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Expression patterns of glycine transporters (*xGlyT1*, *xGlyT2*, and *xVIAAT*) in *Xenopus laevis* during early development

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Abstract

Glycine, a major inhibitory neurotransmitter in the vertebrate nervous system, not only functions in synaptic signaling, but has also been implicated in regulating neuronal differentiation, neuronal proliferation, synaptic modeling, and neural network stability. Elements of the glycinergic phenotype include the membrane-bound glycine transporters (GLYT1 and GLYT2), which remove glycine from the synaptic cleft, and the vesicular inhibitory amino acid transporter (VIAAT or VGAT), which sequesters both glycine and GABA into synaptic vesicles. Here, we describe the spatial and temporal expression patterns of *xGlyT1, xGlyT2,* and *xVIAAT* during early developmental stages of *Xenopus laevis*. *In situ* hybridization reveals that *xGlyT1* is first expressed in early tailbud stages in the midbrain, hindbrain, and anterior spinal cord; it extends posteriorly through the spinal cord and appears in the forebrain, retina, between the somites, and in the blood islands by swimming tadpole stages. *xGlyT2* and *xVIAAT* initially appear in late neurula stages in the anterior spinal cord. By swimming tadpole stages, the expression of these genes appears in the forebrain, midbrain, and hindbrain and extends posteriorly through the spinal cord; *xVIAAT* is also expressed in the retina. Confocal analysis of multiplex fluorescent *in situ* hybridization signal in the spinal cord reveals that *xGlyT1* and *xGlyT2* share little cellular colocalization. While there is significant coexpression between *xVIAAT* and *xGlyT2, xVIAAT* and the GABAergic marker glutamic acid decarboxylase (*xGAD67*), and *xGlyT2* and *xGAD67*, each gene also appears to have discrete, non-colocalized areas of expression.

Keywords: Glycine transporter, *GlyT1*, *xGlyT1*, *GlyT2*, *xGlyT2*; Vesicular inhibitory amino acid transporter, *VIAAT*, *xVIAAT*; Vesicular GABA transporter; *VGAT*; *Xenopus*; *Xenopus laevis*; Nervous system; Development; Neurotransmitter; Spinal cord; Glycine; GABA

1. Results and discussion

As a major inhibitory neurotransmitter in the vertebrate central nervous system (CNS), glycine plays a critical role in motor and sensory processing (reviewed in Legendre, 2001; Zeilhofer, 2005). In addition to its established synaptic function, glycine has been implicated in several developmental processes: it can modulate excitatory neurotransmission by binding to *N*-methyl-D-aspartic acid (NMDA) receptors (reviewed in Betz et al., 2006), promote interneuron differentiation and proliferation (McDearmid et al., 2006), induce elevation in levels of intracellular calcium, regulate synaptic remodeling of some inhibitory pathways (Kandler and Friauf, 1995), and act as a homeostatic regulator of hippocampal network excitability (Zhang et al., 2007). In addition, glycine signaling has been shown to be responsible for the generation of rhythmic swimming movements in lower vertebrates (Soffe et al., 2001).

Essential components of the glycine signaling system include vesicular and membrane transport proteins necessary for the sequestration, release, and reuptake of glycine. Glycine transporter 1 (GLYT1) and glycine transporter 2 (GLYT2), which have been found primarily in glial and neural cells, respectively, function as membrane-bound

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transporters that reuptake glycine from the synaptic cleft (Betz et al., 2006). The vesicular inhibitory amino acid transporter (VIAAT or VGAT), the only known vesicular transporter for glycine as well as γ-aminobutyric acid (GABA), takes up glycine and GABA into synaptic vesicles for release into the synapse (Wojcik et al., 2006). Because of the widespread use of *Xenopus laevis* for investigating all aspects of early neural development – neural induction and patterning (reviewed in De Robertis, 2006), phenotype determination and differentiation (Gamse and Sive, 2000), homeostatic regulation (reviewed in Borodinsky and Spitzer, 2006), and the generation of sensory and motor processing, including glycine-mediated spinal movement (Soffe et al., 2001) – it is an important model for characterizing the expression of these transporters. In previous developmental studies using other vertebrates, the analyses of *GlyT1* and *GlyT2* expression in early development have focused predominantly on the developing spinal cord (Adams et al., 1995; Higashijima et al., 2004; Zafra et al., 1995), with less detailed and sometimes contradictory characterization in other regions of the developing CNS (Adams et al., 1995; Cui et al., 2005; Higashijima et al., 2004; Jursky and Nelson, 1996). A survey of *VIAAT* expression using only whole mount analysis has been performed during mid to late embryonic development in mouse (Oh et al., 2005); no characterization of this gene has been performed in lower vertebrates. We have therefore isolated the *X. laevis* glycine transporter 1 (*xGlyT1*), glycine transporter 2 (*xGlyT2*), and vesicular inhibitory amino acid transporter (*xVIAAT*) and have analyzed their expression patterns throughout early development.

1.1. xGlyT1, xGlyT2, and xVIAAT sequences

In order to characterize the sequence of *X. laevis* glycine transporter 1, RT-PCR and subsequent 3′- and 5′-RACE products were cloned and sequenced. From these sequences, a 2218 bp cDNA sequence (GenBank Accession Number EU117185) containing a 1902 bp open reading frame was constructed. The deduced amino acid sequence predicts a protein of 633 amino acids in length that shows high identity to the deduced protein sequences for GLYT1 in several species (Table 1). Based on this similarity, we have designated this sequence *xGlyT1*.

Likewise, RT-PCR and RACE products were cloned and sequenced to characterize the sequence of *X. laevis* glycine transporter 2. A 2997 bp cDNA sequence (GenBank Accession Number EU117186) containing a 2373 bp open reading frame was assembled. The deduced amino acid sequence predicts a protein of 791 amino acids in length that shows high identity to the deduced protein sequences for GLYT2 in several species (Table 1). Based on this similarity, we have designated this sequence *xGlyT2*.

In order to clone the *X. laevis* vesicular GABA transporter, RT-PCR was employed, producing a 1482 bp fragment that, after sequencing, was found to share 99.4% identity with the predicted open reading frame of *X. laevis* hypothetical protein MGC68938 mRNA (GenBank Accession Number BC057733). This hypothetical mRNA sequence predicts an amino acid sequence highly similar to VIAAT amino acid sequences in several species (Table 1), leading us to call the cloned fragment *xVIAAT*.

Table 1
Percentage identity of the amino acid sequences between GLYT1, GLYT2, and VIAAT orthologs

	GLYT1	GLYT2	VIAAT
Human	80.6	77.1	88.8
Mouse	80.7	76.0	89.1
Chick	81.6	79.4	92.2
Zebrafish	77.1	69.7	87.0
X. tropicalis	93.7	87.3	95.4

Left column represents GLYT1 percent identities between *X. laevis* and human (GenBank Accession Number NP008865.2), mouse (NP032161.2), chick (NP001026450.1), zebrafish (NP001025244.1), and *X. tropicalis* (Joint Genome Project model identification number 213484). Middle column represents GLYT2 percent identities between *X. laevis* and human (GenBank Accession Number NP004202.2), mouse (NP683733.1), chick (XP420906.2), zebrafish (NP001009557.1), and *X. tropicalis* (Joint Genome Project model identification number 247213). Right column represents VIAAT percent identities between *X. laevis* and human (GenBank Accession Number NP542119), mouse (NP033534), chick (XP417347), zebrafish (NP001074170), and *X. tropicalis* (NP001004943).

1.2. Expression patterns of xGlyT1, xGlyT2, and xVIAAT

Whole mount *in situ* hybridization and histological analyses were used to determine the expression patterns of *xGlyT1*, *xGlyT2*, and *xVIAAT* during early development. *xGlyT1* transcripts are first detected in early tailbud stage embryos – earlier in embryogenesis than that which has been reported for the *GlyT1* ortholog in zebrafish (Cui et al., 2005; Higashijima et al., 2004) but in agreement with the earliest stages of ortholog expression in mouse (Adams et al., 1995) – with prominent expression in the midbrain and anterior spinal cord as well as weak expression in the hindbrain (Fig. 1D). By late tailbud stages, the expression pattern extends posteriorly in the spinal cord and appears in between the somites (Figs. 1G and 2I). Immunohistochemistry for glial fibrillary acidic protein (Johnston and Gooday, 1991) has revealed that these regions of *xGlyT1* expression correspond with populations of peripheral glial cells which may be found in regions between the somites (data not shown). This particular characteristic of *GlyT1* expression has not been reported in other species at similar stages of development. Transient expression appears in the ventro-lateral diencephalon and ventral retina and disappears following hatching stages (Figs. 1G and 2A, B). At hatching stages, *xGlyT1* expression continues to intensify and expand posteriorly in the spinal cord and between the somites, and it begins to increase in the posterior-most region of the midbrain (Fig. 1J and M). Transcripts appear at low levels in a small region of the ventricular layer in the medial diencephalon (Fig. 2B). Additionally, they are present in a ventral domain of the embryo (Fig. 1M) corre-