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Progress toward acetate supplementation therapy for Canavan disease: Glyceryl triacetate administration increases acetate, but not N-acetylaspartate levels in brain

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Use of GTA for acetate supplementation to the brain

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Abstract

Canavan Disease (CD) is a fatal genetic neurodegenerative disorder caused by mutations in the gene for aspartoacylase, an enzyme that hydrolyzes N-acetylaspartate (NAA) into L-aspartate and acetate. Because aspartoacylase is localized in oligodendrocytes, and NAA-derived acetate is incorporated into myelin lipids, we hypothesize that an acetate deficiency in oligodendrocytes is responsible for the pathology in CD, and we propose acetate supplementation as a possible therapy. In our preclinical efforts toward this goal we studied the effectiveness of orally administered glyceryl triacetate (GTA) and calcium acetate for increasing acetate levels in the murine brain. The concentrations of brain acetate and NAA were determined simultaneously after intragastric administration of GTA. We found that the acetate levels in brain were increased in a dose- and time-dependent manner, with a 17-fold increase observed at 1-2 hr in 20-21 day old mice at a dose of 5.8 g/kg GTA. NAA levels in the brain were not significantly increased under these conditions. Studies using mice at varying stages of development showed that the dose of GTA required to maintain similarly elevated acetate levels in the brain increased with age. Also, GTA was significantly more effective as an acetate source than calcium acetate. Chronic administration of GTA upto 25 days of age did not result in any overt pathology in the mice. Based on these results and the current FDA approved use of GTA as a food additive, we propose that it is a potential candidate for use in acetate supplementation therapy for CD.

Introduction

Canavan disease (CD) is a fatal hereditary disorder of the brain that is caused by mutations in the gene for the enzyme aspartoacylase (ASPA) (Kaul *et al.* 1994). ASPA acts to deacetylate N-acetylaspartate (NAA), thus generating free acetate in the brain. The clinical symptoms of the disease include poor head control, macrocephaly, marked developmental delay, hypotonia, optic atrophy, seizures, and death in early childhood (Adachi *et al.* 1973). The pathologies associated with CD include cortical and subcortical spongy degeneration, widespread myelin defects and hypertrophy and hyperplasia of astrocytes (Matalon *et al.* 1995). Biochemical analyses have shown that hypomyelination is a characteristic feature of CD (Kamoshita *et al.* 1968). Determination of the elevated levels of urinary NAA is the most reliable diagnostic procedure for CD, because CD patients were found to have 10-100 fold higher excretion of NAA in the urine than normal (Kvittingen *et al.* 1986).

CD is prevalent among Ashkenazi Jews with the two predominant mutations of C693A and A854C being found in 98% of the Jewish CD patients examined. Mutations in the ASPA gene occur less frequently among non-Jewish patients. The mutations among the non-Jewish patients are different and more diverse; the most common mutation is a C914A substitution, and this allele has been observed in 35.7% in non-Jewish European individuals with Canavan disease (Kaul *et al.* 1994). Other mutations in non-Jewish patients can randomly reside on exons 1-6. Currently there is no effective treatment for CD, and children with the disorder are treated symptomatically, especially with respect to reducing the incidence and severity of seizures past the age of three.

NAA is an abundant (5-10 mM) amino acid derivative present in the mammalian nervous system (Tallan *et al.* 1956) and is found predominantly in neurons (Moffett and Namboodiri 1995). It is known that the acetyl moiety of NAA is incorporated into fatty acids and lipids associated with myelin (D'adamo and Yatsu 1966; Burri *et al.*1991; Mehta and Namboodiri 1995; Chakraborty *et al.* 2001) and that the rate of this incorporation is highest during the period of heightened postnatal CNS myelination (D'Adamo *et al.* 1968; Burri *et al.*1991). The developmental appearance of NAA and its synthesizing and metabolizing enzymes correlate with the period of active CNS myelination. Autosomal recessive defects in the ASPA gene give rise to NAA accumulation in the nervous system that is associated with brain edema and a progressive loss of oligodendrocytes and myelin (Matalon *et al.* 1995). These and other observations led to our hypothesis that a deficiency in the supply of NAA-derived acetate results in decreased synthesis of myelin-related fatty acids and lipids, and this lipogenic insufficiency causes CD (Mehta and Namboodiri 1995; Namboodiri *et al.* 2000). Recently, we tested this hypothesis by studying acetate levels and the rate of myelin lipid synthesis in the mouse model of CD and showed that acetate levels were reduced, and lipid synthesis was impaired at the stage of peak postnatal CNS myelination (Madhavarao *et al.* 2005).

Rarely are fatal genetic disorders treatable by means of dietary supplementation, but in the case of Canavan disease, we propose that acetate supplementation during the first several years of life can prevent much of the pathogenesis. In our preclinical studies on the possible use of dietary supplementation therapy for CD we have examined GTA and calcium acetate as potential exogenous sources for providing acetate to the brain.

GTA is a non-toxic glyceryl tri-ester of acetic acid that is widely used as a solvent and plasticizer in perfumery, tanning, dyes, as a food additive, a gelatinizing agent in cosmetics and is also used in external medicine. Biochemical studies on GTA have shown that GTA is hydrolyzed *in vivo* by all tissues of mammals, indicating that it will be readily metabolized even when large doses are administered (Bach *et al.*1970). Calcium acetate is currently approved as a drug for the treatment of kidney disease to control high blood phosphate levels (Schiller *et al.*1989). However, administration of calcium acetate causes gastrointestinal irritation, making its use for the treatment of CD problematic. In the present study we used a modified method (Patience and Thomas 1982) to analyze the concentrations of brain acetate and NAA simultaneously in order to ascertain if GTA is a good candidate for delivery of acetate to the brain, and to ensure that brain NAA levels are not increased by GTA treatment, which could exacerbate CD pathology.

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Methods

Materials

p-Bromophenacyl-8 reagent (0.1 mmol/ml *p*-bromophenacylbromide and 0.005 mmol/ml crown ether in acetonitrile) was purchased from Pierce Biotechnology (Rockford, IL). GTA (fw: 218.21, liquid, density: 1.158), calcium acetate, NAA, sodium acetate and HPLC grade acetonitrile were obtained from Sigma Aldrich Co. (St. Louis, MO). Cation exchange resin (AG 50W-X8) was purchased from Bio-Rad (Hercules, CA). [1-^{14}C] acetyl-coenzyme A and L-[U-^{14}C] aspartic acid were obtained from Amersham Biosciences (Piscataway, NJ). Liquid scintillation cocktail was from Beckman Coulter, Inc. (Fullerton, CA). Deionized and filtered Super Q water (Millipore, Bedford, MA, USA) was used for all purposes. [^{14}C] Acetate was prepared from [1-^{14}C] acetyl-coenzyme A (Namboodiri and Klein 1979) and [^{14}C] N-acetylaspartate was prepared from L- [U-^{14}C] aspartic acid as described previously (Madhavarao *et al.* 2002).

Animal studies

Male C57BL/6 mice of varying stages of development were obtained from Charles River Laboratories (Wilmington, MA). The animals were allowed free access to food and water, and newborn mice were kept with their mothers until they were weaned. Three to five animals/group were used in each set of experiments. Different doses (2.9, 4.3, 5.8 and 7.2 g/kg animal weight) of GTA and 26.5 mmol/kg animal weight of calcium acetate dissolved in water were administered intragastrically to mice at 20-21 day old, and the animals were sacrificed 1 hr after administration. A group of 20-21 day old mice were given 5.8 g/kg GTA, and were sacrificed at different intervals (1hr, 2hr, 4 hr, 8 hr, and 12 hr). We also analyzed acetate concentrations after GTA feeding in mice at varying

stages of development. Mice aged 7-8 days and 13-14 days were administered two different doses of GTA; 1.45 and 2.9 g/ kg and were sacrificed after 1 hr. Mice aged 20-21 days and adults were given 2.9 and 5.8 g/kg GTA and were also sacrificed after 1 hr. Brains and livers were removed, frozen on dry ice and stored at -80 ^{o}C until used. For toxicity studies with chronic GTA administration, starting from the 7th day of age, a group of 5 animals were fed GTA orally two times a day until 25 days of age. The dose of GTA was 2.9 g/kg animal weight until 15 days of age, and thereafter the dose was increased to 5.8g/kg animal weight to achieve the maximal increase of acetate level in the brain. All animal procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Animals.

Tissue Extraction

Brain and liver homogenates (20% w/v) were prepared in methanol:water (90:10 vol), by sonication at 0-4 ^{o}C in an Ultrasonic Homogenizer (4710 Series, Cole-Parmer Instruments Co., Chicago, IL) with the microtip probe limit set at 5 and duty cycle set at 30 %. The homogenate was then centrifuged at 10,000 rpm for 30 min. The supernatant was lyophilized and the residue dissolved in water and passed through a cation-exchange column (AG 50W-X8). The eluate and two column washings were pooled, adjusted to pH 7-8 with 10 mM KOH and lyophilized again for derivatization.

Preparation of phenacyl esters

p-Bromophenacyl ester derivatives of acetate and NAA were prepared using *p*-bromophenacylbromide and crown ether (a phase transfer catalyst) in acetonitrile (Durst *et al.* 1975; Persson *et al.*1991). Briefly, 100 µl of acetonitrile and 40 µl of *p*-bromophenacylbromide reagent were added to the lyophilized tissue extract prepared as

above in a micro centrifuge tube (each vial containing 200 µl equivalent of the 20% tissue homogenate) and the vials were tightly capped. The samples were vortex mixed and kept in a water bath at 80°C for 1 hr with occasional stirring. The vials were then cooled, the solvent removed by lyophilization, and the samples reconstituted in the mobile phase and applied to HPLC.

HPLC analysis of phenacyl esters of acetate and NAA

HPLC analysis was performed isocratically using a Hewlett Packard HPLC system (Series 1100). Separation of phenacyl derivatives was carried out on a reverse phase column (Bio-Advantage 100 C18, 5µm, 250 × 4.6 mm) with a mobile phase of acetonitrile:water (50:50 by vol) at 25 °C. The flow rate was 1.0 ml/min with detection at 250 nm, and the signal was processed by the integrator. The dried phenacyl esters were dissolved in the mobile phase solvent mixture and 100 µl was injected over the column. The peaks of phenacyl esters of acetate and NAA from the tissues were identified via their retention times in relation to the internal standards sodium acetate and NAA.

For recovery studies, known amounts of radiolabeled acetate and NAA were added to the tissue extract with 90 % methanol, and the samples were processed as described above. The HPLC fractions corresponding to phenacyl derivatives of acetate and NAA were collected, pooled separately, and the radioactivity was determined using a Beckmann Liquid Scintillation counter system. The percentage recovery was calculated based on the amount of radioactivity added to the extracts.

Statistical analysis

All data are presented as means ± SD. In the dose response and time course studies, the data were analyzed for significance by one way ANOVA to determine

differences between groups and Dunnet post-hoc analysis (alpha=.05) was used to compare all the groups against the control. The data analyses were carried out using SPSS 11.0 software. In all other experiments statistical analysis was carried out using Student's t test and the values are compared each other. P values of 0.05 or less were considered significant.

Results

The modified assay method used in the present study involved the extraction of the tissue using 90% methanol, purification using cation-exchange column chromatography, derivatization using *p*-bromophenacylbromide and separation/estimation using reverse phase HPLC. By using this method, the levels of acetate and NAA in nanogram quantities were determined simultaneously in the brains of mice at various stages of development following intragastric administration of GTA and calcium acetate. Figure 1 shows representative HPLC chromatograms of phenacyl derivatives of acetate and NAA standards and the corresponding peaks in brain tissue samples. Under the HPLC conditions used (50:50 acetonitrile/ water as the mobile phase with a 1 ml/min flow rate), phenacyl derivatives of acetate and NAA had retention times of ~8.8 and ~18.0 minutes, respectively. The peak at ~13 minutes corresponds to the reaction byproduct, *p*-bromophenacylchloride (Patience and Thomas 1982) and the peak at ~15.5 minutes corresponds to the unreacted excess reagent, *p*-bromophenacylbromide. The byproduct of the reaction, *p*-bromophenacylchloride, is non-reactive for esterification purposes and does not compete with the reagent. All other peaks appeared in the chromatogram are unknown, but do not interfere with the detection of acetate and NAA. This method is highly sensitive, with concentrations of 10 ng of both acetate and NAA being within detection limits under the described conditions.

The recovery of acetate and NAA was 95±2 % and 93±3 % respectively without tissue homogenate and 89±4 % and 77±3 % respectively with brain tissue homogenate. The recovery rates were also determined using the internal standard method with non-radiolabeled standards of acetate and NAA, and similar results were obtained.

Figure 2 shows the acetate levels in brain and liver, and the NAA levels in the brains of 20-21 day old mice that received intragastric administration of varying doses of GTA. The animals were sacrificed one hour after GTA administration. The increase of free acetate in the brain at a dose of 2.9g/kg body weight of GTA was approximately 6-fold. This further increased to about 17-fold on a doubling of the dose, but did not increase additionally at the higher dose of 7.2 g/kg body weight. The liver acetate levels showed a similar pattern of increase under these conditions. The NAA levels detected in the brain were not significantly changed even at the highest concentration of GTA administered, perhaps reflecting the relatively lower *K*m of acetyl CoA for aspartate N-acetyltransferase, the biosynthetic enzyme of NAA (Madhavarao *et al.* 2003). The ED_{50} of GTA, or the dose required to achieve half of the maximum level of acetate in the brain (8.19µmol/g tissue), was calculated to be 4.389 g/kg animal weight, whereas in the liver the value was 4.458 g/kg in 20-21 day old mice.

Figure 3 shows the time courses for increases in acetate levels in the brains and livers, and NAA levels in the brains of 20-21 day old mice in response to a single dose of (5.8 g/kg) of GTA. Free acetate levels in both liver and brain were increased about 16-fold at 1hr. The elevated level of acetate was maintained for up to 2 hours, the acetate levels at 1 hr and 2 hrs after GTA administration were found to be significantly higher than the control values ($p<0.0001$) while at other time points the differences were not significant compared to the control values based on Dunnet's post hoc analysis. Eight hours following GTA administration, the brain acetate level remained elevated at about 2-fold, whereas the liver level was approaching normal. Brain acetate levels remained slightly but significantly higher at 12 hours as compared with controls. The level of NAA

in the brain at different time intervals following GTA administration remained unchanged through the 12 hr time course following GTA administration. The half life of GTA-derived acetate was calculated for brain and liver tissue based on the level of acetate at 1 hr (the time point at which maximum acetate level was attained) and at 4 hrs. (the time point at which the level of acetate decreased to a comparable level to control). The values were found to be 70.8 minutes in the brain and 64.8 minutes in the liver.

Figure 4 compares the effectiveness of GTA with calcium acetate as a method for delivering additional acetate to the brain. Equimolar concentrations of GTA and calcium acetate were administered intragastrically to 20-21day old mice and the animals were sacrificed after 1hr. The results show that GTA is significantly more effective than calcium acetate in increasing acetate levels in both brain and liver. This may reflect the ability of GTA to penetrate cell membranes readily due to its highly hydrophobic nature. It should be noted that GTA has 33.3% more acetate content than calcium acetate on an equimolar basis. The calcium acetate dose used in our study (26.5mmol/kg) is equivalent to 3.86g/kg GTA in terms of acetate content. From the dose-response study (Fig.2), it is clear that 3.86g/kg GTA delivers significantly higher levels of acetate to the brain than 26.5mmol/kg of calcium acetate.

Table 1 shows the concentrations of acetate and NAA in the brain and the level of acetate in the liver of mice at varying stages of development. The concentrations were computed by the internal standard method from peak area ratios using curves of acetate and NAA standards combined with tissue samples, as compared with tissue samples alone. The amount of free acetate in whole brain extracts of adult mice used in these experiments was found to be 0.7 ± 0.05 μmol/g of tissue which is consistent with

previous reports (Colon *et al.* 1987; Kiselevski *et al.* 2003). The NAA concentrations in brains of adult mice was found to be 8.8 ± 0.5 μmol/g of tissue which is also consistent with an earlier report (Tallan *et al.* 1956). As described earlier, NAA showed a substantial concentration increase during development (Tallan 1957; Koller and Coyle 1984; Florian *et al.* 1996), while we found that acetate is only moderately increased in the brains of adult mice as compared with developing mice.

The effect of GTA administration on the levels of acetate in brain and liver during different developmental stages of mice is given in Figure 5. The results obtained show that the dose of GTA required to deliver a significant amount of acetate to the brain increases with age. For example, a dose of 1.45 g/kg animal weight is sufficient to cause a 5-fold increase in 7-8 day old mice but only about 2-fold increase in13-14 day old mice. No significant increase in brain acetate is detected under this condition in 17-20 day old or adult mice. At a dose of 2.9 g/kg GTA, brain acetate levels increased approximately 17-fold in 7-8 day old, 12-fold in 13-14 day old, 7-fold in 17-20 day old and 5-fold in adult mice. The pattern of increasing acetate in the liver was similar to that in the brain.

In order to determine if chronic GTA supplementation has overt toxic effects, a group of five 7-day old mice were fed GTA orally two times a day until 25 days of age (2.9 g/kg animal weight until day 15, and after that 5.8g/kg). At the end of the 25 day period, the mice were examined for apparent pathologies, and were weighed. The GTA fed animals survived the experimental period in good health. They had normal appearance and behavior throughout with an absence of diarrhea. At termination, their plasma was clear, and the animals did not develop fatty livers. Autopsies revealed normal appearing stomach and intestine, and the data in figure 6 demonstrate that the GTA-fed

animals exhibited a small increase in weight, possibly due to the increased caloric intake associated with GTA feeding (Bailey *et al.* 1991; 1989; 1993).

Discussion

The results presented here demonstrate two important issues: (1) GTA is a more effective source for acetate delivery to the brain than calcium acetate and (2) NAA levels are not increased when acetate levels are increased as high as 17-fold in the brain. Given the low toxicity of GTA and its FDA approval for human use, GTA represents a good candidate for use in the proposed acetate supplementation therapy for CD. Further, GTA did not elicit any noticeable toxic effects, and did not cause the overt gastrointestinal irritation associated with high doses of calcium acetate.

Earlier studies from our laboratory showed that there was nearly an 80% reduction in free acetate levels in the brains of ASPA-/- mice as compared with wild-type mice, but the levels in kidney and liver were not reduced relative to controls (Madhavarao *et al.,* 2005). These findings demonstrate that some lipogenic pathways in the brain are unique, and that in contrast to tissues such as liver, NAA is a significant source of free acetate in the brain during development. Further, they demonstrate that myelin lipid synthesis in the brain derives a substantial portion of the requisite acetate from NAA *via* ASPA-mediated catalysis. Gene therapy using adeno-associated virus has been used in the tremor rat, a genetic model of CD, and ASPA activity was found to be expressed in CNS neurons following treatment. NAA levels were also reduced in the brain, however motor functions in these animals remained unimproved (Klugmann et al. 2005). A major drawback of gene therapy is that ASPA protein is not expressed in oligodendrocytes, and myelin lipid synthesis remains dysfunctional. As such, we propose acetate supplementation with GTA as a therapy for Canavan disease, and the bases for the proposed use are summarized below.

First, myelination in the human CNS does not commence until early postnatal life, so postnatal diagnosis could be followed by immediate dietary acetate supplementation. Second, earlier studies have shown that free acetate is almost as effective as the acetyl moiety of NAA for fatty acid/lipid synthesis during myelination (D'Adamo and Yatsu 1966; D'Adamo *et al*. 1968; Burri *et al.*1991; Mehta and Namboodiri 1995; Chakraborty *et al.* 2001). Third, in the developing rat approximately10% of radiolabeled acetate administered is incorporated into brain lipids, whereas about 5% is incorporated into lipids in the liver (Dhopeshwarker and Mead 1973). Because oligodendrocytes are the cells that express aspartoacylase (Kirmani *et al.* 2002; Madhavarao *et al*. 2004), they are the target cells for acetate supplementation therapy in CD. The question of whether acetate levels are significantly increased in oligodendrocytes after GTA administration remains to be answered. However, the high Km values for acetate uptake systems in the blood-brain barrier (Terasaki et al. 1991) and in glial cells (Waniewski and Martin 1998) suggest that a significant proportion of blood acetate would be delivered to the brain, where it could be taken up by glia, including oligodendrocytes. As noted above, in the developing rat it has been shown that approximately 10% of the radiolabeled acetate administered systemically is incorporated into myelin lipids in the brain, demonstrating that a portion of the administered acetate is reaching oligodendrocytes where myelination takes place (Dhopeshwarker and Mead 1973).

The superior effectiveness of GTA over calcium acetate for increasing brain acetate levels is of interest. GTA is thought to be at least partly hydrolyzed in the gut by the action of nonspecific esterases (Bach *et al.,* 1970). However, our finding that brain acetate levels are far significantly higher with GTA as opposed to calcium acetate on an

equimolar basis cannot be accounted for by the increased (33.3%) acetate content of GTA. This suggests that a portion of GTA is transported unhydrolyzed from the gut to blood, from where it can easily enter cells due to its hydrophobic nature. GTA was more effective at the earlier stages of development, possibly reflecting the lower activity levels of nonspecific esterases in the gut, due to their slower developmental profiles.

Regarding toxicity concerns of GTA, the Cosmetic Ingredient Review Expert Panel (Fiume *et al.* 2003) concluded that although there are no available reproductive and developmental toxicity data, GTA is metabolized to glycerol and acetic acid, which are not developmental toxins, and hence the use of GTA does not present a significant risk. GTA is also used as a food and drug additive, and has been studied as a parenteral nutrient (Bailey *et al.* 1991; 1989; 1993). One potential problem with dietary acetate supplementation for CD infants is that GTA has a strongly bitter taste, and relatively large amounts would have to be administered orally on a twice daily basis. It is possible that sweeteners could be used to overcome this difficulty.

No significant increases in NAA concentrations were observed in the present investigation, even when brain acetate levels were increased 17-fold. This finding may have clinical significance for CD patients, who are reported to have 10-100 fold higher excretion of NAA in the urine than normal (Kvittingen *et al.* 1986; Hagenfeldt *et al.* 1987; Matalon *et al.* 1988) and a 0-3 fold increase in NAA levels in the brain as compared with normal individuals (Wittsack *et al.* 1996). One hypothesis on the pathogenesis of CD is that the increased brain NAA concentrations result from a lack of catabolic activity and the excess NAA plays an etiological role in the disease, possibly due to brain edema (Basolw 2003). If this hypothesis is found to be correct, an increase

in brain NAA concentrations during acetate supplementation therapy would likely exacerbate the pathology. Given our present findings that NAA levels remain unchanged with GTA feeding, increased brain edema should not be a major concern of acetate supplementation therapy for CD. A possible explanation for the lack of increase in NAA concentrations is that the biosynthetic enzyme for NAA has a relatively low Km for acetyl CoA (Madhavarao *et al.* 2003). This would mean that the NAA synthetic enzyme would be saturated at relatively low concentrations of acetyl CoA, thus limiting NAA production when acetate concentrations were increased. Further, product feedback inhibition by the increased levels of NAA in CD patients should help maintain NAA synthesis at lower levels (Moreno et al., 2001). In addition it is noteworthy that our simple HPLC analytical technique can detect NAA at nanogram levels, providing a simple and inexpensive method which can be used to clinically diagnose CD.

Finally, additional optimization studies are required before the initiation of preclinical trials in CD mice. For example, the optimal dose and dosing frequency required to maintain increased acetate levels in the brain need to be determined. The minimally increased level of acetate in the brain that is required to correct the acetate deficit in oligodendrocytes remains unclear. One immediate consequence of continuous GTA treatment involves possible acidosis caused by continuous CO_2 production via the oxidation of acetate. This issue should also be addressed before large-scale clinical trials can begin. However, it is encouraging to note that the acetate supplementation for CD could be reduced or discontinued by the end of the second year after birth, because myelin synthesis is nearly complete at that stage of development.

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Footnotes:

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Figure Legends

Fig. 1 HPLC elution patterns of phenacylesterderivatives of brain extract of (A) mouse fed 5.8 g/kg body weight of GTA and sacrificed after 1 hr; (B) untreated control mouse and (C) standards, sodium acetate and NAA. Acetate and NAA had retention times of ~8.8 and ~18.0 minutes, respectively.

Fig. 2 Brain and liver acetate levels, and brain NAA levels 1 hr after feeding varying doses of GTA. Mice of age 20-21 day (~ 8 g body weight) were used in these studies.Values are mean ± SD of samples from 3-5 animals per group. *$p<0.0001$.

Fig. 3 Levels of acetate and NAA at various time points after GTA administration. Acetate levels in brain and liver, and NAA levels in brain were determined in mice (20-21 day old, weighing ~8 g) after intra-gastric administration of GTA at 5.8 g/kg animal weight. Animals were sacrificed at various intervals following the feeding. Values are mean ± SD of samples from 3-5 animals per group. * $p<0.0001$.

Fig. 4 Effect of equimolar GTA and calcium acetate (CA) on acetate levels in the liver and brain. Mice (20-21day old) were fed (26.5 mmol/kg) of either GTA or CA and acetate levels were determined in tissues after 1 hr following administration. *$p<0.01$ **$p<0.0001$.

Fig. 5 Acetate level in (A) brain and (B) liver of mice at different stages of development after intragastric administration of varying doses of GTA. The symbol '♣' indicates missing data. Values are mean ± SD of samples from 3-5 animals per group. *$p<0.01$ and **$p<0.0001$.

Fig. 6 The body weights for groups of mice fed GTA from 7th day to 25th day orally and their corresponding controls. Values are mean ± SD of samples from 5 animals per group. Statistical analysis was carried out using Student's t test and values were compared with corresponding controls. *$p<0.01$ and **$p<0.001$.

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Table 1. Levels of acetate in brain and liver, and of NAA in brain at different stages of development in mice (3-5 animals per group). Values given are means ± SD.

mouse age in days	acetate (µmol/g tissue)		NAA (µmol/g tissue)
	Brain	Liver	
7/8	0.46±0.03	0.5±0.05	2.7±0.5
13/14	0.44±0.03	0.55±0.05	4.5±0.5
20/21	0.45±0.05	0.52±0.05	7.7±0.5
adult	0.7±0.05	0.61±0.05	8.8±0.5

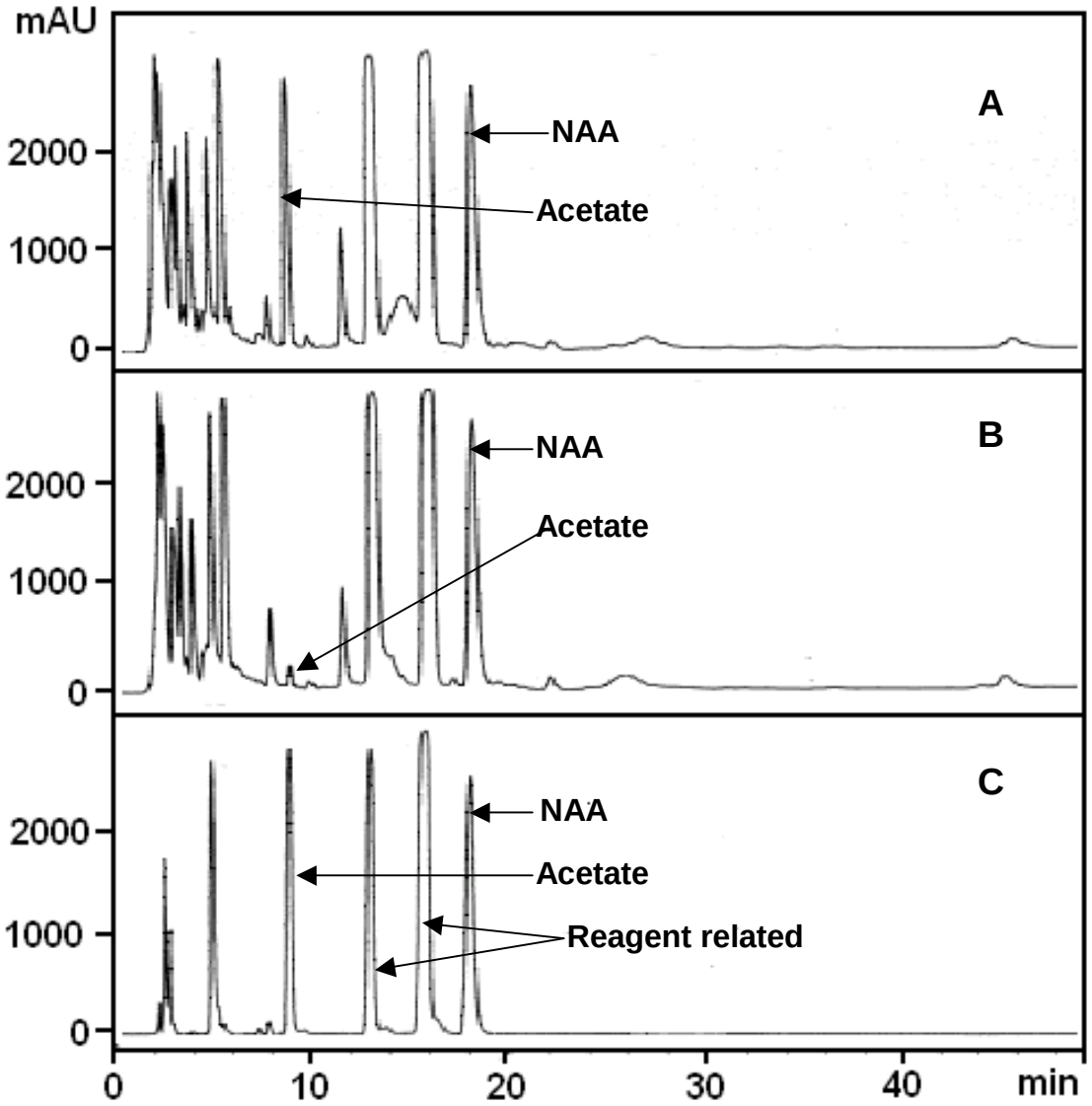

FIGURE 1

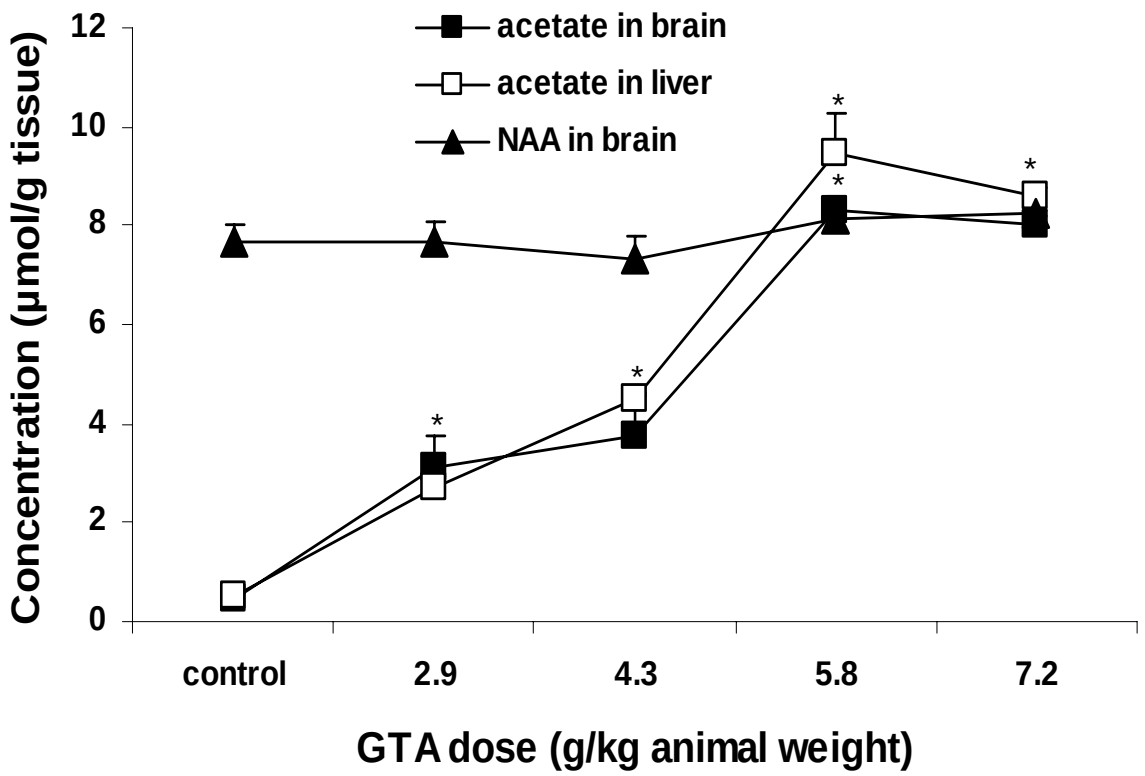

FIGURE 2

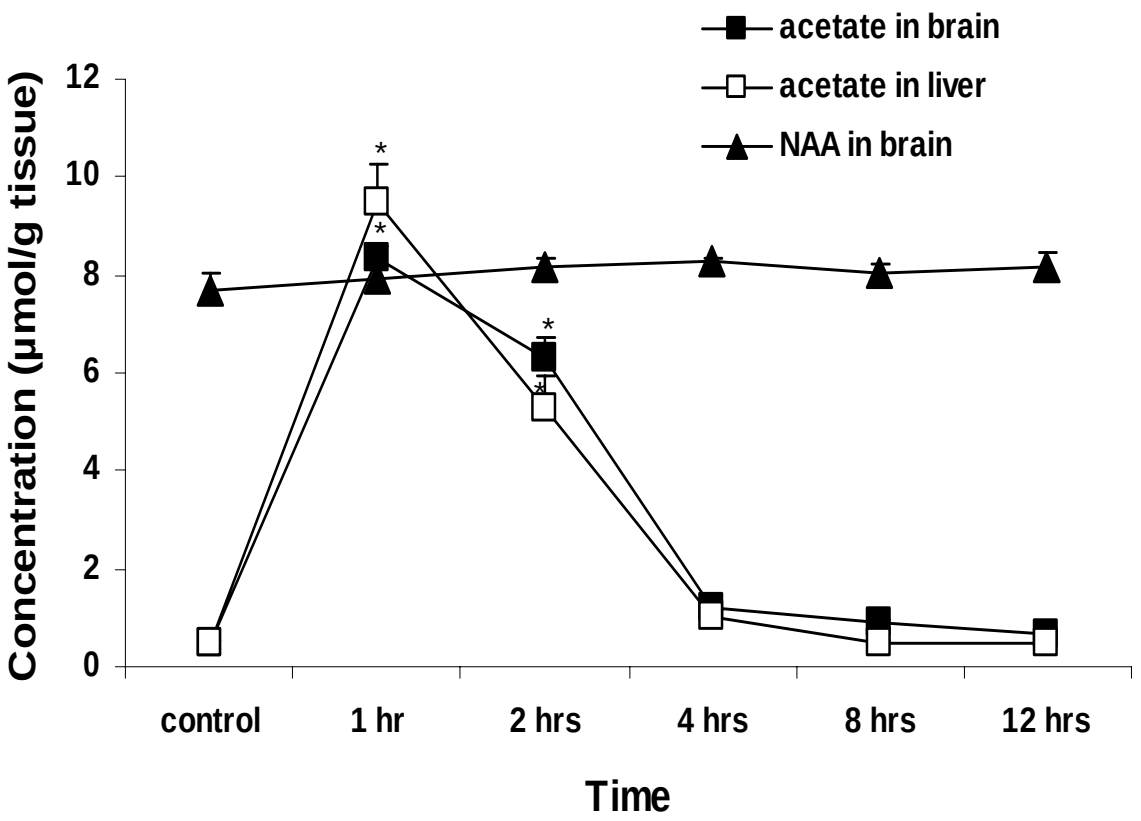

FIGURE 3

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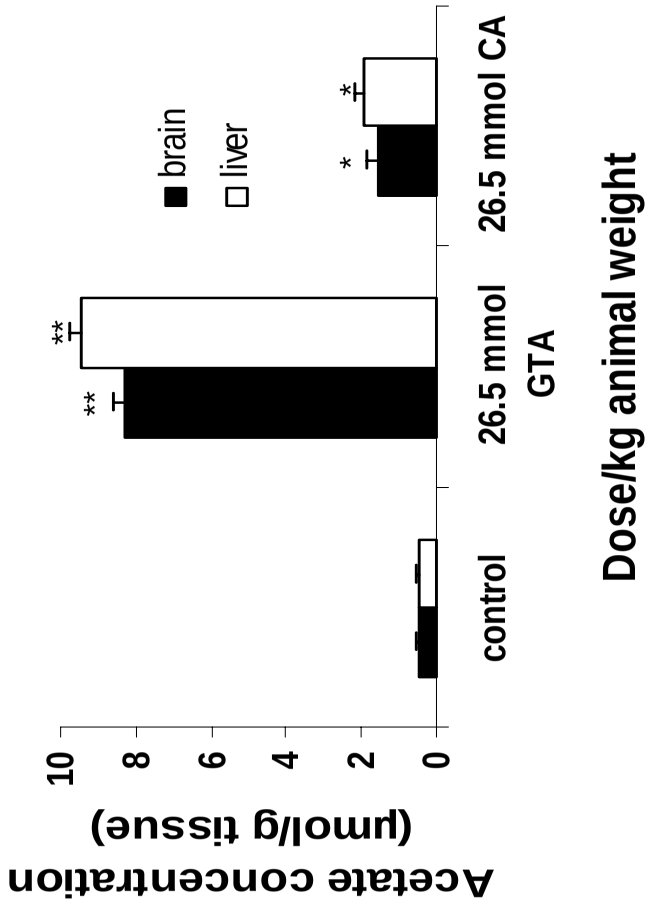

Figure 4

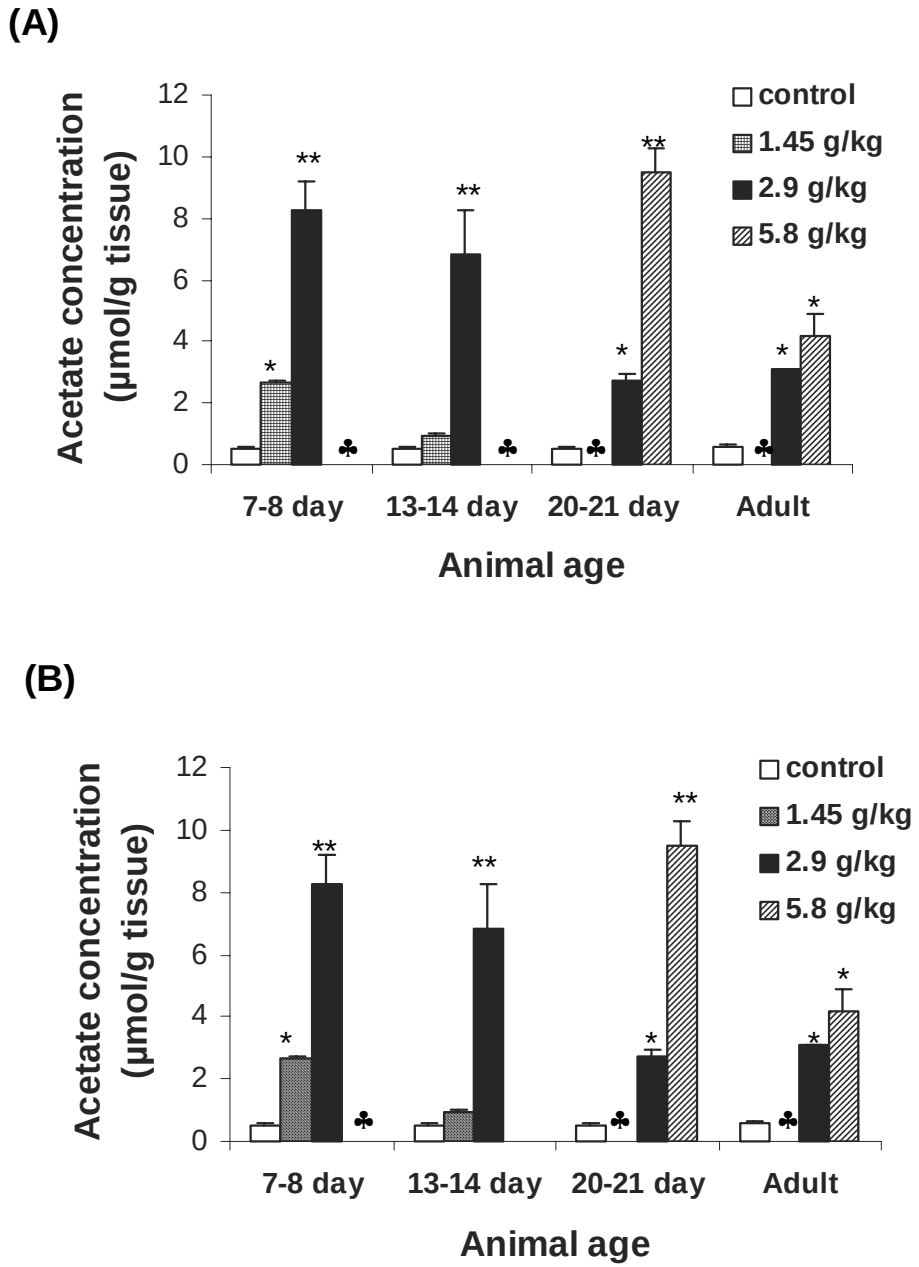

Figure 5

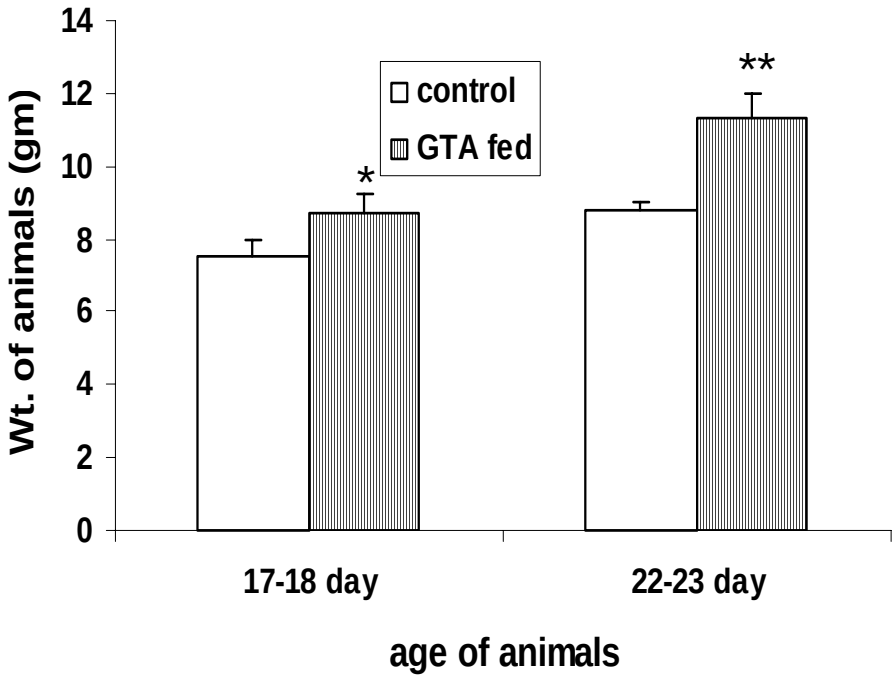

FIGURE 6