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Neonatal Asphyxia in Rats: Acute Effects on Cerebral Kynurenine Metabolism

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ABSTRACT

Two tryptophan metabolites, the anti-excitotoxic N-methyl-D-aspartate (NMDA) receptor antagonist kynurenic acid (KYNA) and the free radical generator 3-hydroxykynurenine (3-HK), have been proposed to influence neuronal viability in the mammalian brain. In rats, the brain content of both KYNA and 3-HK decreases immediately after birth, possibly to ensure normal postnatal functioning of NMDA receptors. Because complications of birth asphyxia have been suggested to be associated with anomalous NMDA receptor function, we examined the acute effects of an asphyctic insult on the brain levels of KYNA and 3-HK in neonatal rats. Asphyxia was induced in animals delivered by cesarean section on the last day of gestation, using the procedure introduced by Bjelke *et al.* (Brain Res 543: 1–9, 1991). KYNA and 3-HK levels were determined in the brain at seven time points between 10 min and 24 h after asphyxia. Up to 6 h, asphyxia caused 160–267% increases in KYNA levels. In the same tissues, 3-HK levels decreased (significantly at five of the seven time points), demonstrating an asphyxia-induced shift in kynurenine pathway metabolism toward the neuroprotectant KYNA. This shift might constitute the brain's attempt to counter the ill effects of birth asphyxia. Furthermore, the transient increase in the brain KYNA/3-HK ratio in these animals might be causally related to the well-documented detrimental long-term effects of asphyxia. **(*Pediatr Res* 50: 231–235, 2001)**

Abbreviations

3-HK, 3-hydroxykynurenine
KYNA, kynurenic acid
NMDA, *N*-methyl-D-aspartate

In newborn humans, birth asphyxia often causes rapid alterations in brain structure and function, which are in turn responsible for the development of cerebral palsy, mental retardation, and other catastrophic chronic conditions (1–4). These acute changes, and the subsequent evolution of permanent brain dysfunction, have been examined in numerous investigations using both invasive and noninvasive methods (cf. ref. 5–7 for review). These studies were complemented by preclinical work in experimental animal models, which allow superior scrutiny of asphyxia-induced behavioral, cellular, and molecular abnormalities and provide opportunities to test therapeutic interventions (8, 9).

An increasingly popular rat model of relatively mild birth asphyxia involves delivery of the uterine horn by cesarean section on the final day of gestation and the subsequent submersion of the uterus in 37°C water for 15–20 min (10). Thus, animals undergoing birth asphyxia have been shown to present with characteristic biochemical changes in the brain in the short and long term (11–15) and with distinct behavioral abnormalities as adults (10, 16, 17).

Because it can be reasonably assumed that most pathologic sequelae of birth asphyxia are triggered by brain changes that occur in the immediate aftermath (*i.e.* within hours) of the insult, and potential remedies are likely to be most effective during this initial period, many experimental studies have focused on early postasphyctic events. This work has revealed, for example, rapid changes in brain metabolism, cellular pH, and tissue levels of neurotransmitters such as dopamine and glutamate (11, 18). The latter effects, together with indications of benefits of antiglutamatergic interventions (19), have led to the suggestion that hyperglutamatergic, and perhaps excitotoxic, mechanisms play a major role in asphyxia-related brain pathology (20, 21).

Glutamatergic neurotransmission in the brain is governed not only by a spectrum of ionotropic and metabotropic glutamate receptors (22) but also by a number of different endogenous receptor ligands. One of these, the tryptophan metabolite KYNA, is a receptor antagonist and has a particularly high affinity for the glycine coagonist site of the NMDA receptor (23). Notably, through their common metabolic precursor L-kynurenine, KYNA is also closely related to 3-HK, a free radical generator with proexcitotoxic properties (24–26).

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KYNA has pronounced neuroprotective properties as evidenced, for example, by its ability to reduce hypoxic damage in immature animals (27, 28).

In several mammalian species, the brain levels of KYNA are exceptionally high during late gestational stages and decrease precipitously at birth (29–31). In rats, the only species examined to date, there is also a rapid and substantial postnatal reduction in the brain concentration of 3-HK (31). We therefore speculated that KYNA, in particular, might provide protection against hyperglutamatergic (asphyctic, hypoxic) injuries during birth. This might involve rapid mobilization of brain KYNA in the face of an insult. In a first approach to address this hypothesis, we decided to examine the early effects of birth asphyxia on the cerebral tissue content of KYNA and 3-HK in rats.

METHODS

Materials. KYNA and 3-HK were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of the highest commercially available purity.

Animals. Time-pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Raleigh, NC, U.S.A.) and kept at a 12 h/12 h light-dark cycle with free access to food and water. The study was approved by the Institutional Animal Care and Use Committee of the University of Maryland.

Birth asphyxia. Birth asphyxia was performed using a slight modification of the model introduced by Bjelke *et al.* (10). Time-pregnant rats on the last day of gestation (embryonic day 22, E22) were decapitated, and an abdominal incision was made to isolate both uterine horns (cesarean section). Subsequently, the two uterine horns were rapidly (in 10–15 s) separated by ligation. One uterine horn was then immediately submerged in a beaker filled with a 37°C saline solution. After 15 min of asphyxia, pups were removed from the uterus, and their umbilical cords were ligated (time 0). Animals delivered from the second uterine horn in an identical fashion served as controls. Tactile stimulation was used in all neonatal rats to facilitate respiration. After recovery at 37°C for up to 60 min, all pups were placed with a surrogate mother. Survival was 84% (51/61) in the asphyctic group and 100% (76/76) in the control group.

Pups were killed by decapitation at various times up to 24 h after delivery. The forebrain was rapidly dissected out, frozen on dry ice, and stored at −70°C until the day of the assay.

Behavioral assessments. After delivery, animals were observed for behavioral changes. Between 10 min and 2 h, the following parameters were recorded as described (13, 14): skin color (scored from 0 to 3, with 3 indicating pink, *i.e.* normal, color), gasping, (scored by the presence or absence of mouth opening), vocalization (scored by the presence or absence of vocalization upon handling of the animal), muscle tone (rated on a scale from 0 to 3, with 3 indicating a strong resistance of a hind limb when the limb was flexed), and spontaneous activity (scored from 0 to 4, with 4 indicating intense movements and wriggling, 3 indicating movements of all body parts, 2 indicating movements of two body parts, and 1 indicating movement of front legs or hind legs, or head alone).

Determination of brain KYNA. Frozen brain tissue was thawed and sonicated (1:10 wt/vol) in distilled water. Two hundred microliters of the tissue homogenate was acidified with 50 μL of 6% perchloric acid and centrifuged at 13,000 × *g* for 10 min. Then, 125 μL of the resulting supernatant was diluted with mobile phase (1:1, vol/vol; see below), and 200 μL was injected onto a 3-μm C_{18} reverse-phase HPLC column (80 × 4.6 mm, ESA, Chelmsford, MA, U.S.A.) using an autoinjector. KYNA was eluted at 1 mL/min with a mobile phase containing 0.2 M zinc acetate and 3.5% acetonitrile, titrated to pH 6.2 with glacial acetic acid. KYNA was detected fluorometrically (excitation wavelength: 344 nm; emission wavelength: 398 nm) using a Perkin Elmer LC240 fluorescence detector (Perkin Elmer, Beaconsfield, UK) (32).

Determination of brain 3-HK. A 50-μL aliquot of the tissue homogenate used for KYNA determination was acidified with 12.5 μL of 6% perchloric acid. Samples were kept on ice for 10 min before centrifugation (10 min, 13,000 × *g*). Then, 20 μL of the resulting supernatant was injected onto a 3-μm C_{18} reverse-phase HLPC column (80 × 4.6 mm, ESA) using a refrigerated autoinjector (ISS 200, Perkin Elmer). 3-HK was eluted isocratically at 20°C at 1 mL/min using a mobile phase containing 2% acetonitrile, 0.9% triethylamine, 0.59% phosphoric acid, 0.27 mM sodium EDTA, and 8.9 mM heptane sulfonic acid. 3-HK was detected electrochemically (Coulochem 5100A, ESA) using an analytical cell with the oxidation voltage set at +0.20 V (33).

Protein determination. The protein content of tissue homogenate was determined according to the method of Lowry *et al.* (34).

Data analysis. Behavioral responses were compared using χ^2 tests for vocalization and gasping, and the Kruskal-Wallis (Wilcoxon rank) test for responses measured as ordered categories. Levels of KYNA and 3-HK were analyzed using two-way ANOVA with time and asphyxia as main effects, and tests for asphyxia by time interaction. Posthoc *t*-tests were performed comparing asphyctic and nonasphyctic rats at individual time points.

RESULTS

Behavioral observations. In agreement with the literature (13, 14), a 15-min episode of asphyxia had pronounced and immediate behavioral effects in neonatal rats. Several of the parameters listed in Table 1 correspond to the Apgar score used for newborn babies (7). At 10 min after birth, asphyctic pups had a pale skin color compared with the pink color of control animals. Moreover, asphyctic animals gasped for air, and seemed to vocalize less upon handling. At this early interval, the pups also did not show voluntary limb or body movements and had a flaccid tone. In contrast to the other acute behavioral effects of asphyxia, changes in skin color and vocalization did not attain statistical significance. Starting at 30 min and up to 2 h (the latest time point assessed for behavioral changes), differences between asphyctic and control animals became progressively less pronounced. Thus, at 30 min, asphyctic animals had stopped gasping and vocalized normally upon handling but were still hypotonic and hypoactive. By 1 h,

muscle tone and spontaneous activity, too, had returned to control values (Table 1).

Effect of birth asphyxia on brain KYNA levels. Brain KYNA concentrations in control animals declined dramatically immediately after birth and gradually decreased further during the first 24 h of life (Fig. 1). A period of asphyxia resulted in a significantly higher level of brain KYNA compared with controls between 10 min and 6 h, but not 24 h after the insult. The average increase over this period was 493 ± 44 fmoles/mg protein ($F = 128.2$, $df = 1, 112$, $p < 0.05$), with no significant variation ($F = 1.04$, $df = 5, 107$, $p > 0.05$). By 24 h, however, there was no elevation in KYNA levels in asphyctic animals compared with controls. At each of the six time points tested (# 6 h), KYNA levels in the asphyctic group were approximately 2-fold higher than in controls ($p < 0.05$, unpaired t test).

Effect of birth asphyxia on brain 3-HK levels. In control animals, the brain content of 3-HK decreased postnatally, but a significant decline from embryonic levels was not observed until 6 h after birth (Fig. 2). In asphyctic animals, brain 3-HK levels tended to be lower than in controls. Overall, ANOVA revealed an average difference of 524 ± 91 fmoles 3-HK/mg protein between asphyctic animals and controls ($F = 33.4$, $df = 1, 124$, $p < 0.05$), which did not vary significantly ($F = 1.56$, $df = 6, 118$, $p > 0.05$) over the 24-h period. At individual postnatal time points, the asphyxia-induced decrease reached statistical significance at 10 min, 60 min, 90 min, 2 h, and 24 h ($p < 0.05$, unpaired t test).

KYNA/3-HK ratio. Up to 24 h after delivery, the ratio between KYNA and 3-HK in the brain of asphyctic animals was between 1.9- and 3.7-fold higher than in controls. This increase reached statistical significance at all time points # 6 h (Table 2).

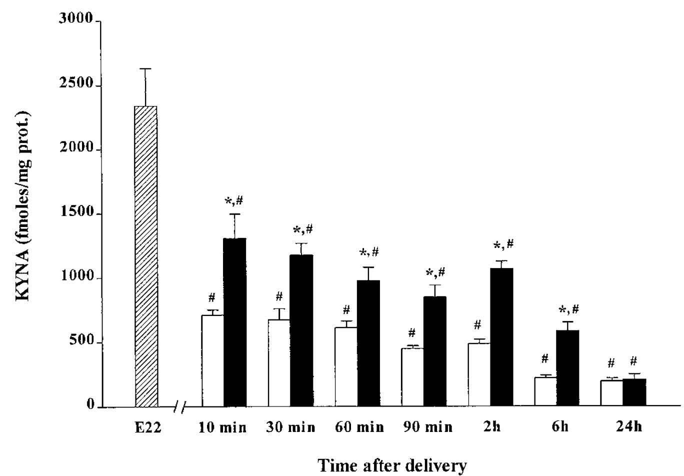

Figure 1. Brain KYNA levels in control (□) and asphyctic rats (■) at various time points after delivery. Data are the mean ± SEM of 5–16 animals per postnatal time point. "E22" ($n = 5$) depicts the brain KYNA level in animals delivered by cesarean section on embryonic d 22 and killed immediately. Experiments were performed as described in the text. *$p < 0.05$ *vs* controls, #$p < 0.05$ *vs* E22 (unpaired t tests).

DISCUSSION

Using an established rat model in which relatively moderate asphyxia is caused by submerging the isolated uterus for 15 min at 37°C in a saline bath (10), the present study constituted an initial effort to determine the possible significance of the high KYNA and 3-HK concentrations that are seen in the embryonic brain of nonhuman primates, sheep, and rats (29–31). The results demonstrated that birth asphyxia has opposite effects on the brain tissue content of KYNA and 3-HK during

Table 1. *Behavioral observations at various times after delivery*

	10 min	30 min	60 min	90 min	2 h
Skin					
Control	3.00 ± 0.0	3.00 ± 0.0	3.0 ± 0.0	3.00 ± 0.0	3.00 ± 0.0
Asphyxia	2.87 ± 0.07	2.94 ± 0.06	3.00 ± 0.0	3.00 ± 0.0	3.00 ± 0.0
χ^2 ($df = 1$)	2.88	0.90	0.00	0.00	0.00
p Value	0.24	>0.99	>0.99	>0.99	>0.99
Gasping					
Control	0/22	0/18	0/14	0/10	0/6
Asphyxia	24/24	0/20	0/16	0/12	0/8
χ^2 ($df = 1$)	46.00	0.00	0.00	0.00	0.00
p Value	<0.001	>0.99	>0.99	>0.99	>0.99
Vocalization					
Control	18/22	18/18	14/14	10/10	6/6
Asphyxia	16/24	18/20	16/16	12/12	8/8
χ^2 ($df = 1$)	1.37	0.00	0.00	0.00	0.00
p Value	0.24	>0.99	>0.99	>0.99	>0.99
Muscle tone					
Control	1.95 ± 0.05	2.61 ± 0.12	3.00 ± 0.0	2.90 ± 0.10	2.67 ± 0.21
Asphyxia	0.87 ± 0.07	1.90 ± 0.14	2.56 ± 0.13	2.50 ± 0.15	2.75 ± 0.16
χ^2 ($df = 1$)	38.54	11.72	7.72	3.62	0.11
p Value	<0.001	<0.001	0.007	0.14	>0.99
Spontaneous activity					
Control	1.91 ± 0.11	2.67 ± 0.11	3.43 ± 0.14	3.70 ± 0.15	3.50 ± 0.22
Asphyxia	0.04 ± 0.04	1.25 ± 0.19	2.56 ± 0.13	3.58 ± 0.15	3.50 ± 0.19
χ^2 ($df = 1$)	39.15	21.61	12.50	0.31	0.70
p Value	<0.001	<0.001	<0.001	0.67	>0.99

p Values and chi-squares from Wilcoxon rank test for ordered categories or Pearson chi-squares for yes/no responses. All parameters were rated as described in the text, using 22 control and 24 asphyctic animals. Mortality in the asphyctic group was 4% (1/24).

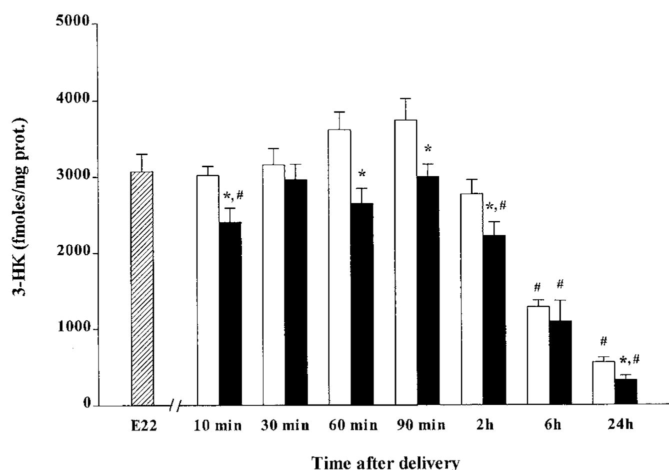

Figure 2. Brain 3-HK levels in control (□) and asphyctic rats (■) at various time points after delivery. Data are the mean ± SEM of 5–16 animals per postnatal time point. "E22" (n = 5) depicts the brain 3-HK level in animals delivered by cesarean section on embryonic d 22 and killed immediately. Experiments were performed as described in the text. *$p < 0.05$ *vs* controls, #$p < 0.05$ *vs* E22 (unpaired t tests).

the first 24 h after the insult. Thus, asphyxia substantially attenuated the dramatic reduction in brain KYNA that is normally observed shortly after birth, but further advanced the postnatal decrease in brain 3-HK levels. Taken together, the experimental injury therefore led to acute, 2- to 3-fold increases in the KYNA/3-HK ratio in the brain.

KYNA and 3-HK are both primary degradation products of L-kynurenine and thus metabolites of competing branches of the kynurenine pathway of tryptophan catabolism (35). In adults, chronic changes in the brain concentrations of both compounds occur under several pathologic conditions, but abnormal levels of one metabolite are not necessarily accompanied by changes in the other. For example, the brain content of 3-HK and its downstream metabolites surge dramatically in immunocompromised humans and animals, probably due to infiltrating macrophages and the activation of microglia (36, 37). Because these cells contain only small amounts of kynurenine aminotransferases, the biosynthetic enzymes of KYNA that are preferentially localized in astrocytes (38, 39), KYNA levels in the same tissues increase only moderately (36). In contrast, cerebral kynurenine metabolism along both branches of the pathway is stimulated in chronically lesioned brain or spinal cord, probably because both astrocytes and microglia are abundant in neuron-depleted tissue (39–41).

Table 2. *Brain KYNA/3HK ratio at various times after delivery*

	Control	Asphyxia	Δ(%)
10 min	0.24 ± 0.02	0.58 ± 0.10*	+242
30 min	0.21 ± 0.02	0.40 ± 0.02*	+190
60 min	0.17 ± 0.01	0.39 ± 0.06*	+229
90 min	0.13 ± 0.01	0.29 ± 0.04*	+223
2 h	0.19 ± 0.02	0.55 ± 0.07*	+289
6 h	0.18 ± 0.02	0.66 ± 0.18*	+367
24 h	0.34 ± 0.05	0.80 ± 0.28	+235

Data are the mean ± SEM of 5–16 animals per time point.
* $p < 0.05$ *vs.* controls (unpaired t test).

An acute excitotoxic insult to the adult rat brain causes a rapid increase in the *de novo* synthesis of KYNA and a concomitant reduction in 3-HK formation (42). Moreover, KYNA levels in the brain rise substantially within 1–2 h after the induction of seizure activity (43, 44) or after a focal excitotoxin injection (39). These data suggest that a shift in kynurenine pathway metabolism, favoring the mobilization of the neuroprotectant KYNA and diminishing the formation of the toxin 3-HK (25), might constitute a common defense mechanism of the brain to acutely combat injuries. While the cellular and molecular mechanisms underlying this rapid metabolic shift toward an increased KYNA/3-HK ratio and enhanced KYNA availability are currently not understood, the present results indicate that they also operate in the neonatal brain. Because of the very high prenatal brain content of KYNA, however, the effect of birth asphyxia manifested itself as an attenuation of the precipitous postnatal decline rather than an absolute increase in KYNA levels.

NMDA receptor activation does not only play a central role in excitotoxicity (45) but is also critically involved in cognitive functions (46). In the immature brain, NMDA receptors are crucial for synapse development (47) and for the modulation of neuronal migration (48). Conceptually, the high content of the NMDA receptor antagonist KYNA in the embryonic brain might therefore provide an antiexcitotoxic (*i.e.* antihypoxic) defense during birth, whereas the swift decline in brain KYNA immediately after birth would assure minimal interference with developmentally essential postnatal NMDA receptor functions. Notably, fluctuations in the brain content of KYNA have indeed been demonstrated to influence NMDA receptor function (49–51), especially when combined with an opposite deflection of cerebral 3-HK levels (52). The present data therefore raise the question of the functional consequences of the asphyxia-induced, rapid increase in the cerebral KYNA/3-HK ratio. It is conceivable that this transient metabolic change, which is accompanied by an elevation in the levels of the neuroprotective neuronal fuel lactate (11, 53, 54), augments endogenous neuroprotection but at the same time impedes normal NMDA receptor function in the neonate (55). Experiments to examine these possibilities, as well as the potential role of these early biochemical changes in the long-term effects of birth asphyxia (6–8, 10–16, 19), are currently in progress in our laboratory.

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