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Research report

Expression and functional analysis of Na^+-dependent glutamate transporters from zebrafish brain

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ABSTRACT

High-affinity excitatory amino acid transporters (EAATs) regulate extracellular glutamate levels. Zebrafish (*Danio rerio*) provides an excellent model to study the function of different neurotransmitter systems. Although the identification of the EAAT family is well established in the mammalian central nervous system (CNS), EAAT-related genes and their expression profile in zebrafish have not yet been reported. Here we identify and describe the expression profile of EAATs-related genes and functional properties of glutamate uptake in three major brain structures from zebrafish (telencephalon, optic tectum and cerebellum). Searches on zebrafish genome databases and a phylogenetic analysis confirmed the presence of several EAAT-related genes (EAAT2, EAAT3, three EAAT1 paralogs and two EAAT5 sequences). All sequences identified were expressed in the structures analyzed. EAAT2 and EAAT3 were the most prominent glutamate transporters expressed in all brain areas. A uniform expression was observed for EAAT1A, whereas higher EAAT1B transcript levels were detected in telencephalon. Lower amounts of EAAT1C transcripts were observed in cerebellum when compared to other structures. No EAAT4-related sequence was found in the zebrafish genome. The EAAT5A expression was similar to EAAT5B in the telencephalon, while EAAT5B was less expressed than EAAT5A in optic tectum and cerebellum. Moreover, the glutamate uptake was significantly higher in optic tectum, which indicates functional differences within zebrafish brain structures. Altogether, the study of glutamate uptake in zebrafish could be important to evaluate the modulation of glutamatergic signaling through pharmacological and toxicological studies.

1. Introduction

Glutamate is the most widespread excitatory neurotransmitter in the mammalian CNS, being involved in many aspects of brain function such as learning and memory [48,26], development and ageing [50,7], and environmental adaptation [11]. However, besides its essential roles in brain activity, the neurotransmitter glutamate may be potently toxic (excitotoxicity), when present in high concentrations in the synaptic cleft [11,64,29], and it has been shown that this excitotoxic effect is involved in various acute and chronic neurological disorders [32,33,53,58]. Thus, the clearance of extracellular glutamate, mainly mediated by sodium-dependent transport into astrocytes [1,52,64], is an essential parameter involved in the physiological/excitotoxic tonus of the glutamatergic system.

The EAATs represent a protein family that displays considerable homology (50–60% at the amino acid level) [6]. To date, five structurally distinct subtypes of excitatory amino acid transporters have been identified and characterized in the mammalian brain: EAAT1 [56], EAAT2 [40], EAAT3 [27], EAAT4 [18] and EAAT5 [4]. EAAT1 is primarily an astroglial transporter and the main transporter pro-

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Table 1
PCR primer design.

EAAT member	Primers (5′–3′)	Tm (°C)	Cycles	PCR product (bp)	GenBank accession numbers	ZFIN ID (ZDB-GENE)
EAAT1A	TGTCACGTCACGAGCTGCGCTC (F) ACAAGAAAACAGTGGACCGCTCG (R)	57	30	321	mRNA BC063233 Protein AAH63233	030131-2159
EAAT1B	AGAAACCGCGGTCGCGCAGC (F) TGCCAATGAACACTGCGATGAAGG (R)	57	30	396	mRNA XM_679025 Protein XP_684117	—
EAAT1C	CGTGATCTTCACCGTGGCTGCTG (F) AGTTGACGTTCTCTGTCGCATTGACC (R)	57	35	497	mRNA NM_001109703 Protein NP_001103173	071004-45
EAAT2	GCTGTCTGGAGGAGAACCTGGGCATTG (F) TCATCTCGATGTCGTCAGTCTTCCCGTG (R)	61	30	432	mRNA BC056751 Protein AAH56751	030131-7779
EAAT3	GGATGGAACTGCACTGTATGAAGCGGTG (F) GGACGATTCCAGCACCGTAGGCGTC (R)	61	30	287	mRNA NM_001002666 Protein NP_001002666	040718-414
EAAT5A	CGTTTGGCATTGTGTTTCTGGTGGCTG (F) TGAGCGATAAATATGGCCGCCACAG (R)	61	35	376	mRNA XM_678579 Protein XP_683671	—
EAAT5B	ATCGTCCTCACTTCAGTGGGTTTGC (F) GCAAAGGTTGTAACAGGCGGTGG (R)	57	35	330	mRNA XM_687808 Protein XP_692900	—
β-actin	GTCCCTGTACGCCTCTGGTCG (F) GCCGGACTCATCGTACTCCTG (R)	54	35	678	mRNA AAC13314 Protein AF057040	000329-1

tein present during CNS development [20]. EAAT2 is one of the two most abundant glutamate transporters in the adult CNS [30], and is an astroglial transporter expressed postnatally and responsible for up to 90% of all glutamate transport in adult tissue [45,61,63,31]. EAAT3, a neuronal glutamate transporter found at high densities on postsynaptic membranes, is present most notably in the hippocampus, cerebellum, and basal ganglia [21]. EAAT4 is a glutamate transporter largely limited to the Purkinje cells of the cerebellum [18,21], whereas EAAT5 is found primarily in the retina on photoreceptors and bipolar cells [4,43].

The zebrafish is a small freshwater teleost that has recently attained a pre-eminent position in biomedical research, being considered an important and emerging vertebrate model in many fields of biology including neuroscience [22]. This species exhibits genetic and anatomic conservation in relation to both mice and humans and a high degree of genetic homology [5,13]. Moreover, the zebrafish appears to be an attractive organism for high throughput screening applications, e.g., mutagenesis screening, forward genetics or drug discovery efforts applied to neurotoxicity tests [68,39].

Recently, the choice of this animal model to investigate some aspects of brain neurotransmission, including the glutamatergic system, has become more common. Studies have demonstrated the distribution and function of ionotropic glutamate receptors in the olfactory bulb [15,59], as well as embryonic expression of NMDA receptor subunit genes [10]. The glutamatergic modulator MK-801 was employed to examine behavioral parameters [57] as well as the role of glutamatergic receptors in learning and memory processes [36]. Furthermore, vesicular glutamate transport has been described in mutant zebrafish larvae, playing a key role in visual perception and behavior [54,37].

Although some parameters of the glutamatergic signaling in zebrafish have already been characterized, the expression and functional profile of glutamate transporters have not yet been reported. Theories about vertebrate neural and behavioral bases propose that brain evolution occurred in successive stages and that these bases have been conserved through phylogenesis. However, recent developmental, neuroanatomical and functional data indicate that the brain and behavioral evolution may have been more conservative than previously thought [47].

To provide new insights into the primary characteristics of the glutamatergic system in zebrafish, the aims of the present study were to identify the relative gene expression profiles of distinct members of the EAAT family and to carry out a preliminary investigation of some parameters of glutamate uptake in three brain structures in this species: the telencephalon, optic tectum, and cerebellum.

2. Experimental procedures

2.1. Materials

Reagents were purchased from Sigma Chemical CO (St. Louis, MO, USA). L-[^{3}H]glutamate (specific activity 30 Ci mmol^{-1}) was purchased from Amersham International, UK. Platinum Taq DNA polymerase, TRIzol reagent, and SuperScript™ First-Strand III (Synthesis System for RT-PCR) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Animals

Adult wild-type zebrafish (*Danio rerio*) of both sexes (3–6 months-old) were obtained from a commercial supplier (Delphis, RS, Brazil). All fish were acclimated to their new environment for at least 2 weeks in a 50-l thermostated aquarium. The water was kept at 26 ± 2 °C under a 12-h light–dark controlled photoperiod and the animals were fed with commercial flake fish food twice a day. They were used according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, being healthy and free of any signs of disease. All procedures in the present study were approved by the Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS), protocol number 477/05-CEP.

2.3. Phylogenetic analysis and primers design

EAAT members were identified by NCBI Blast searches of GenBank, using the well-known *Homo sapiens* and *Rattus norvegicus* proteins as queries. The obtained sequences (supported by mRNA or EST data) were compared with the zebrafish protein database of the Zebrafish Information Network (ZFIN) (University of Oregon, Eugene, OR 97403-5274; World Wide Web URL: http://zfin.org) and the sequences were aligned using the ClustalX program [62]. A phylogenetic tree was constructed according to the Neighbor-Joining method [49] using proportional (p) distance with the MEGA 2.1 program [46]. In order to compare the zebrafish deduced amino acid sequences, an alignment was performed using ClustalX. Regions with low scores for similarity among the sequences were used to search for specific primers, which were designed using the program Oligos 9.6. The primer specificities were checked by comparing each primer with the zebrafish genome to confirm that it would recognize only its specific target sequence. Thus, the strategy adopted for the design of the primers avoided cross-amplification. The optimal conditions for primer annealing were determined (Table 1) and the β-actin primers were designed as described previously [9].

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

In order to obtain distinct brain structures from zebrafish, the animals were cryoanaesthetized and further euthanized by decapitation. Total RNA was isolated from telencephalon, optic tectum, and cerebellum using the TRIzol® reagent in accordance with the manufacturer's instructions. The purity of the RNA was spectrophotometrically determined by calculating the ratio between absorbance values at 260 and 280 nm and its integrity was confirmed by electrophoresis through a 1.0% agarose gel. Afterwards, all samples were adjusted to 160 ng/μl and cDNA species were synthesized using SuperScript™ III First-Strand Synthesis SuperMix (Synthe-