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Increased Density of Hippocampal Kainate Receptors but Normal Density of NMDA and AMPA Receptors in a Rat Model of Prenatal Protein Malnutrition

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ABSTRACT

The postnatal development of excitatory amino acid receptor types including kainate, *N*-methyl-D-aspartate (NMDA), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) was assessed in the hippocampus, entorhinal cortex, and adjacent neocortex in normal and prenatally protein malnourished rats ages 15, 30, 90, and 220 postnatal days by quantitative autoradiography. Tritiated ligands used to measure binding site density were ^{3}[H]kainate, ^{3}[H]MK-801, and ^{3}[H]AMPA, respectively. Kainate receptors showed statistically significant increases in binding density in stratum lucidum of CA3 (hippocampal mossy fiber zone) in 90- and 220-day-old malnourished rats compared with age- and sex-matched controls but not in 15- or 30-day-old malnourished rats. Compared with previous anatomic studies, these results are mostly in agreement with a significantly decreased hippocampal mossy fiber plexus in 15-, 90-, and 220-day-old rats but not in 30-day-old rats. These results suggested that the increased density of postsynaptic kainate receptors located mainly on proximal apical dendrites of CA3 pyramidal cells may be compensatory to decreased glutamate release due to the reduction in mossy fiber plexus. In contrast, the density of putative NMDA and AMPA receptors quantified in prenatally malnourished rats was comparable to the density quantified in age- and sex-matched control rats, as were all three receptor types in entorhinal cortex and adjacent neocortex. Thus, the selectivity of the compensation of ^{3}[H]kainate-labeled mossy fiber plexus in adult but not in early postnatal developing malnourished rats may help ensure continued breeding and survival of the species under otherwise adverse environmental conditions. J. Comp. Neurol. 456:350–360, 2003.

Indexing terms: ligand binding; autoradiography; glutamatergic; undernutrition; mossy fibers (stratum lucidum of CA3)

In human populations, moderate to severe periods of malnutrition before the first 2 years of life are associated with delays in cognitive development and poor school performance in children (for review, see Galler and Ross, 1993; Hall et al., 2001). Animal models of human malnutrition have long been employed to assess malnutrition effects on brain development. Malnutrition during the prenatal period in rats has been shown to alter postnatal development of the brain despite nutritional rehabilitation begun at birth. Alterations in behavior, anatomy, chemistry, and physiology extend into the postnatal period and continue into adulthood.

Tonkiss et al (1998) provided behavioral evidence that the excitatory amino acid system may be altered in prenatally protein malnourished rats. These rats displayed an increased sensitivity to MK-801, an *N*-methyl-D-aspartate (NMDA) receptor channel blocker, in a differen-

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tial reinforcement of low rates of an operant schedule task. A number of earlier studies in prenatally malnourished rats showed that fast excitatory and synaptic plasticity components of the excitatory amino acid system are altered. The magnitude and duration of interneuronally mediated inhibition are significantly increased in prenatally protein malnourished rats when compared with controls (Austin et al., 1992). Long-term potentiation (LTP) and kindling, both of which assess long-term changes in synaptic plasticity, are also affected in prenatally malnourished rats at the perforant path/dentate granule cell synapse (Austin et al., 1986; Bronzino et al., 1986, 1990; Morgane et al., 1993, 1995; for review, see Galler et al., 1996). Bortolotto et al. (1999) described the role of kainate receptors in synaptic plasticity. By using a selective antagonist at neuronal kainate receptors containing the glutamate receptor (GluR) 5 subunit, those investigators found that the receptors function as the induction trigger for long-term changes in synaptic transmission.

Significant alterations in hippocampal anatomy suggest the probability of some generalized receptor re-localization. Microscopic analysis of hippocampal subfields and laminae has demonstrated that hippocampal pyramidal cells and dentate gyrus granule cells do not develop normally in prenatally malnourished rats as demonstrated by reductions in cell soma size, complexity of dendritic branching, and decreased number of dendritic spines (Diaz-Cintra et al., 1991, 1994; Cintra et al., 1997a). The mossy fiber plexus, which is the excitatory dentate gyrus granule cell axonal projection to areas CA4 and CA3 of the hippocampal formation, is significantly deficient in total rostrocaudal extent and volume in prenatally malnourished rats at ages 15, 90, and 220 postnatal days but not at 30 days (Cintra et al., 1997b). Because kainate receptors are especially abundant in stratum lucidum of CA3, changes on the mossy fiber plexus may affect density of kainate receptors located presynaptically on mossy fiber terminals or postsynaptically on proximal dendrites of CA3 pyramidal cells. Recently, the same group of investigators reported that prenatal protein malnutrition in 220-day-old rats produces long-lasting significant decreases in the volume of the mossy fiber system suprapyramidal bundle and in the numerical density of mossy fiber–CA3 asymmetrical synapses, suggesting a reduction in the total number of this synapse type (Granados-Rojas et al., 2002). Granados-Rojas and colleagues suggested that there may be long-term disruption in the progression of developmental programs controlling synaptogenesis and/or synaptic consolidation in the hippocampus of prenatally protein malnourished rats.

Cossart et al. (2001) reported that kainate receptors are also present on presynaptic γ-aminobutyric acidergic (GABAergic) terminals contacting hippocampal interneurons and that their activation increases GABA release. They also demonstrated that application of kainate or glutamate (but not of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate [AMPA] or NMDA) increased the frequency of mIPSCs recorded in CA1 interneurons, suggesting that kainate may selectively control the communication between hippocampal interneurons by increasing their mutual inhibition. Thus, to investigate the effect of prenatal protein malnutrition on the density and distribution of three types of glutamatergic receptors in the hippocampus, entorhinal cortex, and adjacent neocortex, we used ^{3}[H]kainate for kainate receptors, ^{3}[H]MK-801 to label NMDA receptors, and ^{3}[H]AMPA to label AMPA (quisqualate) receptors in 15, 30, 90, and 220 postnatal day control and prenatally protein malnourished rats. A preliminary report of this study was presented in abstract form (Fiacco et al.,1999).

MATERIALS AND METHODS

Animal model

The breeding facility at the Center for Behavioral Development and Mental Retardation at Boston University School of Medicine (J. R. Galler, MD, Director) was responsible for maintaining the animal colony. Virgin viral and antibody-free female albino Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) 60 to 70 days old were given ad libitum access to isocaloric diets containing 25% or 6% casein (Tonkiss and Galler, 1990). Females placed on a 6% or 25% casein diet were allowed 5 weeks before mating to adapt to the diet. After mating, the females continued to receive their respective diets until parturition. Litters culled to eight pups each were fostered to form two groups: those born to mothers provided the 6% casein diet were fostered to dams provided the 25% casein diet throughout (offspring designated 6/25); and those born to dams fed the 25% casein diet were fostered to other lactating dams fed the 25% casein diet (offspring designated 25/25). To eliminate potential litter effects, only one male rat was taken from each litter. All offspring were given ad libitum access to Purina rat chow after weaning. The young rats were housed in polycarbonate microisolator cages (Lab Products Inc., Maywood, NJ) and kept in temperature-, light-, and humidity-controlled conditions. To prevent experimenter bias, a code was used for all experimental and control rats.

Tissue processing and [^{3}H]ligand binding

Six male 25/25 and six male 6/25 rats from each of four ages (15, 30, 90, or 220 days) were perfused intracardially with cold (5–10°C) Krebs-Henseleit buffer (n = 24 25/25 rats and n = 24 6/25 rats). Brains were harvested immediately after each perfusion and then flash frozen by slow immersion into −60°C isopentane. The brains were removed from the isopentane and stored at −80°C. Brains were sectioned horizontally at 15 μm on a Hacker/Brights motorized cryostat after a 20- to 30-minute equilibration period from −80°C storage to the −20°C cryostat chamber temperature. Serial sections were cut and thaw-mounted onto prelabeled gelatin or poly-L-lysine subbed slides, dried on a warm 37°C plate, loaded into a slide box, and stored at −20°C until processed for ligand binding (Blatt et al., 1994).

On the slide in vitro ^{3}[H]ligand binding was performed for kainate receptors by using ^{3}[H]kainate (^{3}[H]KAI, specific activity 58.0 Ci/mmol), for NMDA receptors by using [^{3}H]MK-801 (specific activity 23.9 Ci/mmol), and for AMPA receptors by using ^{3}[H]AMPA (specific activity 40.6 Ci/mmol; New England Nuclear, Boston, MA). A detailed summary of assay conditions for each ligand is presented in Table 1. Tissue sections from normal rats were always processed together through the same receptor binding assays with sections from prenatally protein malnourished rats. Total binding (i.e., specific plus nonspecific binding) was achieved by incubating the tissue sections in a single

TABLE 1. Glutamatergic Receptor Binding Assays

Receptor[1]	^{3}[H]-ligand	Concentration	Buffer[2]	Blocker[3]	References
Kainate	Kainate	15 nM	50 mM Tris citrate (pH 7.0); preincubate for 10 minutes in buffer at room temperature, incubate for 50 minutes at 0°C, rinse 3 × 10 seconds in buffer at 0°C	500 μM kainate	Monaghan et al. (1986)
NMDA	MK-801	7 nM	20 mM HEPES-K, 5 mM K-EDTA, 100 μM glutamate, 100 μM glycine (pH 7.5); incubate for 3 hours at 37°C, rinse for 30 minutes in buffer at 0°C[4]	200 μM ketamine	Subramaniam and McGonigle (1991)
AMPA	AMPA	30 nM	50 mM Tris HCL, 2.5 mM $CaCl_2$, 100 mM K^+ thiocyanate (pH 7.2); preincubate for 10 minutes in buffer at 0°C, incubate for 30 minutes at room temperature, rinse 2 × 10 seconds in buffer at 0°C	100 mM quisqualate	Monaghan et al. (1984), Porter and Greenamyre (1994)

[1]AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; NMDA, *N*-methyl-D-aspartate.
[2]Appropriate ions are included in each assay buffer to maximize specific binding (see references).
[3]Sections incubated with ligand plus displacer represent "nonspecific" binding.
[4]Rinse buffer contains no glutamate or glycine.

concentration of radioactive ligand. Nonspecific binding was obtained by incubating the sections in the radioactive ligand plus competitive cold displacer at a concentration approximately equal to 100 kD of the ^{3}[H]ligand (Table 1). A series of sections adjacent to those used to measure total binding was incubated in nonspecific binding conditions. In these assay conditions, the amount of nonspecific binding was less than 5% of the total. Specific binding was determined by subtracting the images of nonspecific binding from the total binding images. At the conclusion of the incubation and rinse steps, slides were rapidly dried under a stream of cool air and stored overnight in desiccator jars. The slides were loaded into x-ray cassettes (Rigidform, Titan, Boston, MA) with a set of tritium microscales (Amersham, Piscataway, NJ) and apposed to each tritium-sensitive film for 2 weeks (AMPA), 4 weeks (kainate), or 6 weeks (NMDA). The films were developed using Kodak D19 developer and Rapidfix, both designed for use with ^{3}H-sensitive film.

Data analysis of ligand binding

Five "total" and five "nonspecific" images of the sections from each of the three regions (hippocampus, entorhinal cortex, and neocortex) from each case and tritium microscales from film were digitized into a computer with the use of a digital camera and a software program for quantitative autoradiography (Inquiry, Loats Associates, Westminster, MD). The number of cases used per age group for a given ligand was determined by critical review of the five equally spaced sections from each region, i.e., cases were included only if all selected sections were of outstanding quality and clearly focused on the film. Therefore, the number of cases used at each age and ligand varied from four to six cases, which is indicated on the graphs. For results not illustrated (NMDA and AMPA at 15, 30, and 220 days only), sections from six control and six malnourished cases were used for each assay. The tritium standards were used to construct a calibration table in the computer, which converted optical density measurements taken within specific regions of the tissue into femtomoles (fmol) of tritiated ligand bound per milligram of tissue. Fifteen different anatomic regions within the hippocampus and the deep and superficial entorhinal and neocortical layers were sampled for optical density (Fig. 1A shows numbered regions; Fig. 1B shows the corresponding sample lines in specific hippocampal subfields and laminae in an autoradiographic section labeled with ^{3}[H]kainate as an example). The molecular layer of CA1–3, prosubiculum, and subiculum was sampled very close to the hippocampal fissure in all sections, thereby representing the outermost portions of distal pyramidal cell dendrites (Fig. 1A,B). Samples placed in superficial entorhinal and neocortex included layers II–III, and those in deep entorhinal and neocortex cortex were placed in layer V (Fig. 1A,B).

Femtomole values obtained from all sections were transferred to a spreadsheet and sorted by age and anatomic region (e.g., 15 days, CA3mf, CA3ml, etc.; see caption to Fig. 1 for abbreviations). Within each anatomic region, femtomole values from sections from the same rat were averaged together (intra-animal mean values). Intra-animal means from rats of the same nutrition and age group were then averaged to generate inter-animal mean values. Standard error was calculated from the standard deviation of the inter-animal means. The data were analyzed separately by age group with a two-way nested model repeated measures analysis of variance with custom interactions and a Bonferroni post hoc analysis on DataDesk (Kirk, 1982). An overall effect of prenatal malnutrition (nutrition effect, nutr) was considered significant at $P \leq 0.05$. Individual subfields reached a level of significance after the Bonferroni test at $P \leq 0.05$ only when a significant nutrition × subfield interaction (nutr*sbd) was found after the initial analysis of variance ($P \leq 0.05$). The data presented in all graphs are expressed as the actual values of fentomoles per milligram of tissue measured with the use of Inquiry, without the use of correction factors.

RESULTS

Kainate binding sites

Figure 1B shows the pattern of ^{3}[H]kainate binding in a horizontal section through the hippocampus and adjacent cortex (example is from a 90-day control rat). Note that the highest binding region, the mossy fiber zone (stratum lucidum), is labeled 6 in Figure 1A and is seen as an upside-down U shape in Figure 1B.

Postnatal day 15. In the control rats, binding sites for ^{3}[H]kainate were abundant in stratum lucidum (mossy fiber layer) of CA3 (1,831 fmol/mg tissue), nearly double the density measured in the next highest region of binding, stratum moleculare of the subiculum (Fig. 2A). A modest density of binding (>500 fmol/mg tissue) was quantified in the hilus and molecular layer of the dentate gyrus, the pyramidal cell layer of the subiculum, and the deep layers of entorhinal cortex and neocortex (Fig. 2A). In prenatally malnourished rats, the density of ^{3}[H]kainate binding sites was not significantly different in any of the measured hippocampal, entorhinal, or neocortical lami-

nae, although slight elevations of density were detected in most regions. In stratum lucidum, the highest density of ^{3}[H]kainate binding site density was quantified in prenatally malnourished rats (mean = 2,145 fmol/mg tissue but due to inter-animal variability did not obtain statistical significance compared with measures in control rats (mean = 1,831 fmol/mg tissue).

Postnatal day 30. In normal rats at 30 postnatal days, the mean measured density of ^{3}[H]kainate binding in stratum lucidum was 2,566 fmol/mg tissue, considerably higher than the density quantified in 15-day animals (compare Fig. 2A with 2B). Similar to the 15-day analysis, there was no statistical significance in the mossy fiber zone between 30-day malnourished and control rats, and most other regions had lower ^{3}[H]kainate binding at this age (<500 fmol/mg tissue).

Postnatal day 90. In control rats, the quantitative distribution of ^{3}[H]kainate binding in the hippocampus, entorhinal cortex, and neocortex of 90-day normal rats was very similar to the distribution and densities measured at 30 days (compare Fig. 2B with 3A). In malnourished rats, the mean binding site density in the mossy fiber zone (2,638 fmol/mg tissue) was significantly higher than the measured density in control rats (2,330 fmol/mg tissue; $P = 0.0122$ nutr*sbd, $P < 0.001$ Bonferroni; Fig. 3A, asterisk indicates statistical significance).

Postnatal day 220. In control rats, a similar pattern of binding was found, with the highest density in the mossy fiber zone (Fig. 3B). Similar to the 90-day results, there was significantly greater ^{3}[H]kainate mean binding density in the mossy fiber zone of 220-day prenatally protein malnourished rats (2,763 fmol/mg protein) compared with 220-day control rats (2,447 fmol/mg protein; $P = 0.0298$ nutr*sbd, $P < 0.001$ Bonferroni; Fig. 3B, asterisk indicates statistical significance).

Summary of ^{3}[H]kainate binding in the hippocampal mossy fiber zone

As seen in Figure 4, across the four age groups, statistically significant differences in ^{3}[H]kainate binding in malnourished versus age- and sex-matched control rats were found at 90 and 220 days, with elevated binding also found at 15 days but normal levels of binding at 30 days. The overall level of density was lowest at 15 days in control and malnourished rats, leveled off for the malnourished rats at 30, 90, and 220 days, but showed a much different pattern in control rats. The density of [^{3}H]kainate binding in control rats increased markedly from days 15 to 30, decreased slightly at 90 days, and increased slightly at 220 days. ^{3}[H]kainate binding in prenatally malnourished rats increased significantly over normal rats at 90 days ($P = 0.0122$ nutr*sbd, $P < 0.001$ Bonferroni) and at 220 days ($P = 0.0298$ nutr*sbd, $P < 0.001$ Bonferroni), as indicated by asterisks on the graph in Figure 4.

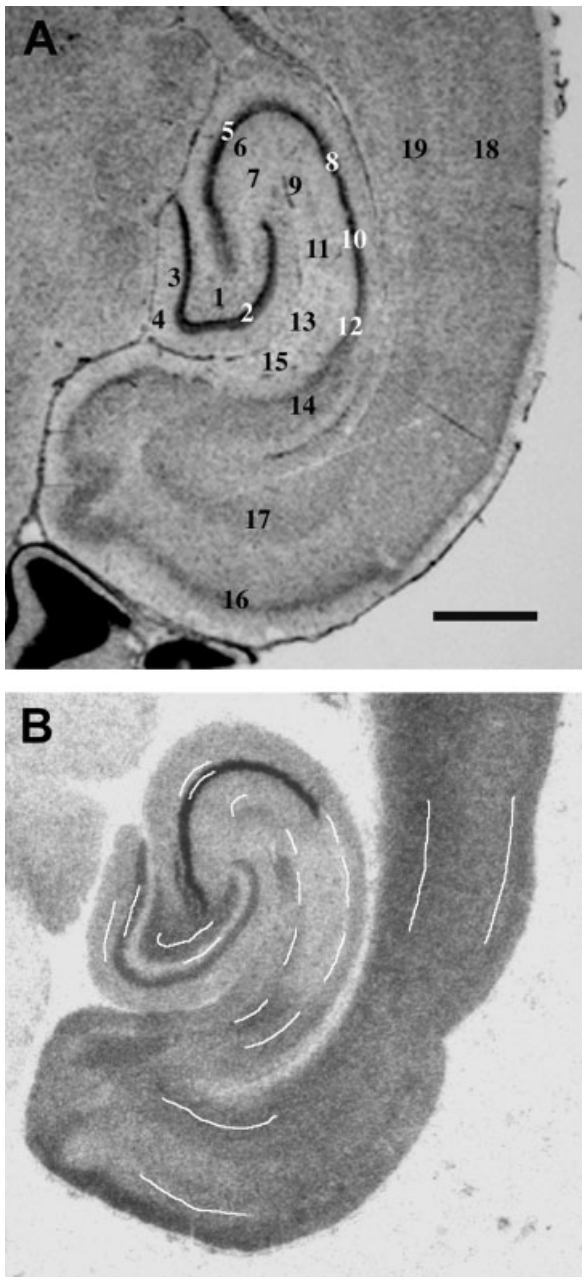

Fig. 1. Sampling technique. Fifteen regions of interest in the hippocampus, including laminae from six hippocampal subfields, and deep and superfical layers of entorhinal cortex and neocortex, were sampled with a line tool from the Inquiry densitometry computer software. The optical density of the region beneath the line sample was converted to femtomoles (fmol) of ligand bound per milligram of tissue by a calibration table constructed from the tritium microscales (see Materials and Methods). The numbered regions in **A** correspond to the line samples in **B** from a horizontal section of a 90-day-old normal rat labeled with ^{3}[H]kainate. The placement of samples in entorhinal cortex correspond to layers II–III (16) and V (17), and that in neocortex correspond to layers II–III (18) and V (19). 1, Hilus of the dentate gyrus (hilus); 2, dentate gyrus granule cell layer (dggc); 3, inner third of the dentate gyrus molecular layer (dgit); 4, outer two-thirds of the dentate gyrus molecular layer (dgott); 5, stratum pyramidale of CA3 (CA3py); 6, mossy fiber zone or stratum lucidum of CA3 (CA3mf); 7, stratum moleculare of CA3 (CA3ml); 8, stratum pyramidale of CA2 (CA2py); 9, stratum moleculare of CA2 (CA2ml); 10, stratum pyramidale of CA1 (CA1py); 11, stratum moleculare of CA1 (CA1ml); 12, stratum pyramidale of prosubiculum (prospy); 13, stratum moleculare of prosubiculum (prosml); 14, stratum pyramidale of subiculum (subpy); 15, stratum moleculare of subiculum (subml); 16, superficial layers of entorhinal cortex (ecsup); 17, deep layers of entorhinal cortex (ecdeep); 18, superficial layers of neocortex (neosup); 19, deep layers of neocortex (neodeep). Scale bar = 1 mm.

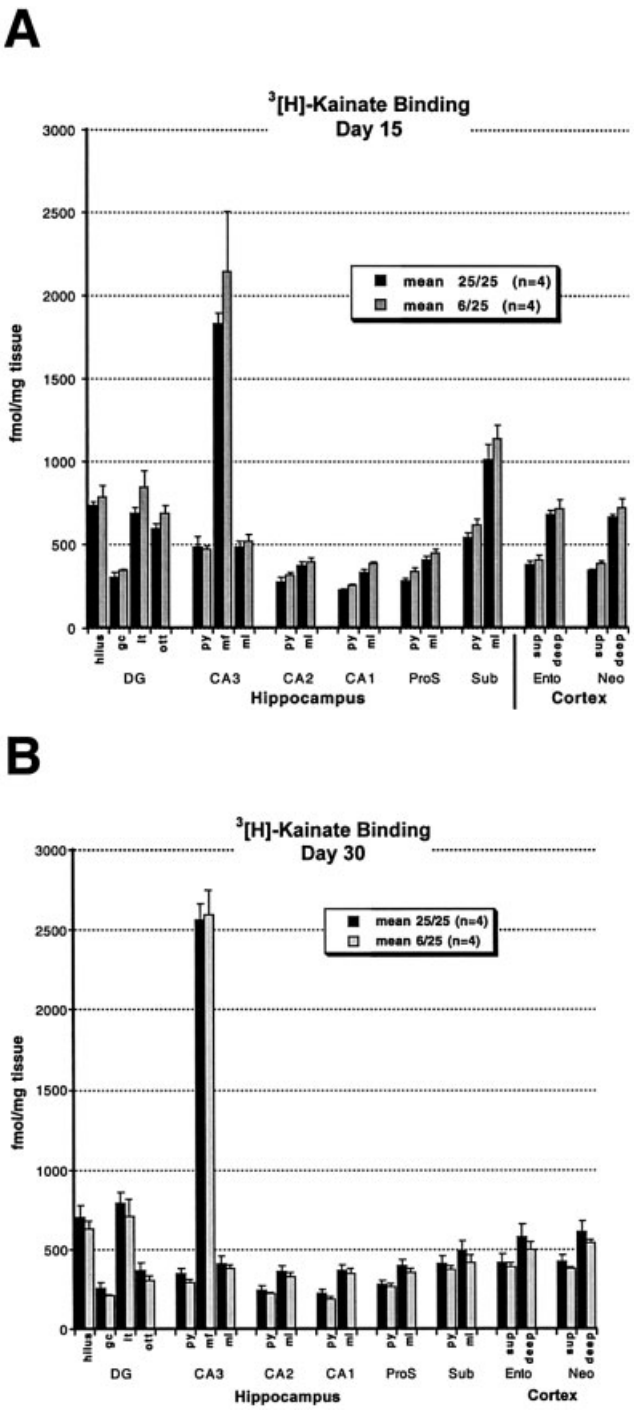

Fig. 2. The density of ^{3}[H]kainate binding in normal and prenatally malnourished rats at 15 (**A**) and 30 (**B**) days. In A and B, the highest binding region is in the mossy fiber zone of CA3 (stratum lucidum). None of the hippocampal subfields or laminae or the laminae of entorhinal and neocortex showed statistical significance when compared. See Figure 1 for guide to abbreviations.

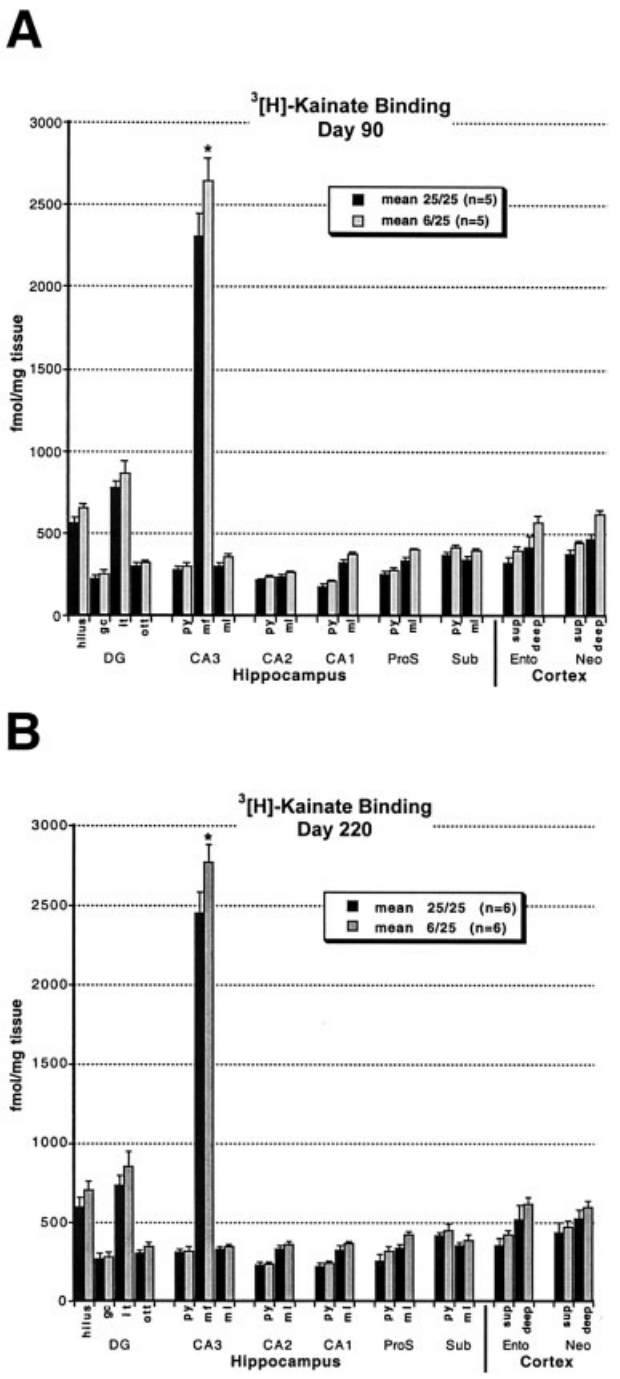

Fig. 3. The regional pattern of ^{3}[H]kainate binding to putative kainate receptors at 90 (A) and 220 (B) days. **A:** In 90-day-old malnourished rats, the mean binding site density in the mossy fiber zone (2,638 fmol/mg tissue) was significantly increased over the measured density in control rats (2,330 fmol/mg tissue; $P = 0.0122$ nutrition $\times$ subfield, $P < 0.001$ Bonferroni; asterisk indicates statistical significance). **B:** Similar to the 90-day results, there was significantly greater ^{3}[H]kainate mean binding density in the mossy fiber zone of 220-day prenatally protein malnourished rats (2,763 fmol/mg protein) than in controls (2,447 fmol/mg protein; $P = 0.0298$ nutrition $\times$ subfield, $P < 0.001$ Bonferroni). See Figure 1 for guide to abbreviations.

AMPA binding sites

At any of the four ages tested, ^{3}[H]AMPA binding site density in prenatally protein malnourished versus control

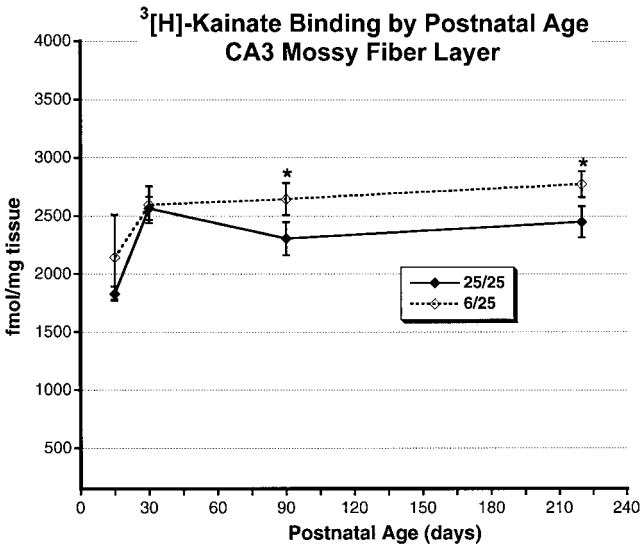

Fig. 4. Development of [3][H]kainate binding sites in stratum lucidum (mossy fiber layer) of CA3 in prenatally malnourished (6/25) and control (25/25) rats across four postnatal ages (15, 30, 90, and 220 days). Density of [3][H]kainate binding sites in 6/25 rats was elevated over control values at 90 days by 12.9% ($P = 0.0122$ nutrition × subfield, $P < 0.001$ Bonferroni) and at 220 days by 11.7% ($P = 0.0298$ nutrition × subfield, $P < 0.001$ Bonferroni) as indicated by asterisks on the graph but not at day 15 or 30. These results may indicate a compensatory response of kainate receptors to reduced mossy fibers in adult prenatally protein malnourished rats.

rats did not show any marked or statistically significant differences in any of the hippocampal, entorhinal, or neocortical regions measured. A gray-scale image taken from tritium-sensitive film for [3][H]AMPA binding sites at 90 days (control section) is shown in Figure 5A. [3][H]AMPA had the highest density binding of the three ligands used in the present study, with highest binding in the molecular layer of the dentate gyrus and in the CA1 and prosubicular subfields. An example of the quantified [3][H]AMPA binding densities at 90 days is shown in Figure 6A. The [3][H]AMPA binding at the other ages was similar to that at 90 days except that stratum pyramidale of CA1 had the highest binding density at 15 days.

NMDA binding sites

Similar to the [3][H]AMPA results, there were no statistically significant differences in [3][H]MK-801 binding of putative NMDA receptors across the four ages studied in prenatally protein malnourished versus control rats in any of the hippocampal laminae and subfields, entorhinal cortex, or neocortex. An example of a gray-scale image taken from film for [3][H]MK-801 binding (90-day control section) is shown in Figure 5B and quantified in Figure 6B. At 30, 90, and 220 days, the highest binding region was in the molecular layer of the dentate gyrus in the inner third and outer two-thirds (see Fig. 6B for day 90). Similar to the [3][H]AMPA binding, there were some differences at 15 days; however, for [3][H]MK-801 binding, the highest binding at day 15 was in the mossy fiber zone of CA3.

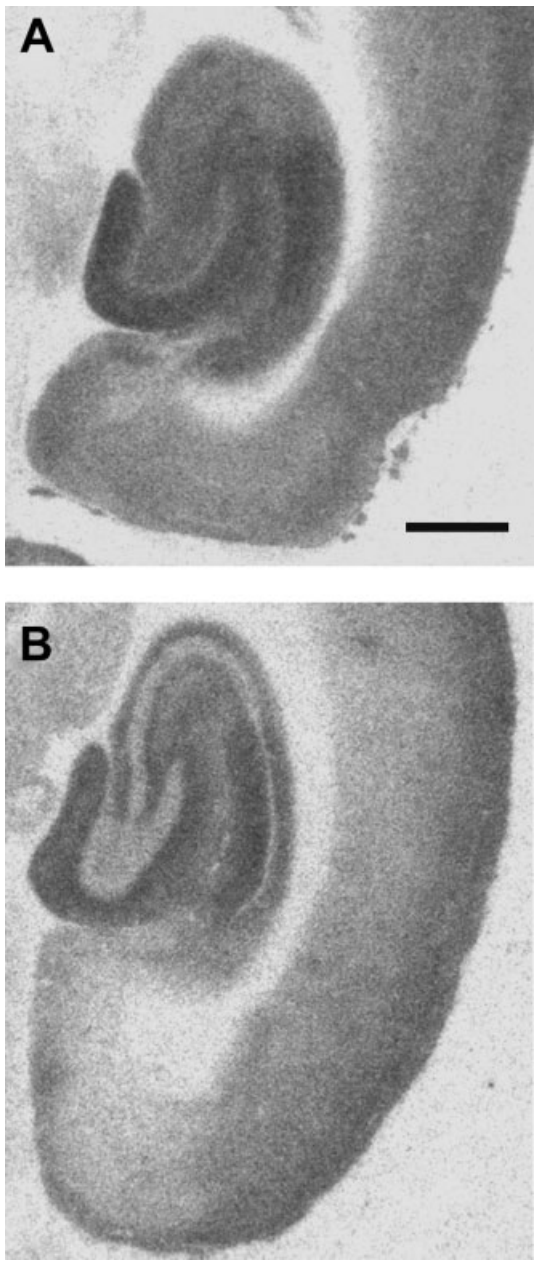

Fig. 5. Distribution of [3][H]AMPA (**A**) and [3][H]MK-801 (**B**) binding to putative NMDA receptors in 90-day control rats. Regions of highest receptor content appear dark in the gray-scale image of horizontal sections taken off tritium-sensitive film. For [3][H]AMPA, high binding regions were in the molecular layer of the dentate gyrus and in the CA1 and prosubicular subfields. For [3][H]MK-801, the density of binding sites was highest in the dentate gyrus molecular layer, mossy fiber layer of CA3, superficial layers of neocortex, and molecular layer of prosubiculum. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; NMDA, *N*-methyl-D-aspartate. Scale bar = 1 mm.

DISCUSSION

To understand the significance of the finding of an increased density of [^{3}H]kainate binding in the mossy fiber zone in the prenatally protein malnourished rats at days 90 and 220 but not at days 15 or 30, we discuss the issue of structural and functional localization of these receptors.

Normal distribution and development of ^{3}[H]kainate receptors in the mossy fiber zone: presynaptic or postsynaptic localization?

Binding of ^{3}[H]kainate was highest in stratum lucidum (the mossy fiber layer) of CA3 as compared with binding in all other cortical subfields and is in agreement with previously reported binding studies (Monaghan and Cotman, 1982; Represa et al., 1987; Ulas et al., 1990; Bahn et al., 1994). Represa et al. (1987) provided evidence that ^{3}[H]kainate binding sites are presynaptic on mossy fiber terminals, because the majority of high-affinity ^{3}[H]kainate binding disappears after destruction of dentate gyrus granule cells (for review, see Wisden and Seeburg, 1993). Additional evidence for presynaptic high-affinity kainate receptors has been provided by electrophysiology studies (Malva et al., 1995; Chittajallu et al., 1996). Normally, presynaptic kainate receptors function to modulate calcium influx in nerve terminals in CA3 (Malva et al., 1995) and/or biphasically regulate synaptically released glutamate in the hippocampus (Chittajallu et al., 1996). In contrast, another electrophysiologic study found a different functional role for kainate receptors, suggesting a postsynaptic component in the hippocampus. Vignes and Collingridge (1997) demonstrated that high-frequency stimulation of mossy fibers in rat hippocampal slices, in the presence of selective antagonists for AMPA, NMDA, and GABA receptors, activates an inward current in CA3 neurons that has a pharmacology typical of kainate receptors. They suggested that previous experiments in which CNQX (6-cyano-7-nitro-quinoxalene-2,3-dione) was used to block AMPA receptor synaptic activation also antagonized kainate receptor-mediated responses. Castillo et al. (1997) reported that repetitive activation of the hippocampal mossy fiber pathway, after application of a blocking agent specific for AMPA receptors (GYKI 35655), generates a slow excitatory synaptic current that greatly augments the excitatory drive of CA3 pyramidal cells. These results directly demonstrated the presence of kainate receptors localized to the proximal dendrites of CA3 pyramidal cells (Castillo et al., 1997). Additional support for a postsynaptic localization of kainate receptors in CA3 has been provided by reports in which the expression and immunocytochemical localization of kainate receptor subunits were measured in the rat (Wisden and Seeburg, 1993; Bahn et al., 1994; Petralia et al., 1994c; Fogarty et al., 2000). Pyramidal neurons of CA3 have been shown to express kainate receptor subunit mRNA (GluR6, KA-1, KA-2; Wisden and Seeburg, 1993; Bahn et al., 1994; Fogarty et al., 2000). Kainate receptor subunits KA-1 (Fogarty et al., 2000) and KA-2 and GluR6-7 (Petralia et

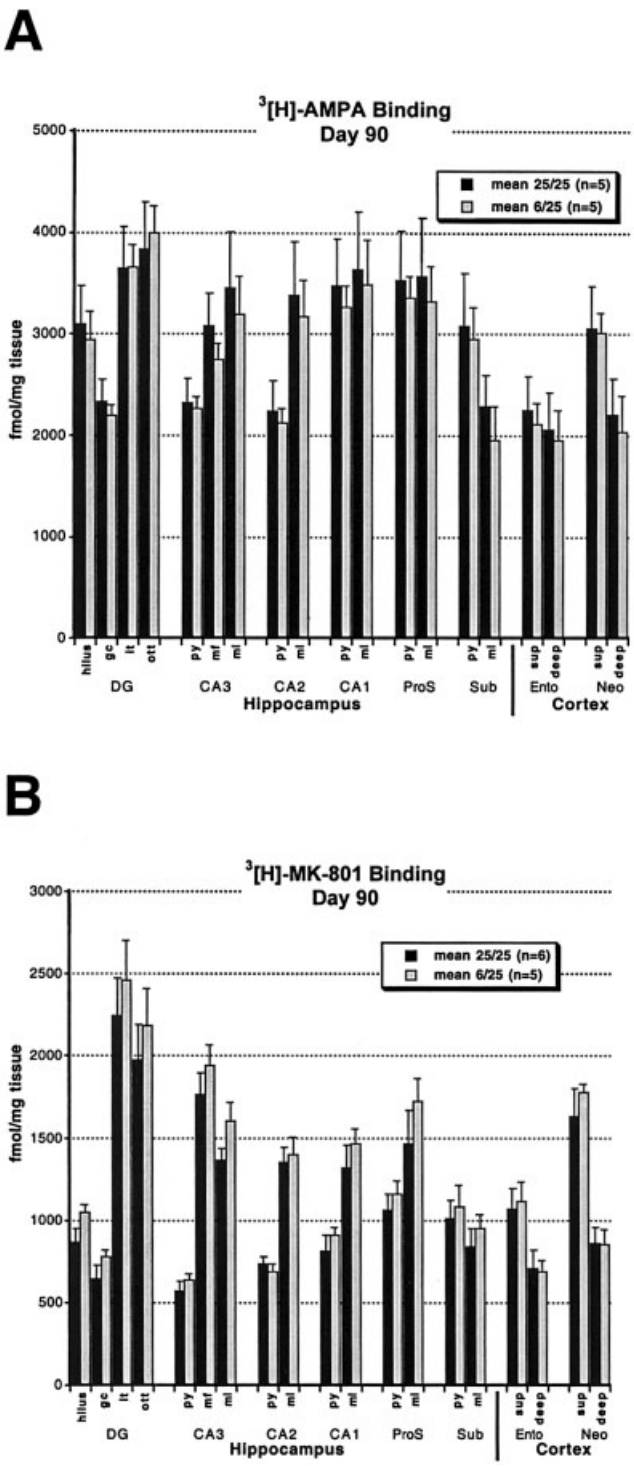

Fig. 6. Density of binding sites for ^{3}[H]AMPA at 90 days (A) and for ^{3}[H]MK-801 binding sites (B) in the hippocampus, entorhinal cortex, and neocortex in adjacent sections from a prenatally malnourished rat. **A:** ^{3}[H]AMPA had the highest density binding of the three ligands used in the present study, with highest binding in the molecular layer of the dentate gyrus and in the CA1 and prosubicular subfields. At any of the four ages tested, ^{3}[H]AMPA binding site density in prenatally protein malnourished versus control rats did not show any marked or statistically significant differences in any of the hippocampal, entorhinal, or neocortical regions measured when compared with sex- and age-matched controls. **B:** The highest binding region was in the molecular layer of the dentate gyrus, in the inner third and the outer two-thirds. Similar to the ^{3}[H]AMPA results, there were no statistically significant differences in ^{3}[H]MK-801 binding of putative NMDA receptors across the four ages studied in prenatally protein malnourished versus control rats in any of the hippocampal laminae and subfields, entorhinal cortex, or neocortex. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; NMDA, *N*-methyl-D-aspartate. See Figure 1 for guide to abbreviations.

al., 1994c) also have been labeled immunocytochemically in CA3 pyramidal cells.

Development of ^{3}[H]kainate binding sites in control and prenatally protein malnourished rats

The density of ^{3}[H]kainate receptor binding was significantly affected by prenatal protein malnutrition at 90 and 220 days. The measured changes in ^{3}[H]kainate binding site density are not likely to be attributed to differences in tissue quench of tritium between control and prenatally malnourished rats, because mossy fibers are unmyelinated. The significant differences in ^{3}[H]kainate binding in adult prenatally malnourished rats is also unlikely due to the prenatal diet of reduced protein and thus amino acids. Patel et al (1975) used ^{14}[C]leucine as a postnatal precursor after prenatal protein malnutrition and found that, by 21–35 days after birth, the rate of conversion of the leucine carbon into proteins including glutamate and glutamine is restored to normal. This restoration in part may account for the normal density of ^{3}[H]kainate receptors at the earlier ages (15 and 30 days) and the ability to upregulate at the later ages (90 and 220 days).

Morphologic studies by Cintra et al. (1997b) have demonstrated a significantly reduced mossy fiber plexus in 15-, 90-, and 220-day but not in 30-day prenatally malnourished rats with the use of Timm's heavy metal (zinc) stain in this same animal model. More recently, Granados-Rojas et al. (2002) conducted a Timm's stain and stereologic study in 220-day-old control and prenatally malnourished rats and found a significantly decreased total volume of the mossy fiber system suprapyramidal bundle in the malnourished group. They also reported a significant decrease in the total numerical density of mossy fiber–CA3 asymmetrical synapses in 220-day adult prenatally protein malnourished rats as compared with controls (Granados-Rojas et al., 2002). In the present study, the increased ^{3}[H]kainate binding in stratum lucidum of prenatally malnourished rats at 90 and 220 days may indicate a compensatory response of postsynaptic kainate receptors to offset a diminished mossy fiber afferent plexus. This hypothesis seems reasonable in terms of the proposed role of postsynaptic kainate receptors in CA3, to generate a slow excitatory synaptic current that greatly augments the excitatory drive of CA3 pyramidal cells (Castillo et al., 1997). Physiologic studies in this animal model have consistently demonstrated a net enhancement of inhibitory processing in the hippocampus, especially in the dentate gyrus and CA3 regions in young adult (90 and 120 days) prenatally protein malnourished rats (Austin et al., 1986, 1992; Bronzino et al., 1986, 1990). This observation suggests that the compensatory increase in ^{3}[H]kainate density in adult prenatally malnourished rats does not in itself restore the normal balance of inhibition-excitation in CA3.

This disturbance in inhibition in the hippocampus of prenatally malnourished rats may be functionally restricted to the mossy fiber–CA3 synaptic region, although Luebke et al. (2000) reported an increase in the frequency of $GABA_A$ receptor-mediated mIPSCs in CA1 pyramidal neurons in adult (90–120 days) prenatally protein malnourished rats. However, a more recent physiologic study in the CA1 hippocampal region by the same group (Moker et al., 2001) reported no significant difference in the frequency of $GABA_A$ receptor-mediated mIPSCs in CA1 when modulated by the benzodiazepine agonist zolpidem. This finding suggests that there may not be significant functional consequences at a single-cell level in the CA1 region of the rat hippocampus measured in vitro. Negative findings were also reported by Rushmore et al. (1998) who found no significant differences in mIPSCs in the perforant pathway or in the Schaffer collateral to CA1 pyramidal cell synapse.

DeBassio et al. (1996) in this same animal model reported an enhancement in postnatal generation of granule cells in the dentate gyrus at 30 days that might also contribute to the normal density of kainate receptors at that age. In contrast, they did not find similar increases in granule cell generation in prenatally malnourished rats at 15 days, which might contribute to the decreased mossy fiber plexus measured by Cintra et al. (1997b). Hippocampal granule cell neurogenesis is now known to continue well into adulthood, with rates of neurogenesis being dependent on levels of circulating adrenal steroids (Cameron and Gould, 1994), and serotonin via activation of the $5\text{-}HT_{1A}$ receptor (Gould, 1999). Interestingly, our previous study in 220-day prenatally protein malnourished rats demonstrated a 15–20% decrease in $5\text{-}HT_{1A}$ receptor density when using ^{3}[H]8-OH-DPAT (8-OH-2-di-n-propylamino tetralin) in the mossy fiber zone of CA3, a 20% decrease in 5-HT uptake sites when using ^{3}[H]citalopram in the mossy fiber zone, stratum radiatum, and moleculare of CA3, and a decreased 5-HT fiber plexus throughout the hippocampus (Blatt et al., 1994). Thus, the decreased mossy fiber plexus in adult malnourished rats may be due in part to a decreased granule cell neurogenesis due to altered 5-HT input and/or pre- or postsynaptic 5-HT receptors, so the upregulation of kainate receptor density may be in response to the decreased mossy fiber input to CA3.

In the prenatally protein malnourished rats, it is not clear why, with a reduced mossy fiber plexus, that sprouting probably does not occur as a compensatory response to restore the normal mossy fiber plexus. Mossy fibers do exhibit much plasticity and have extensive sprouting after brief seizure episodes that induce long-lasting changes reminiscent of classic LTP and establish new synaptic contacts (Ben-Ari and Represa, 1990). Upregulation of particular types of metabotropic glutamate receptors, mGLuR1 and mGluR5, have been induced by kindling and via intraperitoneal kainate injections in two rat models of temporal lobe limbic seizures (Blumcke et al., 2000). In another recent study, a new selective antagonist at neuronal kainate receptors containing the GluR5 subunit, LY382884, that does not affect NMDA or AMPA receptors at particular concentrations, was found to prevent the induction of mossy fiber LTP, which is independent of NMDA receptors (Bortolotto et al., 1999). Thus, upregulation of kainate receptors in the mossy fiber zone of prenatally protein malnourished rats may be necessary to act as an induction trigger for long-term changes in synaptic transmission through the CA3 region of the hippocampus.

Activation of presynaptic glutamatergic metabotrophic receptors at GABAergic terminals may lead to a decrease in transmitter release (Poncer et al., 1995; Rodriguez-Moreno and Lerma, 1998; Semyanov and Kullmann, 2000). The recent discovery that kainate receptors are present on presynaptic GABAergic terminals contacting hippocampal CA1 interneurons in adult rats suggests a

new important role in modulating interneuron communication (Cossart et al., 2001). Application of kainate and not AMPA or NMDA increased the mIPSCs, increased GABA and evoked release at interneuron–interneuron synapses (Cossart et al., 2001). It is not known whether such control with kainate receptors of interneuron activity occurs in other regions of the hippocampus, but because interneurons are distributed throughout the hippocampus and are rich in group III metabotrophic glutamate receptors, it is likely that these effects are more widespread, leading to decreases of GABAergic transmission among interneuronal populations (Semyanov and Kullman, 2000; Cossart et al., 2001).

Development of ^{3}[H]MK-801–labeled NMDA receptors in prenatally protein malnourished rats

In marked contrast to the kainate receptor findings, the density and distribution of postsynaptic NMDA receptors did not change across any of the ages studied, suggesting that perforant path afferents and outer dendrites of hippocampal principal cells are not a primary target of the prenatal insult. This possibility also suggests that alterations in LTP at the perforant path–dentate gyrus granule cell synapse measured electrophysiologically in adult prenatally malnourished rats of this model (Austin et al., 1986; Austin-LaFrance et al., 1991; Morgane et al., 1995) are probably not due to altered NMDA receptor density. However, specific characteristics of the NMDA receptor may be altered in these animals. For example, in a rat model of neonatal hypoxia, Otoya et al. (1997) reported altered glutamate/glycine (glu/gly) stimulation of MK-801 binding in 40-day rats without altered ^{3}[H]MK-801 binding density in buffer free of glu/gly. These changes were attributed to possible differences in the subunit composition of NMDA receptors in hypoxic as opposed to normal brains detected by increased sensitivity of glu/gly to NMDA receptors of particular subunit assemblies (Otoya et al., 1997). Steiger et al. (2002) reported alterations in $GABA_A$ receptor gene expression in prenatally protein malnourished rats. Those investigators found a reduction in β2, β3, and γ2L $GABA_A$ receptor subunits in the septum, which may account for the decreased sensitivity to the benzodiazepine chlordiazepoxide in malnourished rats. Thus, although there were no detectable differences in any of the regions sampled, our study is limited by the nonspecificity of ^{3}[H]MK-801 for particular subtypes of NMDA receptors, leaving possible subtle changes in NMDA receptor subtypes in prenatally malnourished rats undetected.

Development of ^{3}[H]AMPA binding sites in prenatally protein malnourished rats

Similar to the findings of ^{3}[H]MK-801 binding in cortex of prenatally malnourished rats, there were no significant region-specific differences in density of ^{3}[H]AMPA binding in the hippocampus, entorhinal cortex, or neocortex in prenatally malnourished versus age-matched normal rats. The normal distribution of quantified ^{3}[H]AMPA binding sites (Rainbow et al., 1984; Nielsen et al., 1990) in the present study was very similar to the distribution pattern for ^{3}[H]MK-801 binding and NMDA-sensitive ^{3}[H]glutamate binding, suggesting a distribution overlap of NMDA and AMPA receptors. Light and electron microscopic immunocytochemical localizations of AMPA and NMDA receptor subunits support a possible colocalization of the two receptor types (Petralia and Wenthold 1992; Petralia et al., 1994a,b). Colocalization of the two glutamatergic receptor types may be important for the establishment and maintenance of hippocampal LTP (Muller et al., 1988; Bekkers and Stevens 1989) and/or to complement one another in a dual-component excitatory postsynaptic potential (Spruston et al., 1995).

CONCLUDING REMARKS

In prenatally protein malnourished rats, our data demonstrated a vulnerability of kainate receptor density and distribution across adult postnatal ages tested (90 and 220 days), but that AMPA and NMDA receptors appear unaffected by the nutritional insult at any of the tested ages. The observed increases in ^{3}[H]kainate binding density within stratum lucidum likely was due to the statistically significant decreased mossy fiber plexus reported at those ages (Cintra et al., 1997b). An enhanced granule cell production at 30 days might account for the normal mossy fiber plexus at that age and normal ^{3}[H]kainate binding density in these animals and may occur as a compensatory response as the animals begin to reach reproductive age. Because kainate receptors with the GluR5 subunit and possibly the GluR6 subunit (Bortolotto et al., 1999) are involved in the induction of mossy fiber LTP, this kainate receptor-mediated component of excitatory synaptic transmission is an important component in adaptive hippocampal synaptic plasticity. Whether such adaptive plasticity is altered in prenatally protein malnourished rats is not known. The recent discovery (Cossart et al., 2001) that hippocampal kainate receptors may play an important role in the regulation of interneuron–interneuron GABAergic transmission suggests functional heterogeneity of kainate receptors that can influence intrahippocampal network function according to its location and the type of receptor activated. Because physiologic inhibition in the hippocampus has been repeatedly reported in this animal model, it is interesting to speculate that the net inhibition may be the result of altered kainate receptor modulation of GABAergic interneuron activity. We are currently investigating the number and distribution of populations of GABAergic interneuron types in malnourished rats by quantifying the numbers that express different types of calcium binding proteins and neuropeptides. Such a study might provide further insight into the underlying basis of which intrahippocampal pathways might be functionally altered in prenatally protein malnourished rats.

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