Research report

Prenatal choline supplementation increases NGF levels in the hippocampus and frontal cortex of young and adult rats

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Abstract

Female Sprague–Dawley rats received ~300 mg/kg per day of choline chloride through their drinking water on days 11 of pregnancy through birth and the level of nerve growth factor (NGF) in the hippocampus and frontal cortex of their male offspring was measured at 20 and 90 days of age. Prenatal choline supplementation caused significant increases in hippocampal NGF levels at 20 and 90 days of age, while levels of NGF in the frontal cortex were elevated in choline-supplemented rats at 20 days of age, but not 90 days of age. These results suggest that increases in NGF levels during development or adulthood may be one mechanism underlying improvements in spatial and temporal memory of adult rats exposed to elevated levels of choline chloride perinatally.

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1. Introduction

Dietary supplementation with choline chloride during the perinatal period (embryonic days 12–17 and/or postnatal days 16–30) causes long-lasting improvement in spatial memory as determined using radial-arm maze [59,61–63,84] and water maze tests [68,75–77] as well as temporal memory tests [58,60,63]. For example, adult rats that have been supplemented with choline in utero show increased memory capacity [59] and precision [61] when tested on the radial-arm maze as compared to rats that have been given a standard choline-sufficient diet throughout life. Furthermore, perinatal supplementation with choline protects against memory impairments associated with normal aging [7], prenatal alcohol exposure [78], and epileptic seizures [89]. Together, these studies suggest that manipulation of the perinatal availability of choline has lasting organizational consequences on the neural substrates of memory and may alter the restorative or protective capacity of the adult brain.

Choline supplementation during early development causes enduring alterations in anatomy, chemistry and physiology of brain regions known to contribute to normal memory function. Notable among the many effects of perinatal choline supplementation are changes in basal forebrain, hippocampal, and cortical cholinergic markers including choline acetyltransferase (ChAT) activity and muscarinic receptor density [63], acetylcholine (Ach) release [20], and amplitude of Ach-mediated excitatory potentials within the hippocampus [66]. Furthermore, hippocampally-projecting basal forebrain cholinergic neurons expressing the low-affinity receptor for nerve growth factor [42,43] are larger and more spherical in rats that were supplemented with choline during the prenatal sensitive period [52,84].

The exact neurobiological mechanism by which perinatal choline supplementation causes long-term neural and behavioral changes remains unclear, though there are a
number of significant changes in the developing brain immediately following choline supplementation. For example, rates of apoptosis in the hippocampus and basal forebrain of 18-day-old fetuses are inversely related to maternal choline intake between embryonic days 11 and 17 [38]. Conversely, cell division in these regions is increased by elevations of maternal choline intake [2]. Furthermore, rat pups that experience choline supplementation in utero show elevations in hippocampal levels of phospholipase D (PLD) activity as well as reductions in acetylcholinesterase (AchE) activity during early postnatal development [20,21,37]. The extent to which these and other alterations caused by perinatal choline supplementation drive the permanent changes in brain organization and behavior is not clear.

It is interesting to note the similarities between the effects of perinatal choline supplementation and the effects of perinatal manipulations of NGF. During early development, the basal forebrain cholinergic neurons that ultimately innervate the hippocampus and cortex are dependent on NGF for their differentiation and survival [46,82]. Intraventricular administration of NGF to the neonatal rat causes an increase in basal forebrain and hippocampal levels of ChAT [19,33,64]. Conversely, administration of NGF antibodies or mutations of the gene encoding NGF cause a decrease in cholinergic markers [22,50,80]. Behaviorally, rats that receive intraventricular NGF on postnatal days 12 and 13 show both a decrease in the age at which they first exhibit adult-like patterns of performance in the water maze and an improvement in working memory assessed with the radial arm maze in adulthood [9]. A similar phenomenon occurs following perinatal choline supplementation with supplemented rabbits exhibiting a dopaminergic cue use ~3 days earlier than controls [73] and making fewer working memory errors on the radial arm maze in adulthood [59,61–63,84]. Together, these findings suggest that perinatal choline supplementation may increase brain levels of NGF during early development and that this increase may drive the behavioral and neuroanatomical changes evident in choline-supplemented rats.

As adults, rats that received perinatal choline supplementation perform more accurately on tests of spatial memory [59,61–63,84] and exhibit a multitude of physiological changes including an increase in the size of basal forebrain cholinergic neurons that are immunoreactive for the low-affinity NGF receptor [52,84]. These neurons, responsive to perinatal choline manipulation, are integral components of the neural circuitry underlying spatial memory. Fimbria-fornix lesions destroy the axonal projections from the basal forebrain neurons to the hippocampus [32,88] and prevent the retrograde transport of NGF from the hippocampus to the basal forebrain [47,49] resulting in significant impairments in performance on the radial-arm maze [17,18] and Morris water maze [25,74]. Age-related atrophy of basal forebrain cholinergic neurons is also associated with deficits in spatial memory [28,44]. The administration of NGF via either intracranial infusion or implantation of NGF-secreting cells has been shown to improve the memory deficits associated with both fimbria-fornix lesions and normal aging, as well as reduce lesion-induced or age-related atrophy of basal forebrain cholinergic neurons [26,27,29,30,35,55,56,83,85]. Together, these data suggest that perinatal choline may increase brain levels of NGF in adulthood and that this increase may contribute to the long-term neural and behavioral effects of supplementation.

The present study examines the effects of perinatal choline supplementation on NGF levels in the hippocampus and frontal cortex using a two-site enzyme immunoassay. The hippocampus and frontal cortex both receive projections of cholinergic neurons that are affected by choline supplementation [86–88], both of these regions are rich in NGF [70], and both are critical for performance of a variety of learning and memory tasks. During the 3rd postnatal week, cortical and hippocampal levels of NGF protein and mRNA rapidly increase, peaking around day 21 before declining 20–30% and then rising to adult values [46,48]. To determine whether prenatal choline supplementation alters this developmental pattern of NGF expression, tissue from the frontal cortex and hippocampus was analyzed in choline-supplemented and control rats at 20 days of age. To determine whether choline supplementation leads to alterations in NGF levels in adulthood, supplemented and control animals were sacrificed at 90 days of age and hippocampal and cortical NGF levels were analyzed. The supplementation protocol used in this study is similar (though slightly longer: embryonic days 11 to birth versus embryonic days 12–17) to that which causes improvements in spatial memory [62,63,61], inoculation against age-related decline in spatial memory [7], and hypertrophy of basal forebrain cholinergic neurons [52,84]. Because of the changes in spatial memory associated with the infusion of NGF during development, adulthood, and aging, and the morphological response of neurons to the infusion of NGF, we hypothesized that prenatal supplementation with choline may alter the levels of hippocampal and cortical NGF during early development and/or adulthood and that these may be factors leading to cellular hypertrophy and the physiological changes underlying improvements in memory.

2. Materials and methods

2.1. Materials

Choline chloride was provided by Syntex Agricultural. Other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Animals

A total of 18 Sprague–Dawley albino females (strain Crl:CD(SD)BR-CD) (Charles River, Kingston, NY) were
received 8 days pregnant, and housed with lights on between 8:00 am and 8:00 pm. Beginning at 5:00 pm on embryonic day 11 the drinking water of eight dams was supplemented with a 5-ml/l solution of 70% choline chloride (approximate intake: 300 mg/kg per day), delivered in a 0.05-M saccharin solution. This served as the only source of drinking water for the duration of pregnancy. Ten additional dams were given saccharin-sweetened drinking water with no added choline chloride. On the day after birth, choline-treated and control pups were marked by toe clipping, and litters were culled to five males and five females. All pups were cross-fostered to dams that had received no choline chloride during pregnancy. Litters were weaned at 23 days of age and were rehoused in same-sex pairs. Male pups from each treatment group were sacrificed by decapitation on postnatal days 20 and 90. Each brain was rapidly removed and the hippocampus and frontal cortex were quickly dissected and frozen on dry ice. Four control and five supplemented rats were coated with 100 ng BDNF or NT3 [23]. Immunoplates (Nunc, Maxisorb F96) for all comparisons was maintained at 0.05. GAM antibodies for NGF that do not cross-react with further examined using Fisher’s LSD test. The alpha level al. [65] and Weskamp and Otten [81] and used IG3 and factors. Significant main effects and interactions were formed on NGF levels in hippocampal tissue and frontal cortex tissue with age and treatment as between-subject factors. Significant main effects and interactions were further examined using Fisher’s LSD test. The alpha level for all comparisons was maintained at 0.05.

2.3. NGF ELISA

The NGF ELISA protocol was adapted from Mobley et al. [65] and Weskamp and Otten [81] and used IG3 and GAM antibodies for NGF that do not cross-react with BDNF or NT3 [23]. Immunoplates (Nunc, Maxisorb F96) were coated with 100 μl of goat anti-mouse NGF polyclonal antibody (GAM) at a concentration of 20 μg/ml in coating buffer (3 mM NaH2PO4, 355 mM NaHCO3, 16 mM Na2CO3, pH 9.6), and the plates were incubated on a shaker overnight at 4 °C. The GAM solution was removed, the plates washed with washing buffer (10 mM phosphate buffered saline (PBS), pH 7.4, and 0.05% Tween 20) and incubated on a shaker at room temperature for 1 h with 200 μl of a blocking buffer containing 5% fetal calf serum (FCS) in PBS. The plates were then washed three times with washing buffer. Samples were prepared by sonicating frozen tissues for 60 s 1:10 (w/v) in sample buffer (0.5% bovine serum albumin, 20 KIU/ml aprotinin, 0.1 mM phenylmethylsulfonylfluoride, and 0.1 mM benzethonium chloride in PBS), centrifuging for 30 min at 15,000×g at 4 °C, then adding Tween 20 (final concentration of 0.05%) to the supernatant.

NGF (generously provided by W.C. Mobley) was serially diluted to produce a standard curve with concentrations of: 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25.0, 50.0, and 100 pg/100 μl. Standards and sample solutions were each added to four replicate wells and were left on the plate overnight at 4 °C. The plates were then washed with PBS, 100 μl of rat anti-mouse NGF monoclonal antibody was added (IG3; 0.012 mg/ml in 1% FCS, 0.1% Tween 20 and PBS), and the plates were incubated overnight at 4 °C. After the plates were washed with PBS, 100 μl biotinylated goat anti-rat antibody suitable for the detection of mouse antibodies (Vector, 0.3 g/ml in PBS containing 1% FCS, and 0.1% Tween 20) was added to each well, and the plates were incubated overnight at 4 °C. Plates were then washed three times in PBS, and incubated for 2 h at room temperature with 100 μl HRP-streptavidin (Zymed, 1:2000 in PBS containing 1% FCS). Plates were then washed three times with PBS and developed with 100 μl/well of a freshly made solution containing 0.04% O-phenylenediamine and 0.012% hydrogen peroxide in a phosphate–citrate buffer (pH 5.0) by incubating the foil-covered plates for 20 min at room temperature. The reaction was stopped by adding 50 μl of 2.5 M H2SO4 to each well. The plates were lightly agitated during all incubations.

Optical densities were measured with an MR 600 Microplate Reader (Dynatech Laboratories) using a 490-nm filter and a 590-nm reference filter. The optical densities of the samples fell in the linear part of the standard curves and were therefore used to estimate the concentration of NGF in the samples.

2.4. Data analysis

Separate analyses of variance (ANOVAs) were performed on NGF levels in hippocampal tissue and frontal cortex tissue with age and treatment as between-subject factors. Significant main effects and interactions were further examined using Fisher’s LSD test. The alpha level for all comparisons was maintained at 0.05.

3. Results

Prenatal choline supplementation increased hippocampal NGF levels by ~25–30% in both 20- and 90-day-old rats (Fig. 1A). Furthermore, hippocampal NGF levels of 90-day-old rats were significantly higher than levels of 20-day-old rats, regardless of prenatal treatment. There was no interaction between age and prenatal treatment as choline supplementation increased hippocampal NGF levels similarly in 20- and 90-day-old rats.

NGF levels in frontal cortex samples from 20- and 90-day-old rats were also affected by both age and treatment (Fig. 1B). Prenatal choline supplementation caused an ~16-fold increase in frontal cortex NGF levels of 20-day-old rats but no difference between choline-supplemented and control rats was evident at 90 days of age. While NGF levels in the frontal cortex of control rats increased between 20 and 90 days of age, levels in choline-supplemented rats decreased between these ages.

4. Discussion

The present results show that prenatal choline supplementation causes elevations of NGF concentration in the hippocampus of juvenile and adult rats as well as a
Fig. 1. Concentration (mean±S.E.M., ng/g wet tissue) of NGF in hippocampus and frontal cortex of control and choline-supplemented rats at 20 and 90 days of age. (A) Hippocampal NGF levels were significantly higher in choline-supplemented animals at both 20 and 90 days of age. An ANOVA with treatment and age as between-subject factors revealed main effects for treatment ($F(1, 15)=17.40, P<0.001$) and age ($F(1, 15)=6.60, P<0.05$) but no interaction ($F(1, 15)=0.05, P=0.82$). Post-hoc tests confirmed significant increases in hippocampal NGF levels at 20 and 90 days of age relative to controls ($^*P<0.05$). (B) Frontal cortex NGF levels were significantly higher in choline-supplemented animals at 20 days of age, but not at 90 days of age. An ANOVA with treatment and age as between-subject factors revealed main effects for both age ($F(1, 15)=4.72, P<0.05$) and treatment ($F(1, 15)=11.87, P<0.01$). These factors also showed a significant interaction ($F(1, 15)=14.30, P<0.01$). Post hoc tests revealed that frontal cortex NGF levels were significantly elevated by choline supplementation when measured on day 20, but not on day 90 ($^*P<0.05$).

transient elevation of NGF concentration in the frontal cortex of juvenile rats. Choline supplementation-induced increases in hippocampal NGF levels were apparent on days 20 and 90 while NGF levels in the frontal cortex were an order of magnitude larger in supplemented rats on day 20, but returned to control levels by day 90. It should be noted that the level of NGF in hippocampus and cortex of our control rats falls within the ranges previously reported for Sprague–Dawley rats [36,48]. It is presently unclear how choline supplementation causes an elevation in NGF levels or whether the elevations in NGF are directly related to the reported improvements in memory [59,61–63,68,84]. It is possible, however, that this developmental exposure to elevated levels of NGF differentially organizes the brain resulting in an improvement in the neural circuitry underlying memory in adulthood. Intracerebral injections of NGF on postnatal days 12 and 13 cause an improvement in spatial learning and memory assessed both on day 22 and at 6 months of age [9]. Interestingly, NGF administration at an earlier age (postnatal days 2 and 3) causes impairments in water maze performance that persist to at least 2 months of age [10].
indicating a potential sensitive period for the beneficial effects of NGF. This finding has led some researchers to suggest that the consequences of elevations of NGF (either exogenous or resulting from choline supplementation) may be dependent on the maturational state of the neural tissue [68]. Growing evidence indicates that the high-affinity trkA NGF receptor and the low-affinity p75 NGF receptor may play different roles with regard to neuronal survival. For example, NGF binding to trkA homodimers or heterodimers of trkA and p75 promotes cell survival and differentiation while binding to p75 homodimers leads to apoptosis (for review see Refs. [3,4,11,15,16,57]). Different maturational states of the neural tissue (e.g. differential expression of these receptors during early development) may be one factor contributing to the sensitive periods for the effects of choline supplementation on NGF levels. While we have yet to determine the developmental time course of choline-induced elevations in NGF, we do know that there are sensitive periods for the behavioral and neural effects of dietary choline supplementation [62,68] and it is possible that these sensitive periods are related to differential maturational states of the neural tissue and associated patterns of receptor expression.

The elevation of NGF levels apparent in the hippocampus of choline-supplemented rats on postnatal day 20 persists into adulthood though it is unclear how this change might contribute to prenatal choline supplementation induced changes in cognition. Certainly intraventricular administration of NGF to healthy rats causes a change in ChAT activity [31] and viral-vector induced increases in NGF result in a variety of changes in cholinergic markers accompanied by improvements in spatial learning [12–14]. Exogenous NGF administration also facilitates the induction of long-term potentiation [5,6,41], a cellular model of learning and memory. Furthermore, behavioral manipulations that elevate hippocampal levels of NGF have been shown to improve performance on spatial memory tasks in young adult rats. For example, long-term exposure to enriched environments results in increased NGF protein and mRNA levels in the hippocampus [67,79] and improves performance on both the radial-arm maze [40] and Morris water maze [67]. The beneficial effects of NGF on spatial memory in rats that have experienced brain damage via fimbria-fornix transection or fluid-percussion have also been well documented [24,39,71,72,83] and exogenous NGF has been shown to improve memory deficits in aged rats as well as reduce atrophy of basal forebrain cholinergic cells [29,30,34,53,54]. Together, these findings suggest that the elevation of hippocampal NGF levels apparent in prenatally choline-supplemented rats may play an important role in the improvements in learning and memory exhibited by these animals.

Choline serves a variety of functions during development and it is unclear exactly how choline supplementation acts to cause lasting changes in NGF levels or cognition. Choline is a precursor of membrane phospholipids, phosphatidylcholine and sphingomyelin [92], as well as a precursor for the neurotransmitter acetylcholine and the signaling phospholipids, platelet-activating factor and sphingosylphosphorylcholine [8]. It is possible that the supplemental choline is incorporated into cell membranes during development, resulting in increased demand for trophic support and elevations in NGF levels. However, the increase in the size of neuronal soma is restricted to basal forebrain cholinergic neurons [52,84] so a general choline-induced increase in cell size driving elevations in NGF is unlikely. Clearly choline availability during development has the potential to exert wide-ranging influences on nervous system development. Choline availability is inversely related to rates of apoptosis in both PC12 cell cultures and fetal rat hippocampus and septum [1,38,91,90]. In addition, perinatal choline supplementation is associated with an increase in hippocampal phospholipase D (PLD) activity [37]. Given the extensive functions of choline during development we have not yet identified the specific mechanisms responsible for the increases in NGF levels resulting from prenatal choline supplementation.

The present data raise a number of interesting questions regarding the mechanism by which choline supplementation induces its effects on NGF and behavior. For example, we currently know very little about the source of the elevation in NGF. This increase may be due to an increase in the number of NGF-producing cells in the hippocampus and cortex, an increase in the rate of NGF synthesis within those cells, or a decrease in the transport of NGF to target tissues. The latter mechanism, however, is unlikely given the hypertrophy of basal forebrain cholinergic neurons in choline-supplemented rats [52,84]. Analysis of the time-course of changes in NGF levels along with examination of changes in NGF mRNA levels in choline-supplemented animals will improve our understanding of the source of NGF elevations. This initial study examined only male rats and it is unclear whether choline supplementation causes a similar increase in NGF levels in female rats. Interestingly, the effect of choline supplementation on memory and cellular morphology of basal forebrain neurons is greater in males than in females [84]. Given the sex difference that exists in the rate of maturation of the septo-hippocampal system [51] and the sex difference in the developmental expression of NGF receptors [45], it will be interesting to determine whether choline-supplemented female rats show an increase in NGF levels similar to that of males. Finally, we have looked only at NGF levels in hippocampus and frontal cortex. It is unclear how prenatal choline supplementation affects NGF levels in other NGF-rich regions of the brain such as the striatum, cerebellum, and olfactory bulb [46,65,69,70]. Examining these tissues will provide important information regarding the specificity of these effects and will aid our understanding of the potential mechanisms involved.

In summary, we have demonstrated that prenatal choline
supplementation causes (a) elevations of NGF in the hippocampus that persist to at least postnatal day 90 and (b) elevations in the frontal cortex that are present at 20 days of age but are no longer evident at 90 days. These changes are part of a complex collection of changes that persist throughout the lifespan and may underlie improvements in multiple types of memory. Together these findings stress the importance of understanding the mechanisms by which early nutritional experiences may influence brain organization and subsequent behavior.

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References


[27] W. Fischer, K. Victorin, A. Bjorklund, L.R. Williams, S. Varon, F.H. Gage, Amelioration of cholinergic neuron atrophy and spatial memory deficits in multiple types of memory. Together these findings stress the importance of understanding the mechanisms by which early nutritional experiences may influence brain organization and subsequent behavior.

[28] H. Gnahn, F. Hefti, R. Heumann, M.E. Schwab, H. Thoenen,


[63] W.H. Meck, R.A. Smith, C.L. Williams, Organizational changes in cholinergic activity and enhanced visuospatial memory as a function of choline administered prenatally or postnatally or both, Behav. Neurosci. 103 (1989) 1234–1241.


[72] G. Sinson, B.R. Perri, I.Q. Trojanowski, E.S. Flamm, T.K. McIn-


