist et al. (1961), in experiments on *Saccharomyces cerevisiae*, first

described a transaminase reaction involving AAD and AKA that was suspected to play a role in lysine biosynthesis. However, it was not clear whether AAD aminotransferase activity is catalyzed by a discrete enzyme or whether the activity is catalyzed by an already characterized aminotransferase. Subsequently, Matsuda and Ogur (1969) showed that yeast contains two aminotransferases that catalyze the reversible transamination between AAD and α -KG to form AKA and glutamate. The authors named these aminotransferases glutamate-AKA transaminase I and II (GKAT I and GKAT II). These GKAT isozymes were shown to be distinct from aspartate aminotransferase and two forms of alanine aminotransferase (Matsuda and Ogur 1969).

The first indication of the presence of a mammalian AADT (Figs. 4, 5, AADT) came from the discovery in rat liver mitochondria of an enzyme capable of catalyzing transamination between AAD and α -KG (Nakatani et al. 1970). The purified enzyme was found to possess a loosely bound PLP cofactor. Even though the first indications were that AADT is a mitochondrial enzyme, enzyme activity was later demonstrated to be present in both the cytosolic and mitochondrial fractions of rat liver and kidney (Tobes and Mason 1975, 1977). Cytosolic activity is consistent with a role in the pipercolate pathway and mitochondrial activity is consistent with a role in the saccharopine pathway.

Aminotransferases often exhibit wider specificity than first realized, leading to some confusion in the literature regarding nomenclature, and AADT is an example (Figs. 4, 5). An enzyme designated kynurenine aminotransferase (KAT) that catalyzes transamination between L-kynurenine (Kyn) and α -KG was shown to be present in *Pseudomonas fluorescens* by Miller et al. in 1953 and in animal tissues by Mason in 1954. However, Tobes and Mason (1975, 1977) convincingly showed that AADT and KAT are identical enzymes in the rat. [This enzyme is now designated as KAT II. As noted above, KAT I is identical to GTK and KAT III is identical to GTL. An additional KAT (KAT IV) is identical to mitochondrial aspartate aminotransferase (Guidetti et al. 2007).] Tobes and Mason (1975, 1977) found that rat liver possesses mostly mitochondrial KAT activity, whereas in rat kidney the supernatant fraction is more active. AAD was demonstrated to be a better substrate than Kyn. Takeuchi et al. (1983) also confirmed that AADT from rat liver mitochondria is identical to KAT. These authors showed that enzyme activity is strongly inhibited by Hg^{++} as well as by *p*-chloromercuribenzoate, suggesting that a crucial cysteine is in or near the active site. Once again AAD was found to be a better substrate than Kyn.

The relationship between AADT and KAT, however, may be more complicated than the above discussion

suggests. Deshmukh and Mungre (1989) reported that bovine kidney possesses appreciable AADT activity but minimal KAT activity, suggesting that different enzymes catalyze the two reactions. In spite of evidence noted above suggesting that AADT and KAT are identical enzymes, Mawal and Deshmukh (1991) separated two enzymes chromatographically from rat liver supernatant fractions and claimed that AAD and Kyn transamination are due to two distinctly different enzymes, seemingly throwing into doubt the work of Tobes and Mason (1975, 1977). However, a soluble rat kidney enzyme with dual AADT/KAT activities was identified, cloned, and functionally expressed by Buchli et al. (1995). The predicted amino acid sequence showed no evidence of a mitochondrial signaling sequence. Among the α -keto acids tested when Kyn was employed as the amino acid substrate, the recombinant enzyme was shown to have highest specific activity with α -KG and relatively poor activity with pyruvate. The authors also showed that this Kyn- α -KG aminotransferase is distinct from KAT I/GTK. Previously, two AADT isoenzymes were purified from human liver (AADT I and II) by Okuno et al. (1993). AADTI was shown to be localized to the supernatant (cytosol) fraction and AADTII to the mitochondria. AADTI was shown to be highly specific for AAD and glutamate as amino acid substrates and α -KG and AKA as α -keto acid substrates. On the other hand, AADTII was shown to possess KAT activity. AADTI was shown to have a high K_m (20 mM) for AAD and a low K_m (1.4 mM) for glutamate. AADTII, on the other hand, was found to have a low K_m (0.25 mM) for AAD and a high K_m (12.5 mM) for glutamate. AADTII was also shown to have a low K_m (0.95 mM) for Kyn. The human gene (AADT) encoding AADT was subsequently identified and localized to chromosome 4q32.2 (Goh et al. 2002). Human AADT was found to be 72 and 73 % identical to the mouse and rat KAT II orthologues, respectively. The gene is suspected to code for AADTII and possesses a mitochondrial signaling peptide. Recombinant human AADT/KAT II, as noted above, has a broad amino acid and α -keto acid specificity (Han et al. 2008). Among the amino acids tested as substrates, the highest catalytic efficiency (K_{cat}/K_m) was noted with AAD ($196 \text{ min}^{-1} \text{ mM}^{-1}$) followed by Kyn ($126 \text{ min}^{-1} \text{ mM}^{-1}$) (Han et al. 2008). The predicted mitochondrial signal sequence is the first N-terminus 22 amino acid, but a recombinant enzyme that lacked this sequence showed no enzyme activity (Han et al. 2010). Further research is required to ascertain how the enzyme activity is apportioned between mitochondria and cytosol. A cytosolic subcellular localization is consistent with its role in the pipercolate pathway and a mitochondrial location with the saccharopine pathway.

The initiation of the Kyn metabolic pathway, through the action of indoleamine 2,3-dioxygenase (IDO) or

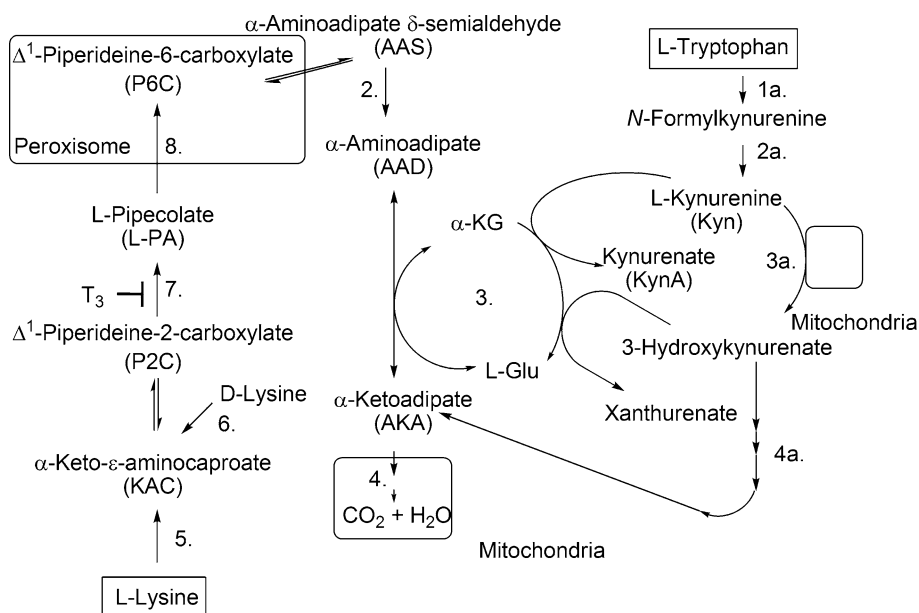


Fig. 4 The interconnection of L-lysine and L-tryptophan degradative pathways in mammals. *Enzymes:* 2–8 as per Figs. 1 and 2; 1a indoleamine dioxygenase/tryptophan dioxygenase (IDO/TDO); 2a kynurenine formylase; 3a kynurenine monooxygenase (depicted as occurring on the mitochondrial outer membrane); 4a enzymes catalyzing the conversion of 3-hydroxykynurenate to α -keto adipate (AKA). Note that as discussed in the text, the conversion of L-lysine to KAC in the pipecolate pathway may be catalyzed by KAT III/GTL. The most important aminotransferase that catalyzes the conversion of

α -amino adipate to α -keto adipate in the pipecolate pathway for L-lysine degradation is the same enzyme that catalyzes the conversion of kynurenine to kynurenate [i.e., α -amino adipate aminotransferase/kynurenine aminotransferase II (AADT/KAT II); enzyme 3 in this figure and Figs. 1, 2]. Thus, any perturbation in one pathway will influence the other. This interconnection between lysine and tryptophan degradative pathways has implications in psychiatric as well as neurological illnesses as discussed in the text

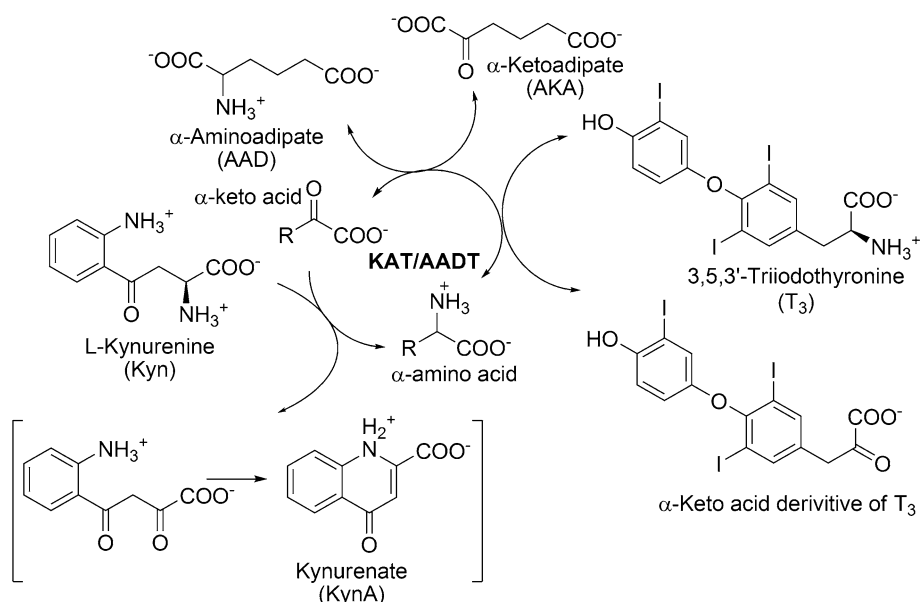


Fig. 5 The remarkable amino acid substrate versatility of α -amino adipate aminotransferase/kynurenine aminotransferase II (AADT/KAT II). Not only is the enzyme active with AAD and Kyn, but it can also transaminate thyroid hormones (e.g., T_3) and halogenated tyrosines as discussed in the text. The thyroid hormone T_3 is a strong inhibitor of CRYM/KR, and AADT/KAT II activity is also potentially

regulated by circulating thyroid hormones. In the text, we postulate that thyroid hormones play a pivotal role in the development of the pipecolate pathway in mammalian brain. The biological importance of T_3 transamination is unknown although it has been suggested that KAT (i.e., KAT II) may be important for the metabolism of thyroid hormones (Tobes and Mason 1978)

tryptophan 2,3-dioxygenase (TDO), is known to suppress the immune response and in particular to prevent rejection of the developing fetus (Gonzalez et al. 2008). The *N*-formylkynurenine formed by the action of either IDO or TDO is rapidly degraded to form Kyn by the action of kynurenine formylase, an enzyme that is abundant in most mammalian tissues (Mehler and Knox 1950). The Kyn pathway undergoes marked developmental changes in fetal brain. Kyn and KynA levels in rat and primate fetal brain are markedly high in comparison to post-natal brain, with a gradual decrease in Kyn as well as KynA levels during the course of gestation and a subsequent rapid decrease after birth (Beal et al. 1992). The high level of Kyn pathway activity in fetal brain and subsequent decrease during development are similar to the changes noted for the high levels and subsequent decrease in expression of LKR/SDH (Rao et al. 1992) (the first enzyme activities in the lysine saccharopine pathway) as discussed in Sect. 2. The decrease in Kyn and KynA levels are also inversely related to the developing ascendancy of the pipecolate pathway as the dominant lysine degradation pathway in adult mammalian brain (Chang 1976). KynA is known to inhibit neurite outgrowth by NMDA receptor stimulation in granule cells in culture (Pearce et al. 1987). We suggest that the high levels of LKR/SDH observed in fetal brain neurons may represent a compensatory mechanism to overcome this inhibition by supplying a *de novo* source of glutamate.

A day before birth in rat fetal brain there is a spike in Kyn and KynA levels (Beal et al. 1992) similar to that observed for rat fetal brain pipecolate (Kim and Giacobini 1985; Nishio and Segawa 1983) as well as NADPH-dependent cytosolic thyroid hormone binding (Suzuki et al. 1991). At day 1 post-delivery in rat brain, KynA levels decrease to adult levels (Beal et al. 1992). This finding is in marked contrast with the formation of *L*-pipecolate (as discussed in Sect. 5), where expression of NADPH-dependent T_3 -binding protein (and by inference CRYM/KR) only reaches detectable levels a few days before birth in fetal rat brain (Suzuki et al. 1991). There is a rapid increase in neurite outgrowth and development of excitatory synapses after birth (Lin et al. 2002), which corresponds to the period of rapid decreases in KynA levels. Beal et al. (1992) also showed that brain levels of nor-epinephrine, dopamine, and DOPAC increase at a time that correlates with the spikes seen with CRYM/KR and also Kyn and KynA.

In Sect. 3, we presented arguments suggesting that the first enzyme(s) in the pipecolate pathway for lysine degradation may be KAT III/GTL. On the other hand, KAT II/AADT is well documented to be involved downstream in the catabolism of both tryptophan and lysine. GTK/GTL are also believed to be responsible for the biosynthesis of

the CRYM/KR sulfur-containing cyclic ketimine enzyme substrates (such as AECK and LK) (Pensa et al. 1989). One of the cyclic ketimines, namely, LK (as mentioned in Sects. 5 and 6), has been shown to cause neurite extension in duck dorsal root ganglia and motor neuron-like cells (Hensley et al. 2010, 2011). We hypothesize that a neuro-differentiation effect may be a generalized characteristic of all the cyclic ketimines and that activation of the pipecolate pathway may be a critical factor in this rapid post-natal neuronal development.

Thyroid hormones play an important regulatory role in the pipecolate pathway as discussed in Sect. 5. They may also play a role in regulation of KAT II/AADT enzyme regulation. Levels of metabolites in the tryptophan/kynurenine pathway are markedly altered in thyroxine-treated rat liver, with a threefold increase in Kyn and KynA levels (Okamoto et al. 1971). KAT activity increased proportionally. Okamoto et al. (1971) suggested that this effect is due to an increase in *de novo* KAT synthesis. Tobes and Mason (1978) showed that KAT (i.e., KAT II) not only functions to transaminate both AAS and Kyn, but also effectively transaminates halogenated tyrosines (such as 3,5-diiodotyrosine) as well as, if not better than, Kyn or AAS, and suggested that halogenated tyrosine transaminase and KAT (KAT II) are identical enzymes based on their kinetic observations (Tobes and Mason 1978). It had earlier been suggested that halogenated tyrosine transaminase is identical to thyroid transaminase (Nakano 1967; Nakano and Danowski 1964; Nakano et al. 1964). The remarkable activity of KAT II as a KYN/AAD/ T_3 aminotransferase is shown in Fig. 5. The marked increase in fetal thyroid hormone production before and after birth in rats, thus, may be causally related to the spikes observed in KynA and pipecolate formation at this time through induction of CRYM/KR expression.

The activity of KAT II/AADT is important in preventing neurotoxicity and neurodegeneration as it regulates the levels of AAD, KynA, and possibly thyroid hormones in the brain. AAD is a 6-carbon dicarboxylic acid homologue of *L*-glutamate, which is known to be toxic to glial cells and is taken up by astrocytes prior to exerting its gliotoxic effects (Huck et al. 1984). AAD is also thought to interfere with glutamate uptake into astrocytes (Tsai et al. 1996). In astrocyte cultures, KynA synthesis is inhibited by AAD as well as by *L*-pipecolate (Chang et al. 1997). In contrast to the toxicity demonstrated by AAD, KynA is a neuroprotectant and an endogenous inhibitor of excitatory glutamate NMDA receptors (Stone 1993). Therefore, the biosynthesis of KynA in the brain and the regulation of KAT enzymes are of utmost importance. In this regard, *L*-cysteine sulfinate, an endogenous intermediate in sulfur-containing amino acid metabolism, has been found to be a selective AADT/KAT II enzyme inhibitor and is suspected of

modulating KAT II activity as well as AAD levels in the brain (Kocki et al. 2003).

Changes in brain KynA levels have been noted in various neurological and psychiatric conditions. For example, in Alzheimer's disease brain, a significant increase in AAD synthesis was found coupled with a decrease in KAT activity (Yang et al. 1995). An intriguing finding is that KynA levels are increased in the cerebrospinal fluid and cortex of schizophrenia patients (Erhardt et al. 2001; Schwarcz et al. 2001). As noted elsewhere in this review, AKA formed by KAT II/AADT is a common intermediate in L-tryptophan as well as in L-lysine catabolic pathways, and it is reasonable to suggest that the metabolism of the one amino acid will influence the metabolism of the other. This relationship may explain the observed positive therapeutic response observed with L-lysine supplementation in a cohort of schizophrenia patients (Wass et al. 2011). It is possible that a decrease in the activity of the pipecolate pathway for lysine metabolism plays a role in schizophrenia. A decrease in this pathway will result in lower amounts of AAD and hence less inhibition of KAT II, leading to increased KynA. Thus, the positive effect of lysine in schizophrenic patients may be due to stimulation of the pipecolate pathway in brain leading to decreased KynA. The CRYM/KR substrates P2C and AECK have been noted in Sect. 6 as also of possible importance in schizophrenia by virtue of their strong inhibition of DAAO.

Thus far our discussion of L-lysine degradation has been restricted to biological processes in the pipecolate and saccharopine pathways that terminate at AKA formation. Further degradation occurs only in the mitochondria, namely, subsequent formation of glutaryl-CoA and eventual catabolism to CO₂ in the TCA cycle. These steps are not discussed in the present review. Detailed reviews of this pathway may be found, for example, in Benevenga and Blemings (2007) and Sauer et al. (2011).

Summary

The pipecolate pathway has evolved as the main L-lysine degradative pathway in adult mammalian brain, whereas in the developing brain and extracerebral tissues the saccharopine pathway predominates. The reason for this dichotomy is not fully understood, but we are slowly beginning to understand the importance of the pipecolate pathway in brain metabolism and function. We predict that the discovery of an enzyme in mammalian forebrain (namely CRYM/KR) that is capable of effectively reducing the intermediate metabolite P2C to L-pipecolate will be a significant step in advancing our knowledge of the pipecolate pathway and its neurological/neurochemical importance. P2C (as well as other cyclic ketimine substrates of KR)

may play a crucial role in brain development, metabolism, and electrophysiology/neurotransmitter regulation. The finding that T₃ strongly regulates CRYM/KR enzyme activity suggests a previously unsuspected inter-relationship between amino acid metabolism and the endocrine system. With the completion of the human genome project, scientists were at first astounded by the relatively low number of genes coding for proteins (~21,000). However, it is now apparent that many gene-encoded proteins exist in multiple forms, and many possess multiple and often disparate roles. Two pivotal enzymes in the lysine degradation pathways, namely, CRYM/KR and AASDH, are examples of "moonlighting" enzymes that have evolved to possess two or more disparate functions.

The mechanism by which L-lysine is oxidized to KAC/P2C is unknown. One possibility is that the reaction is catalyzed by KAT III/GTL. If verified, inasmuch as Kyn is an important metabolite in L-tryptophan metabolism, the involvement of an aminotransferase that catalyzes transamination of both L-lysine and L-kynurenine would suggest a close relationship between lysine degradation and tryptophan degradation. Changes in the concentration of the product of Kyn transamination, namely, KynA, are implicated in many neurological and psychiatric disorders. We suggest that any discussion as to the relevance of the tryptophan-Kyn degradation pathway in neurodegeneration and illness should also take into account its "close relation", namely, the lysine degradation pathway.

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Conflict of interest The authors declare that they have no conflict of interest.

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