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Role of feline maternal taurine nutrition in fetal cerebellar development: An immunohistochemical study

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Summary. We report the effects of four levels of maternal dietary taurine on the cerebellum of 45-day gestation fetuses. As we have previously reported for newborn and 8-week-old kittens, maternal dietary taurine content has a profound effect also on fetal cerebellum. Fetuses from queens fed the lowest amount of taurine had the greatest density of granule cells, probably because of smallest brain size, and had a high proportion of morphological abnormalities. Somewhat surprising was the observation that the fetuses from the lowest maternal dietary taurine group had the highest proportion of taurine-positive granule cells. In addition, these results confirm the vulnerability of developing fetal brain to its intrauterine environment.

Keywords: Amino acids – Taurine – Cerebellum – Development – Immunohistochemistry

Introduction

Interest in taurine as a nutrient began in the early 1970's with the observation that cats fed taurine-free diets suffered progressive retinal photoreceptor degeneration and eventual blindness (Hayes et al., 1975; Schmidt et al., 1976). The last two decades have witnessed the physiological status of taurine move from that of an enigmatic metabolic end product to an essential nutrient for the cat and possibly also for other species, including man. More recent studies have found that dietary taurine deprivation during pregnancy and lactation has a number of additional adverse consequences for developing kittens, including increased neonatal mortality, neurological abnormalities, and abnormal development of the cerebellum and visual cortex (Sturman et al., 1985a; Sturman et al., 1985b; Sturman et al., 1985c; Palackal et al., 1986; Palackal et al., 1988; Sturman et al., 1986). Taurine also plays a role in heart function, and can prevent or cure feline dilated cardiomyopathy (Pion et al., 1987). The nutritional importance of taurine for cats is now widely accepted, and commercial cat foods now contain taurine supplements. Taurine is found

in meat and fish but not in foods of vegetable origin. Cats, as obligate carnivores, obtain large amounts from their natural food sources, but they have a very low capacity to synthesize taurine from other sulfur-containing amino acids. Although it has been assumed that cats are unique in their dependence on dietary sources of taurine, levels of taurine-synthesizing enzymes are even lower in humans than in cats (Jacobsen and Smith 1968). We and others have shown that less than 50% of pregnancies in females fed a taurine-free diet reach term, the remainder being terminated by resorbtion or abortion (Sturman et al., 1986; Messing and Sturman 1993; Dieter et al., 1993). Abnormal relaxin and progesterone values have been reported in pregnant taurine-deficient cats (Dieter et al., 1993). Of those pregnancies that reach term, one-third of the kittens are stillborn, and less than half of those born live survive to weaning. We have described in detail the neurological abnormalities that are present in such surviving kittens, which include thoracic kyphosis, abnormal and/or delayed cerebellum maturation, and abnormal visual cortex and dorsal root-spinal cord development (Sturman et al., 1985a; Sturman et al., 1985b; Sturman et al., 1985c; Palackal et al., 1986; Palackal et al., 1988; Imaki et al., 1986; Xu et al., 1992; Xu et al., 1993). The use of diets containing some, but insufficient, taurine have shown that feline reproductive performance can be marginally improved over that of females consuming a taurine-free diet, that surviving kittens are not obviously neurologically impaired, and that tissue taurine concentrations are intermediate. The results obtained to date clearly establish a dietary content of 0.05% taurine in a dry, purified diet as the minimum necessary prevent taurine depletion in adult cats and as the minimum consistent with normal feline pregnancy and outcome of the progeny (Burger and Barnett 1982; Sturman and Messing 1991). In (1986, the National Research Council) recommended a minimum dietary requirement of 0.04% taurine for kittens and 0.05% taurine for pregnant cats, both based on dry, purified diets. These recommendations have been complicated by observations that cats fed typical commercial heat-processed diets containing as much as 0.15% taurine became taurine-depleted and suffered from many of the associated clinical complications (Odle et al., 1993; Hickman et al., 1992; Pion et al., 1987; Earle and Smith 1991; Fox and Sturman 1992).

We have further demonstrated for newborn taurine-deficient kittens that the total thickness of the visual cortex and each of the individual laminae measured, except for layer I, are significantly smaller than in kittens from taurine-supplemented mothers (Xu et al., 1992). The total number of neurons and glia did not differ in any of the layers and the numerical density of cells was greater in taurine-deficient kitten visual cortex in all layers except for layer I. Using our taurine antiserum we found a band of staining near the pial surface and further investigation using electron microscopy and electron microscopic immunohistochemistry showed this region to be packed with dendrites and axons and that virtually all of the taurine-like immunoreactivity was present within these structures. No such band of staining was evident for GABA or glutamate, further emphasizing the special role of taurine in development. Recent quantitative morphometric measurements on frontal cortex from the same newborn kittens show fewer differences resulting from taurine

deficiency (Lu et al., 1994). It seems likely that those regions of the brain with the greatest density of neurons are most likely to suffer abnormalities during development.

This report describes a study in which the cerebellum of cat fetuses from mothers fed different amounts of taurine was examined.

Methods

Animals

Breeding of mature queens was performed at the Nutrition and Pet Care Colony at the University of California Davis. Four dietary groups were used, consuming diets containing 0.09%, 0.06%, 0.04% and 0.01% taurine on a dry matter basis. Queens were bred over a 2.5 year period and those which completed 2 term pregnancies with live kittens at birth were bred a third time. The third pregnancy was terminated at approximately days 45 gestation by ovariohysterectomy. Thirty-seven queens satisfied these criteria, approximately equally divided amongst the four diet groups. Ovariohysterectomies were performed as previously described (Wilson and Hayes 1983). Briefly, queens were anesthetized using a ketamine (2.5 to 5.5 mg/kg IV)/diazepam (0.12 to 0.275 mg/kg IV) cocktail and intubated immediately. Inhalation anesthesia was maintained throughout surgery using 0.15% to 0.25% isoflurane and 0.2 (1 L/min). Following anesthesia and surgical preparation, a ventral midline incision was made and the gravid uterus immediately identified. Each ovarian pedicle was ligated with 3-0 chromic gut suture then transected. The uterine body was clamped, ligated and transected similarly. The incision was routinely closed using 3-0 vicryl and 3-0 nylon suture. The queens were returned to the colony upon recovery. The excised uterus was opened and the fetal tissues removed immediately upon completion of each ovariohysterectomy. All fetuses had died prior to removal from the uterus. The number of kittens, embryonic tissue and empty gestational sacs were recorded from each uterus. Body weight, crown-rump length, gender, and gross morphology were recorded for each fetus and embryo. The fetuses used in this experiment were day 45 gestation and were placed in a chilled container for transport and processing. Within 1 hour of removal from the uterus, the head was removed from the body by sharp dissection. The calvarium was opened to expose brain tissue and placed into chilled fixative (2.5% glutaraldehyde and 1.0% paraformaldehyde solution in a 0.1 M phosphate buffer). Samples were stored at 5°C until shipped for processing.

Immunohistochemistry

Immunogens were synthesized by the method of (Campistron et al., 1986), starting with 100 mg of taurine [or γ-aminobutyric acid (GABA), glutamate, β-alanine, etc.), and coupling with glutaraldehyde to bovine serum albumin (BSA) or poly-L-lysine (PL, MW 30-70kD, Sigma Chemical Co., St. Louis, Missouri, U.S.A.).

Rabbits were immunized by first injecting 500 μg of immunogen emulsified in Freund's complete adjuvant, followed every 4 weeks by an injection of 500 μg immunogen emulsified in Freund's incomplete adjuvant. Injections alternated amino acid-glutaraldehyde-BSA and amino acid-glutaraldehyde-PL. Two rabbits were injected subcutaneously and two intramuscularly. Blood was collected 7 and 21 days after each injection and serum frozen in 1 ml batches.

The serum was characterized for titre and cross-reactivity using the ELISA method (Lake and Verdone-Smith 1989). High, stable titres of reactivity against amino acid-glutaraldehyde-BSA were reached after three injections (>1:64,000 dilution). The anti-taurine serum had a small cross-reactivity to GABA-glutaraldehyde-BSA and to β-alanine-glutaraldehyde-BSA. This reactivity was removed by preadsorption with both

conjugates and resulted in little loss of taurine-glutaraldehyde-BSA activity. Antibodies to the other immunogens were similarly treated prior to use.

Tissue was embedded in paraffin and serial 6μm sagittal sections cut and mounted on glass slides. Regular histological staining were H and E, Crysol Violet and bodian silver staining. Sections were deparaffined by warming at 60°C for 1 hour, and 3 × 5 min in a histoclear bath, followed by graded ethanol incubation. Staining with the antisera (1 to 2,000 dilution) was carried out at 4°C overnight and visualized using the peroxidase-conjugated avidin/DAB method (Dako Corp., Carpinteria, California, U.S.A.). Control sections were included in every batch, those in which the antisera were preabsorbed with the immunogen prior to processing, and others replacing the primary antiserum with preimmune serum at similar dilutions. Control slides showed on visible staining after processing, whereas slides prepared with the antisera showed brown reaction products. Counterstaining, when employed, was with haematoxylin. Photomicrographs were taken on a Zeiss Axiophot.

Morphometric analysis

For morphometric analysis, the vermis (V) of cerebellum (see Fig. 1) were chosen for quantitative counting due to the morphological changes happening in this area. Therefore, crystal violet stained sections were visualized under a microscope with a ×100 objective lens and a ×100 ocular lens fitted with a micrometer grid divided into 100 squares, each corresponding to 100μm^2 on the section. Two areas were chosen from each cerebellum, equal to 2 mm^2 of tissues to count the nucleus. The areas covered was from the pial surface to the white matter. The nuclei with irregular shapes, lost nucleus membranes, and enlarged size were counted as abnormal nuclei. Using camera lucida, the perimeters of all nuclei identified within the grid and those lying on margins were drawn on Xerox paper. Each paper covered 1 mm^2 area. Nuclear profiles on each paper were traced by a stylus on an digitizing tablet using SigmaScan (Jandel Scientific) version 3.90. Measurements of the areas of the nuclei in crystal violet stained sections and in taurine immunostained sections were calculated. The total cells in each animal, the normal and

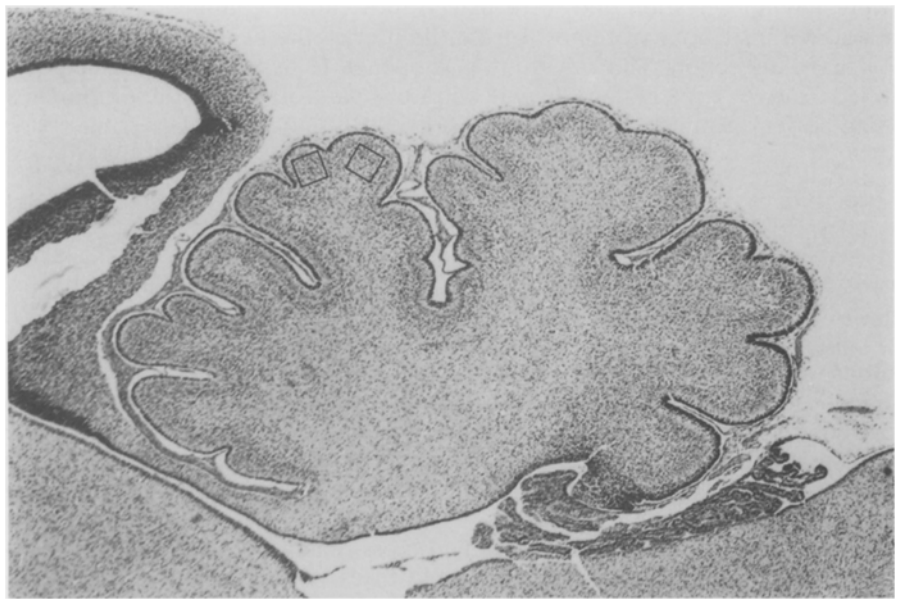

Fig. 1. 6μm sagittal section of fetal cerebellum stained and quantitative. Counting areas with cresyl violet. The boxes in vermis V include the most morphological changes. ×35

abnormal cells, and taurine positive cells all were counted from 2 mm^2 area. The statistics were calculated by ANOVA.

Results

As we have previously reported for newborn term kittens, the number of fetuses and their weight was proportional to the amount of taurine in the maternal diet. Taurine concentrations in blood, plasma and muscle of the queens at parturition also reflected the dietary taurine content as found previously for adult females.

There were a number of differences in cerebellar granule cells between the groups as measured in vermis V (Fig. 1). The density of granule cells was greatest in the 0.01 and 0.09 groups although the proportion of abnormal cells increased with decreasing dietary taurine content (Table 1, Fig. 2). Such abnormal granule cells had significantly increased nuclear area in the two lowest dietary taurine groups. There was a significantly greater proportion of granule cells staining positively with the taurine antibody in the lowest dietary taurine group (Figs. 3 and 4, Table 1). The nuclear area of the taurine-

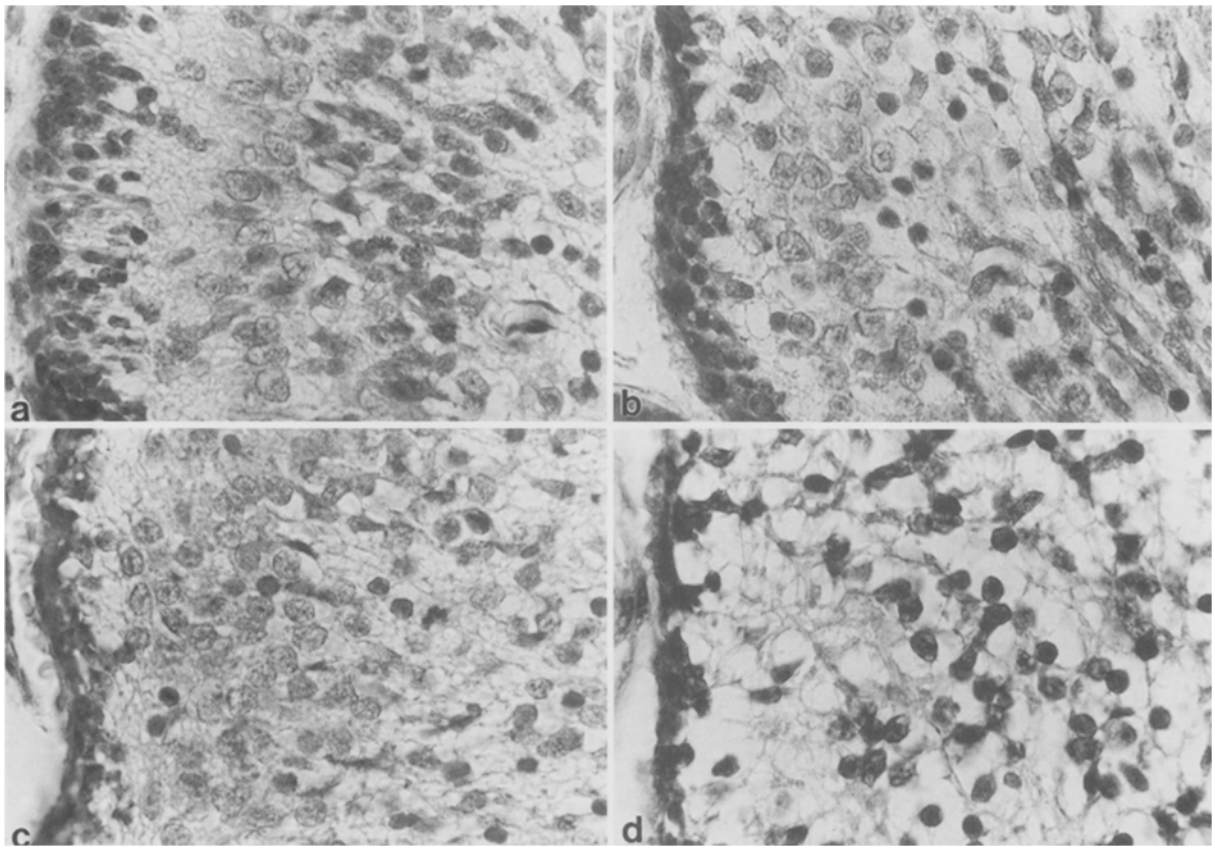

Fig. 2. 6 μm sagittal section of fetal cerebellum stained with cresyl violet. From mothers fed (**a**) 0.01% taurine (**b**) 0.04% taurine (**c**) 0.06% taurine (**d**) 0.09% taurine, ×855

Table 1. Characteristics of granule cells in fetal cerebellum

Taurine in diet %	0.09	0.06	0.04	0.01
Number of cells/mm^2	76.16 ± 9.00[e]	56.41 ± 14.84	64.95 ± 12.75	81.75 ± 5.38[a]
% of normal cells	98.83 ± 0.97	81.26 ± 15.20	73.18 ± 19.78[b]	70.85 ± 4.21[b]
Nuclear area of normal cells μm^2	22.45 ± 1.17	20.07 ± 3.08	19.21 ± 4.18	22.11 ± 0.92
Nuclear area of abnormal cells μm^2	23.13 ± 13.73	23.21 ± 12.20	28.09 ± 4.47[c]	31.53 ± 2.74[c]
% taurine-positive cells	26.35 ± 7.62	25.20 ± 14.43	31.16 ± 16.87	47.70 ± 6.64[d]
Nuclear area of taurine-positive cells μm^2	32.01 ± 1.21	31.66 ± 3.17	35.07 ± 6.71	32.06 ± 2.74
Nuclear area of taurine-negative cells μm^2	23.59 ± 1.72	23.08 ± 1.55	22.84 ± 3.54	24.78 ± 5.48

[a] Significantly different ($p < 0.05$) from 0.06 and 0.04; [b] Significantly different ($p < 0.05$) from 0.09; [c] Significantly different ($p < 0.05$) from 0.09 and 0.06; [d] Significantly different ($p < 0.05$) from 0.09 and 0.06; [e] Significantly different ($p < 0.05$) from 0.06.

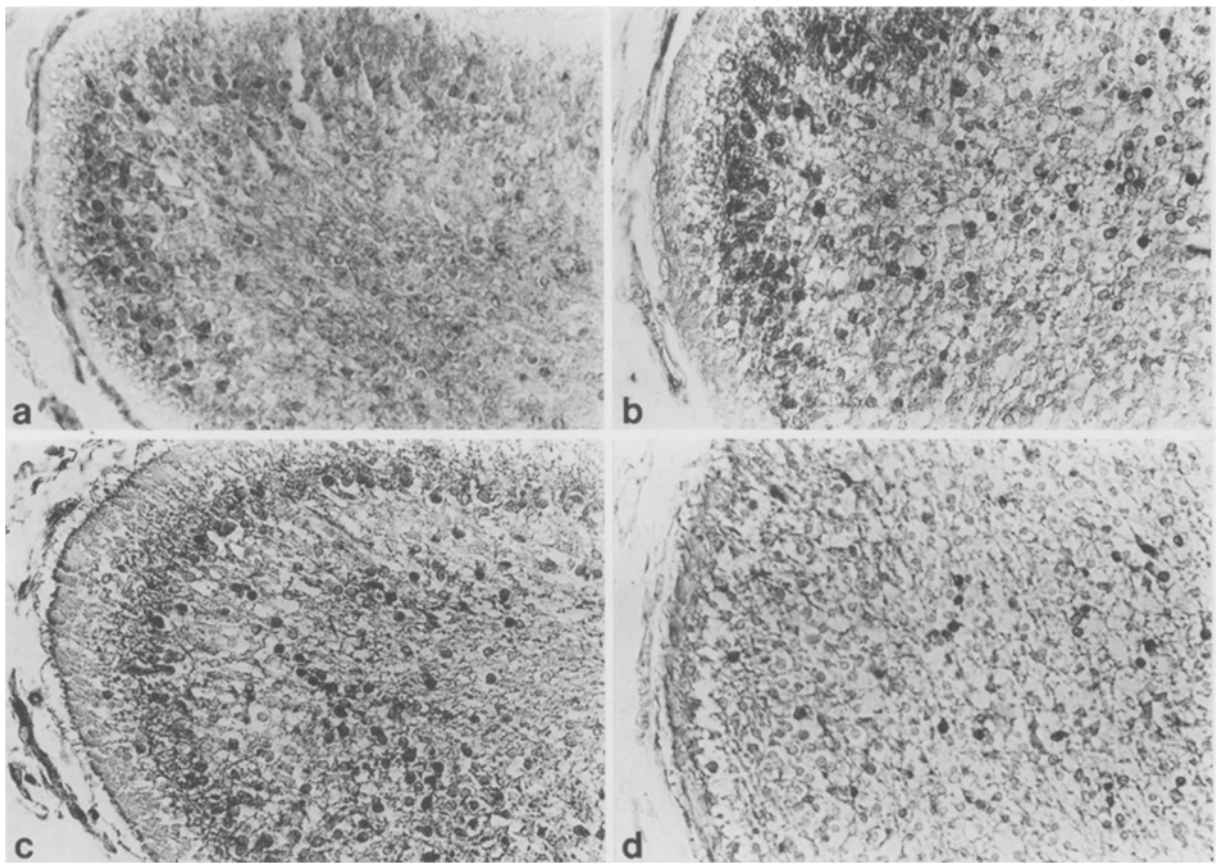

Fig. 3. 6μm sagittal section of fetal cerebellum stained with taurine antibody. From mothers fed (**a**) 0.01% taurine (**b**) 0.04% taurine (**c**) 0.06% taurine (**d**) 0.09% taurine. ×340

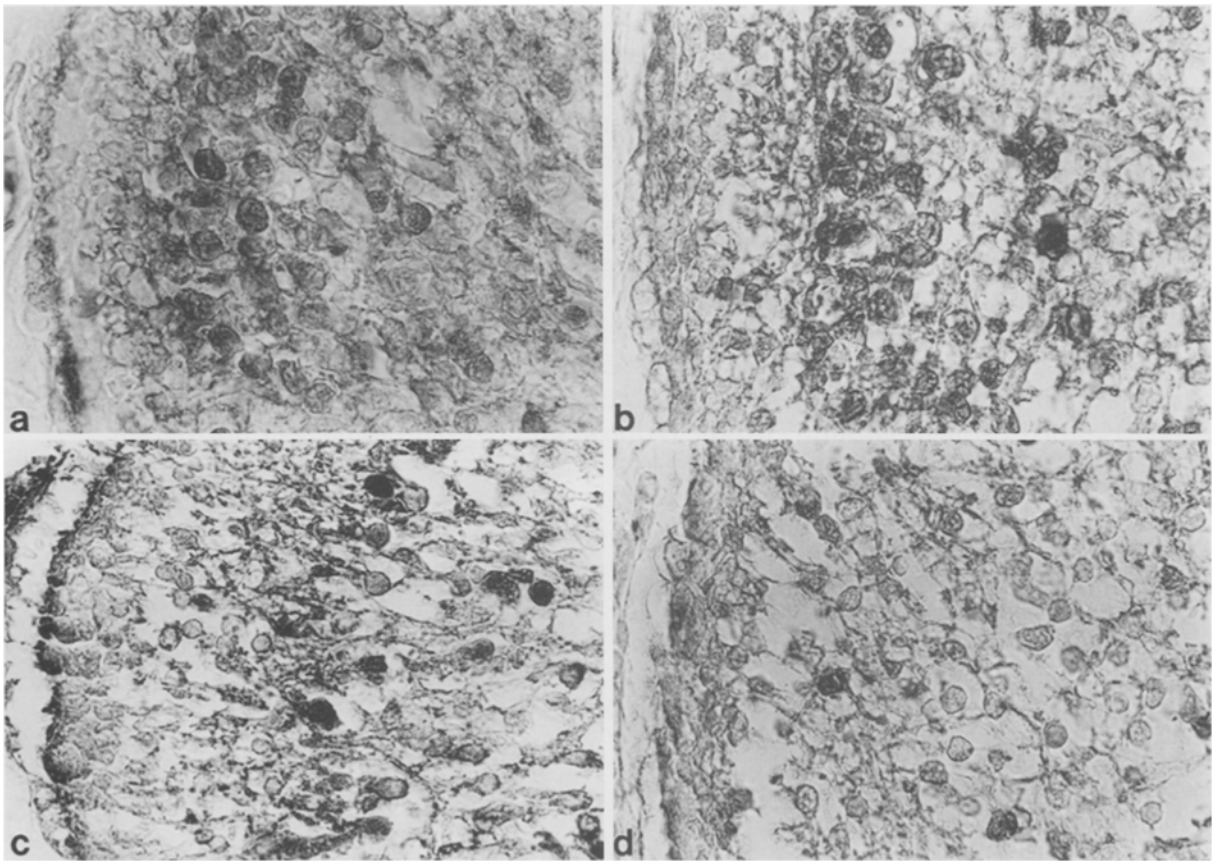

Fig. 4. Same as Fig. 3 at higher magnification. ×855

positive cells was greater in all dietary groups than that of the taurine-negative cells.

Discussion

Previous reports have documented excessive fetal wastage during pregnancies of taurine-deficient queens, and the abnormalities occurring in surviving kittens. We report abnormalities observed in the cerebellum of fetuses at 45 days gestation. As expected, the most significant differences were found with fetuses from the lowest dietary taurine group, although not all of the differences were as expected. The density of granule cells was high, possibly reflecting a reduced brain size compressing the cells, and a high proportion of cells exhibited morphological abnormalities such as swelling, irregular shape of the nucleus, loss of nuclear granules and general enlargement of the nucleus. While there was no difference in the nuclear area of normal granule cells between the diet groups, the nuclear area of the abnormal granule cells was higher in the lowest dietary groups. These observations indicate that the consequences of a low maternal dietary taurine result in more abnormal fetal

cerebellar granule cells which have a greater degree of damage. Somewhat unexpected was the finding of a significantly greater proportion of granule cells staining positively with the taurine antibody in the lowest dietary taurine group. In this group particularly taurine-positive cells included many of the abnormally swollen cells, and may be the result of such cells sequestering taurine to improve their intracellular osmolarity. In general, taurine-positive cells were larger, whether abnormal or not.

The large proportion of abnormal granule cells present in fetuses 45 days after gestation probably are the precursors of the postnatal cerebellar granule cells in kittens from taurine-deficient mothers which have delayed cell division, delayed migration from the external granule cell layer to the internal granule cell layer, and delayed differentation. Some of these fetal cells undoubtedly die, accounting for the reduced number of granule cells postnatally.

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