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Carnosine and anserine homeostasis in skeletal muscle and heart is controlled by β-alanine transamination

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Key points

- Using recombinant DNA technology, the present study provides the first strong and direct evidence indicating that β-alanine is an efficient substrate for the mammalian transaminating enzymes 4-aminobutyrate-2-oxoglutarate transaminase and alanine-glyoxylate transaminase.
- The concentration of carnosine and anserine in murine skeletal and heart muscle depends on circulating availability of β-alanine, which is in turn controlled by degradation of β-alanine in liver and kidney.
- Chronic oral β-alanine supplementation is a popular ergogenic strategy in sports because it can increase the intracellular carnosine concentration and subsequently improve the performance of high-intensity exercises. The present study can partly explain why the β-alanine supplementation protocol is so inefficient, by demonstrating that exogenous β-alanine can be effectively routed toward oxidation.

Abstract The metabolic fate of orally ingested β-alanine is largely unknown. Chronic β-alanine supplementation is becoming increasingly popular for improving high-intensity exercise performance because it is the rate-limiting precursor of the dipeptide carnosine (β-alanyl-L-histidine) in muscle. However, only a small fraction (3–6%) of the ingested β-alanine is used for carnosine synthesis. Thus, the present study aimed to investigate the putative contribution of two β-alanine transamination enzymes, namely 4-aminobutyrate-2-oxoglutarate transaminase (GABA-T) and alanine-glyoxylate transaminase (AGXT2), to the homeostasis of carnosine and its methylated analogue anserine. We found that, when transfected into HEK293T cells, recombinant mouse and human GABA-T and AGXT2 are able to transaminate β-alanine efficiently. The reaction catalysed by GABA-T is inhibited by vigabatrin, whereas both GABA-T and AGXT2 activity is inhibited by aminooxyacetic acid (AOA). Both GABA-T and AGXT2 are highly expressed in the mouse liver and kidney and the administration of the inhibitors effectively reduced their enzyme activity in liver (GABA-T for vigabatrin; GABA-T and AGXT2 for AOA). *In vivo*, injection of AOA in C57BL/6 mice placed on β-alanine (0.1% w/v in drinking water) for 2 weeks lead to a 3-fold increase in circulating β-alanine levels and to significantly higher levels of carnosine and anserine in skeletal muscle and heart. By contrast, specific inhibition of GABA-T by vigabatrin did not affect carnosine and anserine levels in either tissue. Collectively, these data demonstrate that homeostasis of carnosine and anserine in mammalian skeletal muscle and heart is controlled by circulating β-alanine levels, which are suppressed by hepatic and renal β-alanine transamination upon oral β-alanine intake.

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Abbreviations AGXT2, alanine-glyoxylate transaminase; AOA, aminooxyacetate; CARNS, carnosine synthase; GABA-T, 4-aminobutyrate-2-oxoglutarate transaminase; HCD, histidine-containing dipeptide; MSA, malonate semi-aldehyde; PBS, phosphate-buffered saline; SAL, saline; TauT, taurine transporter.

Introduction

Carnosine is a versatile dipeptide, composed of β-alanine and L-histidine. Anserine (β-alanyl-$N\pi$-methyl-histidine) and ophidine/balenine (β-alanyl-$N\tau$-methyl-histidine) are two methylated analogues of carnosine, collectively called histidine-containing dipeptides (HCDs). HCDs are mainly present in mammalian skeletal muscle and neuronal tissue and, to a smaller extent, in the heart, liver and kidney (Boldyrev *et al.* 2013). Skeletal muscles of all mammals, except humans, possess both carnosine and a methylated analogue (anserine or ophidine). In human muscles, carnosine is the only HCD (5–8 mM), with ~2-fold higher concentrations in fast-twitch fibres than slow-twitch fibres (Harris *et al.* 1998; Kendrick *et al.* 2009). Several physiological properties of carnosine are relevant to muscular function and homeostasis, such as pH buffering, anti-oxidant capacity, increasing Ca^{2+} sensitivity and inhibiting protein glycation (Boldyrev *et al.* 2013; Blancquaert *et al.* 2015).

The major pathways involved in carnosine metabolism are synthesis from and hydrolysis to its constituent amino acids, by carnosine synthase (CARNS) (Drozak *et al.* 2010) and carnosinases (Teufel *et al.* 2003), respectively. β-alanine has been shown to be the rate-limiting precursor for carnosine synthesis in human muscle cells (Harris *et al.* 2006). Because HCDs are present in meat and fish, the daily dietary intake of these dipeptides in an omnivorous diet is considered to affect the availability of β-alanine and therefore possibly also the muscle carnosine content. Accordingly, chronic oral β-alanine supplementation (4–6 g day^{-1} for 4–10 weeks) was found to increase muscle carnosine content by 40–80% (Harris *et al.* 2006; Hill *et al.* 2007; Baguet *et al.* 2009). By contrast, a vegetarian diet is free of HCDs and long-term vegetarians may have somewhat lower muscle carnosine contents compared to omnivores (Everaert *et al.* 2011).

However, considering the high amounts of HCDs present in mammalian muscles and the significant roles that they fulfill, carnosine and anserine homeostasis probably do not depend entirely on the nutritional supply of β-alanine. Moreover, herbivores also show a high muscle HCD content (Dunnett & Harris, 1999; Boldyrev *et al.* 2013), although both β-alanine and carnosine are absent in plants and, consequently, from the herbivorous diet. This implies the existence of endogenous pathways that synthesize β-alanine, such as uracil degradation. Similarly, pathways may exist to degrade β-alanine and maintain β-alanine levels and, subsequently, HCD levels within homeostatic limits.

Under conditions where exogenous β-alanine supply exceeds the need and/or capacity to synthesize HCDs, β-alanine is probably degraded and used as an energy source. This was recently suggested by Stegen *et al* (2013), who found that daily orally ingested β-alanine as an ergogenic supplement has a very high whole body retention (only <2% was excreted in urine) and only a small fraction of the exogenous β-alanine is taken up by the human muscles to be converted into carnosine (3–6%). Moreover, Pihl & Fritzson (1955) reported that more than 90% of the injected C^{14}-labelled β-alanine in rats was recovered in the expired CO_2 in 5 h, suggesting that β-alanine can be metabolized elsewhere, most probably as a carbon source for energy provision through oxidation. As a result of this, β-alanine supplementation, which recently became very popular among athletic populations as a result of its ergogenic potential (Hill *et al.* 2007; Derave *et al.* 2007), is a rather impractical process, requiring athletes to take large doses of β-alanine every day over several weeks to induce HCD loading, resulting in a total ingested dose that is an order of magnitude higher than the genuine amount of β-alanine required to synthesize dipeptides.

To enter the citric acid cycle and provide energy, the amine group of β-alanine can be removed through a transamination resulting in the formation of the keto-acid malonate semi-aldehyde (MSA). Mostly based on enzymatic assays in cell extracts, two mitochondrial enzymes are known to catalyse this reaction: 4-aminobutyrate-2-oxoglutarate transaminase (EC 2.6.1.19; also known as GABA-T or β-alanine-2-oxoglutarate transaminase) (Ito *et al.* 2001) and alanine-glyoxylate transaminase (EC 2.6.1.44; also known as AGXT2 or β-alanine-pyruvate transaminase) (Rodionov *et al.* 2014). Vigabatrin is a known selective irreversible inhibitor of GABA-T (Lippert *et al.* 1977), whereas aminooxyacetate (AOA) is known to inhibit all pyridoxal-5′-phosphate-dependent enzymes, including GABA-T and AGXT2 (John *et al.* 1978; Tamaki *et al.* 1990; Horváth & Wanders, 1995). Interestingly, the administration of AOA has already been reported to increase urinary, liver, kidney and plasma β-alanine levels in rats by 27-, 15-, 10- and 3-fold, respectively (Baxter & Roberts, 1961; Kurozumi *et al.* 1999). These results suggest that inhibiting both the transaminase enzymes (GABA-T and AGXT2) might be an efficient strategy for

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counteracting the β-alanine catabolism in rodents. However, to date, the effect of AOA on tissue HCD levels and the effect of vigabatrin on both β-alanine and HCD metabolism have not been determined.

The present study aimed to test the hypothesis that β-alanine is degraded by the transaminase enzymes GABA-T and AGXT2 and that this reaction regulates tissue HCD homeostasis. The present study first aimed to demonstrate that β-alanine is a suitable substrate for both transaminase enzymes by means of recombinant DNA technology, and also that vigabatrin is an inhibitor of GABA-T, whereas AOA inhibits both GABA-T and AGXT2 activity towards β-alanine. In addition, the study aimed to determine the tissue mRNA expression of β-alanine transaminases and their role in muscle HCD metabolism upon oral β-alanine intake.

Methods

Ethical approval

The experimental protocol was approved by the Ethics Committee for Animal Research at Ghent University and followed the Principles of Laboratory Animal Care.

Part 1: *In vitro* enzymatic experiments

Cloning and expression of mouse GABA-T and AGXT2 in HEK293T cells. GABA-T and AGXT2 were PCR-amplified using cDNA from mouse liver using Phusion High-Fidelity DNA Polymerase, cloned in pEF6/myc-HisA plasmid and expressed in HEK293T cells as C-terminal His_6-tagged proteins as described previously (Veiga-da-Cunha *et al.* 2014). HEK293T cell extracts from three independent experiments ($n = 3$) were prepared 48 h after transfection, by removing the medium, washing the plates with phosphate-buffered saline (PBS) and collecting the cells from each plate in 0.5 ml of extraction buffer without Triton X-100. The cells were then lysed by freezing twice in liquid nitrogen and genomic DNA was removed by treating the lysates with DNase I (125 U ml^{-1}). The extracts were stored at –80 °C before analysis of the recombinant proteins by SDS-PAGE/western blotting and measurement of enzymatic activities.

Assays of GABA-T and AGXT2 transaminase activities. GABA-T activity was measured using a spectrophotometric assay based on the sequential transamination and glutamate dehydrogenase reaction, which couples the reduction of iodonitrotetrazolium to a purple iodonitrotetrazolium-formazan dye that absorbs at 490 nm. The reaction was followed at 30°C in a mixture (1 ml) containing 50 mm Tris (pH 8.5), 1 mM ADP-Mg^{2+}, 5 μM pyridoxal-phosphate, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM NAD^+, 0.5 mg ml^{-1} BSA, 75 μM INT, 1 mM α-ketoglutarate, 2.5 mM GABA or β-alanine, 1.5 U of recombinant diaphorase from *Clostridium kluyveri* (500 U ml^{-1}) and 10 U of beef liver glutamate dehydrogenase (5000 U ml^{-1}). Vigabatrin (0.5 mM) and AOA (2 μM) were added to the activity assay and the reaction was started by the addition of HEK293T cell extracts. Appropriate blanks in the absence of GABA or β-alanine were run in parallel. The concentrated stock of diaphorase that was used in the assay (10 mg ml^{-1}) was prepared in 50% glycerol, 0.2 M Tris (pH 7), 0.54 mM flavin mononucleotide and 0.25 mg ml^{-1} BSA and stored at –20 °C.

AGXT2 activity was measured in a two-step assay using alanine dehydrogenase to measure L-alanine formed during the AGXT2 transamination of DL-β-aminoisobutyrate (or β-alanine) in the presence of pyruvate. In the first step (0.2 ml), the assay mixture contained 25 mM Tris (pH 8), 2 μM pyridoxal-phosphate, 2 mM EGTA, 0.25 mg ml^{-1} BSA, 1 mM pyruvate and 5 mM DL-β-aminoisobutyrate or β-alanine. Vigabatrin (0.5 mM) and AOA (2 μM) were added to the activity assay and the reaction was started by the addition of 30 μl of HEK293T cell extracts and left to proceed for 4 h at 37 °C before stopping (5 min at 80 °C). Appropriate blanks in the absence of DL-β-aminoisobutyrate or β-alanine were also run in parallel. In the second step, the L-alanine produced was quantified in an end-point assay performed in 0.8 ml of mixture containing 0.15 ml of the first reaction mixture in freshly prepared 20 mM Tris/0.5 M hydrazine buffer (pH 9), 0.7 mM EDTA and 0.9 mM NAD^+. The reaction was started by the addition of 5 μl (2 U) of recombinant alanine dehydrogenase from *Bacillus cereus* (>350 U ml^{-1}) and the change in absorbance at 340 nm was monitored for each sample.

Part 2: Animal nutritional intervention study

Animal care and experimental protocol. A total of 66 male C57BL/6 mice (8 weeks old) were used in this study, divided over six groups. Upon arrival, mice were allowed to acclimatize to their new surrounding for 10 days before the start of the 2 week intervention period. All animals were allowed free access to food (standard chow not containing carnosine or derivatives) and water at room temperature and were exposed to a 12 : 12 h light/dark cycle.

Mice were randomly divided in groups and underwent different treatments (Table 1). Mice received different drinks depending on the amount of β-alanine dissolved in the drinking water (ranging from 0, 0.1, 0.6 and 1.2% w/v). Mice from the 0.1% β-alanine supplementation group were further divided in subgroups based on daily S.C. injections with β-alanine transaminase inhibitors: vigabatrin, AOA or saline (SAL) as a control. Vigabatrin (Sabril; Lundbeck, Deerfield, IL, USA) was administered at a dose of 500 mg (kg body weight)$^{-1}$ in aqueous solution

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Table 1. Characteristics of mice in the nutritional intervention study (Part 2)

%BA in DW	Injection solution	*n*	BW start (g)	Δ BW (g)	Drinking volume (ml mouse^{-1} day^{-1})	Total dose (g BA mouse^{-1})
0%	Saline	15	27.0 ± 1.5	1.5 ± 0.8	6.34 ± 1.24	/
0.1%	Saline	15	25.0 ± 1.9§	1.3 ± 1.2	4.91 ± 0.79 §	0.069 ± 0.011
	Vigabatrin	15	25.5 ± 2.3	–0.4 ± 0.9*	4.39 ± 0.51*	0.061 ± 0.007
	AOA	8	26.8 ± 1.9	–0.5 ± 0.9*	5.76 ± 0.14*	0.081 ± 0.002
0.6%	Saline	7	26.3 ± 3.2	1.5 ± 0.8	5.25 ± 0.35 §	0.441 ± 0.030
1.2%	Saline	6	26.7 ± 1.5	1.6 ± 1.4	4.15 ± 0.99 §§	0.776 ± 0.108

§$P < 0.05$ and §§$P < 0.001$ *vs*. 0% β-alanine – SAL and *$P < 0.05$ *vs*. 0.1% β-alanine – SAL. Data are the mean ± SD. BA, β-alanine; BW, body weight; DW, drinking water.

[50 mg vigabatrin (ml saline)$^{-1}$ or a 10 μl injection volume (g body weight)$^{-1}$]. AOA (Sigma, St Louis, MO, USA) was administered in a dose of 10 mg (kg body weight)$^{-1}$ in aqueous solution [1 mg AOA (ml saline)$^{-1}$ or a 10 μl injection volume (g body weight)$^{-1}$]. The same injection volume was used for saline. Drinking water was refreshed at least three times a week and body weight and drinking volume per cage (two or three animals) were monitored.

The last inhibitor injection was performed 3 h prior to dissection. Mice were anaesthetized by an I.P. infusion of 80% xylazine–20% ketamine [5 μl (g body weight)$^{-1}$]. After careful dissection of soleus, tibialis anterior and gastrocnemius muscles and blood collection by cardiac puncture, mice were killed by cervical dislocation. Kidneys, liver, heart and brain were dissected and urine was collected from the bladder. Any visible connective or fat tissue was removed from the tissues and all samples were quickly frozen in liquid nitrogen and stored at –80 °C. Blood samples were centrifuged at 16,000 *g* for 5 min at 4 °C and serum was stored at –80 °C.

Preparation of mouse liver extracts. Mouse liver extracts ($n = 3$) were prepared by homogenizing frozen samples in 3 volumes (w/v) of extraction buffer (50 mM potassium phosphate buffer, pH 7, 0.1% Triton X-100, 25 μM pyridoxal-phosphate and 5 μg ml^{-1} of leupeptine and antipaine), followed by centrifugation (16,000 *g* for 20 min at 4 °C) and collection of the supernatant containing soluble proteins. Liver extracts were stored at –80 °C.

Assays of GABA-T and AGXT2 transaminase activities. GABA-T and AGXT2 activity was measured as described above, except the reaction was started by addition of mouse liver extract.

mRNA expression of carnosine-related enzymes and transporters in mouse tissues by means of quantitative PCR. Total RNA from mouse skeletal muscles, heart, liver, kidney and brain was isolated using the TriPure Isolation Reagent (Roche, Basel, Switzerland) followed by purification with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). An on-column DNase treatment was performed using the RNase-Free DNase Set (Qiagen). RNA was quantified using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA purity was assessed using the A_{260}/A_{280} ratio. Using a blend of oligo(dT) and random primers, 500 ng of RNA was reversed transcribed with the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's instructions. Quantitative PCR was carried out on a Lightcycler 480 system (Roche) using an 8 μl reaction mix containing 3 μl of template cDNA (1:10 dilution), 300 nM forward and reverse primers and 4 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The cycling conditions comprised a polymerase activation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Primer sequences (Table 2) of most genes of interest [CARNS, taurine transporter (TauT) and GABA-T] are available in the literature (Everaert *et al.* 2013). The primer sequence for AGXT2 was newly designed using Primer Express, version 3.0 (Applied Biosystems). Sequence specificity was confirmed using NCBI Blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To control the specificity of amplification, data melting curves were inspected and PCR efficiency was calculated for AGXT2. Normalized gene expression values were calculated by dividing the relative gene expression values (calculated by the ΔCt method) for each sample by the expression values of the geometric mean of Ppia, Rplp0 and GAPDH as selected by GeNorm (Vandesompele *et al.* 2002).

Quantification of β-alanine and GABA by means of high-performance liquid chromatography. Brains were dissolved in PBS (1 mg wet weight brain 10 μl^{-1}) for homogenization. Brain homogenates, serum and urine were deproteinized using 35 % sulphosalicylic acid and centrifuged (5 min at 16,000 *g*). Deproteinized supernatant was mixed with AccQ Fluor Borate buffer and reconstituted Fluor Reagent (1:7:2) from the AccQTag

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Table 2. Primers used in quantitative PCR analysis

Function	Gene symbol	Forward primer (5′- to 3′) Reverse primer (5′- to 3′)	Source
Carnosine synthesis	CARNS	TGA-TAG-GCC-CCT-ACT-GAG-TAA-GGT TCA-GTG-TCC-TTG-GCA-GGG-TAT	Everaert *et al.* (2013)
β-alanine transport	TauT	TGG-CCG-ACA-GCA-TTC-CA GCC-TTC-TCT-AAG-GTG-CCT-TCC-T	Everaert *et al.* (2013)
β-alanine transaminase	GABA-T	CCT-TCA-TGG-GTG-CTT-TCC-A CAA-AGG-AAG-GGA-TGT-CAA-TCT-TG	Everaert *et al.* (2013)
	AGXT2	GAT-AGG-CTG-CCA-ATC-AAC-AAT-GT TGC-ACT-GGA-GAA-TCT-CGA-CAA	Primer express
Reference genes	Ppia	CAA-ATG-CTG-GAC-CAA-ACA-CAA-ACG GTT-CAT-GCC-TTC-TTT-CAC-CTT-CCC	RTprimerDB
	Rplp0	GGA-CCC-GAG-AAG-ACC-TCC-TT GCA-CAT-CAC-TCA-GAA-TTT-CAA-TGG	RTprimerDB
	GAPDH	CAC-CAT-CTT-CCA-GGA-GCG-AG CCT-TCT-CCA-TGG-TGG-TGA-AGA-C	RTprimerDB

chemistry kit (Waters, Milford, MA, USA). The same method was applied to the combined standard solutions of β-alanine (Sigma) and GABA (Sigma). The derivatized samples were applied to a Waters high-performance liquid chromatography system comprised of an AccQTag column (3.9 × 150 mm, 4 μm) and fluorescence detector (excitation/emission wavelength: 250/395 nm). The column was equilibrated with buffer A [10% eluent A (Waters)–90% H_2O], buffer B (100% acetonitrile) and buffer C (100% H_2O) at a flow rate of 1 ml min^{-1} at room temperature. Urinary β-alanine values were normalized to creatinine using the creatinine assay kit (Sigma).

Quantification of histidine-containing dipeptides by liquid chromatography–mass spectrometry. Tissue carnosine and anserine levels were measured using a Micromass ZMD mass spectrometer (Waters) (Baba *et al.* 2013). Homocarnosine was also measured in the brain. Briefly, the tissues were homogenized in PBS buffer containing the protease inhibitor (1:100) and internal standard tyrosine-histidine. The homogenates were centrifuged at 16,000 *g* for 10 min. The pellets were discarded and the supernatant was precipitated by perchloric acid. The samples were neutralized by ammonium hydroxide and diluted in 90% H_2O, 10% acetonitrile and 0.1% heptafluorobutyric acid. The peptides were separated by reverse phase elution with a polar RP column protected by a polar RP guard column. The solution was infused into the mass spectrometer in the positive ion mode. The spectrometer was calibrated using NaCsl with the calibration routine included in MassLynx, version 3.4 (Waters). Samples were diluted in 70% water:30% acetonitrile and the solution was infused using a glass syringe and a Harvard infusion pump at a rate of 10 μl min^{-1}. Tuning conditions were capillary 2.9 kV, cone 34 V, extractor 9 V, Rflens 0.9 V, source temperature 100 °C, desolvation gas 200C, low mass resolution 15.2, ion energy 0.3 V multiplier 650 relative setting. The acquisitons for carnosine (parent ion 227 Da, daughter ion 110 Da) anserine (parent ion 241 Da, daughter ion 109.2 Da), homocarnosine (parent ion 241 Da, daughter ion 109.22 Da) and tyrosine histidine (parent ion 319 Da and daughter ion 110.22 Da) were taken in the multiple reaction monitoring mode. The limits of detection for carnosine and anserine are 0.00367 nmol and 0.0303 nmol, respectively. The limits of quantification for carnosine and anserine are 0.011 nmol and 0.0917 nmol, respectively.

GABA-T activity in brains. GABA-T activity was measured in brain samples according to the method of Awad *et al.* (2007). Brain samples were stored at –80 °C until homogenization in 10 volumes of chilled buffer of the composition: 20% glycerol, 0.13% Triton X-100, 0.1 mM glutathione, 1 mM Na_2EDTA, 10 mM K_2HPO_4, 0.1 mM pyroxidal-5′-phosphate and acetic acid (pH 6.8). The homogenates were frozen and thawed once before adding to the incubation medium (20 μl) on a 96-well plate. The incubation medium (180 μl) consisted of 100 mM potassium pyrophosphate, 3.5 mM 2-mercaptoethanol, 0.01 mM pyroxidal-5′-phosphate, 5 mM 2-oxoglutarate and 4 mM NAD^+. The samples were pre-incubated for 15 min at 37 °C before the addition of GABA (10 mM final concentration). The rate of the enzymatic reaction was determined by measuring NADH production at 37 °C for 10 min within the linear range, using a i-control (infinite 200 Pro) spectrophotometer (Tecan, Männedorf, Switzerland) (excitation/emission wavelength: 360/465 nm). Enzymatic activity was calculated relative to a control sample

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(distilled H_2O instead of GABA) using a NADH standard curve.

Statistical analysis

Data are reported as the mean ± SD. $P \leq 0.05$ was considered statistically significant. The body weight at start, change in body weight and drinking volume were evaluated separately for the 4 oral β-alanine supplementation doses on the one hand and the 0.1% β-alanine groups treated with different inhibitors on the other. One-way ANOVA followed by a *post hoc* Tukey's test in the case of a significant group effect was used. A general linear model repeated measures ANOVA was used to evaluate body weight over time (start to end). The dose–response effect of different dosages of β-alanine supplementation on HCD loading and the effect of treatments with different inhibitors on enzyme activity and tissue metabolites was evaluated by one-way ANOVA followed by a *post hoc* Tukey's test in the case of a significant group effect. For the urine and blood parameters, an independent sample *t* test was used to evaluate the effect of 0.1% β-alanine supplementation compared to 0% β-alanine and a one-way ANOVA and, subsequently, a *post hoc* Tukey test was used to evaluate the effect of treatments with different inhibitors compared to the 0.1% β-alanine group. Correlations between serum β-alanine levels and tissue HCD content were obtained by means of Pearson correlations. All statistical analyses were performed using SPSS, verison 22.0 (IBM Corp., Armonk, NY, USA).

Results

Activity of recombinant mouse GABA-T and AGXT2 in HEK293T cell extracts and effect of vigabatrin and AOA

The enzymatic activity of GABA-T was detected in transfected cell extracts and was similar in the presence of 2.5 mM GABA and β-alanine (Fig. 1*A*), confirming that *in vitro* β-alanine was a good substrate for GABA-T. In this case, the activity measured in the presence of 2.5 mM GABA was decreased by 75% and 86% when 0.5 mM vigabatrin or 2 μM AOA, respectively, was added to the assay (Fig. 1*C*). Similarly, cell extracts containing recombinant AGXT2 showed enzymatic activity in the presence of 5 mM β-alanine and DL-β-aminoisobutyrate

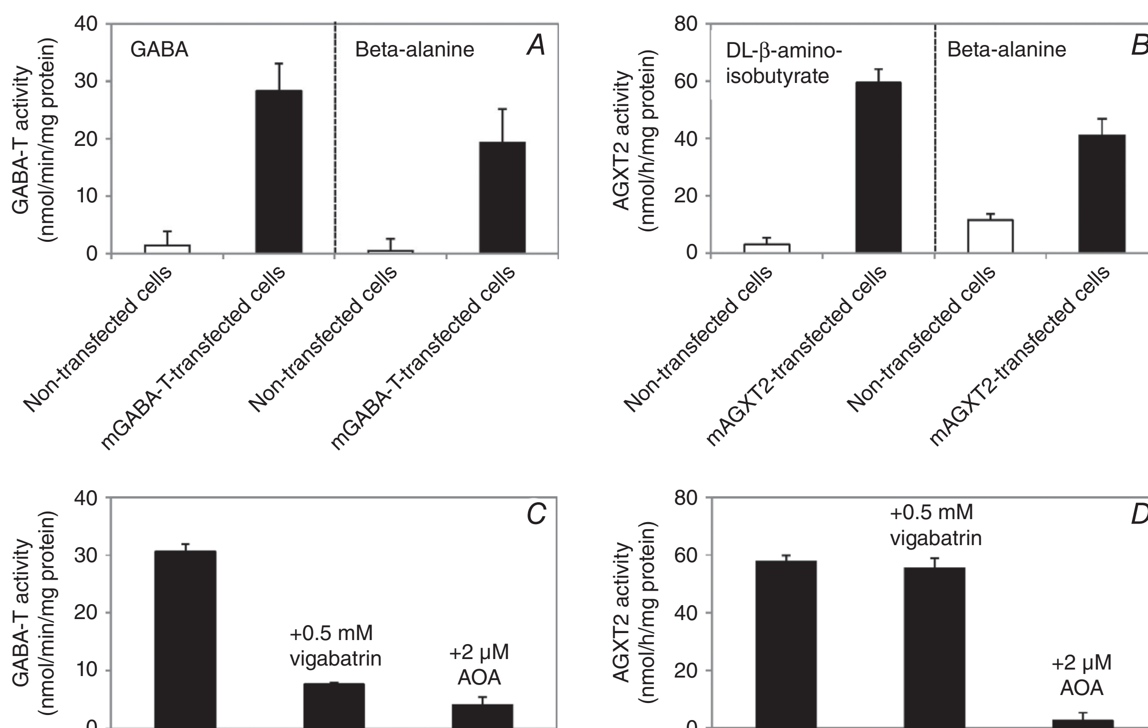

Figure 1. Activity of recombinant mouse GABA-T and AGXT2 (mGABA-T and mAGXT2) in cell extracts of HEK293T and inhibition by vigabatrin and aminooxyacetate (AOA)
HEK293T cells were transfected or not with plasmids expressing mouse GABA-T (*A* and *C*) or AGXT2 (*B* and *D*). The enzymatic activities of recombinant GABA-T and AGXT2 were assayed *in vitro*, using the corresponding cell extracts, in the presence of 2.5 mM GABA and 5 mM DL-β-aminoisobutyrate, respectively (*A* and *B*). The effect of vigabatrin (0.5 mM) and AOA (2 μM) on the enzymatic activities was tested by adding the inhibitors directly to the assay mixture as described in Methods (*C* and *D*). Values are the mean ± SD of three independent measurements.

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(Fig, 1*B*) but, in contrast to GABA-T, AGXT2 activity was only inhibited by AOA (95% inhibition) and not by vigabatrin (Fig. 1*D*). Similar results were obtained with recombinant human GABA-T and AGXT2 (data not shown).

mRNA expression profiles of carnosine-related enzymes and transporters in different mice tissues

The TauT, responsible for transmembrane transport of β-alanine, was ubiquitously expressed in all murine tissues investigated. CARNS, which is the enzyme responsible for carnosine and homocarnosine synthesis, was expressed mainly in the brain and the striated muscles, with highest mRNA levels in glycolytic (gastrocnemius and tibialis anterior muscles) rather than oxidative muscles (soleus, heart) (Fig. 2*A* and *B*). The β-alanine transaminating enzymes GABA-T and AGXT2 showed highest mRNA expression in the liver and kidney, low mRNA expression in oxidative muscles, and even lower expression in glycolytic muscles. However, GABA-T, but not AGXT2, was also clearly expressed in the brain (Fig. 2*C* and *D*).

Effect of vigabatrin and AOA on GABA-T and AGXT2 activity in liver extracts

Because both GABA-T and AGXT2 were highly expressed in liver, we tested the effects of *in vivo* administration of vigabatrin and AOA on the *ex vivo* activity of these enzymes in liver extracts. In agreement with the results found for the recombinant enzymes (Fig. 1), the administration of AOA resulted in the strong inhibition of both GABA-T (–83%) and AGXT2 (–99%) enzymatic activities, whereas vigabatrin was a more specific inhibitor of GABA-T activity (–81%) and only minimally affected the activity of AGXT2 (–26%) (Fig. 3*A* and *B*).

Dose–response relationship of β-alanine supplementation

Oral β-alanine supplementation (0.1% β-alanine w/v in drinking water for 2 weeks) had no effect on the HCD storage in either gastrocnemius or the soleus muscle (Fig. 4*A* and *B*). However, supraphysiological doses of 0.6% and 1.2% of β-alanine led to significant increases in total muscle HCD content. In soleus muscle, which is characterized by low amounts of carnosine and anserine, 3- to 4-fold increases were found ($P \le 0.001$ for 0.6% and 1.2% compared to 0% β-alanine). In gastrocnemius, a 40–50% increase in the levels of HCDs was found in mice supplemented with 0.6% and 1.2% β-alanine ($P = 0.004$ and $P = 0.017$ compared to 0% β-alanine). The lack of increase in muscle HCD concentrations with 0.1% β-alanine suggests that, at these levels of oral supplementation, all the ingested β-alanine is transaminated. Subsequent inhibitor experiments were therefore performed in mice supplemented with this dose of β-alanine.

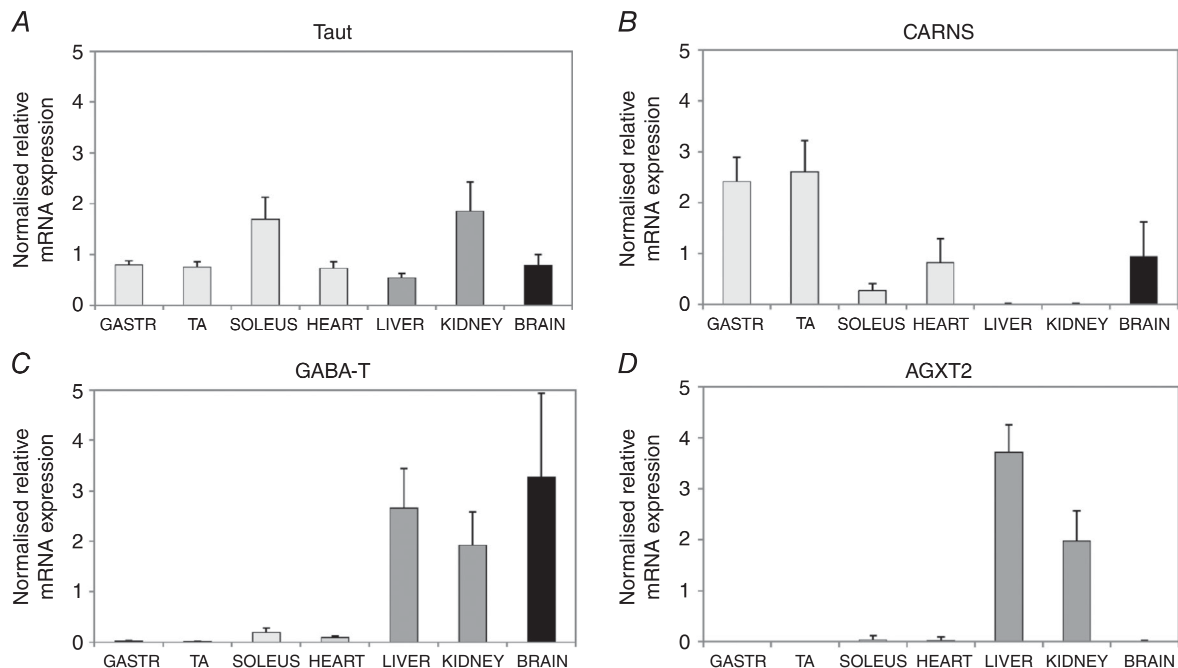

Figure 2. mRNA expression profile of carnosine-related enzymes and transporters in different mice tissues (three skeletal muscles, heart, liver, kidney and brain)
mRNA expression profile is shown for TauT (panel *A*), CARNS (panel *B*), GABA-T (panel *C*) and AGXT2 (panel *D*) GASTR, gastrocnemius; TA, tibialis anterior. Values are the mean ± SD of six measurements for each tissue.

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Effect of inhibitors on serum and urinary β-alanine levels

Although there was no significant effect of 0.1% β-alanine supplementation by itself, serum and urinary β-alanine levels were significantly increased when this low amount of oral β-alanine was combined with AOA (+218%, $P = 0.009$ and +250%, $P = 0.001$ *vs.* 0.1% β-alanine – SAL for serum and urine, respectively). Vigabatrin did not significantly affect serum, nor urinary β-alanine levels (+84%, $P = 0.486$ and +4.0%, $P = 0.997$ *vs.* 0.1% β-alanine – SAL for serum and urine, respectively) (Fig. 5*A* and *B*). Individual data points are shown in Fig. 5 to demonstrate the large inter-individual variation that was observed.

Effect of inhibitors on tissue HCD levels

Supplementation of 0.1% β-alanine did not affect the HCD levels in any of the investigated tissues. However, AOA coadministration led to significantly higher HCD loading in the different muscles (Fig. 6*A*–*D*). The highest effects were found in the soleus muscle and the heart, with increases of 128% and 541%, respectively ($P < 0.001$ *vs.* 0.1% β-alanine – SAL). In soleus and heart, but not in the more glycolytic muscles, serum β-alanine was positively correlated with HCD levels ($r = 0.537$, $P = 0.008$ for soleus, $r = 0.570$, $P = 0.007$ for heart). The more glycolytic muscles also showed significantly (or trending to significance) higher HCD loading with AOA administration (+105%, $P = 0.011$ and +21%, $P = 0.056$ *vs.* 0.1% β-alanine – SAL for tibialis anterior and gastrocnemius, respectively), although the effects were absent in the kidney and brain (Fig. 6*E* and *F*). Furthermore, no effects of vigabatrin on carnosine and anserine levels were found in any of the investigated tissues.

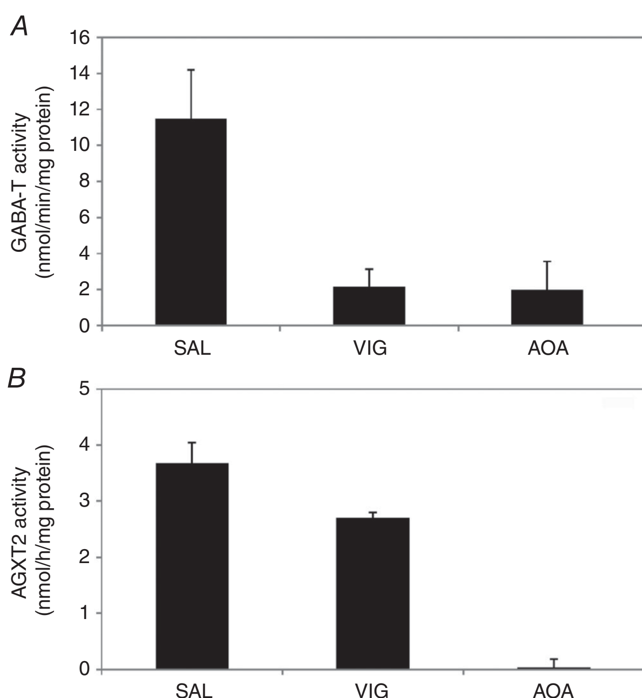

Figure 3. Effect of vigabatrin and AOA on GABA-T and AGXT2 activity in liver extracts
Effect of administration of vigabatrin or AOA on the *ex vivo* activity of β-alanine transaminating enzymes (GABA-T and AGXT2) measured in liver extracts from mice supplemented with 0.1% β-alanine. GABA-T activity (*A*) was measured with 2.5 mM GABA and 1 mM α-ketoglutarate and AGXT2 (*B*) with 5 mM DL-β-aminoisobutyrate and 1 mM pyruvate. The effect of vigabatrin and AOA on the liver enzymatic activities is the result of the 14 day treatment that the mice were subjected to (prior to the preparation of the extract) and not to the addition of the inhibitors directly to the assay mixture, as described in the Methods. VIG, vigabatrin. Values are the mean ± SD of three independent measurements.

Brain GABA-T activity, brain GABA and homocarnosine levels and serum GABA

Brain GABA-T activity was significantly decreased with administration of vigabatrin or AOA (–82% and –88%, respectively; $P < 0.001$) (Fig. 7*A*). B-alanine supplementation did not significantly affect brain GABA levels but led to a significant decrease in brain homocarnosine levels (–29%, $P = 0.05$ *vs.* 0% β-alanine – SAL). With vigabatrin and AOA administration, both brain GABA and homocarnosine levels were significantly increased (brain GABA: $P < 0.001$ *vs.* 0.1% β-alanine – SAL for vigabatrin and AOA, brain homocarnosine: $P = 0.015$ and $P = 0.027$ *vs.* 0.1% β-alanine – SAL for vigabatrin and AOA, respectively) (Fig. 7*B* and *C*). Serum GABA was not affected by β-alanine supplementation and was only significantly elevated when vigabatrin was administered (+369%, $P = 0.002$ *vs.* 0.1% β-alanine - SAL), whereas AOA administration had no effect (Fig. 7*D*).

Body weight and drinking behaviour

Body weight at the start of the intervention was similar between groups, except for the 0.1% β-alanine – SAL group, which showed a significantly lower body weight compared to the control group ($P = 0.044$) (Table 1). Change in body weight was not significantly different between groups supplemented with different amounts of β-alanine, although their body weight over time did show a significant increase compared to the body weight at the start ($P < 0.05$ for all four groups). By contrast, change in body weight was different between the 0.1% supplemented groups treated with different inhibitors. Body weight gain over time was significantly lower in vigabatrin and AOA treated groups compared to 0.1% β-alanine – SAL group ($P < 0.001$ and $P = 0.002$, respectively). In addition, the daily drinking volume of all β-alanine supplemented groups was significantly lower compared to the control

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group (P < 0.05 for 0.1% and 0.6% β-alanine and P < 0.001 for 1.2% β-alanine *vs.* 0% β-alanine) (Table 1). Mice treated with vigabatrin drank significantly less and mice treated with AOA drank significantly more than the 0.1% β-alanine – SAL group (P = 0.05 and P = 0.007, respectively).

Discussion

The present study aimed to clarify the contribution of β-alanine degradation by GABA-T and/or AGXT2 to the metabolism and the homeostasis of HCDs in various mice tissues. Selective GABA-T inhibition by vigabatrin caused a moderate, non-significant elevation of circulating β-alanine concentrations, indicating that GABA-T is probably involved in the degradation of β-alanine upon oral ingestion in mice. Nevertheless, the rise in plasma β-alanine was not sufficient to elevate HCD content in tissues that express CARNS (i.e. skeletal muscles and heart). Administration of AOA, which inhibits GABA-T to the same degree as vigabatrin and additionally inhibits AGXT2, resulted in much larger circulating and urinary β-alanine levels. In turn, this had a marked positive effect on tissue HCD content. This effect was most pronounced in oxidative-type striated muscles (soleus and heart), less pronounced in glycolytic-type muscles (gastrocnemius and tibialis anterior) and absent in the kidney or the brain. The overview of our current understanding of the β-alanine metabolism, based on the results of the present study, is illustrated in Fig. 8.

It is currently assumed that β-alanine is transaminated into MSA mainly by GABA-T and AGXT2. Enzymatic assays have already indirectly demonstrated that these enzymes can transaminate β-alanine in liver, kidney and brain extracts (Tamaki *et al.* 1990; Kontani *et al.* 1998; Ito *et al.* 2001), although the present study provides the first strong direct evidence using pure proteins obtained by recombinant DNA technology (Fig. 1*A* and

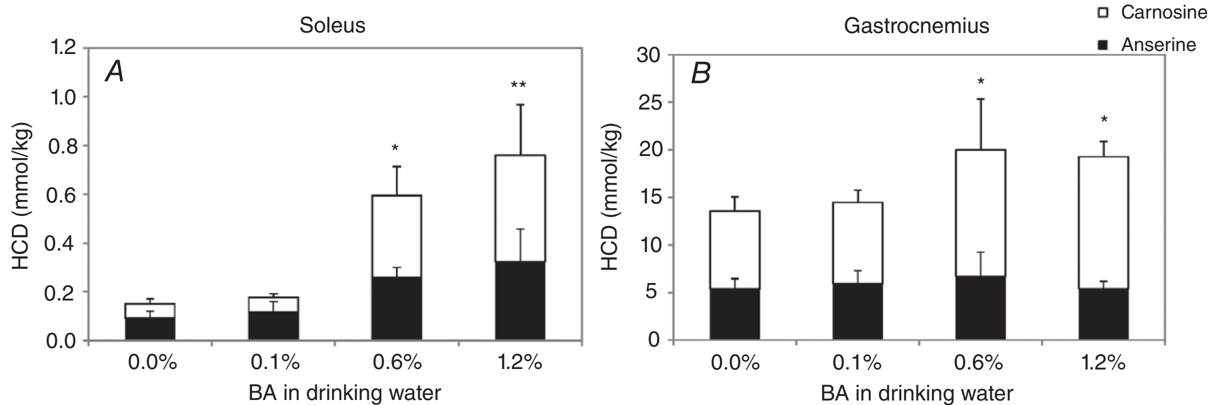

Figure 4. Dose-response relationship of β-alanine supplementation in soleus and gastrocnemius
Dose–response relationship between the amount (w/v) of β-alanine (BA) added to the drinking water (0%, 0.1%, 0.6% or 1.2%) and the HCD levels for musculus soleus (*A*) and musculus gastrocnemius (*B*). $^{*}P$ < 0.05 and $^{**}P$ < 0.001 *vs.* 0% β-alanine – SAL. Significant differences refer to total HCD content (sum of carnosine and anserine). Values are the mean ± SD.

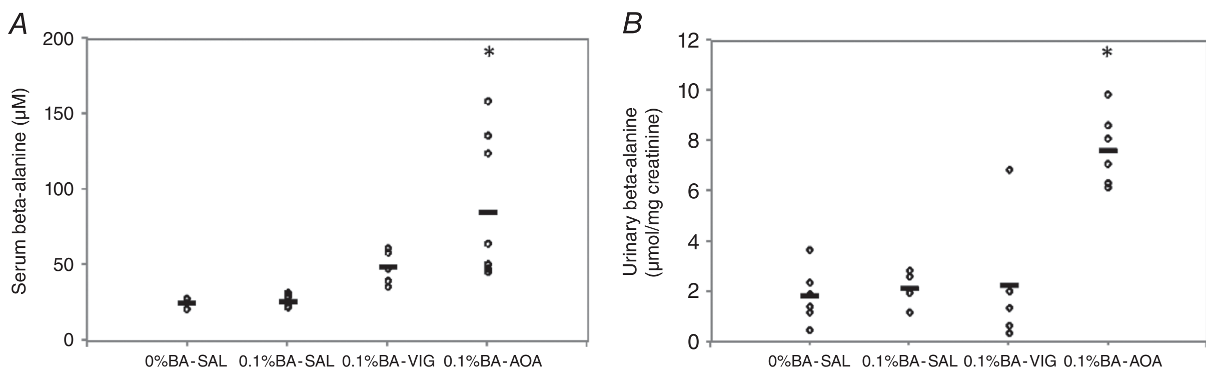

Figure 5. Effect of inhibitors on serum and urinary β-alanine levels
Effect of oral 0.1% β-alanine (BA) supplementation combined with SAL, vigabatrin (VIG) or AOA, on serum (*A*) and urinary (*B*) β-alanine levels. $^{*}P$ < 0.05 *vs.* 0.1% β-alanine – SAL. Symbols represent individual results (circles) and the mean (dash).

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B). Furthermore, using this approach, we could confirm that GABA-T, but not AGXT2, is inhibited by vigabatrin and that both these enzymes are inhibited by AOA (Fig. 1*C* and *D*). Although, on the basis of these data, we cannot exclude the involvement of other enzymes in this process, to our knowledge, there are no other known mammalian enzymes that can transaminate β-alanine. The finding that circulating levels of β-alanine were markedly affected when both GABA-T and AGXT2 were inhibited *in vivo* further supports this notion.

Gene expression of β-alanine transaminating enzymes is shown to be high in liver and kidney and GABA-T, unlike AGXT2, is also expressed in the brain of control mice (Fig. 2*C* and *D*). In line with the *in vitro* results in transfected HEK293t cells, *ex vivo* GABA-T and AGXT2 activity in liver extracts was affected in the same way following *in vivo* administration of vigabatrin and AOA (Fig. 3*A* and *B*). Specific GABA-T inhibition by vigabatrin, however, did not significantly affect circulating β-alanine levels, whereas inhibition of both enzymes by AOA led to a marked increase in these levels *in vivo* (Fig. 5*A*), suggesting that the combined inhibition of both these enzyme increases the circulating levels of this amino acid.

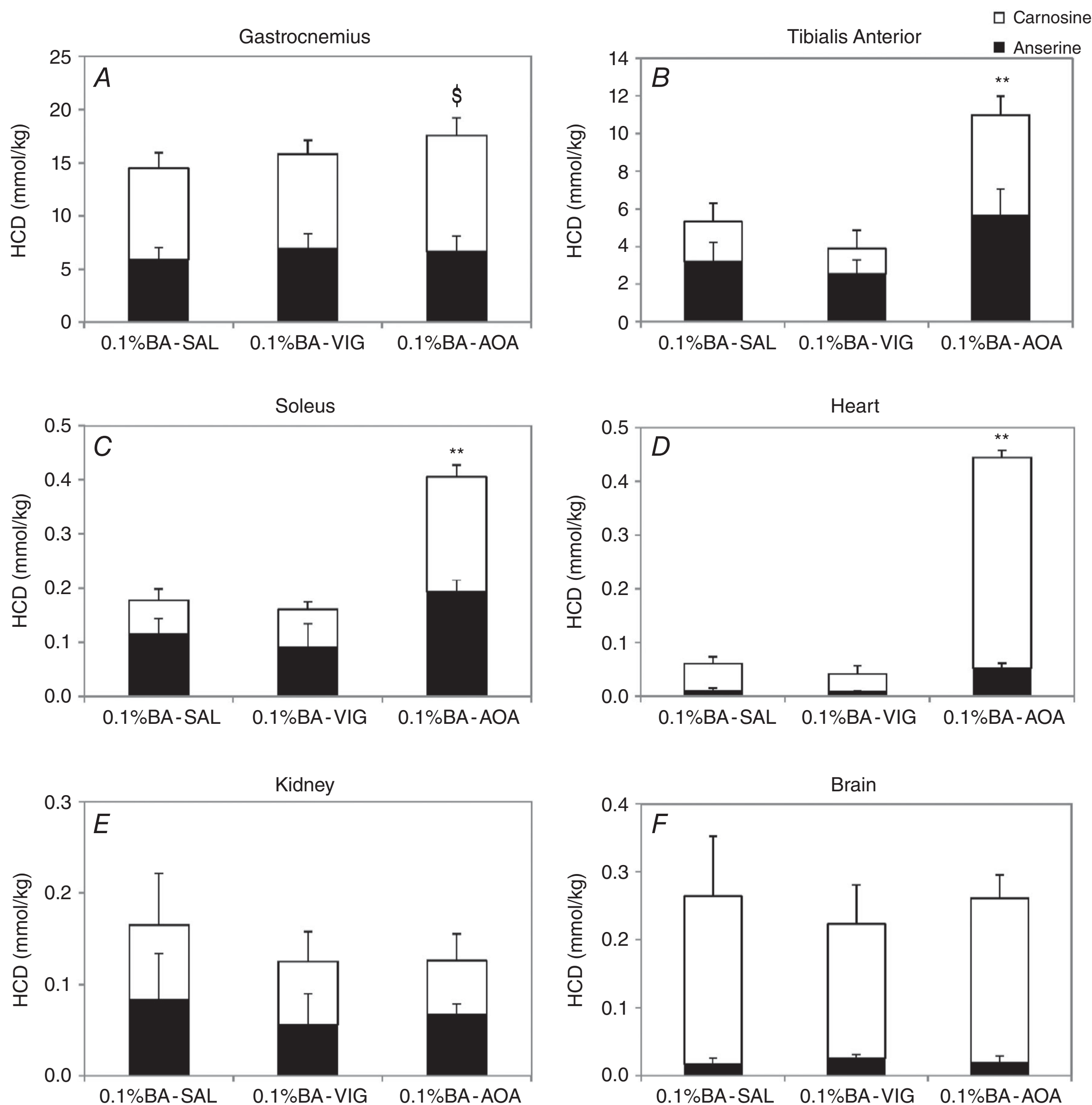

Figure 6. Effect of 0.1% β-alanine (BA) supplementation combined with SAL, vigabatrin (VIG) or AOA on tissue HCD levels
HCD levels are shown for gastrocnemius and tibialis anterior (panel *A-B*), soleus and heart (panel *C-D*), kidney (panel *E*) and brain (panel *F*). $^{**}P < 0.001$; $^{\$}P > 0.05$ and < 0.1 *vs.* 0.1% β-alanine – SAL. Significant differences refer to total HCD content (sum of carnosine and anserine). Values are the mean ± SD.

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To demonstrate that the transamination of β-alanine is involved in HCD homeostasis, we searched for a suitable oral β-alanine dose that did not increase muscle HCD content on its own, meaning that all exogenously provided β-alanine is fully routed toward oxidation rather than dipeptide synthesis. Of the three different doses tested, we found that muscle HCD levels remained stable only with the lowest dose (0.1% β-alanine), whereas both 0.6% and 1.2% β-alanine increased HCD content above the physiological set-point, suggesting that the β-alanine transaminases (GABA-T and AGXT2) are saturated and no longer able to metabolize all circulating β-alanine (i.e. fail to avoid a rise in circulating β-alanine) (Fig. 4*A* and *B*). Hence, to ensure that the β-alanine transaminases are not saturated, we performed all subsequent *in vivo* experiments with the lowest dose (0.1% β-alanine).

In accordance with the results showing that circulating β-alanine levels were increased only upon inhibition of both transaminase enzymes, only AOA administration led to significantly elevated HCD levels in gastrocnemius, tibialis anterior, soleus and heart (Fig. 6*A*–*D*). This effect was absent in kidney, presumably because CARNS is not highly expressed in this tissue (as assumed from the mRNA expression profile). However, specific GABA-T inhibition by vigabatrin did not have an effect on the HCD content in any of the tissues. Because we did not have an inhibitor specific for AGXT2, which does not act on GABA-T, the contribution of GABA-T to the β-alanine metabolism remains unclear. However, our results are consistent with the idea that either GABA-T does not play an important role in the transamination process or AGXT2 is able to compensate for the loss in GABA-T activity when vigabatrin is administered. Nevertheless, when taken together, these data clearly demonstrate that β-alanine transaminases are involved in tissue HCD homeostasis.

As noted above, we found that both GABA-T and AGXT2 are highly expressed in liver and kidney but show very low mRNA expression in the different muscles. However, the *in vivo* effects of AOA were most pronounced in oxidative-type muscles (soleus and heart) and less pronounced in glycolytic-type muscles (tibialis anterior and gastrocnemius). Because GABA-T and AGXT2 are mitochondrial enzymes and oxidative muscles have more mitochondria compared to glycolytic muscles, it is reasonable to propose that, in addition to oxidation in liver and kidney, β-alanine can also be locally oxidized in these muscle cells. Figure 2*C* and *D* shows the low but detectable mRNA expression of GABA-T and AGXT2 in the soleus and the heart, but not in the tibialis anterior and gastrocnemius muscles. Hence, even though it appears that β-alanine oxidation takes place mainly in liver and kidney, additional peripheral β-alanine degradation could possibly take place in oxidative muscles. Using radioactive labelled β-alanine, Tamaki *et al.* (1980) demonstrated that β-alanine is more stable in rat gastrocnemius (half-life of 2.27 h) compared to liver (half-life of 0.41 h) but

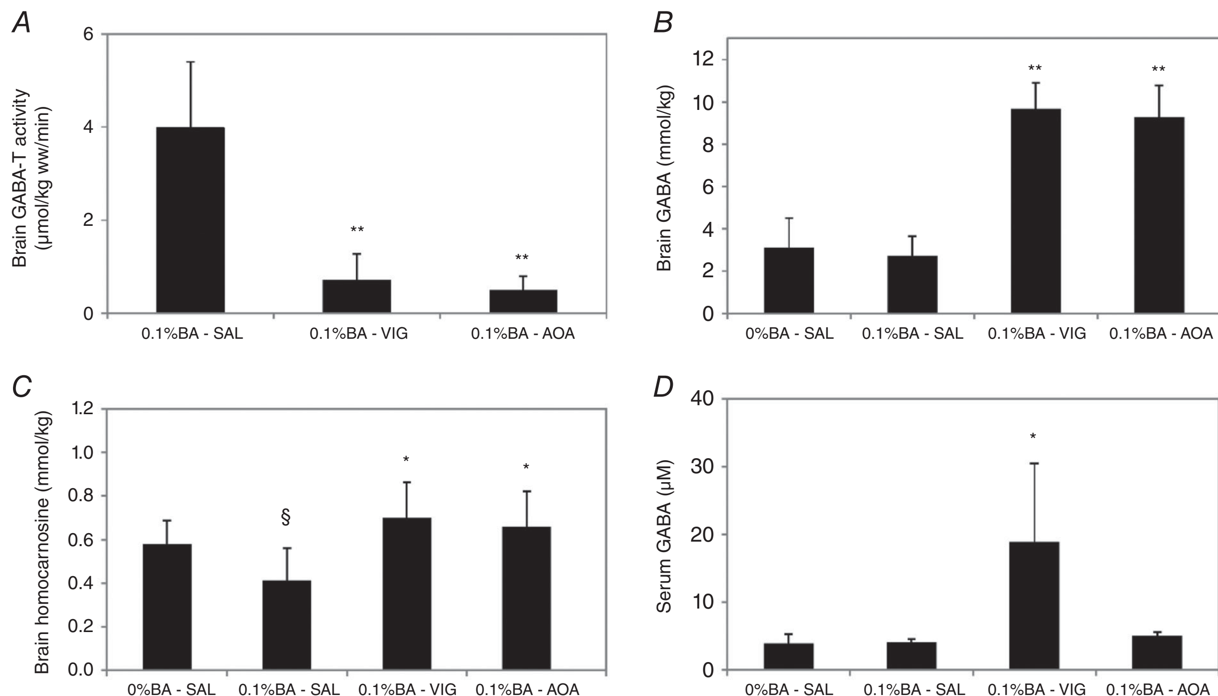

Figure 7. Effect of 0.1% β-alanine (BA) combined with SAL, vigabatrin (VIG) or AOA on brain GABA-T activity (*A*) and concentrations of brain GABA (*B*), brain homocarnosine (*C*) and serum GABA (*D*)
$^*P < 0.05$ and $^{**}P < 0.001$ *vs.* 0.1% β-alanine – SAL; §$P \leq 0.05$ *vs.* 0% β-alanine – SAL. Values are the mean ± SD.

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still much less stable than muscle carnosine (half-life of 28 days), suggesting that the peripheral degradation of β-alanine is possible. This possibility is further supported by a recent study (Hatazawa *et al.* 2015) demonstrating that, in comparison with wild-type mice, GABA-T is upregulated (by 4.0-fold) in the muscles of transgenic mice overexpressing skeletal muscle specific peroxisome proliferator-activated receptor gamma coactivator 1α. Furthermore, muscle β-alanine, carnosine and anserine levels were decreased in these transgenic mice (ranging from 0.04- to 0.15-fold). These findings support the hypothesis that, in oxidative muscles, intramuscular β-alanine is converted into acetyl-coenzyme A (via MSA) and subsequently enters the citric acid cycle.

By far the largest effect of β-alanine transaminase inhibition was found in the heart, showing 10-fold higher HCD levels when 0.1% β-alanine was administered simultaneously with the transaminase inhibitor AOA (Fig. 6*D*). To our knowledge, this is the first study showing an intervention that can elevate HCD content in the heart. A recent study by Swietach *et al.* (2013) demonstrated that HCDs can act as diffusible Ca^{2+}/H^+ exchangers in cardiomyocytes because both calcium ions and protons can competitively bind to proteins and dipeptides, such as the HCDs. Ca^{2+} signalling regulates many cell functions and is modulated by H^+ ions, suggesting that spatial Ca^{2+}/H^+ coupling is probably of general importance in cell signalling and function. An additional pathophysiological relevance of carnosine was recently reported by Baba *et al.* (2013), who demonstrated that carnosine plays an important role in detoxifying reactive aldehydes and promotes functional recovery in the ischaemic heart. Taken together, these recent studies indicate an important role of HCDs in the heart and suggest that an increase in HCD levels could positively influence cardiac function and the resistance of the heart to ischemic injury.

In the present study, we found that, upon moderate dietary β-alanine exposure, β-alanine transaminases can degrade all excess exogenous β-alanine to maintain tissue HCD homeostasis. Hence, to elevate muscle HCD content, it may be necessary to first saturate these enzymes to achieve significant HCD loading. This condition is probably not met under normal human dietary situations. However, the selective ingestion of β-alanine in high doses, as in human athletes ingesting 4–6 g of pure β-alanine for several weeks, can saturate the transaminases and lead to elevated muscle HCD content. The activity of β-alanine transaminating enzymes probably explains the low muscular uptake and loading efficiency (3–6%) of β-alanine supplementation, as calculated by Stegen *et al.* (2013). Because β-alanine is first routed towards the degradation pathway, sufficient β-alanine needs to be ingested, resulting in a rather inconvenient

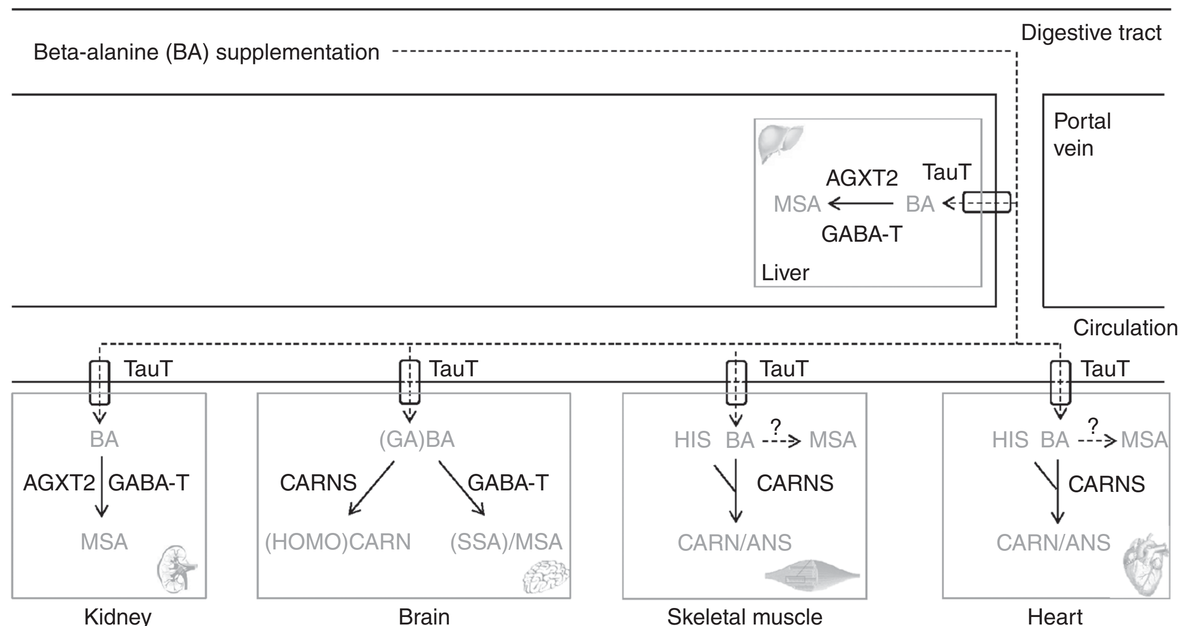

Figure 8. Overview of the current understanding of the β-alanine metabolism
β-alanine transaminating enzymes GABA-T and AGXT2 are expressed at mRNA level in liver and kidney, converting β-alanine to MSA. CARNS is mainly expressed in the brain, skeletal muscles and heart. Inhibiting GABA-T and AGXT2 leads to increased circulating levels of β-alanine, which can be taken up in different tissues by the TauT and subsequently converted to carnosine and anserine. ANS, anserine; BA, β-alanine; (HOMO)CARN, (homo)carnosine; HIS, histidine; SSA, succinate semi-aldehyde.

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supplementation protocol. This is fortified by case reports in which enhanced β-alanine and carnosine levels are present in the plasma and muscles, respectively, which is suggested to be attributed to genetic deficiencies in the β-alanine transaminase enzymes (Scriver *et al.* 1966; Jaeken *et al.* 1984). However, improving the efficiency of the β-alanine supplementation protocol in healthy humans by inhibiting transaminases appears to be difficult because of the toxicity (AOA) and side-effects (vigabatrin) of these inhibitors and the multiple other metabolic pathways in which these transaminases are involved.

The metabolism of β-alanine, as determined in the present study, closely resembles the metabolism of another non-proteinogenic amino acid: GABA. GABA is an inhibitory neurotransmitter present in the brain and, similar to β-alanine, a substrate for GABA-T. Interestingly, GABA is also a substrate for CARNS, synthesizing homocarnosine when combined with L-histidine. It has been shown that the administration of vigabatrin results in a dose-dependent increase in GABA and homocarnosine levels in the brains of both rodents (Jung *et al.* 1977) and humans (Petroff *et al.* 1998, 1999). In addition, AOA has similar effects on GABA levels in the brain (Wallach, 1961; Gelder, 1966; Löscher & Frey, 1978). In the present study, we confirm that GABA-T is expressed at the mRNA level in the brain and inhibition by vigabatrin leads to increased GABA concentrations (Fig. 7*B*), suggesting that GABA-T regulates GABA homeostasis. AGXT2 is not involved in this process. Brain carnosine and anserine were not affected by the administration of vigabatrin or AOA, probably as a result of the high GABA concentrations, with which β-alanine has to compete to occupy CARNS. However, the metabolism of carnosine in the brain is not yet fully understood, although it probably does not depend upon the activity of hepatic transaminases.

The results of the present study show that mice treated with transaminase inhibitors (vigabatrin or AOA) have a reduced body weight gain during the intervention compared to mice treated with saline. This observation is in accordance with other studies (Howard *et al.* 1980; Gale & Iadarola, 1980) and is presumably related to the disturbance of GABA homeostasis, which plays a role in the hypothalamic regulation of food intake.

The present study has some limitations. First, mRNA expression was used to estimate the protein expression and activity of the main enzymes in the different mice tissues. However, mRNA expression is not always in agreement with the amount of mRNA that is effectively translated into protein. Our data on mRNA expression should therefore be considered as an estimation for protein expression and function. However, when available, our expression data are in agreement with the existing literature and probably provide qualitative information on the tissue distribution of the relevant enzymes. Second, two inhibitors were applied in the present study: vigabatrin as a specific inhibitor for GABA-T and AOA as a non-specific inhibitor (inhibiting both GABA-T and AGXT2). Unfortunately, no specific inhibitor for AGXT2 was available. Therefore, we cannot compare the implications of inhibiting only AGXT2 *vs.* inhibiting both enzymes on the metabolism of β-alanine in mice.

In summary, the results of the present study suggest that the homeostasis of the HCDs carnosine and anserine in cardiac myocytes and skeletal muscle is dependent on the circulating availability of β-alanine. In turn, homeostasis of circulating β-alanine is, in the case of excess dietary β-alanine intake, dependent on the degradation of β-alanine in liver and kidney, which express GABA-T and AGXT2 as the main mammalian enzymes capable of metabolizing β-alanine. The present study highlights the importance of β-alanine transamination in tissue HCD homeostasis and thereby contributes to a better understanding of the mammalian β-alanine and carnosine metabolism.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

LB, JS, SS, WC, MVDC, WD and IE conceived and designed the experiments. LB, SPB, SK, JS, SB, WC, AAB, HJD, AB, JD, BV, MVDC, WD and IE collected, analysed and interpreted the data. LB, SPB, SK, JS, SS, SB, WC, AAB, HJD, AB, JD, BV, MVDC, WD and IE drafted the article and revised it critically for important intellectual content. All authors have read and approved the final submission. All authors agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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