Microtubule-Associated Protein 2 and Synaptophysin in the Preterm and Near-Term Ovine Fetal Brain and the Effect of Intermittent Umbilical Cord Occlusion

Reproductive Sciences
2015, Vol. 22(3) 367-376
© The Author(s) 2014
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719114529371
rs.sagepub.com

Marie J. Czikk, MD, MSc¹, Stephanie Totten, BScN²,
Robert Hammond, MD³, and Bryan S. Richardson, MD⁴

Abstract

We have determined the change in immunoreactivity (IR) for microtubule-associated protein 2 (MAP-2) and synaptophysin (SYN) as markers for dendritic and presynaptic nerve development, respectively, in the ovine fetal brain with advancing gestation and in response to intermittent umbilical cord occlusion (UCO), which might then contribute to adverse neurodevelopment. Fetal sheep (control and experimental groups preterm at 111-115 and near term at 132-138 days of gestation; term = 145 days) were studied over 4 days with UCOs performed by inflation of an occluder cuff for 90 seconds every 30 minutes for 3 to 5 hours each day. Animals were then euthanized and fetal brains assessed for IR of MAP-2 and SYN. In control animals, the IR of SYN increased in the gray matter with advancing gestation consistent with a developmental increase in presynaptic vesicles and/or nerve terminals as expected; however, the IR of MAP-2 decreased in all brain regions studied, suggesting concurrent refinement in dendritic branching and spine development. Intermittent UCO as studied with marked but limited hypoxemia resulted in a decrease in IR of SYN for the brain regions of the preterm animals when protein turnover is higher and indicates decreased presynaptic vesicle formation; whereas, MAP-2 IR was selectively increased in the hippocampus CA1 and thalamus of the near-term animals, consistent with reactive dendritic change and heightened vulnerability for neuronal injury. As such, intermittent cord compressions in the ovine fetus can impact protein markers for dendritic and presynaptic nerve development depending on their timing, which might then lead to alterations in synapse formation and neuronal circuitry.

Keywords

brain development, synaptogenesis, hypoxia

Introduction

Neuronal connectivity with the formation of functional synapses and fine-tuning of neuronal specificity is an important aspect of brain development underlying learning and memory in postnatal life. This is a highly dynamic process involving axonal growth, synapse, and dendritic spine formation that is dependent on the availability of growth factors and in particular brain-derived neurotrophic factor (BDNF), new protein synthesis and increased energy metabolism, and activity-dependent synapse remodeling, whereby the more competitive connections are preferentially stabilized while others are eliminated.¹⁻⁹ For prenatal brain developers such as sheep and primates and for humans as perinatal brain developers, the most rapid phase of synapse formation is initiated in the latter half of pregnancy.⁹⁻¹² As such, adverse pregnancy conditions that disrupt regulatory mechanisms of synapse formation may lead to altered development with later cognitive impairment as seen in humans born growth restricted with increased risk

of minimal brain dysfunction, attention-deficit disorders, and possibly schizophrenia and autism.¹³⁻¹⁷

Variable fetal heart rate (FHR) decelerations suggestive of umbilical cord compression and resultant fetal hypoxemia are

¹ Department of Obstetrics & Gynaecology, University of Toronto, Toronto, Canada

² Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Canada

³ Department of Pathology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Canada

⁴ Departments of Obstetrics and Gynecology, Physiology and Pharmacology, and Pediatrics, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Canada

Corresponding Author:

Bryan S. Richardson, Department of Obstetrics and Gynecology, London Health Sciences Centre, Victoria Hospital, 800 Commissioners Road E, London, Ontario, N6A 5W9.
Email: bricharl@uwo.ca

seen in 2% to 10% of antenatal FHR recordings near term and are associated with increased incidence of nuchal cord evident at delivery^{18,19} and in turn lower birth weight.²⁰ As such, chronic/intermittent cord compression likely occurs during some human pregnancies, with the potential to affect longer term growth and development, including that of the brain. In this regard, the association of infants with a symptomatic and/or tight nuchal cord at delivery and the later development of subclinical neurodevelopmental deficits²¹ and cerebral palsy²² implicates a role for chronic intermittent cord occlusion insults.

We have previously shown that intermittent umbilical cord occlusion (UCO) of short duration through the later part of pregnancy in the ovine fetus has minimal effects on measures of cellular necrosis²³ and apoptosis²⁴ within the brain. However, decreases in low-voltage electrocortical activity²⁵ and BDNF immunoreactivity (IR)²⁶ have been noted and might impact growth processes within the brain, as seen in preterm animals with a selective decrease in structural proteins.²⁷ This tolerance of the fetal brain for cord occlusion insults is consistent with the apparent ability of the brain to protect cell membrane integrity and thus viability by shutting down nonessential metabolic activity.²⁸ Although protecting from cell death, the intermittent disruption of nonessential metabolic activity over longer antenatal time periods may then have consequences for altered growth processes as seen with the decrease in intermediate filament proteins²⁷ and likely extending to other proteins with high rates of turnover.

The high-molecular weight isoforms microtubule-associated proteins (MAPs) 2a and 2b are localized to the neuronal cell body and dendrites in the developing brain and have become an established marker for dendritic development.^{29,30} Synaptophysin (SYN) is an integral glycoprotein localized to the membrane of presynaptic vesicle boutons and has been used as a marker protein of presynaptic nerve development.^{31,32} In the present study, we have determined the IR of MAP-2 and SYN in the preterm and near-term ovine fetal brain after several days of intermittent cord occlusion insults to test the hypothesis that this insult will lead to a reduction in synapse formation.^{26,27} We have also determined the regional and developmental change in MAP-2 and SYN IR in these animals with advancing gestation to test the hypothesis that this will reflect maturational events with an expected increase in synapse formation over this time period.

Materials and Methods

Surgical Procedures

A total of 17 preterm (107–108 days gestation, 9 control, and 8 experimental) and 16 near-term (128–131 days gestation, 8 control, and 8 experimental) fetal sheep of mixed Western breed were prepared surgically for the study (term = 148 days). The surgical procedures and postoperative care have been described previously.^{23–25} In the preterm group animals, polyvinyl catheters (Bolab, Lake Havasu City, Arizona) were

placed in the right and left axillary arteries for monitoring fetal arterial blood pressure and for blood sampling; in the cephalic vein for administration of antibiotics; and in the amniotic cavity for pressure recording. In the experimental fetuses, a silicon rubber inflatable occluder cuff (In Vivo Metric, Healdsburg, California) was placed around the proximal portion of the umbilical cord and secured to the abdominal skin. The volume required for complete inflation of the occluder cuff was determined at the time of surgery. The near-term group animals were similarly instrumented, with a polyvinyl catheter additionally placed in the trachea for monitoring fetal breathing movements and with stainless steel electrodes implanted biparietally on the dura for the recording of electrocortical activity and through the lateral orbital ridge of the zygomatic bone of each eye for the recording of electro-ocular activity. The catheters, filled with heparinized saline, and the umbilical cord occluder were exteriorized to the flank of the ewe. In both animal groups, a polyvinyl catheter was additionally placed in the maternal femoral vein for administration of fluids. Postoperatively, the ewes were maintained in metabolic cages suitable for continuous monitoring and were allowed free access to food and water. A 3-day postoperative recovery period was allowed prior to initiation of experiments. Animal care followed the guidelines provided by the Canadian Council on Animal Care and experiments received ethics approval by the University of Western Ontario Council on Animal Care.

Experimental Protocol

Both preterm and near-term animals were studied similarly over a 4-day period. In experimental group animals, following a 1-hour control period, either 7 (days 1 and 4) or 11 (days 2 and 3) complete UCOs were carried out for 90 seconds every 30 minutes. Control animals did not receive UCOs. The time of day for study and the frequency, duration, and intensity of UCOs were similar in the preterm and the near-term animals.

Fetal arterial blood was sampled in both preterm and near-term experimental animals on days 1 and 4 for the first and last occlusions, 5 minutes prior to, 60 seconds into, and 5 minutes following occlusion. Fetal arterial blood was also sampled 1 hour following the final occlusion on each of these days. On days 2 and 3, fetal arterial blood was sampled 5 minutes prior to the first, sixth, and last occlusion. Fetal arterial blood was sampled from the control animals at similar time points except for the two 60-second cord occlusion samples on days 1 and 4. After the last blood sample on days 1 to 3, the fetus was transfused with an equivalent amount of maternal blood to correct for blood loss (~20 mL on day 1; ~12 mL on days 2 and 3). All blood samples were analyzed for P_{O_2} , P_{CO_2} , and pH using an ABL-500 blood gas analyzer (Radiometer Medical, Copenhagen, Denmark) with temperature corrected to 39.5°C.

Following the last blood sample on day 4 of study, the ewe and fetus were killed using an overdose of barbiturate (30 mg sodium pentobarbital intravenously; MTC Pharmaceuticals, Cambridge Canada) and an autopsy was done to confirm positioning of the catheters and the umbilical cord occluder.

Tissue Collection and Immunohistochemistry

Fetal brain tissue was collected, perfusion fixed, blocked in paraffin, and sectioned as described previously.²³ Other fetal tissues were also collected and either frozen in liquid nitrogen or placed in fixative for analysis in concurrent studies.

A standardized coronal section of the cerebral hemispheres taken through the mamillary bodies and including the hippocampus and thalamus was produced for each fetal brain. Immunoreactivity for MAP-2 and SYN was localized on the slides using the ABC immunohistochemical technique (Vectastain, Vector Labs, Burlingame, California) as described previously.²⁷ Primary antisera used were monoclonal mouse anti-bovine MAP-2 (Sigma, St Louis, Missouri, initial dilution 1:1000) and monoclonal mouse anti-bovine SYN (Dako Corporation, Carpinteria, California; initial dilution 1:20). Antigen retrieval was used for SYN staining with incubation in 10 mmol/L citrate buffer in a steam cooker for 25 minutes and then cooling for 25 minutes. All slides for each primary antibody were stained on the same day using the same solutions in order to minimize variation in intensity of stain. Negative controls included substitution of the primary antiserum with nonimmune serum to rule out nonspecific binding and confirmed absence of staining. Positive controls included staining tissue sections that were known to be positive for MAP-2 and SYN.

Quantification of Immunohistochemistry

Morphometric analysis was performed using a transmitted light microscope (Axioscope, Zeiss, Jena, Germany) at 400 $\times$ magnification and with the use of image analysis software for quantification as previously reported²⁶ (Northern Eclipse 2.0, Empix Imaging Co, Mississauga, Canada). Brain regions selected for analysis were those we had previously studied for changes in BDNF and tyrosine kinase receptor (TrkB)²⁶ and included cerebral mid-parasagittal gray matter (layers 1, 2/3, and 5/6), periventricular white matter, thalamus, and hippocampus (CA1 and dentate gyrus). Four to six high-power fields (HPFs) were analyzed for each region in each animal, and the values for each region then averaged for each animal. Values for the parasagittal gray matter layers 1, 2/3, and 5/6 were further averaged to obtain 1 gray matter value for each animal. Before measuring, the image analysis system was calibrated for the appropriate magnification settings used and all measurements were adjusted for uneven lighting and background staining. Each HPF included in the analysis was visualized and analyzed as images in Northern Eclipse. Each image was first converted to an 8-bit gray scale which changes the color of the stain to gray pixels. Each pixel reflects the inverse intensity of the stain (ie, darker staining objects are converted into lower arbitrary gray scale units). This conversion then allowed a threshold of staining to be selected, thereby allowing for discrimination of what was deemed to be specific staining from background staining.

The IR of MAP-2 was quantified through the selection of a uniform threshold for all brain regions which would include the

staining in the cell bodies and neuronal processes. This was accomplished by obtaining gray scale samplings of positive-stained areas from multiple brain regions of preterm and near-term control and UCO animals and testing for specificity against the negative and positive controls. Appropriate gray scale ranges for positive staining for area measurements were thus established and a uniform threshold applied to the calibrated images of all brain regions. The intensity of stain in gray scale units was calculated, with the data reported as average intensity of MAP-2 for the brain regions of the different animal groups. Because a lower gray scale value represents a greater intensity of stain, the average intensity is presented in the text and graphically as 1/gray scale unit. This transformation was performed following statistical analysis and was solely for the purpose of better illustrating changes in the true intensity of stain (ie, a greater intensity of stain is thereby reflected in a higher arbitrary value).

The IR of SYN is reported as the fractional area positively stained (ie, above the threshold chosen) for SYN and is expressed as a percentage of the total image area. The IR of SYN varied considerably across regions, with an abundance of staining in gray matter and scant staining in white matter. In order to quantify and compare staining between control and experimental groups in the preterm and near-term animals, images were converted to gray scale and a threshold was selected and applied in a similar manner to that described for MAP-2 but for each region separately. This allowed for a more accurate and realistic comparison for each individual region between control and experimental animals. As such, comparison was not made between the regions, only between experimental groups. All tissue staining and scoring of IR was performed in a blinded fashion to gestational age and to control/experimental groups. Cell counts were also done for all MAP-2 and SYN brain regions imaged since their abundance can also be impacted by neuronal density.

Data Analysis

Results obtained from all animals are presented as grouped mean $\pm$ standard error of the mean. Data sets were tested for normal distribution using the Kolmogorov-Smirnov test. In the preterm and near-term groups, the blood gas and pH values were analyzed using a repeated measures analysis of variance followed by a Student *t*-test with Bonferroni correction for multiple comparisons. The IR of MAP-2, SYN, and cell count data were analyzed using unpaired *t*-tests for differences between the animal groups. For all analyses, statistical significance was assumed as $P < .05$. As noted, the behavioral state and cerebral necrotic and apoptotic cell injury, structural protein, and BDNF findings for the animal groupings have been previously reported.²³⁻²⁷

Results

In both preterm and near-term animal groups, cord occlusion caused a large decline in fetal arterial Po_2 (preterm: $24.1 \pm$

Figure 1. Photomicrographs from the gray matter layer 2/3 of (A) preterm control and (B) near-term control group animals and from the hippocampus CA1 region of (C) near-term control and (D) near-term UCO group animals. MAP-2 immunoreactivity is seen in the cytoplasm of the neuronal cell bodies and processes, appearing as a fine network of positive staining in the neuropil. MAP-2 indicates microtubule-associated protein 2; UCO, umbilical cord occlusion. Scale bars = 50 μ m.

0.5 to 6.7 ± 0.8 mm Hg, $P < .01$; near term: 19.9 ± 0.7 to 7.6 ± 0.8 mm Hg, $P < .01$), and pH (preterm: 7.36 ± 0.01 to 7.30 ± 0.01 , $P < .01$; near term: 7.35 ± 0.01 to 7.30 ± 0.01 , $P < .01$) and a rise in P_{CO_2} (preterm: 52.3 ± 0.6 to 60.2 ± 1.0 mm Hg, $P < .01$; near term: 53.5 ± 0.7 to 61.1 ± 1.1 mm Hg, $P < .01$) as studied on days 1 and 4 but returned to control values after the occluder release. Baseline pre-cord occlusion measurements of blood gases and pH were unaltered over the course of each day of study for both the preterm and the near-term control and experimental group animals. However, baseline prefirst cord occlusion pH values did show a small fall over the 4 days of study in each of the study groups (preterm control 7.37 ± 0.01 to 7.32 ± 0.01 , $P < .01$; preterm cord occlusion: 7.38 ± 0.01 to 7.34 ± 0.02 ; near-term control: 7.36 ± 0.01 to 7.34 ± 0.01 , $P < .05$; near-term cord occlusion: 7.39 ± 0.02 to 7.35 ± 0.01 , $P < .05$).

In the control group animals with advancing gestational age from preterm to near-term, there was a significant decrease in total number of cells/area imaged in the gray matter layers 2/3 and 5/6, hippocampal dentate gyrus (both $P < .01$), and in the thalamus ($P < .05$). However, there was no difference in the total number of cells/area imaged between the occlusion

group animals and respective control group values for any of the brain regions studied.

Immunoreactivity of MAP-2

As studied, IR of MAP-2 was evident in both the cytoplasm of the neuronal cell bodies and the processes that appeared as a fine network of positive staining in the neuropil (Figure 1), and given its ubiquitous presence was analyzed as intensity of staining. The IR of MAP-2 for all brain regions studied was significantly decreased with advancing gestational age between the preterm and the near-term control group animals, gray matter 19%, white matter 9%, hippocampal CA1 10% and dentate gyrus 13%, and thalamus 21% ($P < .05$ to $P < .01$; Figure 2). This developmental decrease in IR of MAP-2 continued to be significant and much the same when normalized to the total number of cells/area imaged excepting that for the hippocampal CA1 region where this was still decreased, but no longer significant, and the gray matter layer 1 and periventricular white matter where cell numbers were too low to allow for meaningful analysis.

Figure 2. Bar graph of MAP-2 immunoreactivity expressed as intensity in gray scale units⁻¹ × 10³ in the different brain regions of the preterm (open bars) and near-term (light gray bars) control group animals. **P* < .05; ***P* < .01. MAP-2 indicates microtubule-associated protein 2.

Intermittent cord occlusion in the preterm group animals resulted in no significant change in IR of MAP-2 for any of the brain regions studied (Figure 3). Conversely, in the near-term animals, there was a significant increase in IR of MAP-2 in the cord occlusion animals compared to the controls in the hippocampal CA1 region and in the thalamus (*P* < .01 and *P* < .05, respectively; Figure 3).

Immunoreactivity of SYN

As studied, IR of SYN occurred in a widespread granular pattern within the neuropil consistent with localization of the presynaptic boutons (Figure 4), and given its more confined presence was analyzed as area of staining. The IR of SYN in the gray matter was significantly increased with advancing gestational age between the preterm and the near-term control group animals by 37% on average (*P* < .05) but with no significant change for the other brain regions studied (Figure 5). This developmental increase in IR of SYN within the gray matter was even greater in layers 2/3 and 5/6 when normalized to the total number of cells/area imaged, by 2-fold on average (both *P* < .01), but still with no significant change for the hippocampus CA1 and dentate gyrus and thalamus where cell counts were sufficient for meaningful analysis.

Intermittent cord occlusion in the preterm group animals resulted in a decrease in IR of SYN in all of the brain regions which was significant for the white matter (*P* < .05), the hippocampal CA1 region (*P* < .01), and the thalamus (*P* < .01) when compared to that of the control group animals (Figure 6).

Conversely, in the near-term animals there was no significant change in IR of SYN in the cord occlusion animals for any of the brain regions studied (Figure 6).

Discussion

Sheep and primates with peak growth velocity for the brain prior to birth are known as prenatal brain developers, whereas humans with peak growth velocity for the brain at birth are known as perinatal brain developers.¹⁰ These species with much of their early brain development occurring in utero are thereby more vulnerable to adverse pregnancy conditions and altered development with the potential for longer term neurologic sequelae. The current study used a model of intermittent cord occlusion to examine effects on MAP-2 and SYN as measures of synapse formation in the preterm and near-term ovine fetus which might then contribute to altered brain development. Although each cord occlusion insult resulted in a marked but limited drop in fetal arterial Po₂, no cumulative blood gas or pH changes were observed through each day of study in either the preterm or near-term animal groups, thus ensuring longer term survival and relevance of such study in terms of subsequent growth and development, including that of the brain. Although arterial pH did show a small fall over the 4 days of study possibly due to the chronic instrumentation, this was marginal and seen in all study groups and accordingly should not be contributory to the MAP-2 and SYN differences between the UCO and the control animals. Fetal brains were collected 1 hour after the last cord occlusion on day 4 with the changes in MAP-2 and SYN noted thereby presumed to reflect a cumulative response to chronic intermittent UCOs over the 4 days of study.

In the preterm and near-term control group animals, MAP-2 IR was seen to decrease in all brain regions examined with advancing age from ~0.75 to 0.90 of gestation, while IR of SYN was seen to increase in the gray matter over this time period. To date, there has been little study of synapse formation in the ovine species from a developmental standpoint, although the prominent occurrence of the juvenile MAP-1β and MAP-2c isoforms at this time suggests that neuronal outgrowth and synapse maturation have not been completed.³³ Indeed, as a prenatal brain developer a rapid increase in the density of synapses would be expected toward term as is seen in primates.^{10,11} Accordingly, the developmental increase in IR of SYN presently noted in the gray matter likely reflects an increase in the amount of presynaptic vesicles per synapse and/or a structural increase in presynaptic nerve terminals^{31,32} and mainly in the gray matter at this time since synapse formation in the cortex matures later than in subcortical areas.³⁴ Conversely, the developmental decrease in IR of MAP-2 in all brain regions was unexpected to the extent that presynaptic vesicle formation and postsynaptic dendritic branching occur concurrently,^{3,12} and the MAP-2 antibody herein used specifically targets the 2a and 2b isoforms and thereby dendritic processes, albeit with some staining of neuronal cell bodies at this stage of development.²⁹ As such, this decrease in IR of MAP-2

Figure 3. Bar graph of MAP-2 immunoreactivity expressed as intensity in gray scale units⁻¹ × 10³ in the different brain regions of the preterm control (open bars) and UCO (dark gray bars) and near-term control (light gray bars) and UCO (closed bars) animal groups. **P* < .05; ***P* < .01. MAP-2 indicates microtubule-associated protein 2; UCO, umbilical cord occlusion.

may instead reflect the change from an abundance of dendritic branching during early development with a decrease in size and numbers thereafter, but with a concurrent increase in the number of dendritic spines, which are morphologically distinct and more stable structures and better align with the developmental increase in functional synapses.⁴ Some of the decrease in IR of MAP-2 may also reflect the growth of the brain over this time period with a shift from neurogenesis to gliogenesis¹⁰ and a decrease in neuronal cell density, although the decrease in IR of MAP-2 continued to be evident even after normalizing for the cell counts imaged, thereby controlling for the developmental decrease in overall cell density. Intermittent cord occlusion over a 4-day period with marked but limited hypoxemia resulted in a significant increase in IR of MAP-2 in the CA1 and thalamus brain regions of the near-term animals but with no changes for any of the brain regions of the preterm animals. Reduction in IR of MAP-2 is widely accepted as an early marker of neural injury after hypoxic-ischemic insult and has also been shown in newborn rats³⁵ and piglets.³⁶ However, this is usually in association with some degree of necrotic/apoptotic cell loss and thereby indicating more severe and lethal brain injury, which was not the case in the present study as we have previously reported.^{23,24} Prolonged hypoxia in fetal sheep at midgestation³⁷ and induced growth restriction in fetal sheep³⁸ and guinea pigs³⁹ has been shown to alter dendritic morphology with decreased branching and elongation which might also be expected to decrease IR of MAP-2 although this has yet to be studied. Although the present findings of an increase in IR of MAP-2 in the near-term animals with the intermittent UCOS

is therefore somewhat surprising, this may instead reflect the sublethal nature of these insults and segmental dendritic swelling or beading as seen *in vitro* in neuronal cell cultures after oxygen/glucose deprivation.⁴⁰ The increase in IR of MAP-2 as a reflection of reactive dendritic change with the UCOS was greatest in the hippocampal CA1 region which might well be expected with this region particularly sensitive to hypoxic-ischemic insult during fetal development and more so closer to term.^{38,41}

Contrary to the MAP-2 findings, intermittent UCOS over 4 days resulted in a decrease in IR of SYN in all brain regions of the preterm animals which was significant for the white matter, CA1, and thalamus but with no changes for any of the brain regions of the near-term animals. This would indicate a differential effect of these sublethal insults on presynaptic vesicle formation and dendritic branching and spine formation and thereby their regulatory mechanisms, to the extent that IR of SYN and MAP-2 broadly reflects these respective aspects of synaptogenesis. Although there has been considerable study of dendritic morphology and/or MAP-2 in the fetus or perinate in response to hypoxic conditioning as noted, there has been no parallel study of presynaptic vesicle formation and/or related protein markers such as SYN. The present findings with a decrease in IR of SYN in the preterm animals but no change in the near-term animals are similar to that we have reported for intermediate filament proteins²⁷ and BDNF and its high-affinity receptor TrkB²⁶ in these animals. This decrease in BDNF expression in response to intermittent hypoxic insult during the brain's development is likely protective to the extent that BDNF/TrkB-mediated effects on developing neurons,

Figure 4. Photomicrographs from the gray matter layer 2/3 of (A) preterm control and (B) near-term control group animals, and from the hippocampus CA1 region of (C) preterm control and (D) preterm UCO group animals. SYN immunoreactivity is seen in a widespread granular pattern within the neuropil. SYN indicates synaptophysin; UCO, umbilical cord occlusion. Scale bars = 50 μ m.

including synapse formation, require increased protein synthesis thereby enhancing energy demand.^{4,8} It is thereby likely that protein synthesis as an energy-using process is also disrupted with intermittent hypoxic insults and more so during earlier brain development, given the higher protein synthesis/degradation at this time,²⁶ thereby accounting for the decrease in intermediate filament proteins for the preterm animals that we previously reported²⁷ and the decrease in IR of SYN now shown. Of note, the sublethal nature of the UCO insults studied while sufficient to impact growth processes within the brain^{26,27} have minimal effect on cellular necrosis/apoptosis^{23,24} consistent with the lack of any change in cellular density herein observed and accordingly should not be contributory to the present UCO-related findings.

In the present study, we have determined the developmental change in IR of MAP-2 and SYN in the ovine fetal brain with advancing gestation and in response to intermittent cord compression with marked but limited hypoxemia and no cumulative acidosis to ensure longer term survival. Immunohistochemical methods use relative saturation methodology resulting in imprecise relationships between the amount of a given protein and its IR and thereby in quantification techniques using image analysis that are semiquantitative at best.

Figure 5. Bar graph of SYN immunoreactivity expressed as percentage area positively stained in the different brain regions of the preterm (open bars) and near-term (light gray bars) control group animals. * $P < .05$. SYN indicates synaptophysin.

Figure 6. Bar graph of SYN immunoreactivity expressed as percentage area positively stained in the different brain regions of the preterm control (open bars) and UCO (dark gray bars) and near-term control (light gray bars) and UCO (closed bars) animal groups. * $P < .05$; ** $P < .01$. SYN indicates synaptophysin, UCO, umbilical cord occlusion.

Furthermore, the quantitative relationship between the amount of MAP-2 and the SYN (and thereby in their IR even if precisely measured) and dendritic branching versus dendritic spines, and presynaptic vesicle boutons versus synaptic contacts, respectively, is unknown and likely changes with the brain's development.^{4,42} Despite these limitations, when determining the intensity of staining as herein with MAP-2, or area of staining as herein with SYN, immunohistochemistry still serves as a useful method of quantification of the distribution of a given protein and by inference, the relative quantity in a brain region. Accordingly, the present findings support a developmental increase in presynaptic vesicles and/or nerve terminals in the gray matter of the ovine fetal brain over the latter part of pregnancy as expected but with concurrent refinement in dendritic branching and spine development which might then underlie the later elimination of redundant synapses.^{1-3,9}

Fetal growth restriction which is known to be associated with chronic hypoxemia⁴³ is a risk factor for a range of neurologic adversities which may have a common basis with altered neuronal connectivity involving synapse and dendritic formation.^{13-17,44,45} This has led to considerable animal modeling in fetal sheep and guinea pigs and showing altered presynaptic and dendritic development with induced growth restriction.^{38,39,46} We now add to these findings showing that intermittent UCO as studied also impacts marker proteins for synaptogenesis, with IR of SYN overall decreased in the preterm animals when protein turnover is higher^{26,27} and indicating decreased presynaptic vesicle formation; whereas, IR of MAP-2 was selectively increased in the hippocampus and thalamus of the near-term animals, consistent with heightened

vulnerability for neuronal injury at this time.^{38,41} Most importantly, these findings indicate that intermittent cord compressions as herein studied in the ovine fetus have the potential to disrupt synapse formation in the absence of gross neuronal injury. It remains to be determined whether such insults if more frequent and/or severe can lead to persistent changes in neural circuitry and underlie the increased risk of later subclinical neurodevelopmental deficits reported in infants with a symptomatic or tight nuchal cord at delivery.²¹

Acknowledgments

We thank Dr Tenille Paulsen, Ms Karen Nygard, Mr Jack Homan, Mr Brad Matuszewski, and Ms Jennifer Ryder for technical assistance and Mr Larry Stitt for statistical support.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Supported by grants from the Canadian Institutes of Health Research (BSR). BSR is the recipient of a Canada Research Chair Tier I in Fetal and Neonatal Health and Development.

References

- Whitford KL, Dijkhuizen P, Polleux F, Ghosh A. Molecular control of cortical dendrite development. *Annu Rev Neurosci.* 2002;25:127-149.

2. Scheiffele P. Cell-cell signaling during synapse formation in the CNS. *Annu Rev Neurosci.* 2003;26:485-508.
3. Hua JY, Smith SJ. Neural activity and the dynamics of central nervous system development. *Nat Neurosci.* 2004;7(4):327-332.
4. Shen K, Cowan CW. Guidance molecules in synapse formation and plasticity. *Cold Spring Harb Perspect Biol.* 2010;2(4):a001842.
5. Vanderhaeghen P, Chen HJ. Guidance molecules in axon pruning and cell death. *Cold Spring Harb Perspect Biol.* 2010;2(6):a001859.
6. Coffey ET, Akerman KEO, Courtney MJ. Brain derived neurotrophic factor induces a rapid upregulation of synaptophysin and tau proteins via the neurotrophin receptor TrkB in rat cerebellar granule cells. *Neurosci Lett.* 1997;227(3):177-180.
7. Tang SJ, Schuman EM. Protein synthesis in the dendrite. *Philos Trans R Soc Lond B Biol Sci.* 2002;357(1420):521-529.
8. Burkhalter J, Fiumelli H, Allaman I, Chatton JY, Martin JL. Brain-derived neurotrophic factor stimulates energy metabolism in developing cortical neurons. *J Neurosci.* 2003;23(23):8212-8220.
9. Penn AA, Shatz CJ. Brain waves and brain wiring: the role of endogenous and sensory-drive neural activity in development. *Pediatr Res.* 1999;45(4 pt 1):447-458.
10. McIntosh GH, Baghurst KI, Potter BJ, Hetzel BS. Foetal brain development in the sheep. *Neuropathol Appl Neurobiol.* 1979;5(2):103-114.
11. Bourgeois JP. Synaptogenesis, heterochrony and epigenesis in the mammalian neocortex. *Acta Paediatr Suppl.* 1997;422:27-33.
12. Huttenlocher PR, Dabholkar AS. Regional differences in synaptogenesis in human cerebral cortex. *J Comp Neurol.* 1997;387(2):167-178.
13. Low J, Handley Derry MH, Burke SO, et al. Association of intrauterine fetal growth retardation and learning deficits at age 9 to 11 years. *Am J Obstet Gynecol.* 1992;167(6):1499-1505.
14. Berg AT. Indices of fetal growth-retardation, perinatal hypoxia-related factors and childhood neurological morbidity. *Early Hum Dev.* 1989;19(4):271-283.
15. Dalman C, Jones PB, Murray RM. Signs of asphyxia at birth and risk of schizophrenia. Population-based case-control study. *Br J Psychiatry.* 2001;179:403-408.
16. Cannon M, Jones PB, Murray RM. Obstetric complications and schizophrenia: historical and meta-analytic review. *Am J Psychiatry.* 2002;159(7):1080-1092.
17. Hultman CM, Sparén P, Cnattingius S. Perinatal risk factors for infantile autism. *Epidemiology.* 2002;13(4):417-423.
18. Anyaegbunam A, Brustman L, Divon M, Langer O. The significance of antepartum variable decelerations. *Am J Obstet Gynecol.* 1986;155(4):707-710.
19. Hoskins IA, Frieden FJ, Young BK. Variable decelerations in reactive nonstress tests with decreased amniotic fluid index predict fetal compromise. *Am J Obstet Gynecol.* 1991;165(4 pt 1):1094-1098.
20. Osak R, Webster K, Bocking A, Campbell KJ, Richardson B. Nuchal cord evidence at birth impacts on fetal size relative to that of the placenta. *Early Hum Dev.* 1997;49(3):193-202.
21. Clapp JF, Lopez B, Simonean S. Nuchal cord and neurodevelopmental performance at 1 year. *J Soc Gynecol Investig.* 1999;6(5):268-272.
22. Nelson KB, Grether JK. Potentially asphyxiating conditions and spastic cerebral palsy in infants of normal birth weight. *Am J Obstet Gynecol.* 1998;179(2):507-551.
23. Rocha E, Hammond R, Richardson B. Necrotic cell injury in the preterm and near-term ovine fetal brain after intermittent umbilical cord occlusion. *Am J Obstet Gynecol.* 2004;191(2):488-496.
24. Falkowski A, Hammon R, Han V, Richardson B. Apoptosis in the preterm and near-term ovine fetal brain and the effect of intermittent umbilical cord occlusion. *Brain Res Dev Brain Res.* 2002;136(2):165-173.
25. Kawagoe Y, Green L, White S, Richardson B. Intermittent umbilical cord occlusion in the ovine fetus near term: effects of behavioral state activity. *Am J Obstet Gynecol.* 1999;181(6):1520-1529.
26. Nishigori H, Mazzuca DM, Nygard KL, Han VK, Richardson BS. BDNF and TrkB in the preterm and near-term ovine fetal brain and the effect of intermittent umbilical cord occlusion. *Reprod Sci.* 2008;15(9):895-905.
27. Rocha E, Totten S, Hammond R, Han V, Richardson B. Structural proteins during brain development in the preterm and near-term ovine fetus and the effect of intermittent umbilical cord occlusion. *Am J Obstet Gynecol.* 2004;191(2):497-506.
28. Kaneko M, White S, Homan J, Richardson B. Cerebral blood flow and metabolism in relation to electrocortical activity with severe umbilical cord occlusion in the near-term ovine fetus. *Am J Obstet Gynecol.* 2003;188(4):961-972.
29. Tucker RP. The roles of microtubule-associated proteins in brain morphogenesis: a review. *Brain Res Brain Res Rev.* 1990;15(2):101-120.
30. Johnson GV, Jope RS. The role of microtubule-associated protein 2 (MAP-2) in neuronal growth, plasticity, and degeneration. *J Neurosci Res.* 1992;33(4):505-512.
31. Eastwood SL, Burnet PW, McDonald B, Clinton J, Harrison PJ. Synaptophysin gene expression in human brain: a quantitative in situ hybridization and immunocytochemical study. *Neuroscience.* 1994;59(4):881-892.
32. Tarsa L, Goda Y. Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons. *Proc Natl Acad Sci U S A.* 2002;99(2):1012-1016.
33. Schwab M, Antonow-Schlorke I, Kühn B, et al. Effect of antenatal betamethasone treatment on microtubule-associated proteins MAP1 β and MAP2 in fetal sheep. *J Physiol.* 2001;530(pt 3):497-506.
34. Morest DK. The growth of dendrites in the mammalian brain. *Z Anat Entwicklungsgesch.* 1969;128(4):290-317.
35. Ota A, Ikeda T, Ikenoue T, Toshimori K. Sequence of neuronal responses assessed by immunohistochemistry in the newborn rat brain after hypoxia-ischemia. *Am J Obstet Gynecol.* 1997;177(3):519-526.
36. Lingwood BE, Healy GN, Sullivan SM, Pow DV, Colditz PB. MAP2 provides reliable early assessment of neural injury in the newborn piglet model of birth asphyxia. *J Neurosci Methods.* 2008;171(1):140-146.
37. Rees S, Breen S, Loeliger M, McCrabb G, Harding R. Hypoxemia near mid-gestation has long-term effects on fetal brain development. *J Neuropathol Exp Neurol.* 1999;58(9):932-945.

38. Rees S, Breen S, Cock M, Mallard C, Stringer M, Harding R. Fetal brain injury following prolonged hypoxemia and placenta insufficiency: a review. *Comp Biochem Physiol A MollIntegr Physiol.* 1998;119(3):653-660.
39. Dieni S, Rees S. Dendritic morphology is altered in hippocampal neurons following prenatal compromise. *J Neurobiol.* 2003;55(1):41-52.
40. Park JS, Bateman MC, Goldberg MP. Rapid alterations in dendrite morphology during sublethal hypoxia or glutamate receptor activation. *Neurobiol Dis.* 1996;3(3):215-227.
41. Drury PP, Bennet L, Booth LC, Davidson JO, Wassink G, Gunn AJ. Maturation of the mitochondrial redox response to profound asphyxia in fetal sheep. *PLoS One.* 2012;7(6):e39273.
42. Calhoun ME, Jucker M, Martin LJ, Thinakaran G, Price DL, Mouton PR. Comparative evaluation of synaptophysin-based methods for quantification of synapses. *J Neurocytol.* 1996;25(12):821-828.
43. Cox WL, Daffos F, Forestier F, et al. Physiology and management of intrauterine growth retardation: a biologic approach with fetal blood sampling. *Am J Obstet Gynecol.* 1988;159(1):36-41.
44. West AE, Greenberg ME. Neuronal activity-regulated gene transcription in synapse development and cognitive function. *Cold Spring Harb Perspect Biol.* 2011;3(6).
45. Stamou M, Streifel KM, Goines PE, Lein PJ. Neuronal connectivity as a convergent target of gene $\times$ environment interactions that confer risk for autism spectrum disorders. *Neurotoxicol Teratol.* 2013;36:3-16.
46. Rees S, Harding R, Walker D. An adverse intrauterine environment: implications for injury and altered development of the brain. *Int J Dev Neurosci.* 2008;26(1):3-11.