Acamprosate reduces ethanol drinking behaviors and alters the metabolite profile in mice lacking ENT1

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Abstract

Acamprosate is clinically used to treat alcoholism. However, the precise molecular functionality of acamprosate in the central nervous system remains unclear, although it is known to antagonize glutamate action in the brain. Since elevated glutamate signaling, especially in the nucleus accumbens (NAc), is implicated in several aspects of alcoholism, we utilized mice lacking type 1 equilibrative nucleoside transporter (ENT1), which exhibit increased glutamate levels in the NAc as well as increased ethanol drinking behaviors. We found that acamprosate significantly reduced ethanol drinking of mice lacking ENT1 (ENT1$^{-/-}$) while having no such effect in wild-type littermates. We then analyzed the basal and acamprosate-treated accumbal metabolite profiles of ENT1$^{-/-}$ and wild-type mice using *in vivo* 16.4T proton magnetic resonance spectroscopy (MRS). Our data show that basal glutamate+glutamine (Glx), glutamate, glutamine and *N*-acetylaspartatic acid (NAA) levels are increased in the nucleus accumbens (NAc) of ENT1$^{-/-}$ compared to wild-type mice. We then found that acamprosate treatment significantly reduced Glx and glutamine levels while increasing taurine levels in the NAc of only ENT1$^{-/-}$ compared to their saline-treated group while normalizing other metabolite compared to wild-type mice. This study will be useful in the understanding of the molecular basis of acamprosate in the brain.

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Research Highlights

- Acamprosate reduced ethanol drinking in mice lacking ethanol-sensitive ENT1.
- Using 16.4T MRS, we detected increased basal glutamate levels in ENT1 null mice.
- Acamprosate reduced glutamine and Glx levels in the NAc of ENT1 null mice.
- Acamprosate increased taurine levels as well in the NAc of ENT1 null mice.

INTRODUCTION

Acamprosate (Campral®) is a taurine homologue, which has been FDA-approved and is clinically used to treat alcoholism [34]. However, the effect of acamprosate is not uniform in all patients, with some patients responding better to this drug than others [15]. Therefore, understanding the molecular function of acamprosate and how brain metabolites are affected by its administration will help in the prediction of a successful reduction in alcohol drinking in humans [12,18].

Adenosine is one of the main inhibitory neuromodulators that regulates glutamate levels in response to alcohol [1]. An ethanol-sensitive nucleoside transporter, type 1 equilibrative nucleoside transporter (ENT1), regulates adenosine levels in response to acute ethanol treatment [16]. Human genetic association studies indicate that allelic variants of *SLC29A1* (human ENT1) are associated with an alcohol abuse phenotype in women [10]. A number of recent studies also suggest that ENT1 gene expression is inversely correlated with ethanol drinking and sensitivity to the acute intoxicating effects of ethanol in several rodent models [1,5,19,24,25]. Specifically, mice lacking ENT1 (ENT1$^{-/-}$) exhibit reduced ataxic and hypnotic effects of acute ethanol intoxication as well as increased ethanol drinking compared to wild-type littermates [4,5,17]. Based on our previous studies, these behaviors appear to be mediated by constitutively increased glutamate levels in the NAc of ENT1$^{-/-}$ mice [4,5,17]. Since chronic ethanol treatment and withdrawal are known to increase extracellular glutamate levels in several brain regions [22,23], ENT1$^{-/-}$ mice may be used as a model representing adenosine-mediated, constitutively-increased glutamate signaling in alcoholism [4,5,17].

Here we report that acamprosate significantly reduces ethanol drinking in ENT1$^{-/-}$ mice. Using *in vivo* 16.4T [^{1}H] MRS, we investigated the basal and acamprosate treated metabolite profile in the NAc of ENT1$^{-/-}$ mice. Our results indicate that acamprosate may reduce ethanol drinking by normalizing Glx levels and increasing taurine levels in the NAc of ENT1$^{-/-}$ mice.

MATERIALS AND METHODS

ENT1$^{-/-}$ mice were generated as described previously [5]. F1 generation mice were generated by crossing 129X1/SvJ background heterozygous ENT1 mice with C57BL/6J wild-type mice. The C57BL/6J and 129X1/SvJ mice were purchased from the Jackson laboratory (Bar Harbor, ME). The heterozygous F1 mice were then crossed to generate F2 generation mice. Only 8-16 week old male F2 generation homozygote wild-type and ENT1$^{-/-}$ mice were used in all experiments. Animal care and handling procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committees in accordance with NIH guidelines.

The effect of acamprosate on oral alcohol self-administration and preference were examined using a modified two-bottle choice drinking experiment [13]. To examine the effect of acamprosate on ethanol consumption and preference, mice were given 200 mg/kg (*i.p.*) acamprosate (Estechpharma, Korea) every 12 h for four additional days after mice steadily consumed 10% ethanol in a two-bottle choice drinking experiment.

In vivo magnetic resonance spectroscopy (MRS) was performed on a 16.4 T (700 MHz) Bruker Avance III 700WB spectrometer with an 89 mm vertical bore (Bruker BioSpin, Billerica, MA). Mice were anesthetized in an isolated chamber using 3.5% isoflurane. For the basal metabolite level measurements, mice were injected with saline (*i.p.*) and to analyze the effect of acamprosate, mice were given a 200 mg/kg *i.p.* injection of acamprosate. Mice were immediately placed in the anesthesia chamber for 5-10 min following injections and then inserted into the probe for scanning. For both experiments, the mouse typically spent about 95 minutes inside the magnet (5 minutes preparation time and 90 minutes scan time). Accumbal metabolites were quantified using LCModel Version 6.2-2 software [20]. Metabolites were quantified as percent ratio to total creatine (creatine + phosphocreatine) levels as an internal control to standardize for animal and instrumental variations as well as for the total number of cells analyzed within the volume of interest (VOI). Pilot fast low-angle shot (FLASH) images were recorded for placement of an 8 μl ($2 \times 2 \times 2$ mm^3) accumbal VOI [28]. Then, method specific local magnet field homogenization was performed. A point-resolved spectroscopy (PRESS) sequence was used with a repetition time (TR) of 1768 ms, an echo-time (TE) of 10 ms, and a number of averages (NA) of 3072. Consistent spectral resolution was ensured by considering LCModel calculated Cramér-Rao lower bounds. LCModel quantified glutamate + glutamine (Glx) with a Cramér-Rao lower bounds of less than 10%; *N*-acetylaspartatic acid (NAA), glutamate, glutamine, glycerophosphocholine + phosphocholine (GPC + PCh), guanosine, taurine, γ-aminobutyric acid (GABA), *myo*-inositol, and phosphocreatine with a Cramér-Rao lower bounds of less than 20%; and *N*-acetylaspartylglutamic acid (NAAG), alanine and lactate with a Cramér-Rao lower bounds of less than 35% in all spectra.

All data are presented as the mean ± SEM (standard error of the mean). Data were analyzed by two-tailed *t*-test, one-way or two-way repeated measures ANOVA. Results were considered significantly different when $p < 0.05$.

RESULTS

We examined whether acamprosate could reduce ethanol drinking behaviors in ENT1$^{-/-}$ mice in a two-bottle drinking experiment. When analyzing the drinking behaviors of ENT1$^{-/-}$ and wild-type mice we found that acamprosate significantly reduced ethanol consumption (Figure 1B; $n = 16$ per genotype) and preference (Figure 1C; $n = 15$~16 per genotype) of ENT1$^{-/-}$ mice compared to respective saline injection periods in a two-bottle choice drinking experiment. As reported, ENT1$^{-/-}$ mice consume more ethanol than wild-type mice under basal conditions [5]. Thus, to examine the antidipsotrophic effect of acamprosate, we analyzed the data after normalization by baseline. For ethanol consumption, one-way repeated measures ANOVA on ENT1$^{-/-}$ mice indicated a significant effect of acamprosate treatment in ENT1$^{-/-}$ mice ($F_{2,24} = 8.395$, $p = 0.002$) but not in wild-type littermates. For ethanol preference, one-way repeated measures ANOVA on ENT1$^{-/-}$ mice also indicated a significant effect of acamprosate treatment in ENT1$^{-/-}$ mice ($F_{2,22} = 3.712$, $p = 0.041$) but not in wild-type littermates. These data indicate that acamprosate treatment (*i.p.*) is effective in reducing ethanol consumption and preference in ENT1$^{-/-}$ mice but not in wild-type littermates. Additionally, during the post-injection phase (the four days following the acamprosate administration period), ethanol drinking returned to levels similar to that of baseline in ENT1$^{-/-}$ mice.

Since acamprosate is capable of altering the drinking behaviors of ENT1$^{-/-}$ mice, we investigated whether acamprosate treatment could alter the brain metabolite profile of mice lacking ENT1 using *in vivo* proton MRS. Figure 2A is a representation of the position of the 8 μl VOI on 1.0 mm thick coronal and sagittal sections of the mouse brain using a rapid acquisition relaxation enhanced (RARE; RARE factor = 4, TE = 9.6 ms, TR = 4000 ms, NA

= 4) sequence for increased resolution. The data from three consecutive 30 min acquisitions were added together to maximize spectral sensitivity and resolution making the total scan time 90 min (Figure 2B). Spectra were fit using LCModel. Figure 2C is the LCModel best fit metabolite profile of the example spectrum and the residual difference.

In the present study using *in vivo* 16.4T MRS, we measured elevated basal total glutamate levels in the NAc of ENT1$^{-/-}$ mice compared to wild-type littermates (Figure 3A and Table 1). When comparing wild-type and ENT1$^{-/-}$ mice metabolite levels ($n = 10 \sim 12$ per genotype and metabolite), two-tailed *t*-test illustrated that ENT1$^{-/-}$ mice display elevated glutamate +glutamine (Glx; $p = 0.01$), glutamate ($p = 0.04$), glutamine ($p = 0.009$) and NAA ($p = 0.008$) levels in the NAc while other metabolites were not altered (Figure 3B and Table 1). According to our previous work, elevated basal glutamate levels in the NAc might contribute to some of the observed behavioral phenotypes of mice lacking ENT1 including reduced ataxic and hypnotic effects of acute ethanol intoxication as well as increased voluntary ethanol consumption compared to wild-type littermates [5].

Since acamprosate is thought to regulate glutamate levels or neurotransmission [8,14,21,26], we analyzed the effect of acamprosate treatment on the metabolite profile of ENT1$^{-/-}$ mice using *in vivo* 16.4T MRS. We found that acamprosate treatment significantly reduced Glx and glutamine levels and increased taurine and glycerophosphocholine+phosphocholine (GPC+PCh) levels compared to saline treated ENT1$^{-/-}$ mice, while having no such effect in wild-type mice (Figure 3 and Table). When comparing acamprosate treatment and an equivalent volume of saline in wild-type mice, no significant alterations in any metabolite levels were observed (Figure 3 and Table 1). However, when analyzing acamprosate treatment compared to an equivalent volume of saline in ENT1$^{-/-}$ mice, two-tailed *t*-test indicated a significant difference between acamprosate treatment and saline treatment for Glx ($p = 0.003$), glutamine ($p = 0.011$), taurine ($p = 0.003$) and GPC+PCh ($p = 0.024$; Figure 3 and Table 1). Taurine was also significantly increased during acamprosate treatment in ENT1$^{-/-}$ mice compared to acamprosate treatment in wild-type mice ($p = 0.042$). Additionally, two-way ANOVA indicated an effect of genotype ($F_{1,42} = 3.849$, $p = 0.05$) and treatment ($F_{1,42} = 5.215$, $p = 0.028$) without a significant interaction between genotype and treatment. Taken together, these results indicate that reduced Glx and glutamine levels and/or increased taurine levels in the NAc might be important in the regulation of ethanol consumption.

Discussion

Our results indicate that basal accumbal glutamate+glutamine (Glx), glutamate and glutamine are significantly increased in ENT1$^{-/-}$ mice compared to their wild-type littermates. Deletion or inhibition of ENT1 increases extracellular glutamate levels by inhibiting presynaptic adenosine A$_1$ receptors (A1R) [5] and/or astrocytic excitatory amino acid transporter type 2 (EAAT2) [33]. Therefore, it is likely that the increased basal accumbal glutamate levels of ENT1$^{-/-}$ mice compared to wild-type mice measured by MRS are at least in part, owing to increased extracellular glutamate levels. Basal glutamine, as well as Glx, levels were also significantly increased compared to wild-type littermates which may be a result of the astrocytic glutamate-glutamine cycle [1]. We have also found that *N*-acetylaspartatic acid (NAA) is significantly increased in the NAc of ENT1$^{-/-}$ mice compared to wild-type littermates. Some studies suggest that NAA is important as a measure of neuronal function and structural integrity, while reduced levels are implicated in neurodegenerative disorders [29]. Decreased cortical NAA is also correlated with neuronal loss, relative decreased viability, and/or impaired function [9]. In contrast, elevated striatal NAA levels may reflect increased neuronal activity [11] which is consistent with the

observation of increased glutamate excitatory postsynaptic currents in the NAc of ENT1$^{-/-}$ mice [5].

Acamprosate treatment successfully antagonized glutamate/glutamine signaling in the NAc of only ENT1$^{-/-}$ mice. Since ENT1$^{-/-}$ mice appear to mimic either a chronic ethanol treated or withdrawn status, it is possible that an addictive phenotype such as elevated glutamate levels is required for acamprosate to be effective [14,27]. Consistently, in clinical studies, acamprosate treatment has been shown to reduce glutamate levels in the anterior cingulate of detoxified alcohol-dependent patients as well as in the cortex of healthy individuals, both measured by MRS [2,30]. While other recent studies show that acamprosate may interact with glycine receptors in the NAc and attenuate dopamine release, which may have an effect on alcohol consumption in rats [3]. Moreover, as ethanol enhances taurine-activated glycine receptor function [32], the blockade of glycine transporter-1 has been shown to reduce relapse-like alcohol drinking in rats [31]. However, the effect of acamprosate on specific receptors still awaits further investigation.

The present study is also consistent with a microdialysis study, which showed that acamprosate increased taurine levels in the NAc of rats [6]. Because a taurine injection (45 mg/kg, *i.p.*) blocks accumbal glutamate levels in rats [7], our findings suggest that increased taurine levels may reduce glutamate levels in ENT1$^{-/-}$ mice. Thus, acamprosate-induced elevated taurine levels might contribute to the normalization of glutamate or glutamine which could be associated with decreased voluntary ethanol consumption of ENT1$^{-/-}$ mice.

Since the voxel used is a cube, it is likely other brain regions, especially some portions of the dorsal striatum, might be included within the selected VOI. Nonetheless, the majority of the VOI consisted of the NAc. In future studies, it would be interesting to analyze the metabolite profiles and drug effects in other brain regions including the anterior cingulate or dorsal striatum. The long-term effect of acamprosate on the accumbal metabolite profile as measured by MRS also awaits further investigation.

In summary, our findings demonstrate that acamprosate treatment reduced ethanol drinking of ENT1$^{-/-}$ mice. Then, using *in vivo* 16.4T [^{1}H] MRS, we quantified several brain metabolites related to ethanol intoxication and consumption. Our findings indicate that Glx and glutamine levels were normalized while taurine levels were significantly increased by acamprosate administration. This study suggests that manipulating taurine levels or the receptors associated with taurine metabolism could be a potential therapeutic target for alcohol use disorders and *in vivo* MRS could be useful to diagnose or monitor the therapeutic process in humans.

Acknowledgments

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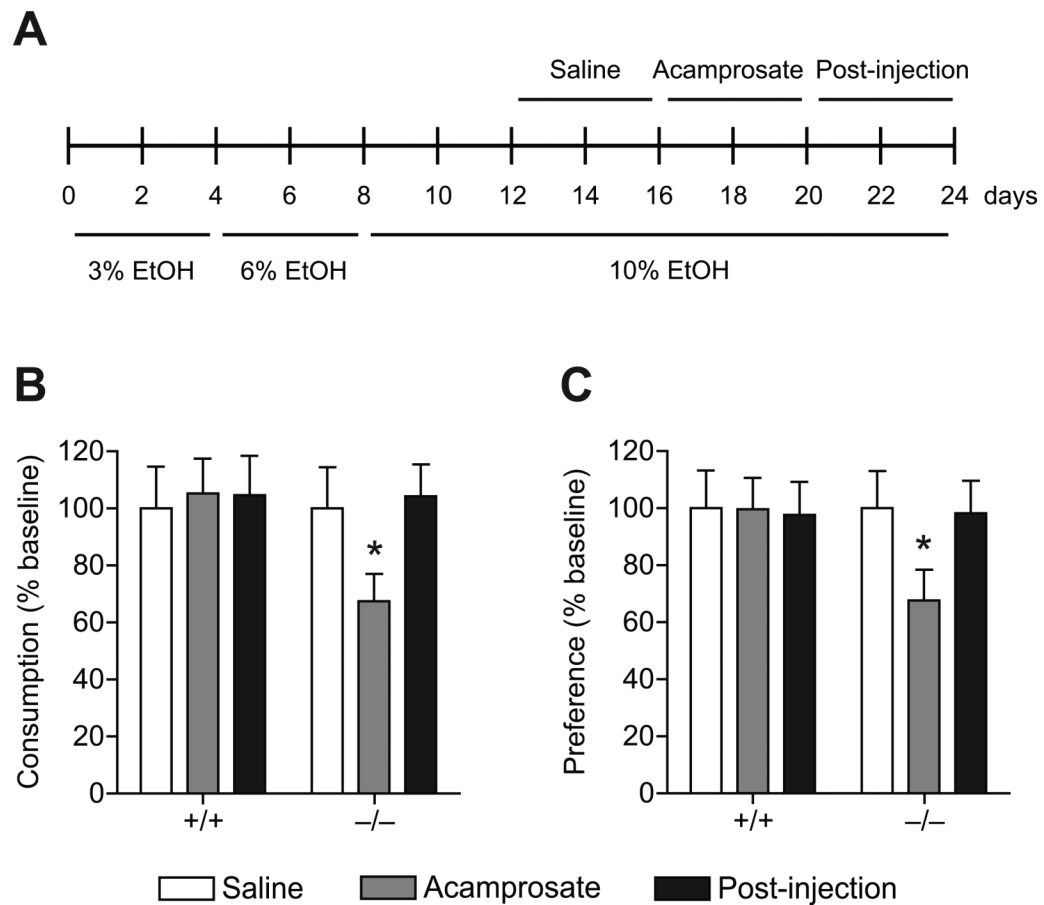
Figure 1.
Reduced ethanol consumption and preference by acamprosate administration in ENT1$^{-/-}$ mice. (A) Mice were given 12 days to acclimate to a 10% (v/v) ethanol concentration. Then mice were injected with saline and acamprosate (4 days each) during the two-bottle drinking experiment. The post-injection period refers to the 4 days following the acamprosate injection period. (B) Reduced ethanol consumption and (C) preference in ENT1$^{-/-}$ mice during the acamprosate administration period compared to the saline injection period. $n = 15\sim16$ mice per genotype and per treatment group. All data are expressed as mean ± SEM. *$p < 0.05$ compared to respective saline treatment groups by one-way repeated measures ANOVA.
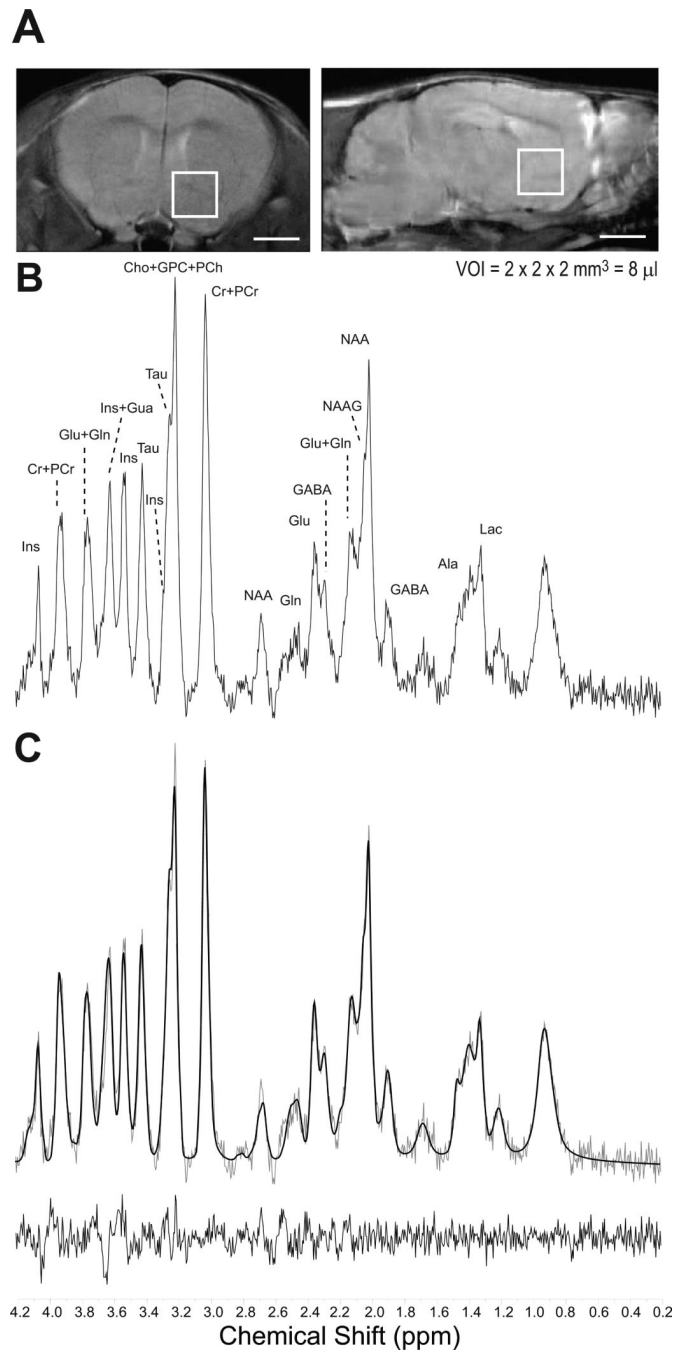
Figure 2.
Metabolite profile from the mouse nucleus accumbens using *in vivo* 16.4T MRS and
LCModel. (A) Representative RARE images of 1.0 mm thick coronal and sagittal brain
sections where the 8 μl volume of interest (VOI) is centered on the nucleus accumbens.
Scale bar = 2 mm. (B) Representative brain metabolite spectrum acquired using *in vivo*
16.4T. See Table 1 for abbreviation of labeled brain metabolites. (C) LCModel best fit of the
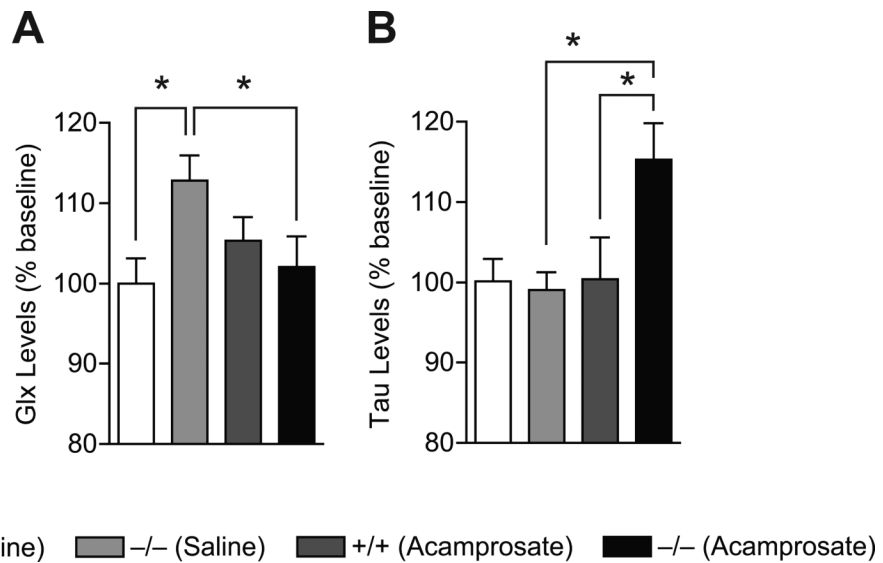brain metabolite spectrum in Fig. 2B and residual difference.

Figure 3.
The effect of acamprosate on basal accumbal metabolite levels of wild-type and ENT1$^{-/-}$ mice presented as percent baseline. (A) Basal glutamate+glutamine (Glx) is significantly increased in ENT1$^{-/-}$ mice compared wild-type mice and acamprosate treatment normalizes this increase. (B) Acamprosate administration significantly increases taurine (Tau) levels in ENT1$^{-/-}$ mice compared to ENT1$^{-/-}$ mice basal levels and compared to wild-type acamprosate treatment levels. n = 10~11 mice per genotype, treatment and metabolite. All data are expressed as mean ± SEM. *$p < 0.05$ by two-tailed t-test.

Table 1

Effect of acamprosate treatment in the NAc

Metabolites	Wild-type		ENT1$^{-/-}$	
	Saline	Acamprosate	Saline	Acamprosate
N-acetylaspartatic acid (NAA)	0.76 ± 0.03 (12)	0.77 ± 0.03 (10)	0.87 ± 0.02[*] (12)	0.77 ± 0.04 (12)
Glutamate (Glu)	0.89 ± 0.03 (10)	0.94 ± 0.03 (10)	0.98 ± 0.03[*] (10)	0.92 ± 0.03 (10)
Glutamine (Gln)	0.40 ± 0.01 (10)	0.39 ± 0.02 (10)	0.45 ± 0.01[*] (10)	0.37 ± 0.02[†] (10)
Glutamate+Glutamine (Glx)	1.26 ± 0.04 (10)	1.33 ± 0.04 (10)	1.43 ± 0.04[*] (10)	1.29 ± 0.05[†] (10)
Glycerophosphocholine+Phosphocholine (GPC+PCh)	0.34 ± 0.01 (12)	0.33 ± 0.01 (10)	0.35 ± 0.01 (12)	0.31 ± 0.01[†] (12)
Taurine (Tau)	1.22 ± 0.03 (10)	1.23 ± 0.06 (10)	1.21 ± 0.03 (12)	1.41 ± 0.06[*,†] (11)
N-acetylaspartylglutamic acid (NAAG)	0.17 ± 0.02 (10)	0.12 ± 0.01 (4)	0.15 ± 0.01 (6)	0.16 ± 0.01 (7)
Alanine (Ala)	0.26 ± 0.04 (8)	0.19 ± 0.01 (7)	0.21 ± 0.02 (9)	0.21 ± 0.02 (9)
γ-aminobutyric acid (GABA)	0.62 ± 0.04 (12)	0.61 ± 0.04 (10)	0.68 ± 0.03 (12)	0.65 ± 0.04 (12)
Guanosine (Gua)	0.59 ± 0.04 (11)	0.57 ± 0.03 (10)	0.53 ± 0.02 (12)	0.56 ± 0.03 (11)
Lactate (Lac)	0.40 ± 0.03 (10)	0.48 ± 0.11 (7)	0.49 ± 0.06 (10)	0.46 ± 0.04 (6)
myo-Inositol (Ins)	1.08 ± 0.05 (12)	0.97 ± 0.10 (10)	1.12 ± 0.04 (12)	1.02 ± 0.03 (12)
Phosphocreatine (PCr)	0.84 ± 0.04 (12)	0.87 ± 0.05 (10)	0.77 ± 0.03 (11)	0.84 ± 0.04 (12)

Metabolites are measured relative to total creatine. All data are expressed as mean ± SEM. Number in parentheses is number of mice used in statistical analyses based on Cramér-Rao lower bounds. See "Materials and Methods" for detailed inclusion criteria for statistical analyses.

[*] $p < 0.05$ compared to wild-type mice within each treatment group by two-tailed *t*-test.

[†] $p < 0.05$ compared to saline treated group within each genotype by two-tailed t-test.