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REVIEW ARTICLE

Neuroprotective role of taurine during aging

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Abstract Aging of the brain is characterized by several neurochemical modifications involving structural proteins, neurotransmitters, neuropeptides and related receptors. Alterations of neurochemical indices of synaptic function are indicators of age-related impairment of central functions, such as locomotion, memory and sensory performances. Several studies demonstrate that ionotropic GABA receptors, glutamate decarboxylase (GAD), and somatostatinergic subpopulations of GABAergic neurons are markedly decreased in experimental animal brains during aging. Additionally, levels of several neuropeptides co-expressed with GAD decrease during aging. Thus, the age-related decline in cognitive functions could be attributable, at least in part, to decrements in GABA inhibitory neurotransmission. In this study, we showed that chronic supplementation of taurine to aged mice significantly ameliorated the age-dependent decline in spatial memory acquisition and retention. We also demonstrated that concomitant with the amelioration in cognitive function, taurine caused significant alterations in the GABAergic and somatostatinergic system. These changes included (1) increased levels of the neurotransmitters GABA and glutamate, (2) increased expression of both isoforms of GAD (65 and 67) and the neuropeptide somatostatin, (3) decreased hippocampal expression of the β3 subunits of the $GABA_A$ receptor, (4) increased expression in the number of somatostatin-positive neurons, (5) increased amplitude and duration of population spikes recorded from CA1 in response to Schaefer collateral stimulation and (6) enhanced paired pulse facilitation in the hippocampus. These specific alterations of the inhibitory system caused by taurine treatment oppose those naturally occurring in the aging brain, suggesting a protective role of taurine in this process. An increased understanding of age-related neurochemical changes in the GABAergic system will be important in elucidating the underpinnings of the functional changes of aging. Taurine supplementation might help forestall the age-related decline in cognitive functions through interaction with the GABAergic system.

Keywords Taurine · Aging · Somatostatin · GABA · GAD · Passive avoidance

Abbreviations

GAD	Glutamate decarboxylase
GABA	γ-Aminobutyric acid
SST	Somatostatin
DEPC	Diethylpyrocarbonate

Introduction

Aging of the brain is characterized by several neurochemical modifications involving structural proteins,

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neurotransmitters, neuropeptides and related receptors (Marczynski 1998). Alterations of neurochemical indices of synaptic function have been considered as indicators of age-related impairment of central functions, such as locomotion, memory and sensory performances. GABA (γ-aminobutyric acid) is the primary inhibitory neurotransmitter in the central nervous system and is present in neurons in all brain regions. The ratio of glutamatergic to GABAergic neurons in the neocortex is approximately 5:1, and disruption of this ratio can present in neurological disorders (Lehmann et al. 2012). A number of GABAergic parameters reportedly undergo changes during senescence (Araki et al. 1996). Of importance, ionotropic GABA receptors are markedly decreased in experimental animal brains during aging (Govoni et al. 1980; Hunter et al. 1989; Milbrandt et al. 1996). Additionally, a significant age-related decrease in glutamate decarboxylase (GAD 65 and 67), the enzyme responsible for GABA synthesis, is observed in the cortex and hippocampus of aged rats relative to their young adult cohorts, suggesting a plastic down-regulation of normal adult inhibitory GABA neurotransmission (Marczynski 1998). Consistent with this, functional studies in primate visual and auditory cortices establish sensory coding changes suggestive of altered inhibitory processing in aged animals (Yang et al. 2008). Such age-related loss of normal adult GABA neurotransmission in the auditory cortex would likely alter temporal coding properties and could contribute to the loss in speech understanding observed in the elderly (Mendelson and Ricketts 2001; Ling et al. 2005). Thus, the age-related central sensory processing deficits could be attributable, at least in part, to decrements in GABA inhibitory neurotransmission (Caspary et al. 1990, 2002). Indeed, the auditory midbrain shows significant age-related changes related to GABA neurotransmission (Banay-Schwartz et al. 1989a, b; Caspary et al. 1990; Gutierrez et al. 1994; Raza et al. 1994; Caspary et al. 1995; Milbrandt et al. 1996). Furthermore, different subpopulations of GABAergic neurons such as somatostatin- and parvalbumin-containing neurons are reduced in aged rats (Kuwahara et al. 2004a, b). These observations seem to indicate that age-related changes in GABAergic function maybe an important determinant of cognitive function. In the present study, therefore, we focused on the biochemical alteration in the GABAergic system, the major inhibitory neurotransmitter system.

The neonatal brain contains high levels of taurine (Huxtable 1989, 1992; Sturman 1993; Kuriyama and Hashimoto 1998). As the brain matures its taurine content declines and reaches stable adult concentrations that are second to those of glutamate, the principal excitatory neurotransmitter in the brain. Taurine levels in the brain significantly increase under stressful conditions (Wu et al. 1998), suggesting that taurine may play a vital role in neuroprotection. A possible mechanism of taurine's neuroprotection lies in its calcium modulatory effects. We have shown that taurine modulates both cytoplasmic and intra-mitochondrial calcium homeostasis (El Idrissi and Trenkner 1999, 2003, 2004). Furthermore, taurine serves as an agonist of $GABA_A$ receptors (Quinn and Harris 1995; Wang et al. 1998; del Olmo et al. 2000; Mellor et al. 2000; El Idrissi and Trenkner 2004; El Idrissi et al. 2003). Further, we reported that mice chronically fed taurine express elevated levels of the neurotransmitters GABA and glutamate and the GABA synthesizing enzyme glutamate decarboxylase (GAD) (El Idrissi et al. 2005). GAD exists in two isoforms (GAD65 and GAD67) and with different subcellular distributions (Denner and Wu 1985; Spink et al. 1983; Erlander et al. 1991). GAD67 is typically found in the cytoplasm, whereas GAD65 is associated with vesicles at axon termini (Esclapez et al. 1994). The severity of loss of GAD65 is associated with increase in pain (Zhang et al. 2011), anxiety (Kash et al. 1999), and hearing (Burianova et al. 2009). Loss of GAD67 expression leads to decreased axonal branching and synapse formation (Chattopadhyaya et al. 2007) and is implicated in schizophrenia (Curley et al. 2013). Thus, we hypothesize that the age-dependent deterioration in GABAergic function and the resulting decline in cognitive function could be ameliorated by supplementing exogenous taurine.

Using this paradigm of taurine supplementation, we will gain significant understanding of the mechanisms by which taurine influences the inhibitory GABAergic systems in the brain and explore the potential of taurine in reversing the age-dependent alteration in the inhibitory system. Most importantly, the identification of specific age-dependent alterations in the GABAergic system will enhance our understanding of the basis for the long-lasting altered cellular and synaptic properties that contribute to the decline in cognitive function, characteristic of senescence.

Methods

Animals

All mice used in this study were FVB/NJ males. Mice were housed in groups of three in a pathogen-free room maintained on a 12 h light/dark cycle and given food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY, and were in conformity with National Institutes of Health Guidelines. The number of mice sufficient to provide statistically reliable results was used in these studies.

Passive avoidance

Using a passive avoidance paradigm, we tested the ability of mice to learn to avoid an electrical shock in young and old mice. We define for our purposes two populations of mice: young (2 months) and old (16 months). It is generally thought that the age selected for young mice is developmentally equivalent to the human at 18 months. The drinking water of young mice was supplemented with taurine (0.05 %) for 4 weeks, whereas the old mice's water was supplemented with the same concentration of taurine for 8 months. Controls received only water. The passive avoidance apparatus employs both bright and dark compartments with a computer-controlled guillotine-type door between them. The delivery of electric shocks, the raising and lowering of the door, and the latencies at which the animals stepped into the dark from the initial bright compartment were controlled by the computer. Each mouse was gently placed in the light compartment for 10 s, after which the guillotine door was raised. The amount of time the mouse waited before crossing to the dark (shock) compartment was recorded as the latency. Mice were given six trials a day for 5 days and after repetitive training, learning was measured. Figure 1 shows that the performances of young and old mice during the 5 days of training. The trial ended when the mouse waited more than 180 s to cross to the other side, or if it received an electrical shock in the dark side after crossing. Once the mouse crossed with all four paws to the next compartment, the door was closed and a 1.5 mA foot shock was delivered for 5 s. The tester was blind to the genotype of the mice.

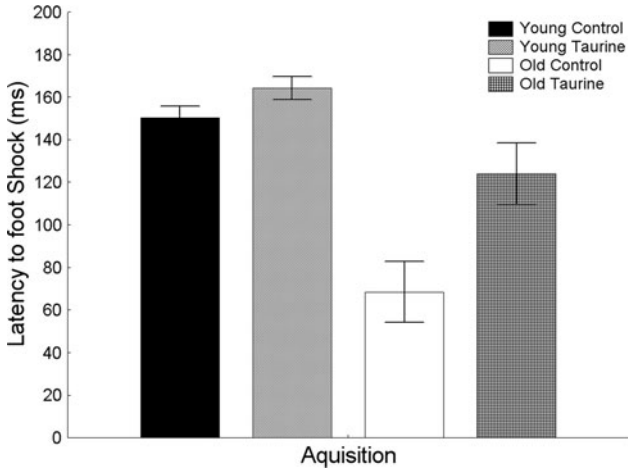

Fig. 1 Acquisition of a passive avoidance task in young and old mice. Sustained dietary intake of taurine in drinking water improved acquisition and retention in the aged group. Each animal was familiarized with the behavioral apparatus for 2–3 min the day before the training session. All training and testing were carried out between 08:00 and 12:00 h. Data represent the mean ± SEM of latencies to the end of the trial on the fifth day of training (6 trials a day) obtained from 10 young and 10 aged mice. A two-way analysis of variance (ANOVA) showed that there was significant effect of age [$F(1,277) = 64.345$, $p < 0.0001$] and a significant effect of treatment [$F(1,277) = 20.920$, $p < 0.0001$]. The interaction between age and treatment was also significant [$F(1,277) = 7.6122$, $p < 0.005$]. Tukey HSD post hoc test revealed that old control group was significantly ($p < 0.0001$) different from all other groups. Chronic supplementation of taurine significantly ($p < 0.0001$) enhanced acquisition in the old but not in the young group

Spatial position learning

Position learning was evaluated in a cross-shaped water maze as previously described (Decker and McGaugh 1989; Dumas and Rabe 1994; Dobkin et al. 2000). The cross maze is similar to the more commonly used Morris maze that has been found to be hippocampally dependent (Cho et al. 1999; Logue et al. 1997). A maze of clear plastic in the shape of a cross was placed in a circular tub (1 m diameter) of opaque 25 °C water. A hidden escape platform was located 1 cm below the surface of the water at the end of the East (E) arm of the cross. A variety of small objects were fixed around the tub as distal visual cues. For each trial, the mouse was placed in the North (N), South (S), or West (W) arm of the cross (the order determined semi-randomly) and allowed a maximum of 60 s to swim to the escape platform in the E arm. Swimming into an arm other than E was an error. Only swimming directly to the platform without entering the N, S or W arms was a correct response. When the mouse swam to the escape platform after an error, it was not scored as a correct response. When the mouse found the escape platform within the 60 s, it was allowed to remain there for 20 s. If the mouse did not find the escape platform in 60 s, it was gently pushed onto the platform and left there for 20 s. After each trial, the mouse was removed from the platform and held for 10 s before being placed in another arm for the next trial. Each mouse was given six of these "massed" trials on each of 6 consecutive days. The 6-day acquisition training was followed 4 weeks later by one 6-trial day of retention. The tester was blind to the genotype of the mice.

Western blotting

Brains were dissected within 3 min of the killing and frozen on dry ice. Total soluble and membrane bound proteins were extracted from each region (Benke et al. 1999). For ionotropic $GABA_A$ receptor β3 subunit (GABARB), 40 μg of protein from each region was loaded onto a 12.5 % polyacrylamide-SDS gel, separated by electrophoresis, and transferred onto PVDF membrane (Millipore, Bedford, MA, USA). Membranes were sequentially probed with two monoclonal antibodies: MAB341 (1:1,000 dilution, Chemicon, Temecula, CA, USA) that recognizes the β3 subunit of GABARB and an antibody (clone AC-15,

Sigma, St. Louis, MO, USA) that recognizes β-actin (1:100,000 dilution) as a control for protein loading. For GAD detection, 10 μg of protein from each region was used and membranes were simultaneously probed with two antibodies: a rabbit polyclonal (1:5000 dilution of AB1511, Chemicon, Temecula, CA, USA) that recognizes both GAD65 and GAD67 and anti β-actin as above. For somatostatin 28/24 (SST) detection, the membrane was probed with two antibodies: a rabbit polyclonal (1:1,000 dilution, Chemicon, Temecula, CA) that recognizes both isoforms (SST28 and SST14) and anti β-actin as above. After incubation with the primary antibodies overnight at 4 °C, alkaline phosphatase-linked goat anti-mouse or anti-rabbit secondary antibodies (1:5,000 dilution, Sigma, St. Louis, MO, USA) were applied and the membranes were subsequently incubated with CDP-Star (NEB, Beverly, MA, USA) reagent according to the supplier's instructions. For quantification, membranes were exposed to X-ray film for 0.5–20 min and 600 dpi scans of these films were analyzed with AIDA software (Raytest, Wilmington, NC, USA). GABARB, GAD and SST levels were normalized to actin and a density ratio (GABARB/actin, GAD/actin or SST/actin) was calculated for each sample. Each set of blots included a serial dilution of brain protein that was used to identify the exposures where the densities were in the linear range for actin and GABARB, GAD or SST.

Immunohistochemistry

Mice were anesthetized with pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by 4 % paraformaldehyde. Brains were cryopreserved in 30 % sucrose, frozen and then sectioned on a Cryostat. Cryosections (30 μm) were collected in a 12-well plate containing cryoprotectant (30 % glycerol, 30 % ethylene glycol and 40 % PBS). Sections were immunostained after blocking in 0.3 % Triton X 100 and 5 % normal goat serum. The primary antibodies employed for western blot recognized both denatured and native structures, so were again employed in the immunohistochemical analyses. The β3 subunit of GABARB (MAB341) was used at 1:500 dilution; somatostatin 28/24 (SST) was used at 1:500 dilution). Both primary antibodies were diluted in PBS containing 0.3 % Triton X-100 and 5 % normal goat serum and applied to free-floating sections for 24 h at 4 °C with gentle agitation. Immunoreactivity was detected with secondary antibodies (Molecular Probes/Invitrogen) conjugated with fluorophores and diluted 1:500 in the same buffer. A goat anti-mouse IgG was used to detect GABARB and was conjugated with Alexa Fluor 488 and a goat anti-rabbit IgG coupled with Alexa Fluor 568 was employed for indirect visualization of SST. Immunostaining was visualized by confocal microscopy using a Leica SP2 AOBS. For comparison of tissue from controls vs. treated animals, gain and offset were identical for each assay.

EGFP GABAergic interneurons for analysis of alterations in the inhibitory system

To better elucidate how specific interneuronal subtypes contribute to both normal and pathological brain functions, it is essential that these neurons be easily identifiable during experimental manipulations. To facilitate the study of GABAergic inhibitory interneurons, we used transgenic mice that selectively express the enhanced derivative of the auto-fluorescent protein, green fluorescent protein (EGFP) in a subpopulation of GABAergic neurons (Fig. 7). In these mice, an upstream regulatory region from the murine Gad1gene (Oliva et al. 2000), which codes for the 67 kDa form of GAD, was used to drive EGFP expression, as this gene appears to be ubiquitously expressed in GABAergic neurons. Thus, by examining the pattern of EGFP expression, we can directly observe changes that occur in GABAergic neurons, which serve to validate the biochemical observations. In these mice, induction of hippocampal and cortical EGFP expression was found to begin at approximately postnatal day 5 (Oliva et al. 2000). This developmental onset of EGFP expression temporally coincides with the terminal differentiation of GABAergic interneurons and the onset of expression of many of the macromolecules (e.g., somatostatin) that delineate subpopulations of mature GABAergic neurons (Naus et al. 1988; Bergmann et al. 1991; Jiang and Swann 1997).

Analysis of the number of GAD/EGFP neurons in the brain

Genotypes of the male mice were determined by PCR. The brains of GAD/EGFP males were prepared and sectioned in the coronal plane. Sections separated by 180 μm spanning the whole brain were stained with a fluorescent red Nissl stain to facilitate identification of brain structures. Images were captured at low magnification (largest field of view) with a Zeiss Axioskop2 microscope equipped with AxioCam MRc5 digital camera. The number of EGFP-expressing neurons in each image was counted by individuals who were blind to the genotype of the mice.

Slice preparation

Hippocampal slices (400 μm) were prepared using an automated Leica tissue chopper. Artificial cerebrospinal fluid (aCSF) consisted of the following composition (mM): NaCl 124, KCl 3, $CaCl_2$ 2.4, $MgSO_4$ 1.3, NaH_2PO_4 1.25, $NaHCO_3$ 26, and glucose 10 (gassed with 95 % O_2/5 %

CO_2, pH 7.4). Slices were prepared on ice-cold aCSF and calibrated for 45 min at RT in oxygenated aCSF prior to recording.

Recording and stimulation

Extracellular recordings of evoked field excitatory post-synaptic potentials (fEPSPs) were measured by placing the stimulating bipolar platinum electrode in the perforant path close to the suprapyramidal blade of the DG in the outer third of the molecular layer. Recording electrodes (tip impedance 1–5 MΩ) were placed in the middle or outer of the molecular layer of CA1 to record fEPSPs. This stimulation paradigm allowed us to assess both the evoked responses and the overall integrative properties of the whole hippocampus. Stimulation parameters for test pulses (0.05 Hz) were standardized across slices by setting current and pulse width to evoke a fixed percentage of maximal EPSP amplitude or slope in any given data set. Similarly, stimulus intensity for plasticity induction was standardized for all data sets. The current that produced about 40 % of maximal responses (usually 100–150 μA) was used throughout the experiment for monitoring test pulses. Data were sampled at 100 kHz and band-pass filtered between 0.3 Hz and 3 kHz. All extracellular voltage inputs were amplified X 500.

RNA preparation

RNA was prepared from tissue samples as described in the manufacturer's manual (TRIzol Reagent; Invitrogen 15596-026). Briefly, tissue samples were homogenized in 1 ml of TRIzol Reagent per 100 mg of tissue using a glass-Teflon for 1 h. After centrifugation, RNA was extracted with chloroform and precipitated with isopropyl alcohol. Finally, samples were re-suspended in 100 μl of diethyl-pyrocarbonate (DEPC)-treated H_2O.

Preparation of cDNA and real-time PCR analysis

Equal amounts (10 μg) of total RNA were treated with RNase-free DNase (Qiagen cat.#79254) at 37 °C for 1 h, and purified by phenol/chloroform (3:1) extraction and ethanol precipitation. 1 μg of pure RNA was used in SYBR GreenER Two-Step qRT-PCR kit (Invitrogen cat#11765-100) for the first strand cDNA synthesis and real-time PCR preparation as described in manufacture's manual.

The real-time PCR primers are described in the Table 1. All experiments were repeated twice, and in each experiment, PCRs were done in triplicate in a 7500-sequence detection system (Applied Biosystems). Target DNA sequence quantities were estimated as described previously (Ford et al. 2007; Zhang et al. 2009; Wimalarathna et al. 2011; Hong et al. 2012). Briefly, target DNA sequence quantities were estimated from the threshold amplification cycle number (CT) using Sequence Detection System software (Applied Biosystems). A ΔCT value was calculated for each sample by subtracting their CT value from the CT value for the corresponding GAPDH to normalize the differences in cDNA aliquots. Each relative mRNA level was then expressed as $2^{(-\Delta CT)} \times 100$ % of GAPDH.

Table 1 Oligonucleotides used in the real-time qRT-PCR

Oligonucleotides	Sequence
GAPDH *ORF*	
Forward primer	5′-ACAGGGTGGTGGACCTCATG-3′,
Reverse primer	5′-GTTGGGATAGGGCCTCTCTTG-3′
$GABA_A$ β3 *ORF*	
Forward primer	5′-CCACGGAGTGACAGTGAAAA -3′
Reverse primer	5′-CACGCTGCTGTCGTAGTGAT-3′
GAD65 *ORF*	
Forward primer	5′-GGTCAACTTCTTCCGCATGGT-3′
Reverse primer	5′-TGTCCGAGGCGTTCGATT-3′
SST *ORF*	
Forward primer	5′-GAG CCC AAC CAG ACA GAG AA-3′
Reverse primer	5′-GAA GTT CTT GCA GCC AGC TT-3′

Statistical analysis

All values are presented as mean ± SEM. Differences between pairs of means were addressed by the two-tailed Student's *t* test. Analysis of variance was used followed by the Tukey HSD post hoc test, when necessary, for multiple comparisons. Differences were considered significant for $p < 0.05$.

Results

Age-dependent decline in learning

As taurine is a $GABA_A$ receptor agonist and senescence is characterized by a decline in GABAergic neurotransmission, we supplemented taurine in drinking water to determine if chronic taurine intake alleviates the age-dependent decline in cognitive function. Using the passive avoidance paradigm, we tested the ability of mice to learn to avoid an electrical shock. Our assays were performed in young (2 months) and old (16 months) mice. Figure 1 represents the mean performances (expressed in the latency to foot shock) of young and old mice during the last days of training (day 5). Old control mice preformed significantly lower ($p < 0.001$) than young mice, indicating a lower degree of learning. Supplementation of taurine had no

significant effects on young mice, presumably due to the abundance of GABAergic neurons in the hippocampus. However, aged mice supplemented with taurine showed a significant increase ($p < 0.001$) in learning when compared to age-match controls. Furthermore, old mice supplemented with taurine approached learning levels observed in the young group, with no significant difference observed between the taurine-treated old mice and the young controls (Fig. 1). These data indicate that taurine supplementation to old mice had a beneficial role in learning related to this specific task. The lack of effect in the young group could be due to the abundance of GABAergic neurons and represents a ceiling effect of the test.

Taurine prevents the age-dependent decline in spatial learning

Aging is associated with Ca^{2+} dysregulation and decline in GABAergic system. Both of these functions are regulated by taurine. We explored whether taurine could reduce aging-related cognitive decline. We evaluated spatial position learning using cross-shaped water maze. The cross maze is a visuospatial place-learning task in which the mice can use visual cues to learn the location of an escape platform. Although the two tests have similarities, the brain regions specific for learning the cross maze have not been determined and may well be different. In the cross maze, the alternative paths are much more restricted than in the Morris maze, there is a strong "right and wrong" aspect to the test and errors can be scored. Behavioral features that may not be apparent in the Morris maze may be revealed in cross maze analysis.

Using the cross-shaped water maze as an assay for spatial learning, we found that in the 8 month-old group taurine significantly ($p < 0.0001$) improved both acquisition and retention.

In the 16-month-old group supplementation of taurine significantly ($p < 0.0001$) improved retention but no acquisition of spatial place learning (Fig. 2). Together, the data suggest that taurine treatment a) has no detrimental effects on the young mice and may possibly improve learning, and b) provides a benefit to older mice especially in retention of previously learned memory tasks.

Taurine-fed mice have elevated levels of glutamate decarboxylase

Because glutamate is a precursor for GABA biosynthesis, this concomitant increase in the level of both neurotransmitters was accompanied by a significant ($p < 0.001$) increase in the expression of both isoforms of glutamate decarboxylase (GAD), the enzyme that catalyzes the

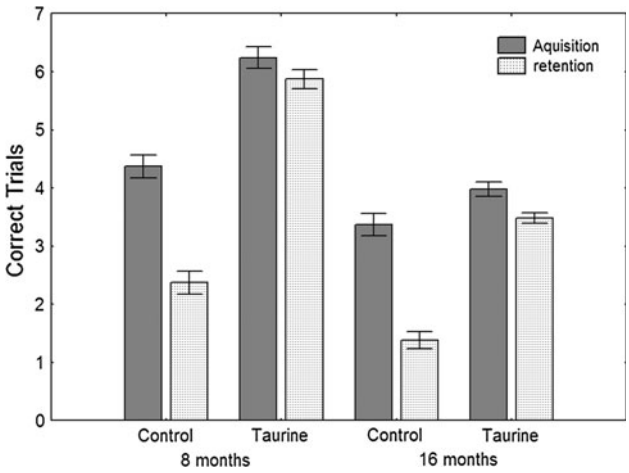

Fig. 2 Taurine improves hippocampal-dependent spatial learning and memory: bars represent the mean ± SEM of correct responses (finding the hidden platform) on the last day of acquisition and the day of retention (4 weeks later). A three-way analysis of variance (ANOVA) showed that there was significant effect of age [$F(1,484) = 172.12$, $p < 0.0001$], a significant effect of treatment [$F(1,484) = 302.31$, $p < 0.0001$] and a significant effect of the memory tested (acquisition *vs* retention) [$F(1,484) = 112.29$, $p < 0.0001$]. The interaction between age and treatment was significant [$F(1,484) = 43.963$, $p < 0.0001$]. The interaction between age and memory tested was not significant [$F(1,484) = 21739$, n.s.]; however, the interaction between age and treatment was significant [$F(1,484) = 43.963$, $p < 0.0001$]. Finally, the interaction between age × treatment × memory did not reach significance [$F(1,484) = .71025$, n.s.]. Tukey HSD post hoc test revealed that the 16-month mice controls performed significantly ($p < 0.05$) lower than 8 months controls in both acquisition and retention tasks. Taurine significantly ($p < 0.0001$) improved acquisition in the 8 months group but not in the 16 months. However, taurine significantly ($p < 0.0001$) improved retention in both age groups. There were 7 control and 7 taurine mice at 8 months, and 13 controls and 14 taurine animals at 16 months

conversion of glutamate to GABA, as shown by western blotting (Fig. 3). We also examined the mRNA levels for GAD65 in response to taurine supplementation and found that taurine leads to a significant increase in GAD65 gene expression (Fig. 4). Taurine results in the transcriptional up-regulation of GAD65 in the cortex, cerebellum, brain stem and diencephalon ($p < 0.05$). However, the GAD65 gene expression in the hippocampus was not affected by chronic taurine treatment.

During aging, decreased GAD expression and the resulting bioavailability of GABA are consistent with previous observations and constitute a potential substrate for the decline in cognitive functions. Since taurine reversed the age-dependent decrease in GAD expression and GABA levels, it is plausible to speculate that taurine supplementation may reverse the GABA-dependent decrease in cognitive function observed with aging through prevention in the decline of GABA levels observed with aging.

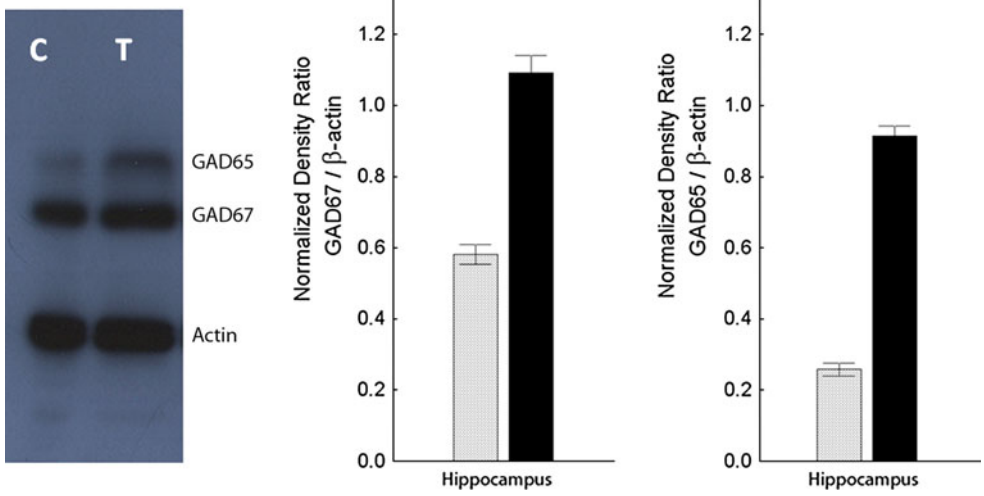

Fig. 3 Representative Western blot showing the expression levels of (GAD 65 and 67) in the hippocampus: Densitometry analysis showed that the expression level in taurine-fed mice is 66 ± 8 % (mean ± SEM) higher as compared to controls ($p < 0.005$) when normalized to actin expression. $n = 4$ in each group

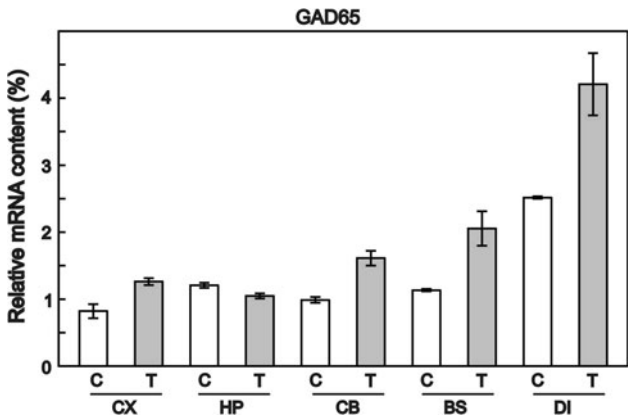

Fig. 4 Taurine supplementation up-regulates GAD65 mRNA expression levels: The relative expression levels were 0.82 ± 0.11, 1.21 ± 0.04, 0.99 ± 0.04, 1.13 ± 0.02, and 2.52 ± 0.02 % under control conditions for cortex (CX), hippocampus (HP), cerebellum (CB), brainstem (BS) and diencephalon (DI), respectively. The relative expression levels were 1.26 ± 0.05, 1.05 ± 0.04, 1.61 ± 0.11, 2.05 ± 0.26, and 4.21 ± 0.46 % under Taurine conditions for CX, HP, CB, BS and DI, respectively. Supplementation with taurine resulted in the transcriptional up-regulation of GAD65 at CX, CB, BS and DI region, but not at HP region

$GABA_A$ receptor expression in the hippocampus of taurine-fed mice

To investigate further biochemical alterations of the inhibitory system in taurine-fed mice, we analyzed $GABA_A$ receptor expression in the hippocampus from untreated controls and taurine-fed mice with a monoclonal antibody that recognizes the β3 subunits of the $GABA_A$ receptor. When we compared the expression levels in the hippocampi of taurine-fed mice and untreated age-matched controls, we found a reduction in β3 subunits expression by both IHC and western blotting (Fig. 5). Expression of these subunits was significantly reduced by approximately 35 % in the hippocampus (Fig. 5b). We also examined the mRNA levels for the β3 subunit of the $GABA_A$ receptors in response to taurine supplementation and found that taurine leads to a significant decrease in β3 subunit gene expression (Fig. 6). Taurine treatment resulted in the transcriptional down-regulation of β3 subunit in all brain regions examined except the diencephalon (Fig. 7).

Expression of EGFP in taurine-fed mice

Using these transgenic mice, we tested if the observed increase in GAD expression was due to an actual increase in the number of EGFP-, and thus, GAD 67-positive neurons. To answer this question, we supplemented taurine (0.05 %) in the drinking water for 4 weeks and counted the number of EGFP-positive neurons in the cortex of these mice. We selected cortical coronal sections because of the relatively constant surface area from section-to-section within the coronal plane. We counted the number of EGFP-positive neurons in approximately 100 coronal sections from each mouse comprising the parieto-temporal lobes. We used for this study four pairs of taurine-fed and age-matched controls (siblings). Figure 8 shows that taurine-fed mice have a significant increase in the number of EGFP-positive neurons in the cortex compared to age-matched controls.

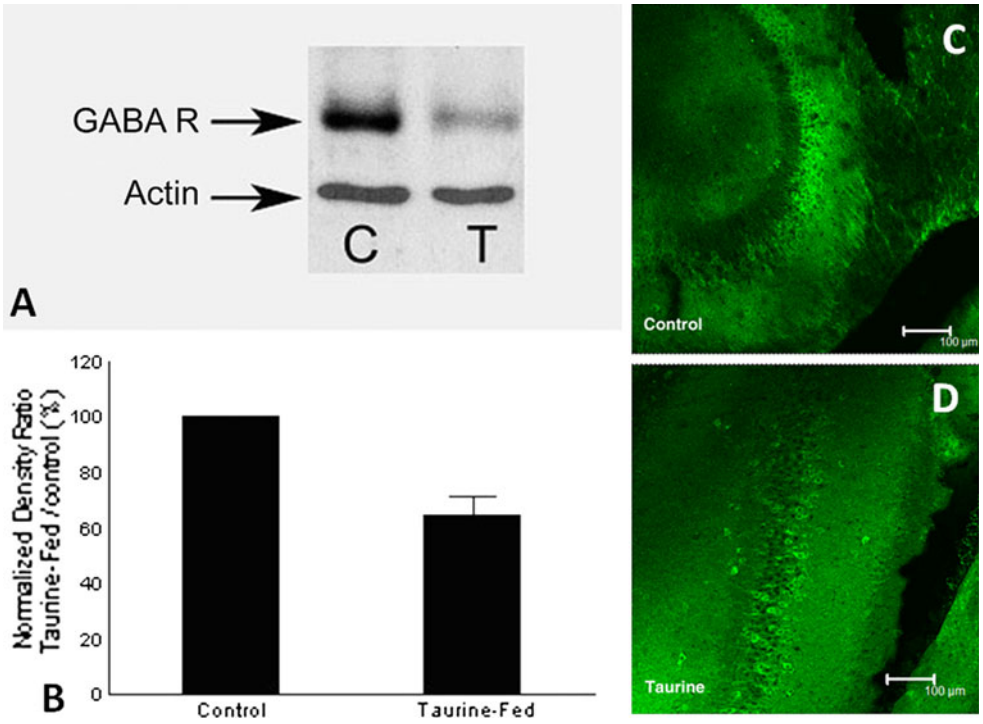

Fig. 5 Taurine reduces $GABA_A$ β3 subunit expression: **a** Representative western blot of $GABA_A$ receptor β3 subunit and β actin expression in the hippocampus of (C) untreated control and (T) taurine-fed mice. All mice were tested at 2 months of age. Taurine was supplemented in water for 4 weeks before dissecting the brains. **a** Western blots were probed sequentially with a mouse monoclonal antibody that recognized the β3 subunits of the $GABA_A$ receptor and a monoclonal antibody that recognized β actin. **b** Densitometry analysis showed that the expression level in taurine-fed mice is 35 ± 11 % (mean ± SEM) lower as compared to untreated controls when normalized to actin expression ($p < 0.05$). Four FVB/N controls and four littermates of taurine-fed mice were analyzed in this study. Samples were obtained from the same hippocampi used to determine GAD expression (Fig. 3). **c** and **d** Representative images of hippocampi showing $GABA_A$ receptor β3 subunit immunoreactivity in the CA3 region. Reduced immunoreactivity in brains from taurine-fed mice. *Calibration bar* 100 µm

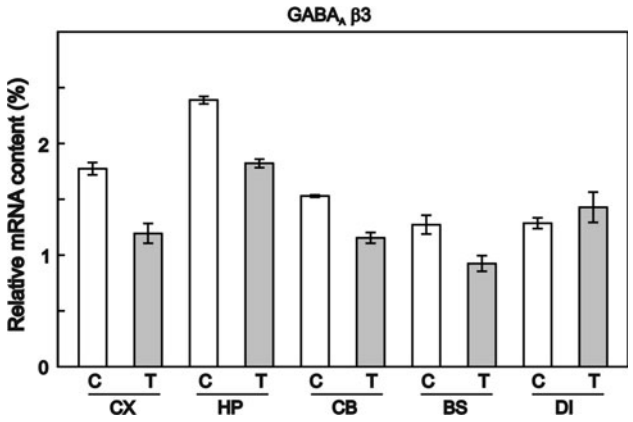

Fig. 6 Taurine supplementation down-regulates $GABA_A$ receptor β3 subunit mRNA expression levels: The relative expression levels were 1.77 ± 0.05, 2.39 ± 0.03, 1.53 ± 0.01, 1.27 ± 0.08, and 1.29 ± 0.05 % under control conditions for CX, HP, CB, BS and DI, respectively. The relative expression levels were 1.19 ± 0.09, 1.82 ± 0.04, 1.15 ± 0.05, 0.92 ± 0.07, and 1.43 ± 0.14 % under Taurine conditions for CX, HP, CB, BS and DI, respectively. Chronic supplementation of taurine resulted in the transcriptional down-regulation of $GABA_A$ β3 in CX, HP, CB, and BS regions, but not at DI region

What can these EGFP-positive neurons tell us about the brains of taurine-fed mice?

The increased expression of EGFP-positive neurons in the brains of taurine-fed mice may indicate a GABAergic marker whose expression is altered by taurine. To determine the identity of this marker, we used fluorescence immunohistochemistry for numerous markers of GABAergic interneurons and found that somatostatin expression was significantly increased in taurine-fed as compared to control mice (Fig. 9).

Further evidence for the interaction of taurine with the somatostatinergic system was obtained from experiments measuring SST gene expression in response to chronic taurine supplementation. We found that when mice were supplemented with taurine, SST gene expression was significantly up-regulated (Fig. 10) in the cortex, diencephalon, brainstem and cerebellum. However, the expression levels were decreased in the hippocampus. The differential effect of taurine on SST mRNA in the hippocampus and the discrepancy between increased protein expression and

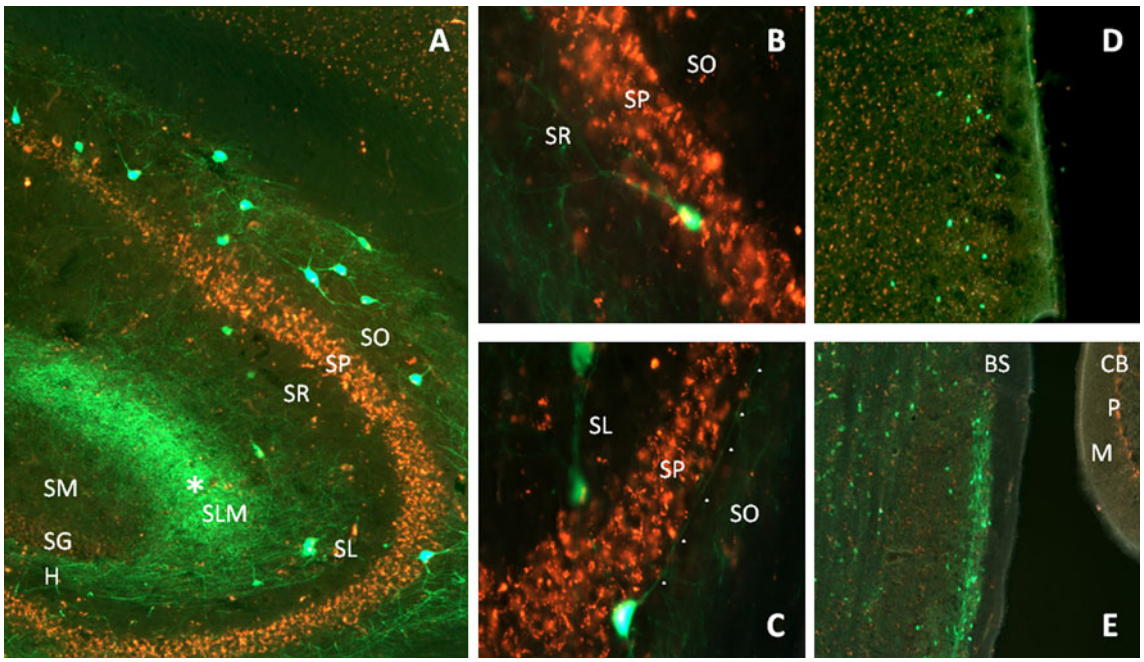

Fig. 7 Pattern of EGFP expression found in the brain of 2 months old EGFP transgenic mice. **a** Low magnification image of the hippocampus, asterisk denotes the plexus of EGFP-expressing axonal terminals in SLM (stratum lacunosum-moleculare) of area CA3. Cells in SO (stratum oriens) of CA1 (**b**), and area CA3 (**c**), both show processes that can be followed for a long distance from the cell body (*dots*). **d** Photomicrograph illustrating the pattern of EGFP expression in the somatosensory cortex, which is typical of all cortical areas. The laminar specificity of EGFP expression in the primary cortex is illustrated in this 30-µm-thick section: EGFP-expressing somata are restricted mainly to layers II–IV and upper layer V. **e** Pattern of EGFP expression in the *BS* brainstem and *CB* cerebellum. At 2 months old, the cerebellum does not show any EGFP-positive neurons. Additional abbreviations: *SP* stratum pyramidale, *SR* stratum radiatum, *SM* stratum moleculare, *SG* stratum granulosum, *H* hilus of the dentate gyrus, *SL* stratum lucidum, *GL* internal granule cell layer, *P* Purkinje cell layer, *ML* external molecular layer. These are images of a 30-µm-thick sagittal brain section from a homozygous mouse. It should be noted that the EGFP fluorescence shown in these images is intrinsic fluorescence and not the product of a fluorophore-labeled antibody immunoreaction. All sections were stained with a red fluorescent Nissl stain (Molecular Probes) to facilitate visualization of neuronal structures. Images were captured with Zeiss Axioskop2 microscope equipped with AxioCam MRc5 digital camera. Magnifications: **a**, **d** and **e** ×10; **b** and **c** ×40 dry objective

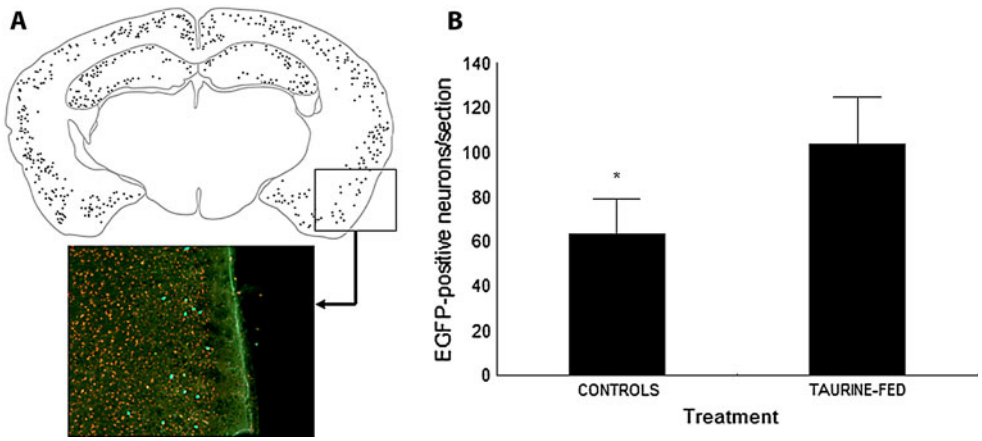

Fig. 8 Taurine induces an increase in the number of EGFP-positive neurons. **a** Laminar specificity of EGFP expression demonstrated in primary cortex from a 30-µm-thick section. This expression pattern typifies the one seen in all cortical areas. EGFP-expressing somata are restricted mainly to layers II–IV and upper layer V. Magnifications ×10 objective. **b** Comparison of EGFP expression in control and taurine-fed mice. Bars represent the mean ± SD of EGFP-positive neurons obtained from four controls and four taurine-fed mice ($n \approx 400$, 100 sections per brain). The increase in the number of EGFP-positive neurons in the taurine-fed mice was significant ($p < 0.05$)

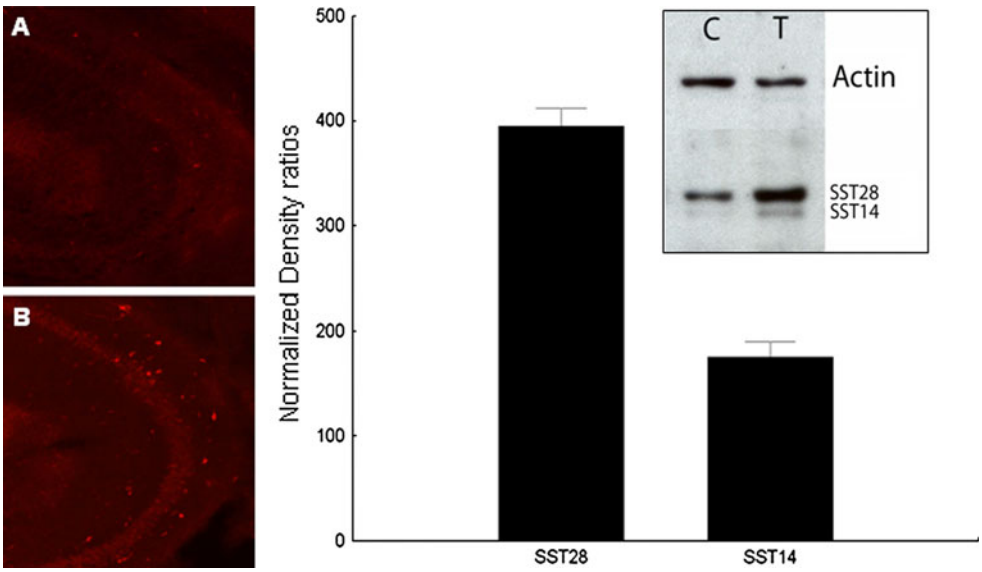

Fig. 9 Somatostatin Immunoreactivity in the hippocampus: Representative images from the hippocampus from control (**a**), and taurine-fed mice (**b**) showing SST immunoreactivity in SST-positive interneurons. These are images of a 30-µm-thick sagittal brain cryosections showing CA3 region of the hippocampus. The level of SST expression in the hippocampi of controls and taurine-fed mice was analyzed by WB. Representative WB showing the expression levels of SST (both isoforms). Taurine supplementation resulted in a fourfold increase in SST28 and ~twofold increase in the SST protein levels

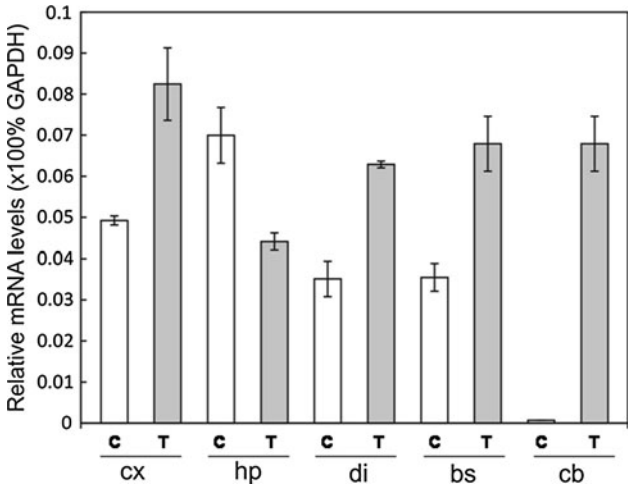

Fig. 10 Taurine supplementation up-regulates SST mRNA expression levels. Supplementation of taurine to mice resulted in a global transcriptional up-regulation of SST gene expression. Interestingly, the hippocampus showed a reduction in SST gene expression in the presence of taurine, whereas the cerebellum showed the highest level of up-regulation. Consistent with the protein levels, control mice had very reduced SST expression in their cerebellum

decreased mRNA levels in response to taurine is not currently understood and needs further investigation.

It is interesting, however, to see an increase in the number of somatostatin-positive neurons within this short period of treatment with taurine (i.e., 4 weeks). This timing is inconsistent with neurogenesis, migration and differentiation. Thus, we suggest that treatment with taurine caused some GAD-positive neurons to additionally acquire the new phenotype of somatostatin expression. We suggest that the resulting dual phenotype (GAD and somatostatin) after taurine supplementation is a compensatory mechanism to increase excitability and reduced expression of GAD observed during aging. Somatostatin plays an important role in modulating neuronal excitability and the population of somatostatin-containing neurons has been shown to decline with age (Kuwahara et al. 2004a). Therefore, this funding further suggests that taurine may be beneficial in opposing the neurochemical alterations observed during the brain aging process.

Chronic taurine feeding increases hippocampal excitability

To further characterize the effects of taurine on the GABAergic system, we electro-physiologically investigated the evoked potentials of CA1 hippocampal pyramidal cells in response to afferent Schaeffer collateral fibers stimulation since the hippocampus is a very important neuronal substrate for learning and memory. We primarily focused on the bursting activity recorded extracellularly in the stratum pyramidale of CA1 since such data represent the best measure of the level of synchrony in the local area network. Orthodromic stimulation of the Schaefer collaterals in hippocampal slices from control mice evoked a single population spike (Fig. 11). Whereas stimulation of hippocampal slices from taurine-fed mice evoked a typical epileptiform burst composed of 4–5 population spikes, similar to those recorded from hippocampal slices of mice treated with kainic acid (Bernard and Wheal 1995).

These electrophysiological data from hippocampal slices of taurine-fed mice show increased hippocampal excitability and suggest a lower threshold for LTP induction. This in turn might explain the improved acquisition and recall in taurine-fed mice.

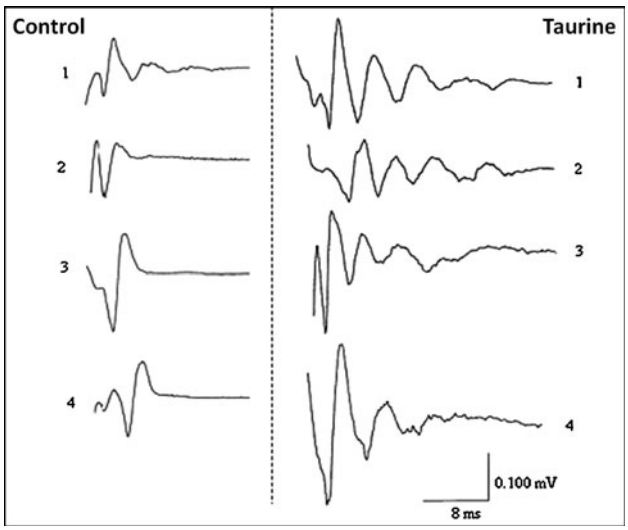

Fig. 11 Chronic taurine feeding increased hippocampal excitability. The figure shows four different traces (*1*, *2*, *3* and *4*) that were recorded from hippocampal slices. The slices were obtained from eight different animals—four controls shown on the left and four taurine-fed shown on the right. The traces are for hippocampal population spikes that were recorded from CA1 and were evoked by stimulating Schaefer collaterals. The population spikes duration and amplitude were significantly increased in the taurine-fed mice

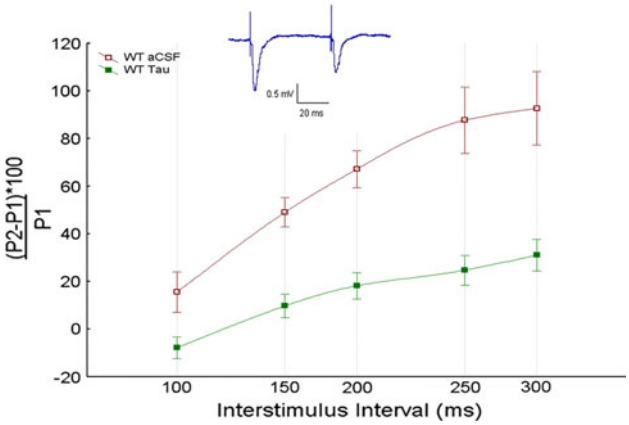

Fig. 12 Taurine affects paired pulse facilitation in the hippocampus: Paired pulse stimulation at inter-stimulus intervals (ISI) of 100 and up to 300 ms consistently yielded facilitation as determined by the increase in the second EPSP (p2) compared to the first (p1). Addition of taurine (20 μM) to slices resulted in a consistent depression of the second evoked response at all ISI used. Each data point represents mean ± SEM of EPSP obtained following the paired pulse stimulation and expressed as [(p2-p1)/p1 × 100]. Data were obtained from 3 brains in each group and at least 5 slices per brain

Taurine enhances short-term plasticity in the hippocampus

To confirm our previous findings on taurine's role as a GABA agonist, we tested the role of taurine in electrophysiological responses in the hippocampus. In hippocampal slices, paired pulse stimulation at inter-stimulus intervals (ISI) of 100 ms and up to 300 ms consistently yielded facilitation (Fig. 12) as determined by the ratio of the evoked response to the second stimulus (p2) compared to the first stimulus (p1). Consistent with the agonistic role of taurine on $GABA_A$ receptors, bath application of taurine (20 μM) resulted in a paired pulse depression of the responses, confirming our previous work on taurine's role in synaptic plasticity (El Idrissi et al. 2010). As a $GABA_A$ taurine application to hippocampal slices resulted in activation of $GABA_A$ receptors and hyperpolarization of $GABA_A$ receptors-expressing neurons, which comprise all principal cells of the hippocampus. This leads to reduced threshold for neuronal firing. Thus, under these conditions paired pulse stimulation resulted in a depression of neuronal response.

Discussion

We found an age-dependent decline in learning and memory as measured by acquisition and retention of a passive avoidance task and cross-shaped water maze between young and old mice. Taurine supplementation in drinking water significantly increased the performances of aged mice as compared to untreated controls. Furthermore, we found several biochemical changes in the brain of taurine-fed mice that oppose those induced by the naturally occurring aging process. The brains of taurine-fed mice have elevated levels of both the excitatory and inhibitory neurotransmitters (glutamate and GABA, respectively; El Idrissi and Trenkner 2004) and the GABA synthesizing enzyme, glutamate decarboxylase (GAD). The levels of somatostatin and somatostatin-positive neurons were increased in the hippocampus and cortex of taurine-fed mice. These biochemical changes are contrasting those naturally induced by aging and suggest that taurine improves learning and memory in aged mice through amelioration of the age-dependent decline in GABAergic function. Interestingly, electrophysiological recordings from hippocampal slices prepared from the brain of taurine-fed mice showed an increase in the amplitude and duration of population spikes recorded from CA1 in response to Schaefer collateral stimulation. Such increased excitability of hippocampal slices of taurine-fed mice is consistent with lower threshold for LTP induction (El Idrissi et al. 2010), which would explain the increased learning in these mice. These observations indicate that taurine might improve learning and retention in aged mice through up-regulation of GABAergic function. However, several issues remain to be resolved. There were no differences in the young group between taurine-fed and controls. This is possibly because of the ceiling effect observed

at young ages on the behavioral tests we employed. Taurine, on the other hand, induced several biochemical changes to the inhibitory GABAergic system at early ages that could be beneficial in aging. The main question, therefore, is at what time in development we have to intervene to forestall the deterioration, and improve the function of the GABAergic system so we can see a benefit on cognitive functions at old age.

GAD, the enzyme responsible for GABA synthesis in GABAergic neurons, has two isoforms, 65 and 67 kDa (GAD65 and GAD67), encoded by different genes (Erlander et al. 1991). The expression of both isoforms has been shown to be activity-dependent (Ramirez and Gutierrez 2001; Nishimura et al. 2001) and to be influenced by the effectiveness of GABAergic inhibition (Ribak et al. 1993). We have previously shown that this decrease in $GABA_A$ receptor expression had a functional significance on seizure threshold (El Idrissi et al. 2003). We found that taurine-fed mice were susceptible to pharmacologically induced seizures mainly with kainic acid (Huxtable 1992) and resistant to isoniazide-induced seizure (El Idrissi and L'Amoreaux 2008) an inhibitor of GAD activity. These pharmacological observations landed support for the biochemical observations in terms of changes in protein expression.

Since reduced $GABA_A$ receptor expression would increase excitability, the increased GAD expression could be a compensatory mechanism for reduced efficacy of the inhibitory system. This is particularly interesting because increased GAD can be a compensatory response to the increased excitability (El Idrissi and Trenkner 2004; Ramirez and Gutierrez 2001) that would be the net result of decreased GABAergic inhibition.

The concomitant increase in glutamate with GABA in the brain of taurine-fed mice might also explain the reduced threshold for seizure induction. If there is more glutamate release (following depolarization with kainic acid), the excess glutamate will lead to sustained activation of glutamate receptors resulting in high-frequency discharge that typifies seizure activity. Alternatively, chronic taurine treatment may facilitate seizures through desensitization of the inhibitory system, primarily through $GABA_A$ receptors. Chronic-elevated concentrations of taurine in the brain would lead to a desensitization of $GABA_A$ receptors or a down-regulation of their expression. Consistent with this we found that chronic treatment with taurine resulted in a down-regulation of the expression of the β subunit of the $GABA_A$ receptors. Therefore, taurine modulates seizure threshold through the interaction with $GABA_A$ receptors and chronic interaction may lead to functional modifications in the GABAergic system. Furthermore, we found that the taurine content of the brain was similar between taurine-fed and control mice (El Idrissi et al. 2003). These findings indicate that taurine levels in the brain are highly regulated but might be differentially compartmentalized between intracellular, extracellular, neuronal and non-neuronal cells. We suggest that taurine-fed mice have elevated extracellular taurine levels, which would lead to sustained activation or at least binding to $GABA_A$ receptors. Such a chronic interaction of taurine with $GABA_A$ receptors may lead to down-regulation of $GABA_A$ receptor function or expression. In response to these changes, there is increased synthesis of GABA by GABAergic neurons, as compensatory mechanism to reduced post-synaptic inhibition. Consistent with this, brains of taurine-fed mice have the highest GABA content, increased GAD expression and reduced expression of the β subunit of the $GABA_A$ receptors.

The use of transgenic mice that selectively express the enhanced derivative of the auto-fluorescent protein, green fluorescent protein (EGFP) in a subpopulation of GABAergic neurons allowed us to further investigate these biochemical changes in the GABAergic system. In these mice, an upstream regulatory region from the murine *Gad1* gene (Oliva et al. 2000), which codes for the 67 kDa form of the GABA synthesizing enzyme, GAD, was used to drive EGFP expression, as this gene appears to be ubiquitously expressed in GABAergic neurons. Thus, by examining the pattern of EGFP expression, we can directly observe changes that occur in GABAergic neurons, which will serve to validate the biochemical observations and will allow us to determine the type of neuro-architectural changes that occur in response to hypersensitivity. Since reduced $GABA_A$ receptor expression leads to increased excitability, one would expect an increase in GAD expression as a compensatory mechanism for reduced efficacy of the inhibitory system.

Interestingly, however, is the finding that taurine supplementation resulted in an increase in the somatostatin-positive neurons. The expression of somatostatin has been shown to decline during aging and it plays a crucial role in brain excitability and function. Electrophysiological studies in the hippocampus have shown that somatostatin has inhibitory actions on the spontaneous activity of pyramidal cells and that bath application of the peptide induces dendritic hyperpolarization (Schwarzer et al. 1995). Somatostatin-containing neurons in the hippocampus play an important role in hippocampal excitability and epilepsy. Somatostatin suppressed chronic susceptibility to kainic acid seizures in rats (Perez et al. 1995). Hippocampal somatostatin also retards the acquisition of generalized seizures in electrically kindled rats (Monno et al. 1993). Somatostatin is preferentially released from neurons during seizures (Bartfai et al. 1988; Hokfelt 1991; Vezzani et al. 1993), and marked changes in the expression of somatostatin mRNA, the levels of the peptide and its receptors

occur after experimentally induced seizures and in human epileptic tissue (Laming et al. 1989; Paul et al. 1981). Intracerebral injections of somatostatin, its analogues or somatostatin-specific antibodies affect seizures and epileptogenesis in rats (Vezzani and Hoyer 1999). Furthermore, there is an inverse relationship between somatostatin content in the entorhinal cortex and interictal paroxysmal activity in the hippocampus of human epileptics (Deutch et al. 1991), suggesting that this peptide has inhibitory action on seizures, through modulation of recurrent excitation, similar to that described in the rat hippocampus (Manfridi et al. 1991; Monno et al. 1993; Perez et al. 1995). This is consistent with the data presented here, showing that somatostatin expression and the number of somatostatin-immunoreactive cells are up-regulated in taurine-fed, which show hyper-excitability.

Somatostatin mediates its inhibitory modulatory effects on synaptic transmission through inhibition of ionic currents, in that it decreases the probability of a neuron firing an action potential. In rat CA1 hippocampal pyramidal neurons, somatostatin has inhibitory action, including hyperpolarization of the membrane at rest through increase in the voltage-insensitive K + leak current. Somatostatin also augments the voltage-sensitive K^+ M current (Moore et al. 1988). In these cells, somatostatin inhibits N-type Ca^{2+} current (Ishibashi and Akaike 1995), thus reducing the probability of neurotransmitter release. Indeed, somatostatin reversibly depresses evoked EPSCs and reduced the frequency of miniature EPSCs (Sun et al. 2002).

Unlike classical neurotransmitters, release of neuropeptides, including somatostatin, is generally thought to depend on high-frequency neuronal discharge. Thus, somatostatin may be preferentially released from neurons during certain forms of rhythmic oscillations or elevated neuronal activity (Vezzani and Hoyer 1999). This is in turn consistent with the observation of enhanced learning in taurine-fed mice. This could be due at least partially to the reduced LTP induction, increased GAD and somatostatin expression in these mice.

The neonatal brain contains high levels of taurine (Huxtable 1989, 1992; Sturman 1993). As the brain matures its taurine content declines and reaches stable adult concentrations that are second to those of glutamate, the principal excitatory neurotransmitter in the brain. Taurine levels in the brain significantly increase under stressful conditions (Wu et al. 1998), suggesting that taurine may play a vital role in neuroprotection. A possible mechanism of taurine's neuroprotection lies in its calcium modulatory effects (El Idrissi and Trenkner 1999, 2003, 2004) and agonistic role on $GABA_A$ receptors (El Idrissi et al. 2003; El Idrissi and Trenkner 2004).

Taurine plays a role in neurotransmission, but taurine does not satisfy the criteria of a classical neurotransmitter. However, there is increasing evidence supporting a functional interaction between GABA, glycine and taurine (El Idrissi and Trenkner 2004; Kuriyama and Hashimoto 1998). Taurine has been shown to increase plasma membrane chloride conductances by affecting bicuculine-

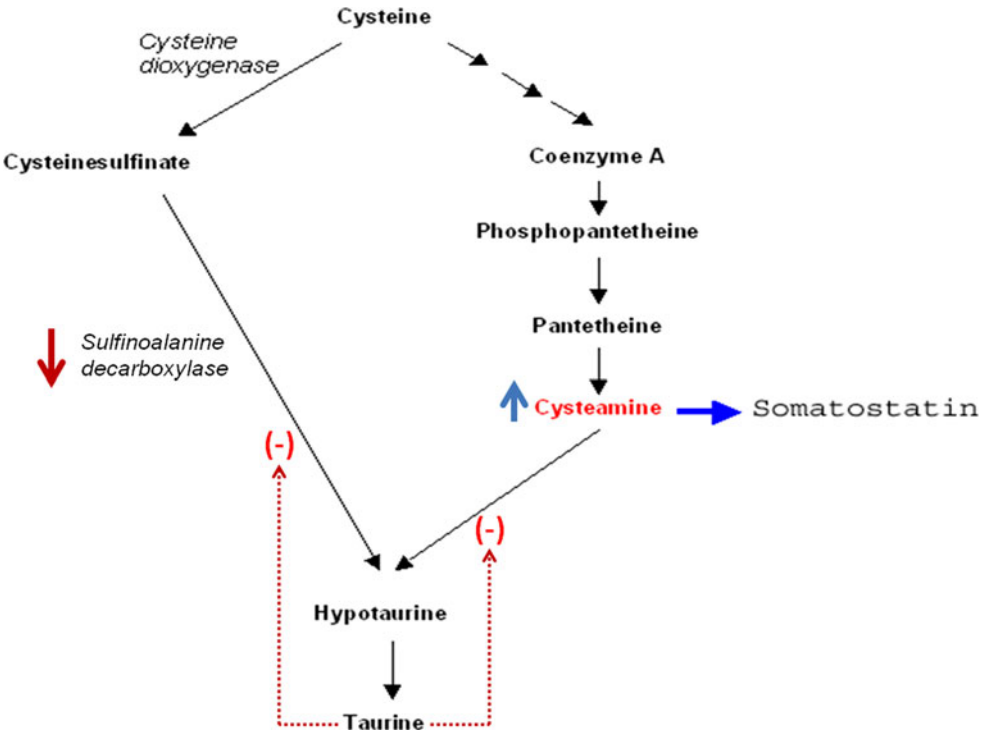

Fig. 13 Alternative pathway of taurine biosynthesis and potential site of product-mediated inhibition: when mice are supplemented with taurine, the endogenous biosynthetic pathways are down-regulated through product-mediated inhibition. Elevated levels of circulating taurine may inhibit the formation of hypotaurine from its precursors cysteinsulfinate and cysteamine. The later is a very potent reducing agent (commercially available as Mercaptamine©) and interacts with SST making it biologically inactive through the breakdown of the two disulfide bonds. Through compensatory mechanisms, SST-positive interneurons upregulate SST expression

sensitive chloride channels (del Olmo et al. 2000; Mellor et al. 2000). Taurine acts as a partial agonist of $GABA_A$ receptors in synaptic membranes (Quinn and Harris 1995). In addition to modulating neuronal transmission, the observed effects of taurine on the up-regulation of somatostatin expression are not well understood and could be mediated at the transcription level. There are two pathways for taurine biosynthesis. The conversion of cysteine into hypotaurine and taurine requires the enzyme sulfinoalanine decarboxylase (CSAD). Supplementation of taurine to mice has been shown to inhibit CSAD both at the mRNA and protein level (J.E. Dominy, personal communication). The alternative pathway for taurine biosynthesis involves the production of cysteamine. Cysteamine is a potentially significant intermediate in taurine biosynthesis (Pitari et al. 2000). The cysteamine moiety is derived from cysteine during co-enzyme A degradation (Fig. 13). Pantetheinase is a ubiquitous enzyme that in vitro has been shown to recycle pantetheine (vitamin B5) and to produce cysteamine. Vanin-1 gene, which encodes pantetheinase, is widely expressed in mouse tissues. Vanin-1 null mice have no detectable free cysteamine, indicating the importance of this pathway in cysteamine production and taurine biosynthesis.

We hypothesize that supplementation of taurine in the drinking water leads to a build-up of circulating cysteamine. Such a build-up would lead to a reduction of the disulfide bonds of the somatostatin making biologically inactive. In response to such a decrease in the levels of biologically active somatostatin, there is an overcompensation of somatostatin expression. This is consistent with the observation that the levels of somatostatin are drastically increased 24 h after a single injection of cysteamine (30 mg/kg; not published). Therefore, chronic supplementation of taurine leads to a build-up of circulating cysteamine, which tends to increase the levels of somatostatin through a compensatory mechanism (Fig. 13).

In summary, aging is responsible for a vast array of neurochemical changes in the brain. The culmination of these factors leads to a decline in cognitive functions as measured by a decline in hippocampal-dependent learning and retention. Through supplementation of taurine in drinking water, we could reverse both biochemical and behavioral parameters affected by aging, suggesting, therefore, that taurine may be beneficial to brain function especially during aging.

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Conflict of interest The authors declare that they have no conflict of interest.

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