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Short communication

Prenatal choline deficiency decreases the cross-sectional area of cholinergic neurons in the medial septal nucleus

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Abstract

Levels of dietary choline in utero influence postnatal cognitive performance. To better understand this phenomenon, forebrain cholinergic neurons were studied in the 8–9 month old offspring of dams fed a control or choline-deficient diet from EDs 11–17. Serial sections were immunostained with antibodies against p75, a cholinergic marker. Neuronal morphology was analyzed in the basal forebrain, a heterogeneous area composed of several structures including the medial septal nucleus (MSN), nucleus of the diagonal band (DB), and the nucleus basalis of Meynert (NB). Neuronal cross-sectional areas were selectively reduced in the MSN of choline-deficient animals, compared to controls, but cell counts were not altered. Our findings suggest that cholinergic medial septal neurons may be selectively vulnerable to in utero choline deficiency.

Theme: Development and regeneration

Topic: Nutritional and prenatal factors

Keywords: Medial septal nucleus; p75 Neurotrophin receptor; Choline; Neuronal area

Behavioral [1–3], biochemical [4,5], and electrophysiological evidence [6–8] shows that choline availability during gestation modifies brain development. The availability of choline to pregnant dams influences the cognitive abilities of adult offspring when it is administered during embryonic days (EDs) 11–17 [1,9]. Choline supplementation during this period causes long-lasting facilitation of spatial [2,10,11] and temporal memory [11,12], and increases the size of p75 neurotrophin receptor-immunoreactive neurons in the MSN/DB [3]. Conversely, choline deficiency during the sensitive prenatal period alters neuronal survival in hippocampal regions of the fetus [13–17], decreases the ability of hippocampal CA1 neurons of young and aging rats to exhibit LTP [6], and causes specific impairments in memory and attention of rats and mice [11,18].

In the present study, we examined the morphology of BF cholinergic neurons in the adult offspring of dams treated with a choline-deficient diet. The results of behavioral studies utilizing these animals have been reported elsewhere [1,9]. Specifically, we examined the effect of choline deprivation during EDs 11–17 on the cross-sectional area of neuronal cell bodies in the rat MSN, DB and NB, and then on cell number in the MSN. This experiment

Abbreviations: AD, Alzheimer's disease; BF, basal forebrain; DB, diagonal band; ED, embryonic day; LTP, long-term potentiation; MSN, medial septal nucleus; NB, nucleus basalis of Meynert; PBS, phosphate-buffered saline; PD, postnatal day; p75, low-affinity neurotrophin receptor; TrkA, high-affinity nerve growth factor receptor

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was designed to test the hypothesis that choline deficiency could alter the neuroanatomy of cholinergic neurons. The possibility of such a change seemed plausible to us, because choline is required for many cellular processes, including integrity of cellular membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signaling and lipid transport/metabolism [19]. We chose to examine the effect of choline deficiency in the cholinergic neurons of the BF because many of these neurons have particularly prominent projections to the hippocampus, an area essential for learning and memory [20]. Furthermore, BF cholinergic neurons are selectively vulnerable to cell death in Alzheimer's disease [21]. Neurons of the MSN project widely throughout the telencephalon via septohippocampal, septo-bulbar and septo-cortical pathways. In particular, these neurons terminate in the subiculum, entorhinal cortex, the CA1 and CA2 fields of the hippocampus proper, and the polymorphic and molecular layers of the dentate gyrus [22,23].

Dams were fed a choline-deficient or control diet during EDs 11–17. After 8–9 months, the male offspring of these dams were sacrificed, and cholinergic neurons in the BF were identified immunocytochemically using a polyclonal antibody to the p75 neurotrophin receptor, a well-established marker of forebrain cholinergic neurons [24].

Timed-pregnant Sprague–Dawley CD-strain rats were obtained from Charles River Laboratories (Kingston, NY, USA) at 9 days of gestation. Dams were housed individually in clear polycarbonate cages (27.9×27.9×17.8 cm^2), and were subjected to a 12-h light/dark cycle, with lights on at 7 a.m. All dams were fed an AIN-76A purified synthetic diet containing 1.1 g/kg choline chloride (Dyets, Bethlehem, PA, USA) and water ad libitum until the evening of gestational day 11. At this time, one half of the dams remained on this diet (Control) and one half was switched to a modified AIN76A diet that contained no choline (Deficient). The manipulation of dietary choline was terminated on the morning of ED 18, at which time all dams were given the Control diet.

Deficient and Control groups did not differ significantly with regard to the amount of diet or water consumed during the treatment period. At birth, offspring from Deficient and Control dams were cross-fostered in mixed-treatment litters of 10 pups, to dams that had been fed a Control diet. The use of untreated dams as foster mothers prevented the introduction of a confounder factor, due to the lasting effects (theoretical) of choline deficiency on dams' milk composition or behavior. Pups were weaned at 24 days postnatal and were housed five per cage from PD 30–PD 45, and two per cage thereafter. Weaned offspring were fed the Control diet and permitted to drink water ad libitum until they were sacrificed at 259–274 days of age. During their lifespan, rats in both treatment groups were tested in a variety of non-spatial, behavioral tasks (not reported here).

Because the rat septohippocampal system exhibits sexual dimorphism with regard to cholinergic enzymes [25], and prenatal choline supplementation appears to have a smaller effect on memory in female rats compared to males [3], only male offspring were used in this study. Adult male rats were anesthetized deeply with pentobarbital to minimize pain or discomfort, in accordance with NIH publication No. 80-23. Brain tissue was fixed in vivo by transcardial perfusion with 150 ml of normal saline, followed by 300 ml of buffered 4% paraformaldehyde. Brains were removed carefully, post-fixed for 4 h in 4% paraformaldehyde at 4 °C, then cryoprotected in a graded series of 10 and 20% glycerol–2% dimethylsulfoxide, in 0.1 M PBS, pH 7.3.

Forebrains were serially sectioned at a thickness of 50 μm, using a freezing microtome. In all cases, an adjacent section was stained for Nissl substance (cresyl violet) to identify the cytoarchitectonic boundaries within the BF. Every third section was stained for p75 neurotrophin receptor and an adjacent section was stained with a Nissl stain (see Fig. 1). Standard immunohistochemical procedures were used as previously reported [26]. Sections were stained using an anti-p75 neurotrophin receptor antibody (catalog No. AB1554, Chemicon, Temecula, CA, USA). The neuronal cross-sectional area measurements were made in coronal sections using Optimas™ (MediaCybernetics) image analysis software. Nuclear boundaries of the p75-stained sections were confirmed with adjacent Nissl-stained sections and the rat stereotaxic atlas of Paxinos and Watson [34]. The boundary between the MSN and the vertical limb of the DB was distinguished by a line connecting the anterior commissures (Fig. 1A). Technicians blinded to the treatments performed all quantitative morphometry.

Neuronal cross-sectional area measurements: Cross-sectional area measurements were made in the brains of 15 animals in the Control group and 15 in the Deficient group. Calibrated areal measurements were made in the MSN, DB and NB in all animals. Measurements were made by video microscopy of six randomly selected sections from each animal. As many as 100 individual neurons were measured in the MSN, DB and NB of each animal. A p75-immunoreactive neuron was included for measurement when a nuclear profile could be observed (see Fig. 1B). Neuronal profiles were defined by the maximum diameter of the soma and by a straight line across the base of the axon hillock (see Fig. 1A, inset). To minimize variability in the neuronal population selected for inclusion, all neuronal tracings were performed by a single operator. As the operator traced soma profiles on a video image, the software marked each profile, preventing more than one measurement of a single profile. The field of view was moved manually in a systematic 'left to right, down, and right to left' pattern until either 100 soma measurements were made, or until all sections had been scanned. Once a section had been scanned and the profiles traced, the software calculated the area of traced profiles in square

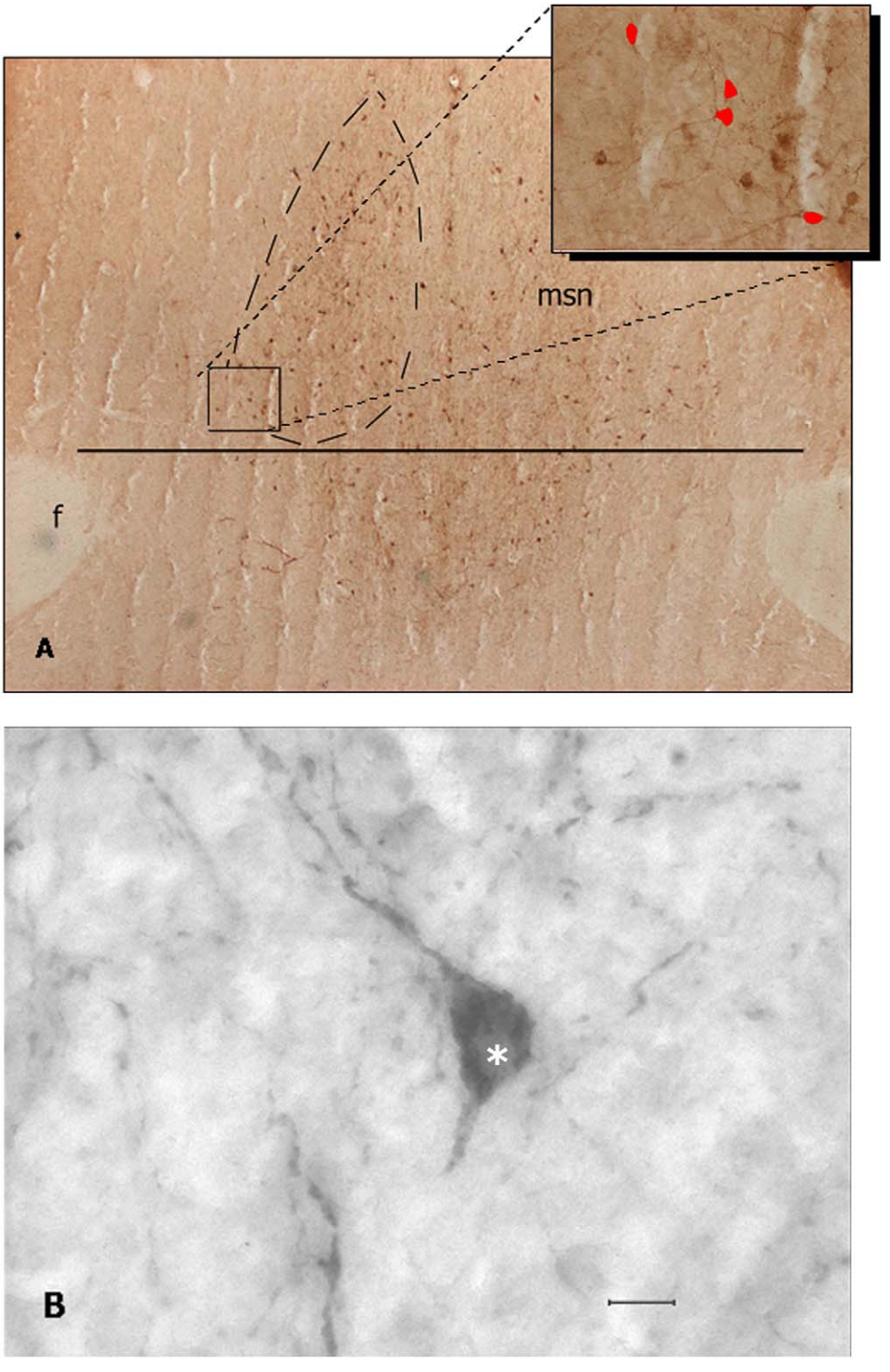

Fig. 1. (A) Photomicrograph of the medial septal area in the forebrain of the rat. The boundaries of the medial septal nucleus are indicated by the dashed line (MSN). The horizontal line indicates the plane between the medial septal nucleus above and the vertical limb of the diagonal band below. The fornix is indicated by the letter f. (A, inset) The inset shows the area indicated by the square at higher magnification and also shows the appearance of a microscopic field after the cellular outline has been traced and counted by the software (red profiles). (B) Black and white photomicrograph showing an example of a p75 neurotrophin receptor-immunoreactive neuron in the medial septal nucleus. The ghost profile of the nucleus can be seen (asterisk). Scale bar=10 μm.

microns and exported the data to a Microsoft Excel spreadsheet for subsequent analysis. Approximately 1,200 neuronal profiles were measured in each basal forebrain structure (MSN, DB, NB) from each treatment group (the Control and Deficient groups). The mean neuronal cross-sectional areas per animal were calculated, and then those

values were averaged to produce a mean cross-sectional area for each group of 15 animals. The mean neuronal areas in each basal forebrain structure were compared in Control and Deficient rats by two-sample *t*-test. SigmaPlot 2002 (SPSS, Chicago, IL, USA) and Prophet Vr. 5.0 (NCRR/NIH) were used for statistical analyses. In addition, frequency distribution histograms of average neuronal cross-sectional areas were generated for each treatment group to assess the nature of any changes that might be detected.

Neuronal cell counts: Unbiased estimates of the numbers of p75-immunoreactive neurons were obtained from sections corresponding to 15 animals in the Control group, and 15 animals in the Deficient group. Systematic random sampling methods were employed using the Optical Disector method [27], in conjunction with Neurolucida Stereo Investigator software (MicroBrightfield Inc., Colchester, VT, USA). The latter software produces counting grids as a video overlay on the section image when viewed through a microscope. Once neurons in an individual counting brick are tallied, the software automatically moves the section to the next counting location.

Cross-sectional area analyses showed that, in comparison to the Control group, the Deficient group exhibited a 9% reduction in the average cross-sectional area of p75-immunoreactive neurons in the medial septal nucleus ($P=0.016$) (see Fig. 2). Prenatal choline deficiency did not change the average cross-sectional areas of p75-immunoreactive neurons in the DB or NB. In the MSN, mean cross-sectional areas ranged from 139 to 186 μm^2 for the Control group, and from 124 to 185 μm^2 for the Deficient group. Frequency distribution histograms (Fig. 3) of the average neuronal cross-sectional area were constructed for the each of the two treatment groups. In comparison to the Control group, the mean neuronal cross-sectional areas

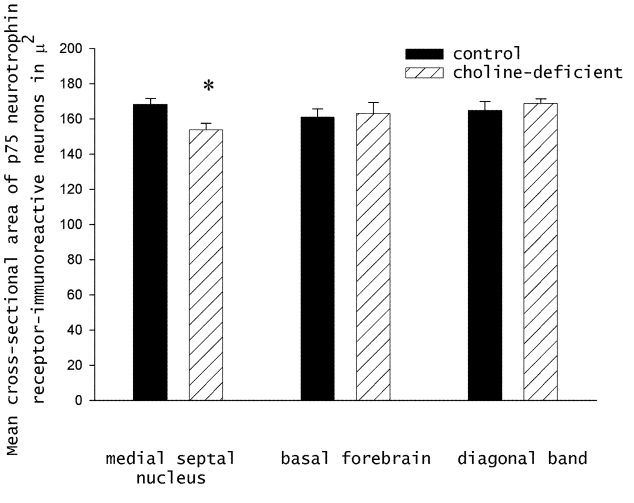

Fig. 2. Mean cross-sectional area of p75 neurotrophin receptor-immunoreactive neurons in the adult offspring of dams fed a control or choline-deficient diet during embryonic days 11–17. Brain regions examined: MSN, NB and DB. Error bars represent the standard error.

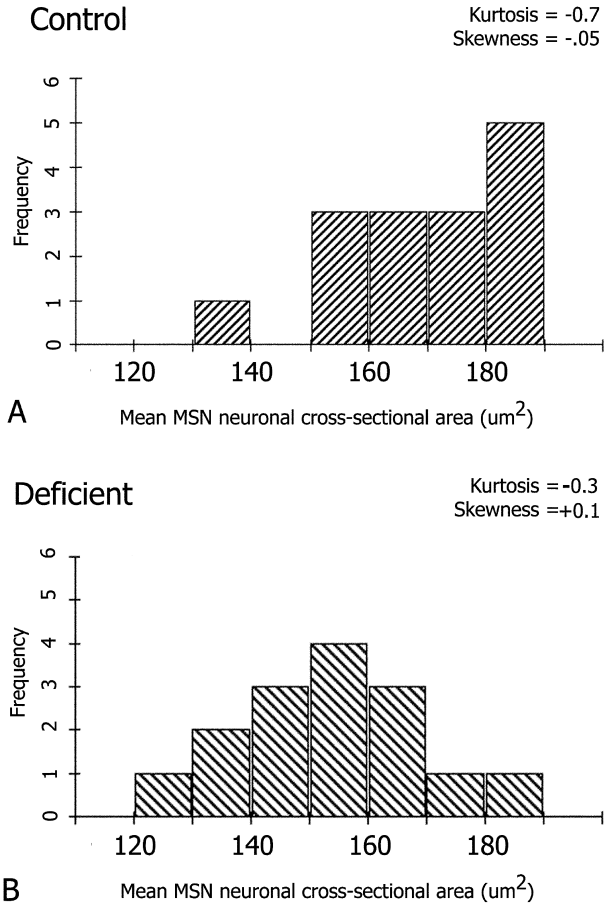

Fig. 3. Frequency distribution histograms of the mean cross-sectional area of medial septal neurons in 15 Control animals (A) and 15 Deficient animals (B). In each graph, kurtosis and skewness values are provided. See text for additional details.

were shifted downward in the Deficient group (Deficient group mean=153.6 μm^2, Control group mean=168.1 μm^2), and the neuronal population was characterized by a tighter and more symmetrical distribution about the mean (skewness=+0.1). Both frequency distribution histograms show a negative kurtosis, but kurtosis was roughly halved in the Deficient group in comparison to the Control group (Deficient group kurtosis=−0.3, Control group kurtosis=−0.7). Fig. 3 suggests that prenatal choline deficiency may cause an overall shift to a smaller neuronal size, and may normalize the distribution of cross-sectional area measurements for neuronal soma.

Stereological methods were also used to assess whether choline deprivation caused a change in the number of MSN neurons. In the MSN, the number of p75-immunoreactive neurons did not vary significantly between the Deficient and Control groups (see Fig. 3).

Although choline serves as a substrate for many biological molecules, most of this nutrient is converted into phospholipids for use in membrane synthesis [19]. Therefore, one might expect choline deprivation to cause a reduction in phospholipid synthesis, and conceivably, such

a reduction could manifest itself as a generalized effect across multiple brain regions. However, we saw a specific effect of choline deficiency in the medial septal nucleus. The mechanism by which choline deprivation causes decreased neuronal size is unknown; our finding may be the result of a primary developmental failure in the MSN or a secondary failure in a *target* of the MSN such as the dentate gyrus.

The behavioral consequences of choline deficiency may or may not be attributable to decreased neuronal size. However, there is some evidence to suggest that larger neuronal size is associated with improved memory function. Perinatal choline supplementation, which has been shown to enhance the performance of rats on a 12-arm radial maze, increases the size of p75 neurotrophin receptor-immunoreactive neurons in the MSN/DB by 8–15% [3]. In CA1 pyramidal neurons of the hippocampus, choline supplementation increases both soma size and dendritic branching [28]. Conversely, we observed that the size of p75 neurotrophin receptor-immunoreactive neurons is decreased in choline-deficient rats, which show deficits in tasks of sustained attention and temporal processing relative to controls [11,18]. Decreased neuronal size is also associated with other conditions characterized by cognitive deficits, including AD [29] and docosahexaenoic acid deficiency [30–33].

In conclusion, we have identified a possible anatomical correlate of choline deficiency in the rat, namely a decrease in the size of p75 neurotrophin receptor-immunoreactive MSN neurons. These observations are significant since they were made using the brains of animals that had undergone behavioral testing showing specific impairments in memory and attention of rats and mice [11,18]. Our findings suggest that during a sensitive period of fetal development, decreased choline availability causes long-lasting changes in the morphology of cholinergic neurons in the medial septal nucleus. It remains to be determined whether changes in neuronal soma size are specifically related to, or causal of, the behavioral deficits seen after a similar period of choline deficiency.

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