Ornithine-δ-Aminotransferase Inhibits Neurogenesis During Xenopus Embryonic Development

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Purpose. In humans, deficiency of ornithine-δ-aminotransferase (OAT) results in progressive degeneration of the neural retina (gyrate atrophy) with blindness in the fourth decade. In this study, we used the Xenopus embryonic developmental model to study functions of the OAT gene on embryonic development.

Methods. We cloned and sequenced full-length OAT cDNA from Xenopus oocytes (X-OAT) and determined X-OAT expression in various developmental stages of Xenopus embryos and in a variety of adult tissues. The phenotype, gene expression of neural developmental markers, and enzymatic activity were detected by gain-of-function and loss-of-function manipulations.

Results. We showed that X-OAT is essential for Xenopus embryonic development, and overexpression of X-OAT produces a ventralized phenotype characterized by a small head, lack of axial structure, and defective expression of neural developmental markers. Using X-OAT mutants based on mutations identified in humans, we found that substitution of both Arg 180 and Leu 402 abrogated both X-OAT enzymatic activity and ability to modulate the developmental phenotype. Neurogenesis is inhibited by X-OAT during Xenopus embryonic development.

Conclusions. Neurogenesis is inhibited by X-OAT during Xenopus embryonic development, but it is essential for Xenopus embryonic development. The Arg 180 and Leu 402 are crucial for these effects of the OAT molecule in development.

Keywords: Xenopus, embryos, development, neuralization, ventralization

Pyroline-5-carboxylate (P5C), the precursor and immediate degradative product of proline, participates in a redox shuttle in which the cycling of proline and P5C transfers reducing (oxidizing) potential into and out of mitochondria.1-2 In the brain, proline enters neurons via a high-affinity transporter3,4 and proline acts as a neurotransmitter by inhibiting glutamatergic neurons. Mutations in proline dehydrogenase coding for proline oxidase results in neurologic defects in animals5 and is associated with susceptibility to schizophrenia.6,7 Studies into the mechanism of this association can take advantage of two other pathways that generate P5C. Pyroline-5-carboxylate synthase generates P5C from glutamate,8 and mutations in the gene encoding this enzyme have been associated with neurodegeneration, catacares, and connective tissue disease.9,10 but mechanistic studies in this disorder have only begun. On the other hand, ornithine-δ-aminotransferase (OAT), which produces P5C from ornithine,8 has been intensively studied, and defects in OAT result in a well-characterized clinical disorder with a defect in neural tissue.11 Ornithine-δ-aminotransferase is a pyridoxal phosphate-dependent mitochondrial matrix enzyme expressed in most mammalian tissues, including the kidney, liver, small intestine, and retina. Ornithine-δ-aminotransferase catalyzes the reversible transamination of ornithine to glutamic-γ-semialdehyde, an intermediate in proline and glutamate metabolism.12 In humans, deficiency of OAT is inherited as an autosomal recessive trait and results in gyrate atrophy (GA) of the choroid and retina, a progressive chorioretinal degeneration, with blindness occurring by the fourth decade.12-14 Simell and Takki15 reported that hyperornithinemia and overflow ornithine were present in GA patients, and Valle et al.16 showed that OAT deficiency was the primary cause of GA. Subsequently, the human OAT gene was cloned and mapped (10q26), and more than 60 mutations causing GA have been identified.14,17-21 Two mouse models with OAT deficiency, one generated by gene targeting22 and a spontaneous variant,23 were found to have conditions similar to those in GA patients, including chronic hyperornithinemia, massive orthinuria, and progressive retinal degeneration. Studies of the OAT-deficient mouse also revealed that an arginine-restricted diet substantially reduced plasma ornithine levels and completely prevented retinal degeneration.24 Treatment for GA is still a daunting task,
OAT Inhibits Embryonic Neurogenesis in *Xenopus*

**METHODS**

All experimental procedures were approved by the Animal Care and Use Committee of National Cancer Institute-Frederick, National Institutes of Health. The protocol is #99-084 entitled, “Signaling mechanisms in *Xenopus* embryos.” All experiments are in compliance with the ARVO policy for the Use of Animals in Ophthalmic and Vision Research.

**Animals**

Adult *Xenopus laevis* were bred according to guidelines outlined in the *Xenopus* book. Embryos were incubated at 22°C in MMR medium (0.1 M NaCl, 2.0 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 5 mM HEPES, pH 7.8, 0.1 mM EDTA), and staged according to the external morphology.

**Cloning of the X-OAT and Plasmid Construction**

The *Xenopus* homologue of OAT was cloned by RT-PCR. We used 3 μg total RNA isolated from *Xenopus* oocytes and an oligo-dT adapted primer to convert mRNA into cDNA according to the procedure described in the 3′ RACE System for rapid amplification of cDNA ends kit (GibcoBRL, Rockville, MD, USA). The PCR used a gene-specific primer designed from the X-OAT fragment (GenBank, Accession #AW199859) and the abridged universal amplification primer obtained from the kit (primers: forward, 5′-ATGCCTTCACCTGATCCAGA-3′; reverse, 5′-GGCCACCGCGTCGACACTAGTCA-3′). The cDNA was ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA) and then sequenced. The full-length cDNA was amplified using the primers containing a Psi I or Sal I restriction endonuclease site (forward, 5′-CCTCCTGCAGATGTTTCCAAAATCTCCA-3′; reverse, 5′-TACGGTGCACTTTAGAGACAGGAGGTTT-3′). The full-length PCR product (1.32 kb) was digested with restriction endonucleases (Pst I and Sal I) and then cloned into pBluescript II SK-plasmid for sequence. The consensus cDNA nucleotide sequences (X-OAT) were found to be highly homologous to those reported in human, mouse, rat, and *Drosophila* genes.

**Mutagenesis and In Vitro Translation**

Mutations were generated using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer’s instructions. Based on homologies to human OAT and mutations in *G. elegans*, we made the following four mutations: X-OATR180T (M1), X-OATL402P (M2), X-OATV420P (M3), and X-OATK292N (M4) (Fig. 1). All constructs were sequenced to verify mutations and to ensure that no additional mutations were introduced. The mutants were subcloned into pSP64TEN as previously reported, and capped mRNA was prepared using the MEGAscript in vitro transcription kit (Ambion, Austin, TX, USA). The translation reaction was conducted in a TNT-coupled reticulocyte lysate system (TNT Kit, Promega) in the presence of [35S]methionine (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). The products of the translation reaction were subjected to SDS-PAGE, and dried gels were analyzed by autoradiography.

**Whole-Mount In Situ Hybridization**

Embryos were collected from stages 2 to 40 and analyzed for X-OAT expression. In situ hybridization studies were performed using a digoxigenin-labeled (Boehringer-Ingelheim, Ingelheim, Germany) antisense RNA probe or a fluorescein-labeled (Boehringer-Ingelheim) antisense RNA probe. Both probes were synthesized from pBluescript II SK–X-OAT plasmids linearized with Pst I by using T7 RNA polymerase. Digoxigenin was detected using alkaline phosphatase–conjugated anti-digoxigenin antibodies (Boehringer-Ingelheim) and 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium substrates.

**Microinjections and Explant Culture of Embryonic Tissues**

The X-OAT construct and mutants of X-OAT were subcloned into pSP64TEN vector for in vitro synthesis of capped sense mRNA using the Sp6 transcription kit (Ambion), according to the manufacturer’s instructions. The synthetic RNAs were quantitated by ethidium bromide staining with reference to standard RNA. Embryos of *X. laevis* were obtained by artificial insemination after induction of females with 500 U human chorionic gonadotropin. The developmental stage was designated according to our previous publication. Embryos at the two-cell stage were injected into the animal pole, and at the four-cell stage, they were injected in the dorsal marginal zone with various mRNAs. Animal caps (ACs) were dissected from the injected embryos at stages 8.5 to 9.0 and cultured at 22°C in 67% Leibovitz’s L-15 medium (Life...
Technologies, Inc., Bethesda, MD, USA) with 7 mM Tris-HCl (pH 116.7.5) and gentamycin (50 μg/mL) to various stages before being harvested for RT-PCR. Noteworthy in these studies was the concentration of L-arginine at 335 mg/L and glutamine at 201 mg/L. There was no added ornithine in this medium.

Morpholino Antisense Oligonucleotides

The X-OAT morpholino antisense oligonucleotide (X-OAT-Mo), a 25-mer oligo, was designed against the 5' untranslated region of Xenopus OAT, immediately adjacent to the initiation start site with the following base sequence 5'-TGGATTAGTTTGAGAAAGCATTCTGG-3' (Gene Tools, LLC, Philomath, OR, USA). Doses of 5 to 10 ng X-OAT-Mo per embryo were injected into the animal pole area of two-cell stage embryos or ventral marginal zone at the four-cell stage. A sense control morpholino oligonucleotide (Co-Mo) composed of base sequence 5'-CCAGAATGCTTTCCAAACTAATCCA-3' (Gene Tools, LLC) was injected as a control at the same concentration.

Reverse Transcriptase PCR

Total RNA was extracted from cultured AC explants with TRIzol reagent (Life Technologies, Inc.), following the manufacturer’s instructions. Reverse transcriptase PCR was performed using a Superscript Preamplification System (Life Technologies, Inc.). Primers were designed as follows: X-OAT: forward, 5'-CGCGTGGCTATGGCTTCTTT-3', and reverse, 5'-GGAGGAGCCAGCCGGGATAAT-3'. Otx2: forward, 5'-GGATGGATTTGTTGCACCAGTC-3' and reverse, 5'-CACTCTCCAGCTCACTTCTC-3'. Hoxb9: forward, TACTTACGGGCTTGGA, and reverse, 5'-AGCGTGTAACCAGTTGGCTG-3'. Polymerase chain reaction conditions were as follows: 5 minutes at 95°C for 1 cycle; 1 minute at 94°C, 45 seconds at 60°C, and 1 minute at 72°C for 32 cycles. Polymerase chain reaction products were subjected to electrophoresis on Tris acetate EDTA gels and visualized by ethidium bromide staining. Although the data from individual experiments are shown, the results were confirmed in multiple experiments in all cases.

Ornithine-δ-Aminotransferase Enzyme Activity

Ornithine-δ-aminotransferase activities from the ACs (stage 22) were measured with a radioisotopic assay. The ACs dissected from embryos injected with β-gal, X-OAT (X-OAT-WT, X-OATR180T, X-OATR180T/L402P, X-OATL402P, X-OATK292N) and X-OAT-Mo mRNAs were sonicated in 100 mM potassium phosphate (pH 8.0). Protein was determined by Lowry assay. The reaction mixture contained 0.5 μCi L-ornithine-C14 (Amersham Pharmacia Biotech, Inc.), 0.7 mM unlabeled ornithine, 0.14 μM pyridoxal phosphate, 0.1 M potassium phosphate (pH 8.0), and 0.7 mM α-ketoglutarate. The AC extracts were incubated with reaction mixture at room temperature (21°C) for 60 minutes, then 50 μL orthoamino-benzaldehyde solution was added to terminate the reaction.

**Figure 1.** Comparison of human and *Xenopus* OAT amino acid sequences. Arrows indicate locations of X-OAT mutations generated as described in the Methods section.
**RESULTS**

**Nucleotide and Amino Acid Sequences and Phylogenetic Analysis**

The OAT isolated from *Xenopus* oocytes in this study was designated X-OAT (GenBank AY005479) and has a 1520-bp open reading frame (ORF) encoding an OAT monomer of 439 residues (Fig. 1). Compared with human OAT, X-OAT showed 80% identity at the amino acid level. We also compared the sequence of X-OAT to that of OAT from mouse, rat, *C. elegans*, and *Drosophila*. The amino acid alignment of OAT is highly conserved in all species examined (data not shown).

**X-OAT Gene Spatio-Temporal Expression During Embryonic Development and Expression Patterns in Various Adult Tissues**

The gene expression pattern of X-OAT in various adult tissues of a male *Xenopus* was investigated by RT-PCR. We examined the following tissues: brain, liver, kidney, heart, testis, lung, muscle, stomach, small intestine, and large intestine, plus dorsal and ventral skin. Our results show that X-OAT is expressed in nearly all tissues and the results are consistent with the findings in mouse, rat, and human expression patterns, with some minor differences. We found an elevated X-OAT expression in the brain and testis of adult *Xenopus* (Fig. 2A). The temporal and spatial patterns of X-OAT expression were investigated by RT-PCR and whole-mount in situ hybridization. Reverse transcriptase PCR by semiquantitative analysis revealed that almost the same level of X-OAT was expressed from the oocyte to the tadpole stage (stage 40) (Fig. 2B). Whole-mount in situ hybridization revealed that X-OAT was ubiquitously expressed in all regions of the oocyte (Fig. 3A). In the early neural development stage, X-OAT initially localized in the dorsal side (Fig. 3B), especially in the neural plate. In the tadpole stage, X-OAT was predominantly expressed in the brain and spinal cord (Fig. 3C).

**Consequences of Perturbation of X-OAT Expression in Early Development**

To understand the role of X-OAT during embryonic development, we inhibited X-OAT expression by microinjecting 5 or 10 ng X-OAT-Mo into the animal pole of the two-cell stage embryos individually, then culturing the embryos in 30% MMR. The gastrulation defects were observed with 5 ng X-OAT-Mo–injected embryos at late gastrulation stage (stage 13). The 10 ng X-OAT-Mo–injected embryos died at early gastrulation stage (stage 10) (Figs. 4A–C). To assess the embryonic activity of X-OAT hyperexpression, we injected synthetic sense RNA encoding X-OAT into the animal pole of two-cell stage embryos (Fig. 5A) or into the dorsal marginal zone of four-cell stage embryos (Fig. 5B). Injection into either site elicited a similar phenotype of ventralizing malformations, with small heads, well-patterned ventral side, and short tails with the absence of apparent axial structures (56%, *n* = 50, injected into animal pole; 68%, *n* = 50, injected into dorsal marginal zone) (Figs. 5A, 5B).

**Overexpression of X-OAT Inhibits Neurogenesis**

The morphological changes induced by X-OAT led us to consider whether exogenous X-OAT affected neural development. After X-OAT mRNA (1 ng) was injected into the animal pole of the two-cell stage embryos, ACs were dissected at stages 8.5 to 9.0 and cultured in 67% Leibovitz’s L-15 medium containing activin (10 ng/mL) and retinoic acid (10⁻⁵ M) until they reached the equivalent of stage 22. Activin, a member of the TGF-β superfamily, can induce differentiation of almost all mesodermal tissue. Retinoic acid, a derivative of vitamin A, is a potent teratogen and affects the differentiation of many cells in vitro. In vertebrates, retinoic acid is apparently involved in the development of the central nervous system. Therefore, both activin A and retinoic acid are good candidates for neural- and mesoderm-inducing factors. As shown in Figure 6, ACs dissected from embryos injected...
with X-OAT-Mo or control β-gal developed a neural-like phenotype: slightly swollen with one side white and the other black (Figs. 6A, 6C). In contrast, X-OAT sense-injected ACs developed an epidermal-like phenotype, mostly round and evenly brownish (Fig. 6B). To advance our understanding of the role of the X-OAT gene in neurogenesis, an analysis of molecular markers was performed by RT-PCR on RNA extracted from explants of embryos. At the two-cell stage, 1 ng of mRNAs encoding X-OAT sense or 5 ng X-OAT-Mo was injected into each of the two blastomeres of Xenopus embryos. The ACs were dissected at stages 8.5 to 9.0 and cultured until the equivalent of stage 22. The results in X-OAT-expressing ACs showed that the pan-neural marker NCAM were decreased (Fig. 7A). In addition, anterior neural
marker Otx2\textsuperscript{44,45} and posterior neural marker HoxB9\textsuperscript{46} were also decreased or absent (Fig. 7B). These results were consistent with the phenotypic changes of ACs observed in Figure 6.

**Analysis of Functional Activity of the X-OAT Mutants**

We artificially generated four mutants, X-OAT\textsuperscript{R180T}, X-OAT\textsuperscript{R180T/L402P}, X-OAT\textsuperscript{L402P}, and X-OAT\textsuperscript{K292N}, because these mutations in humans result in GA (Fig. 8A). The mutants were confirmed by DNA sequencing. Messenger RNA was synthesized from wild-type (WT) and from each of the mutants. The mutants were translated at an efficiency similar to that for the WT X-OAT (Fig. 8B), suggesting that these mutations did not affect translatability. Thus, we used these mRNAs for the biological studies described below. To investigate the functional activity of the X-OAT during Xenopus embryonic development, we injected each of the mutants into the animal pole of the two-cell stage embryos. The injected embryos were allowed to develop until the tadpole stages, and the dorsoanterior index (DAI) was scored.\textsuperscript{47} As shown in Table 1, the embryos injected with X-OAT\textsubscript{WT}, X-OAT\textsubscript{R180T}, X-OAT\textsubscript{L402P}, or X-OAT\textsubscript{K292N} mRNA were malformed with DAI of 2.5 (68%, simple sizes: 34/50), 2.3 (74%, simple sizes: 37/50), 2.8 (62% simple sizes: 31/50), and 2.6 (72%, simple sizes: 36/50), respectively. In contrast, the X-OAT\textsubscript{R180T/L402P} mRNA-injected embryos, like the \beta-gal-injected control embryos, remained normal with DAI 5.0 for both (Fig. 9A). We also analyzed the neutralization-inhibiting activity of the X-OAT mutants in ACs. As shown in Figure 9B, the X-OAT\textsubscript{WT}, X-OAT\textsubscript{R180T} (M1), X-OAT\textsubscript{L402P} (M3), and X-OAT\textsubscript{K292N} (M4) mRNA-injected ACs developed an epidermal-like phenotype: basically round without a clear white/black delineation. On the other hand, the X-OAT\textsubscript{R180T/L402P} (M2) mRNA-injected ACs, like the \beta-gal-injected control ACs, developed a neural-like phenotype: slightly swollen with one side white and the other side black (cement gland) (Fig. 9B). To confirm these results, we examined the expression of the pan-neural marker NCAM using RT-PCR assay. The NCAM was expressed only in the ACs injected with mRNA containing both R180T and L402P mutations (Fig. 9C). These results suggest that the double mutation was crucial for mitigating the neutralization inhibitory activity of X-OAT.

**Enzyme Activity of OAT**

We sought insight into the mechanism of X-OAT as a neutralization inhibitor by correlating this inhibitory activity with the catalytic activity of the protein encoded by the various mutant X-OATs harvested from the ACs. We used a specific radioisotopic assay and performed the assays in triplicate. Because there was considerable dilution of the injected X-OAT mRNA by stage 22, the level of enzyme activity measured in the ACs injected with X-OAT\textsubscript{WT} was only 44% higher than that in the ACs injected with \beta-gal (125.3 + 2.0 and 86.6 + 22.0 pmol/h·mg, respectively). Nevertheless, it was readily apparent that the ACs injected with X-OAT\textsubscript{R180T/L402P} were similar to those in the ACs injected with \beta-gal. By contrast, the ACs injected with X-OAT\textsubscript{R180T}, X-OAT\textsubscript{L402P}, and X-OAT\textsubscript{K292N} had enzyme activities only slightly lower than that of ACs injected with X-OAT\textsubscript{WT}, but ACs injected with X-OAT-Mo had lower than that ACs injected with \beta-gal (Fig. 10; Table 2). The pattern showed that the enzyme activities in the ACs correlated with the ability to inhibit the neutralization phenotype and endogenous enzyme activities are necessary for normal embryo development.

**DISCUSSION**

The Xenopus embryo developmental model provided an opportunity to evaluate the biological function of OAT in this context. *Xenopus* OAT was found to be highly homologous to OAT of human, mouse, rat, *C. elegans*, and *Drosophila*. Among these species, OAT was highly conserved at the amino acid level. The X-OAT is expressed in almost all Xenopus adult tissues and at every stage of early embryonic development. In situ hybridization studies showed that X-OAT expression was especially prominent in the neural cord at the tailbud stage (Fig. 3C). In loss-of-function studies, embryonic development arrested at the gastrulation stage, suggesting that maternal OAT is essential for *Xenopus* embryonic development. In gain-of-function studies, overexpression of X-OAT ventralized *Xenopus* embryos. To corroborate these morphologic findings, we examined neural development induced by retinoic acid and activin in the early stages of embryonic development by analyzing relevant molecular markers in explants. The pan-
neural marker NCAM, the anterior neural marker Otx2, and the posterior neural marker HoxB9 were all markedly decreased by exogenous X-OAT expression. These results are consistent with the ventralizing phenotype and suggest that OAT inhibits neurogenesis in ectodermal explants induced by retinoic acid and activin. It is of considerable interest that X-OAT may be involved in neural development. Although humans without OAT activity have no apparent developmental abnormality, they develop GA, which is associated with degeneration of the neuroretina and visual defects. In these individuals, neuromuscular and central nervous system abnormalities also have been described.48,49 Thus, the abnormalities in *Xenopus* embryonic
development with either loss- or gain-of-function in OAT may represent a more severe expression not found in mammalian species with overlapping metabolic pathways. There are examples of genes and their homologues, respectively, implicated in late-onset human disease, which play a critical developmental role in lower organisms. 29,30 Although the genetic or metabolic mechanisms of OAT on development remain unknown, the findings of OAT on *Xenopus* embryonic development may serve as a model for the neurological changes in GA. The more than 60 OAT mutations resulting in

**FIGURE 8.** Translation of WT and mutated X-OATs. The introduced mutations are shown with their designations (A). The M2 has both the M1 and M3 mutations. M1: X-OATR180T; M2: X-OATR180T/L402P; M3: X-OATL402P; M4: X-OATK292N; WT: X-OATWT. Synthetic WT and mutated X-OAT mRNA were translated in a TNT-coupled reticulocyte lysate system (Promega) with [35S] methionine. Products were analyzed by autoradiography on an SDS Tris-HCl gel. A 32 kDa band appeared at similar intensity in all the lanes loaded with the translation products (B) and the densitometric analysis (C).

**TABLE 1.** Comparison of Dorsalizing Activity of X-OAT and Its Mutants

<table>
<thead>
<tr>
<th>mRNA Injected</th>
<th>DAI Score</th>
<th>%</th>
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<tbody>
<tr>
<td>X-OATWT</td>
<td>2.5</td>
<td>68 (n: 34/50, d: 8/50)</td>
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</tr>
<tr>
<td>X-OATR180T</td>
<td>2.3</td>
<td>74 (n: 37/50, d: 5/50)</td>
<td></td>
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<tr>
<td>X-OAT R180T/L402P</td>
<td>5.0</td>
<td>92 (n: 46/50, d: 4/50)</td>
<td></td>
<td></td>
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<tr>
<td>X-OATL402P</td>
<td>2.8</td>
<td>62 (n: 31/50, d: 9/50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-OATK292N</td>
<td>2.6</td>
<td>72 (n: 36/50, d: 7/50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-gal</td>
<td>5.0</td>
<td>96 (n: 48/50, d: 2/50)</td>
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Note: 1 ng mRNA encoding X-OAT or each of the X-OAT mutants was injected into the animal pole at two-cell stage embryos. The embryos were allowed to develop to tailbud stage and were scored for DAI. A normal embryo was scored 5, a completely ventralized embryo was scored 0, and a completely dorsalized embryo was scored 10. Other intermediate phenotypes were scored between 0 and 10, according to their degree of ventralization or dorsalization. n, the number of embryos; d, embryos that died.

**FIGURE 9.** Phenotype observations and RT-PCR analysis of ACs expressing WT and mutated X-OAT; β-gal or 1 ng mRNA encoding WT or each of the X-OAT mutants was injected into the animal pole at the two-cell stage. (A) Embryos were grown to tadpole stage for photography. (a) β-gal (normal: 48/50, died: 2/50), (b) X-OATWT (normal: 8/50, ventralization: 5/50, died: 5/50), (c) X-OATR180T (normal: 8/50, ventralization: 37/50, died: 5/50), (d) X-OATL402P (M2) (normal: 46/50, died: 4/50), (e) X-OATL402P (M3) (normal: 10/50, ventralization: 31/50, died: 9/50), or (f) X-OATK292N (M4) (normal: 7/50, ventralization: 36/50, died: 7/50). (B) The ACs were dissected at stage 8.5 to 9.0 and cultured to equivalent of stage 22. The ACs were harvested for photography: (a) β-gal (neurolization 8/8), (b) X-OATWT (normal: 8/8), (c) X-OATR180T (M1) (normal: 8/8), (d) X-OATL402P (M2) (neurolization: 7/8, died: 1/8), (e) X-OATL402P (M3) (normal: 8/8) or (f) X-OATK292N (M4) (normal: 7/8, died: 1/8), or (C) for RT-PCR analysis of NCAM expression with EF-1α as control.
GA in humans provided a starting point for our analysis of the functional relationship of X-OAT in the *Xenopus* embryonic model.\(^{17,35}\) We constructed X-OAT mutations analogous to the mutations in humans that cause the GA phenotype and loss of catalytic activity. However, in comparison with X-OATWT, single mutations resulting in X-OATR180T, X-OATL402P, and X-OATK292N did not mitigate the inhibition of neuralization activity (i.e., they inhibited neuralization the same as WT). However, when both R180T and L402P mutations were present in X-OATR180T/L402P, the inhibitory effect on neuralization was abrogated (Figs. 9A–C). Of the aforementioned mutations used, all were situated in the conserved region of the OAT gene;\(^{12}\) in cells from GA patients, these mutated genes yielded normal amounts and size of OAT mRNA. The R180T and L402P mutant alleles from Finnish patients with GA were described by Mitchell et al.\(^{35}\) In studies of OAT from these patients, these investigators found that L402P apparently destabilizes the structure of the enzyme, leading to degradation and decreased amounts of immunoreactive protein. On the other hand, R180T results in an inactive enzyme without reducing the amount of OAT antigen.\(^{11,35}\)

Because of the divergence between humans and *Xenopus* in the phenotypic consequence of these mutations, it was important to characterize the effect of these mutations on enzyme activity in *Xenopus*. Using a specific radioisotopic assay, we determined OAT activity in extracts from ACs (stage 22) injected with various X-OAT mRNAs at the two-cell stage. As shown in Figure 9, embryos injected with X-OATWT increased their OAT activity approximately 45\% over controls injected with β-gal. The dilutional loss of mRNA up to stage 22 probably limited the increase to this modest level, and it is likely that the relative contribution of exogenous X-OAT was considerably greater at earlier stages. Strikingly, the effect of the mutations on enzyme activity, or rather the lack of effect of certain mutations, paralleled the failure of these mutations to mitigate the profound alterations in developmental phenotype. X-OATR180T, X-OATL402P, and X-OATK292N had little effect on enzyme activity. However, the double mutant X-OATR180T/L402P appeared to have completely lost enzyme activity and was no different from control explants injected with β-gal. In the study by Brody et al.\(^{17}\) using GA fibroblasts, and also in the study using lymphocytes from Finnish GA patients,\(^{35}\) OAT mutants R180T and R154L have no measurable enzyme function but have OAT antigen. In humans, the loss of activity due to these point mutations is likely due to loss of protein conformation and stability.\(^{50,51}\) It may be that this loss of conformation is temperature-sensitive (i.e., loss of conformation and activity occurs at 57°C for human OAT but not for either embryonic development at 14–18°C or embryonic activity at 24°C). The double mutation, on the other hand, which resulted in loss of both measured enzyme activity and the ability to modulate phenotype, most likely yielded a protein without active conformation at the relevant temperatures. This explanation, however, does not account for the retention of activity in X-OATK292N because lysine 292 is the binding site for pyridoxal phosphate. We will undertake additional studies to distinguish these physical and catalytic properties of mutant OAT at various temperatures.

Nevertheless, the enzymatic activity residing in the various X-OAT mutants followed the same pattern as their ability to ventralize and inhibit neuralization (Figs. 9A–C) and suggests that the modulation of the developmental phenotype resides in the metabolic activity of X-OAT. This enzyme catalyzes reactions at the central juncture in the pathways of amino acid intermediary metabolism\(^{11}\) and produces glutamic-γ-semialdehyde, which is in tautomeric equilibrium with P5C. The

**Table 2.** Enzyme Activities of X-OAT in ACs Expressed β-gal, X-OAT, and X-OAT Mutants

<table>
<thead>
<tr>
<th>Gene Expression</th>
<th>Net DPM</th>
<th>X-OAT Activity, pmol/h·ug Protein</th>
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<tr>
<td>β-gal</td>
<td>2933.24 ± 54.67</td>
<td>76.42 ± 0.88</td>
</tr>
<tr>
<td>X-OAT WT</td>
<td>5255.68 ± 116.79*</td>
<td>150.66 ± 3.64*</td>
</tr>
<tr>
<td>X-OAT M1</td>
<td>4062.04 ± 57.14*</td>
<td>101.7 ± 4.79†</td>
</tr>
<tr>
<td>X-OAT M2</td>
<td>2974.61 ± 54.13*</td>
<td>84.68 ± 1.14†</td>
</tr>
<tr>
<td>X-OAT M3</td>
<td>5808.28 ± 85.09*</td>
<td>109.58 ± 1.42*</td>
</tr>
<tr>
<td>X-OAT M4</td>
<td>6577.63 ± 35.20*</td>
<td>110.58 ± 0.89*</td>
</tr>
<tr>
<td>X-OAT M5</td>
<td>2741.41 ± 151.57</td>
<td>46.16 ± 2.43*</td>
</tr>
</tbody>
</table>

Data are mean ± SD. The assays were performed in triplicate. *P < 0.001, compared with β-gal. †P < 0.01, compared with β-gal.

**Figure 10.** Differential X-OAT enzyme activities in ACs expressing β-gal, X-OAT-Mo, X-OATWT, and mutants. Messenger RNA (1 ng) from X-OATWT or mutants was injected into the animal pole at the two-cell stage. The ACs were dissected at stage 8.5 to 9.0 and cultured to equivalent of stage 22.

The ACs were harvested in PBS buffer (16 ACs per group) for protein concentration measurement and X-OAT enzyme activity assay (see Methods). All groups were assayed in triplicate. P values are compared between β-gal and each OAT by Student’s t-tests. *P < 0.001, compared with β-gal; **P < 0.01, compared with β-gal; †P < 0.01, compared with β-gal.
OAT Inhibits Embryonic Neurogenesis in Xenopus

Recent studies have shown that the metabolic cycling of proline and P5C by proline oxidase and P5C reductase, respectively, may play important roles in several disease processes. The expression of proline oxidase responds to p53,52 PPAR-activating ligands,53 and rapamycin,54 representing respective signaling due to genotoxic, inflammatory, and nutrient stress.55,56 Doimo et al.57 found that 5-aminomimidazole-4-carboxamidine ribonucleoside markedly stimulates OAT expression, thus representing a possible treatment for a subset of GA patients with hypomorphic alleles. Overexpression of proline oxidase (POX) results not only in cell cycle blockade and apoptosis,58 but also produces ATP for cell maintenance.53 Mutations resulting in loss of POX activity increase the risk for schizophrenia,7,59 and the disappearance of POX during neurogenesis suggests that it functions as a cancer-suppressor protein.58 Just how these demonstrated mechanisms in carcinogenesis relate to the observed developmental abnormalities remain unclear. The absence of OAT in humans does not have a clinical phenotype until later in life. We supposed that is because the genes of the proline-arginine-glutamate pathway are epistatic. Neurodevelopmental abnormalities are associated with mutations in P5C synthase, which converts glutamate to P5C.9,10 Investigators have recently reported that neurological abnormalities are also associated with mutations in P5C reductase, which converts P5C back to proline, and morpholino knockdown of Pycr1 in both Xenopus and zebrafish, cause developmental defects.60 These more recent findings further support the importance of this pathway in neurodevelopment. Whatever the mechanism, our findings provide robust, direct evidence that perturbations of P5C metabolism of ornithine and arginine from proline and P5C, which is essential for proliferation and differentiation.64,65 Al-

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