Activity and protein localization of multiple glutamate transporters in gestation day 14 vs. day 20 rat placenta

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Activity and protein localization of multiple glutamate transporters in gestation day 14 vs. day 20 rat placenta. Am. J. Physiol. 274 (Cell Physiol. 43): C603–C614, 1998.—Concentrative absorption of glutamate by the developing placenta is critical for proper fetal development. The expression of GLAST1, GLT1, and EAAC1 was greater throughout the day 14 vs. day 20 rat choriovitelline placenta. Steady-state mRNA levels were greater at day 14 vs. day 20 for all transporters. Immunohistochemistry determined that the expression of GLAST1, GLT1, and EAAC1 was greater throughout the day 20 placenta and was asymmetric with respect to cellular localization. EAAT4 protein was not detected. System XAG, known to be capable of D-aspartate-inhibitable and Na+-coupled glutamate transport (system XAG), was evaluated in day 14 vs. day 20 rat choriovitelline placenta. Steady-state mRNA levels were greater at day 20 for all transporters. Immunohistochemistry determined that the expression of GLAST1, GLT1, and EAAC1 was greater throughout the day 20 placenta and was asymmetric with respect to cellular localization. EAAT4 protein was not detected. System XAG activity was responsible for most of the Na+-dependent glutamate uptake and was greater in day 20 than in day 14 apical and basal membrane subdomains of the labyrinth syncytiotrophoblast. Greater quantities of EAAC1 and GLAST1 protein were identified on day 20, and quantities were greater in basal than in apical membranes. GLT1 expression, unchanged in apical membranes, was decreased in basal membranes. These data correlate transporter mRNA and protein content with transport activity and demonstrate an increasing capacity for glutamate absorption by the developing placenta.

Developmental regulation; spongiotrophoblast tissue; labyrinth tissue; system XAG; anionic amino acid transport

Three primary functions of the placenta are mediation of the passage of nutrients from the maternal to the fetal circulation, facilitation of endocrine communication between maternal and fetal units (36), and absorption of end products of fetal metabolism, thereby establishing metabolic cycles between the fetus and placenta (13). In the rat, two placentas develop during gestation (21 days) for each fetus, the choriovitelline (visceral yolk sac) and the choriovitelline. By day 14 (the start of the last trimester), the choriovitelline placenta has regressed (36), leaving the choriovitelline placenta as the primary unit of maternal-fetal substrate exchange during the last trimester of pregnancy. The choriovitelline placenta is composed of trophoblasts that arise from the trophectoderm. Mural trophoderm cells invade the uterine stroma, inducing formation of maternal decidual tissue from the stromal cells (36). Other cells of the trophoderm differentiate into giant trophoblast, spongiotrophoblast, syncytiotrophoblast, and vacuolated glycogen cells and are organized into the junctional and labyrinth zones. From dam to fetus, these morphological regions are organized in the following order: maternal decidual tissue, the junctional zone giant cell layer, junctional zone spongiotrophoblasts and vacuolated glycogen cells, and labyrinth syncytiotrophoblasts, the predominant cell type of the labyrinth zone.

In the rat labyrinth tissue, trophoblast cells are arranged in three distinct layers with respect to blood supply. Layer I is composed of fenestrated syncytiotrophoblasts that face maternal blood. Because of the fenestration, these cells are not thought to represent a barrier to nutrient transfer. Layer II syncytiotrophoblasts form a contiguous epithelium, with their apical membranes facing the maternal blood. This apical domain is thought to be responsible for active extraction of nutrients from the maternal bloodstream. Layer III syncytiotrophoblasts also form a contiguous syncytia, and the basal membrane surface faces the fetal mesenchyme and vasculature (4, 23). The layer III basal membrane is responsible for both the release of extracted maternal nutrients into the fetal circulation and the active extraction of nutrients from the fetal circulation into the placental tissue. The labyrinth zone trilaminar syncytia increases in tissue mass until day 20 of gestation (4) and represents the structural and functional barrier to substrate passage between maternal and fetal circulations.

In contrast to most amino acids, for normal growth the fetus does not require a continuous supply of glutamate directly from the maternal circulation. Instead, the fetus is dependent on placental absorption of maternal glutamine and removal of selenoperoxidase glutamate to establish the placental-fetal glutamine/glutamate cycle, which is essential for the proper growth and development of both the placenta and fetus (25, 27, 39). Along with glutamine absorbed by the placenta from the maternal circulation, glutamine synthesized and released by the placenta is absorbed by the fetal liver and deaminated to glutamate as a source of nitrogen (25). Glutamate is then released back into the fetal circulation and actively extracted by the placenta. Much of the returned glutamate is oxidized for metabolic fuel and is also used to generate NADPH for placental fatty acid and steroid synthesis, thus sparing glucose for use by the fetus (25, 39). Additionally, active placental absorption of circulating fetal glutamate may serve to protect the fetus from potentially neurotoxic levels of glutamate (24). Conse-
near-term (tamate absorption during the final trimester of pregnancy) was to characterize the expression and localization of the proteins responsible for glutamate uptake in the placenta. The concentration of glutamate measured in placental tissue (in mM) exceeds that measured in maternal or fetal blood (in μM) (5). Na$^+$-dependent, K$^+$-coupled, D-aspartate-inhibitable glutamate transport activity [system X$_{AG}$ (9)] has been identified in both the apical (14) and basal (24) membranes of labyrinth zone syncytiotrophoblasts from human placenta and is responsible for concentrating glutamate in the placenta. Four cDNAs for proteins capable of mediating high-affinity Na$^+$-coupled anionic amino acid transport (GLAST1, GLUT1, EAAC1, and EAAT4) have been cloned (7, 26, 30, 37). The EAAT4 transporter appears to have unique properties relative to the other three in that its expression in oocytes induces a glutamate/aspartate-dependent chloride channel activity (7). The detection in human placental tissue of a single mRNA species for GLAST1 (17, 26), GLUT1 (26, 35), EAAC1 (26), and EAAT4 (7) suggests that if translated and transferred to the plasma membrane, these proteins would contribute to the observed system X$_{AG}$ activity. In the rat, we have identified Na$^+$-dependent glutamate transport in near-term (day 20) placental tissue (21).

The proteins responsible for increased placental glutamate absorption during the final trimester of pregnancy have not been described. The purpose of this research was to characterize the expression and localization to specific cell types of glutamate transporter mRNA, protein, and activity at two specific time points of gestation, day 14 and day 20. These days were chosen because they represent the start and end of the last trimester of gestation in rat placenta, the period of greatest placental and fetal growth in rats and humans.

**MATERIALS AND METHODS**

Reagents and materials. [3H]glutamate was purchased from DuPont NEN. 32P-labeled isotopes and all other reagents for immunoblot analyses were purchased from Amer sham (Arlington Heights, IL) unless otherwise noted. Nitrocellulose filters (0.45 mm) were used for transport assays (Millipore, Bedford, MA). Preliminary experiments were performed using antibodies generously supplied by Dr. Baruch Kanner (GLT1) and Dr. Wilhelm Stoffel (GLAST1), but all of the data shown were generated with antibodies prepared in our laboratories. All other reagents and chemicals were obtained from either Sigma (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), or Gibco BRL (Gaithersburg, MD). Timed pregnant Sprague-Dawley rat dams were obtained from Zivic-Miller (Zelienople, PA).

Collection of chorionallantoic placenta. These studies were approved by the Institutional Animal Care and Use Committee of the University of Florida. Animals were housed in a temperature-controlled room with 12:12-h light-dark cycles and had ad libitum access to water and rat chow. On the indicated day of gestation, the dams were anesthetized with pentobarbital sodium and the fetal-placental units were removed. Whole placenta were used for RNA isolation and immunohistochemical analyses after removal of extraembryonic membranes, fetal membranes, and fetuses. For membrane vesicle preparations, the labyrinth zone was dissected from the remainder of the placenta, which was discarded.

Isolation of RNA. For any given RNA isolation, 7–10 placentas/litter were snap-frozen and then ground to a powder under liquid nitrogen. Total RNA was extracted from the powder (31) after homogenization with five strokes in a glass tube with a motor-driven Teflon pestle. Poly(A)$^+$-selected mRNA was isolated from 500 μg total RNA using the Poly A Tract System (Promega, Madison, WI).

Glutamate transporter protein reverse transcription-polymerase chain reaction. To obtain cDNA probes for rat GLAST1 and EAAT4, reverse transcription-polymerase chain reaction (RT-PCR) of rat brain and placental mRNA was performed. PCR primers were chosen, based on published sequences of the glutamate transporters rat GLAST1 (5’TGGATTTGCTCC-GACCG-3’, 5’-GTTGCATACACATATCAACC-GG-3’) and human EAAT4 (5’-TGCCCCATATCAGCTCATTACC3C-3’, 5’-TGCACGCTATAAGGGC-3’). Rat brain cDNA was used as a positive control to optimize the PCR conditions. Thermal cycling using Taq DNA polymerase included 25 cycles at 94°C for 1 min, 60°C for 1.5 min, and 72°C for 2 min. All reactions contained 4 mM MgCl$_2$.

RT-PCR products were electrophoresed on a 1.2% agarose gel, capillary transferred to 0.45-μm nylon membrane as described in Northern analysis for Northern blots, and hybridized with 32P-labeled antisense oligomer probes (GLAST1, 5’TGGTACCGTGATAATGTGGTATGC-3’, EAAT4, 5’-GCTTCAAACAGTTCAAGACGCAGTACAGC-3’) that were internal to the original RT-PCR digonucleotides and that were radiolabeled by a 5’ terminal exchange reaction (2). The blots were washed for 2 min in 5X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) at 55°C and then twice for 1 min in 1X SSC at 55°C and were exposed to autoradiograph film. The specificity of both oligomer probes for their cDNA was determined by demonstrating the lack of cross-hybridization of the radiolabeled oligomers with RT-PCR cDNAs for the other members of the glutamate transporter family (data not shown).

Cloning of partial-length rat GLAST1 and EAAT4 cDNA. After cloning into pCR2.1 (Invitrogen, San Diego, CA) of the GLAST1 and EAAT4 rat brain RT-PCR products, we determined the identity of the cDNAs by multiple-restriction analyses (GLAST1) or sequenced (EAAT4) by dideoxy-mediated chain-termination sequencing by a core facility at the University of Florida.1 Sequence alignments and analyses were performed using PCGene software (Intelligenetics, Mountain View, CA).

Northern analysis. Five micrograms of poly(A)$^+$-selected mRNA were loaded per lane and subjected to 1% agarose gel electrophoresis in the presence of 0.02 M formaldehyde. The RNA was transferred by downward capillary action (21) to 0.45-μm nylon membranes; covalently cross-linked by ultraviolet light; hybridized with individual 32P-labeled cDNA probes in solutions that contained 1% bovine serum albumin, 7% sodium dodecyl sulfate (SDS), 0.5 M Na$_2$HPO$_4$ (pH 7.2), and 1 mM EDTA for 15 to 18 h at 67°C; and then washed for 4×15 min in a 67°C solution that contained 40 mM Na$_2$HPO$_4$ (pH 7.2), 0.1% SDS, and 1 mM EDTA. The presence of GLAST1 and EAAT4 mRNA was tested using the partial-length rat cDNAs described above. Full-length rat EAAC1 (2.2 kb) (40) and GLUT1 (2.1 kb) (30) cDNAs were used to

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1 The nucleotide sequence reported in this paper resides in the GenBank/EMBL data bank with accession number U80915.
prepare probes to determine the presence of transporter mRNAs. A cathepsin B cDNA probe (Dr. Harry Nick, Univ. of Florida) was used to detect the corresponding mRNA as a loading control for all blots. Cathepsin B mRNA does not increase in rat placenta from day 14 to day 20 (29). The radiolabeled cDNA probes were prepared by random-priming extension using [32P]dCTP and a kit from GIBCO BRL. For each transporter, two blots containing poly(A) <sup>+</sup>RNA isolated from day 14 and day 20 placentas of two different litters were probed for mRNA.

**Membrane vesicle isolation.** The placentas from 8 to 10 dams were pooled, and isolation, characterization, and protein content of both apical and basal plasma membrane vesicles were performed as previously described (20, 21). Marker enzyme profiles were similar at days 14 and 20 (20, 28).

**Vesicle transport assay.** A nitrocellulose filter assay (21) was used to measure [3H]glutamate uptake by isolated membrane vesicles. Vesicles were preloaded with 100 mM KCl by a freeze-thaw cycle, followed by incubation at 4°C for 30 min. Uptake was performed at 37°C in uptake buffer [10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-KOH, pH 7.5, 10 mM MgCl<sub>2</sub>, and 0.2 mM CaCl<sub>2</sub>] containing either 125 mM NaCl or 100 mM KCl. All uptake buffers were adjusted to isosmolarity (310 mosM) with sucrose. [3H]glutamate was added in tracer amounts (25 µCi/ml) to unlabeled substrate to achieve a final concentration of 1 µM except when otherwise noted. When indicated, 500 µM of unlabeled D-aspartate was added to the uptake media to confirm the presence of system X<sub>AG</sub> activity (16, 37). To quantify the amount of nonspecific [3H]glutamate binding in each uptake buffer, 3 ml of 4°C stop solution [100 mM NaCl or KCl, 10 mM HEPES-tris(hydroxymethyl)aminomethane (HEPES-Tris), pH 7.4, and 100 mM sucrose] were added to [3H]glutamate in the uptake mixture before being mixed with the membranes. These “blank” values were subtracted from each experimental observation. For both the actual assays and these blanks, the membranes were then filtered, and the amount of [3H]glutamate was quantified by liquid scintillation counting.

The Na<sup>+</sup>-dependent uptake velocities were calculated as the difference between uptake of glutamate in the presence of NaCl and KCl and are represented as the means ± SE of at least three uptake assays (pmol·mg protein<sup>-1</sup>·20 s<sup>-1</sup>). System X<sub>AG</sub> activity was calculated as the difference between total Na<sup>+</sup>-dependent uptake of 1 µM glutamate and Na<sup>+</sup>-dependent uptake in the presence of 500 µM D-aspartate.

Generation and characterization of rat EAAC1 antibody. The sequence encoding the COOH-terminal 120 amino acids of rat EAAC1 was cloned into the pMALc2 prokaryotic expression vector, as described in MATERIALS AND METHODS. The protein was expressed in E. coli and purified by amylose resin chromatography. The purified protein was used to raise antibodies in rabbits, and the resulting polyclonal antibodies were used to detect EAAC1 protein in rat brain and placenta by immunoblotting.
expression vector according to the manufacturer’s directions (New England Biolabs, Beverly, MA) to produce a maltose binding protein (MBP)/EAAC1 fusion protein. After expression, amplification, and purification (2), the MBP/EAAC1 fusion protein was concentrated and combined with Freund’s adjuvant (Sigma) for polyclonal antibody production by immunization of rabbits at CoCalico Biological (Reamstown, PA). Immunoglobulin G (IgG) was purified by caprylic acid and ammonium sulfate precipitations (11) from preimmune and immune sera before use in immunoblot analysis.

To demonstrate the specificity of the MBP/EAAC1 polyclonal antibody for rat EAAC1 glutamate transporter protein, the amount of EAAC1 was assayed in human epithelial kidney (HEK) 293c18 cells (Invitrogen) that were transfected with vector only (pDR2) or with vector containing EAAC1 cDNA (pDR2/EAAC1), as described in MATERIALS AND METHODS (left). Specificity of immunoreactive response was tested by preabsorption of polyclonal EAAC1 antibody with 20 µg/ml of antigen maltose binding protein (MBP)/EAAC1 fusion protein (middle) or MBP alone (right) before immunoblotting.

Immunoblot analysis. Placental day 14 and day 20 membrane vesicle proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis (19) and then electrotransferred to a 0.45-µm nitrocellulose membrane (Schleicher & Schuell, Keene, NH). For the detection of EAAC1 protein, blots were probed with 43 ng IgG/ml of the EAAC1 polyclonal antibody or preimmune sera in blocking solution (1% nonfat dry milk and 2% casein hydrolysate in 10 mM Tris·HCl, pH 7.5, and 300 mM NaCl) for 1 h at room temperature with agitation. Horseradish peroxidase-conjugated protein A was used to detect immunoreactive bands by visualization with a chemiluminescence kit (Amersham). For the detection of GLAST1, EAAT4, and GLT1 proteins, blots were probed with rat GLAST1 (320 ng IgG/ml), rat GLT1 (68 ng IgG/ml), or human EAAT4 (540 ng IgG/ml) antibodies as described previously (10, 33).

Immunohistochemical analysis. Immunohistochemical analyses for glutamate transporters were performed using modifications to the protocol of Campbell-Thompson and McGuigan (3). Within one preparation, immunohistochemis-

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**Fig. 3.** Specificity of a polyclonal antibody for rat EAAC1 glutamate transporter protein. A plasma membrane-enriched fraction (30 µg lane) was subjected to electrophoresis and then immunoblotted for presence of EAAC1 protein from human epithelial kidney 293c18 cells that were transfected with vector only (pDR2) or with vector containing EAAC1 cDNA (pDR2/EAAC1), as described in MATERIALS AND METHODS (left). Specificity of immunoreactive response was tested by preabsorption of polyclonal EAAC1 antibody with 20 µg/ml of antigen maltose binding protein (MBP)/EAAC1 fusion protein (middle) or MBP alone (right) before immunoblotting.

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**Fig. 4.** Northern blot analysis of mRNA in gestation day 14 and day 20 rat placenta encoding glutamate transporter proteins. Poly(A)⁺ placental RNA (5 µg lane) was analyzed for glutamate transporter mRNAs by Northern analysis. Blots were separately hybridized with [³²P]cDNA probes [0.9–1.2 × 10⁷ counts·min⁻¹·cpm⁻¹] specific for each transporter. Subsequently, each of the blots was hybridized with [³²P]-labeled cathepsin B (CB) cDNA probe (1.2–1.4 × 10⁵ cpymol) to test for equal loading of mRNA. For EAAC1, GLAST1, and GLT1, autoradiographs were exposed for 1 day at room temperature, 5 days at −70°C, and 3 days at −20°C, respectively. Because day 14 placental mRNAs (14) for EAAC1, GLAST1, and GLT1 were not visible at same exposure times as their day 20 counterparts, corresponding autoradiographs shown for these mRNAs were the result of exposures at −70°C for 15 days, 15 days, and 5 days, respectively. For EAAT4, both day 14 and day 20 mRNAs are shown on same autoradiograph, which was exposed for 15 days at −70°C. Likewise, cathepsin B mRNAs are shown for same autoradiographs, each exposed for 1–2 h at room temperature. All autoradiographs are representative of independent experiments using poly(A)⁺ RNA isolated from 2 separate litters each for day 14 and day 20 placentas.
except that before incubation with tissue sections, the antibodies were incubated overnight at 4°C in blocking solution with 24 mM MBP/EAAC1 fusion protein for the EAAC1 antibody or 5 mM of the appropriate antigen peptide for the GLAST1, EAAT4, or GLT1 antibodies.

Statistical analysis. Differences between mean vesicle uptake values and densitometric values for protein quantities were determined utilizing Student’s two-tailed t-test or Z scores.

RESULTS

Cloning of partial-length rat GLAST1 and EAAT4 cDNA. A single RT-PCR product was identified for GLAST1 (658 base pairs (bp)) and EAAT4 (699 bp) from both brain and placental mRNA (data not shown). The identity of the GLAST1 cDNA (658 bp) was confirmed by multiple restriction analyses at known sites of the rat sequence (37). The rat EAAT4 product was sequenced (Fig. 1) and showed 90.4% nucleic acid identity as well as 93.1% identity and 97.0% predicted similarity of amino acid sequence with the human EAAT4 protein sequence (7). There are no previously published reports of the rat EAAT4 sequence, but a full-length clone for rat EAAT4 has been isolated independently (Rothstein, unpublished observations) that contains a

![Graph representing the ratio of glutamate transporter protein mRNA content in gestation day 14 vs. day 20 rat placentas. Bars represent proportional increase in amount of day 20-to-day 14 mRNA detected by Northern analysis, as described in Fig. 4, and are mean ratios of all detected mRNA transcripts of 2 separate experiments (EAAC1, 20.0 and 16.9; GLT1, 12.2 and 8.38; GLAST1, 5.22 and 6.76; EAAT4, 4.99 and 2.32). Day 20-to-day 14 ratios were determined by quantifying amount of hybridized mRNA from day 14 and day 20 placenta detected on same autoradiographs. The mRNA hybridized was quantified using an LKB laser scanning densitometer and corrected for loading variations by normalizing to the cathepsin B signal (21).

Control experiments were performed to ensure that absorbance was in linear range of film and densitometer.

Antibody treatment and control experiments were performed on the same slide, using two serial sections of placenta. Control experiments to test for nonspecific antibody reactions were performed as above except that either the primary or secondary antibody was omitted. Control experiments to ensure specific staining for GLUT1, GLAST1, EAAC1, and EAAT4 proteins were also performed as described above except that before incubation with tissue sections, the antibodies were incubated overnight at 4°C in blocking solution with 24 mM MBP/EAAC1 fusion protein for the EAAC1 antibody or 5 mM of the appropriate antigen peptide for the GLAST1, EAAT4, or GLUT1 antibodies.

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Fig. 5. Ratio of glutamate transporter protein mRNA content in gestation day 14 vs. day 20 rat placentas. Bars represent proportional increase in amount of day 20-to-day 14 mRNA detected by Northern analysis, as described in Fig. 4, and are mean ratios of all detected mRNA transcripts of 2 separate experiments (EAAC1, 20.0 and 16.9; GLT1, 12.2 and 8.38; GLAST1, 5.22 and 6.76; EAAT4, 4.99 and 2.32). Day 20-to-day 14 ratios were determined by quantifying amount of hybridized mRNA from day 14 and day 20 placenta detected on same autoradiographs. The mRNA hybridized was quantified using an LKB laser scanning densitometer and corrected for loading variations by normalizing to the cathepsin B signal (21).

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Demonstration of human EAAT4 polyclonal antibody recognition of rat brain EAAT4. A polyclonal anti-EAAT4 antibody has been generated (8) against the COOH-terminal last 30 amino acids of human EAAT4. This sequence differs from rat by only one amino acid and detects a single 73-kDa rat protein, a size similar to the predicted molecular size of human EAAT4 (7). The immunoreactivity was completely inhibited when the antibody was preabsorbed with the polypeptide antigen (Fig. 2).

Characterization of polyclonal antibody against rat EAAC1 transporter protein. The EAAC1 polyclonal antibody recognized a predominate band of ~68 kDa and a minor band of ~57 kDa in a plasma membrane-enriched fraction from HEK 293c18 cells that had been transfected with rat EAAC1 cDNA (Fig. 3). These bands were completely inhibited when the antibody was preabsorbed with the MBP/EAAC1 fusion protein but not with MBP alone. The larger band is approximately the same size as that reported for rat brain (69 kDa) (33), whereas the smaller band is similar to the predicted core size of the rat EAAC1 protein (56.8 kDa) (40).

Glutamate transporter mRNA size and quantity. Northern blot analysis was used to quantify the steady-state levels of transporter mRNA of day 14 or day 20 placenta (Fig. 4). For GLAST1, mRNA species of 4.1

Fig. 7. Immunoblot analysis of gestation day 14 and day 20 rat placental plasma membranes for specific glutamate transporters. Detection of glutamate transporter proteins on day 14 and day 20 of gestation in apical and basal plasma membranes was performed by immunoblotting as described in MATERIALS AND METHODS. Blots shown are representative of independent experiments using protein samples from 3 different membrane isolations. Width of detectable protein bands are different because presence of EAAC1 was probed using blots containing 5-mm-wide lanes, whereas GLAST1 and GLT1 were probed using a miniblot apparatus that has 1.2-mm-wide lanes. In most cases, exposures shown are more intense, for photographic clarity, than those used for the quantitative densitometry summarized in Fig. 8.

Fig. 8. Quantitative analysis of immunoblot data for glutamate transporters in placental apical and basal plasma membranes. Densitometric analysis of EAAC1, GLAST1, and GLT1 transporter proteins detected in gestation day 14 (open bars) and day 20 (solid bars) placentas from 3 independent experiments, 1 of which is represented in Fig. 7, was performed on all immunoreactive bands, using an LKB laser densitometer. Control experiments were performed to ensure that absorbance values were in linear range of densitometer and film. Data were normalized to day 14 apical values of each protein and represent means ± SE (n = 3), in arbitrary units, of protein content in placental apical and basal membrane vesicles. *P < 0.02 and **P < 0.01 vs. EAAC1 in day 14 apical membranes; †P < 0.04 vs. EAAC1 in day 14 basal membranes; ‡P < 0.07 vs. EAAC1 in day 20 apical membranes; §P < 0.06, ‡P < 0.09, and †P < 0.05 vs. GLAST1 in day 14 apical membranes; ‡P < 0.21 vs. GLAST1 in day 20 apical membranes; ‡P < 0.26 and †P < 0.006 vs. GLT1 in day 14 apical membranes; ‡P < 0.02 vs. GLT1 in day 14 basal membranes; †P < 0.001 vs. GLT1 in day 20 apical membranes.
and 2.4 kb were detected. The 4.1-kb mRNA has been detected in rat brain (37) and human brain and placenta (26). The existence of the second species observed at 2.4 kb has not been reported previously. For EAAC1, three mRNA species of 4.0, 2.8, and 2.2 kb were detected in poly(A)$^+$ mRNA isolated from day 20 placentas, consistent with similar transcript sizes in numerous tissues (16, 21, 26, 40). For EAAT4, a strong signal of 2.9 kb was detected, whereas in rat brain, the same probe detected a single band of 2.3 kb (data not shown). In humans, the mRNA for EAAT4 is reported to be 2.4 kb in brain and placenta (7). For GLT1, three mRNA species of 4.0, 2.8, and 2.2 kb were detected in poly(A)$^+$ mRNA isolated from day 20 placentas, consistent with similar transcript sizes in numerous tissues (16, 21, 26, 40). For EAAT4, a strong signal of 2.9 kb was detected, whereas in rat brain, the same probe detected a single band of 2.3 kb (data not shown). In humans, the mRNA for EAAT4 is reported to be 2.4 kb in brain and placenta (7). For GLT1, transcripts of 2.8 and 2.0 kb were detected in day 14 placentas. In day 20 placentas, a third band of 10.0 kb in size was also expressed, as reported for both human brain and placenta (17, 18). All three of these GLT1 mRNA species were observed in adult rat brain (Fig. 3).

Densitometric analysis (Fig. 5) revealed that the proportional increase in the mRNA transcripts from day 14 to day 20 placentas was $\sim 18 \times$ for EAAC1, 10$\times$ for GLT1, 6$\times$ for GLAST1, and 4$\times$ for EAAT4. These results indicate that the magnitude of developmentally regulated expression of mRNA differs among the proteins encoding system X$_{AG}$ activity in the rat placenta.

Determination of system X$_{AG}$ activity at gestational day 14 vs. day 20. The uptake of glutamate was examined in both apical and basal syncytiotrophoblast plasma membrane subdomain vesicles derived from day 14 and day 20 rat placentas to determine whether the capacity for glutamate uptake increased as gestational age advanced. The apical and basal subdomains face the maternal and fetal circulations, respectively. The contribution of system X$_{AG}$ activity was evaluated by comparing the Na$^+$-dependent uptake of 1 µM glutamate in the presence and absence of 500 µM D-aspartate (Fig. 6). This concentration of D-aspartate completely inhibits system X$_{AG}$ activity in human placental membrane vesicles (14), and the Michaelis constants for D-aspartate of either GLT1 or GLAST1 expressed in oocytes are $< 10 \mu M$ (7, 16).

The total Na$^+$-dependent glutamate uptake by day 20 apical membrane vesicles was 134% greater than that measured in day 14 membrane vesicles (Fig. 6). In basal membrane vesicles, glutamate uptake by day 20 membranes was 47% greater than by day 14 vesicles. D-Aspartate did not inhibit the Na$^+$-dependent uptake of glutamate into apical vesicles derived from day 14 placentas. In contrast, D-aspartate inhibited by 68% the Na$^+$-dependent glutamate uptake by day 20 apical vesicles. In basal membranes, D-aspartate inhibited glutamate uptake in day 14 vesicles by 86% and in day 20 vesicles by 85%. The data in Fig. 6 also illustrate the effect of gestational age within and between apical and basal plasma membranes on D-aspartate-inhibitable

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**Fig. 9.** Immunohistochemical localization of GLT1 protein in day 14 and day 20 rat placenta. Immunohistochemical analysis of day 14 (A and C) and day 20 (B, D-F) placentas was performed as described in MATERIALS AND METHODS using primary antibodies (170 ng IgG/ml) against GLT1. E: peptide inhibition (5 mM) to document specificity of immunoreaction. D, decidual basal cells; G, junctional zone giant cells; J, junctional zone spongiotrophoblast cells; L, labyrinth zone syncytiotrophoblast cells. Original magnification of light-microscope photographs: $\times 32$ (A, B, E), $\times 64$ (C and D), or $\times 312$ (F).
transport (system \(X_{\text{AG}}\) activity). In basal membranes, system \(X_{\text{AG}}\) activity, already at a relatively high level on day 14, increased 36% by day 20. By day 20, apical system \(X_{\text{AG}}\) activity had increased from a nondetectable level in day 14 vesicles to a level approximately equal to that observed for day 14 basal membranes.

Determination of glutamate transporter protein regulation with gestational age. To determine whether GLAST1, GLT1, EAAC1, or EAAT4 proteins were present in placental apical and basal plasma membrane vesicles, immunoblot analysis was performed (Fig. 7). For EAAC1, a predominant immunoreactive species of 78 kDa in day 20 apical and basal membrane vesicles was detected. That the migration weight of the placental immunoreactive band was ~10 kDa greater than that observed in membrane vesicles prepared from HEK 293c18 cells (Fig. 3) indicates that the pattern of glycosylation of EAAC1 protein may differ between cell types.

For GLT1, immunoreactive bands of ~108, 148, and >208 kDa were observed in both day 14 and day 20 apical and basal plasma membrane subdomains. In contrast, only the 108-kDa band was observed in apical membranes. This pattern of multiple immunoreactive GLT1 protein bands has been reported previously in rat brain tissue homogenate (10) and in cortical synaptic membrane proteins (6). For GLAST1, a single band of 164 kDa was detected in both apical and basal membranes under a variety of conditions.2 Probing of replicate blots with GLT1 or GLAST1 antibodies that had been preabsorbed with the corresponding antigen peptides or with nonimmune IgG resulted in complete loss of all the immunoreactive bands (data not shown). Transfection of L cells with either GLAST1 or GLT1 cDNA resulted in expression of proteins with similar migration sizes (Rothstein, unpublished observations).

We consistently saw this band, whether freshly isolated or frozen membranes were used, whether 6 M urea or 10 or 100 mM dithiothreitol replaced 2-mercaptoethanol as the reducing agent, and regardless of the heating temperature (39°C, 65°C, and 100°C) and length of heating (5 and 15 min) of the samples before SDS-polyacrylamide gel electrophoresis analysis. In liver tissue homogenates and plasma membrane preparations, we detected a similar immunoreactive band of ~160 kDa (Matthews, unpublished research). Therefore, the GLAST1-immunoreactive band may represent either a placental tissue homodimer with a different glycosylation profile from that of the brain or a complex of GLAST1 protein with another unknown protein. Alternatively, the 160-kDa band for GLAST1 as well as the larger multiple bands observed for GLT1 may represent dimeric species that were induced by the oxidation of monomers during in vitro manipulations, as has been recently described (12).
In contrast to EAAC1, GLT1, and GLAST1, the protein corresponding to EAAT4 was not detected in either plasma membrane subdomain at day 14 or day 20 (Fig. 2). As demonstrated in Fig. 2, EAAT4 was recognized in rat brain tissue.

To quantify the effect of gestational age on the amount of GLAST1, GLT1, and EAAC1 proteins, densitometric analyses (summarized in Fig. 8) were performed on immunoblots from three independent vesicle preparations of each gestational age, one of which is represented in Fig. 7. The relative amount of EAAC1 detected in apical plasma membranes was ~15× greater on day 20 than the nominal amount detected on day 14. Similarly, the amount of GLAST1 protein was ~10× greater in day 20 than in day 14 apical membranes. These increases were statistically significant for both proteins. In contrast, the ~25% increase in total GLT1 protein content for apical membrane from day 14 to day 20 was not statistically significant. In basal membranes, the amount of EAAC1 protein on day 20 was ~9× greater than on day 14. Although the amount of GLAST1 protein increased by 56% from day 14 to day 20, this increase was not statistically significant for the mean of three independent experiments. In contrast to the increase for both EAAC1 and GLAST1 basal membrane proteins with gestational age, the amount of GLT1 protein decreased by ~66% from day 14 to day 20. In conjunction with the data in Fig. 6, these data indicate clearly that the EAAC1 and GLAST1 transporters contribute to, if not completely account for, the parallel increase in system X_{AG} activity in the apical and basal plasma membrane subdomains from day 14 to day 20 of gestation.

The quantitative distribution of the three detected GLT1 polypeptides in basal membranes changed with gestation. Whereas day 14 membranes primarily contained the >208-kDa polypeptide, the day 20 basal membranes had nearly equal quantities of the >208- and 148-kDa polypeptides, with a slightly lesser amount of the 108-kDa polypeptide. As stated above, these multiple bands for GLT1 have been observed by independent investigators in other tissues, are peptide inhibitable, and are recognized as cDNA-dependent expression products. It has been reported that these bands are aggregates of the same polypeptide (12). However, it is possible that one or more of the multiple bands represents immunoreactivity with a protein(s) other than GLT1. If this is the case, then the magnitude of the immunohistochemical reaction in day 14 and day 20 placental tissue would be the result of multiple proteins. However, the interpretation that the increase...
Table 1. Zonal distribution of anionic amino acid transporters in rat placenta

<table>
<thead>
<tr>
<th>Protein</th>
<th>Decidua</th>
<th>Giant</th>
<th>Junctional</th>
<th>Labyrinth</th>
<th>Relative Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAC1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>D = J &gt; G &gt; L</td>
</tr>
<tr>
<td>GLAST1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>D = L &gt; J</td>
</tr>
<tr>
<td>GLT1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>D = J &gt; L &gt; G</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>J &gt; L = G = D</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>J &gt; J &gt; G = L</td>
</tr>
</tbody>
</table>

The presence (+) or absence (−) of proteins known to be capable of system XAG anionic amino acid transport in tissue sections of gestation day 14 and day 20 choioallantoic placenta, from at least 2 placentas from different litters, was determined by immunohistochemical analysis as described in Materials and Methods and Results and as illustrated in Figs. 4–6. The presence of EAAC1, GLAST1, GLT1, and EAAT4 proteins in maternal decidua tissue (D) and placental junctional zone giant cell layer (G), junctional zone spongiotrophoblasts (L), and labyrinthine zone syncytiotrophoblasts (J) and their relative increase (†), decrease (‡), or no change (NC) from day 14 to day 20 of gestational age are indicated. *Very weak immunoreactivity.

In GLT1 antibody immunoreactivity from day 14 to day 20 is limited to the spongiotrophoblast would not change (see below). Collectively, if one compares the transporter protein content in apical with basal plasma membrane subdomains, the absolute amount of EAAC1, GLT1, or GLAST1 was greater at the basal surface at both day 14 and day 20.

Cellular distribution of transporter protein throughout the choioallantoic placenta. To determine placentation distribution of the transporter proteins and to determine whether increasing gestational age affects the pattern of protein localization, immunohistochemical analysis was performed on thin tissue sections of day 14 and day 20 rat choioallantoic placentas (see Figs. 9–11). No immunoreactivity within maternal or fetal red blood cells was observed for any of the antibodies. Immunostaining of day 14 placental sections with antibodies against GLT1 revealed light immunoreactive staining in decidual tissue (Fig. 9A) and junctional zone spongiotrophoblast cells (Fig. 9, A and C). Even less immunoreactivity was detected in labyrinth zone syncytiotrophoblast (Fig. 9C) and junctional zone giant (Fig. 9A) cells. By day 20, compared with day 14, the GLT1 immunoreactive response increased in the spongiotrophoblast cells (Fig. 9, B, D, and F) and to a lesser extent in decidual cells (Fig. 9B) and giant cells (Fig. 9, B and D). In contrast, little if any staining was detected in the labyrinth syncytiotrophoblasts (Fig. 9, B and D). The specificity of the GLT1 staining was demonstrated by the lack of reaction when the antibody was incubated with its antigen peptide before incubation with placental sections (Fig. 9E).

Weak immunoreactivity was observed in nearly all day 14 placental cells when tissue was incubated with antibody against GLAST1 (Fig. 10, A and C). The nucleus of some cells appeared immunoreactive (Fig. 10A), and a punctate-staining-pattern GLAST1 reactivity was evident in the spongiotrophoblast and labyrinth syncytiotrophoblast cells that overlay a more diffuse general staining (Fig. 10C). By day 20, this punctate pattern of GLAST1 immunoreactivity had increased considerably in the cell bodies and nuclei of maternal decidua and placental trophoblast cells (Fig. 10, B, D, and F). Preabsorption of GLAST1 antibody with its peptide antigen before immunohistochemical staining resulted in no immune reaction (Fig. 10E).

All cells of day 14 and day 20 tissue sections were immunopositive for the presence of EAAC1 (Fig. 11). Punctate immunoreactive staining of EAAC1 was localized at the outer boundary of the cells, within or near the plasma membrane (Fig. 11F). In day 14 placental sections, the intensity of the EAAC1 immunoreactive response was much greater in decidual and junctional zone giant and spongiotrophoblast cells than in labyrinth syncytiotrophoblast cells (Fig. 11, A and C). By day 20, however, the intensity of the immunoreaction for EAAC1 in labyrinth syncytiotrophoblast cells was greater than that in the decidual cells and junctional zone giant cells but equal to that in the junctional zone spongiotrophoblast cells (Fig. 11, B, D, and F). The positive immunohistochemical reaction to EAAC1 was completely inhibited when the antibody was preabsorbed to its antigen (Fig. 11E).

The immunohistochemical data corresponding to Figs. 9–11, representing hundreds of sections from placentas of several litters, were reviewed independently (by Matthews, Beveridge, and Novak) and are summarized in Table 1. Increasing placental gestation results in the selective alteration in expression of EAAC1, GLAST1, and GLT1 transport proteins within maternal decidua and placental trophoblast cells. The EAAT4 protein was not detected in any cells of day 14 or day 20 placentas (data not shown), consistent with the lack of EAAT4 protein detection by immunoblotting (Fig. 7).

**DISCUSSION**

Both glucose and glutamate are important sources of metabolic energy for the placenta (13, 15, 25). Therefore, placental reliance on glutamate may become especially important to spare maternally derived glucose for fetal consumption during the last trimester, when fetal growth is rapid and gluconeogenesis is nominal (13, 15). This study describes changes in the expression profiles of mRNA and protein content and activity of four different glutamate transporters in the rat choioallantoic placenta. Collectively, these transporters result in increased capacity for glutamate absorption at the start (day 14) and end (day 20) of the last trimester of gestation. Immunoblot and immunohistochemical staining revealed distinct, region-specific expression of EAAC1, GLAST1, and GLT1 proteins throughout the choioallantoic placenta, consistent with an increase in their mRNA levels. In contrast, EAAT4 protein was not detected in any cell type by immunoblot
or immunohistochemical analysis despite the increase of detectable mRNA for EAAT4 from day 14 to day 20. This may be the result of posttranscriptional regulation of mRNA availability for translation, slow translational rates, and/or maintenance of steady-state EAAT4 protein levels that are below detection.

An important understanding gained from this study is that the relative increase at day 20 vs. day 14 of system XAG activity in the apical and basal plasma membranes of the labyrinth syncytiotrophoblast was achieved by the differential expression of three distinct transport proteins capable of system XAG activity. The greater expression of system XAG activity and proteins in the basal than in the apical plasma membrane subdomains at day 14 could be in response to a greater substrate supply, given that the average concentration of glutamate in fetal rat blood during the last trimester of gestation is twice that of maternal blood (5). In apical membranes, the significant increase in system XAG activity was paralleled by an increase in EAAC1 and GLAST1 but not GLT1 protein content. In basal membranes, the slight increase in system XAG activity was coincident with large increases of EAAC1 and GLAST1 proteins and a large decrease in GLT1 protein content. Overall, this differential regulation of glutamate transporter expression probably reflects increased reliance on maternal sources of glutamate as a metabolic fuel as gestation progresses.

Most of the Na\textsuperscript{+}-dependent glutamate transport measured in day 20 apical and day 14 and day 20 basal membrane vesicles was mediated by system XAG activity (9), as reported in apical and basal sncytiotrophoblast plasma membranes of human placenta (14, 24). In contrast, all of the day 14 apical Na\textsuperscript{+}-dependent activity was D-aspartate insensitive, an activity not previously documented in the placenta. Given that the amount of D-aspartate-insensitive glutamate transport activity was not affected by gestational age in either plasma membrane subdomain, the transporter protein(s) responsible for this activity may be constitutively expressed.

In the placenta, the production of NADPH through glutamate catabolism is thought to represent an important source of anabolic energy for steroid synthesis because of the low activity of the pentose-phosphate shunt (27). A primary function of junctional zone giant cells is the production of steroid hormones to coordinate placental and fetal development. This process follows a very specific temporal pattern (36). Therefore, it is likely that these cells benefit from a parallel increase in the expression of glutamate transporter protein. In support of this hypothesis, the expression of all three glutamate/aspartate transporter proteins was increased in the junctional zone giant cells at day 20 vs. day 14 of gestation. In an analogous manner, placental glutamine synthetase activity is known to increase during the last trimester (5, 32) at a time of increased glutamate absorption capacity by the placenta (Fig. 6). When considered in terms of placental-to-fetal nitrogen shuttling, these two observations also suggest that a coordinated metabolism exists that facilitates the shuttling of nitrogen to fetal liver in the form of glutamine.

Differentiated placental trophoblast cells are capable of high levels of glutamate absorption and metabolism. In vitro, the human placenta is capable of absorbing 49% of the glutamate present in fetal perfusate and is thought to metabolize 80% of that absorbed (34). In an ovine in vivo model, the placental trophoblast has been shown to extract nearly 90% of circulating fetal glutamate in a single circulatory passage (39), to rapidly metabolize absorbed glutamate by decarboxylation and oxidation (25), and to return ~6% of the absorbed glutamate to the fetus as glutamine by the action of placental glutamine synthetase (25). Collectively, the increased capacity of both junctional and labyrinth zone cells to absorb more maternally derived glutamate would result in an increase in the amount of oxidizable substrate to meet the metabolic energy needs for growth, thus sparing glucose for transfer to the fetus. Both apical and basal plasma membrane GLUT-1 glucose transporter protein content increases during the third trimester in rat, indicating that mechanisms to mediate an increased transepithelial glucose flux exist (38).

Not all of the physiological reasons underlying the differential pattern of glutamate transporter expression by the placenta can be explained by our current understanding of placental metabolism. The reported Michaelis constants of GLAST1, EAAC1, and GLT1 proteins expressed in Xenopus laevis oocytes or COS-7 cells do not seem to differ significantly within these particular expression models (1, 16, 26), although little is known regarding the individual kinetic behavior of these transporters in placental tissue. Therefore, research that fully characterizes specific glutamate transporters, as expressed in placental cells, will be necessary to place our data in the proper physiological context. Nonetheless, the enhanced system XAG activity in the apical plasma membrane, mediated by increased EAAC1 and GLAST1 protein content, presumably reflects the need for the transport characteristics that these two transporters possess and that GLT1 does not. It is intuitive that the specific populations of cells within these tissues have differing needs for anionic amino acid transport capacity, given their differing metabolism and exposures to fluctuations of intra- and intercellular glutamate and aspartate concentrations.

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