Research Report

Prenatal choline deficiency increases choline transporter expression in the septum and hippocampus during postnatal development and in adulthood in rats

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ABSTRACT

Supplementation of maternal diet with the essential nutrient, choline, during the second half of pregnancy in rats causes long-lasting improvements in spatial memory in the offspring and protects them from the memory decline characteristic of old age. In contrast, prenatal choline deficiency is associated with poor performance in certain cognitive tasks. The mechanism by which choline influences learning and memory remains unclear; however, it may involve changes to the hippocampal cholinergic system. Previously, we showed that the hippocampi of prenatally [embryonic days (E) 11–17] choline-deficient animals have increased synthesis of acetylcholine (ACh) from choline transported by the high-affinity choline transporter (CHT) and reduced ACh content relative to the control and to the E11–17 choline-supplemented rats. In the current study, we found that, during postnatal period [postnatal days (P) 18–480], prenatal choline deficiency increased the expression of CHT mRNA in the septum and CHT mRNA and protein levels in the hippocampus and altered the pattern of CHT immunoreactivity in the dentate gyrus. CHT immunoreactivity was more prominent in the inner molecular layer in prenatally choline-deficient rats compared to controls and prenatally choline-supplemented animals. In addition, in all groups, we observed a population of hilar interneurons that were CHT-immunoreactive. These neurons are the likely source of the hippocampal CHT mRNA as their number correlated with the levels of this mRNA. The abundance of hippocampal CHT mRNA rose between P1 and P24 and then declined reaching 60% of the P1 value by P90. These data show that prenatal availability of choline alters its own metabolism (i.e., CHT expression). While the upregulated CHT expression during the period of prenatal choline deficiency may be considered as a compensatory mechanism that could enhance ACh synthesis when choline supply is low, the persistent upregulation of CHT expression subsequent to the brief period of prenatal deprivation of choline in utero might be beneficial during choline deficiency in adulthood.

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1. Introduction

The availability of the essential nutrient, choline, during gestation in rodents causes changes in brain organization that are apparent in the fetus, in the young, in the adult, and in the aged animal (Blusztajn, 1998; McCann et al., 2006; Meck and Williams, 2003; Zeisel, 2004). In the most studied model that employs pregnant rats consuming diets of varying choline content during the 7-day period of the second half of gestation (embryonic days E11–17), animals that were supplemented with choline are characterized by improved spatial and temporal memory and improved attention at a young age and in adulthood (Meck et al., 1988, 1989; Meck and Williams, 1997a,b,c, 1999). These animals do not exhibit the normally occurring age-related decline in memory performance, i.e., they are protected from the cognitive deficits of old age (Blusztajn, 1998; Meck and Williams, 2003). Taken together these data suggest that cognitive decline is not an inevitable outcome of old age, but rather can be prevented by increasing the supply of choline during a critical period of prenatal development. In contrast, rats deprived of choline during the E11–17 period have deficits in certain memory tasks (Meck and Williams, 1997c). One of the physiological functions of choline is to serve as the precursor of the neurotransmitter, acetylcholine (ACh) in cholinergic neurons. The rate-limiting factor in the synthesis of ACh is the supply of choline (Blusztajn and Wurtman, 1983), and our previous studies provided evidence that, in young rats, this process is profoundly affected by the availability of choline *in utero* in the septohippocampal cholinergic neurons that are thought to participate in the mechanisms underlying learning, memory, and attention (Gold, 2003; Sarter et al., 2003). Specifically, the prenatally choline-supplemented rats derived a high proportion of choline for ACh synthesis from the membrane-bound choline pool in the form of phosphatidylcholine (Cermak et al., 1998; Holler et al., 1996), whereas prenatally choline-deficient rats were characterized by upregulated transport of choline from the extracellular space catalyzed by the high-affinity choline transporter, CHT (Cermak et al., 1998). The latter result could be due to either increased CHT expression or its activation (Sarter and Parikh, 2005) as choline transport catalyzed by CHT can be rapidly stimulated by increased neuronal firing (Collier and Ilson, 1977; Murrin and Kuhar, 1976; Polak et al., 1977; Sherman et al., 1978) that appears to be mediated by a recruitment of CHT molecules from a synaptic vesicular pool to the plasma membrane resulting in an increased V_{max} of choline transport without a change in K_t (Ferguson et al., 2003). In the current study we found that prenatal choline deficiency is associated with increased CHT mRNA expression in the septum (where the cell bodies of the cholinergic septohippocampal neurons reside) and increased CHT mRNA and protein levels in the hippocampus. The CHT-immunoreactive septohippocampal projection, which terminates in the inner molecular layer of the dentate gyrus, is augmented in the choline-deficient animals and CHT-immunoreactive local circuit neurons in the hilus of the hippocampus are in many cases more prominent than in controls and choline-supplemented animals.

2. Results

Initially, we examined CHT expression in the medial septum on P18, P90, and P480 (16 months old) by RT-PCR (Fig. 1). At each of the three ages, prenatally choline-deficient animals had the highest level of CHT mRNA expression with the most significant difference on P480. The medial septal cholinergic neurons project to the hippocampus and, consistent with CHT mRNA expression in the septum, the levels of CHT protein in the hippocampus were at least 2-fold higher in prenatally choline-deficient rats as compared to the other two groups on both P18 and P480 (Fig. 2). Previous studies have noted that a small number CHT-expressing neurons can also be found in the hippocampus, specifically the hilus of the dentate gyrus (Misawa et al., 2001). Moreover, we previously reported the presence of CHT mRNA in the hippocampus (Berse et al., 2005). We therefore performed a developmental study of CHT mRNA expression in the hippocampus (Fig. 3). During the first postnatal week CHT mRNA expression increased and then plateaued from P8 to P24. Subsequently there was a dramatic drop in CHT mRNA levels that reached a nadir by P34, followed by a new, apparently stable, plateau until P90. The latter level of CHT was approximately 50% of that seen at the peak.
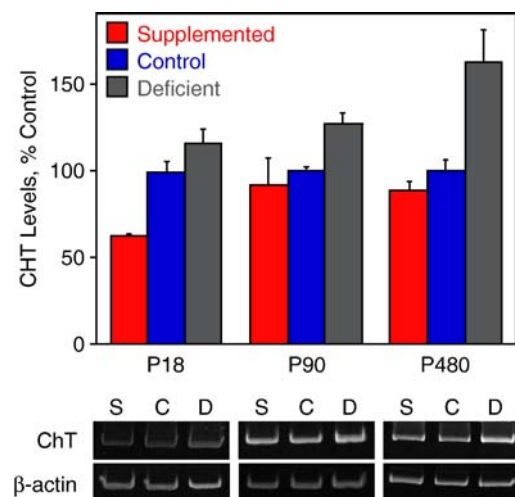

Fig. 1 – CHT mRNA expression in the medial septum. Septal RNA from P18, P90 and P480 rats was used for RT-PCR of CHT and β-actin. CHT levels were normalized using β-actin levels and are presented as means ± SEM ($n = 4$ per group on P18 and P90, $n = 6$ per group on P480). The levels of CHT were significantly different among the three groups of animals on each day as determined by ANOVA ($p < 0.005$, $p < 0.05$, and $p < 0.01$, respectively). The ANOVA was followed up with Tukey tests at a 5% procedure wise error rate. Prenatally choline-deficient animals had a significantly higher amount of CHT mRNA compared to prenatally choline-supplemented animals on all days and to control animals at P480. On P18, the CHT mRNA levels were significantly higher in the control animals than prenatally choline-supplemented animals. S, prenatally choline-supplemented; C, control; D, prenatally choline-deficient.
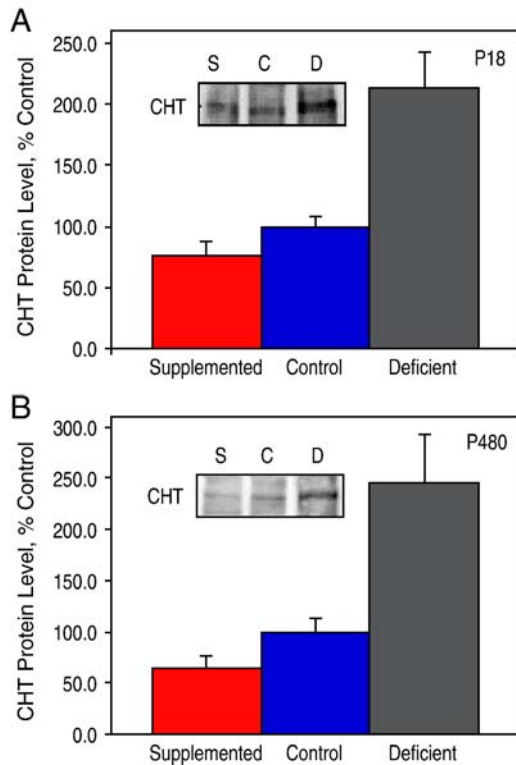
Fig. 2 – CHT protein levels in the hippocampus. Hippocampal lysates from P18 (A) and P480 (B) rats were used for Western blot analysis of CHT. CHT protein levels are presented as means±SEM, $n=4$ per group for P18 and $n=6$ per group for P480. The levels of CHT were significantly different among the three groups of animals on both days as determined by ANOVA ($p<0.005$, and $p<0.01$, respectively). The ANOVA was followed up with Tukey tests at a 5% procedure wise error rate. On both P18 and P480, prenatally choline-deficient animals had a significantly higher amount of CHT protein compared to control animals and to prenatally choline-supplemented animals. S, prenatally choline-supplemented; C, control; D, prenatally choline-deficient.

In the hippocampal formation, CHT immunoreactivity was primarily located in non-pyramidal neurons in CA4 and in fibers and terminals in the supragranular plexus and inner molecular layer of the dentate gyrus (Fig. 4). Less prominent fiber staining was seen in the stratum pyramidale and stratum oriens of CA1–3. In P18 animals that experienced reduced dietary choline availability *in utero*, CHT immunoreactivity was more prominent in the inner molecular layer of the dentate gyrus and CHT-immunoreactive neurons were more prominent in CA4 compared to control and supplemented groups despite similar staining intensity patterns among the three groups in other brain regions (Fig. 4). Quantitation of CHT-immunoreactive neurons in CA4 showed a significant difference among the three groups of P18 animals with supplemented animals having a lower density than either deficient or controls (Fig. 5A). The CHT mRNA expression in the hippocampus of the same P18 animals was determined. Prenatally choline-supplemented animals had a significantly lower amount of CHT expression as compared to that of the

other two groups of animals (Fig. 5B). Regression analysis showed a significant correlation between the hilar cell counts and CHT mRNA in the hippocampus (Fig. 5C).

At P72, the differences in CHT immunoreactivity among the three groups were less consistent but a similar pattern of altered CHT immunoreactivity was seen in deficient animals in some cases (Fig. 6). There were no distinguishable differences in CHT-immunoreactive basal forebrain neurons among the three groups at P18 or P72 (Figs. 4C, F, I and 6C, F, I). Analysis of P72 animals did not show a significant difference in cell density among the three groups (Fig. 7A). Because the P72 animals were perfused, the tissue could not be used for RNA extraction and parallel analysis of CHT mRNA as performed on the P18 animals. As an alternative, hippocampal tissue from rats that were similar in age (P64) was used to measure the expression level of CHT. Prenatally choline-deficient animals had 2- to 3-fold higher amounts of CHT mRNA than animals from each of the other two groups (Fig. 7B). These results suggest that the prenatally choline-deficient animals maintain a higher level of CHT in the hippocampus, possibly by increased CHT expression per cell rather than a higher number CHT-positive cells.

3.   Discussion

The data presented here indicate that the previously observed upregulation of CHT activity in hippocampal slices from the prenatally choline-deficient rats (Cermak et al., 1998) can be accounted for by the increased expression of CHT in these animals. Prenatal choline deficiency caused a long-term
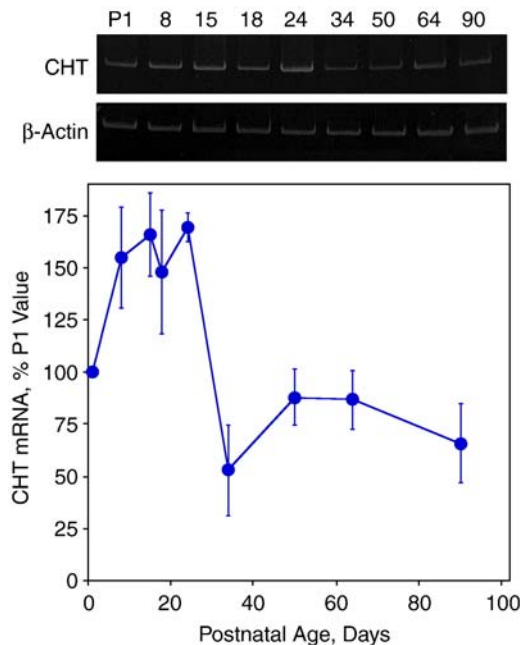

Fig. 3 – Developmental changes in CHT expression in the hippocampus of control rats. Hippocampal RNA from P1, 8, 15, 18, 34, 50, 64 and 90 rats was used for RT-PCR of CHT and β-actin. CHT levels were normalized using β-actin levels and are presented as means±SEM, $n=6$ per group.
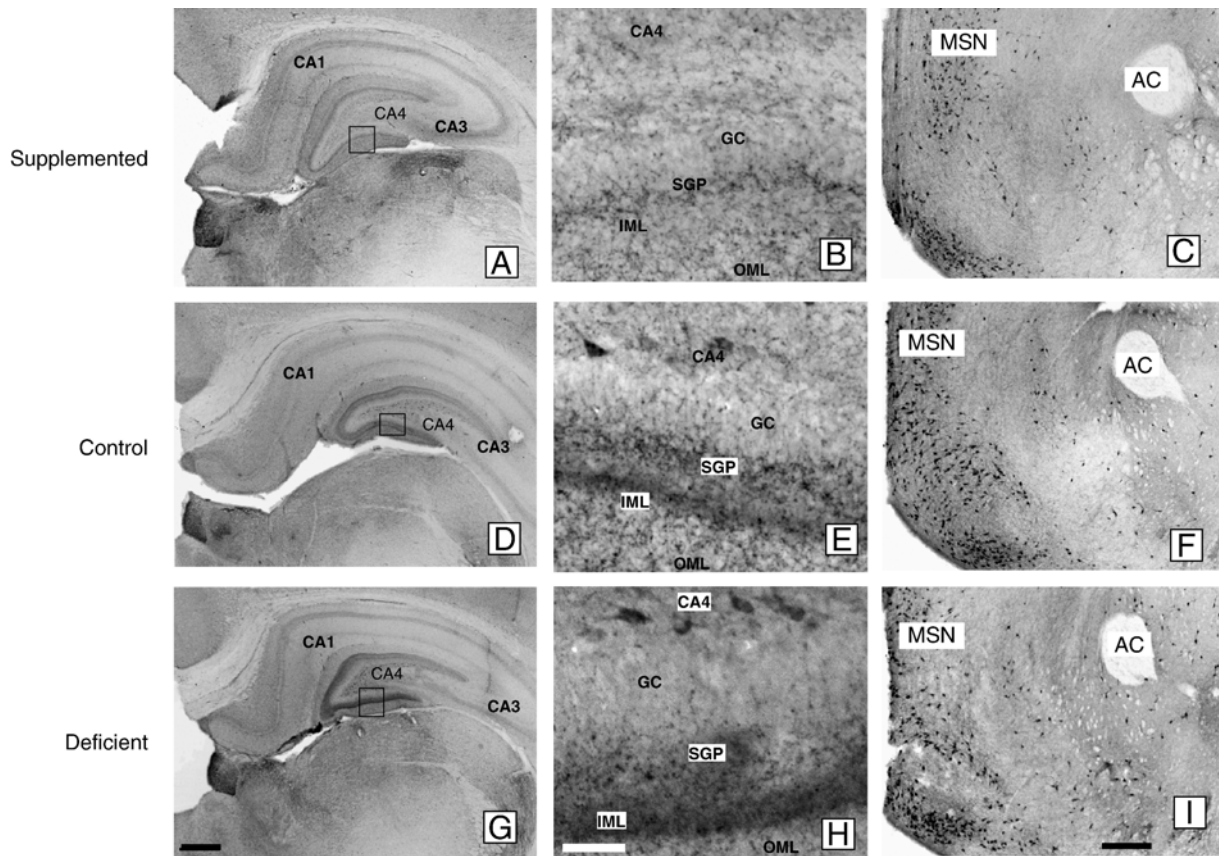
Fig. 4 – CHT immunocytochemistry in the hippocampus and medial septum of P18 rats. Prenatally choline-supplemented (A, B, C), control (D, E, F), deficient (G, H, I) animals. GC, granular cell layer; SGP, supragranular plexus; IML, inner molecular layer; OML, outer molecular layer; MSN, medial septal nucleus; AC, anterior commissure. The pattern of CHT immunoreactivity in the molecular layer of the dentate gyrus differed among the three groups and CHT-immunoreactive neurons were generally more prominent in CA4 in the deficient group (G, H) and less prominent in the supplemented group (A, B) compared to controls (D, E) despite similar staining intensities in other brain regions such as the medial septal nucleus (C, F, I). Magnification bars: A, D, G—500 μm; B, E, H—50 μm; C, F, I—500 μm.

upregulation of the abundance of CHT mRNA in the septum and hippocampus. CHT protein levels were also dramatically increased in the hippocampi from the prenatally choline-deficient rats as compared to the control and prenatally choline-supplemented animals. These biochemical findings were further corroborated by the increased prominence of CHT immunoreactivity in the inner molecular layer of the dentate gyrus in prenatally choline-deficient animals. However, the number of CHT-immunoreactive neurons in the medial septal nucleus was similar among the three groups of rats, suggesting that the increased hippocampal fiber immunoreactivity in the prenatally choline-deficient animals is likely due to plasticity and enhancement of the axonal projections, synaptic terminations, and/or CHT protein levels in the nerve terminals of neurons originating from the medial septum (Mesulam et al., 1983). Interestingly, previous studies showed that prenatal choline deficiency reduced (McKeon-O'Malley et al., 2003), while supplementation caused an increase (Williams et al., 1998) in the average size of the medial septal cholinergic neurons as assessed by immunostaining with antibodies against the p75 neurotrophin receptor, NGFR. Given that both CHT and NGFR are markers for the

same population of medial septal cholinergic neurons, it appears that prenatal choline availability has a profound impact on the development of these cells with low choline consumption resulting in upregulated CHT expression and reduced size of these neurons, while high choline intake causing an increase in the neuronal size, possibly mediated by the previously reported elevated expression of hippocampal nerve growth factor (Sandstrom et al., 2002)—a trophic protein for these neurons (Hefti et al., 1984; Koliatsos et al., 1991; Mobley et al., 1986). In contrast to the profound impact of prenatal choline deficiency on CHT expression, prenatal choline supplementation had a minor effect on CHT with significantly reduced levels of its mRNA relative to the control animals observed only at P18 in the septum and hippocampus.

In behavioral studies, prenatally choline-supplemented adult rats are characterized by improved performance relative to prenatally deficient and control animals in tasks measuring spatial memory, temporal processing, and attention (Brandner, 2002; Meck et al., 1989; Meck and Williams, 1997a,b,c, 1999; Schenk and Brandner, 1995). Prenatally choline-deficient animals are normal in many behavioral tests, however, they exhibit impairments in attentional and demanding memory
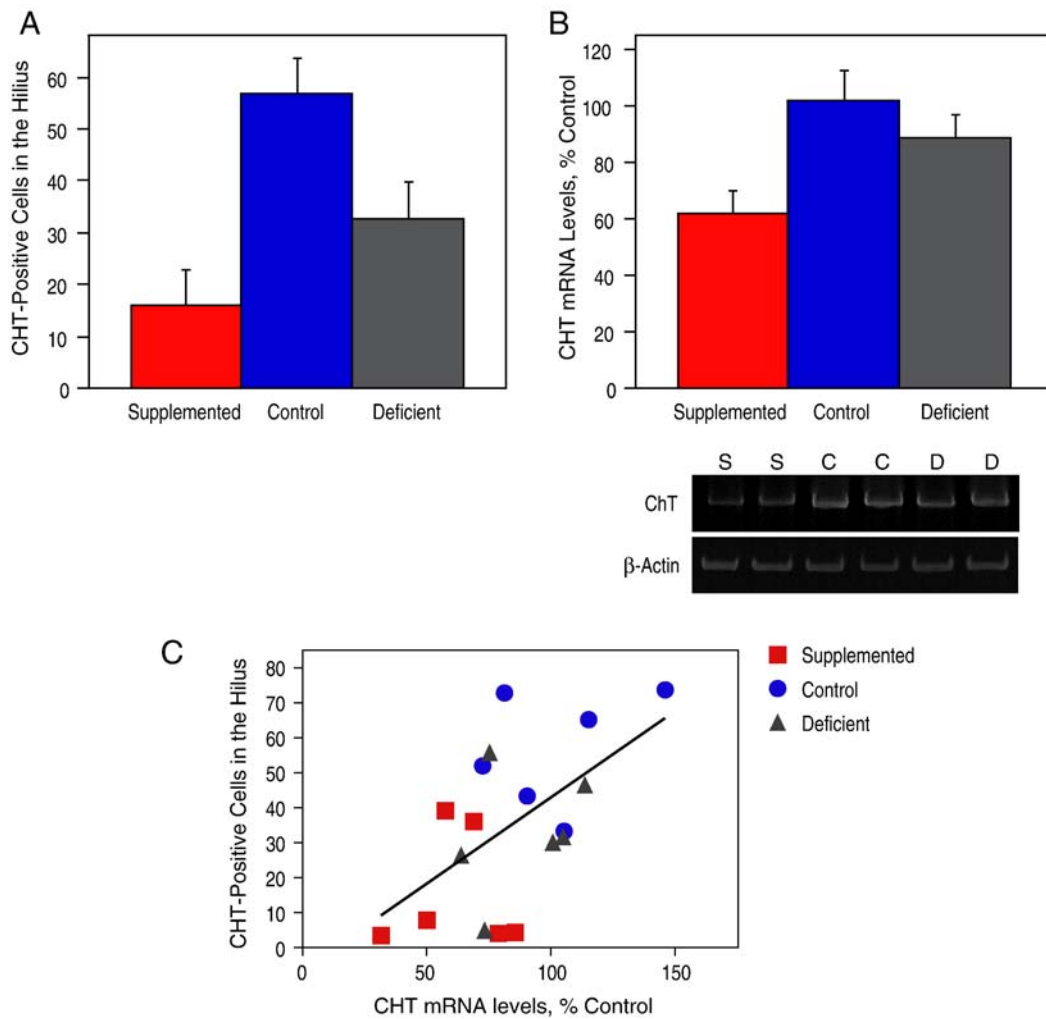
Fig. 5 – Correlation between the number of CHT-positive cells in the hilus and the level of CHT mRNA in the hippocampus at P18. (A) CHT-positive neuronal perikarya in the CA4 regions of the hippocampal formation of P18 animals were quantitated and analyzed using a semi-automated computerized morphometry system. The number of CHT-positive cells was significantly different among the three groups of animals as determined by ANOVA ($p < 0.005$). The ANOVA was followed up with Tukey tests at a 5% procedure wise error rate. Control animals had a significantly higher number of CHT-positive cells compared to prenatally choline-supplemented animals. (B) Hippocampal RNA from P18 rats was used for RT-PCR of CHT and β-actin. CHT levels were normalized using β-actin levels and are presented as means ± SEM ($n = 6$ per group). The levels of CHT were significantly different among the three groups of animals as determined by ANOVA ($p < 0.05$). The ANOVA was followed up with Tukey tests at a 5% procedure wise error rate. Prenatally choline-deficient and control animals had a significantly higher amount of CHT mRNA compared to prenatally choline-supplemented animals. (C) A significant correlation between the number of CHT-positive cells in the hilus and the level of CHT mRNA in the hippocampus was observed ($R = 0.567$ and $p < 0.05$). S, prenatally choline-supplemented; C, control; D, prenatally choline-deficient.

tasks (Meck and Williams, 1997c, 1999), and, unlike the prenatally choline-supplemented rats that are characterized by enhanced hippocampal long-term potentiation (LTP) studied *in vitro* in slice preparations, these animals have LTP deficits (Jones et al., 1999; Pyapali et al., 1998), possibly as a result of diminished slice viability. NMDA receptor-mediated neurotransmission was also reduced in hippocampal slices from prenatally choline-deficient rats as compared to controls (Montoya and Swartzwelder, 2000). Moreover, phosphorylation of hippocampal mitogen-activated protein kinase (MAPK) and cAMP response element binding protein (CREB) evoked by NMDA, glutamate, or depolarizing concentrations of potas-

sium was reduced by prenatal choline deficiency and increased by prenatal choline supplementation (Mellott et al., 2004). These data suggested that prenatal availability of choline has long-term effects on multiple neuronal systems. We tested this hypothesis using gene expression profiling with oligonucleotide microarray techniques (Mellott et al., in press) and found alterations in the expression of multiple genes that were, in most cases, transient occurring during the P15–P34 period. Prenatally choline-deficient rats had the highest expression of calcium/calmodulin-dependent protein kinase (CaMK) IIβ, protein kinase Cβ2, and GABA$_B$ receptor 1 isoforms c and d in the hippocampus. Prenatally choline-
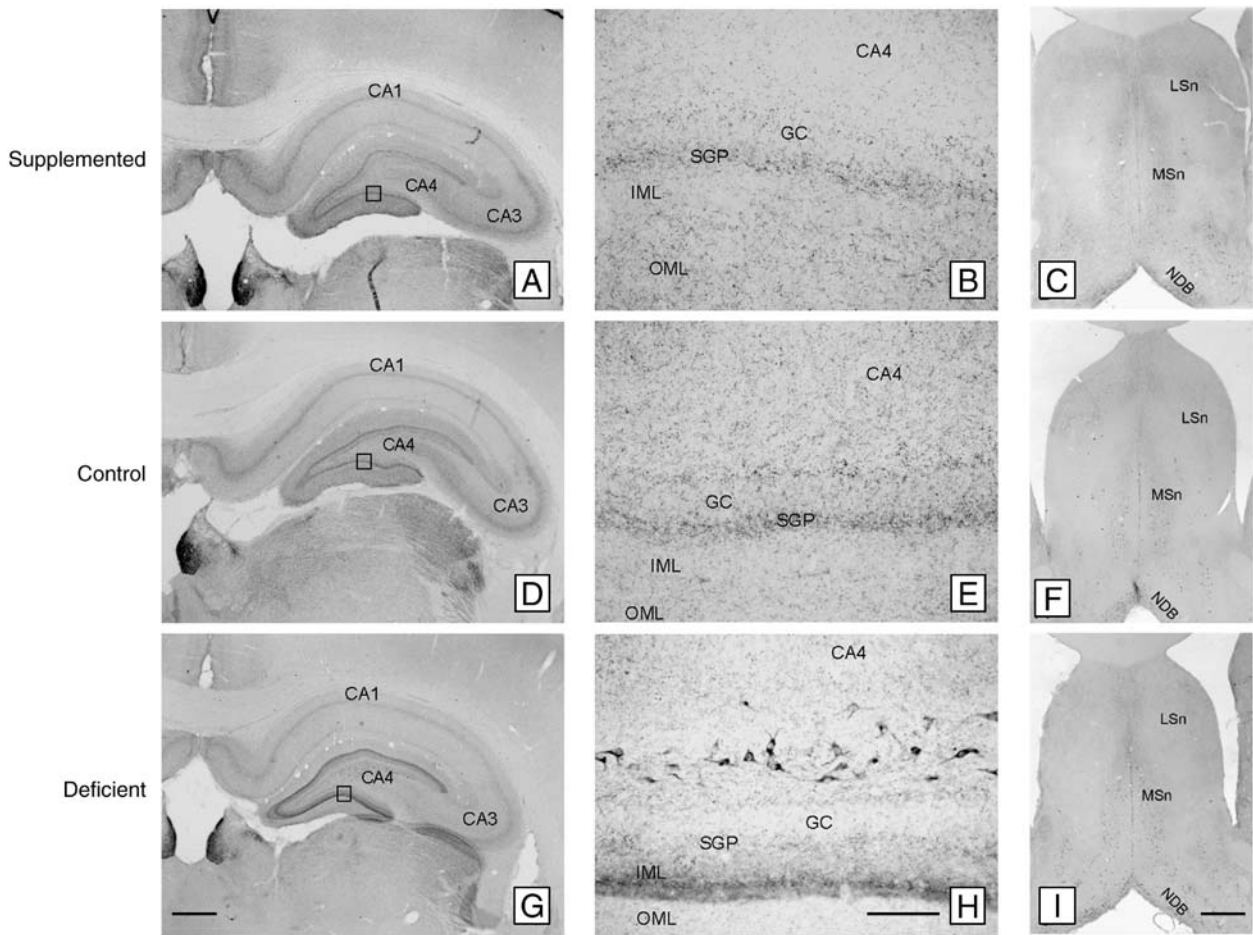
Fig. 6 – CHT immunocytochemistry in the hippocampus and medial septum of P72 rats. Prenatally choline-supplemented (A, B, C), control (D, E, F), and deficient (G, H, I) animals. GC, granular cell layer; SGP, supragranular plexus; IML, inner molecular layer; OML, outer molecular layer; MSn, medial septal nucleus; LSn, Lateral septal nucleus; NDB, nucleus of the diagonal band. The pattern of CHT immunoreactivity seen at P18 persisted at P72. CHT immunoreactivity was more prominent in the inner molecular layer of the dentate gyrus and CHT-immunoreactive neurons were generally more prominent in CA4 in the deficient group (G, H) compared to the supplemented (A, B) and control (D, E) groups despite similar staining intensities in other brain regions such as the medial septal nucleus (C, F, I). The prominence of inner molecular layer staining generally paralleled the number CHT-immunoreactive hilar neurons even in the face of case to case variability (B, E, H). Magnification bars: A, D, G—500 μm; B, E, H—50 μm; C, F, I—500 μm.

supplemented rats had the highest expression of CaMKI and insulin-like growth factor II in the cortex and of the transcription factor Zif268/EGR1 in the cortex and hippocampus.

Upregulation of CHT expression also occurs under conditions that can be collectively described as "hypocholinergic." Mice heterozygous for a mutation in the gene encoding the ACh-synthesizing enzyme, choline acetyltransferase (ChAT), have 1.5- to 2-fold increase in CHT mRNA and protein expression, as well as activity, as compared to the wild-type animals (Brandon et al., 2004). This upregulation of CHT contributes to the apparently normal behavioral phenotype and ACh turnover in the ChAT heterozygotes. Similarly, overexpression of the ACh-hydrolyzing enzyme, acetylcholinesterase (AChE), in mice, doubled the striatal binding of a CHT-specific ligand, hemicholinium-3 (Beeri et al., 1997). Perhaps surprisingly, AChE knockout mice had increased CHT protein expression in the striatum (Volpicelli-Daley et al., 2003). It is possible that in the latter case ACh levels are

high at the expense of free choline and that this relative choline deficiency results in high CHT expression. CHT-null mice die shortly after birth due to apnea caused by the deficit in the cholinergic neurotransmission at the neuromuscular junctions of the respiratory muscles (Ferguson et al., 2004). CHT heterozygous mice are normal in multiple behavioral tasks (Bazalakova et al., 2006). However, these animals have a spontaneous phenotype, i.e., they show more rearing behavior in the open field than the wild-type littermates (Bazalakova et al., 2006). They also display early fatigue on a treadmill (Bazalakova et al., 2006) suggesting that a single CHT allele is insufficient to support normal physical performance. In addition, CHT heterozygotes have reduced levels of hippocampal, striatal, and cerebral cortical ACh concomitant with elevations in the free choline concentrations (Bazalakova et al., 2006). The latter observations are consistent with the model of Farber et al. who estimated that even though cholinergic neurons in the striatum constitute only 1% of all
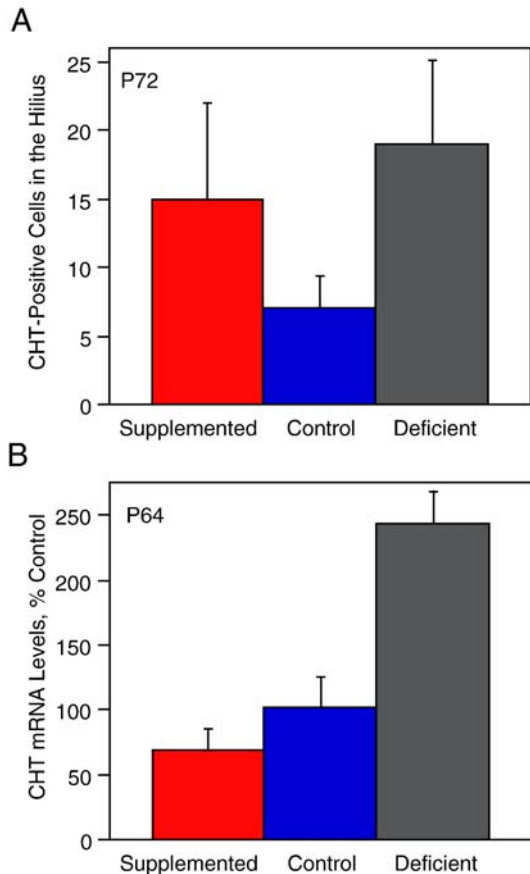
Fig. 7 – CHT-positive cells in the hilus at P72. (A) CHT-positive neuronal perikarya in the CA4 regions of the hippocampal formation of P72 animals were quantitated and analyzed using a semi-automated computerized morphometry system. There were no significant differences between the groups. (B) Hippocampal RNA from P64 rats was used for RT-PCR of CHT and β-actin. CHT levels were normalized using β-actin levels and are presented as means±SEM ($n=6$ per group). The levels of CHT were significantly different among the three groups of animals as determined by ANOVA ($p<0.0005$). The ANOVA was followed up with Tukey tests at a 5% procedure wise error rate. Prenatally choline-deficient animals had a significantly higher amount of CHT mRNA compared to prenatally choline-supplemented and control animals.

cells, it is the activity of CHT expressed by these neurons that accounts for 60% of the total choline uptake in this tissue (Farber et al., 1996). Similar conclusions have been reached by Parikh and Sarter (2006) based on *in vivo* studies. Thus, when CHT activity is reduced, such as that occurring in the CHT heterozygotes, ACh levels drop and, the presumably extracellular, brain free choline levels are allowed to rise (Bazalakova et al., 2006).

The current public databases list 96 single nucleotide polymorphisms (SNPs) within the human *CHT* gene (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=60482&chooseRs=all). Only one of those *CHT* alleles, occurring with an approximately 6% frequency among Ashkenazi Jews and North American

Whites (Bazalakova et al., 2006; Okuda et al., 2002), produces an amino acid substitution in the CHT protein (I89V). This results in a 40–50% reduced velocity of choline transport as compared to the wild type, when measured in a cell culture expression system, although no phenotype has been reported to date. Multiple additional SNPs are found near the promoter regions and in the 3′ untranslated region of the CHT mRNA. Given the highly plastic regulation of CHT expression, its subcellular distribution (Ferguson and Blakely, 2004), and posttranslational regulation (Gates et al., 2004) it is possible that these polymorphisms may be associated with subtle phenotypes in the human population.

In addition to describing modulation of brain CHT expression by prenatal nutrition, our data provide new information on the intrinsic hippocampal CHT-immunoreactive neurons. Consistent with the studies of Misawa et al. (2001), we find CHT-positive hilar neurons in the rat. However, when we counted these neurons at the level of the habenular nucleus, their number varied greatly among the animals, from very few to over 100 cells (Fig. 5). Given a remarkably high correlation between the number of these neurons observed with immunohistochemical techniques and the levels of CHT mRNA measured in the contralateral hippocampus, the data suggest that these CHT-immunoreactive neurons are the most likely source of the hippocampal CHT mRNA. Moreover, the current data confirm our previous studies in mice showing a decline in the hippocampal abundance of CHT mRNA during the early postnatal period (Berse et al., 2005). It is not clear at present if these hilar CHT-positive cells also express ChAT, and are thus *bona fide*, cholinergic. Several (Kanaya-Ida and Ben Ari, 1989; Levey et al., 1984; Matthews et al., 1987; Wainer et al., 1985), but not all (Blaker et al., 1988; Ichikawa et al., 1997), investigators have previously observed ChAT-positive neurons in the rat hilus. These, possibly cholinergic (Kanaya-Ida and Ben Ari, 1989; Levey et al., 1984; Matthews et al., 1987; Wainer et al., 1985), and CHT-immunoreactive hilar neurons (polymorphic layer of the dentate gyrus) could also contribute local projections in the dentate gyrus and thus be the source of the increased CHT immunoreactivity observed in the inner molecular layer of the prenatally choline-deficient rats. The overall lower number of the hilar CHT-positive cells identified at P72 (Fig. 7) as compared to P18 (Fig. 5) roughly parallels the reduction in CHT mRNA that normally occurs during development as shown in Fig. 3. The lower cell counts at P72 could either reflect cell death or reduce *CHT* gene expression in cells that do not die. It is possible that choline supplementation interferes with this normal developmental pattern and results in persistent expression of CHT or survival of CHT-positive neurons.

The mechanism of the long-term regulation of CHT expression by prenatal supply of choline remains to be elucidated. One possibility is that it is related to the function of choline as a donor of methyl groups. The availability of the metabolic methyl groups is known to affect DNA methylation in a cell (see below) and, because the DNA methylation patterns are passed on to cell progeny following mitosis, the patterns established in embryogenesis may persist throughout lifetime. Choline is a source of methyl groups for enzymatic methylations (Blusztajn, 1998; Zeisel, 2006) because its metabolite betaine is used to synthesize methionine and

subsequently S-adenosylmethionine—a methyl group donor for most biological methylation reactions including the methylation of cytidines in CpG dinucleotide sequences of DNA (Waterland, 2006). This cytidine methylation of CpG-rich sequences near promoters of multiple genes can modulate transcription. In general hypermethylated DNA binds transcriptional repressors whereas hypomethylation stimulates transcription by preventing this binding (Li, 2002; Paulsen and Ferguson-Smith, 2001; Reinhart et al., 2002; Robertson and Wolffe, 2000; Tucker, 2001). Thus, low availability of choline during the time of neurogenesis of the basal forebrain cholinergic neurons (E13–E19 in the rat, Koh and Loy, 1989) could lead to hypomethylation of regulatory regions within the CHT gene causing its long-term overexpression. Analysis of the rat CHT gene reveals the presence of a CpG island (chr9:133805-134277; UCSC Genome Browser http://genome.ucsc.edu/) near its promoter, consistent with this model. Indeed, animals fed diets deficient in methyl donors (choline and methionine) had hypomethylated DNA (Locker et al., 1986; Niculescu et al., 2006; Tsujiuchi et al., 1999; Wainfan et al., 1989) and in choline-deficient mouse fetuses, global and gene-specific DNA methylation was decreased in the ventricular and subventricular zones of hippocampal Ammon's horn and this correlated with the induction of expression of Cdkn3, a gene known to be silenced by DNA methylation (Niculescu et al., 2006). Together with previous findings, the current data show that the availability of an essential nutrient, choline, in utero causes changes in its own turnover in the hippocampus later in life. In prenatally choline-deficient animals, this results in efficient recycling of choline for ACh synthesis catalyzed by upregulated expression of CHT (Cermak et al., 1998). Prenatally choline-supplemented rats, instead, exhibit greater reliance on phosphatidylcholine-stored choline for ACh synthesis (Cermak et al., 1998). These adaptations seem appropriate for the periods when choline availability is altered (i.e., prenatally), however, they are long-lasting, i.e., they are observed at a time when all animals consume the control diet, months after the termination of treatment. Therefore, elevated dietary choline supply in adult animals that were prenatally choline-deficient could result in increases of ACh synthesis, whereas it might have a lesser effect in prenatally choline-supplemented animals. Conversely, choline deficiency in adults that were prenatally choline-deficient might be well tolerated due to upregulation of CHT. Thus, cholinergic neurotransmission may be differentially sensitive to the availability of choline in adulthood, based on its supply in utero.

4. Experimental procedures

4.1. Animal subjects

Pregnant Sprague–Dawley rats (Charles River Laboratories) were divided into three groups: choline-deficient, control, and choline-supplemented. During days 11–17 of pregnancy, the rats were fed an AIN76A diet (Bieri, 1980; Bieri et al., 1977) (Dyets Inc., Bethlehem, PA) that contained no choline (deficient), 7.9 mmol/kg of choline (control), or 35.6 mmol/kg of choline (supplemented). Following embryonic day 17, all rats were returned to a control AIN76A diet. Offspring also consumed a control diet once weaned at P25.

For immunohistochemical studies male subjects were used. For all other studies equal number of males and females per dietary group was used. Four to six individual offspring (each from a different litter) were used per group. At various postnatal ages, offspring were anesthetized and the septum and hippocampus were removed rapidly on ice. For protein, the tissue was immediately frozen on dry ice and stored until later use. For RNA, the brain tissues were immediately homogenized in cold guanidine thiocynate solution and placed on dry ice. First, the RNA was extracted using phenol/chloroform method and then precipitated. The RNA pellet was resuspended in RNase-free water. The quantity of RNA was determined using Quant-iT™ RiboGreen® RNA assay kit (Molecular Probes) and the Victor^3 multi-label plate reader (Perkin Elmer Life Sciences).

4.2. Reverse transcriptase PCR

RNAs were used for reverse transcriptase PCR using Superscript™ One-Step RT-PCR with Platinum® Taq (Invitrogen Life Technologies). First strand cDNA synthesis was performed with 25 ng of total RNA (except 50 ng of hippocampal RNA for CHT), oligo dT primer and reverse transcriptase at 48 °C (45 min). Primers used for PCR include β-actin (Forward: CACAGCTGAGAGGGAAATC, Reverse: TCAGCAATGCCTGGG-TAC), and CHT (Forward: CGGGGAACCATTGAATTCGTTGAA-GTCTAC, Reverse: GGGGCAAGCTTCCACTTTCAA ATAGATACT). PCR was performed using Platinum Taq DNA polymerase with a denaturing step for 2 min at 94 °C, followed by 32 cycles for β-actin or 36 cycles for CHT of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, and terminated by an elongation step at 72 °C for 7 min. PCR products were displayed on a 10% polyacrylamide gel and stained with ethidium bromide. PCR products were visualized with Kodak Image Station 440 and product intensities were quantified using Kodak software.

4.3. Western blot analysis

For Western blot analysis, whole tissue extracts were prepared by adding lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet NP-40, 10% glycerol, 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mg/ml leupeptin, 2 mg/ml aprotinin, 2 mg/ml pepstatin) to a frozen sample of hippocampal tissue, gently sonicating, incubating for 15 min on ice, and briefly centrifuging to clear. The extracts were normalized for total protein and subjected to SDS-PAGE. After transfer of protein to an Immobilon P membrane (Millipore), the membrane was blocked with 5% nonfat dry milk in 1× Tris-buffered saline (TBS) containing 0.1% Tween 20 for 1 h and then probed with affinity-purified rabbit anti-CHT polyclonal antibody AB5966 (1:1000) (Chemicon) overnight. The antibody/antigen complexes on the membranes were detected using a peroxidase-conjugated anti-rabbit IgG (1:5000) and visualized using the enhanced chemiluminescence method (Western Lightning, Perkin Elmer) and a Kodak Image Station 440. Digitized images of immunoblots were quantified using Kodak ID software.

4.4. Immunocytochemistry

For 18-day-old male rats, six animals per dietary group were anesthetized. The brain was rapidly removed and bisected through the midsagittal plane. The left hemibrain was dissected for neurochemistry and the right was submerged in phosphate-buffered PLP fixative (4% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate; pH 7.4) for immunocytochemistry. Brains were post-fixed for 24 h in PLP at 4 °C, then cryoprotected in a graded series of 10% and 20% glycerol/ 2% dimethylsulfoxide, in 0.1 M PBS, pH 7.3. For 72-day-old male rats, groups of four from each of the three dietary groups were deeply anesthetized with pentobarbital and euthanized by transcardial perfusion with 150 ml of normal saline, followed by 300 ml of PLP. Brains were removed, post-fixed for 4 h in PLP at 4 °C, and then cryoprotected as described above.

Brains were serially sectioned at 50 μm, using a freezing microtome. Every fifth section was stained with affinity-purified rabbit anti-CHT polyclonal antibody AB5966 (1:1000) (Chemicon) for P72 rats or anti-CHT polyclonal antibody (1:5000) (generous gift from Dr. Blakely) (Ferguson et al., 2003) for P18 rats. Standard immunohistochemical procedures were used as previously reported (McKeon-O'Malley et al., 2003).

CHT-positive neuronal perikarya in the CA4 regions of the hippocampal formation of P18 and P72 animals were quantitated and analyzed using a semi-automated computerized morphometry system (MicroBrightField, Colchester, VT, Neurolucida™ and StereoInvestigator™, Burlington, VT). All CHT-immunoreactive profiles in the CA4 were mapped in 3–4 step sections spaced at 250-μm intervals at the level of the habenular nucleus and confirmed by visual inspection.

4.5. Statistical analyses

Data were analyzed by analysis of variance (ANOVA). If significant effects were found data were further analyzed by Tukey multiple comparison test to determine individual group differences. Analyses were performed with the statistical program SYSTAT (SYSTAT Software Inc., San Jose, CA).

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