Protective Effects of Prenatal Choline Supplementation on Seizure-Induced Memory Impairment

Yili Yang,1 Zhao Liu,1 Jennifer M. Cermak,2 Pushpa Tandon,1 Matthew R. Sarkisian,1 Carl E. Stafstrom,3 John C. Neill,4 Jan K. Blusztajn,2 and Gregory L. Holmes1

1Department of Neurology, Center for Research in Pediatric Epilepsy, Harvard Medical School, Children’s Hospital, 2Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, Massachusetts, 3Department of Neurology, University of Wisconsin School of Medicine, Madison, Wisconsin, and 4Southampton College of Long Island University, Southampton, New York

Choline is an essential nutrient for rats and humans, and its availability during fetal development has long-lasting cognitive effects (Blusztajn, 1998). We investigated the effects of prenatal choline supplementation on memory deficits associated with status epilepticus. Pregnant rats received a control or choline-supplemented diet during days 11–17 of gestation. Male offspring [postnatal day 29 (P29)-32] were tested for their ability to find a platform in a water maze before and after administration of a convulsant dose of pilocarpine at P34. There were no differences between groups in water maze performance before the seizure. One week after status epilepticus (P41–P44), animals that had received the control diet prenatally had a drastically impaired performance in the water maze during the 4 d testing period, whereas prenatally choline-supplemented rats showed no impairment. Neither the seizures nor the prenatal availability of choline had any effect on hippocampal choline acetyltransferase or acetylcholinesterase activities. This study demonstrates that prenatal choline supplementation can protect rats against memory deficits induced by status epilepticus.

Key words: pilocarpine; water maze; hippocampus; epilepsy; seizures; learning; behavior

Status epilepticus is a serious cause of morbidity in both children and adults (Aicardi and Chevrie, 1990; De Lorenzo et al., 1992). Animal studies have confirmed the clinical finding that prolonged seizures can lead to a variety of adverse effects on cognition, learning, and behavior (Stafstrom et al., 1993; Liu et al., 1995; Holmes, 1997). Therapeutic methods that could prevent or reduce this seizure-related brain dysfunction are clearly needed. The possibility that events of early life, including nutrition during development, might influence the severity of memory impairment evoked by seizures deserves particular attention, because, if true, it could lead to nutrition-based preventive strategies.

Recent studies have focused on one such strategy involving dietary administration of a nutrient, choline, during perinatal development. Choline is an essential nutrient for animals and humans (Zeisel and Blusztajn, 1994; Blusztajn, 1998), and its adequate supply is particularly important during fetal development, when the organism grows rapidly. Pregnancy and lactation are the periods of highest dietary demands for choline because large amounts of this compound are transferred from the mother to the offspring via placenta and milk (Zeisel and Blusztajn, 1994). Choline serves as a precursor of phospholipids, phosphatidylcholine, lyso phosphatidylcholine, choline plasmalogens, and sphingomyelin, essential components of all membranes (Zeisel and Blusztajn, 1994). It is also the precursor for the biosynthesis of the neurotransmitter acetylcholine (Blusztajn and Wurtman, 1983).

Previous studies have shown that supplementation with choline during both prenatal and postnatal development in rats causes long-lasting improvements in retention on passive avoidance tasks (Ricceri and Berger-Sweeney, 1998), spatial memory tasks (Meck et al., 1988, 1989; Schenk and Brandner, 1995; Meck and Williams, 1997c, 1999; Tees, 1999), and of timing and temporal memory (Meck and Williams, 1997a,b), possibly because of improved memory capacity. We hypothesized that this increase in memory capacity might constitute a sufficient “cognitive reserve” to make prenatally choline-supplemented animals resistant to seizure-induced memory impairment.

We compared the performance of rats receiving supplemental choline during gestation with controls in a water maze after status epilepticus. Status epilepticus was induced by pilocarpine (Cavalleiro et al., 1991). The behavior, EEG findings, and spontaneous recurrent seizures in these models resemble those seen in human temporal lobe epilepsy (Turski et al., 1983; Liu et al., 1995). Previous studies from our laboratory have demonstrated...
that after pilocarpine- or kainic acid (KA)-induced status epilepticus adolescent and adult rats have significant deficits, compared with controls, in behavior and learning (Stafstrom et al., 1993; Liu et al., 1995). Here we report that prenatal choline supplementation in rats protects against seizure-evoked memory impairment.

**MATERIALS AND METHODS**

*Study design and animals.* Pregnant Sprague Dawley rats (Charles River Laboratories, Cambridge, MA) (n = 27) were randomly assigned to either choline supplementation (n = 15) or control diet (n = 12). From embryonic day 11 (E11) to E17 the choline chloride (Sigma, St. Louis, MO) (25 mM choline chloride + 50 mM saccharin; choline group) or saccharin (50 mM saccharin; control diet group) was delivered in tap water that was given to all of the rats as their only source of drinking water. After the period of supplementation (E17) the dams received standard tap water without choline supplementation. Throughout the study all rats had ad libitum access to a standard choline diet (AIN-76A; a purified diet from Dyets, Inc.; 1.1 gm choline chloride/kg). This supplementation paradigm was similar to that originally described by Meck et al. (1989). The litters were culled to male pups and divided into the following four groups: group 1 (n = 42), prenatal choline supplementation/pilocarpine (Chol/Pilo); group 2 (n = 36), control diet/pilocarpine (Control/Pilo); group 3 (n = 13), prenatal choline supplementation/saline (Chol/Sal); and group 4 (n = 15), control diet/saline (Control/Sal).

Rats were kept in plastic cages on a 12 hr light/dark cycle. Procedures were approved by the Animal Care Committee of Children’s Hospital and in accordance with guidelines set by the National Institutes of Health.

Pilocarpine hydrochloride (Sigma) was freshly dissolved in 0.9% saline and administered intraperitoneally at P34 at a dosage of 180 mg/kg. Control rats received equal volume injections of normal saline. The rats were observed for behavioral changes for 4 hr after the pilocarpine injection.

Rats underwent behavioral testing using the Morris water maze test on two occasions: (1) before pilocarpine injection (P29–P32) and (2) 1 week after pilocarpine or saline injection (P41–P44). After the behavioral tests, rats received an overdose of sodium pentobarbital (80 mg/kg). For the biochemical testing, rats were anesthetized with ether and decapitated. The brains were rapidly removed from the skulls, frozen by immersion in isopentane, sectioned at 20 μm in the coronal plane, and stained with cresyl violet. Slides were examined for cell loss in the hippocampus by an observer blind to the treatment group. The severity of the lesion was established on a scale of 0 to 3 (Mikati et al., 1994; Bolanos et al., 1998). A score of 0 indicated no lesion; 1 indicated mild cell loss in CA1 or CA3 subfields; 2 indicated moderate cell loss in CA1 or CA3 with preservation of the general cellular architecture; and 3 indicated marked cell loss with complete disruption of the normal cellular architecture. Scores for each animal were averaged. In addition to hippocampal damage, the brains were examined for lesions in the amygdala and in the entorhinal and piriform cortices.

*Histology.* After the completion of all behavioral testing, rats were anesthetized with ether and decapitated. The brains were removed from the skulls, frozen by immersion in isopentane, sectioned at 20 μm in the coronal plane, and stained with cresyl violet. Slides were examined for cell loss in the hippocampus by an observer blind to the treatment group. The severity of the lesion was established on a scale of 0 to 3 (Mikati et al., 1994; Bolanos et al., 1998). A score of 0 indicated no lesion; 1 indicated mild cell loss in CA1 or CA3 subfields; 2 indicated moderate cell loss in CA1 or CA3 with preservation of the general cellular architecture; and 3 indicated marked cell loss with complete disruption of the normal cellular architecture. Scores for each animal were averaged. In addition to hippocampal damage, the brains were examined for lesions in the amygdala and in the entorhinal and piriform cortices.

*Data analysis.* All group means are given with the SEM. Differences between mean latencies of groups with multiple measures were compared using ANOVA with repeated measures. All neurochemical assays were performed in triplicate. ChAT and AChE values from hippocampus homogenates were evaluated by two-way ANOVA to test the main effects of choline and pilocarpine. If a significant difference was found, data were further analyzed by Fisher’s or Tukey’s multiple comparison tests to determine individual group differences. The unpaired t test was also used to compare litter size and pup body weight in the choline and control diet groups and to compare means of escape latencies on individual days in the water maze. A significant level was defined as p < 0.05 (two-sided) for all comparisons.

**RESULTS**

Both the control and choline-supplemented pregnant rats drank the water without adverse effects on body weight of offspring or litter size. There was no difference in the amount of water intake: the choline-supplemented rats drank an average of 30.9 ± 3.0 ml/d; controls drank 31.7 ± 1.6 ml/d (t = 0.246; p = 0.809; df = 26). Litter size was 11.8 ± 0.6 pups in the choline group and 12.9 ± 0.4 pups in the control group (t = 1.36; p = 0.189; df = 26). Mean body weights of the pups were also similar (6.35 ± 0.07 gm in the choline group and 6.28 ± 0.07 gm in the control diet group; t = 0.745; p = 0.458; df = 105).

**Behavioral effects of pilocarpine injection**

A decrease in activity, chewing, eye blinking, “wet dog shakes”, and head nodding occurred within 15–30 min of the injection. This was followed by upper-extremity clonus, rearing, and generalized convulsions. There was no difference in mortality rate in the rats receiving choline supplementation and those receiving control diets [choline-supplemented, 19/42 (45.2%); control diet, 15/36 (41.7%)]; χ² = 0.001; p = 0.994). None of the rats that received saline (Chol/Sal and Control/Sal) had seizures or died.

**Water maze**

Figure 1A shows the mean escape latencies in the water maze when the rats were studied between P29 and P32 before status epilepticus. Both groups of animals learned the task, as evidenced...
by the reduction in escape latencies over the 4 d training period from ~300 to 100 sec (controls, \( F_{(3,200)} = 54.529, p < 0.001 \); choline-supplemented, \( F_{(3,210)} = 34.861, p < 0.001 \)). The water maze is a relatively simple test of spatial reference memory, and consistent with previous studies (Skjei et al., 1995), there were no differences in the water maze performance between the two groups during this training period (\( F_{(1,104)} = 0.074; p = 0.785 \)).

When the animals were retested in the water maze 1 week after the pilocarpine-induced status epilepticus there was a dramatic difference found between the choline-supplemented and control animals (\( F_{(1,80)} = 25.602; p < 0.001 \)) (Fig. 1B). After the pilocarpine-induced seizures, the performance of the noncholine-treated control animals drastically deteriorated. On the fourth day of testing before pilocarpine, the mean escape latency was 115.71 ± 6.27 sec; after the pilocarpine the mean escape latency on the first day of retraining was 208.97 ± 30.63 (\( t = 2.855; p = 0.009 \); df = 23). Their performance improved during the 4 d of testing (Fig. 1, compare A, B, black bars), but not between days 3 and 4 of testing \( [F_{(3,80)} = 4.323; p < 0.001] \); days 1 and 2 differed significantly \( (p < 0.05) \) from days 3 and 4; no difference between days 3 and 4, suggesting that these animals could not be rehabilitated by training. Conversely, in the choline-supplemented animals the mean latency to platform on the fourth day of water maze testing before pilocarpine was 122.22 ± 7.07 and 92.40 ± 7.63 sec on the first trial of water maze testing after pilocarpine \( (t = 0.127; p = 0.127; \text{df} = 27) \). Despite having had pilocarpine-induced seizures, the performance (total escape latencies) of the prenatally choline-supplemented animals improved markedly after retraining (Fig. 1, compare A, B, gray bars). In fact, the total escape latencies of choline-supplemented animals after pilocarpine (Chol/Pilo) was indistinguishable from the performance of a set of control animals that did not receive pilocarpine (Chol/Sal) \( F_{(1,25)} = 1.789; p = 0.185 \). In summary, there was a saving of previous training in the water maze in rats who received choline supplementation. In contrast, there was a loss of previous training, as shown by a very large increase in latencies after seizures in animals that did not receive prenatal choline supplementation.

There were no differences in water maze performance between the two groups that did not receive pilocarpine (Chol/Sal and Control/Sal groups) either before \( F_{(1,26)} = 0.641; p = 0.43 \) or after repeated water maze testing after saline injections \( F_{(1,260)} = 2.156; p = 0.146 \). As can be seen by comparing Figure 1, A and C, both groups of animals that did not receive pilocarpine had a memory effect with shorter latencies to the platform during the second series of trials than during the first trial \( (\text{Chol/Sal}, F_{(1,35)} = 123.81, p < 0.001]; \text{Control/Sal}, F_{(1,35)} = 73.96, p < 0.001 \).

**Neurochemical assays**

In our previous studies we found that prenatal choline supplementation reduced hippocampal ChAT and AChE activities in juvenile rats (up to P27), but that it had no effect in older animals (Cermak et al., 1998, 1999). In the current study we measured the activities of these enzymes at P78. Consistent with previous studies prenatal choline supplementation failed to alter ChAT and AChE activity at this age. The long-term effects of pilocarpine-evoked seizures on hippocampal ChAT and AChE have not been studied previously. ChAT activity, measured 1 month after seizures, tended to be reduced in the pilocarpine-treated animals. However, this effect did not reach statistical significance \( (\text{ANOVA}, p = 0.08 \text{for all groups}; \text{or} \ t \text{test for the control group only}, p = 0.075) \). Similarly, pilocarpine treatment did not alter AChE activity in the hippocampus.

**Histology**

Cell loss was significantly different among the four groups of rats (choline-supplemented with pilocarpine, nonsupplemented with...
pilocarpine, choline-supplemented without pilocarpine, and non-supplemented with pilocarpine; Kruskal–Wallis = 13.994; \( p = 0.003 \), because of the cell loss found in CA3, CA1, and the hilus in the two groups receiving pilocarpine. Figure 4 provides examples of histological changes in CA3 and CA1 in the control and choline-supplemented rats. The mean pathology score in the two groups receiving pilocarpine did not differ significantly (Chol/Pilo, 1.07; Control/Pilo, 1.00; Kruskal-Wallis, 0.045; \( p = 0.831 \)). Mild cell loss was also seen in the amygdala and entorhinal and piriform cortices in both groups that received pilocarpine. No qualitative differences were seen between the two groups. No lesions were detected in the animals that did not receive pilocarpine. Considering the significant differences in water maze performance between the controls and choline-supplemented rats, the lack of significant histological damage was surprising and suggests that the histological techniques used lack sensitivity for detection of damaged neuronal circuits.

DISCUSSION

Compared with control animals, prenatal supplementation with choline dramatically improved the performance in a spatial maze task after status epilepticus. The beneficial effects of choline were striking. Seven days of prenatal choline supplementation prevented the status epilepticus-induced impairment of memory in the water maze. The benefits of choline were seen only in the animals that had status epilepticus. No differences in water maze performance were found between the choline-supplemented and controls when tested before the status epilepticus nor were any differences found between choline-supplemented and control animals who did not receive pilocarpine and were tested a second time.

The mechanism by which choline supplementation resulted in preservation of memory is not clear. Prenatal choline supplementation has widespread effects on brain function through several mechanisms. In this study, choline was administered between E11 and E17, a critical period for development of the cholinergic system in rat brains (Armstrong et al., 1987; Brady et al., 1989; Semba and Fibiger, 1989). The finding that perinatal choline supplementation can alter the rostrocaudal distribution and size of cells in the medial septal nucleus and the nucleus of the diagonal band of Broca (Loy et al., 1991) is consistent with reports that choline can affect forebrain cytogenesis, migration, and neuronal survivability (Albright et al., 1999a,b). Moreover, choline supplementation during this period alters multiple functional indices of the septohippocampal cholinergic system, thought to be critical for the processes of attention, learning, and memory (Fibiger, 1991; Muir et al., 1992, 1994; Berger-Sweeney et al., 1994; Voytko et al., 1994; Chiba et al., 1995; Jones et al., 1995; Acquas et al., 1996; Baxter et al., 1997). Specifically during the first four postnatal weeks the activities of ChAT, AChE, and ACh synthesized from choline transported by the sodium-dependent high-affinity choline transporter are reduced in the hippocampus of the perinatally choline-supplemented rats relative to controls (Cermak et al., 1998, 1999). In contrast, depolarization-evoked ACh release is higher in the choline-supplemented animals (Cermak et al., 1998). The latter observation, together with the reduced AChE activity (Cermak et al., 1999), suggest that intrasynaptic ACh concentrations and dwell times may be increased, resulting in enhanced cholinergic neurotransmission. The observations that ACh turnover in prenatally choline-supplemented animals is relatively slow, but that cholinergic neurotransmission is well maintained (as evidenced by ro-
bust ACh release), suggested that the pool of choline used for the synthesis of ACh in these animals may include that stored in membrane phosphatidylcholine, and may be generated by the hydrolysis of phosphatidylcholine catalyzed by phospholipase D. Consistent with the latter possibility, we found that, at a young age, hippocampal phospholipase D activity was twofold higher in prenatally choline-supplemented rats relative to control animals (Holler et al., 1996). Furthermore, Loy et al. (1991) found that prenatal and postnatal choline supplementation resulted in medial septal cell bodies that were larger, rounder, and more uniform than controls and the p75 neurotrophin receptor-positive cells (presumably cholinergic neurons) in the diagonal band of the prenatally choline supplemented rats were larger than those of controls (Williams et al., 1998).

In addition to its effect on the cholinergic system, choline supplementation also influences other systems. For example, prenatal dietary choline supplementation has also been found to decrease the threshold for induction of long-term potentiation in young and adult rats (Pyapali et al., 1998; Jones et al., 1999), a process that is mediated by glutamatergic neurotransmission (Malenka and Nicoll, 1999). Jones et al. (1999) found that a significantly larger percentage of slices from choline-supplemented rats displayed LTP at 50% stimulus intensity (compared with control and choline-deficient rats). Changes in LTP threshold may be responsible for the enhancement of visuospatial memory obtained after prenatal choline supplementation. Whereas choline supplementation may have protective effects on other systems, including visual and motor systems, rather than central memory processes, memory is used here simply as a concept that refers to the organism behaving as it did earlier under similar stimulus conditions. Prenatal choline supplementation undoubtedly has multiple effects on the developing organism beyond memory-dependent behavioral performances and hippocampal cells (Blusztajn, 1998). In the present experiments there were no obvious differences in the motor behavior of the choline-supplemented and the nonsupplemented animals. Further experiments are necessary to determine more specifically how choline protects behavioral performances after seizures.

The effects of choline supplementation on the developing nervous system are complex. Behavioral effects appear to be dependent both by the time the choline is supplemented and when the animal is tested as well as type of memory test studied. Nevertheless, this study demonstrates that even a short period of choline supplementation also influences other systems. For example, prenatal and postnatal choline supplementation results in medial septal cell bodies that were larger, rounder, and more uniform than controls and the p75 neurotrophin receptor-positive cells (presumably cholinergic neurons) in the diagonal band of the prenatally choline supplemented rats were larger than those of controls (Williams et al., 1998).

In addition to its effect on the cholinergic system, choline supplementation also influences other systems. For example, prenatal dietary choline supplementation has also been found to decrease the threshold for induction of long-term potentiation in young and adult rats (Pyapali et al., 1998; Jones et al., 1999), a process that is mediated by glutamatergic neurotransmission (Malenka and Nicoll, 1999). Jones et al. (1999) found that a significantly larger percentage of slices from choline-supplemented rats displayed LTP at 50% stimulus intensity (compared with control and choline-deficient rats). Changes in LTP threshold may be responsible for the enhancement of visuospatial memory obtained after prenatal choline supplementation. Whereas choline supplementation may have protective effects on other systems, including visual and motor systems, rather than central memory processes, memory is used here simply as a concept that refers to the organism behaving as it did earlier under similar stimulus conditions. Prenatal choline supplementation undoubtedly has multiple effects on the developing organism beyond memory-dependent behavioral performances and hippocampal cells (Blusztajn, 1998). In the present experiments there were no obvious differences in the motor behavior of the choline-supplemented and the nonsupplemented animals. Further experiments are necessary to determine more specifically how choline protects behavioral performances after seizures.

The effects of choline supplementation on the developing nervous system are complex. Behavioral effects appear to be dependent both by the time the choline is supplemented and when the animal is tested as well as type of memory test studied. Nevertheless, this study demonstrates that even a short period of choline administration during gestation can have marked protective effects when the animals are subjected to the stress of status epilepticus. Thus, we postulate that one of the benefits of appropriate choline nutrition during the perinatal period may be reduced vulnerability of cognitive function to brain insults such as those elicited by epilepsy.

REFERENCES


Meck WH, Smith RA, Williams CL (1989) Organizational changes in cholinergic activity and enhanced visuospatial memory as a function of choline administered prenatally or postnatally or both. Behav Neurosci 103:1234–1241.


Yang et al. • Effects of Choline on Memory after Seizures