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Undernutrition and Overnutrition in the Neonatal Rat: Long-Term Effects on Noradrenergic Pathways in Brain Regions

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ABSTRACT. To determine whether neonatal nutrition influences development of CNS noradrenergic systems, litter sizes were manipulated at birth to produce undernutrition (16–17 pups/litter) or overnutrition (five to six pups) and compared to rats reared in normal litter sizes (10–11 pups). Studies were conducted throughout the preweaning period in which nutrition was manipulated, as well as during postweaning nutritional rehabilitation. Sparing of brain growth occurred, evidenced by much smaller changes in brain region wt than in body wt. Similarly, neonatal malnutrition produced major deficits in norepinephrine levels in peripheral sympathetic pathways, but levels in the brain remained within normal limits. Development of [^{3}H]norepinephrine synaptosomal uptake, a biochemical index for presynaptic terminals, was unimpaired by malnutrition; indeed, higher uptake values were seen than in the control population. Nevertheless, norepinephrine turnover was severely attenuated during nutritional restriction and the effect persisted into adulthood; the deficit was greater in the cerebral cortex than in the cerebellum, despite the fact that cerebellar growth showed less sparing. Development of binding capabilities of noradrenergic receptors, particularly the α_2- and β-subtypes, were also adversely affected in cerebral cortex, again suggestive of a deleterious effect on synaptic function. Animals exposed to neonatal overnutrition showed only slight effects on brain region wt or norepinephrine levels, but did display some suppression of [^{3}H]norepinephrine synaptosomal uptake and enhancement of norepinephrine turnover; changes in receptor binding capabilities in the overnourished animals were attributable to the small alterations in brain region wt. These data indicate that neonatal nutrition alters presynaptic and postsynaptic markers of noradrenergic function that remain abnormal even when nutritional rehabilitation occurs. (*Pediatr Res* 27: 191–197, 1990)

Abbreviation

ANOVA, analysis of variance

The role of prenatal and postnatal nutrition in maintenance of normal growth and development is an issue of intense interest. A variety of studies on animal models of early malnutrition has demonstrated the phenomenon of "brain sparing," wherein the CNS undergoes substantially less growth impairment and structural disruption than does the rest of the organism (1–3). However, studies of central catecholaminergic mechanisms suggest that abnormal nutritional status has a substantial deleterious effect on neuronal and synaptic function. During dietary restriction, norepinephrine levels and some indices of neuronal activity show moderate degrees of attenuation (4–8); although whole brain norepinephrine levels tend to be restored to normal in adulthood (9–11), there are at least two reports that deficits in receptor binding sites and synaptic activity may persist if malnutrition is maintained into adulthood (12, 13).

Recent work on factors other than nutrition has demonstrated the existence of critical periods for the "programing" of synaptic activity (14–18), thus suggesting that early nutritional alterations could similarly have a lasting influence on CNS function despite nutritional rehabilitation. In the current study, we have manipulated litter sizes in neonatal rats to produce postnatal food restriction or enhancement during the preweaning stage and have then assessed maturational indices for central noradrenergic systems not only during the period in which food intake was manipulated, but also into young adulthood. Two different brain regions were examined, representing one that tends to be effectively growth-spared during malnutrition (cerebral cortex) and one that is less well-spared (cerebellum) (19), and the results compared with noradrenergic projections to heart and kidney, peripheral tissues whose growth does not display sparing (19, 20). In addition to transmitter levels, we assessed norepinephrine turnover as an index of neuronal activity (21–23), synaptosomal uptake of [^{3}H]norepinephrine as an index of proliferation of synaptic terminals (24, 25), and development of three major subtypes of noradrenergic receptor binding sites. Our results indicate that, despite the sparing of brain growth, early neonatal malnutrition may induce functional deficits in noradrenergic pathways that persist despite subsequent nutritional rehabilitation.

MATERIALS AND METHODS

Animal treatments and tissue dissection. Timed pregnant Sprague-Dawley rats (Zivic-Miller Laboratories, Allison Park, PA) were housed individually in breeding cages and allowed food and water *ad libitum*. Immediately after birth, pups from all litters were pooled, randomized, and redistributed into three litter size groups: normal litter (10–11 pups, "control" group), small litter (five to six pups, "overnourished" group), or large litter (16–17 pups, "undernourished" group). For each experiment, pups were chosen at random from several cages within each group, with approximately equal proportions of males and females. The remaining rats were then randomized again within each group to maintain their respective litter sizes. All animals were weaned at 23 d of age. Previous work with this nutritional model has demonstrated that maternal care is adequate and neonatal stress is absent (19, 26). The different types of determinations used

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here represented a total of approximately 150 pregnant rats divided after birth into 45–65 litters per nutritional grouping.

Brains were removed and dissected as follows: blunt cuts were made through the cerebellar peduncles, whereupon the cerebellum (including flocculi) was lifted from the underlying tissue; a cut was then made rostral to the thalamus to obtain the cerebral cortex.

Catecholamine levels and turnover. Animals in the three different groups were given α-methyl-*p*-tyrosine methyl ester HCl (300 mg/kg intraperitoneally); rats used for basal catecholamine levels received equivalent volumes of saline. α-Methyl-*p*-tyrosine inhibits tyrosine hydroxylase, the rate-limiting step in catecholamine biosynthesis, and the disappearance of endogenous catecholamines alter administration of this drug, thus estimates the turnover rate (21–23). Animals were killed 3 h later, a time point chosen because a major proportion of norepinephrine depletion occurs within this span in developing rat brain (23). Tissues were homogenized in 0.1 N $HClO_4$ containing 3,4-dihydroxybenzylamine as an internal standard, centrifuged at 26 000 x *g* for 10 min, and supernatant solutions were stored at -30°C. Norepinephrine was first isolated by alumina adsorption and then analyzed using HPLC with electrochemical detection (22). All values were corrected for recovery of the internal standard. Turnover values were determined as the difference between saline and α-methyl-*p*-tyrosine-treated animals. Basal norepinephrine content was also evaluated in heart and kidney for comparison with brain regions.

Synaptosomal uptake of [^{3}H]norepinephrine. Tissues were disrupted in a smooth-glass homogenizer fitted with a Teflon pestle in 9 vol of 0.3 M sucrose containing 25 mM Tris HCl (pH 7.4) and 10 μM iproniazid and sedimented at 1000 x *g* for 10 min. Synaptosomal uptake was then assessed in aliquots of the supernatant solution, using a modified Krebs-Henseleit medium and a final concentration of 0.1 μM l-[^{3}H]norepinephrine. Incubations lasted 5 min at 37°C and blanks were handled identically except that 0.1 mM cocaine HCl was added to the incubation mixture to inhibit the specific uptake. As described previously (25), labeled synaptosomes were trapped by filtration.

Adrenergic receptor binding sites. Tissues for receptor binding studies were frozen on dry ice and stored at -80°C; preliminary studies indicated no degradation of receptor binding characteristics during storage. Crude membrane fractions were prepared by the method of Witkin and Harden (27). Tissues were homogenized (Polytron, Brinkmann Instruments, Westbury, NY; setting of 5.5 x 15 s) in 40 vol of ice-cold 145 mM NaCl, 2 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.5) and centrifuged at 40 000 x *g* for 15 min. The pellets were washed twice by resuspension (Polytron) in homogenization buffer and recentrifugation. The final pellet was dispersed with a Teflon-to-smooth-glass homogenizer (four up-down strokes maximum) in 3 vol (based on original wet wt of tissue) of 250 mM sucrose, 2 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5) and used for ligand binding studies (see below) and for protein analysis by dye-binding of Coomassie brilliant blue. Radioligands were then incubated with the tissue membrane preparations in a total volume of 0.35 mL. It was not practicable to determine binding at a full range of ligand concentrations because of limitations of amounts of tissue and the numbers of tissues to be analyzed, and thus we used a single ligand concentration corresponding to the approximate k_d (28); this would be sensitive to age- or nutritionally induced changes in either k_d or B_{max}. Incubations were stopped by dilution with 6 to 7.5 mL of ice-cold buffer, followed by rapid vacuum filtration onto Whatman GF/C filters (Whatman Inc., Clifton, NJ), which were then washed with an additional 6 to 7.5 mL of buffer. Nonspecific binding was defined as binding of radioligand in the presence of an excess concentration of a specific displacing agent (5 μM phentolamine for α_1-and α_2-receptors, 100 μM *dl*-isoproterenol for β-receptors).

α_1-Receptor binding was determined with [^{3}H]prazosin (28). Aliquots of membrane preparation (50 to 500 μg of protein, depending upon age and region) were incubated with 2.2 nM radioligand in 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), at 4°C for 50 min. Nonspecific binding of [^{3}H]prazosin constituted 15–20% of total binding in older animals, but as much as 40% in tissues from very young animals, where receptor density was low. α_2-Receptor binding was evaluated similarly, using 2.5 nM [^{3}H] rauwolscine (28) in a medium containing 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), with incubations lasting 20 min at room temperature; both α_1-and α_2-receptor incubations were stopped and filters were washed with ice-cold 50 mM Tris-HCl, 10 mM $MgCl_2$(pH 7.5). Nonspecific binding of [^{3}H]rauwolscine typically ranged from 25–50%, depending on age and region. β-Receptor binding was assessed with 67 pM [^{125}I]pindolol (28), using 20 to 80 μg of membrane protein in 145 mM NaCl, 2 mM $MgCl_2$, 1 mM sodium ascorbate, 20 mM Tris-HCl (pH 7.5); wash buffer was the same as that in the incubation. Non-specific binding was typically 5–20% of the total.

Materials. Prazosin furoyl-5[^{3}H] (sp act 19.3 Ci/mmol), rauwolscine-methyl[^{3}H] (sp act 78.8 Ci/mmol), *l*-norepinephrine[7-^{3}H] (sp act 14.3 Ci/mmol, diluted with unlabeled compound to 2 Ci/mmol) and [^{125}I]pindolol (sp act 2200 C/mmol) were obtained from Du Pont Medical Products (Wilmington, DE). Phentolamine HCl was obtained from Ciba Pharmaceuticals (Summit, NJ) and cocaine HCl from Merck Sharp & Dohme (West Point, PA). α-Methyl-*p*-tyrosine methyl ester HCl, 3,4-dihydroxybenzylamine HBr, *l*-norepinephrine HCl, *dl*-isoproterenol HCl, and

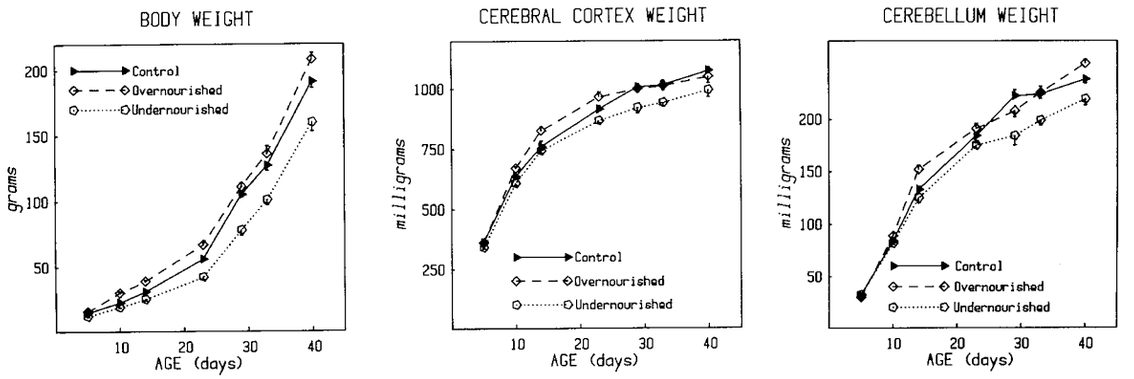

Fig. 1. Effects of nutrition on body and brain region wt. Data represent means and SE of eight animals in each group at each age. For body wt or cerebellum wt, ANOVA indicates that overnourished animals are significantly heavier and have heavier cerebella than controls, and controls are significantly heavier and have heavier cerebella than undernourished animals (main effect of nutrition and interaction of nutrition x age for each). For cerebral cortex wt, ANOVA also indicates that the overnourished group is not significantly distinguishable from controls, although cortex wt in controls are significantly greater than in the undernourished animals (main effect of nutrition).

iproniazid phosphate were purchased from Sigma Chemical Corp. (St. Louis, MO).

Data analysis and statistics. Data are presented as means and SE. Longitudinal studies were analyzed by two-way ANOVA (data log-transformed whenever variance was heterogeneous), using factors of age and nutritional status. For the initial analysis, a global test was used across all three groups (control, overnourished, undernourished); where the global test indicated a significant difference attributable to nutrition or an interaction of nutrition x age, a subsequent two-way ANOVA was then conducted separately comparing overnourished animals and undernourished animals to the controls. Significance for all tests was assumed at the level of $p < 0.05$.

Data for norepinephrine levels and turnover and for [^{3}H] norepinephrine synaptosomal uptake were evaluated both as concentration (per g tissue) and content (per tissue), and receptor binding was analyzed as amount bound per mg protein, per g tissue and per tissue, as determined at the single radioligand concentrations described above. The various representations have different meanings (29): *e.g.* a nutritionally induced decrease in tissue mass that spares norepinephrine-containing terminals, would lead to an increase in norepinephrine uptake per g but no change in uptake per tissue, whereas a specific deficit in norepinephrine terminals would decrease both terms.

RESULTS

As previously reported (19, 20, 26), undernutrition caused by redistribution of neonatal rats into large litters, produced significant growth restriction (Fig. 1). Body wt in the undernourished group became 25% subnormal by weaning, with some postweaning catch-up growth occurring; by 40 d of age, these animals were approximately 15% less than the control body wt. Overnutrition produced by redistribution to small litter sizes produced a corresponding enhancement of body wt gains, reaching 20% more than control by weaning and regressing to 10% more than control by 40 d. Brain-sparing was most evident in the cerebral cortex, where undernutrition produced a regional wt deficit of $\leq$10% and overnutrition had no statistically significant wt effect. The cerebellum was less spared, with maximum wt deficits (undernutrition) or enhancement (overnutrition) of about 15%.

Examination of norepinephrine levels in brain regions, indicated an even greater degree of sparing than did region wt (Fig. 2). In cerebral cortex, neither undernutrition nor overnutrition had any significant effect on norepinephrine concentration. Norepinephrine content in the cerebral cortex was significantly reduced in the undernourished group, but the reduction was less than that seen for tissue wt. Similarly, in the cerebellum, there was no effect of either nutritional manipulation on norepinephrine concentration and a small deficit in content was seen in the undernourished group. These minor effects stand in contrast to the profound attenuation of norepinephrine content in peripheral tissues of undernourished rats (Fig. 3).

Despite the small degree of change in brain region norepinephrine levels caused by altered nutritional status, there were marked effects on norepinephrine turnover (Fig. 4). In the cerebral cortex, undernourished rats showed major deficits in turnover that first appeared at the end of the 2nd postnatal wk, expressed either in terms of concentration (turnover/g tissue) or content (turnover/tissue). Importantly, the differences persisted or worsened after weaning, during the period of catch-up growth. Overnutrition had a smaller, but statistically significant enhanc-

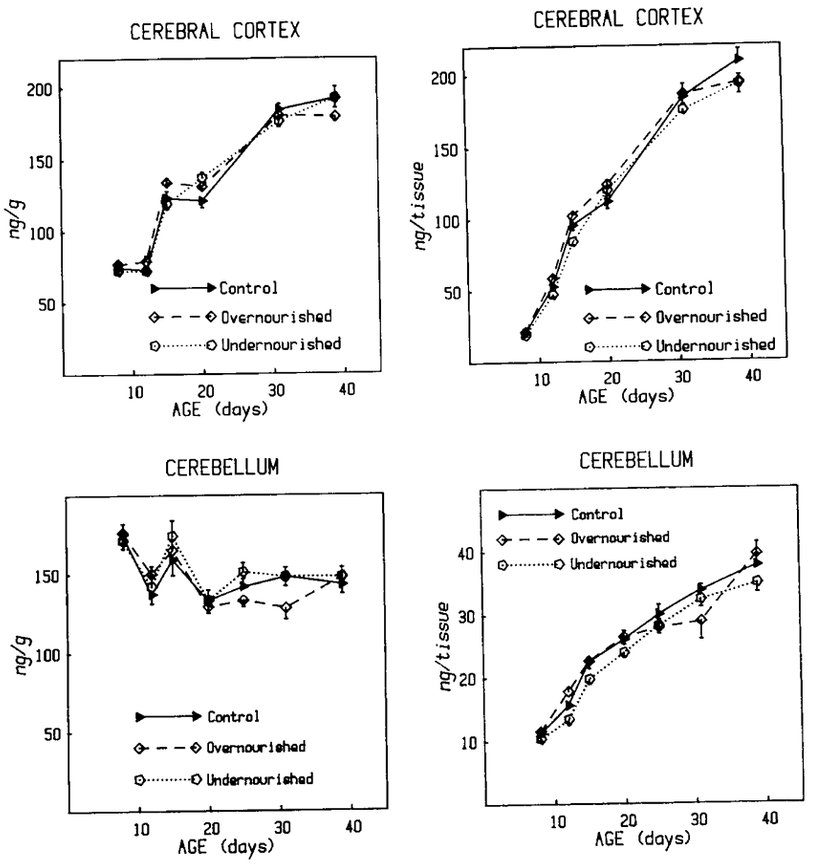

Fig. 2. Effects of nutrition on norepinephrine concentration (*left*) and content (*right*) in cerebral cortex and cerebellum. Data represent means and SE of seven to 10 animals in each group at each age. In either tissue, ANOVA indicates no significant differences in concentration for overnourished or undernourished groups *versus* the control, but a significant overall reduction in norepinephrine content in the undernourished group (main effect of nutrition).

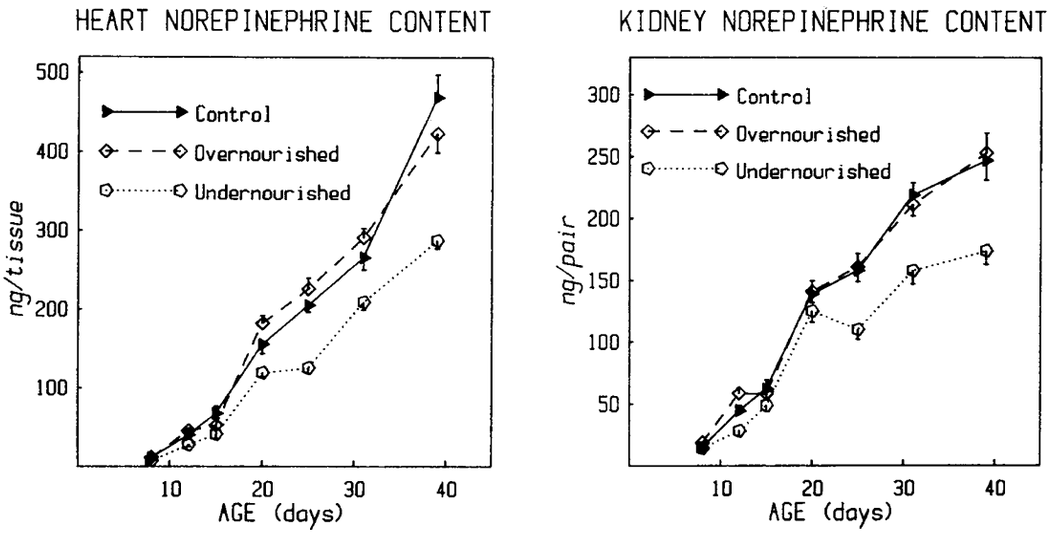

Fig. 3. Effects of nutrition on heart and kidney norepinephrine content. Data represent means and SE of three to 10 animals in each group at each age. For heart, ANOVA indicates a significant lowering in the undernourished group *versus* control (main effect of nutrition) and age-dependent alterations in the overnourished cohort (interaction of nutrition x age). For kidney, ANOVA indicates a significant overall lowering in the undernourished group (main effect of nutrition and interaction of nutrition x age) and elevation in the overnourished group (main effect of nutrition).

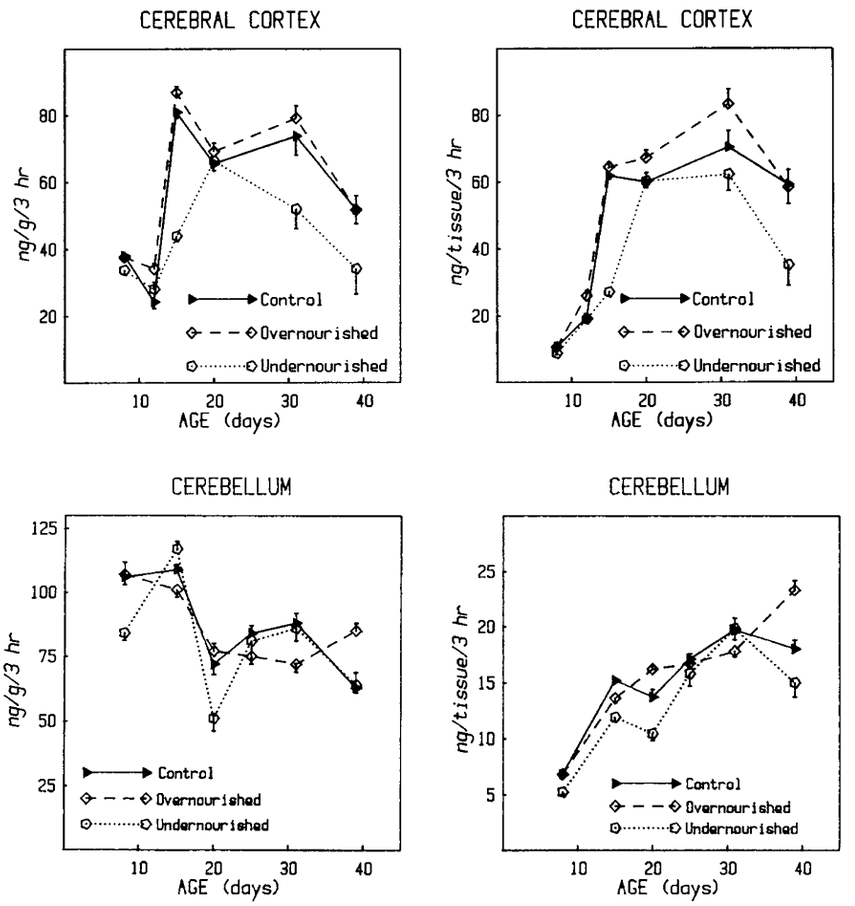

Fig. 4. Effects of nutrition on norepinephrine turnover in cerebral cortex and cerebellum, measured after administration of α-methyl-*p*-tyrosine (see Materials and Methods) and expressed in units of concentration (*left*) or content (*right*). Data represent mean and SE of six to 10 animals in each group at each age. In the cerebral cortex, ANOVA indicates that turnover is reduced in the undernourished group, regardless of representation as concentration or content (main effect of nutrition and interaction of nutrition x age); turnover is enhanced in the overnourished group (main effect of nutrition). In the cerebellum, turnover is significantly reduced in the undernourished group, regardless of representation as concentration (main effect of nutrition and interaction of nutrition x age) or content (main effect of nutrition); turnover in the overnourished group shows only age-dependent alterations (interaction of nutrition x age).

ing effect on norepinephrine turnover in the cerebral cortex. Although the cerebellum showed a greater degree of growth restriction in the undernourished rats, the nutritional effect on norepinephrine turnover was less pronounced and consistent than in the cerebral cortex. Cerebellar norepinephrine turnover measured in terms of concentration was significantly subnormal overall in the undernourished group, but the differences were limited to the preweaning period when nutrition was restricted. The differences were more consistent when expressed as turnover per tissue, due to the greater growth deficits in this region. Overnutrition had small, variable effects on cerebellar norepinephrine turnover.

Examination of synaptosomal uptake of [^{3}H]norepinephrine in brain regions of rats subjected to altered nutrition, revealed a completely different pattern from that of growth, norepinephrine levels, or turnover (Fig. 5). In the cerebral cortex, uptake per g tissue was significantly elevated in the undernourished animals and suppressed in the overnourished group; uptake per region showed the same nutritional effects, albeit with a slightly lower magnitude. Undernutrition produced proportionally larger elevations of [^{3}H]norepinephrine uptake in the cerebellum, expressed either per g tissue or per region; the effect of overnutrition was smaller but statistically significant.

Nutritional status also affected adrenergic receptor binding capabilities, but the effects were less notable than those on norepinephrine turnover or uptake. As shown in Figure 6, binding of the α_1-selective radioligand, [^{3}H]prazosin, was virtually unaffected in cerebral cortex when evaluated per g of tissue. Binding of [^{3}H]rauwolscine (α_2-receptors) and of [^{125}I]pindolol (β-receptors) both showed optimal development in the control group, with lower values obtained consistently in both the overnourished and undernourished animals. In the cerebellum, significant differences in binding were found only for β-receptors, where once again the values were higher in the controls than in either the undernourished or overnourished animals. It should be noted that, because of the regional wt differences caused by nutritional manipulations, the binding differences in both cerebral cortex and cerebellum would tend to resolve in the overnourished group if calculated per region, but would intensify in the undernourished cohort (data not shown). Determination of receptor binding per mg protein yielded essentially the same results as binding per g tissue (data not shown).

DISCUSSION

Data obtained in our study are consistent with the concept that undernutrition spares brain growth and indicates that the sparing includes several important features of central neurochemical development. Animals reared in abnormally large litter sizes exhibited much smaller impairment of brain regional

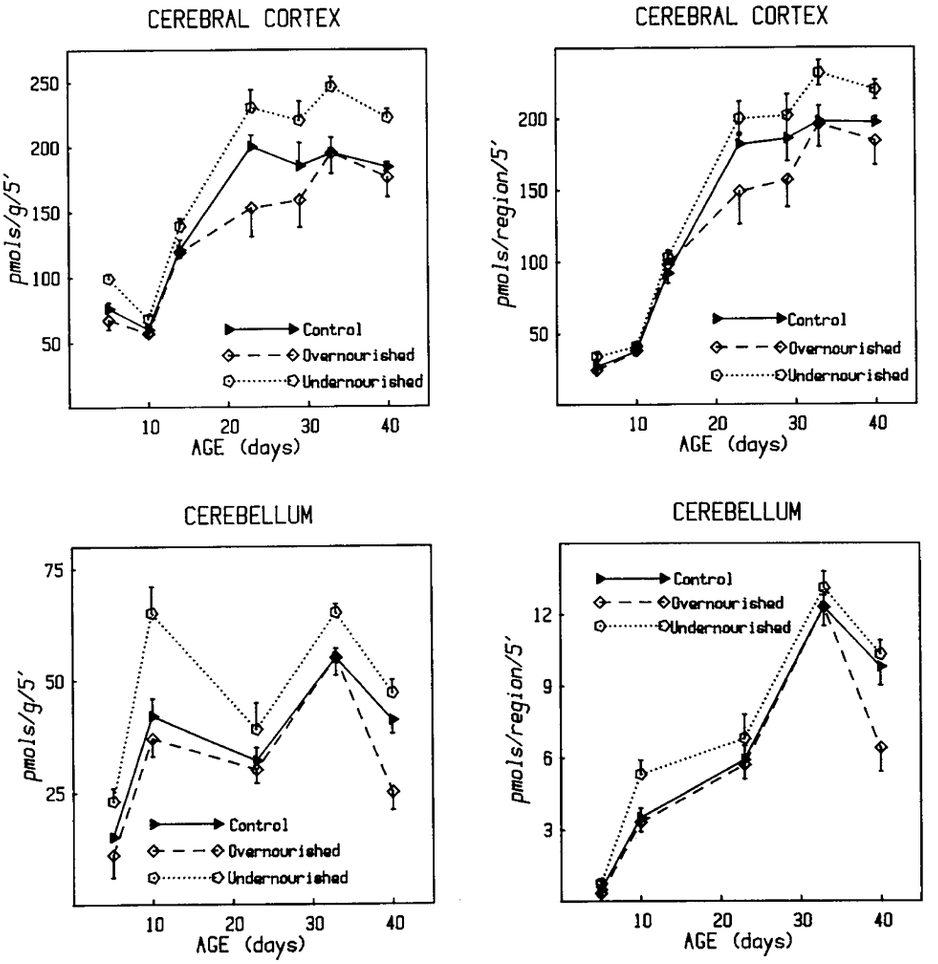

Fig. 5. Effects of nutrition on synaptosomal uptake of [^{3}H]norepinephrine in cerebral cortex and cerebellum, assessed either as uptake per g tissue (*left panels*) or per region (*right panels*). Data represent means and SE of seven to eight animals in each group at each age. In either tissue using either measure, ANOVA indicates greater uptake in the undernourished cohort than in controls, and lower uptake in the overnourished cohort than in controls (main effect of nutrition; also nutrition x age interaction for cerebellum).

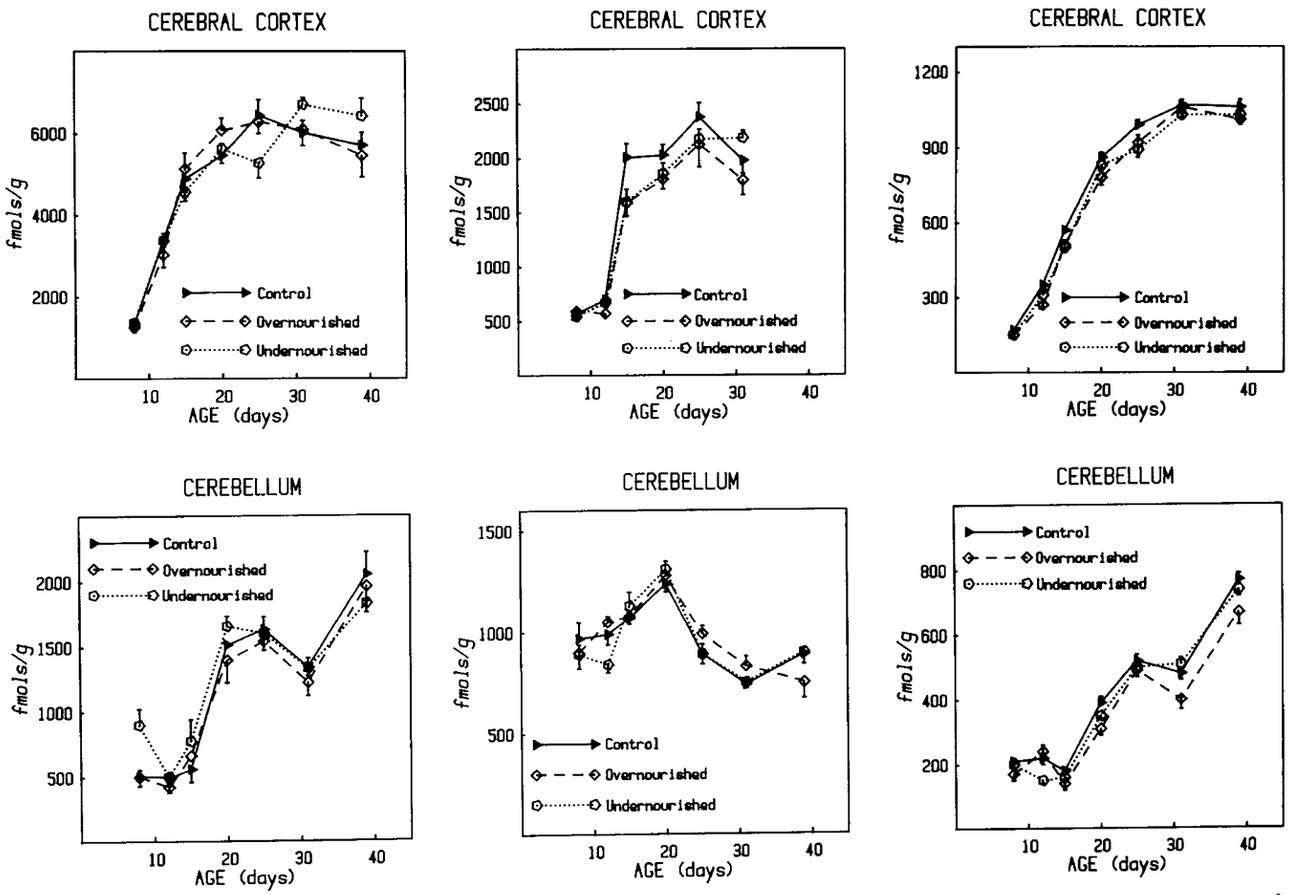

Fig. 6. Effects of nutrition on adrenergic receptor binding capabilities, measured at 2.2 nM [^{3}H]prazosin (α_1-receptors, *left panels*), 2.5 nM [^{3}H] rauwolscine (α_2-receptors, *middle panels*), and 67 pM [^{125}I]pindolol (β-receptors, *right panels*) in cerebral cortex and cerebellum. Data represent means and SE of seven to eight animals in each group at each age. In cerebral cortex, ANOVA indicates a significantly higher value for α_2-and β-receptors (main effect of nutrition) in the controls when compared to either under- or overnourished animals; cerebral cortical α_1-receptors show only a significant nutrition x age interaction in the undernourished cohort *versus* controls. In cerebellum, ANOVA indicates significantly greater values in the controls *versus* either over- or undernourished animals only for β-receptors (main effect of nutrition).

growth than body growth, and, in keeping with earlier results, the cerebral cortex showed greater sparing than did the cerebellum, a region that matures substantially later (1–3, 19). In the face of undernutrition, both brain regions preserved their developmental patterns of norepinephrine levels much better than did the peripheral sympathetic pathways to heart or kidney. Furthermore, examination of synaptosomal uptake of [^{3}H]norepinephrine, a marker for development of noradrenergic nerve terminals (24, 25), indicated higher values in the undernourished neonates than in controls. This suggests that the adverse effects on growth of brain regions are not shared equally among all cellular elements, but rather that neurons are spared relative to other cell types. Selective impairment of growth of nonneuronal cells would leave additional space for synaptic expansion and thus increase the apparent concentration of uptake sites. This could explain why the cerebellum, which displays greater growth impairment than the cerebral cortex, also shows proportionally larger increases in synaptosomal uptake per g tissue. Functionally, an increased synaptosomal uptake capability would also enhance the reuptake of norepinephrine from the synapse, thus representing a mechanism for transmitter conservation despite nutritional shortfalls; availability of dietary tyrosine, the precursor for norepinephrine, has been hypothesized to limit the levels and turnover of the transmitter during development (13).

The decrease in norepinephrine turnover seen here with neonatal undernutrition, may also represent a strategy for conservation of transmitter in the developing brain. By decreasing the utilization of norepinephrine, less demand would be placed on *de novo* synthesis. However, not all of these changes may be advantageous in the long run. Recent work has shown that synaptic activity in the period just before and after birth serves to "program" future responsiveness and that interference with neurotransmission in this period permanently alters synaptic function (14–18). This appears to be the case with neonatal undernutrition, because all of the alterations in synaptosomal uptake and norepinephrine turnover persisted or even intensified in the postweaning period, when food availability was no longer limited and catch-up growth was occurring.

The same type of long-term disruption of developmental programing can account for the deficits in noradrenergic receptor binding capabilities in the malnourished neonates. In adults, decreases in synaptic activity typically result in compensatory up-regulation of receptor binding, but during development synaptic activity instead exerts a positive trophic effect on the number of sites and/or their reactivity to stimulation (14–18). The decrease in noradrenergic activity represented by the reduced transmitter turnover in the undernourished animals, thus probably contributes to the parallel deficits in receptor binding capabilities. The combined effect of decreased norepinephrine in the synapse (decreased turnover and increased reuptake) and impaired postsynaptic reactivity (decreased receptor binding) would render these pathways functionally hypoactive into adulthood, just as occurs with prenatal exposure to opiates (30). The connections among brain growth sparing, conservation of trans-

mitter levels, and long-term interference with patterns of turnover and receptor binding are reinforced by the regional selectivity of the effects of undernutrition: the region that showed the greater sparing of growth (cerebral cortex) also showed greater deficits in turnover and binding.

In view of these findings, it is not surprising that overnutrition produces effects on synaptosomal uptake and norepinephrine turnover in the opposite direction from those caused by undernutrition. Dilution of synapses with other elements would reduce the apparent concentration of uptake sites and receptor binding, and the increased availability of energy and precursor for transmitter would promote norepinephrine utilization. The fact that the alterations caused by overnutrition were less robust, suggests that normal nutritional status is already close to optimal for maintenance of development of central noradrenergic pathways. Indeed, the failure of enhanced nutrition to increase receptor binding indicates that programing of synaptic development probably cannot be significantly advanced solely by manipulating food availability.

In conclusion, these results indicate that the immature brain is capable of making adjustments to preserve growth, transmitter levels, and synaptic development in the face of nutritional deprivation. Although the strategy of reducing norepinephrine utilization and enhancing its recapture by the nerve terminal helps to conserve transmitter levels, this may compromise the future development of synaptic activity and receptor binding capabilities, resulting in long-lasting hypoactivity even when food becomes freely available.

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