ORIGINAL ARTICLE

Cloning, tissue and ontogenetic expression of the taurine transporter in the flatfish Senegalese sole (*Solea senegalensis*)

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Abstract Flatfish species seem to require dietary taurine for normal growth and development. Although dietary taurine supplementation has been recommended for flatfish, little is known about the mechanisms of taurine absorption in the digestive tract of flatfish throughout ontogeny. This study described the cloning and ontogenetic expression of the taurine transporter (TauT) in the flatfish Senegalese sole (*Solea senegalensis*). Results showed a high similarity between TauT in Senegalese sole and other vertebrates, but a change in TauT amino acid sequences indicates that taurine transport may differ between mammals and fish, reptiles or birds. Moreover, results showed that Senegalese sole metamorphosis is an important developmental trigger to promote taurine transport in larvae, especially in muscle tissues, which may be important for larval growth. Results also indicated that the capacity to uptake dietary taurine in the digestive tract is already established in larvae at the onset of metamorphosis. In Senegalese sole juveniles, TauT expression was highest in brain, heart and eye. These are organs where taurine is usually found in high concentrations and is believed to play important biological roles. In the digestive tract of juveniles, TauT was more expressed in stomach and hindgut, indicating that dietary taurine is quickly absorbed when digestion begins and taurine endogenously used for bile salt conjugation may be recycled at the posterior end of the digestive tract. Therefore, these results suggest an enterohepatic recycling pathway for taurine in Senegalese sole, a process that may be important for maintenance of the taurine body levels in flatfish species.

Keywords Taurine transporter · TauT · SLC6A6 · Senegalese sole · Flatfish · Ontogeny

Introduction

Taurine is an amino sulphonic acid that is among the most abundant low molecular weight organic constituents of animal tissues. For instance, taurine alone accounts for approximately 0.1% of total body weight in humans. Although often considered an amino acid (AA), taurine lacks a carboxyl group and is not incorporated into proteins. Instead, taurine is freely distributed throughout cytosol, and is particularly accumulated in excitable tissues. Taurine plays a vital role in several important biological functions such as bile salt conjugation, osmoregulation, membrane stabilisation, modulation of neurotransmitters, antioxidation, and early development of visual, neural and muscular systems (Huxtable 1992).

Due to its abundance in animal tissues, taurine is highly available in carnivorous and omnivorous diets. Several animals may temporarily or permanently depend on a dietary source of taurine, due to limited endogenous rate of taurine biosynthesis caused by an inherent deficiency on the enzyme cysteine sulfinate decarboxylase. For instance, cats are completely unable to biosynthesise taurine and offspring of female cats that are fed taurine-deficient diets may not survive or may develop neurological defects and retinal

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degeneration (Hayes et al. 1975; Sturman 1988). In fish, the ability to biosynthesise taurine varies inter-specifically and throughout ontogenesis (Goto et al. 2001, 2003; Yokoyama et al. 2001; Kim et al. 2003, 2008), indicating that taurine may be essential for various fish species during some developmental stages. This seems to be the case for Japanese flounder (*Paralichthys olivaceus*), which has been shown to have a low capacity for taurine biosynthesis, requiring dietary taurine for normal growth, development and feeding behaviour (Kim et al. 2005, 2008). Based on this, dietary taurine supplementation is recommended on a general basis for flatfish during ontogeny (Takeuchi 2001).

Dietary taurine deficiencies may be particularly critical for flatfish during the larval stage. On one hand, taurine levels remain stable during the yolk-sac and mouth opening stages of flatfish development, suggesting that taurine is incorporated into the free AA pool of the egg before spawning (Rønnestad and Fyhn 1993; Rønnestad et al. 1993). Like in the mammalian embryo, these findings suggest that taurine may have a significant physiological importance for the development of fish embryo (Sturman 1993). Furthermore, the prey of flatfish larvae in the natural environment (e.g. copepods) contain high taurine levels (Conceição et al. 1997; van der Meeren et al. 2008), and high taurine levels in live food were suggested to result in higher growth rates of turbot (*Scophthalmus maximus*) larvae (Conceição et al. 1997). Nevertheless, although dietary taurine supplementation is likely to be beneficial for flatfish larvae, there is no information available on the mechanisms involved in taurine absorption in the digestive tract and the bioavailability of dietary taurine in the early developmental stages.

The research on intestinal taurine transport in vertebrates mainly targets the high-affinity, low-capacity sodium/chloride-dependent taurine transporter (TauT; SLC6A6) (ÓFlaherty et al. 1997). This transporter functions as an active transport system that carries taurine against a concentration gradient, driven by transmembrane sodium and chloride gradients and membrane potentials. TauT has been cloned and characterised in a range of species and tissues, including Madin–Darby canine kidney cells (Uchida et al. 1992), porcine renal proximal tubule cells (Jones et al. 1995), mouse (Liu et al. 1992) and human (Jhiang et al. 1993; Ramamoorthy et al. 1994). In fish, only a few studies have aimed to characterise TauT (Takeuchi et al. 2000; Zarate and Bradley 2007; Kozlowski et al. 2008; Chow et al. 2009), and to our knowledge no work has analysed TauT expression in flatfish species or during fish larval stages. In fact, up to now few works have been done on the molecular characterisation of transporters in teleosts in general (e.g. Verri et al. 2003) and in larvae (Amberg et al. 2008). In mammals, several AA transporters appear at different times during ontogenesis (Buddington and Diamond 1989). Although the ontogeny of fish larvae is not

comparable to foetal mammals, it remains to be explored if this also holds true for teleosts. It has been shown that fish larvae do not have a fully developed digestive system at the onset of exogenous feeding and the digestive/absorption capacities increase along with development (Conceição et al. 2007; Rønnestad et al. 2007). Therefore, since flatfish may require dietary taurine supplementation during the early developmental stages, it is important to determine the functionality of TauT during ontogenesis.

This work aimed to characterise TauT expression during the ontogeny of Senegalese sole (*Solea senegalensis*), a flatfish species with increasing importance for the South-Eastern European aquaculture (Imsland et al. 2003; Conceição et al. 2007) and that seems to require dietary taurine supplementation during the early life stages (Pinto et al. 2010). For this purpose, TauT was cloned from Senegalese sole cDNA and its expression was determined by real time quantitative PCR (qPCR) from first feeding larvae until juvenile stages of development.

Materials and methods

Fish rearing

Senegalese sole (*S. senegalensis*) larvae were reared at UAlg (Faro, Portugal) according to standard procedures until 30 days after hatching (DAH) (Dinis et al. 1999). Larvae were initially reared in 100 L conical cylindrical sand-coloured tanks at a density of 80 larvae L^{-1}. At 16 DAH, benthonic Senegalese sole were transferred to 21 L sand-coloured fibreglass raceways (0.21 m^2; 10 cm water depth; initial larval density of 3,000 individuals m^{-2}). Light/dark cycles of 12:12 h were used throughout the experiment. Water temperature (21.0 ± 1.5°C; mean ± SD), oxygen saturation level (96.1 ± 4.6%) and salinity (33.8 ± 2.6 g L^{-1}) were measured on a daily basis. The Senegalese sole larvae were fed rotifers and *Artemia* enriched with commercial products according to standard rearing procedures (Dinis et al. 1999).

Juvenile Senegalese sole were obtained from a different batch of larvae reared at CCMAR (Faro, Portugal) facilities in a flat-bottom sand-coloured fibreglass tank (2.40 m length; 1 m width; 30 cm depth) at a density of 8 kg m^{-2}. Water supply was provided by a partial-recirculated water system (temperature 21 ± 1°C; salinity 36 g L^{-1}). Fish were fed to satiety with a commercial diet (Dourasoja Balance, Sorgal, S.A.) and fasted for 24 h before sampling.

Fish sampling

Senegalese sole larvae were sampled for total length (TL), dry weight (DW) and metamorphosis pattern analysis at

Table 1 Primer sequences for amplification and expression analysis of taurine transporter in *S. senegalensis*

	Primer sequence 5′ → 3′	Amplification region (bp)	Amplicon length (bp)
TauT cloning	ATGGCACAGAAAGAGAAACTCCA (Fw)	1–23	750
	TTGCTGTTATGTAGACCACTTTCCC (Rev)	727–751	
TauT cloning	TCGCTGTGTGGGTCATCTGC (Fw)	674–693	1,222
	CCTCTCACAGAGAGCGTTCAC (Rev)	1,875–1,895	
TauT qPCR	CCGAAAGCTGTGTCCATGATG (Fw)	1,123–1,143	120
	CAATAGAGGTGATCTGTCCTTCCA (Rev)	1,220–1,243	
eEF1a1 qPCR	GATTGACCGTCGTTCTGGCAAGAAGC (Fw)	–	142
	GGCAAAGCGACCAAGGGGAGCAT (Rev)		

TauT taurine transporter, *eEF1a1* elongation factor 1 alpha isoform 1, *Fw* forward primer, *Rev* reverse primer, *qPCR* real time quantitative PCR

2, 6, 12, 16, 20 and 30 DAH. The metamorphosis pattern was assessed according to Fernández-Díaz et al. (2001). These authors categorised five metamorphic stages, according to the eye migration status: 0, pre-metamorphic (symmetric larvae); 1, early metamorphic (beginning of left eye migration); 2, middle metamorphic (left eye touching the midline of the dorsal surface); 3, middle metamorphic (changing in the swimming plane and left eye migration within the ocular side); and 4, later metamorphic (completion of left eye migration and visibility of orbital arch).

For gene expression analysis whole larvae were collected at the same sampling points as above (2, 6, 12, 16, 20 and 30 DAH). From 12 DAH onwards, additional larvae were dissected to reveal the compartmental distribution of TauT; larvae were separated into three fractions: (1) head (including heart), (2) digestive tract with liver and (3) remaining body. Samples from whole and dissected larvae were rapidly transferred to RNAlater (Ambion) and stored at −80°C for later analysis.

For further analysis of the organ and tissue distribution of the taurine transporter, Senegalese sole juveniles (94.1 ± 18.1 g wet weight; 17.6 ± 0.7 cm total length) were killed with a lethal dose of 2-phenoxiethanol (Sigma, St Louis, MO, USA). The fish were then dissected and the following samples were collected for gene expression analysis: brain, eye, gills, heart, head kidney, spleen, gallbladder, liver, stomach, midgut (divided into four equal fractions) and hindgut (divided into two equal fractions). The samples were stored in RNAlater at −80°C until analysis.

Cloning and sequence analysis of Senegalese sole taurine transporter

Total RNA (RNA) was extracted from midgut of juvenile Senegalese sole using TRI Reagent (Sigma). First-strand cDNA was subsequently synthesised from RNA (3 μg) using oligo (dT) primer from SuperScript III First-Strand Synthesis system for RT-PCR kit (Invitrogen). Amplifications were performed on the resulting cDNA using primer sets designed on Primer premier 5 (Biosoft international) and based upon Common carp (*Cyprinus carpio*) mRNA (Genbank accession No. AB006986.1; Table 1). Amplification reactions were performed using GoTaq PCR reagents (Promega) according to the manufacturer's instructions and by the following protocol: 95°C for 2 min followed by 32 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 90 s and a final termination step of 72°C for 5 min. The amplified PCR products were visualised in an agarose gel (1%) stained with GelRed (Biotium), purified using a QIAquick Gel Extraction Kit (Qiagen) and cloned into the pCR4-TOPO vector (TOPO TA Cloning Kit, Invitrogen). Sequencing was performed at the University of Bergen Sequencing Facility (Bergen, Norway), using BigDye Terminator v3.1 chemistry in a ABI PRISM377 DNA sequencer (Applied Biosystems). Nucleotide sequences were identified using the Basic Local Alignment Search Tool (BLAST) from GenBank database.

The Senegalese sole TauT gene structure was deduced with Spidey software (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey) following identification of intron/exon splices boundary consensus sequences (AG/GT). To confirm the predicted gene organisation, comparisons were performed with TauT sequence in other species using Ensembl database (http://www.ensembl.org/). Conserved domains present in protein sequence were identified using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) and putative transmembrane domains were predicted using TMHMM 2.0, which is part of the Simple Modular Architecture Research Tool. Transmembrane protein 2D topology was created using TOPO2 software (Johns 2010).

Multiple alignment of TauT AA sequences was performed with ClustalX (version 2.0.11) according to the following parameters: Gonnet series matrix, Gap opening penalty 10, Gap extension 0.2 (Thompson et al. 1997; Larkin et al. 2007). Alignments were displayed in GeneDoc (www.psc.edu/biomed/genedoc), edited and percentages of sequence identity and similarity were calculated.

Phylogenetic analysis was performed by Maximum Parsimony (Eck and Dayhoff 1966), using MEGA 4.0 software (Tamura et al. 2007) to build a tree with 1,000 bootstrap replicates based on vertebrate TauT AA sequences. The sequence from the Human solute carrier SLC6A13 (neurotransmitter transporter, GABA; Genbank accession No. ENSP00000339260) was used as an outgroup.

Taurine transporter expression analysis

For analysis of TauT expression, RNA was isolated from Senegalese sole tissues (see "Fish sampling" for further information on sampled tissues). RNA quality was assessed on all samples using Agilent 2100 Bioanalyzer (Agilent Technologies) and only samples with a RNA integrity number (RIN) equal or higher than 7, on a scale of 1–10, were utilised for first-strand cDNA synthesis, since a high RNA integrity seems to be crucial for obtaining meaningful gene expression data (Fleige and Pfaffl 2006). First-strand cDNA synthesis occurred according to the procedures previously described. Senegalese sole TauT mRNA expression was analysed by qPCR using a Chromo4 Continuous Fluorescence Detector (Bio-Rad). The qPCR was performed in a volume of 25 µl containing cDNA generated from 100 ng of original mRNA template, 300 nM of forward and reverse primers and 12.5 µl of SYBR Green PCR master mix (Applied Biosystems). Primers were designed to hybridise in different exons (Table 1), thereby avoiding contamination by genomical DNA and false positive signal in qPCR (Kubista et al. 2006). Genomic DNA contamination was also monitored in every assay using a sample free of reverse transcribed cDNA (minus RT control). The amplification protocol used was: 94°C for 3 min and 40 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. Absence of primer dimers and non-specific products was verified in every qPCR assay by melting curve analysis (temperature reading every 0.2°C from 60°C until 95°C). qPCR analysis was conducted in triplicate reactions for all samples.

Senegalese sole elongation factor 1 alpha isoform 1 (eEF1a1; Genbank accession No. AB326302) was used as an internal reference gene for normalisation of TauT mRNA expression. Amplification of eEF1a1 was performed according to Infante et al. (2008), while cloning and sequencing occurred as previously described. Standard curves (tenfold dilution series) were generated for Senegalese sole TauT and eEF1a1 by plotting the cycle threshold (Ct) obtained in qPCR analysis versus the logarithm of standard copy numbers (CN) and performing a linear regression (Bustin 2000). These curves were used to estimate efficiency in every qPCR assay, showing correlation coefficients higher than 0.99 and a mean efficiency of 98.8 ± 1.9%. Senegalese sole TauT and eEF1a1 copy numbers were calculated for each sample based on the respective standard curve, using the following expression: $CN = (Ct - \text{intercept}) \times (\text{slope})^{-1}$. TauT copy numbers were subsequently normalised: $CNTauT \times CNeEF1a1^{-1}$.

Data analysis

Results were expressed as mean ± standard deviation (SD). Data were initially tested by Levene's test for homogeneity of variances. When homogeneity of variances was observed, detection of group mean differences was tested by one-way ANOVA followed by Tukey's multiple comparison tests. Data were analysed through Kruskall–Wallis and Mann–Whitney U non parametric tests, when mean variances were significantly different across groups. Data regarding Senegalese sole metamorphosis pattern were analysed through Chi-square tests. The significance level was $P \leq 0.05$. All results expressed as a percentage were based on arcsine transformed data (Ennos 2007).

Results

Cloning of Senegalese sole taurine transporter

The full coding cDNA sequence for Senegalese sole TauT was 1,895 bp long, with an open reading frame of 1,878 bp (Genbank accession No. HQ148721) encoding for a putative protein with 625 AA and a predicted molecular weight of 70 kDa (Fig. 1). TauT structure comparisons and hydropathy analysis predicted 14 exons and 12 putative transmembrane helices, respectively (Supplemental Figure 1). Three signature fingerprints were identified among the AA residues of Senegalese sole TauT, corresponding to the sodium/chloride-dependent neurotransmitter transporter superfamily (eight elements), neurotransmitter symporter family (two elements) and taurine transporters (four elements) (Fig. 1 and Supplemental Figure 1).

The multiple AA sequence alignment (Supplemental Figure 2) showed that TauT was very similar among Senegalese sole and the remaining vertebrates, sharing at least 76% identity with mammals and not less than 87% with other teleosts. The intracellular segment 4 (S4) was highly conserved among vertebrates, but changes in AA residues 319 (K) and 322 (C) were found for Senegalese sole (Supplemental Figures 1 and 2). C322 was also found in other fish, reptiles and birds, but a serine replacement was found in this position for mammals.

Two major clades were found in the phylogenetic tree constructed with vertebrate TauT AA sequences, corresponding to a division between mammals and other vertebrates. Senegalese sole TauT was grouped with other teleost species (Supplemental Figure 3).

Fig. 1 Nucleotide and predicted amino acid sequences for *S. senegalensis* taurine transporter (TauT). *Numbers* on the *left* refer to nucleotide (*upper row*) and amino acid (*lower row*) positions. Nucleotide numbering starts on the initiation codon (first ATG), while *asterisk* indicates the stop codon (TGA). Predicted alternating exons are represented by *white and grey colors*. Signature for the sodium/chloride-dependent neurotransmitter transporter superfamily is provided by an eight element fingerprint (*underlined* from I to VIII). Signature for neurotransmitter symporter family is provided by a two element fingerprint represented by *white boxes*, while signature for taurine transporters is provided by a four element fingerprint (*double underlined* from 1 to 4)

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  1  ATGGCACAGAAAGAGAAACTCCAGTGCCTGAAGGATTTCCATAAGGACACT
  1  M  A  Q  K  E  K  L  Q  C  L  K  D  F  H  K  D  T

 52  CTGAAACCATCTCCTGGCAAGAGCCCAGGCACTCGGCCTGAAGATGAAGCA
 18  L  K  P  S  P  G  K  S  P  G  T  R  P  E  D  E  A

103  GAGGGCAAACACCCCCAGCGGGAGAAGTGGGCCAGCAAGTTGGACTTTGTT
 35  E  G  K  H  P  Q  R  E  K  W  A  S  K  L  D  F  V
                      1

154  CTGTCTGTAGCTGGTGGCTTTGTTGGTTTAGGGAATGTGTGGCGTTTCCCG
 52  L  S  V  A  G  G  F  V  G  L  G  N  V  W  R  F  P
                                            I

205  TACCTCTGCTATAAAAATGGTGGAGGTGCATTTCTCATCCCATACTTCATT
 69  Y  L  C  Y  K  N  G  G  G  A  F  L  I  P  Y  F  I
                                            II

256  TTCCTGTTTGGTGGTGGTCTGCCAGTCTTCTTTCTGGAGGTCGCCCTGGGT
 86  F  L  F  G  G  G  L  P  V  F  F  L  E  V  A  L  G

307  CAGTTTACCTCCGAGGGTGGTATCACTTGCTGGGAGAAGCTCTGCCCCATC
103  Q  F  T  S  E  G  G  I  T  C  W  E  K  L  C  P  I

358  TTTACTGGTATTGGTTACGCCTCCATTGTGATCGTTTCCCTTTTGAATATC
120  F  T  G  I  G  Y  A  S  I  V  I  V  S  L  L  N  I
              III

409  TACTACATCGTCATCCTGGCCTGGGGCGTCTACTACCTGTTCCAGTGTTTT
137  Y  Y  I  V  I  L  A  W  G  V  Y  L  F  Q  C  F

460  CAGCCAGAGCTTCCCTGGGCCAAGTGCAATCAGCCGTGGAATACCGATCGC
154  Q  P  E  L  P  W  A  K  C  N  Q  P  W  N  T  D  R

511  TGCATCGAGGACACCTACCGCAATAACAAAACCCTTTGGCTGGCTTCCAAC
171  C  I  E  D  T  Y  R  N  N  K  T  L  W  L  A  S  N
              2

562  ACCTCCAACTTTACTTCCCCTGTCACCGAGTTCTGGGAACGCAATGTGCTG
188  T  S  N  F  T  S  P  V  T  E  F  W  E  R  N  V  L

613  GGAATCAGCAATGGTATTGATGAGATAGGCCCTATTAAATGGGACCTGGCT
205  G  I  S  N  G  I  D  E  I  G  P  I  K  W  D  L  A

664  CTGTGTTTACTGCTCGTCTGGGTCATCTGCTTTTTCTGCATCTGGAAGGGA
222  L  C  L  L  L  V  W  V  I  C  F  F  C  I  W  K  G

715  GTCAGGTCCACTGGGAAAGTGGTCTACATAACAGCAACTTTCCCATTTGTA
239  V  R  S  T  G  K  V  V  Y  I  T  A  T  F  P  F  V
                                            IV

766  ATGCTGATTGTGCTATTGATCCGCGGTGTGACTCTTCCTGGGGCAGCTGAA
256  M  L  I  V  L  L  I  R  G  V  T  L  P  G  A  A  E

817  GGAATTAAATTTTACCTTTACCCAGACCTGACCCGCCTGCAGGACCCAGAG
273  G  I  K  F  Y  L  Y  P  D  L  T  R  L  Q  D  P  E

868  GTGTGGATTGATGCTGGAACCCAGATCTTCTTCTCCTATGCCATTTGTCTG
290  V  W  I  D  A  G  T  Q  I  F  F  S  Y  A  I  C  L

919  GGAGCCATGACATCACTGGGGAGCTACAACAAGTACAGATACAACTGCTAC
307  G  A  M  T  S  L  G  S  Y  N  K  Y  R  Y  N  C  Y

970  AGGGACTGTTTGCTGCTGGGAGGCCTCAACAGCGGTACCAGCTTTGTGTCT
324  R  D  C  L  L  L  G  G  L  N  S  G  T  S  F  V  S
                                      V
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Fig. 1 continued

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1021 GGCTTCGCAATATTTTCCGTCCTGGGCTTCATGGCACAAGAACAAGGGGTG
 341 G   F   A   I   F   S   V   L   G   F   M   A   Q   E   Q   G   V

1072 GACATTGCCGATGTGGCAGAGTCAGGTCCTGGCTTGGCCTTCATCGCCTAC
 358 D   I   A   D   V   A   E   S   G   P   G   L   A   F   I   A   Y

1123 CCGAAAGCTGTGTCCATGATGCCGATGCCAACGCTCTGGGCCATCCTCTTC
 375 P   K   A   V   S   M   M   P   M   P   T   L   W   A   I   L   F
                             VI

1174 TTTATCATGCTGCTGCTTCTGGGCCTCGACAGCCAGTTTGTTGAAGTGGAA
 392 F   I   M   L   L   L   L   G   L   D   S   Q   F   V   E   V   E

1225 GGACAGATCACCTCTATTGTGGATCTGTATCCATCTGTCCTCAGGAGGGGT
 409 G   Q   I   T   S   I   V   D   L   Y   P   S   V   L   R   R   G

1276 TACCACAGAGAGATCTTTATAGCTGTGATTTGCTTCATGAGCTATCTCCTG
 426 Y   H   R   E   I   F   I   A   V   I   C   F   M   S   Y   L   L

1327 GGACTTGCCATGGTAACGAAAGGTGGCATGTATGTGTTTCAACTCTTTGAT
 443 G   L   A   M   V   T   K   G   G   M   Y   V   F   Q   L   F   D

1378 TACTATGCAGCCAGTGGTGTGTGCCTTTTGTGGGTCGCATTCTTTGAATGC
 460 Y   Y   A   A   S   G   V   C   L   L   W   V   A   F   F   E   C

1429 ATTGCTGTAGCCTGGGTTTATGGGAGTGGATAATTTCTATGACGCAGTGGAG
 477 I   A   V   A   W   V   Y   G   V   D   N   F   Y   D   A   V   E
        VII

1480 GATATGATTGGCTACAGACCAAACCCGTGGATGAAATGGAGCTGGACCTTT
 494 D   M   I   G   Y   R   P   N   P   W   M   K   W   S   W   T   F

1531 ATCACACCTGTCCTTTGCATGGGATGCTTTGTCTTTTCCCTGGTGAAGTAC
 511 I   T   P   V   L   C   M   G   C   F   V   F   S   L   V   K   Y
        VIII

1582 AAGCCCTTGACCTATAACAAGGTGTACGAGTATCCTGACTGGGCCATCGGT
 528 K   P   L   T   Y   N   K   V   Y   E   Y   P   D   W   A   I   G

1633 GTGGGCTGGTGTTTGGCTTTGGCCTCCATGATCTGCATCCCATTGGTGATG
 545 V   G   W   C   L   A   L   S   M   I   C   I   P   L   V   M

1684 GTCATCAAGATCTTGCAGTCTGAGGGACCACTGATTGAGAGGATCAAAGCA
 562 V   I   K   I   L   Q   S   E   G   P   L   I   E   R   I   K   A

1735 GTGGCAGCTCCTGCAAAGTCAGGTGTGAGCTCCCGCCCAAAGGAGTACAAC
 579 V   A   A   P   A   K   S   G   V   S   S   R   P   K   E   Y   N

1786 TTGAAGGCCAGCGAGTTGACACAGCCCCTGGACCCAAGCGGGAACAATGGC
 596 L   K   A   S   E   L   T   Q   P   L   D   P   S   G   N   N   G
                                             3

1837 TTGAACAAGCCCACCCACACCATTGTGGAAACAATGATGTGAACGCTCTCT
 613 L   N   K   P   T   H   T   I   V   E   T   M   M   *
            4

1888 GNGAGAGG
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Expression of the taurine transporter during Senegalese sole ontogeny

Senegalese sole larvae grew exponentially during the experimental period, attaining approximately 5 mg DW and 13 mm TL by the end of the first month of development. Eye migration started at 12 DAH, with about 45% of the larvae being found in early metamorphic stage 1 and around 33% at middle metamorphic stage 2 (Fig. 2). At 20 DAH, 95% of Senegalese sole larvae had finished eye migration (later metamorphic stage 4), but this process was only concluded in all larvae at 30 DAH.

The expression of TauT in Senegalese sole larvae increased along the first month of development (Fig. 2). This increase was primarily significant from 6 to 12 DAH, while the highest expression level was found at 30 DAH. In
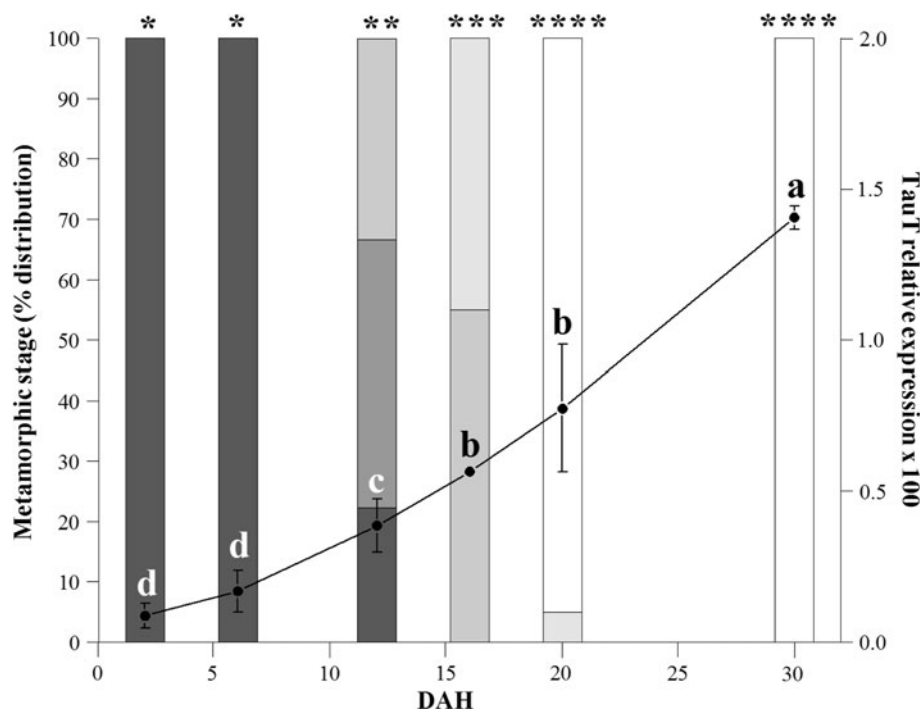
Fig. 2 Metamorphosis pattern and expression of taurine transporter (TauT) in *S. senegalensis* larvae reared under standard procedures. DAH, days after hatching. Results for metamorphosis pattern are expressed as percentage of each metamorphic stage (0 *dark bars*; 1 *dark grey bar*; 2 *light grey bar*; 3 *grey bar*; 4 *open bar*) found at a certain age ($n = 20$). Results for expression of taurine transporter (*filled circles*) are shown as TauT copy:eEF1a1 copy ($n = 3$ pooled samples). Results are given as mean ± standard deviation. Different numbers of *asterisks* represents significant differences for the mean metamorphic stage at a certain age. *Different letters* represent significant differences for the expression of the taurine transporter throughout larval development

dissected larvae, a similar trend was observed in all fractions (head, digestive tract and body), with TauT expression levels significantly increasing from 12 until 30 DAH (Fig. 3). TauT expression decreased within compartments according to the following order: body > head > digestive tract.

In Senegalese sole juveniles, TauT was ubiquitously observed in all examined tissues (Fig. 4). The highest expression levels were found in brain, heart and eye. In the digestive tract, TauT was more expressed in hindgut and stomach. The expression of TauT was not significantly different among midgut or hindgut sections.

Discussion

This study describes the cloning of the full coding cDNA sequence for Senegalese sole TauT. The putative protein was formed by 625 AA, being easily identified by three signatures (sodium/chloride-dependent neurotransmitter transporter superfamily, neurotransmitter symporter family and taurine transporters) and the prediction of 12 transmembrane domains, a characteristic common in all TauT. Senegalese sole TauT exhibited a high similarity with other vertebrate TauT, sharing over 76% identity with mammals

and more than 87% with other teleosts, which strongly suggests a high substrate specificity of transport. Accordingly, the phylogenetic tree constructed for vertebrate TauT grouped Senegalese sole among teleosts, indicating high evolutionary conservation of TauT in fish species.

Several studies have shown that few AA residues in S4 of TauT seem to be involved in the control of taurine transport (Han et al. 2006). Compared to other vertebrates, two noteworthy replacements are found for these AA in Senegalese sole, in positions 319 (R) and 322 (C). In the remaining vertebrates, position 319 is occupied by lysine (K), a positively charged AA that seems to be important for maintaining the loop structure and controlling the gating function of TauT by attracting the negatively charged aspartic acid (D) in position 325 (Han et al. 2006). The replacement of lysine by arginine (R319) in Senegalese sole may not affect this function, since arginine is also a positively charged AA and ion attraction with D325 will possibly be maintained. On the other hand, position 322 is occupied by a cysteine (C322) in fish, reptiles and birds, while in mammals a highly conserved serine is found in this position (S322). This replacement is likely to influence taurine uptake, since gating of TauT activity seems to be controlled by protein kinase C (PKC) phosphorylation of S322. For instance, Han et al. (1999) have shown that the
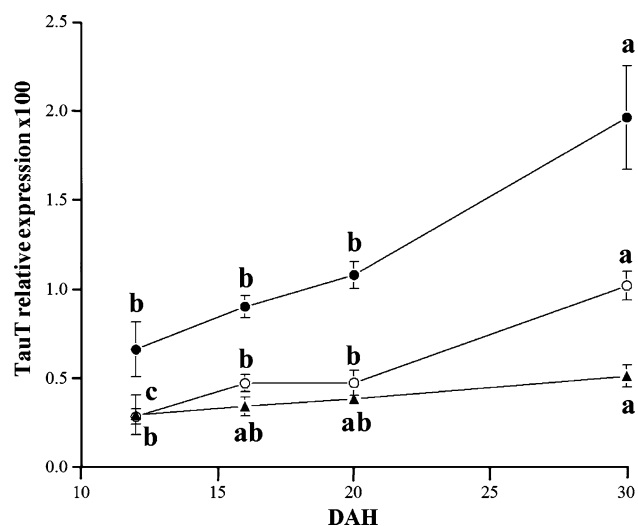
Fig. 3 Expression of taurine transporter (TauT) in dissected *S. senegalensis* larvae reared under standard procedures. DAH, days after hatching. Results for expression of taurine transporter in head (*open circles*), digestive tract (*filled triangles*) and body (*filled circles*) fractions are shown as TauT copy:eEF1a1 copy and given as mean ± standard deviation (*n* = 3 pooled samples). *Different letters* within the same fraction represent significant differences throughout larval development

replacement of S322 by an alanine increased TauT activity by threefold in *Xenopus laevis* oocytes. Since alanine and cysteine phosphorylation by PKC cannot occur, these findings suggest that the replacement of S322 by C322 also result in a higher TauT activity. The biological explanation and consequences of this replacement on TauT activity in mammals compared with fish, reptiles and birds remain unknown and should be clarified.

In Senegalese sole larvae, TauT mRNA was found at the onset of exogenous feeding, and was found to significantly increase during the larval stage and during metamorphosis. A similar trend was observed in all fractions of dissected

larvae (head, digestive tract and body), with TauT expression significantly increasing throughout metamorphosis. These results indicate that metamorphosis is an important developmental trigger to promote taurine transport in Senegalese sole larval tissues. These findings also show that although TauT mRNA can be found in Senegalese sole at the onset of exogenous feeding, it is during metamorphosis that the capacity to transport taurine increases. Nevertheless, it should be kept in mind that factors that influence taurine accumulation include ionic environment, electrochemical charge, post-translational and transcriptional factors (Han et al. 2006). However, Han et al. (2006), and Han and Chesney (1994) suggested that the long term adaptive response to diet is regulated at the mRNA level. Compared to the remaining fractions, TauT expression was very high in Senegalese sole body during the larval stage, indicating a high capacity to transport taurine in muscle during this period. In a developmental stage where growth potential is extremely high (Kamler 1992), this capacity may be important for larval growth, since taurine has been indicated to mediate the uptake of calcium in muscle sarcoplasmatic reticulum, playing a role on excitation–contraction mechanisms of muscle fibres (Huxtable 1992; De Luca et al. 2000), and to induce the uptake of AA into skeletal muscle cells (Huxtable et al. 1987). Since AA are the building blocks for growth (Carter and Houlihan 2001), a high capacity to transport taurine in muscle may be related to an increase of AA retention, leading to a high growth potential, as observed for Senegalese sole larvae reared under dietary taurine supplementation (Pinto et al. 2010). In addition, dietary taurine supplementation has also been reported to simultaneously increase taurine body levels and larval growth performance in other fish species, such as cod (Matsunari et al. 2005), red seabream (Chen et al. 2004) and Japanese flounder (Chen et al. 2005).

Fig. 4 Tissue expression of taurine transporter (TauT) in juvenile *S. Senegalensis*. *Br* brain, *Ey* eye, *Gi* gills, *He* heart, *Hk* headkidney, *SP* spleen, *Gb* gallbladder, *Li* liver, *St* stomach, *Mg* midgut, *Hg* hindgut, *Sec* sections from anterior to posterior. Results are shown as TauT copy:eEF1a1 copy and given as mean ± standard deviation (*n* = 4). *Different letters* represent significant differences among fractions
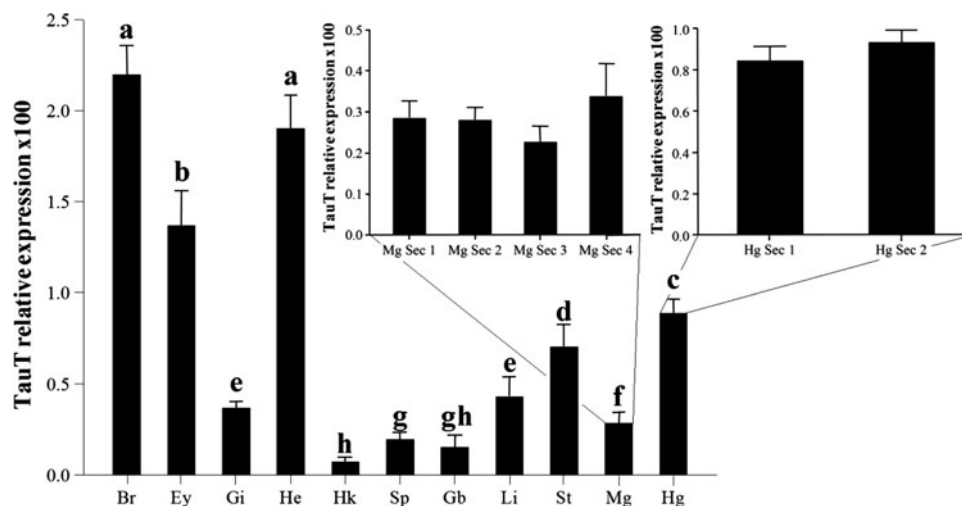
Contrary to what was observed for the body fraction, TauT expression in the digestive tract was lower in the remaining fractions throughout the experimental period. Although these results indicate that in comparison to the remaining fractions, Senegalese sole larvae may have a low ability to transport taurine in the digestive tract, the capacity to absorb dietary taurine at gut level seems to be already established, at least, from the onset of metamorphosis. The current data do not allow us to verify if TauT was expressed in the digestive tract earlier in the development, but it is observed that TauT expression in the digestive tract did not increase as much as in remaining fractions during larval development, suggesting that the capacity to absorb dietary taurine in the digestive tract is likely to be sufficiently high in the early larval stages.

The ubiquitous nature of TauT reported for juveniles of other fish species (Takeuchi et al. 2000, 2001; Zarate and Bradley 2007) was also found in Senegalese sole juveniles, since TauT was expressed in all examined tissues. Among these, TauT expression was particularly high in brain, heart and eye. Not surprisingly, these are organs where taurine can be found in high concentrations and has been suggested to play important roles (Huxtable 1992). For instance, taurine has been suggested to modulate neurotransmitter and synaptic activity, acting as a membrane stabiliser or playing a key role in cell volume regulation, a crucial factor for the normal function of the central nervous system (Huxtable 1989). In heart, taurine functions are mainly related to calcium modulation for cardiac muscle contraction, while in eye taurine has been indicated to prevent retinal degeneration by protecting photoreceptors, acting as a biological mirror behind the retina and having a shielding effect on rod outer segments on protection of high levels of light (Pasantes-Morales et al. 1981).

In the digestive tract of juveniles, TauT was more expressed in stomach and hindgut. Regarding stomach, this high expression of TauT indicates that dietary taurine is readily absorbed when the digestive process begins. It is not known when absorption of dietary taurine in the stomach region may start. Most studies conclude that altricial marine fish larvae do not have a functional stomach at the onset of first feeding (Rønnestad et al. 1999). However, in many fish the differentiation of stomach into a separate compartment with some short-term storage function appears to start prior to the onset of gastric acid and pepsin production as shown in Japanese flounder (Rønnestad et al. 2000). In Senegalese sole it has been suggested that the incipient stomach can already be observed at first feeding (Ribeiro et al. 1999), but the functional characterisation of this structure, including any taurine absorbing capacity, remains to be established. The high expression of TauT found in the hindgut of juvenile Senegalese sole also indicates that this area may be an important site of taurine absorption during the larval stage. The high expression of TauT found in the hindgut also suggests that taurine endogenously used for bile salt conjugation may be reabsorbed at the posterior end of the digestive tract. Therefore, these results suggest an enterohepatic recycling pathway for taurine in Senegalese sole, present at least from the juvenile stage. The enterohepatic recycling pathway of taurine is likely important for controlling taurine losses and maintaining taurine body pool in flatfish species. As observed in cats, the enterohepatic circulation of taurine can be disrupted by anaerobic bacteria in the digestive tract, leading to a deconjugation of taurine from bile salts (Ikeda et al. 1963). Taurine released during this process is reabsorbed or undergoes further bacterial degradation, being lost from the taurine body pool. These losses may be substantial (between 30 and 50%) upon dietary factors that enhance bile secretion or increase taurine degradation by changes in gut flora (Cooke et al. 1988; Hickman et al. 1992). With this in mind, the results obtained in the current study suggest that future research should address understanding the mechanisms underlying the enterohepatic recycling pathway of taurine in flatfish species, as well as controlling losses from the taurine body pool resulting from dietary changes.

In conclusion, this study described the cloning and ontogenetic expression of TauT in the flatfish Senegalese sole. This transporter was highly similar to other vertebrate TauT, but changes in position 322 (S4) indicate that taurine transport may be different in mammals than in fish, reptiles or birds. Results also showed that Senegalese sole metamorphosis is an important developmental trigger to promote taurine transport, especially in larval muscle tissues. Moreover, results indicated that the larval capacity to absorb dietary taurine in the digestive tract seems to be established, at least from the onset of metamorphosis. In juveniles, taurine transport was especially high in brain, heart and eye, organs where taurine has been suggested to play important biological roles. Moreover, results from this study indicate that dietary taurine is quickly absorbed when the digestive process begins and taurine endogenously used for bile salt conjugation may be recycled at the posterior end of the digestive tract. Therefore, these results suggest an enterohepatic recycling pathway for taurine in Senegalese sole, at least in the juvenile stage, a process that may be important for maintenance of the taurine body levels in flatfish species.

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