Dietary Choline Supplementation Improves Behavioral, Histological, and Neurochemical Outcomes in a Rat Model of Traumatic Brain Injury

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Abstract

Novel pharmacological approaches that safely and effectively lessen the degree of neurological impairment following traumatic brain injury (TBI) are sorely needed. Non-invasive approaches that could be used over an extended periods of time might be particularly useful. Previous studies from our lab have hypothesized that TBI-induced decreases in hippocampal and cortical $\alpha 7$ neuronal nicotinic cholinergic receptor (nAChR) expression might contribute to cognitive impairment that follows brain injury. The purpose of this study was to determine whether the low-potency, but selective $\alpha 7$ nAChR agonist choline might be a useful treatment for improvement of neurological outcome in a rat model of TBI. Male Sprague-Dawley rats were exposed to control or choline-supplemented diets for 2 weeks prior to experimental brain injury (1.5-mm cortical contusion injury) and throughout the recovery phase. Dietary choline supplementation resulted in a modest degree of improvement in spatial memory as assessed in the Morris water maze test. In addition, choline treatment resulted in significant cortical tissue sparing, reduced brain inflammation, and normalized some TBI-induced deficits in nAChR expression. The results of this study suggest that $\alpha 7$ nAChR agonists may be useful drugs to enhance recovery following brain injury.

Key words: acetylcholine; alpha 7 nAChR; bungarotoxin; dietary supplementation; Morris water maze; neuroprotection; nicotinic

Introduction

TRAUMATIC BRAIN INJURY (TBI) affects approximately 1.4 million people in the United States each year, and survivors of TBI often experience long-lasting physical and neuropsychiatric impairments. Prior studies have suggested that TBI-related cognitive impairments are perhaps the most detrimental to the quality of life in many long-term survivors of TBI (Thurman et al., 1999; Arciniegas, 2003). Cognitive problems observed following TBI include deficits in arousal/attention, anterograde/retrograde amnesia, and changes in executive function. Unfortunately, the pathophysiology of TBI-related cognitive impairment is not completely understood and not usually clinically treated (Starkstein and Jorge, 2005). TBI-related dysfunction of multiple brain neurotransmitter systems (e.g., glutamate, norepinephrine, acetylcholine) may contribute significantly to cognitive dysfunction following brain injury (McIntosh et al.,

1996; Gaetz, 2004). A transient period of excess cholinergic activity occurs immediately following brain injury, and could contribute to excitotoxicity via effects mediated by nicotinic and muscarinic receptor subtypes (Saija et al., 1988; Dixon et al., 1995). On the other hand, the chronic phase of TBI is associated with a significant decline in brain cholinergic function (Murdoch et al., 1998; Arciniegas, 2003). Animal studies have found that TBI results in reduced basal and evoked release of ACh, decreased choline acetyltransferase activity, and a reduction in the number of high affinity choline uptake sites (Dixon et al., 1994, 1996; Leonard et al., 1994).

Acetylcholine acts on nicotinic and muscarinic acetylcholine receptors, both of which are prominently located in brain regions that are involved with arousal, attention, and cognitive processing. Studies from our lab have postulated that TBI-related deficits in $\alpha 7$ nAChR density may contribute to post-TBI cognitive deficits (Verbois et al., 2003). Al-

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terations in the density or function of nAChRs has also been implicated in pathophysiology of several neurodegenerative conditions that adversely affect cognition, including Alzheimer's disease and Parkinson's disease (Rinne et al., 1991; Guan et al., 2000). Decreased hippocampal $\alpha 7$ nAChR expression has also been found in post mortem tissue obtained from schizophrenic patients (Freedman et al., 1994; Guan et al., 1999). In schizophrenia, a loss of nAChRs may be related to sensory gating deficits, which contribute to impairments in attention that are typical of this patient population (Freedman et al., 2000). If down-regulation of $\alpha 7$ nAChRs contributes to TBI-related cognitive impairment, drugs that are selective $\alpha 7$ agonists may be helpful in ameliorating some measures of cognitive decline.

Choline is an essential nutrient available from a wide variety of nutritional sources with some limited *de novo* synthesis. Choline is important for the synthesis of structural cell membrane phospholipids, other signaling molecules, and a precursor for acetylcholine synthesis (Blusztajn, 1998; Zeisel and Niculescu, 2006). Recent electrophysiological studies have shown that choline is also a full agonist at $\alpha 7$ nAChRs, but not other nicotinic receptor subtypes. Choline-evoked electrophysiological responses from single cells or hippocampal slices are blocked by either methyllycaconitine or α bungarotoxin (BTX), selective $\alpha 7$ antagonists (Alkondon et al., 1997; Albuquerque et al., 1998; Fayuk and Yakel, 2004). Dietary supplementation with choline results in selective increases the density of $\alpha 7$ nicotinic receptors in multiple brain regions, consistent with the actions of a nicotinic cholinergic agonist (Morley et al., 1977; Coutcher et al., 1992; Guseva et al., 2006).

Inflammation is an important participant in secondary damage produced following TBI (Bramlett and Dietrich, 2004; Bazan et al., 2005). Microglia are essential regulators of the central nervous system (CNS) inflammatory response following TBI, and may contribute to secondary tissue damage via both direct and indirect mechanisms. Activated microglia produce cytokines, reactive oxygen species, proteinases, complement proteins and other mediators that contribute to downstream brain damage following TBI (Block and Hong, 2005; Town et al., 2005). Emerging evidence suggests that the $\alpha 7$ nAChR may be important regulator of inflammation in the periphery as well as the central nervous system. Nicotine has been shown to inhibit lipopolysaccharide (LPS)-induced microglial activation via an $\alpha 7$ nAChR specific pathway (Shytle et al., 2004). The isoquinoline carboxamide [3 H]-PK11195 has been shown to bind with high affinity to the peripheral benzodiazepine receptor (PBR). Under normal conditions, CNS binding of [3 H]-PK11195 is restricted to PBRs located exclusively on ependymal cells of the choroid plexus. However, following neurotoxic brain injury, time-specific and brain region-specific increases in [3 H]-PK11195 binding are thought to reflect reactive gliosis involving activated microglia expressing the PBR, possibly as a component of the mitochondrial permeability transition pore (Park et al., 1996; Banati et al., 1997; Casellas et al., 2002). In addition, increased PK11195 binding is observed in other conditions associated with microglial activation, including brain ischemia (Myers et al., 1991), manganese toxicity (Hazell et al., 1999), dopamine neurotoxin exposure (Kuhlmann and Guilarte, 1999), and thiamine deficiency (Casellas et al., 2002). Thus, the purpose of the present study was to test the

hypothesis that dietary choline supplementation would attenuate TBI-induced cognitive deficits, spare cortical tissue and reduce brain inflammation by attenuating microglial activation following injury.

Methods

Animals, Diets, and Surgeries

The study utilized 26 adolescent male Sprague-Dawley rats (Harlan Breeding Laboratories, Indianapolis, IN) that were 28 days old at the start of the study. Rats were housed two per cage, provided *ad libitum* food and water, and maintained on a 12:12 light/dark cycle. Animals were randomly assigned into two dietary exposure groups: standard choline diet (containing 0.2% choline-TD 03118; Harlan Teklad, Madison, WI) and a choline-supplemented diet (2% choline-TD 03119). These studies employed a concentration of 2% choline for dietary supplementation because previous studies have shown that this is a palatable diet that results in significant increases in the number of CNS $\alpha 7$ nAChRs (Coutcher et al., 1992; Guseva et al., 2006). Diets were nutritionally matched with respect to all ingredients except choline. Animals were maintained on these diets for a period of 14 days and then subjected to a controlled cortical impact (CCI) brain injury ($n = 7$ for each dietary group) or sham operation ($n = 6$ for each dietary group). Rats were anaesthetized with isoflurane (2%) and immobilized in a Kopf stereotaxic frame. A craniotomy (6 mm in diameter; Bregma -2.8 , 2.5 mm lateral) was performed using a Michele trephine, and the skull disk was removed with minimal damage to the dura. An electronically controlled piston (5 mm tip diameter; Precision Systems and Instrumentation LLC, Fairfax, VA) was used to administer a 1.5-mm cortical deformation (speed 3.5 mm/sec; 400 msec dwell time) (Hall et al., 2005).

Cognitive Assessment

Spatial memory was assessed using a standard Morris water maze (MWM) paradigm, starting on the 8th day following the injury. Animals were maintained on the various dietary treatments throughout the cognitive testing phase of the study. The test room contained a 127 cm (diameter) $\times$ 56 cm (height) pool with a submerged escape platform (13.5 cm in diameter) at the center of one quadrant; visual cues, distributed throughout the room, helped to aid spatial orientation. All of the cognitive evaluations were videotaped and analyzed using Videomex software (Columbus Instruments, Columbus, OH). Quadrant entry was randomized for different starting positions, and animals were allowed to swim until they found the platform, where they remained for 15 sec. Rats that did not find the platform in 60 sec were manually placed on the platform for 15 sec after each trial. Twenty acquisition trials were administered (four trials per day separated by a 5-min inter-trial interval, continued for 5 days). Four hours following the last acquisition trial, the platform was removed and a 30-sec retention trial was performed. Training data were analyzed using a three-way repeated-measures analysis of variance (ANOVA), with the day of training as a repeated measure. Retention data were analyzed using a two-way ANOVA. Student-Newman-Keuls (SNK) tests were used for all post-hoc analyses.

Histological Evaluation

Histological and neurochemical analyses of the CNS were performed after completion of the behavioral testing. Animals were euthanized, and the brains were immediately removed and frozen in isopentane chilled on dry ice. Brains sections (16 μ M) were prepared using a Lecia CM1850 cryostat (Nussloch, Germany) and mounted onto poly-L-lysine coated slides. One set of sections was stained with cresyl violet and evaluated using a video-based image analysis system (NIH Image) to calculate cortical tissue sparing, as previously described (Scheff and Sullivan, 1999; Verbois et al., 2000). Twelve equally spaced cryosections through the damaged area were used from each animal. The ipsilateral and contralateral sides of the cerebral cortex were carefully circumscribed using lamina 1 and the corpus callosum as boundaries, and tissue volume calculated. The percentage of cortical tissue spared was calculated by dividing the mean cortical volume of the ipsilateral side by that of contralateral side ($\times 100$). Two-way ANOVA was used to analyze the data with dietary group and surgery as independent variables; SNK post hoc tests were used when appropriate.

Receptor Autoradiography

Alpha 7 nicotinic acetylcholine receptors were visualized through α -[125 I]-Bungarotoxin (BTX) autoradiography, as previously described (Sparks and Pauly, 1999). Tissue sections were incubated in 2.5 nmol α -[125 I]-Tyr54-BTX (PerkinElmer Life Sciences, Inc., Boston, MA; specific activity = 102.9 Ci/mmol on the day of the binding) under equilibrium binding conditions. Non- $\alpha 7$ nAChR expression was assessed using 10 pM [125 I]-Epibatidine (specific activity 2200 Ci/mmol; PerkinElmer Life Sciences, Inc., Boston, MA), as described elsewhere (Perry and Kellar, 1995). For assessment of brain inflammation/microglial activation, slides were pre-incubated in 50 mM Tris HCl buffer (pH 7.4) for 15 min at 4°C, and then transferred to incubation buffer containing 50 mM Tris HCl and 2 nM [3 H]-PK11195 (specific activity = 85.5 Ci/mmol; PerkinElmer Boston MA) for 2 h at 4°C. All slides were then washed, dried, and exposed to RayMax Beta high-performance autoradiography film (ICN Biomedicals Inc., Aurora, OH) for appropriate intervals. Binding data were analyzed using NIH image v1.59 on a Power Macintosh connected to a Sony XC-77 CCD camera via a Scion LG-3 frame-grabber. Brain regions for autoradiographic analyses were selected based on previously reported differences in susceptibility to TBI-induced down-regulation of nAChR number, as well as functional contributions of various regions to MWM performance (Leon-Carrion et al., 2000). Binding results were analyzed using a three-way, repeated-measures ANOVA, with hemisphere as a repeated measure; SNK tests were used to make individual comparisons. For simplicity, only binding data from the injured side of the brain are presented.

Results

Cognitive Assessment

Dietary choline supplementation was associated with modest, but significant improvement in the acquisition phase of the MWM test (Fig. 1). ANOVA results revealed significant effects of surgery ($F(1,20) = 11.5$, $p < 0.003$), the day of testing ($F(4,80) = 42.6$, $p < 0.0001$), and a significant Day $\times$

FIG. 1. Dietary choline supplementation enhances performance in the acquisition phase of Morris water maze testing. Analysis of the results by three-way analysis of variance (ANOVA) revealed significant effects of surgery ($F(1,20) = 11.5$, $p = 0.003$) and the day of testing ($F(4,80) = 42.6$, $p < 0.0001$), as well as a significant surgery $\times$ diet $\times$ day interaction ($F(4,80) = 3.26$, $p = 0.02$). On day 3 of testing sham-operated animals on the choline-supplemented diet found the platform faster than sham animals on the standard diet. Brain-injured animals consuming the choline diet had improved performance on day 5 of acquisition testing, compared to controlled cortical impact (CCI) animals on the standard diet. * $p < 0.05$, significantly different from sham operated animals consuming the standard diet; $\dagger p < 0.05$, significantly different from brain-injured animals consuming the standard diet.

Surgery $\times$ Dietary treatment interaction ($F(4,80) = 3.26$, $p < 0.02$). On day 3 sham-operated rats fed the standard diet found the platform slower than sham animals on the choline-supplemented diet ($p < 0.05$). However, considering the fact that latency values of standard diet shams from day 2 and 4 are much lower compared to day 3, the effect seen on day 3 most likely is due to chance alone. Post hoc analysis of daily latencies also revealed that animals on the choline supplemented diet had improved performance on day 5 of acquisition testing compared to injured animals on the normal diet ($p < 0.05$). No significant group differences in swim speed were present during the acquisition phase of testing (data not shown). The results of the memory retention phase of testing showed more consistent improvements in animals supplemented with dietary choline (Fig. 2). CCI animals consuming the normal diet entered the target quadrant fewer times (Fig. 2A, [$F(1,20) = 15.86$, $p < 0.001$]) spent less time over the previous platform area (Fig. 2B, [$F(1,20) = 5.88$, $p < 0.025$]), and had a greater cumulative distance from the goal (Fig. 2C, [$F(1,20) = 11.23$, $p < 0.005$]), compared to sham-operated animals. Post-hoc analysis showed improvement in each of these measurements obtained from brain-injured animals that consumed the choline diet. No significant differences in swim speed between the groups were present during the retention phase (Fig. 2D).

Autoradiographic Studies

The number of $\alpha 7$ nicotinic receptors, as assessed by [125 I]-BTX binding, was altered by the injury as well as by dietary

FIG. 2. Dietary choline supplementation enhances performance on memory retention tests conducted 4 h following the last acquisition trial. Injured animals on the standard diet entered the target quadrant fewer times than animals supplemented with choline (A; $\dagger p < 0.05$). Relative platform time in the probe test was also significantly reduced in traumatic brain injury (TBI) animals consuming the standard choline diet; dietary choline supplementation significantly improved this deficit in memory retention (B; $\dagger p < 0.05$). Over the 30 sec of the retention trial, TBI animals on the standard diet also were further from the goal location (C). There were no treatment differences in swim speed observed during the retention trial (D).

choline supplementation (Table 1). In animals maintained on the standard choline diet, TBI caused a significant reduction in $\alpha 7$ nicotinic receptor binding in the auditory cortex ($F[1,18] = 8.32$, $p < 0.01$), CA1 ($F[1,23] = 5.13$, $p < 0.005$), dentate gyrus ($F[1,19] = 8.8$, $p < 0.008$), hilus of the dentate

gyrus ($F[1,19] = 5.6$, $p < 0.03$), and the superior colliculus ($F[1,18] = 8.3$, $p < 0.01$). Sham-operated animals exposed to a high choline diet had significant increases in BTX binding in nine of the 12 regions analyzed, with significant effects of the diet noted in auditory cortex layers 1–4 ($F[1,18] = 52.4$,

TABLE 1. EFFECTS OF TRAUMATIC BRAIN INJURY (TBI) AND DIETARY CHOLINE SUPPLEMENTATION ON THE DENSITY OF $\alpha 7$ NACHRs AS MEASURED BY [125 I]-BTX-AUTORADIOGRAPHY

Brain region	Standard choline diet		Choline supplemented diet	
	Sham	CCI	Sham	CCI
Auditory cortex layers 1–4	0.30 $\pm$ 0.04	0.27 $\pm$ 0.02**	0.35 $\pm$ 0.03**	0.33 $\pm$ 0.02** $\dagger\dagger$
Auditory cortex layers 5–6	0.41 $\pm$ 0.04	0.40 $\pm$ 0.02	0.49 $\pm$ 0.01**	0.49 $\pm$ 0.03**
Endopiriform nucleus	0.61 $\pm$ 0.05	0.60 $\pm$ 0.05	0.65 $\pm$ 0.05*	0.66 $\pm$ 0.03*
Hippocampus, CA1	0.30 $\pm$ 0.04	0.28 $\pm$ 0.03*	0.31 $\pm$ 0.02	0.31 $\pm$ 0.01
Hippocampus, CA2/3	0.36 $\pm$ 0.03	0.37 $\pm$ 0.03	0.43 $\pm$ 0.03**	0.44 $\pm$ 0.03**
Hilus of dentate gyrus	0.66 $\pm$ 0.07	0.61 $\pm$ 0.06	0.72 $\pm$ 0.04*	0.67 $\pm$ 0.02 $\dagger\dagger$
Dentate gyrus	0.36 $\pm$ 0.02	0.33 $\pm$ 0.04*	0.41 $\pm$ 0.03**	0.38 $\pm$ 0.01 $\dagger$
Superior colliculus	0.74 $\pm$ 0.04	0.68 $\pm$ 0.04**	0.76 $\pm$ 0.04	0.76 $\pm$ 0.01
Stratum oriens	0.41 $\pm$ 0.04	0.41 $\pm$ 0.06	0.45 $\pm$ 0.01*	0.42 $\pm$ 0.03
Ventral lateral geniculate	0.65 $\pm$ 0.04	0.62 $\pm$ 0.05	0.67 $\pm$ 0.05	0.61 $\pm$ 0.1
Posterior hypothalamus	0.64 $\pm$ 0.03	0.65 $\pm$ 0.04	0.68 $\pm$ 0.02	0.69 $\pm$ 0.05*
Ventral posterior thal	0.74 $\pm$ 0.03	0.74 $\pm$ 0.04	0.77 $\pm$ 0.02*	0.78 $\pm$ 0.05*

In animals maintained on the standard choline diet, TBI caused a significant reduction in $\alpha 7$ nicotinic receptor binding in four of 12 brain regions evaluated. Sham-operated animals exposed to a high choline diet had significant increases in BTX binding in nine of the 12 regions analyzed. Choline treatment reversed binding deficits caused by TBI in two brain regions (CA1 and superior colliculus) but not in others (cortex, dentate gyrus, hilus).

* $p < 0.05$, ** $p < 0.01$, significantly different from sham-operated animals maintained on the standard choline diet (SNK).

$\dagger p < 0.05$, $\dagger\dagger p < 0.01$, significantly different from sham-operated animals maintained on the choline-supplemented diet (SNK).

TABLE 2. EFFECTS OF TRAUMATIC BRAIN INJURY (TBI) AND DIETARY CHOLINE SUPPLEMENTATION ON THE DENSITY OF NON- $\alpha 7$ nAChRs AS MEASURED BY [125 I]-EPIBATIDINE-AUTORADIOGRAPHY

Brain region	Standard choline diet		Choline supplemented diet	
	Sham	CCI	Sham	CCI
Auditory cortex layer 2	1.01 $\pm$ 0.12	0.85 $\pm$ 0.15	0.99 $\pm$ 0.06	0.96 $\pm$ 0.1
Auditory cortex layers 3–6	0.61 $\pm$ 0.05	0.53 $\pm$ 0.04	0.63 $\pm$ 0.05	0.62 $\pm$ 0.0
Striatum	1.07 $\pm$ 0.17	1.22 $\pm$ 0.1	1.14 $\pm$ 0.16	1.21 $\pm$ 0.1
Dentate gyrus (medial blade)	0.71 $\pm$ 0.13	0.62 $\pm$ 0.1	0.72 $\pm$ 0.12	0.78 $\pm$ 0.1
Dentate gyrus (lateral blade)	0.49 $\pm$ 0.06	0.42 $\pm$ 0.04	0.50 $\pm$ 0.07	0.53 $\pm$ 0.0
Superior colliculus	2.79 $\pm$ 0.5	3.00 $\pm$ 0.6	2.81 $\pm$ 0.46	2.72 $\pm$ 0.4
VPL/VPM	1.58 $\pm$ 0.29	1.58 $\pm$ 0.15	1.59 $\pm$ 0.3	1.71 $\pm$ 0.2
Medial habenula	4.83 $\pm$ 0.69	5.59 $\pm$ 0.57*	4.51 $\pm$ 0.49	5.26 $\pm$ 0.6*,†
Subiculum	1.19 $\pm$ 0.20	1.22 $\pm$ 0.22	1.10 $\pm$ 0.13	1.14 $\pm$ 0.2
Medial geniculate nucleus	1.68 $\pm$ 0.20	1.61 $\pm$ 0.33	1.40 $\pm$ 0.26	1.57 $\pm$ 0.4
Fasciculus retroflexus	2.84 $\pm$ 0.37	3.06 $\pm$ 0.35	3.01 $\pm$ 0.49	2.88 $\pm$ 0.2

In animals maintained on the standard choline diet, TBI increased binding in only the medial habenula. Choline administration did not generally affect non- $\alpha 7$ nAChR density.

* $p < 0.05$, significantly different from sham-operated animals maintained on the standard choline diet.

† $p < 0.05$, significantly different from sham-operated animals maintained on the choline-supplemented diet (SNK).

$p < 0.0001$); auditory cortex layers 5–6 ($F[1,18] = 60.4$, $p < 0.0001$); endopiriform nucleus ($F[1,18] = 12.8$, $p < 0.002$); CA2/3 ($F[1,19] = 32.9$, $p < 0.0001$); hilus of dentate gyrus ($F[1,19] = 23.0$, $p < 0.0001$); dentate gyrus ($F[1,19] = 18.0$, $p < 0.0004$); posterior hypothalamus ($F[1,17] = 9.4$, $p < 0.007$); ventral posterior thalamus ($F[1,18] = 10.6$, $p < 0.005$). Choline treatment reversed $\alpha 7$ nAChR binding deficits caused by TBI in two brain regions (CA1 and superior colliculus) but not in others. Dietary choline manipulation and CCI had a minimal effects on the density of non- $\alpha 7$ nAChRs as assessed with [125 I]-Epibatidine binding (Table 2). In animals maintained on the standard choline diet, TBI increased non- $\alpha 7$ nAChR binding in the medial habenula ($F[1,20] = 10.8$, $p < 0.004$) only; a similar effect was seen in choline supplemented animals following TBI. Choline administration did not affect the density of non- $\alpha 7$ nAChR expression. Following brain injury, there was a significant increase in the density of [3 H]-PK11195 binding observed in all brain regions evaluated, consistent with widespread microglial activation. Significant effects of the surgery found in auditory cortex ($F[1,22] = 16.9$, $p < .001$), CA1 ($F[1,22] = 63.5$, $p < 0.001$), CA2/3 ($F[1,22] = 34.4$, $p < 0.001$), hilus of dentate

gyrus ($F[1,22] = 44.1$, $p < 0.001$), dentate gyrus ($F[1,22] = 55.7$, $p < 0.0001$), and VPL/VPM ($F[1,22] = 29.2$, $p < 0.001$). Dietary choline supplementation significantly attenuated TBI-induced microglial activation in the auditory cortex, hilus of the dentate gyrus, and the thalamus (Table 3, Fig. 3).

Cortical Tissue Sparing Analysis

Dietary choline supplementation was associated with cortical tissue preservation, consistent with a neuroprotective action. Two-way ANOVA revealed a significant effect of the surgery ($F[1,17] = 122.00$, $p < 0.001$), and significant Diet $\times$ Surgery interaction ($F[1,17] = 4.61$, $p < 0.045$; Fig. 4), without a significant main effect of the diet. A significant decrease in cortical tissue sparing was noted in the injured animals on the standard diet ($p < 0.01$), as well as CCI animals on the choline-supplemented group ($p < 0.05$), compared to their respective shams. However, choline-supplemented animals with TBI had significantly more cortical tissue spared than TBI animals consuming the standard diet ($p < 0.05$).

TABLE 3. EFFECTS OF TRAUMATIC BRAIN INJURY (TBI) AND DIETARY CHOLINE SUPPLEMENTATION ON MICROGLIAL ACTIVATION, AS ASSESSED BY [3 H]-PK11195 AUTORADIOGRAPHY

Brain region	Standard choline diet		Choline supplemented diet	
	Sham	CCI	Sham	CCI
Auditory cortex layers 1–6	2.01 $\pm$ 0.13	4.77 $\pm$ 0.16**	1.73 $\pm$ 0.10	3.41 $\pm$ 0.44††
Hippocampus, CA1	1.36 $\pm$ 0.06	6.45 $\pm$ 2.25**	1.44 $\pm$ 0.16	6.25 $\pm$ 1.46**
Hippocampus, CA2/3	1.45 $\pm$ 0.09	6.58 $\pm$ 2.81**	1.50 $\pm$ 0.08	5.92 $\pm$ 1.86**
Hilus of dentate gyrus	1.43 $\pm$ 0.07	9.00 $\pm$ 2.65**	1.42 $\pm$ 0.08	6.54 $\pm$ 2.71**††
Dentate gyrus	1.52 $\pm$ 0.12	6.35 $\pm$ 1.89**	1.58 $\pm$ 0.03	5.4 $\pm$ 1.03**
VPL/VPM	1.66 $\pm$ 0.38	4.02 $\pm$ 1.02**	1.45 $\pm$ 0.19	3.27 $\pm$ 1.01**†

Following brain injury, there was a significant upregulation of PK11195 binding observed in all brain regions evaluated. Dietary choline supplementation significantly attenuated TBI-induced microglial activation in the auditory cortex, hilus of the dentate gyrus, and the thalamus.

** $p < 0.01$, significantly different from sham-operated animals maintained on the standard choline diet (SNK).

† $p < 0.05$, †† $p < 0.01$, significantly different from sham-operated animals maintained on the choline-supplemented diet (SNK).

FIG. 3. Representative [^3H]-PK11195 autoradiographs depicting the effects of traumatic brain injury (TBI) and dietary choline supplementation on presumed microglial activation. In sham-operated animals, [^3H]-PK11195 binding is restricted to choroid plexus, located in the lateral ventricles (LV) and the third ventricle. At 12 days following brain injury, prominent microglial activation was seen in the cerebral cortex adjacent to the lesion area, throughout the hippocampus (CA1, CA3), and in the ventral posteromedial thalamus (VPM). Dietary choline supplementation significantly reduced [^3H]-PK11195 binding in the cerebral cortex, hilus of the dentate gyrus, and the thalamus.

Discussion

Previous findings from our laboratory have suggested that TBI-induced down-regulation of cortical and hippocampal $\alpha 7$ nAChRs may contribute to cognitive impairment following brain injury. We hypothesize that pharmacological treatments that normalize TBI-induced deficits in $\alpha 7$ nAChR expression may be associated with improvements in cognitive function. In the present study, dietary choline supplementation significantly reduced brain injury-induced spatial learning deficits in MWM during the retention phase of cognitive evaluation, with a trend towards improvement in the acquisition phase. Dietary choline supplementation also attenuated CCI-induced decreases in $\alpha 7$ nAChR expression, reduced brain inflammation and increased cortical tissue sparing. The results of the present studies are in general agreement with others that have shown beneficial effects of the choline precursor, CDP-choline, in humans with neurological disorders (Cacabelos et al., 1996; Clark et al., 1997; Leon-Carrion et al., 2000). The utility and promise of choline as a safe therapy for long term treatment of neurological disorders is currently being evaluated in clinical trials for a number of conditions including, cocaine and marijuana abuse, schizophrenia, bipolar disorder, stroke (ICTUS trial), and traumatic brain injury (COBRIT trial; www.ClinicalTrials.gov).

The neuroanatomical and/or biochemical substrates of choline-induced neuroprotection and cognitive restoration seen in the present study are not certain. Enhanced membrane repair, increased synthesis of acetylcholine, stabilization of cerebrovascular function as well as direct stimulation of $\alpha 7$ nAChRs could all contribute to the beneficial effects of

choline that were observed (Parnetti et al., 2007). Choline's action as a direct-acting $\alpha 7$ nAChR agonist may improve cognitive outcome as this receptor is expressed at high levels in human and rodent hippocampus (Marks et al., 1986; Berg and Conroy, 2002; Tribollet et al., 2004) and has previously been implicated in cognitive processing (Van Kampen et al.,

FIG. 4. Dietary choline supplementation results in significant cortical tissue sparing, compared to a standard rodent diet. In both groups of traumatic brain injury (TBI) animals, there was a significant loss of cortical tissue mass (** $p < 0.01$). However, tissue sparing in choline-treated rats (79% tissue spared) was significantly higher than that measured in control TBI animals (68% tissue spared; $\dagger p < 0.05$).

2004; Buccafusco et al., 2005; Levin et al., 2006; Nott and Levin, 2006). However, in our study, sham-operated animals exposed to the choline diet had no significant enhancement of MWM performance, in spite of significant $\alpha 7$ nAChR up-regulation. Although it is also possible that cognitive performance is maximized in sham-operated animals, dietary choline supplementation alone does not seem to result in a general improvement in spatial memory performance, as we have previously reported (Guseva et al., 2006).

Previous studies evaluating the neuroprotective actions of nicotine have consistently implicated involvement of $\alpha 7$ nAChRs (Carlson et al., 1998; Dajas-Bailador et al., 2000; Pauly et al., 2004). In addition, choline and other $\alpha 7$ selective nAChR agonists have been shown to exert a wide-variety of beneficial properties in animal models of neurodegeneration (Jonhalla et al., 2003; Marrero et al., 2004; Mulholland et al., 2004). Several studies have suggested that the protective actions of $\alpha 7$ nAChR agonists be related to increases in the density of [125 I]-BTX binding sites. Jonhalla and Buccafusco (2001) correlated increased density of $\alpha 7$ nAChRs on differentiated PC-12 cells and $\alpha 7$ mediated neuroprotection against apoptotic cell death induced by neurotrophic factor withdrawal. Acute or chronic activation of $\alpha 7$ nAChRs may cause changes in intracellular signaling that up-regulate pro-survival factors such as Bcl-2 ultimately protecting cells from death via apoptosis or necrosis (Kihara et al., 2001; Marrero et al., 2004).

The neuroprotective actions of choline could also potentially involve interactions with the peripheral cholinergic anti-inflammatory pathway. A study by Wang et al. (2003) established a link between cholinergic activity of the vagus nerve and peripheral inflammation, with central involvement of $\alpha 7$ nAChRs expressed on macrophages. Electrical stimulation of the vagus nerve causes a significant decrease in tumor necrosis factor (TNF) release from macrophages, and the effects of vagal stimulation were blocked by administration of $\alpha 7$ antagonists, and absent in $\alpha 7$ knockout mice. Shytle et al. (2004) showed that exposure to acetylcholine or nicotine reduces inflammatory markers following the administration of lipopolysaccharide, and that this effect was blocked by $\alpha 7$ antagonists. In the present study, dietary choline supplementation significantly attenuated TBI-induced increases in hippocampal and thalamic inflammation as assessed by [3 H]-PK11195 binding to presumed activated microglia. Since choline treatment also results in significant cortical tissue sparing, it might be expected that secondary brain inflammation would be decreased in this group of animals.

One important issue not addressed in the present study is the therapeutic window for choline-induced neuroprotection in the CCI model of traumatic brain injury. Since dietary choline supplementation was administered to animals prior to surgery, and throughout the cognitive evaluation stage, the time frame most important to the beneficial effect of choline is uncertain. Maximal up-regulation of $\alpha 7$ nAChRs prior to injury could have conferred some neuroprotective actions, or stimulation of $\alpha 7$ nAChRs coupled with improved phospholipid profiles following the injury may have been important. It may also be that enhanced choline levels both before, and after the injury may have been necessary to produce the results obtained. Future studies will evaluate the timing of choline administration in attempt to resolve this

issue. In summary, the present study supports the hypothesis that $\alpha 7$ nAChR agonists may be neuroprotective compounds that have potential use in the treatment or prevention of brain neurodegeneration. Establishing the most efficacious timing of administration and potential mechanisms involved with the neuroprotective actions of choline are important next steps.

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Author Disclosure Statement

The authors report no conflicting financial interests.

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