Cerebral leucine uptake and protein synthesis in the
near-term ovine fetus: relation to fetal behavioral state

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impact on synthetic processes within the brain, thus account-
ing for the developmental change in such activity and sug-
gesting a role in the brain’s growth and development. We
have therefore determined the cerebral uptake of leucine and
[14C]leucine during continuous tracer infusion as measures
of leucine metabolism in relation to behavioral state activity,
as well as the regional flux of leucine into brain tissue in the
ovine fetus near term. The cerebral fractional protein syn-
thetic rate and the absolute protein synthetic rate averaged
~20%/day and ~1 g/day, respectively, as measured for the
whole brain, which is considerably higher than anticipated
protein accretion and indicates a high rate of protein turn-
over with protein synthesis closely linked to protein degra-
dation. Measures of protein synthesis were significantly
higher in the pituitary gland, which may be attributed to the
active synthesis and export of peptide hormones from this
region. Cerebral leucine and [14C]leucine uptakes averaged
~630 and ~1,000 nmol·100 g−1·min−1, with the latter
higher than leucine unidirectional flux and thus supporting a
degree of leucine oxidation by the brain. Cerebral leucine
metabolism as studied was affected by behavioral state ac-
tivity, with uptake measurements for both leucine and
[14C]leucine significantly increased during the high-voltage
electrocortical/non-rapid eye movement state by 1.7-fold
and 2.8-fold, respectively, indicating that protein synthesis
and degradation must also be increased at this time, and sup-
porting a role for behavioral state activity in the brain’s growth and
development.

brain development; leucine metabolism

CEREBRAL PROTEIN SYNTHESIS has been studied during
brain growth and development, both pre- and postna-
tally in sheep (1, 24) and postnatally in the rat (6, 26),
because proteins are integral components of structural
elements in brain tissue and with the metabolism of
brain protein directly related to maturational events
(26). Initial study in the near-term ovine fetus by
Schaefer and Krishnamurti (24) using a tyrosine iso-
topic-dilution technique showed high rates of cerebral
protein synthesis with a fractional protein synthetic
rate between 14 and 37%/day and an absolute rate of
synthesis of ~1 g/day. A subsequent study in fetal
sheep by Abrams and colleagues (1) using [14C]leucine
autoradiography reported a rate of leucine incorpora-
tion into brain protein of ~5 nmol·g−1·min−1, with an
overall increase through the latter part of gestation and
into the early postnatal period likely reflecting
cerebral myelination. Postnatal studies in rats have
similarly shown high rates of brain protein synthesis
during early development, with peak values occurring
shortly after birth and gradually decreasing thereafter
(6, 26). While high rates of cerebral protein synthesis
are thus evident during early life in both sheep and
rats in support of the brain’s growth and development,
these rates are considerably greater than anticipated
protein accretion, indicating that protein degradation
must also play an active role in the development of the
brain (6).

The increase in brain weight for the ovine species
during early development occurs in two phases, one up
to 90 days postconception followed by a more rapid and
larger increase thereafter that continues to birth at
~145 days, leading to the classification of sheep as a
prenatal brain developer (14). These two phases ap-
pear to reflect an increase in neuroblast multiplication,
followed by neuronal multiplication and myelination,
and with growth and development of the cerebral
hemispheres in advance of that in the cerebellum. For
the ovine species, well-differentiated electrocortical
(ECOG) patterns as a measure of neurobehavioral de-
velopment are evident from 120 days gestation on-
ward, with initially a high proportion of time in the
low-voltage (LV) ECOG state in the presence of rapid
eye movements (REM), and with a progressive de-
crease in this activity thereafter (27). Observations on

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the maturation of electrocortical patterns in utero or of behavioral activity from birth indicate a similar trend in the development of sleep-wakefulness patterns in humans and other mammals (22, 27), with the rate of such development in relation to birth well correlated with the neuroanatomic maturity of the brain (20).

A behavioral state effect on cerebral metabolic rate is evident for the ovine fetus near term, with a significant increase in cerebral blood flow and in oxygen and glucose uptake during the LV/REM state compared with that of the high-voltage (HV) ECOG state with no rapid eye movements [non-rapid eye movement (NREM)] (21), presumably reflecting an increase in neuronal functional activity and/or synthetic processes within the brain. The relationship of behavioral state activity to biosynthetic processes within the brain during development remains unclear, although recent studies in adult animals have shown higher rates of cerebral protein synthesis to be positively correlated with the occurrence of NREM sleep rather than REM sleep (16, 19). We have therefore determined the cerebral uptake of leucine and of \[^14\text{C}\]leucine during continuous tracer infusion as measures of leucine metabolism in relation to behavioral state activity in the ovine fetus near term. Regional fractional protein synthetic rates and flux of leucine into protein within the brain were also determined from the specific activity of protein-bound \[^14\text{C}\]leucine within brain tissues as additional measures of cerebral protein synthesis.

**MATERIALS AND METHODS**

**Surgical procedure.** Nine fetal sheep were surgically prepared at \(\sim 129\) days gestation (term = 145 days). Anesthesia was induced with an intravenous injection of thiopental sodium (1 g in 40 ml solution; Abbott Laboratories, Montreal, Canada) and was maintained with 1–1.5% halothane in oxygen (Halocarbon Laboratories, Hackensack, NJ). Before fetal surgery, a polyvinyl catheter (V11, Bolak, Lake Havasu City, AZ) was placed in the maternal femoral vein. Using sterile technique, a midline incision was made in the ewe’s lower abdominal wall, and the uterus was palpated to determine fetal position. An incision was then made in the uterus, and the fetal head, neck, and upper body were exteriorized. Polyvinyl catheters (V4, Bolak) were placed in the axillary artery for blood sampling, in the cephalic vein for administration of antibiotics and radiolabeled leucine, in the trachea for monitoring of fetal breathing movements, and in the amniotic cavity for pressure recording. Electrodes of teflon-coated stainless steel wire (Cooner Wire, Chatsworth, CA) were placed biparietally on the dura for ECOG recording, through the lateral orbital ridge of the zygomatic bone of each eye for electrooculogram (EOG) recording, and on the nuchal muscle for electromyographic (EMG) recording. A midline window of bone (\(\sim 2\) cm\(^2\)) was removed from the fetal skull, immediately anterior to the coronal suture; the dura on either side of the superior sagittal sinus was incised, and a transit time flow probe (3 mm, S-series, Transonic Systems, Ithaca, NY) was secured around the vessel. The cutaneous surrounding the probe was filled with bone wax to prevent leakage of cerebral spinal fluid. Another small midline window of bone (\(\sim 0.5\) cm\(^2\)) was removed between the coronal and lambdoid sutures and rostral to the bifurcation of the superior sagittal sinus. A nonocclusive polyvinyl catheter (V4, Bolak) was inserted into the vessel for blood sampling and was directed caudally by \(\sim 1\) cm. Sagittal sinus blood flow \((Q_{ss})\) was briefly monitored intraoperatively to ensure that the flow probe was functioning properly and to observe the effects of subsequent catheter placement in the sagittal sinus on \(Q_{ss}\). After placement of the probe and catheter, the scalp was sewn over. The uterus and abdomen were closed in layers, and all catheters, electrodes, and the Transonic flow probe lead were exteriorized to the flank of the ewe and secured to the ewe’s back in a plastic pouch. The ewes received a long-acting, broad-spectrum antibiotic immediately before surgery (1.2 g oxytetracycline im; LA-200, Rogar, STB, London, Canada) and were given intravenous fluids throughout the surgery (1,000 ml 0.9% saline solution).

**Postoperative care.** After surgery, ewes were placed in metabolic cages suitable for continuous monitoring and were given an analgesic (1.5 ml Banamine). Antibiotics were administered for 3 days postoperatively to the fetus (1,000,000 IU sodium penicillin G iv; Ayerst, Montreal, Canada) and to the amniotic cavity (1,000,000 IU sodium penicillin G). The ewes were allowed 4 days of postoperative recovery, during which maternal and fetal catheters were flushed daily with heparinized saline to maintain patency, and fetal arterial blood was collected for blood gas analysis. Animals were allowed food and water ad libitum, and all surgical, postoperative, and experimental procedures followed the guidelines provided by the Canadian Council on Animal Care and the University of Western Ontario Council on Animal Care.

**Physiological measurements.** On the day of experimental study, strain-gauge transducers (Statham model P-2310, Gould, Oxnard, CA) and a physiograph recorder (model 78D, Grass Instrument, Quincy, MA) were used to record amniotic and tracheal pressures. Fetal ECOG, EOG, and EMG potentials were displayed directly on the chart recorder after passing through a passive band-pass filter, 0.3–30 Hz on the preamplifier for ECOG and EOG recordings, and 10–90 Hz on the preamplifier for EMG recordings. The amplified, filtered ECOG signal was further processed by means of a frequency integrator with the ECOG frequency shift displayed separately on the chart recorder. The mean volume blood flow measurement derived from the flow probe was processed using a Transonic flowmeter (model T208, Transonic Systems) and was displayed on the chart recorder with the normal scale calibration. Blood samples were studied over a 6-h period, with a continuous infusion of L-\[^14\text{C}\]leucine in sterile normal saline into the fetal cephalic vein at 0.15 \(\mu\)Ci/min (in 30 ml normal saline at a rate of 5 ml/h). Arterial blood samples (1 ml) were drawn at 15, 30, 60, and 90 min of infusion to determine that the specific activity of leucine had reached steady state. An arterial blood sample (1 ml) was additionally drawn before initiation of the infusion and was analyzed for blood gases and pH as a measure of fetal well being. After the first 2 h of infusion, spontaneous changes in behavioral state were scored and four LV/REM and four HV/NREM epochs were studied. Cerebral arterial-venous (A-V) difference samples were collected from the axillary artery (1.5 ml) and the sagittal sinus (1.5 ml) at least 5 min into each state epoch, with LV measurements made only if EOG activity was present and in the absence of nuchal muscle EMG activity, and HV measurements made only if EOG activity was absent and in the presence of nuchal muscle EMG activity. The remaining whole blood was then spun (4°C, 10 min at 9,000 \(g\)), and the plasma was titrated off and divided into two equal aliquots for duplicate measurements. All plasma aliquots were frozen at \(-80°C\) for subsequent measurement of leucine concentra-
tion and specific activity and α-ketoisocaprylic acid (α-KIC) concentration.

After completion of the 6-h infusion period, the ewe and fetus were immediately euthanized by an overdose of barbiturate (30 mg pentobarbital sodium iv, MTC Pharmaceuticals, Cambridge, Canada), and an autopsy was performed to verify placement of catheters and to retrieve the flow probe. The fetal brain was rapidly removed, weighed, and dissected into cerebral cortex, brain stem, cerebellum, spinal cord, and pituitary. Dissected regions were fast frozen in liquid nitrogen and stored at −80°C for later analysis of protein-bound and intracellular free leucine specific activity.

Chemical analysis. Blood gases and pH were measured using anABL-500 blood gas analyzer with temperature corrected to 39.5°C (Radiometer, Copenhagen, Denmark). Methods for measurement of plasma leucine concentration and specific activity and α-KIC concentration have been described previously (15). Briefly, plasma (~350 μl) was combined with an equal volume of 6% sulfosalicylic acid containing norleucine (250 μM) and α-ketocaprylic acid (50 μM) (internal standards for leucine and α-KIC, respectively). Samples were vortexed and centrifuged for 6 min at 10,000, and the supernatant was collected. Plasma concentration of leucine or α-KIC was determined by injecting derivatized samples onto an HPLC column (150 × 3.9-mm ID, Resolve C18, 5-mm particle size, column temperature 42°C, Waters, Milford, MA; and 250 × 4.6-mm ID, Ultrasphere ODS, 5-mm particle size, 42°C, Beckman, respectively). The leucine/α-KIC eluate peak was collected and counted for radioactivity (model LS 5000 TD, Beckman Instruments, Fullerton CA). Blood leucine or α-KIC concentration was calculated by the method of peak-height ratio to the internal standards.

Tissue concentrations and specific activity of leucine were measured using a modification of the method described by Horber and colleagues (10). Frozen tissue samples were weighed (~0.07–1.3 g), and 6% sulfosalicylic acid was added. Samples were homogenized on ice for ~40 s and then centrifuged for 40 min at 1,500 g and 4°C, with this procedure repeated two additional times. The pooled supernatant was then frozen for later analysis of intracellular free leucine. The pellet (containing the protein-bound leucine) was dried overnight in a drying oven at 60°C, accurately weighed, hydrolyzed overnight, and then resuspended in 4 ml water.

The supernatant and hydrolyzed protein samples were placed on ion-exchange columns, washed four times with 2 ml 0.01 N HCl, drained to waste, washed with 4 ml water, drained to waste, and finally the leucine fraction was collected by washing four times with 2 ml 25% NH4OH, and dried overnight. To the dried residue, exactly 2 ml of water was added, and the vials were capped, vortexed, and sonicated for 20 min to promote dissolution. Exactly 1 ml was then transferred to a scintillation vial and then counted for radioactivity. For the intracellular free samples (supernatant), 0.5 ml of remaining sample was added to 0.5 ml 250 μM norleucine, and samples were taken to dryness. For the protein-bound samples (hydrolyzed protein), a fraction of the remaining sample was accurately diluted, 0.5 ml of 250 μM norleucine was added, and then it was evaporated to dryness. All samples were then derivatized and processed for HPLC in a similar manner to that previously described for plasma leucine.

Data analysis and mathematical calculations. Q_a as a relative measure of cerebral blood flow was determined every 10 s and averaged over the duration of each A-V difference blood sample draw (~60 s) using a computerized data-acquisition system (CADA, Hartronx Computer Solutions, Toronto, Canada).

The cerebral A-V difference for unlabeled leucine (μmol/l) was added to the A-V difference for unlabeled α-KIC (μmol/l) as α-KIC is a freely interconvertible metabolite of leucine and could possibly contribute to the intracellular leucine pool that is destined to be incorporated into protein (15). This sum was then multiplied by the corresponding value obtained for Q_a determined every 10 s and averaged over the duration of the cerebral A-V difference sample to determine relative cortical leucine uptake

cortical leucine uptake = Q_a × ([14C]Leu)_{a} − ([14C]Leu)_{v}

where subscripts “a” and “v” refer to arterial and venous concentrations, respectively.

The superior sagittal sinus drains regions of the frontal cortex and anterior regions of the parietal cortex as measured in the newborn lamb and represents ~20% of total brain blood flow (7). Cerebral blood flow and leucine uptake per unit mass of brain tissue were then calculated assuming the respective relative values to be reflective of ~20% of brain tissue mass (7), thus allowing for comparison with previous studies. Relative cortical [14C]leucine uptake and cerebral [14C]leucine uptake per unit mass of brain tissue were determined in a similar fashion to that described for the unlabeled leucine using the cerebral A-V difference for [14C]leucine + α[14C]KIC (dpm/ml)

cortical [14C]leucine uptake = Q_sc × ([14C]Leu)_{a} − ([14C]Leu)_{v} − [α-KIC]_{a} + [α-KIC]_{v}

Fractional extraction of leucine and [14C]leucine was calculated as the cerebral A-V difference for leucine or [14C]leucine divided by the respective leucine or [14C]leucine arterial concentration. The cortical metabolic values determined for each of the four LV/REM and four HVNREM state epochs studied were averaged to obtain mean LV/REM and HVNREM values for each animal.

The specific activity of plasma (S_{AP}) leucine (in dpm/nmol) was determined by dividing the concentration of [14C]leucine (dpm/ml) by the corresponding concentration of leucine (nmol/ml). The specific activity of brain tissue protein-bound (S_{APB}) leucine and intracellular free (S_{API}) leucine were similarly determined from the concentration of [14C]leucine (dpm/g) and the corresponding concentration of leucine (nmol/g) in the tissue pellet and supernatant, respectively.

Tissue fractional protein synthetic rate (FSR), which is the percentage of brain protein that is newly synthesized per day, was then calculated for each brain region using respective S_{APB} and S_{API} values and the total infusion time (days) in the following formula (3, 24)

FSR (%/day) = ([S_{APB}/S_{API}]/infusion time) × 100%

At the time of tissue leucine analysis, both the wet weight of the thawed tissue aliquot and the corresponding dried protein pellet weight were accurately determined. The ratio of dried pellet weight to aliquot wet weight was then multiplied by the wet weight of the entire region as determined at the time of postmortem to determine the total dry weight (estimate of the protein content) of that particular region. Estimated absolute protein synthetic rate (g/day) was then calculated for each region as the product of the regional dry weight (g) and the corresponding regional fractional synthetic rate (per day). The rate at which leucine enters fetal protein (unidirectional leucine flux) (μmol·100 g−1·min−1) was calculated for each brain region as the product of the fractional synthetic rate for that region (per day) and the
amount of leucine in protein in that region (Leuprotein) (nmol/100 g) divided by 1,440

unidirectional leucine flux = [FSR × Leuprotein]/1,440

Results obtained for the nine animals are presented as grouped means ± SE. The leucine uptake data reported were analyzed by a Student’s paired t-test for differences between the LV/REM and HV/NREM states, with significance taken at \( P < 0.05 \). The regional fractional protein synthetic data, estimated absolute synthetic rate, and leucine flux data were each analyzed using a one-way ANOVA for repeated measures with a post hoc Tukey test for differences between regions, with significance taken at \( P < 0.05 \).

RESULTS

Fetal arterial blood gases and pH measured within the normal range, with an average \( P_{O_2} \) of 23.3 ± 1.2 mmHg, \( P_{CO_2} \) of 50.3 ± 1.4 mmHg, and pH of 7.37 ± 0.01, indicating that all animals were in good health at the time of experimental study. SAP leucine reached steady-state values by 120 min of infusion. Figure 1 illustrates mean SAP leucine at time 0 and at 15, 30, 60, and 90 min of infusion and throughout the blood sampling period, demonstrating the attainment of plateau values, which averaged 6.7 ± 0.7 dpm/nmol. SAP leucine values measured in the fetal brain tissue at the end of the 6-h infusion period were lower than SAP leucine plateau values, averaging 5.3 ± 0.4 dpm/nmol, while SAP values were considerably lower, averaging 0.27 ± 0.02 dpm/nmol.

Cerebral leucine and \([^{14}C]\)leucine uptake. Blood flow in the superior sagittal sinus was affected by behavioral state activity, as studied at the time of cerebral A-V difference leucine measurements, and was increased in each of the animals during the LV/REM state compared with the HV/NREM state, 19.8 ± 2.3 vs. 15.0 ± 1.7 ml/min \((P < 0.001)\) (Table 1). Accordingly, cerebral blood flow per unit mass of brain tissue was similarly increased, 206 ± 27 vs. 156 ± 20 ml/100 g \(\cdot\)min \(-1\) \((P < 0.002)\) (Table 1). Conversely, the A-V difference for leucine and leucine fractional extraction were increased and to a greater extent during the HV/NREM state, 4.7 ± 1.0 vs. 2.0 ± 0.8 \(\mu\)mol/l \((P < 0.001)\) and 2.1 ± 0.4 vs. 0.8 ± 0.5 \(\%\) \((P < 0.02)\), respectively, but with no measurable A-V difference for \(\alpha\)-KIC during either behavioral state (Table 1). As such, cortical leucine uptake and thus cerebral leucine uptake were both increased during the HV/NREM state compared with the LV/REM state, 77 ± 25 vs. 44 ± 30 nmol/min and 796 ± 266 vs. 467 ± 315 nmol/100 g \(\cdot\)min \(-1\), respectively \((P < 0.02)\) (Table 1). The A-V difference for \([^{14}C]\)leucine and \([^{14}C]\)leucine fractional extraction were also increased during the HV/NREM state and more so than their unlabeled counterparts, 62 ± 9 vs. 20 ± 10 dpm/ml and 4.4 ± 0.5 vs. 1.6 ± 0.8, respectively \((P < 0.01)\).

Table 1. Cerebral leucine and \([^{14}C]\)leucine metabolism

<table>
<thead>
<tr>
<th></th>
<th>LV/REM</th>
<th>(P)</th>
<th>HV/NREM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Q_{sw}, ) ml/min</td>
<td>19.8 ± 2.3</td>
<td>&lt;0.001</td>
<td>15.0 ± 1.7</td>
</tr>
<tr>
<td>Cerebral blood flow, ml/100 g (\cdot)min (-1)</td>
<td>206 ± 27</td>
<td>&lt;0.002</td>
<td>156 ± 20</td>
</tr>
<tr>
<td>Leucine(_{artery}), (\mu)mol/l</td>
<td>215 ± 25</td>
<td>NS</td>
<td>213 ± 25</td>
</tr>
<tr>
<td>Leucine A-V difference, (\mu)mol/l</td>
<td>2.0 ± 0.8</td>
<td>&lt;0.001</td>
<td>4.7 ± 1.0</td>
</tr>
<tr>
<td>(\alpha)-KIC(_{artery}), (\mu)mol/l</td>
<td>24.0 ± 2.8</td>
<td>NS</td>
<td>24.2 ± 2.8</td>
</tr>
<tr>
<td>(\alpha)-KIC A-V difference, (\mu)mol/l</td>
<td>0.2 ± 0.4</td>
<td>NS</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>Cortical leucine uptake, nmol/min</td>
<td>44 ± 30</td>
<td>&lt;0.02</td>
<td>77 ± 25</td>
</tr>
<tr>
<td>Cerebral leucine uptake, nmol/100 g (\cdot)min (-1)</td>
<td>476 ± 315</td>
<td>&lt;0.02</td>
<td>796 ± 266</td>
</tr>
<tr>
<td>Leucine fractional extraction, (%)</td>
<td>0.8 ± 0.5</td>
<td>&lt;0.02</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>([^{14}C])leucine, dpm/ml</td>
<td>1,397 ± 269</td>
<td>NS</td>
<td>1,394 ± 242</td>
</tr>
<tr>
<td>([^{14}C])leucine A-V difference, dpm/ml</td>
<td>20 ± 10</td>
<td>&lt;0.01</td>
<td>62 ± 9</td>
</tr>
<tr>
<td>(\alpha)-[(^{14}C)]KIC(_{artery}), dpm/ml</td>
<td>88 ± 17</td>
<td>NS</td>
<td>93 ± 15</td>
</tr>
<tr>
<td>(\alpha)-[(^{14}C)]KIC A-V difference, dpm/ml</td>
<td>1 ± 1</td>
<td>NS</td>
<td>1.5</td>
</tr>
<tr>
<td>Cortical ([^{14}C])leucine uptake, dpm/min</td>
<td>341 ± 218</td>
<td>0.05</td>
<td>971 ± 199</td>
</tr>
<tr>
<td>Cerebral ([^{14}C])leucine uptake, dpm/100 g (\cdot)min (-1)</td>
<td>3,460 ± 2,303</td>
<td>0.05</td>
<td>10,149 ± 2,266</td>
</tr>
<tr>
<td>([^{14}C])leucine fractional extraction, (%)</td>
<td>1.6 ± 0.8</td>
<td>&lt;0.01</td>
<td>4.4 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n = 6\) due to either failure of the sagittal sinus catheter or technical difficulties with plasma leucine samples. Significance is based on paired data analysis. Cerebral blood flow, leucine uptake, and \([^{14}C]\)leucine uptake were calculated assuming the respective sagittal sinus blood flow \(Q_{sw}\) or cortical uptake values to be reflective of ∼20% of brain tissue mass \((? )\). A-V, arterial-venous; \(\alpha\)-KIC, \(\alpha\)-ketoisocapric acid; LV/REM, low-voltage electrocortical state in presence of rapid eye movement; HV/NREM, high-voltage electrocortical state with no rapid eye movement present; NS, not significant.
but again with no measurable A-V difference for α-[14C]KIC during either behavioral state (Table 1). As such, cortical [14C]leucine uptake and thus cerebral [14C]leucine uptake were also increased during the HV/NREM state and more so than their unlabeled counterparts, 971 ± 199 vs. 341 ± 218 dpm/min and 10,149 ± 2,266 vs. 3,460 ± 2,303 dpm·100 g⁻¹·min⁻¹, respectively (both P = 0.05) (Table 1).

Regional cerebral protein synthesis. Cerebral fractional synthetic rates for protein averaged ~20%/day and were similar as measured for the cerebral cortex, cerebellum, brain stem, and spinal cord, but significantly higher as measured for the pituitary gland at 29 ± 3%/day (P < 0.05) (Fig. 2). The unidirectional flux of leucine into cerebral protein averaged ~0.7 μmol·100 g⁻¹·min⁻¹ and was again similar as measured for the cerebral cortex, cerebellum, and brain stem, but significantly lower as measured for the spinal cord at 0.41 ± 0.03 μmol·100 g⁻¹·min⁻¹, and significantly higher as measured for the pituitary gland at 1.40 ± 0.12 μmol·100 g⁻¹·min⁻¹ (both P < 0.05) (Fig. 2). The estimated absolute synthetic rate for the brain as a whole was ~1 g/day, constituting mainly from the cerebral cortex at 1.0 ± 0.1 g/day, and with considerably smaller contributions from the cerebellum and brain stem at 0.12 ± 0.01 and 0.06 ± 0.01 g/day, respectively.

**DISCUSSION**

The present study utilized a continuous intravenous infusion of L-[1-14C]leucine to investigate leucine metabolism in the near-term ovine fetal brain. This was accomplished with the measurement of tissue protein leucine specific activity, which reflects the unidirectional flux of leucine into cerebral protein, i.e., protein synthesis, and using Fick methodology to measure the uptake of labeled/unlabeled leucine by the brain, which reflects that utilized for protein synthesis/accretion, plus any leucine that is oxidized. A-V differences were collected in both the LV/REM and HV/NREM behavioral states to determine the relationship between cerebral leucine metabolism and behavioral state activity during brain development. The label on L-[1-14C]leucine at the carboxyl position can be measured in leucine that has been incorporated into protein, or it can be followed through the catabolic pathway, which involves the reversible transamination with α-ketoglutarate to form α-KIC, followed by oxidative decarboxylation to form 14CO₂, which diffuses out of the tissue (8). The measurement of tissue SA₆F leucine along with that of the precursor pool at a defined time from the start of the tracer infusion provides a means of estimating the fractional synthetic rate of brain proteins, i.e., the percentage of brain proteins newly synthesized per unit time. The accretion of proteins within the brain in turn represents the difference between the synthesis of protein and the degradation, or breakdown, of protein. The measurement of unlabeled leucine uptake by the brain here studied using Fick methodology will reflect that leucine directed toward protein accretion and any leucine that is oxidized. The infused labeled leucine will also be taken up by the brain and will equilibrate with the intracellular free leucine pool (25). However, depending on the half-life of proteins, which approximates 4 days for the adult brain (12), relative to the infusion time (6 h), there should be limited recycling of labeled leucine out of brain proteins due to degradation over the course of the study. Thus the measurement of labeled leucine uptake by the brain should mainly reflect that leucine directed toward protein synthesis as well as any that is oxidized.

Measures of cerebral protein synthesis in the present study were determined using tissue SA₆F leucine for the precursor pool and, although likely to be more representative than SAP, may in fact underestimate the actual specific activity values of the immediate precursor amino acyl-tRNA. This could occur due to recycling of unlabeled leucine into the intracellular cycle.

![Fig. 2. Fractional synthetic rate (A) and unidirectional flux of leucine into cerebral protein (B). Different letters indicate significance at P < 0.05.](image-url)
free leucine pool from protein degradation and tRNA species that are preferentially charged from the plasma extracellular rather than intracellular free pool (5). As such, measures of cerebral protein synthesis may be overestimated; however, this should be minimal, since the SA\textsubscript{P} leucine values were only slightly lower than the SA\textsubscript{P} leucine plateau values, averaging 5.3 vs. 6.7 dpm/nmol, i.e., an ∼20% difference between the maximum and minimum measures of cerebral protein synthesis when using the SA\textsubscript{P} and SA\textsubscript{P} leucine values for the precursor pool, respectively. The cerebral fractional synthetic rate for protein as measured averaged ∼20%/day for the brain as a whole, which is within the range of values previously reported for the ovine fetus at a slightly younger gestational age by Schaefer and Krishnamurti (24) using tyrosine rather than leucine as the tracer amino acid. The unidirectional flux of leucine into cerebral protein, as measured using the fractional synthetic rate and the concentration of leucine in protein, averaged ∼0.7 μmol·100 g\textsuperscript{-1}·min\textsuperscript{-1}, which is similar to that reported by Abrams and colleagues (1) using leucine autoradiography at ∼5 nmol·g\textsuperscript{-1}·min\textsuperscript{-1} for animals at 130–135 days gestation. The estimated absolute protein synthetic rate here measured for the brain as a whole using the fractional synthetic rate and the dry weight of the brain as an estimate of protein content was ∼1 g/day, which is the same as that reported by Schaefer and Krishnamurti (24). This value is much higher than anticipated protein accretion at ∼36 mg/day as calculated for the cerebral hemispheres, brain stem, and cerebellum of the ovine fetus between 121 and 150 days gestation from the data reported by McIntosh and colleagues (14) relating to protein content within the brain at these developmental time points. As such, the high rate of protein synthesis noted for the ovine fetal brain near term must be closely linked to a rate of protein degradation, which is almost as high as that for synthesis. Study of protein metabolism in the rat brain at 10–20 days postnatal and thus at a developmental stage comparable to that of the near-term ovine fetal brain (14) likewise reveals that protein accretion is the result of a relatively small difference between a high rate of degradation and an even higher synthesis rate, but with these rates now slowing and converging from that evident during the first week postnatal, with a resultant slowing of protein accretion (6).

In the present study, there were no regional differences in the measures of protein synthesis from the cerebral cortex, cerebellum, or brain stem, which one might expect given the heterogeneous makeup of each of these structures and random tissue sampling. However, the findings of Abrams and colleagues (1) with the detailed study of regional protein synthesis within the brain using leucine autoradiography would also indicate that the composite rates for these brain regions will be similar albeit with subregional differences with rates higher in the pineal body and hypothalamic nuclei, and lower in the white matter. Of interest, measures of protein synthesis here studied were significantly higher in the pituitary gland, with the fractional synthetic rate at ∼30%/day and the unidirectional flux of leucine into protein at ∼1.4 μmol·100 g\textsuperscript{-1}·min\textsuperscript{-1}, which may be attributed to the active synthesis and export of peptide hormones from this region (25). Conversely, the unidirectional flux of leucine into protein was significantly lower in the spinal cord at ∼0.41 μmol·100 g\textsuperscript{-1}·min\textsuperscript{-1}, which is similar to that reported by Abrams and colleagues (1) and may reflect slower turnover of structural components within the nervous system compared with those involved in functional attributes.

Cerebral leucine uptake in the present study was determined using A-V differences for plasma leucine because the plasma transport of this amino acid across tissues per unit volume is reported to be at the same rate as blood cellular transport in sheep (9). It is also recognized that α-KIC is interconvertible with leucine within the cell and can move freely between the intracellular and extracellular pools. As such, the uptake of α-KIC could potentially contribute to the intracellular leucine precursor pool (15). However, there was no measurable uptake of α-KIC by the ovine fetal brain, indicating that the contribution of α-KIC to the intracellular leucine pool was minimal. Cerebral leucine uptake as measured for those cortical areas drained by the superior sagittal sinus averaged ∼60 nmol/min and, assuming that this blood flow represents ∼20% of total brain flow (7), results in a cerebral leucine uptake of ∼630 nmol·100 g\textsuperscript{-1}·min\textsuperscript{-1}. This value is threefold higher than that previously reported for the adult sheep brain (18) and is in keeping with a greater amino acid requirement during brain development due to protein accretion and possibly to some degree of oxidative metabolism, although glucose uptake as a metabolic fuel is sufficient to account for the oxidative needs of the ovine fetal brain (4, 11). Of interest, cerebral [\textsuperscript{14}C]leucine uptake averaged ∼6,800 dpm·100 g\textsuperscript{-1}·min\textsuperscript{-1}, which is equivalent to 1,000 nmol·100 g\textsuperscript{-1}·min\textsuperscript{-1} as calculated using the plateau SA\textsubscript{P} leucine value of 6.7 dpm/nmol. This uptake of labeled leucine is 2-fold greater than the unidirectional flux of leucine into cerebral protein as measured here and by Abrams and colleagues (1). To the extent that this uptake measurement reflects leucine directed toward protein synthesis as well as that which is oxidized, a degree of oxidative metabolism is thereby supported.

Cerebral leucine uptake as studied was effected by behavioral state activity, with uptake measurements for both leucine and [\textsuperscript{14}C]leucine significantly increased during the HV/NREM state by 1.7-fold and 2.8-fold, respectively, compared with that of the LV/REM state. Cerebral uptake values were in fact not significantly different from zero during the LV/REM state. Conversely, these values during the HV/NREM state were all significantly different from zero with [\textsuperscript{14}C]leucine uptake at 10,149 dpm·100 g\textsuperscript{-1}·min\textsuperscript{-1} or 1,514 nmol·100 g\textsuperscript{-1}·min\textsuperscript{-1} as calculated using the plateau SA\textsubscript{P} leucine value, ∼2-fold higher than unlabeled leucine uptake at 796 nmol·100 g\textsuperscript{-1}·min\textsuperscript{-1}. It is unlikely that the increased uptake of leucine during HV/NREM is attribu-
utable to changes in the transport of leucine via the blood-brain barrier (BBB) neutral amino acid transporter, as leucine has a high BBB permeability (17) and as there is no change in the arterial plasma concentration of leucine (as measured here) or the other neutral amino acids (23) between the two behavioral states. Assuming that $^{14}$C]leucine oxidation reflects mainly protein synthesis whereas unlabeled leucine uptake reflects protein synthesis plus degradation, i.e., accretion as previously discussed, then both synthesis and degradation must also be increased during the HV/NREM state compared with the LV/REM state. However, the increase in synthesis will also be dependent on the extent of any leucine oxidation by the brain. For example, if 50% of the cerebral leucine uptake were to be oxidatively metabolized, i.e., $\sim 400 \text{ nmol} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$, with the remainder directed toward protein accretion, then that attributed to synthesis vs. degradation would measure $\sim 1,115$ and $715 \text{ nmol} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$, respectively. While this degree of oxidative metabolism would account for $< 2 \mu$mol of oxygen $\cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$, it is of interest that Chao and colleagues (4) found measured oxygen uptake by the ovine fetal brain during the HV/NREM state to be somewhat higher than that accounted for by glucose oxidation alone, 127 vs. 114 $\mu$mol $\cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$, with the probability of additional metabolic substrates including that of lactate at this time. These findings now add to those in adult animals where higher rates of cerebral protein synthesis have been shown to be positively correlated with the occurrence of NREM sleep (16, 19) and further support a role for behavioral state activity in the brain's growth and development. Together, these studies would also indicate that the decrease in the brain's metabolic demand during NREM sleep as seen in the ovine fetus (21) and in other species postnatally, including humans (13), does not result from a decrease in biosynthetic activity and may, in fact, favor the synthesis of new proteins. This would support the restorative theory of sleep (2) whereby energy conservation during NREM sleep favors the anabolic restoration of tissue components.

**Perspectives**

Brain growth and development are characterized by a series of events that include the proliferation and migration of nerve cells, the growth of axons and dendrites, the formation of functional synapses, cell death, myelination of axons, and the fine tuning of neuronal specificity. These maturational events in turn will be intricately linked to protein synthesis in the brain for the provision of structural components and the signaling processes to direct these events. The high rates of protein synthesis herein reported point to a high rate of protein turnover within the brain during development and indicate that growth processes leading to protein accretion at this time must involve extensive tissue remodeling. Behavior state activity, which is well delineated in the ovine fetus near term as a prenatal brain developer, appears to impact on protein metabolism within the brain with an increase in both synthesis and degradation during the HV/NREM state and further supporting a role for behavioral state activity in the brain's growth and development.

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