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Perinatal kynurenine pathway metabolism in the normal and asphyctic rat brain

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Summary. The kynurenine pathway of tryptophan degradation contains several metabolites which may influence brain physiology and pathophysiology. The brain content of one of these compounds, kynurenic acid (KYNA), decreases precipitously around the time of birth, possibly to avoid deleterious N-methyl-D-aspartate (NMDA) receptor blockade during the perinatal period. The present study was designed to determine the levels of KYNA, the free radical generator 3-hydroxykynurenine (3-HK), and their common precursor L-kynurenine (L-KYN) between gestational day 16 and adulthood in rat brain and liver. The cerebral activities of the biosynthetic enzymes of KYNA and 3-HK, kynurenine aminotransferases (KATs) I and II and kynurenine 3-hydroxylase, respectively, were measured at the same ages. Additional studies were performed to assess whether and to what extent kynurenines in the immature brain derive from the mother, and to examine the short-term effects of birth asphyxia on brain KYNA and 3-HK levels. The results revealed that 1) the brain and liver content of L-KYN, KYNA and 3-HK is far higher pre-term than postnatally; 2) KAT I and kynurenine 3-hydroxylase activities are quite uniform between E-16 and adulthood, whereas KAT II activity rises sharply after postnatal day 14; 3) during the perinatal period, KYNA, but not L-KYN, may originate in part from the maternal circulation; and 4) oxygen deprivation at birth affects the brain content of both KYNA and 3-HK 1 h but not 24 h later.

Keywords: Amino acids – Asphyxia – Excitotoxicity – 3-Hydroxykynurenine – Kynurenic acid – N-Methyl-D-aspartate (NMDA)

Introduction

Several metabolites of the kynurenine pathway of tryptophan degradation, in particular the preferential glycine/N-methyl-D-aspartate (NMDA) receptor antagonist kynurenic acid (KYNA), the free radical generator 3-hydroxykynurenine (3-HK) and the excitotoxin quinolinic acid (QUIN), have

neuroactive properties in the mammalian brain (Stone, 1993; Moroni, 1999). Because of their potential role in CNS physiology and pathology, the metabolism and function of these compounds have been examined in some detail during the past two decades. In the course of these studies, most enzymes of the kynurenine pathway were identified and characterized in the rodent and human brain, selective antibodies against these enzymes were used for immunocyto-chemical analyses, and molecular biological methodologies were applied to clone several of the enzymes and examine their expression patterns in the normal and abnormal brain. Cumulatively, these studies have indicated that kynurenine pathway metabolites of astro- and microglial origin may function as modulators of synaptic neurotransmission (Stone, 1993; Schwarcz et al., 1996; Moroni, 1999).

One of the most intriguing discoveries during the study of neuroactive kynurenines was the observation that the brain levels of KYNA are remarkably high during the late fetal period and decrease dramatically around the time of birth. This phenomenon, which has so far been described in rats, monkeys (Beal et al., 1992) and sheep (Walker et al., 1999), led to the speculation that the high brain content of the endogenous NMDA receptor antagonist KYNA at the time of delivery serves a neuroprotective role against possible parturition-related hypoxic-ischemic insults (cf. McDonald et al., 1987; Hattori et al., 1989). Exogenously supplied KYNA indeed provides impressive protection against the effect of experimental neonatal hypoxia (Andiné et al., 1988). It was also hypothesized that the rapid decline of cerebral KYNA levels immediately following birth is necessary to avoid the postnatal blockade of NMDA receptor function, which is known to be critical for normal brain development (Balazs et al., 1988; Simon et al., 1992; Komuro and Rakic, 1993).

In the adult mammalian brain, the formation of KYNA is normally catalyzed primarily by kynurenine aminotransferase II (KAT II), one of the two KATs present in cerebral tissue (Guidetti et al., 1997). Notably, KYNA's bioprecursor L-kynurenine (L-KYN) is also metabolized by kynurenine 3-hydroxylase to produce 3-HK (Erickson et al., 1992). Attempts to study the possible role of KYNA in perinatal brain injury therefore ought to examine these competing branches of L-KYN degradation in parallel. Since the pre- and postnatal development of several elements of the kynurenine pathway has not been described so far, the present study was designed to provide baseline data for some of these parameters in rat tissues. Moreover, analyses of maternal serum and brain were made to clarify the source of L-KYN and KYNA in the developing brain. Finally, we report the results of a preliminary study of the acute effects of birth asphyxia on KYNA and 3-HK levels in the brain.

Materials and methods

Materials

L-Kynurenine sulphate (L-KYN), KYNA and 3-HK were purchased from Sigma Chemical Co. (St. Louis, MO). Dowex 50W cation exchange resin (200–400 mesh, H^+-

form) was obtained from Bio-Rad (Hercules, CA). All other chemicals were of the highest commercially available purity.

[^{3}H]-Kynurenine (specific activity: 9.3 Ci/mmol) was obtained from Amersham (Arlington Heights, IL) and was purified by HPLC as described by Speciale and Schwarcz (1990).

Animals

Timed-pregnant Sprague-Dawley rats were purchased from Charles River (Kingston, NY) and kept in an AAALAC-approved animal facility at a 12h/12h light/dark cycle with free access to food and water. Rat embryos were delivered by Caesarean (C-) section on gestational days 16, 19 and 22 (E-16, E-19 and E-22). Male rats were examined on the day of birth (P-0) and on postnatal days 1, 3, 5, 7, 14 and 28 (P-1 – P-28). Adult male rats (2.5 months-old) were also included in the study. Animals of all postnatal ages were anesthetized with chloral hydrate, and an aliquot of blood was removed through heart puncture. The rats were then transcardially perfused with 10–20 ml ice-cold saline solution and decapitated. Their forebrain (brain minus cerebellum) and liver were immediately dissected out and frozen. In the blood samples, red blood cells were removed by centrifugation, and the resulting supernatant plasma was frozen. Tissues and plasma were stored at $-80°$ until the day of the assay.

Determination of KAT I and KAT II activities

Tissues were thawed and sonicated (1 : 5, w/v) in distilled water. An aliquot of the original tissue homogenate was further diluted (up to 1 : 10, v/v) in a buffer containing 5 mM Tris-acetate (pH 8.0), 50 μM pyridoxal-5-phosphate and 10 mM 2-mercaptoethanol, and dialyzed overnight at 4°C against 4 l of the same buffer. For determination of KAT I, the reaction mixture contained 150 mM 2-amino-2-methyl-l-propanol (AMP) buffer (pH 9.5), 25 nCi [^{3}H]-KYN, 2 μM L-KYN, 1.1 mM pyruvate, 80 μM pyridoxal-5′-phosphate and 80 μl of dialyzed tissue in a total volume of 200 μl. A similar mixture was used to determine KAT II activity, except that 150 mM Tris-acetate buffer (pH 7.0) was substituted for AMP buffer, and 2 mM glutamine was included in order to inhibit KAT I activity. Blanks were prepared by heat inactivation of the dialyzed tissue. Samples and blanks were incubated at 37°C for 20 h. The reaction was terminated by adding 10 μl of 50% (w/v) trichloroacetic acid. 1 ml of 0.1 M HCl was added, and denatured protein was removed by centrifugation. 1 ml of the remaining supernatant was applied to a Dowex 50W cation exchange column (0.5 $\times$ 10 cm, H^+-form), and the column was washed with 1 ml of 0.1 M HCl, followed by 1 ml of distilled water. [^{3}H] KYNA was eluted with 2 ml of distilled water (see Guidetti et al., 1997, for further details).

Determination of kynurenine 3-hydroxylase

Tissues were thawed and sonicated (1 : 5, w/v) in ice-cold Tris-buffer (100 mM Tris, 10 mM KCl, and 1 mM EDTA, pH 8.1). 50 μl of the tissue homogenate were then incubated in the same buffer for 1 h at 37°C with 50 μl of a reaction mixture containing 1 mM NADPH, 3 mM glucose 6-phosphate, 1 U/ml glucose 6-phosphate dehydrogenase, and 2 μM L-KYN in a total volume of 100 μl. Blank samples were obtaining using heat-inactivated tissue. After the incubation, 25 μl of 6% perchloric acid were added, samples were centrifuged (10 min, 11,000 $\times$ g), and 20 μl of the resulting supernatant were subjected to HPLC analysis. 3-HK was eluted and quantitated by electrochemical detection as detailed below.

Determination of L-KYN and KYNA in brain, liver and serum

Tissues were thawed and sonicated 1:10 (w/v) or 1:5 (w/v; from P-0 to P-5) in distilled water. 300 μl of the tissue homogenate were then acidified with 75 μl of 6% perchloric acid. After centrifugation (10 min, 11,000 $\times$ g), an aliquot of the supernatant was diluted with a mobile phase containing 0.1 M ammonium acetate and 2% acetonitrile (pH 4.65), and L-KYN was determined by HPLC analysis with UV detection at 365 nm (Holmes, 1988).

For KYNA measurement, another aliquot of the same supernatant was further diluted 1:2.5 (v/v) with a solution containing 50 mM sodium acetate, 0.25 M zinc acetate and 5% acetonitrile (pH 6.2), and KYNA was measured by HPLC analysis with fluorescence detection (excitation wavelength: 344 nm; emission wavelength: 398 nm) (Swartz et al., 1990b).

Thawed plasma samples were diluted (1:6, v/v) in distilled water. Protein was precipitated by the addition of 6% perchloric acid and removed by centrifugation (10 min, 11,000 $\times$ g). The resulting supernatant (serum) was then diluted with the respective mobile phases, and L-KYN and KYNA were analyzed by HPLC as described above.

Determination of 3-HK in brain and liver

Tissue samples were thawed and sonicated (1:5, w/v) in distilled water. 25 μl of of 6% perchloric acid were then added to 100 μl of tissue homogenate. After centrifugation (10 min, 11,000 $\times$ g), 20 μl of the supernatant were subjected to HPLC using a refrigerated autoinjector. 3-HK was eluted at 30°C using a 8 cm C_{18} HPLC column perfused at 1 ml/min with a mobile phase consisting of 2% acetonitrile, 0.9% triethylamine, 0.59% phosphoric acid, 0.27 mM sodium EDTA and 8.9 mM heptane sulphonic acid. Analysis was performed by using an electrochemical detector (Coulochem, ESA, Chelmsford, MA) connected to an analytical cell with the oxidation voltage set at + 0.20 V (Heyes, 1988).

C-Section and birth asphyxia

On gestational day 22, timed pregnant rats were decapitated, the uterus was isolated from its blood supply, and the pups were immediately delivered (= time 0). Umbilical cords were ligated, and the pups were placed under a heating lamp for about 1 h before being placed with a foster mother.

In separate litters, asphyxia was induced by immediately immersing the intact isolated uterus in a 37°C saline bath for 15 min (Bjelke et al., 1991). The pups were then delivered and ventilated by mechanical stimulation. After recovery under a heating lamp, the pups were placed with a foster mother.

Protein determination

Protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results

Ontogeny of kynurenine pathway metabolites in rat brain and liver

L-KYN concentrations were examined in the rat forebrain and in the liver during pre- and postnatal development (Fig. 1). In both organs, highest concentrations were present between gestational days E-16 and E-22.

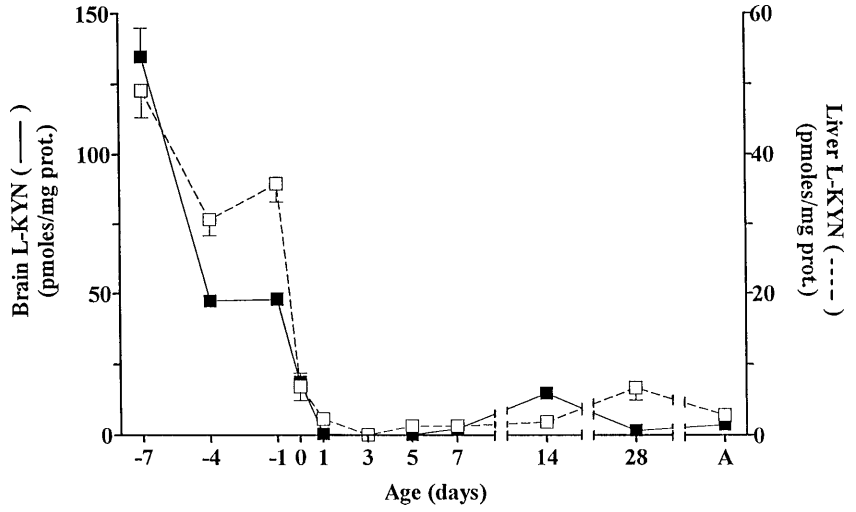

Fig. 1. Endogenous levels of L-KYN in brain (■) and liver (□) during ontogeny. Data are the mean and standard error of the mean (SEM) of determinations in 5–19 animals per age group. In this Fig. and in Figs. 2, 3 and 4, "0" indicates the day of birth, positive numbers represent postnatal days (P) 1 to 28, and "−7", "−4" and "−1" are embryonic days (E) 16, 19 and 22, respectively. *A* Adult. In the brain, L-KYN concentrations were significantly increased prenatally and at P-0 and P-14 compared to the other age groups ($P < 0.05$). In the liver, L-KYN levels were significantly increased prenatally compared to the other age groups ($P < 0.05$) (one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test)

Immediately before birth, cerebral L-KYN levels were approximately 12-fold higher than in the adult. From P-0 to P-7, the concentrations were very low, increasing moderately after the first week of life. A similar trend was observed in the liver, though hepatic tissue mostly contained less L-KYN than the brain.

The ontogeny of endogenous KYNA is illustrated in Fig. 2. In both brain and liver, KYNA levels were highest on the last day of gestation. 24 h after birth, a dramatic reduction was observed in both organs (by 4- and 12-fold, respectively, of E-22 levels). KYNA concentrations then increased gradually and moderately to reach adult levels after the first week of life.

Endogenous concentrations of 3-HK, too, were higher in the prenatal brain and liver as compared to newborn and adult rats (Fig. 3). Notably, from E-16 to E-22 brain levels were 3–4 times higher than liver content. A robust increase in 3-HK levels was observed in the liver on the day of birth. In contrast, there was a marked decrease in brain 3-HK on P-0. Finally, a transient elevation was noted in both liver and brain at P-14.

Ontogeny of kynurenine pathway enzyme activities in the rat brain

Brain KAT I and II and kynurenine 3-hydroxylase were detectable at all ages examined. No significant differences in KAT I activity were noted between E-16 and P-5 (Fig. 4A). However, enzyme activity increased gradually after the first week of life.

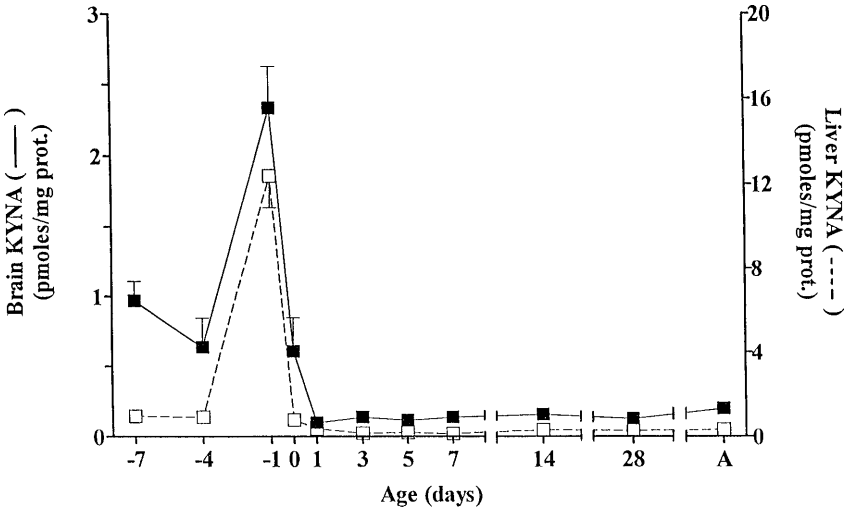

Fig. 2. Endogenous levels of KYNA in brain (■) and liver (□)during ontogeny. Data are the mean and SEM of determinations in 5–19 animals per age group. In the brain, KYNA concentrations were significantly increased at embryonic days (E)16 and 22 compared to all postnatal age groups ($P < 0.05$). In the liver, KYNA levels were significantly increased at E-22 compared to all other age groups ($P < 0.05$) (one-way ANOVA followed by Bonferroni's multiple comparisons test)

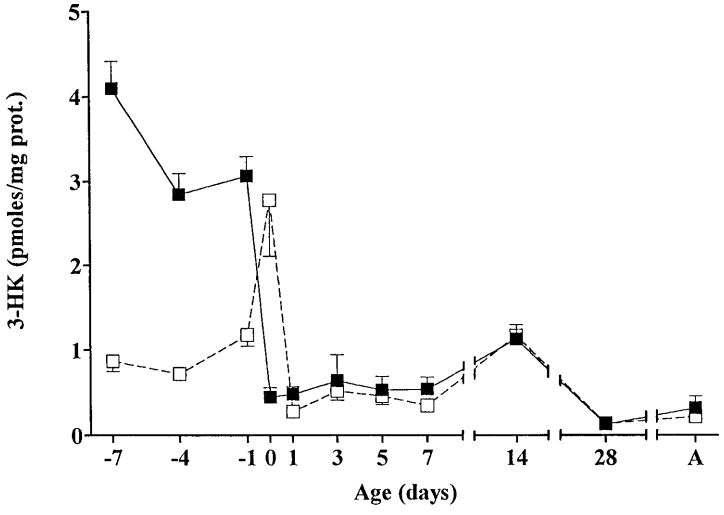

Fig. 3. Endogenous levels of 3-HK in brain (■) and liver (□) during ontogeny. Data are the mean and SEM of determinations in 4–9 animals per age group. In the brain, 3-HK concentrations were significantly increased at all prenatal ages compared to the other age groups ($P < 0.05$). In the liver, the 3-HK level at postnatal day 0 was significantly increased compared to the other age groups ($P < 0.05$) (one-way ANOVA followed by Bonferroni's multiple comparisons test)

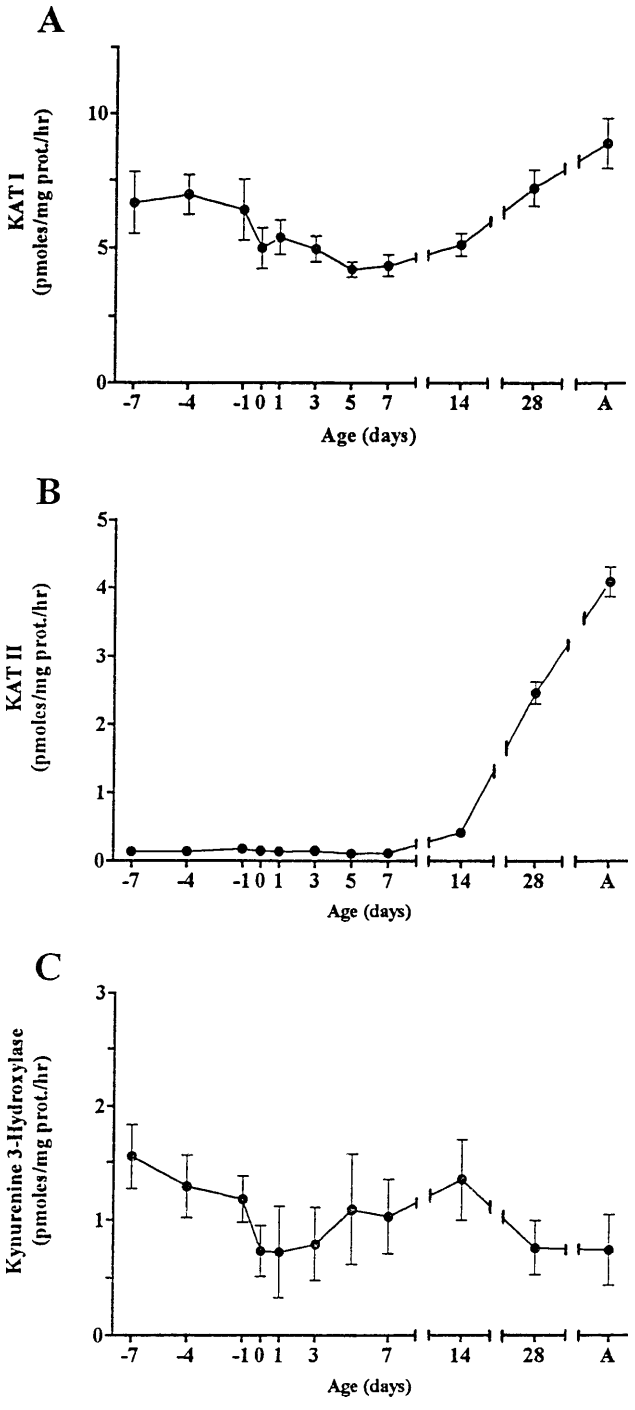

Fig. 4. Enzyme activities in the rat brain during ontogeny. **A** KAT I activity was significantly increased in the adult compared with all postnatal age groups ($P < 0.05$). Data are the mean ± SEM of determinations in 5–12 animals per age group. **B** KAT II activity was significantly increased at postnatal day 28 and in adults compared with other pre- and postnatal age groups. Data are the mean ± SEM of determinations in 5–12 animals per age group. **C** Kynurenine 3-hydroxylase activity. Data are the mean ± SEM of determinations in 5–7 animals per age group (one-way ANOVA followed by Bonferroni's multiple comparisons test)

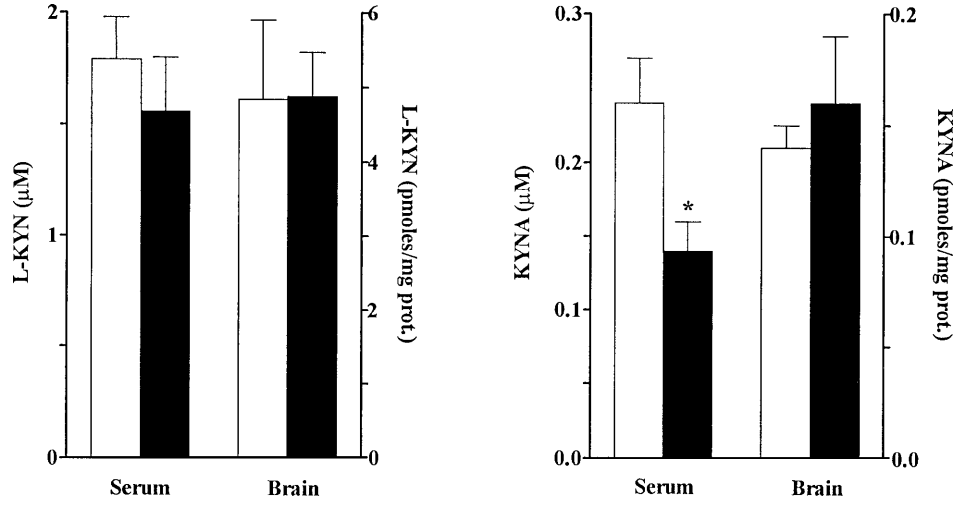

Fig. 5. L-KYN and KYNA levels in maternal serum and brain at embryonic day (E) 19 (open bars; N = 7) and postnatal day 1 (solid bars; N = 6). Data are the mean and SEM. *P < 0.05 compared to E-19 (unpaired Student's t-test)

KAT II activity, measured in the same tissue samples as KAT I, was very low during early ontogeny, but rapidly increased after P-14 (Fig. 4B).

Kynurenine 3-hydroxylase activity decreased moderately from a high level at E-16. However, enzyme activity remained relatively constant between P-0 and adulthood, with a tendency to higher activity at P-14 (Fig. 4C).

L-KYN and KYNA concentrations in maternal serum and brain

Maternal L-KYN and KYNA concentrations were measured in the serum and brain during gestation (E-19) and on the day after birth (P-1). L-KYN concentrations were not statistically different at E-19 compared to postnatal age P-1 either in the serum or in the brain (Fig. 5). However, serum (but not brain) KYNA decreased significantly after birth.

Effect of birth asphyxia on brain KYNA and 3-HK

Birth asphyxia (15 min) increased the brain levels of KYNA measured 1 h later, i.e. on the day of birth (P-0), by 40%. However, KYNA levels were not statistically different from the C-section control group 24 h later (P-1) (Fig. 6). Brain 3-HK levels in the asphyctic group were significantly decreased (by 30%) at P-0. Again, no effect was observed on P-1 (Fig. 6).

Discussion

The present data confirm and extend previous observations on the ontogeny of the kynurenine pathway in mammals. In particular, our studies in the rat

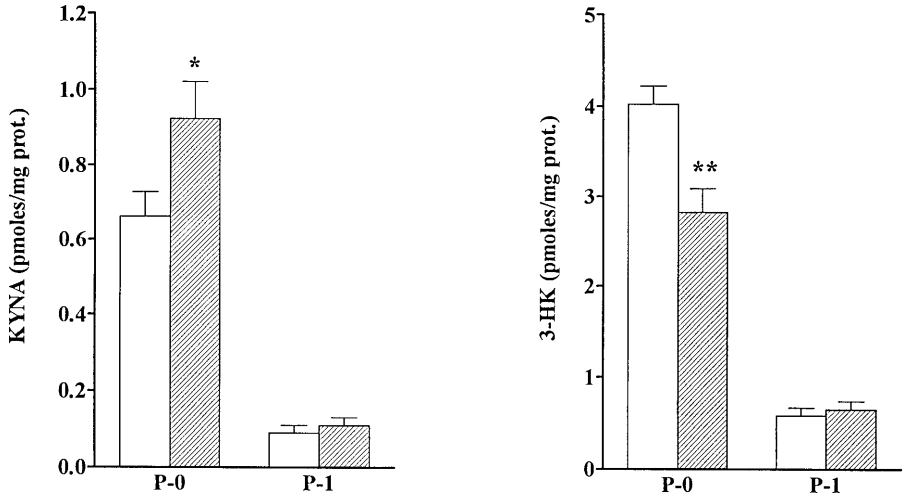

Fig. 6. Effect of birth asphyxia (15 min) on KYNA and 3-HK levels in the brain. The metabolites were measured on postnatal days *(P)* 0 (1 h after birth) and 1 (24 h after birth) in both the Caesarean section control group (open bars) and after asphyxia (hatched bars). Data are the mean and SEM of determinations in 5–13 animals per group. *$P < 0.05$, **$P < 0.01$ vs. C-section controls (unpaired Student's t-test)

demonstrated that exceedingly high tissue levels of L-KYN, KYNA and 3-HK exist in both brain and liver during the last week of gestation compared to the immediate postnatal period and adulthood. In the case of L-KYN and 3-HK, and especially at the earliest timepoint measured (E-16), the brain content exceeded that of the liver several-fold, implying that the presence of these metabolites in various organs is differentially regulated during prenatal development.

To date, the mode of brain entry of circulating kynurenines in immature animals, and the transfer of these metabolites from the mother to the fetus, have not been systematically studied (Kamimura et al., 1991; Eastman and Guilarte, 1992; Kazda et al., 1998). In adult rats, L-KYN, the parent compound of both KYNA and 3-HK, enters the brain from the periphery using the same large neutral amino acid transporter that also recognizes 3-HK as a substrate (Fukui et al., 1991; Eastman et al., 1992). The present data, showing essentially identical L-KYN levels in maternal plasma on days E-19 and P-1, suggest that the extremely high L-KYN concentrations in the fetal brain do not derive from the maternal blood. Rather, L-KYN in the pre-term brain could originate from the placenta, which, in contrast to the brain (Fujigaki et al., 1998), contains very high indoleamine 2,3-dioxygenase activity (Yamazaki et al., 1985). However, it is also possible that L-KYN is a product of tryptophan degradation in the immature brain itself.

Regardless of the origin of L-KYN in the fetal brain, the present results indicate that the high levels of 3-HK are a direct consequence of high L-KYN concentrations. Thus, the shapes of the ontogenetic curves of the two metabolites in the brain are essentially superimposable (cf. Figs. 1 and 3),

and the biosynthetic enzyme of 3-HK, kynurenine 3-hydroxylase, displays considerable activity in the brain prior to birth. Notably, the pre-term pattern of liver 3-HK does not follow the ontogeny of hepatic L-KYN equally well and, in particular, shows a more than 2-fold, transient increase on the day of birth.

In contrast to L-KYN and 3-HK, KYNA is not actively taken up into the adult brain (Fukui et al., 1991), but penetrates the blood-brain barrier to some extent following systemic administration (Gill and Woodruff, 1990; Swartz et al., 1990a; Scharfman and Goodman, 1998). The significant reduction in KYNA in the maternal blood on P-1, which parallels the dramatic decrease in pup brain KYNA immediately after delivery (Beal et al., 1992; Walker et al., 1999 and this study), indicates that at least a proportion of brain KYNA during the immediate perinatal period derives from the mother. This is also in line with the observation that the KYNA concentration in the serum of pregnant rats during the last week of gestation exceeds that of control females (G.C.-B. and R.S., unpublished data). Interestingly, and for unknown reasons, the decline in maternal serum KYNA content shortly after parturition appears to be limited to the periphery since no differences in KYNA (or L-KYN) levels were observed in the maternal *brain* between E-19 and P-1.

In the adult rat brain, the transamination of L-KYN to KYNA is preferentially catalyzed by KAT II (Guidetti et al., 1997), an enzyme that appears to be largely contained in glial cells (Ceresoli-Borroni et al., 1999). KAT I, which is also a glial enzyme (Du et al., 1992; Ceresoli-Borroni, 1999), is less important for KYNA formation in the mature brain due to its basic pH optimum and its ability to transaminate competing, abundant amino acids, such as glutamine, phenylalanine and tryptophan (Okuno et al., 1990). The present data indicate that the relative importance of KAT I and KAT II for cerebral KYNA production may be reversed early during ontogeny. Thus, brain KAT I activity varied only moderately between E-16 and adult levels, while KAT II activity was barely measurable until P-7, but increased sharply during and after the second postnatal week. It is questionable, however, if brain KAT I is indeed solely responsible for high pre-term KYNA levels, since no significant elevation in enzyme activity was recorded between E-19 and P-1 to account for the massive rise in KYNA levels during that period (cf. Figs. 2 and 4A).

Examination of the ontogeny of brain KYNA after P-7 (Moroni et al., 1988 and this study) revealed a steady but relatively modest increase towards adult levels. The brain concentrations of L-KYN and 3-HK, too, changed gradually between P-7 and adulthood, though transient peaks were observed at P-14. Interestingly, similar temporary increments were noted in the cerebral activity of kynurenergic enzymes such as kynurenine 3-hydroxylase (Battie and Verity, 1981 and this report) and 3-hydroxyanthranilate oxygenase (Foster et al., 1986), perhaps indicating a selective activation of the quinolinate branch of the kynurenine pathway two weeks *post natum*.

In a first attempt to assess the possible functional significance of the high prenatal levels of KYNA and 3-HK in the rat brain, we examined the acute

(1 h) and subacute (24 h) effects of birth asphyxia on the brain content of these metabolites. Because of the dependence of cerebral KYNA formation on oxygen and cellular energy (Turski et al., 1989; Hodgkins and Schwarcz, 1998), it seemed conceivable that this treatment would reduce KYNA levels, possibly in conjunction with an increase in 3-HK synthesis, and that these changes would be causally related to the neurochemical and behavioral abnormalities seen in these animals later in life (Loidl et al., 1994; El-Khodor and Boksa, 1998). These hypothetical considerations were based on the established links between KYNA, NMDA receptor function and hypoxic brain damage in neonates (Andiné et al., 1988; Ikonomidou et al., 1989), the fact that a reduction in brain KYNA enhances the vulnerability to excitotoxic insults (Poeggeler et al., 1998), and the pro-excitotoxic properties of 3-HK (Guidetti and Schwarcz, 1999). However, birth asphyxia resulted in a transient *elevation* in brain KYNA levels and a concomitant reduction in brain 3HK content, possibly indicating the stimulation of endogenous defense mechanisms (Fig. 6). In contrast, a recent elegant study in sheep demonstrated a dramatic *decrease* in brain KYNA after prolonged hypoxia *in utero* (Walker et al., 1999). Taken together, it is clearly possible to influence perinatal levels of neuroactive kynurenines in the brain under experimental pathological conditions. However, the effects appear to be determined by the timing and nature of the pathogenic challenge, and may differ between precocial and non-precocial species (Walker et al., 1999). The implications of such fluctuations in kynurenergic metabolism and disposition for neural development – and beyond – need to be carefully evaluated in the years to come.

Acknowledgement

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