Extensive profiling of histidine-containing dipeptides reveals species-specific distribution and metabolism in mice, rats and humans

Short title: Profiling of histidine-containing dipeptides

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Abstract

Histidine-containing dipeptides (HCDs) are pleiotropic homeostatic molecules linked to inflammatory, metabolic and neurological diseases, as well as exercise performance. Using a sensitive UHPLC-MS/MS approach and an optimized quantification method, we performed a systematic and extensive profiling of HCDs in the mouse, rat, and human body (in n=26, n=25, n=19 tissues, respectively). Our data show that tissue HCD levels are uniquely regulated by carnosine synthase, an enzyme preferentially expressed by fast-twitch skeletal muscle fibers and brain oligodendrocytes. Cardiac HCD levels are remarkably low. The low abundant HCD N-acetylcarnosine is enriched in human skeletal muscles. Here, N-acetylcarnosine is continuously secreted into the circulation as the most stable plasma HCD, which is further induced by acute exercise in a myokine-like fashion. Carnosine is preferentially transported within red blood cells in humans but not rodents. We provide a novel basis to unravel tissue-specific, paracrine, and endocrine roles of HCDs in human health and disease.

Teaser: Human muscle releases N-acetylcarnosine at rest and especially during exercise, potentially initiating tissue crosstalk.

Introduction

Carnosine synthase (CARNS1) is presumably the only enzyme in animals capable of synthesizing an abundant class of endogenous dipeptides. The enzyme links L-histidine to either β-alanine or γ-aminobutyric acid (GABA), respectively rendering carnosine or homocarnosine. These parent dipeptides and their methylated (anserine and balenine) and acetylated (N-acetylcarnosine) analogs are collectively called the histidine-containing dipeptides (HCDs).

Since the initial discovery in 1900 by Vladimir Gulevich (*1*), carnosine and the other HCDs have been linked to various physiological functions, mostly serving to preserve redox status and cellular homeostasis (for a full overview, see (*2*)). The most relevant biochemical properties for their functions relate to proton buffering, metal chelation and antioxidant capacity, which further translates to protection against advanced glycation and lipoxidation end products (*3, 4*). The physiological importance of tissue HCD content is underscored by an extensive body of research ranging from enhancement of exercise performance (*5*) to treatment of cardiometabolic (*6, 7*) or neurological diseases (*8*) in rodents. Major differences between animal and human HCD metabolism may be present however, given that high carnosinase (CN1) activity in human, but not rodent, plasma results in rapid degradation of carnosine (*9, 10*).

Nevertheless, even more than 120 years after the initial discovery of carnosine and 10 years after the molecular identification of CARNS1 (*11*), there remains a lack of basic understanding of HCD synthesis, distribution, and metabolism throughout the animal and especially the human body. It is thought that HCDs are primarily expressed in excitable tissues such as skeletal and cardiac muscle and the central nervous system (CNS), but current literature mostly consists of scattered observations focussing on a limited number of tissues or species. Information on cardiac levels is sparse, although HCDs could play an important role in cardiomyocyte homeostasis (*12*). Furthermore, there is unclarity regarding the synthesis and physiological role of HCDs in kidney, lung, liver, and other non-excitable tissues. A first profiling of HCDs in rat tissues from Aldini *et al.* (*13*) did not detect HCDs in non-excitable tissues, although this and other previous endeavours were potentially limited from lower detection sensitivity compared to the currently available technology. For example, the low abundant HCDs anserine, balenine and N-acetylcarnosine have never been extensively characterized in animal or human tissues.

Here, we have performed the first systematic profiling of the five main HCDs, combined with determination of CARNS1 expression levels, in the mouse, rat and human body. Various human tissue samples were collected from live donors, except for post-mortem collected brain regions. We uncovered profound differences in HCD distribution and metabolism between tissues and species. For

instance, we demonstrate that humans have a unique way of circulating HCDs and releasing it from carnosine-synthesizing tissues such as skeletal muscle.

Results

CARNS1 is the unique and rate-limiting enzyme for HCD synthesis

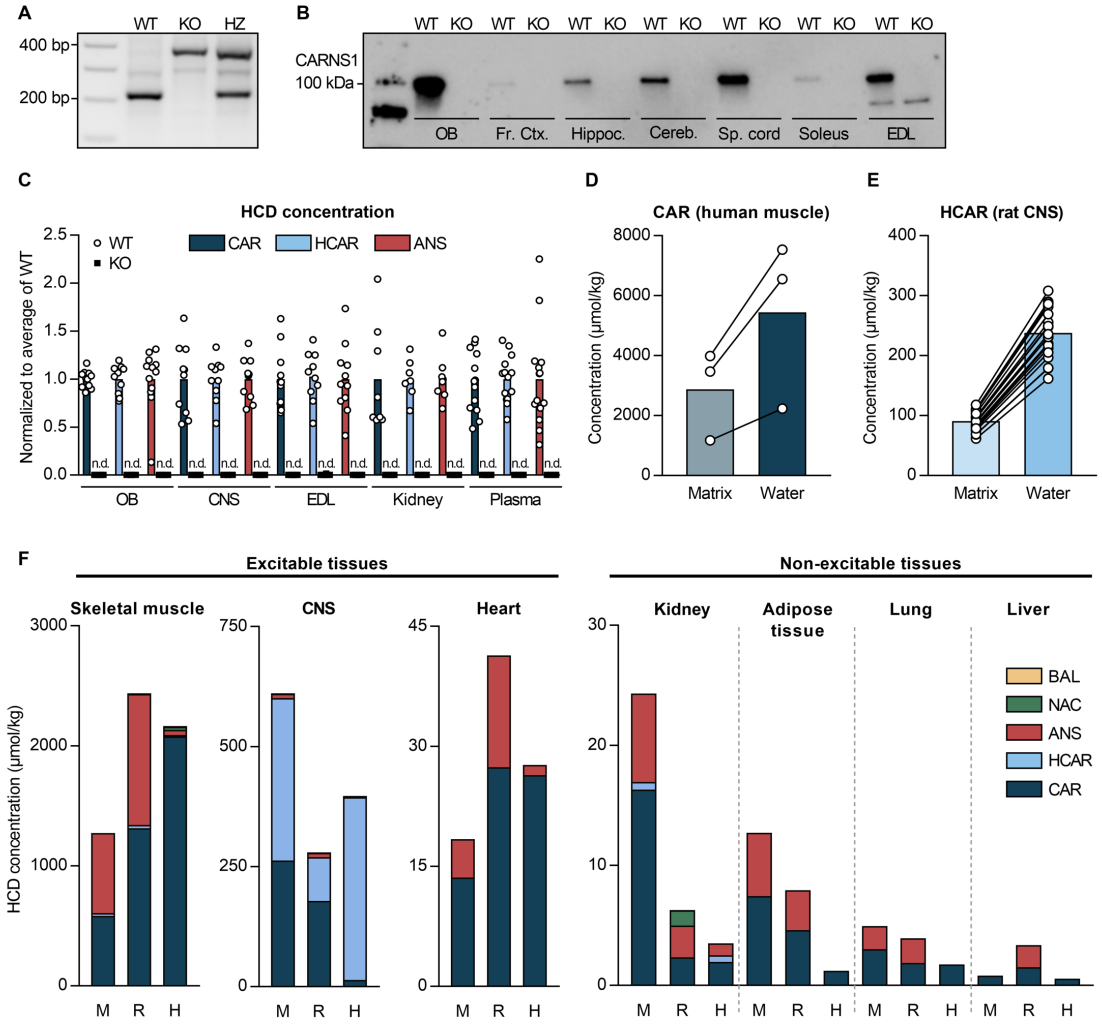
Using whole-body *Carns1*-knockout (KO) mice, we aimed to investigate whether CARNS1 deficiency results in a complete lack of endogenous (homo)carnosine and their derivatives in a variety of tissues, which would imply that CARNS1 is the unique and rate-limiting enzyme for HCD synthesis. CARNS1 was successfully knocked out at the gene (**Fig 1A**) and protein (100 kDa, **Fig 1B**) level, thereby also validating our Western blot antibody for specifically detecting the CARNS1 protein. As described previously, *Carns1*-KO mice displayed normal growth and survival (*14*). The deletion of *Carns1* led to an absence of carnosine and homocarnosine in all investigated tissues (**Fig 1C)**. Similarly, these mice were devoid of the carnosine-derived analogs anserine, balenine and N-acetylcarnosine (of which only anserine is consistently present in mouse tissues, cfr. infra, **Fig 1C**).

In addition, tissue from *Carns1*-KO mice was used to optimise our quantitative UHPLC-MS/MS-based detection of HCDs. Concentration levels in muscle and brain were compared using matrix-matched standard calibration curve preparation in *Carns1*-KO tissue matrix (i.e. muscle or brain homogenates) and water (as current standard practice in HCD research). In water, HCD levels were overestimated by ~2 to 3-fold (**Fig 1D-E**), indicating the importance of utilizing a corresponding blank tissue matrix for HCD quantification. This approach was used for all further analyses in this paper (except human cerebrospinal fluid), rendering the HCD quantifications more accurate than previously reported.

HCDs are not excitable tissue-specific metabolites

Using our sensitive UHPLC-MS/MS method, we performed a systematic profiling of HCDs in various tissues of the mouse, rat, and human body (**Fig 1F**, **Table S1**). Carnosine was the only HCD present in all studied tissues across the three species. Skeletal muscles contained the largest amounts of HCDs, followed by the CNS, up to the millimolar range. However, not all excitable tissue contained large amounts of HCDs, since unexpectedly low HCD levels were observed in the heart of all three species (50-100 times lower than skeletal muscles). These low HCD levels in cardiac tissue better reflect those measured in non-excitable tissues. Besides skeletal muscle, CNS, heart, kidney, adipose, lung and liver

102 tissue (presented in **Fig 1F**), we also found low levels of HCDs in the mouse and rat stomach wall,
103 gallbladder, pancreas, small intestine, colon, thymus, spleen, and eye (**Table S1**).



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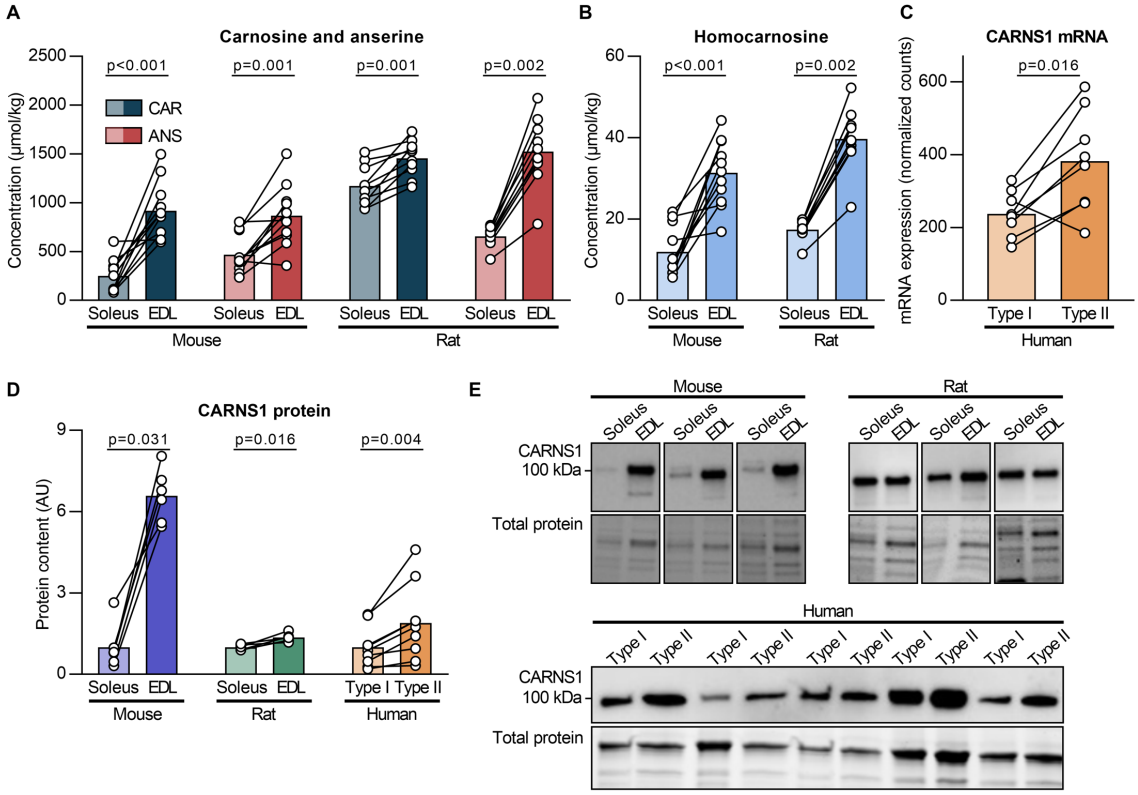
105 **Figure 1. Species- and tissue-specific distribution of histidine-containing dipeptides. (A)** PCR gel and **(B)** Western blot
106 showing the successful knockout of the *Carns1* gene and the absence of CARNS1 protein in mice. **(C)** HCD measurements
107 by UHPLC-MS/MS showing HCDs are absent from various tissues of *Carns1*-KO compared to WT mice. **(D)** Carnosine and
108 **(E)** homocarnosine measured by UHPLC-MS/MS, followed by quantification based on a standard calibration curve
109 prepared in water *vs. Carns1*-KO tissue matrix. **(F)** HCD measurements by UHPLC-MS/MS in skeletal muscle, CNS, heart,
110 kidney, adipose tissue, lung, and liver tissue from mice (M), rats (R) and humans (H). Values were averaged if more than
111 1 type of the respective tissue was present (e.g. soleus and EDL for rodent muscle). ANS, anserine; BAL, balenine; CAR,
112 carnosine; Cereb., cerebellum; CNS, central nervous system; EDL, extensor digitorum longus; Fr. ctx., frontal cortex;
113 HCAR, homocarnosine; Hippoc., hippocampus; HZ, heterozygous; KO, knockout; NAC, N-acetylcarnosine; OB, olfactory
114 bulb; Sp. cord, spinal cord; WT, wild type.

115

116 <u>CARNS1 content drives fiber type-related differences in HCD content in skeletal muscle</u>

117 We next aimed to profile the HCD content in skeletal muscle in more detail, with a focus on potential
118 fiber type-specific differences. In mice and rats, we determined the HCD content in soleus (more
119 oxidative, slow-twitch) and extensor digitorum longus (EDL; more glycolytic, fast-twitch) muscles. Our

120 results confirmed previous reports (*15-17*) that carnosine and anserine content is higher in EDL muscle
121 (**Fig 2A**). Also the homocarnosine content was ~2.5-fold higher in EDL compared to soleus in both mice
122 and rats (**Fig 2B**). To get more insights if CARNS1 content (i.e. HCD production) is the main driver for
123 the fiber type-related differences, we first explored a publicly available human muscle fiber type-
124 specific RNAseq dataset (*18*). These data show a ~2-fold higher *CARNS1* mRNA content in type IIa *vs.*
125 type I fibers (**Fig 2C**). We next determined CARNS1 protein levels in the soleus and EDL muscles from
126 the mice and rats (**Fig 2D-E**). CARNS1 levels were indeed higher in EDL muscles, with a larger difference
127 between soleus and EDL muscles in mice (6.6-fold) than in rats (1.4-fold). To translate mRNA
128 differences in human muscle to the protein level, we used Western blotting on pools of pre-classified
129 type I or type IIa fibers (**Fig S1**). This revealed a ~2-fold higher CARNS1 content in type IIa fibers (**Fig**
130 **2D-E**), consistent with findings at the mRNA level. These results suggest that CARNS1 expression is the
131 main driver regulating the clear fiber type-related differences in HCD content across the three species.



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133 **Figure 2. Muscle and muscle fiber type-specific differences in histidine-containing dipeptides and CARNS1. (A)**
134 Carnosine and anserine and (**B**) measurements by UHPLC-MS/MS in soleus and EDL muscles from mice and rats. **(C)**
135 Human muscle fiber type-specific calculation of *CARNS1* mRNA based on a previously published dataset. **(D)** Protein
136 levels of CARNS1 determined by Western blot in soleus and EDL muscle from mice and rats, and human type I and type
137 II fiber pools. **(E)** Representative Western blot and loading controls. ANS, anserine; CAR, carnosine; EDL, extensor
138 digitorum longus.

139

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141

142 Homocarnosine is the dominant HCD in the CNS, except for the olfactory bulb of rodents

143 Apart from skeletal muscle, HCDs are also highly present in the CNS. By analyzing seven different
144 regions from the mouse, rat and human CNS, we could confirm that the highest levels of carnosine are
145 found in the olfactory bulb in rodents, reaching concentrations of ~1200 µmol/kg tissue, which is
146 similar to or even higher than skeletal muscle carnosine levels (**Fig 3A, Table S1).** In contrast, the
147 human olfactory bulb contained approximately 15 times less carnosine (~80 µmol/kg). In mice and
148 rats, the olfactory bulb was the only CNS region containing more carnosine than homocarnosine, whilst
149 in humans all regions contained more homocarnosine than carnosine. Similar to our findings in human
150 CNS tissue, homocarnosine was also abundantly present in human cerebrospinal fluid (**Fig 3B**).

151 CARNS1 protein levels also showed considerable variability between CNS regions (**Fig 3C-D**). Mice and
152 rats exhibited high expression in olfactory bulb, as well as the spinal cord and medulla oblongata, but
153 not the rest of the CNS. In human tissues, we found lower CARNS1 levels in the olfactory bulb, but
154 instead observed greater amounts in the white matter, thalamus, and frontal cortex.

155 Immunofluorescence was used to further study the localisation of CARNS1 in the CNS. We chose the
156 region exhibiting the greatest CARNS1 protein levels among rodents (mouse olfactory bulb) and
157 humans (white matter). Double-labeling of CARNS1 and OLIG2, an oligodendrocyte lineage marker,
158 revealed that CARNS1 resides in oligodendrocytes of human white matter (**Fig 3E**). Cell markers for
159 microglia (CD68, **Fig S2A**), astrocytes (GFAP, **Fig S2B**) and neurons/axons (NF-H, **Fig S2C**) did not co-
160 localise with CARNS1. In the mouse olfactory bulb, CARNS1 appeared in spherical structures near the
161 surface of the olfactory bulb, i.e. the glomeruli, where olfactory nerve terminals form synapses with
162 dendrites from projection neurons that carry signals into the brain (**Fig 3F**).

163

164 CARNS1 expression scales with tissue HCD levels on a whole-body level

165 To investigate if tissue CARNS1 expression closely relates to tissue HCD levels, CARNS1 protein levels
166 (Western blot) were plotted against HCD concentrations (UHPLC-MS/MS). If the tissue CARNS1 level is
167 the main determinant of tissue HCD levels, a linear relation between both variables is expected. In all
168 non-excitable tissues, no CARNS1 could be detected by Western blot (**Fig 4A-C**). HCD levels in these
169 tissues probably reflect transmembrane HCD uptake. On a whole-body level, CARNS1 scaled with HCD
170 levels in mice (**Fig 4A**), rats (**Fig 4B)** and humans (**Fig 4C**). However, when comparing within organs,
171 more CARNS1 was not always directly linked to a higher HCD concentration. In the human CNS, for
172 example, CARNS1 was 13-fold higher in white matter than in the cerebellum, but both tissues had
173 similar HCD levels. This indicates that although CARNS1 is the only enzyme responsible for HCD

174 synthesis, other factors, such as exchange of HCDs between organs or a high HCD turnover rate
175 (synthesis/degradation), could also influence intracellular HCD levels.

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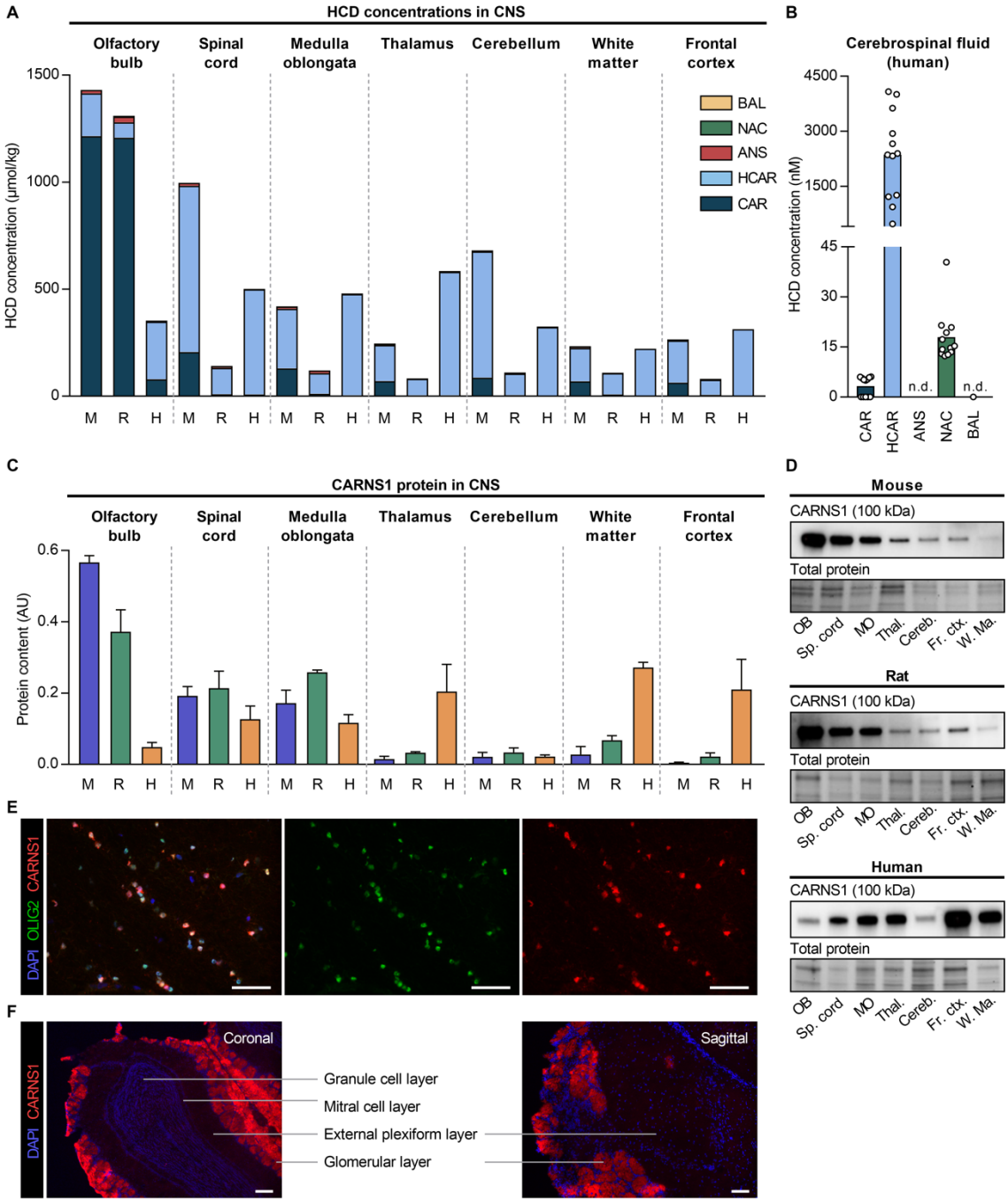

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178 **Figure 3. Region-specific levels of histidine-containing dipeptides and CARNS1 in the mouse, rat and human central**
179 **nervous system. (A)** HCD measurements by UHPLC-MS/MS in seven different central nervous system regions from mice
180 (M), rats (R) and humans (H). **(B)** HCD measurements by UHPLC-MS/MS in human cerebrospinal fluid. **(C)** Protein levels
181 of CARNS1 determined by Western blot in seven different central nervous system regions from mice, rats and humans.
182 Data are mean ± SD. **(D)** Representative Western blot and loading controls. **(E)** Immunohistochemical detection of
183 CARNS1 and OLIG2 in human white matter. **(F)** Immunohistochemical detection of CARNS1 in mouse olfactory bulb. In
184 panels (A), (C) and (D), spinal cord tissue is from the cervical region, mouse white matter is from the corpus callosum,

185 and human frontal cortex is from the superior frontal gyrus. ANS, anserine; AU, arbitrary units; BAL, balenine; CAR,
186 carnosine; Cereb., cerebellum; CNS, central nervous system; Fr. ctx., frontal cortex; HCAR, homocarnosine; MO, medulla
187 oblongata; NAC, N-acetylcarnosine; OB, olfactory bulb; Sp. cord, spinal cord; Thal., thalamus; W. Ma., white matter. Scale
188 bars are 50 µm (E), 100 µm (F).

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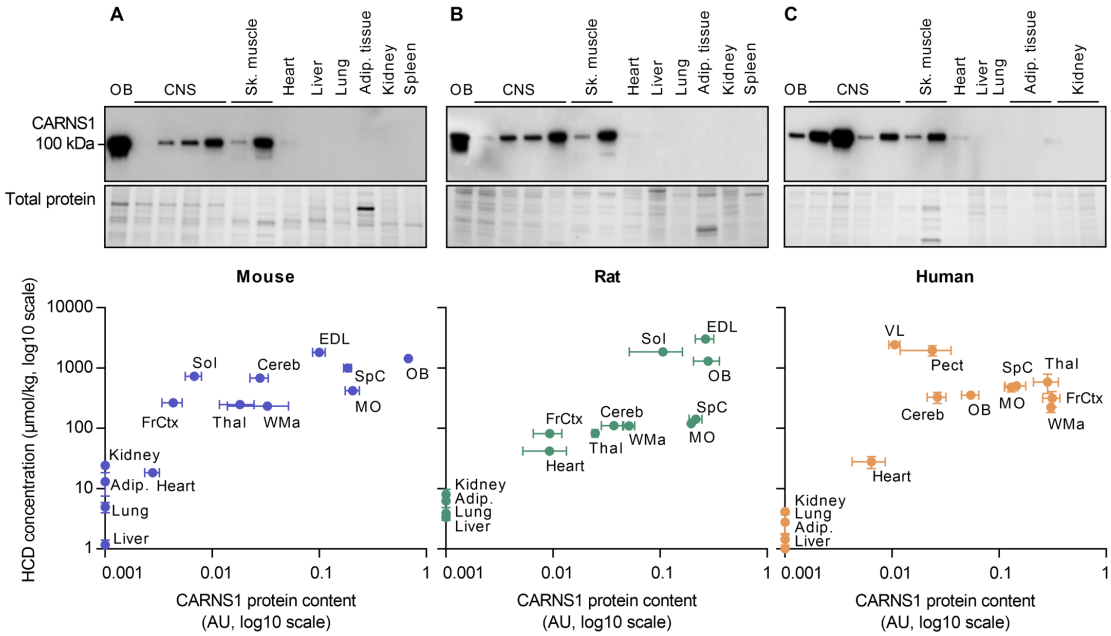

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192 **Figure 4. Association between CARNS1 and tissue histidine-containing dipeptide levels.** Plotted relationship between
193 CARNS1 levels (Western blot) and HCD measurements (UHPLC-MS/MS) in a variety of **(A)** mouse, **(B)** rat, and **(C)** human
194 tissues. The central nervous system (CNS) regions that are shown, besides the olfactory bulb (OB), are frontal cortex
195 (FrCtx), cerebellum (Cereb), spinal cord (SpC), thalamus (Thal), white matter (WMa) and medulla oblongata (MO). Mouse
196 muscles are soleus (Sol) and extensor digitorum longus (EDL). Human muscles are m. vastus lateralis (VL) and m.
197 pectoralis (Pect). Human adipose tissue is subcutaneous and visceral fat (Adip). Human kidney is medulla and cortex.
198 Data are mean ± SEM. AU, arbitrary units.

199

200 <u>N-acetylcarnosine and balenine are mainly found in human skeletal muscle</u>

201 Whilst the parent HCDs (carnosine and homocarnosine) were ubiquitously expressed, most of the
202 examined tissues also contained at least one methylated (anserine or balenine) or acetylated (N-
203 acetylcarnosine) carnosine analog (**Fig 1F**). Rodent skeletal muscles contained by far the highest
204 anserine levels (up to ~1500 µmol/kg in the rat EDL, **Fig 5A**). Besides anserine (~40 µmol/kg, **Fig 5A**),
205 human skeletal muscle also contained N-acetylcarnosine (~25 µmol/kg, **Fig 5B**) and balenine (~5
206 µmol/kg, **Fig 5C**). In fact, human skeletal muscle was the tissue where we observed the highest N-
207 acetylcarnosine and balenine levels. **Fig 5D-F** display the proportion of methylated or acetylated
208 carnosine variants in different species and tissues.

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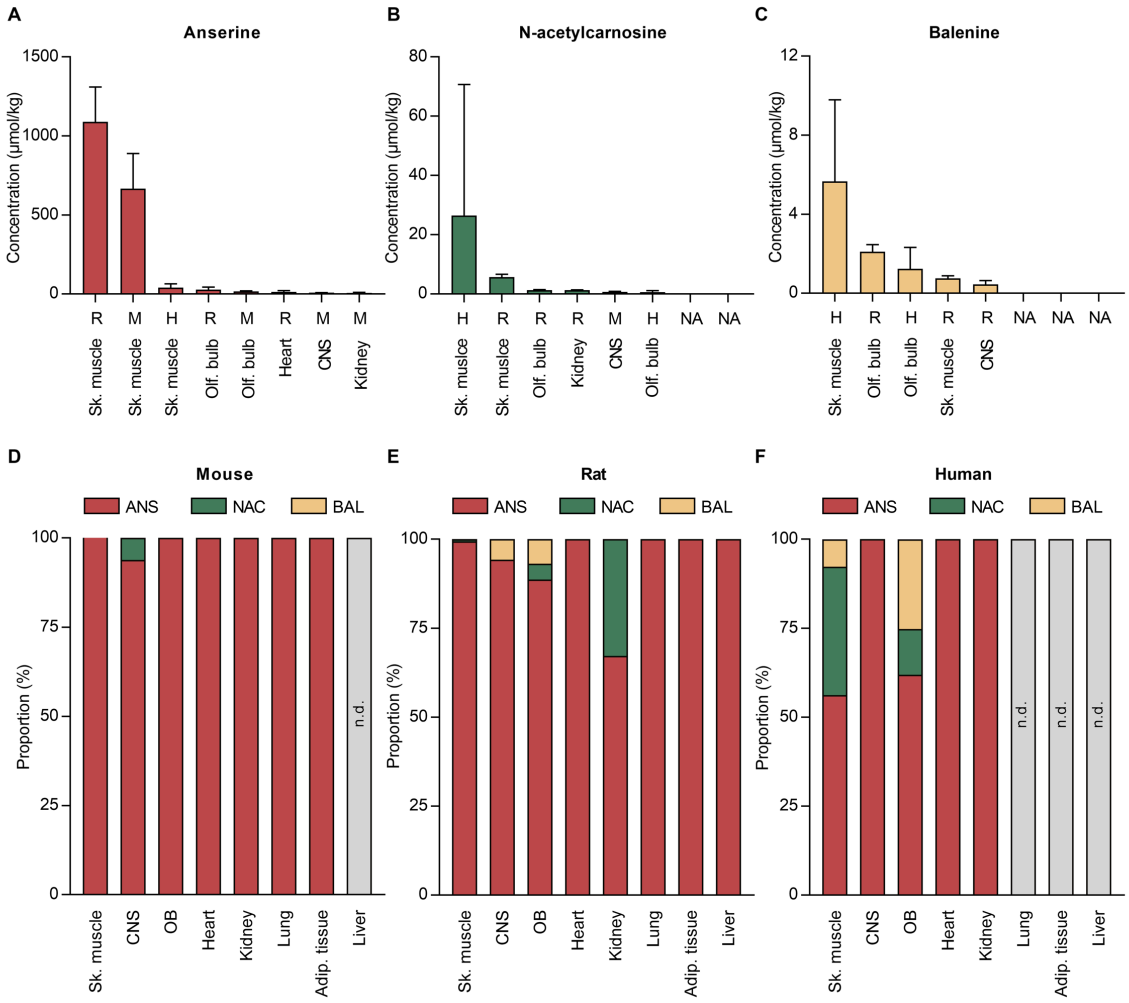

Figure 5. Anserine, N-acetylcarnosine and balenine levels in mouse, rat and human tissues. (A) Anserine, **(B)** N-acetylcarnosine, and **(C)** balenine measurements by UHPLC-MS/MS in mouse (M), rat (R), and human (H) tissues. The figures display the 8 tissues with the highest concentration of each dipeptide. Data are mean ± SD. (**D-F**) Relative proportion of anserine, N-acetylcarnosine, and balenine in **(D)** mouse, **(E)** rat, and **(F)** human tissues. Adip., adipose; ANS, anserine; BAL, balenine; CNS, central nervous system; NAC, N-acetylcarnosine; n.d., not detectable; Olf. bulb, olfactory bulb; Sk., skeletal.

<u>Acetylation of carnosine provides stability in the human circulation, which is not required in red blood cells or rodents</u>

Since rodents and humans differ substantially in presence and activity of the hydrolyzing enzyme CN1 in the circulation (*2, 10*), we attempted to map the circulating content of the five main HCDs. As expected, levels of plasma carnosine and anserine were very high in mice (~1500 nM) and rats (~3500 nM), but in the low nanomolar range in humans (**Fig 6A**). Low levels of homocarnosine could be detected in all 3 species, while balenine was only present in human plasma (**Fig 6A**). Interestingly, N-acetylcarnosine was the most abundant HCD in the human circulation, accounting for ~45% of the total HCDs (**Fig 6B**). Although HCD levels in red blood cells (RBCs) were in the nanomolar range in all 3

species, striking differences were observed compared to plasma (**Fig 6A** and **6C**). For both rodent species, HCD levels were drastically lower in RBCs than plasma (**Fig 6D** and **S3**). On the contrary, human carnosine and anserine levels were higher in every RBC sample compared to plasma, whilst N-acetylcarnosine levels were lower in RBCs than plasma (**Fig 6E**). These data suggest that carnosine in the human circulation is rendered more stable via acetylation to N-acetylcarnosine (which is resistant to hydrolysis by CN1) or via transport inside RBCs.

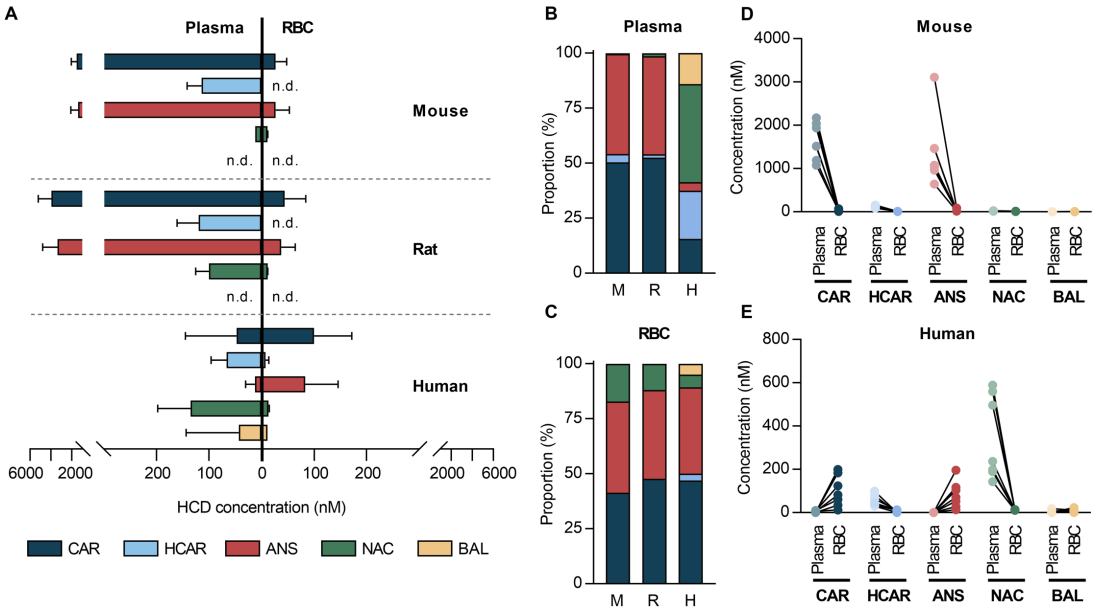

Figure 6. Species differences in histidine-containing dipeptides in plasma and red blood cells. (A) HCD measurements by UHPLC-MS/MS in mouse, rat and human plasma and red blood cells (data are mean ± SD). **(B)** Relative proportion of each HCD in mouse (M), rat (R) and human (H) plasma. **(C)** Relative proportion of each HCD in mouse, rat and human red blood cells. **(D)** Direct comparison of HCDs in plasma and red blood cells collected from the same mice. **(E)** Direct comparison of HCDs in plasma and red blood cells collected from the same human individuals. ANS, anserine; BAL, balenine; CAR, carnosine; HCAR, homocarnosine; NAC, N-acetylcarnosine; RBC, red blood cells.

Oral β-alanine supplementation affects HCDs in skeletal muscle and circulating N-acetylcarnosine levels in humans

We next investigated the effects of chronic supplementation of the rate-limiting precursor β-alanine on the content of all five HCDs in human skeletal muscle. As expected, β-alanine supplementation increased muscle carnosine content (+89%, **Fig 7A** and **S4A**). In addition, we observed increases in muscle anserine (+38%, **Fig 7A** and **S4B),** N-acetylcarnosine (+97%, **Fig 7A** and **S4C**) and balenine (+199%, **Fig 7A** and **S4D)**, and these increases were proportional to the carnosine increase (**Fig 7B**). However, homocarnosine content significantly decreased (-50%) after 12 weeks of β-alanine supplementation (**Fig 7A** and **S4E**). Plasma N-acetylcarnosine increased by 48% after the supplementation period (**Fig 7A** and **S4E**), which correlated at the individual level with muscle

251 carnosine content (**Fig 7C**), suggesting a possible link between intramuscular and circulating HCD levels.
252 In summary, oral β-alanine supplementation is a potent stimulus affecting all HCDs in skeletal muscle,
253 and plasma N-acetylcarnosine may reflect muscle HCD levels under baseline conditions and during β-
254 alanine supplementation.

255

256 <u>N-acetylcarnosine is released from human skeletal muscle during exercise</u>

257 Given this likely relationship between intra- and extracellular HCDs, and given that skeletal muscle is
258 the main active organ during exercise, we explored HCD dynamics during exercise. First, we collected
259 muscle interstitial fluid at rest and during exercise in humans. During exercise, interstitial levels for
260 every HCD increased (**Fig 7D**). This increase could however be primarily caused by sarcolemmal
261 damage following insertion of the microdialysis probe (*19*). To check this, we performed two follow-
262 up experiments. Firstly, *in vitro* human primary muscle cells were electrically stimulated for 2-3 h to
263 simulate muscle contraction. This did not result in secretion of carnosine into the culture medium
264 immediately after the electrical stimulation or following 30 min recovery (**Fig 7E**). Other HCDs could
265 not be detected in the cell culture medium. Secondly, we collected interstitial fluid from mouse skeletal
266 muscle, with a previously published method in which the muscle is not mechanically affected (*20, 21*).
267 Interstitial levels of carnosine and anserine were not higher in exercised mice compared to control
268 mice (**Fig S5A**). Next, we collected samples of the femoral artery and vein at rest and during exercise
269 (from a group of postmenopausal women). Our results clearly indicate a release of N-acetylcarnosine
270 from muscle tissue at rest of ~13 nmol/min (from one leg), which further increased ~2-fold during
271 exercise (**Fig 7F**). No release at rest or during exercise was observed for any of the other HCDs (**Fig 7F**).
272 These results were confirmed using a similar experimental setup in healthy young men, and with more
273 sampling time points during rest, passive movement, exercise and recovery. Here, we again showed a
274 release of N-acetylcarnosine at rest (~14 nmol/min from one leg), which increased 3.4-fold during
275 exercise and quickly returned back to baseline during recovery (**Fig 7G**). Exercise did not induce
276 carnosine release or uptake within RBCs (**Fig S5B**). Also in mice, no changes in carnosine or anserine
277 levels within plasma were observed following 60 min exercise (**Fig S5C**). Taken together, these data
278 indicate that N-acetylcarnosine in humans is likely the major, or most stable, HCD released during
279 exercise in a myokine-like fashion.

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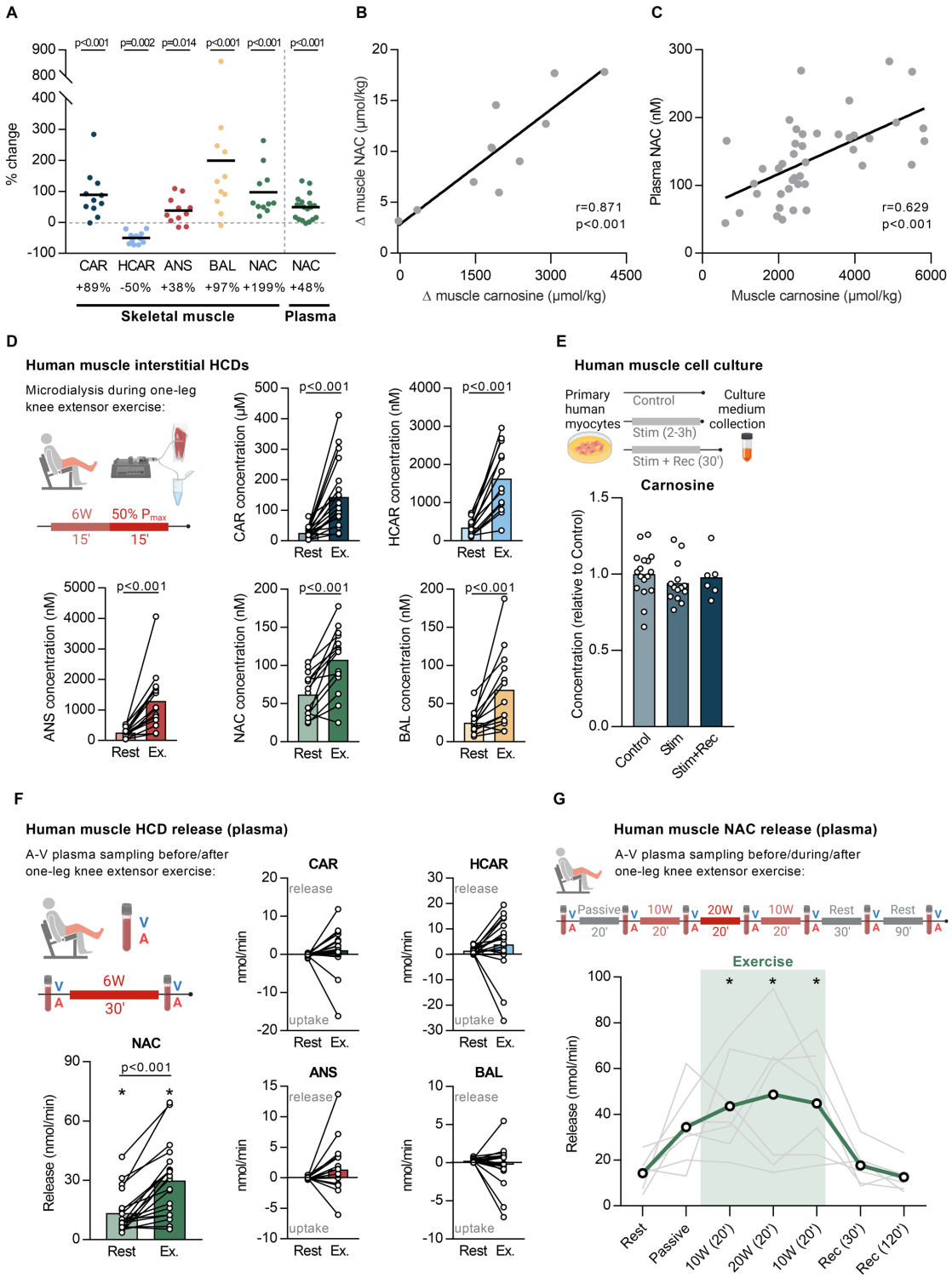

Figure 7. β-alanine supplementation and N-acetylcarnosine release during exercise from human muscle. (A) Changes in HCD levels, measured by UHPLC-MS/MS, in human skeletal muscle (m. vastus lateralis) after β-alanine supplementation. **(B)** Correlation between supplementation-induced changes in skeletal muscle carnosine and N-acetylcarnosine, measured by UHPLC-MS/MS. **(C)** Correlation between skeletal muscle carnosine and plasma N-acetylcarnosine, measured by UHPLC-MS/MS. **(D)** HCD measurements by UHPLC-MS/MS in human skeletal muscle interstitial fluid at rest and following exercise (Ex.). **(E)** Carnosine measurements by UHPLC-MS/MS in culture medium from primary human muscle cells in control condition, after 2-3 h of electrical stimulation (Stim), and after 2-3 h of electrical stimulation followed by 30 min recovery (Stim+Rec). **(F)** HCD measurements by UHPLC-MS/MS in human arterial and venous plasma samples at rest and following exercise (Ex.). Positive values indicate a net release, negative

values indicate a net uptake. Asterisk indicates significantly different from zero release at the respective time point. **(G)** Release of N-acetylcarnosine in human plasma, based on arterio-venous differences, at rest, during passive movement, at different time points during exercise, and up to 120 min recovery (Rec). Positive values indicate a net release, negative values indicate a net uptake. ANS, anserine; A-V; arterio-venous, BAL, balenine; CAR, carnosine; HCAR, homocarnosine; NAC, N-acetylcarnosine.

Discussion

This is the first study to systematically and extensively profile the organ distribution of HCDs and their differences between mice, rats and humans. Although present in all investigated non-excitable tissues in minute amounts, mainly excitable tissues contained high HCD levels in all species. Yet, our data show surprisingly low values in cardiac tissue across species, and a different distribution of HCDs in CNS regions between species. The enzyme CARNS1 is the unique enzyme responsible for endogenous carnosine and homocarnosine synthesis, and is a major determinant for tissue HCD levels. In human CNS white matter, CARNS1 appears restricted to cells from the oligodendrocyte lineage. We also uncover that N-acetylcarnosine is the primary circulating HCD in human plasma and is continuously secreted from skeletal muscle into the circulation, which is further increased by physical exercise. An overview of the main findings is visualized in **Fig 8**.

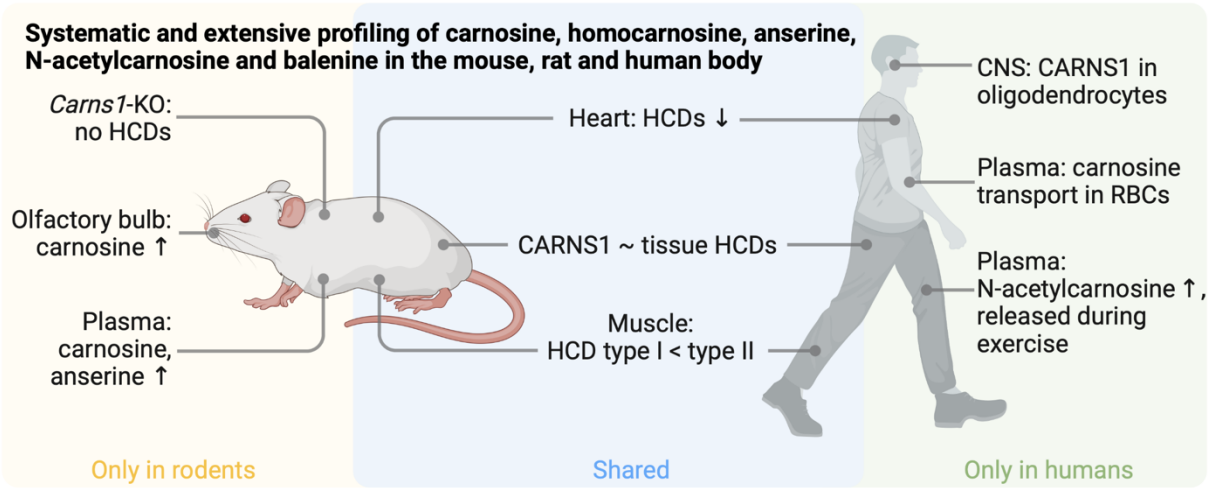

Figure 8. Graphical summary. Visual representation of the main findings, based on our extensive profiling of histidine-containing dipeptides (HCDs) in mice, rats and humans, and related follow-up experiments. Arrow up indicates high abundance, arrow down indicates low abundance. CNS, central nervous system; RBC, red blood cell.

Previous endeavors to profile HCDs mostly focused on rodent tissues, and resulted in fragmented and sometimes contradictory literature (*13, 22-25*). Our systematic approach and very sensitive state-of-the-art UHPLC-MS/MS methodology facilitate direct comparison between tissues and species. Our data contradicted some of the previous findings, e.g. that human muscle contains only carnosine and no other HCDs (*2*), that rat kidney, lung, plasma and liver lack HCDs (*13*), or that homocarnosine is

319 exclusively found in the CNS (*26*), with no presence of carnosine in the human brain or cerebrospinal
320 fluid (*27*). We confirmed that anserine is predominantly found in rodent skeletal muscles (*2, 16*), but
321 add that N-acetylcarnosine and balenine were primarily enriched in human skeletal muscles, although
322 still lower than anserine and (homo)carnosine. From our systematic approach, we calculate that 99.1%
323 of the total amount of HCDs in the human body is found in skeletal muscle tissue, which confirms
324 previous estimations (*28*). Moreover, HCD concentrations in the present study are often different than
325 those previously reported. More specifically, we report lower HCD concentrations in human skeletal
326 muscle than previous studies (*29-31*). This is most likely explained by the use of tissue-specific *Carns1*-
327 KO tissue matrix for our quantification method, which is known to be important for MS-based
328 quantification (*32*). Furthermore, Peters *et al.* reported very high carnosine (1800 µmol/kg) and
329 anserine (4000 µmol/kg) concentrations in human kidney (*23*), which are approximately 1000-fold
330 higher than the concentrations in our dataset (~2 µmol/kg). Besides the use of a different detection
331 technique and quantification method, it is unclear where such differences may have originated from.

332 CARNS1 and HCDs, especially carnosine, have long been recognised as enriched compounds within the
333 olfactory tract of rodents (*33, 34*). In human olfactory bulbs, we found remarkably low levels of
334 carnosine compared to homocarnosine. In addition, this is the first study to unequivocally ascribe
335 CARNS1 expression to a specific cell type within the CNS; outside of the olfactory bulb. Our discovery
336 of CARNS1 localisation within cells of the oligodendrocyte lineage (human white matter) confirms
337 suggestions from recent RNA sequencing databases of the mouse and human CNS that reported
338 *Carns1*/*CARNS1* as an oligodendrocyte-enriched gene (*35, 36*).

339 Traditionally, the highest HCD levels are assigned to the excitable tissues. Though this holds true with
340 respect to skeletal muscle and CNS tissue, it was quite compelling that we found extremely low
341 amounts of HCDs in cardiac muscle tissue. In contrast to previous suggestions that the rat heart
342 contains ~10 mM HCDs (*37*), we report 100-fold lower levels. This was consistently found in all three
343 species we investigated, and is also 50- to 100-fold lower compared to the concentrations in skeletal
344 muscle. This can potentially be attributed to more accurate and sensitive quantification compared to
345 the older technology. Nevertheless, HCDs are thought to play a crucial role in cardiac function and
346 recovery from injury (*12, 38*). For instance, isolated cardiac myocytes from *Carns1*-transgenic hearts
347 were protected against hypoxia reoxygenation injury (*39*), whilst *Carns1*-KO rats have impaired cardiac
348 contractility accompanied by reduced Ca^{2+} peaks and slowed Ca^{2+} removal (*40*). This underscores the
349 physiological importance of HCDs and indicates that even low HCD levels can contribute significantly
350 to cell/organ function and health. It remains to be determined, however, which biochemical properties
351 and physiological roles of these pleiotropic molecules are the most important in different tissues and
352 under different conditions. With respect to cardiomyocytes, it has been proposed that carnosine

353 functions as a Ca^{2+}/H^{+} exchanger to shuttle calcium towards and protons away from the sarcomere site
354 (*41, 42*).

355 The parent HCDs carnosine and homocarnosine share the same synthesizing enzyme CARNS1. This also
356 underlies our observation that oral β-alanine intake leads to increased carnosine but reduced
357 homocarnosine levels in human muscle, implying substrate inhibition between GABA and β-alanine for
358 CARNS1. Additionally, high expression of CARNS1 can lead to high tissue content of either carnosine
359 or homocarnosine, probably dependent of the local precursor availability (GABA *vs.* β-alanine). *Carns1*-
360 KO mice did not produce HCDs, whereas in WT mice, rats and humans, there appears to be a
361 relationship between CARNS1 expression and HCD content on a whole-body level. Moreover, the
362 differences in HCD content between slow- and fast-twitch muscle fibers were paralleled by similar
363 differences in CARNS1 expression. However, the correlation between CARNS1 expression and HCD
364 content was not perfect, suggesting that there might be inter-organ exchange or that there is higher
365 HCD turnover in tissues that have an important role for carnosine consumption/recycling, for example
366 through oxidative stress and reactions with toxic metabolites in pathological conditions (*8*).
367 Alternatively, the fact that CARNS1 shows a preference for β-alanine compared to GABA as a substrate
368 may skew this relationship considering that some tissues contain more carnosine than homocarnosine
369 and vice versa (*11*).

370 Tissues that have no or minimal CARNS1 expression likely rely on transmembrane uptake of HCDs
371 derived from exogenous/dietary sources or from production in CARNS1-expressing organs. It has
372 remained unclear, however, if and how HCDs are transported between tissues. The detection of HCDs
373 in various rodent and human tissues likely illustrates that HCDs can be exchanged between organs with
374 and without synthesizing capacity. Indeed, mice that lack the carnosine transporter PEPT2 have altered
375 (mostly reduced) HCD levels in various organs but increased levels in skeletal muscle tissue, which is
376 capable of synthesizing carnosine itself (*22*). This issue remains largely unclear in humans, in which
377 high activity of the CN1 enzyme quickly degrades circulating carnosine (*2*). It has long been suggested
378 that circulating HCDs are extremely low or absent in human plasma (*43-45*), although more recent
379 reports already detected carnosine (*46, 47*). We now demonstrate that N-acetylcarnosine is the most
380 stable carnosine analog in plasma, indicating that acetylation of the β-alanine residue protects against
381 the hydrolyzing activity of CN1. Thus, N-acetylcarnosine may be the primary HCD that is exchanged
382 between tissues in humans. Interestingly, plasma N-acetylcarnosine levels were correlated to muscle
383 carnosine (and N-acetylcarnosine) levels, possibly indicating that plasma N-acetylcarnosine can serve
384 as a surrogate marker for intramuscular HCD levels. Plasma N-acetylcarnosine levels increased
385 following β-alanine intake, showing that circulating N-acetylcarnosine levels may also be a marker for
386 muscle carnosine/HCD loading. In addition, our data indicate that transport of carnosine in RBCs is an

387 alternative strategy to protect against CN1 in human plasma, as recently suggested (*48*). This was not
388 true for rodents, who had lower HCD levels in RBCs than humans, despite more than 25 times higher
389 plasma HCD levels.

390 It has been proposed that carnosine is released from muscles during periods of contractile activity,
391 potentially serving as a health-promoting myokine. This hypothesis is primarily based on a study in
392 rats, where plasma carnosine levels increased during the dark/active phase when animals were
393 provided with a running wheel (*49*). We report that in humans, N-acetylcarnosine is the only HCD that
394 is consistently released from muscle tissue into plasma at rest, which is further increased during
395 periods of muscular activity (exercise). This opens various new research avenues on N-acetylcarnosine
396 as an exercise-induced myokine (*50, 51*). Future experiments should determine its relevance for
397 exercise training adaptations and cell/organ crosstalk (*52*). The average N-acetylcarnosine release of
398 14.3 nmol/min from non-contracting leg muscles at rest is striking. Extrapolation of this release to the
399 whole body, assuming that all muscles have the same N-acetylcarnosine secretion, suggests that in
400 theory the blood N-acetylcarnosine concentration should increase by 23.9 µM every 24 hours. Despite
401 this continuous and substantial release of N-acetylcarnosine into the circulation, resting plasma N-
402 acetylcarnosine levels only reach 50-350 nM in most subjects, indicating that there is a large
403 uptake/utilization of N-acetylcarnosine in other organs, or urinary excretion. Based on the amount of
404 N-acetylcarnosine release measured in arterio-venous samples from the leg, we also estimated that a
405 daily turnover of 25% of the total muscle N-acetylcarnosine pool is needed to maintain stable muscle
406 N-acetylcarnosine levels, suggesting a rather dynamic HCD homeostasis in human muscle. Specific
407 description of these calculations and used assumptions can be found in the **Supplementary Text**. In
408 muscle interstitial fluid samples, all HCDs appear to increase during exercise. However, it is hard to
409 distinguish physiological exercise-induced release from sarcolemmal rupture caused by insertion of
410 the microdialysis probes (*19*), as also supported by (I) the absence of a carnosine release during
411 electrical stimulation of primary human muscle cells or interstitial fluid sampled from mice post-
412 exercise, and (II) the lack of other HCD release (besides N-acetylcarnosine) in the venous effluent of
413 contracting muscles.

414 Despite being the first study to systematically and extensively study the distribution of HCDs in three
415 species, we acknowledge that the mouse and rat data cannot be fully extrapolated to all mouse and
416 rat strains, since these can differ somewhat (*53*). We also decided to focus on the two parent HCDs
417 (carnosine and homocarnosine) and carnosine's methylated and acetylated analogs (anserine,
418 balenine, N-acetylcarnosine). Other HCD conjugates do exist, but these are mostly very low abundant
419 products from reactions with other (toxic) compounds (e.g. carnosine-propanol or 2-oxo-carnosine
420 (*54-56*)).

421 In conclusion, we extensively profiled the organ distribution of the five main HCDs and discovered new
422 physiological routes of HCD metabolism. Our results can be used to generate various new research
423 hypotheses and highlight that findings derived from animal research on HCDs can not always be
424 translated to humans. In particular, the apparent inter-cell and inter-tissue para- and endocrine
425 regulation of HCDs, as well as its relevance to human health, disease and exercise
426 performance/adaptation, deserve further investigation.

427

428 Materials and methods

429 HCD profiling - Rodent tissue collection

430 All mouse tissues were obtained from an in-house breeding of *Carns1*-KO and WT mice with a C57/BL6
431 background, kindly provided by Prof. M. Eckhardt (*14, 57*). Genotypes of the offspring from
432 heterozygous parents were determined in toe samples using a previously published protocol (*14*).
433 Wistar rats were supplied by Envigo (The Netherlands). Mice and rats were housed under standard
434 room conditions (12h:12h light:dark cycle, 20-24°C, relative humidity 30-60%) and had *ad libitum*
435 access to drinking water and food pellets. For tissue collection, female and male animals were
436 sacrificed at an age of 6-10 w (mice) or 7-8 w (rats) old. Following overdose injection of Dolethal
437 (200 mg/kg, i.p.), blood was collected from the right ventricle, kept in Multivette® 600 K3 EDTA vials
438 on ice, centrifuged (5 min, 3500 rpm), and plasma was stored at -80°C. Before tissue dissection, mice
439 and rats were perfused with 0.9% NaCl solution containing heparin (25 UI/mL) via a left ventricular
440 puncture. For determination of HCD levels by UHPLC-MS/MS and CARNS1 expression by Western blot,
441 tissues were immediately frozen in liquid nitrogen, before being stored at -80°C. For
442 immunohistochemistry, whole mouse brains were carefully placed on a metal plate cooled by dry ice
443 in a foam box for several minutes, wrapped in aluminum foil, and stored at -80°C. Mouse exercise
444 experiments were performed on a treadmill (6 m/min, speed increased every 2 min by 2 m/min until
445 16 m/min, total duration 60 min), and were preceded by a 1-week adaptation period (3 running
446 sessions, gradually increasing exercise intensity and duration). Sedentary mice were placed on a
447 stationary treadmill for 60 min. Immediately after exercise, plasma was obtained and mice were
448 perfused as described above. To collect interstitial fluid, gastrocnemius muscles were placed on 20 µM
449 nylon net filters (Millipore, cat# NY2004700) and centrifuged (10 min, 800 × g) (*20, 21*). All animal
450 procedures were approved by the Ethical Committee on Animal Experiments at Hasselt University
451 (202074B, 202127 and 202145).

452 HCD profiling - Human tissue collection

453 All human samples were obtained after written informed consent.

454 **Human vastus lateralis muscle**: Muscle biopsies were collected from the m. vastus lateralis of healthy,
455 young volunteers using the Bergström needle biopsy technique with suction, as described previously
456 (*58*). One part of the samples was immediately snap-frozen in liquid nitrogen and stored at -80°C until
457 UHPLC-MS/MS analysis. The other part was submerged in 1-1.5 mL of RNA*later* (Thermo Fisher
458 Scientific), stored at 4°C for maximum 48 h and subsequently stored at -80°C for later individual fiber
459 dissection.

460 **Human heart and pectoralis muscle**: Heart and pectoralis muscle samples were collected from
461 patients undergoing open heart surgery under general anaesthesia. Heart samples consisted of the
462 right atrial appendage, harvested at the time of venous drainage cannulation for cardiopulmonary
463 bypass. Samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. For **Fig 1F**, HCD
464 concentrations of vastus lateralis and pectoralis muscle were averaged.

465 **Human kidney:** Kidney samples were collected from patients undergoing radical nephrectomy. In case
466 of kidney cancer, tissue was sampled as far away from the site of the tumor to obtain the healthiest
467 part of the kidney, immediately snap-frozen in liquid nitrogen and stored at -80°C. Medulla and cortex
468 were sampled separately (**Table S1**), but data was later averaged for analysis and visualization.

469 **Human adipose tissue:** Visceral and subcutaneous adipose tissue were sampled from lean and obese
470 individuals during abdominal surgery following an overnight fast (*59*). Tissue pieces were rinsed, freed
471 from visible blood and connective tissue, and snap-frozen in liquid nitrogen. The two subtypes (**Table**
472 **S1**) were later averaged for analysis and visualization.

473 **Human lung**: Lung tissue was obtained from unused healthy donor lungs that were not used for
474 transplantation from the BREATH KULeuven biobank (S51577).

475 **Human liver**: Liver samples were surgically removed from fasted subjects with obesity during gastric
476 bypass surgery. Exclusion criteria were the presence of malignancies, drinking more than two units
477 (women) or three units (men) of alcohol per day, having known liver pathologies other than non-
478 alcoholic fatty liver disease. For the current analysis, five samples with NAS score 0 or 1 were selected
479 (*60*).

480 **Human plasma and RBC**: Blood samples were always collected in pre-cooled EDTA tubes, after a 1-2-
481 day lacto-ovo vegetarian diet to ensure no influence of dietary HCD intake. For plasma, samples were
482 immediately centrifuged (10 min, 3000 × g, 4°C), followed by immediate deproteinization (110 µL of

483 35% 5-sulfosalisylic acid per 1 mL of plasma) and second centrifugation (5 min, 15000 × g, 4°C). Plasma
484 samples were then stored at -80°C. For RBC isolation (*61*), blood tubes were centrifuged for 15 min at
485 120 × g at room temperature. Plasma was carefully removed and 200 µL RBCs were collected in 1.8 mL
486 of ice-cold methanol (55% v/v). Samples were then stored at -80°C.

487 **Human CNS**: Seven different human CNS regions were obtained from The Netherlands Brain Bank
488 (NBB), Netherlands Institute for Neuroscience, Amsterdam (open access www. brainbank.nl). All
489 donors were 'non-demented controls', indicating the absence of neurological and psychiatric disease.
490 From 2 subjects, all 7 regions were available. For immunohistochemical analyses, white matter tissue
491 from healthy controls and unaffected white matter tissue of multiple sclerosis patients from a previous
492 study was used.

493 **Human cerebrospinal fluid**: Cerebrospinal fluid from subjects without neurological disease at the time
494 of sampling was obtained via the University Biobank Limburg (UbiLim), with approval from the Medical
495 Ethics Committee at Hasselt University (CME2021-004). Lumbar cerebrospinal fluid was collected into
496 PPS tubes, kept at 4°C, centrifuged to remove cells (10 min, 500 × g, 4°C), and supernatant was stored
497 at -80°C.

498

499 <u>β-alanine supplementation study in humans</u>

500 Vastus lateralis muscle biopsies obtained via the Bergström needle biopsy technique with suction
501 (n=11 β-alanine, n=11 placebo) and plasma samples (n=19 β-alanine, n=17 placebo) were collected
502 before and after 12 weeks of β-alanine supplementation in patients with COPD (sustained-release
503 CarnoSyn®, NAI). Methodological details have been described previously (*62*). Snap-frozen biopsies
504 were freeze-dried for 48 h, followed by manual removal of non-muscle material (fat, connective tissue,
505 blood) under a light microscope. Effects of β-alanine were analyzed using a two-way repeated
506 measures ANOVA (group *vs*. time) for each HCD separately, followed by Sidak's multiple comparisons
507 tests. Correlations were performed using Pearson correlation (Δ muscle carnosine *vs.* Δ N-
508 acetylcarnosine) or Spearman rank correlation (muscle carnosine *vs.* plasma N-acetylcarnosine).

509

510 <u>Human HCD release experiments</u>

511 **Microdialysis experiment**: Detailed methodology has been described previously (*63*). In short,
512 interstitial samples from m. vastus lateralis were collected using the microdialysis technique at rest
513 and after 30 min of one-legged knee extensor exercise (15 min 6 W, 15 min 50% peak power). Subjects

514 consisted of a group of young (n=7) and old (n=13) healthy men (results are pooled together since no
515 differences between groups could be observed). Concentrations were corrected for probe recovery as
516 determined by relative loss of [2–3H]-labeled adenosine in the dialysate. Interstitial levels during
517 exercise were compared to resting values using a multiple Wilcoxon matched-pairs signed rank test
518 with Holm-Sidak multiple comparison test.

519 **Arterio-venous balance experiment 1**: Arterial and venous samples were collected from the femoral
520 artery/vein at rest and after 30min one-legged knee extensor exercise (6W) in a group of healthy
521 postmenopausal women (n=19). Methodological details can be found in (*64*). Samples were
522 deproteinized with 35% 5-sulfosalisylic acid, as described above, on the day of the UHPLC-MS/MS
523 analysis. Exercise *vs.* resting HCD levels were compared using a multiple Wilcoxon matched-pairs
524 signed rank test with Holm-Sidak multiple comparison test.

525 **Arterio-venous balance experiment 2**: Seven healthy, young men (28 ± 4 years old, BMI of 24 ± 2,
526 VO_{2max} of 49 ± 7 mL/min/kg) participated in this experiment. After passive transport to the lab,
527 catheters were inserted in the femoral artery and vein. Next, arterial and venous samples were
528 collected every 30 min during a 90 min supine resting period. After this, 20 min of passive leg
529 movement was performed, followed by 60 min of active one-legged knee extensor exercise (20 min 10
530 W, 20 min 20 W, 20 min 10 W). Samples were collected at the end of each exercise bout. Finally,
531 arterio-venous samples were collected after 30 min and 2 h of recovery. Plasma and RBC samples were
532 collected as described above. Exercise-induced effects were analyzed using a mixed-effect model with
533 repeated measurements over time, with post-hoc comparison of every time point *vs.* baseline (Holm-
534 Sidak test).

535

536 <u>Primary cell culture experiments</u>

537 Biopsy samples (~150 mg) were obtained from m. vastus lateralis from young, healthy men. Primary
538 skeletal muscle cells were isolated with homemade antibody-coated magnetic beads and cultured as
539 previously described (*65, 66*). Cultured skeletal muscle cells were used for analysis on day 5 or 6 after
540 the onset of differentiation. At this time, most of the myocytes have differentiated into multinucleated
541 myotubes and can easily be identified as muscle cells. Myotubes were starved with media containing
542 0.1% Bovine Serum Albumin (DMEM without phenol, D-glucose and L-glutamine) for 16 hours before
543 experiments. The skeletal muscle cells were electro-stimulated as described previously (*67*), with the
544 minor addition of 5µM (S)-nitro-Blebbistatin (Cayman Chemical, CAS. 856925-75-2) to the stimulation
545 buffer to inhibit the spontaneous contraction of the myotubes (*68*). The cells were stimulated for 2-3
546 h (50 Hz, 0.6s/0.4 s trains, 1 ms pulse width, 10 V, homemade electrical stimulator connected to a

547 Digitimer MultiStim SYSTEM-D330). The extracellular medium was collected immediately or 30 min
548 after the end of stimulation, and medium from non-stimulated control cells was harvested
549 simultaneously. Changes over time were analyzed using a mixed-effect model with repeated
550 measurements over time, with post-hoc comparison of stimulated and stimulated+recovery *vs.* control
551 cells (Holm-Sidak test).

552

553 HCD determination by UHPLC-MS/MS

554 Details of the validation of the in-house developed UHPLC-MS/MS analysis has been described
555 previously (*69*), with the exception that all experiments were performed on a Xevo® TQ-S MS/MS
556 system with 2.5 µL injection volume. The limit of detection was determined to be 5-10 nM (in plasma),
557 corresponding to 0.38-0.76 µmol/kg tissue for our homogenization protocol. Pure carnosine and
558 anserine were kindly provided by Flamma S.p.a. (Chignolo d'Isola, Bergamo, Italy), and pure balenine
559 by NNB Nutrition (Frisco, Texas, USA). Homocarnosine (#33695) and N-acetyl-L-carnosine (#18817)
560 were bought from Cayman Chemical (Ann Arbor, Michigan, USA). UHPLC-MS/MS data extraction and
561 analysis was performed using Masslynx software 4.2 (Waters, Milford, USA).

562 **Tissues**: All tissues were prepared similarly, based on the method described previously (*8*). Frozen
563 tissues were quickly weighed and immediately homogenized in extraction solution (ultrapure water
564 with 10 mM HCl and internal standard carnosine-d4) in a ratio of 95 µL extraction solution per 5 mg
565 tissue in a QIAGEN TissueLyser II (1 min, 30 Hz). The concentration of carnosine-d4 varied according to
566 expected HCD concentrations in the tissues: muscle (20 µM), CNS (5 µM), liver/lung/spleen (0.5 µM)
567 and all other tissues (1 µM). Then, homogenates were centrifuged (20 min, 3000 × g, 4°C).
568 Supernatants were immediately diluted in a 3:1 ratio with ice-cold acetonitrile (-20°C), vortexed and
569 kept on ice for 15min. After a second centrifugation step (20 min, 3000 × g, 4°C), samples were stored
570 at -80°C until the day of the UHPLC-MS/MS analysis. Samples were combined with 75:25
571 acetonitrile:water in a 4:1 ratio before injection in the UHPLC-MS/MS device. For skeletal muscle and
572 CNS, samples were also injected after an extra initial dilution (1:25 for muscle, 1:10 for CNS) for the
573 determination of carnosine (skeletal muscle) and homocarnosine (CNS) content. Standard calibration
574 curves were prepared for each individual run and for each tissue separately in the respective tissue of
575 the *Carns1*-KO mice to account for possible tissue matrix effects (for all three species). Differences
576 between soleus and EDL HCD levels in mice and rats were analyzed using paired t-tests or Wilcoxon
577 signed-rank tests, depending on normality of the data.

578 **Plasma**: Deproteinized plasma (150 µL) was combined with acetonitrile containing 1% formic acid (215
579 µL), 1 µM carnosine-d4 as internal standard (10 µL) and ultrapure water (25 µL). For mouse plasma

580 analysis, volumes were scaled down to available plasma (75 µL). After thoroughly vortexing, samples
581 were centrifuged (15 min, 15000 × g, 4°C). The supernatant was collected and injected in the UHPLC-
582 MS/MS. A standard calibration curve was prepared in a pool of human deproteinized plasma that was
583 collected after a 2-day lacto-ovo vegetarian diet to minimize circulating HCDs (for all three species).

584 **RBC**: First, 190 µL of the RBC samples was combined with 10 µL of internal standard carnosine-d4
585 (2µM) and 10 µL of 75:25 acetonitrile:water (with 1% formic acid). This mixture was vortexed and then
586 centrifuged in a 10 kDa filter (Nanosep® Centrifugal Device with Omega Membrane™, Pall
587 Corporation). The supernatant was subsequently evaporated at 40°C and the droplet resuspended in
588 40 µL of 75:25 acetonitrile:water (with 1% formic acid) before injection in the UHPLC-MS/MS device.
589 A standard calibration curve was prepared in a pool of human RBC samples that were collected after a
590 2-day lacto-ovo vegetarian diet to minimize circulating HCDs (for all three species).

591 **Cerebrospinal fluid**: Human cerebrospinal fluid was treated identically as plasma, but the standard
592 calibration curve was prepared in ultrapure water since no *Carns1*-KO tissue matrix was available,
593 possibly resulting in overestimation of the absolute concentrations.

594 **Interstitial fluid**: Interstitial fluid (15 µL) was combined with 30 µL acetonitrile containing 1% formic
595 acid and 1 µM carnosine-d4 and 10 µL ultrapure water. This mixture was vortexed, centrifuged (15
596 min, 15000 × g, 4°C) and the supernatant was used to inject in the UHPLC-MS/MS. For detection of
597 carnosine in humans, samples were first diluted 1:30. For detection of carnosine and anserine in mice,
598 samples were first diluted 1:500. A standard calibration curve was prepared in Ringer-Acetate buffer
599 as this was used to perfuse the microdialysis probes.

600 **Cell culture medium**: Extracellular medium (150 µL) was mixed with acetonitrile containing 1% formic
601 acid (240 µL) and internal standard carnosine-d4 (2 µM, 10 µL), vortexed and injected in the UHPLC-
602 MS/MS. A standard calibration curve was prepared in DMEM culture medium.

603

604 <u>CARNS1 protein levels by Western blot</u>

605 Tissues were diluted in RIPA buffer (300 µL per 10 mg tissue; 50 mM Tris pH 8.0, 150 mM NaCl, 0.5%
606 sodium deoxycholate, 0.1% SDS, 1% Triton-X100, and freshly added protease/phosphatase inhibitors
607 [Roche]), and homogenized using stainless steel beads and a QIAGEN TissueLyser II (shaking 1 min, 30
608 Hz). Following centrifugation (15 min, 12000 × g, 4°C), supernatants were stored at -80°C. Pierce™ BCA
609 Protein Assay Kit (Thermo Fisher) was used according to manufacturer's instructions to determine
610 protein concentrations (read at 570 nm wavelength). For detection of CARNS1 protein levels, 1 µg of
611 protein was diluted in loading buffer solution (63 mM Tris Base pH 6.8, 2% SDS, 10% glycerol, 0.004%

612 Bromophenol Blue, 0.1 M DTT), heated for 4 min at 95°C, and separated in polyacrylamide gels (4-15%,
613 Mini-PROTEAN TGX, Bio-Rad) at 100-140 V on ice. Next, stain-free gels were imaged following UV
614 exposure to visualise total protein content (ChemiDoc MP Imaging System, Bio-Rad). Proteins were
615 transferred from the gel to an ethanol-immersed PVDF membrane in transfer buffer (30 min, 25 V, 1.0
616 A, Trans-Blot Turbo Transfer System, Bio-Rad). Membranes were briefly washed in Tris-buffered saline
617 with 0.1% Tween20 (TBS-T), and blocked for 30 min using 3% milk powder in TBS-T. Following overnight
618 incubation at 4°C with primary antibodies against CARNS1 (rabbit polyclonal, 1:1000 in 3% milk/TBS-T,
619 HPA038569, Sigma), membranes were washed (3 × 5 min), incubated with secondary HRP-conjugated
620 goat anti-rabbit antibodies (1:5000 in 3% milk/TBS-T) for 60 min at room temperature, washed again
621 (3 × 5 min), and chemiluminescent images were developed in a ChemiDoc MP Imaging System (Bio-
622 Rad) using Clarity Western ECL substrate (Bio-Rad). CARNS1 protein bands were quantified with Image
623 Lab 6.1 software (Bio-Rad), and normalized to total protein content from the stain-free image. Finally,
624 bands were expressed relative to total CARNS1 expression (sum of all bands) of a particular mouse,
625 rat, or human. For muscle, band densities were expressed as fold changes relative to the average
626 density from soleus muscles per blot. Linearity of the signal was determined for every tissue.
627 Differences between soleus and extensor digitorum longus CARNS1 content were analyzed using a
628 paired t-test (rat) or Wilcoxon signed-rank test (mouse), depending on normality of the data.

629

630 <u>CARNS1 protein level in human single muscle fibers.</u>

631 CARNS1 protein levels in type I *vs*. type II muscle fibers were determined based on a previously
632 published method (*70*). Muscle samples in RNA*later* (Thermo Fisher Scientific) were thawed and
633 subsequently transferred to a petri dish filled with fresh RNA*later* (Thermo Fisher Scientific) solution.
634 Individual muscle fibers were manually dissected under a light microscope and immediately
635 submerged in a new 0.5 mL tube with 5 µL ice-cold Laemmli buffer (125 mM Tris-HCl (pH 6.8), 10%
636 glycerol, 125 mM SDS, 200 mM DTT, 0.004% bromophenol blue). Tubes were then incubated for 15
637 min at 4°C, followed by 10 min at 70°C and then stored at -80°C until the next step of the analysis. A
638 total of 40-72 fibers were isolated from 9 biopsies. Next, muscle fiber type (based on myosin heavy
639 chain expression) was determined using dot blotting techniques. For this, 0.5 µL of the muscle fiber
640 lysate was spotted onto two activated and equilibrated PVDF membranes, one for MHC I and one for
641 MHC IIa. After air-drying the PVDF membrane for 30 min, it was re-activated in 96% ethanol and
642 equilibrated in transfer buffer (8 mM Tris-base, 39 mM glycine, 0.015% SDS, 20% ethanol). The next
643 steps are similar to standard Western blotting as described above, with primary antibodies for MHC I
644 (A4.840, 1:1000 in 3% milk in TBS-T, DSHB) or MHC IIa (A4.74, 1:1000 in 3% milk in TBS-T, DSHB). Fiber

645 lysates were classified as type I or IIa fibers based on a positive stain for only the MHC I or IIa antibody
646 (**Fig S1**). Next, fibers of the same type from the same subject were pooled (n=10-26 for type I and n=9-
647 22 for type IIa fibers). CARNS1 protein content in these fiber type-specific samples were determined
648 with standard Western blotting technique as described above, with loading of 5 µL per pool. Band
649 densities were expressed as fold changes relative to the average density from type I fibers per blot.
650 Statistical analysis was performed using a Wilcoxon signed-rank test.

651

652 *CARNS1* mRNA expression

653 The fiber type-specific RNAseq dataset of Rubenstein *et al.* was downloaded from the GEO repository
654 under accession number GSE130977 (*18*). This dataset consists of RNAseq data of pools of type I or
655 type II fibers from m. vastus lateralis biopsies of 9 healthy, older men. For full details on generation of
656 this dataset, we refer to the original publication. Raw counts were normalized with DESeq2 to allow
657 for between-sample comparisons (*71*). First, normalized counts for *MYH2* (ENSG00000125414, type II
658 fibers) and *MYH7* (ENSG00000092054, type I fibers) were extracted and assessed for each fiber pool
659 as purity quality control. Based on this analysis, the fiber pools of one participant were excluded for
660 further analysis. Next, normalized counts of *CARNS1* (ENSG00000172508) were extracted and
661 compared between type I and type II fiber pools within each participant using a Wilcoxon signed-rank
662 test.

663

664 Immunohistochemical detection of CARNS1

665 Sagittal and frontal cryosections (10 µm) were cut from whole mouse brains and human white matter
666 samples. Following acetone fixation (10 min) and blocking (30 min, 10% donkey serum in PBS with 1%
667 BSA), sections were exposed overnight at 4°C to antibodies detecting CARNS1 (rabbit polyclonal, 1:100
668 in PBS with 1% BSA, HPA038569, Sigma). The next day, sections were washed and exposed to
669 complementary secondary antibodies for 60 min (1:500 in PBS with 1% BSA, Thermo Fisher).
670 Fluorescence imaging was performed with a Leica DM4000 B LED (Leica Microsystems). For double-
671 labeling, we used the following antibodies: OLIG2 (1:50, goat polyclonal, AF2418, R&D Systems),
672 neurofilament heavy polypeptide (NF-H, rabbit polyclonal, 1:200, ab8135, Abcam), CD68 (mouse
673 monoclonal, 1:100, M0814 KP1 clone, Dako), GFAP (mouse monoclonal, 1:100, G3893, Sigma).
674 Absence of CARNS1 from *Carns1*-KO brain sections was used as negative control.

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677 Statistical analysis

678 Statistical analyses were performed in GraphPad Prism v9.4 and were described in the appropriate
679 method paragraphs. Significance level was set at α=0.05.

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917 TVDS, JS, SDJ and WD designed the study.
918 TVDS, JS, SDJ, BVer and CH performed the experiments and/or biochemical analyses.
919 TVDS, JS, SDJ, JDB, RVT, KV, DH, TB, BL, CVP, KD, BVan and LG contributed to tissue collection.
920 SC, BO, LG, YH and WD supervised the study.
921 TVDS, JS, SDJ and WD analyzed the data.
922 TVDS, JS and WD drafted the manuscript.
923 All authors approved the final version of the manuscript.

924 **Competing interests**

925 The authors declare that they have no competing interests.

926 **Data and materials availability**

927 All data needed to evaluate the conclusions in the paper are present in the manuscript and
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929 request to the author.