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Maternal Treatment with Glucocorticoids Modulates Gap Junction Protein Expression in the Ovine Fetal Brain

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Abstract

Gap junctions facilitate intercellular communication and are important in brain development. Connexins (Cx) comprise a transmembrane protein family that forms gap junctions. Cx-32 is expressed in oligodendrocytes and neurons, Cx-36 in neurons, and Cx-43 in astrocytes. Although single antenatal steroid courses are recommended for fetal lung maturation, multiple courses can be given to women at recurrent risk for premature delivery. We examined the effects of single and multiple glucocorticoid courses on Cx-32, Cx-36, and Cx-43 protein expression in fetal cerebral cortex, cerebellum, and spinal cord, and differences in connexin expression among brain regions under basal conditions. In the single course groups, the ewes received dexamethasone (6 mg) or placebo as four intramuscular injections every 12 h over 48 h. In the multiple course groups, the ewes received the same treatment, once a week for five weeks starting at 76–78 days of gestation. Connexins were measured by Western immunoblot on brain samples from 105–108 day gestation fetuses. A single dexamethasone course was associated with increases ($P < 0.05$) in cerebral cortical and spinal cord Cx-36 and Cx-43 and multiple courses with increases in cerebellar and spinal cord Cx-36, and cerebral cortical and cerebellar Cx-43. Cx-32 did not change. Cx-32 was higher in cerebellum than cerebral cortex and spinal cord, Cx-36 higher in spinal cord than cerebellum, and Cx-43 higher in cerebellum and spinal cord than cerebral cortex during basal conditions. In conclusion, maternal glucocorticoid therapy increases specific connexins, responses to different maternal courses vary among connexins and brain regions, and connexin expression differs among brain regions under basal conditions. Maternal treatment with glucocorticoids differentially modulates connexins in the fetal brain.

Keywords

Connexins; brain; fetus; gap junctions; glucocorticoids; sheep

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DISCLOSURE

The authors have no duality or conflicts of interests to declare.

Gap junctions are specialized membrane structures that mediate cell-to-cell communication via channels between adjacent cells. These junctions enable direct exchange of intercellular messengers that coordinate tissue homeostasis, proliferation, differentiation, metabolism, cell survival, and death (Bennett et al., 1991, Nagy and Rash, 2000, Bruzzone and Dermietzel, 2006, Eugenin et al., 2012). Connexins comprise a multigene family of transmembrane intercellular channel-forming membrane proteins (Herve et al., 2007). Intercellular communication via gap junctions is particularly important in organs with extensive tight junctions such as brain (Michelle et al., 2012).

Gap junctions couple neurons and glial cells in the central nervous system (CNS) in adults and during development (Rozental et al., 2000). Intercellular communication through gap junction channels is a prominent feature of the developing CNS (Bittman et al., 2002, Bruzzone and Dermietzel, 2006). Moreover, the expression of specific connexin proteins varies among different brain regions, developmental stages, and cell types (Orellana et al., 2009).

Connexin-32, -36, and -43 are the major connexin proteins expressed in the developing brain (Bittman et al., 1997, Bittman et al., 2002). Connexin-32 is found in Schwann cells and oligodendrocytes. It plays an important role in myelin forming cells and in interneuron synchronization (Dermietzel et al., 1989, Scherer et al., 1995, Li et al., 2004, Montoro and Yuste, 2004, Nagy et al., 2004). Gap junctions between neurons are formed by connexin-36 (Sutor and Haggerty, 2005). The principal gap junction protein in astrocytes is connexin-43 (Dermietzel et al., 1991), which has an organizational role during neural development (Katbamna et al., 2004, Bruzzone and Dermietzel, 2006).

We have previously shown that connexin-32 and -43 proteins are expressed in brain from early in fetal life and throughout ovine development. However, the developmental patterns of these connexins differ. Connexin-43 increases from 60% of gestation up to maturity in adults, whereas connexin-32 decreases during development (Sadowska et al., 2009). Furthermore, there is increasing evidence that unopposed connexin hemi-channels or connexons also could play a role in perinatal brain injury by mediating release of paracrine molecules that propagate cell death signals (Davidson et al., 2012a, Davidson et al., 2012b, Davidson et al., 2013a, Davidson et al., 2013b). Therefore, a greater understanding of connexins under various experimental conditions in the brain during fetal development is important.

Antenatal glucocorticoids are routinely given to women at risk for premature labor to reduce the incidence of respiratory distress syndrome in preterm infants (Brownfoot et al., 2013). However, there is ongoing debate regarding the optimal dose, type of glucocorticoid, and regimen to accelerate pulmonary maturation versus potential harmful effects to other organs including the brain (Crowther and Harding, 2007, Stiles, 2007, Brownfoot et al., 2013).

We have previously shown that both single and multiple courses of maternal glucocorticoids reduce blood-brain barrier permeability in the ovine fetus (Stonestreet et al., 2000, Sadowska et al., 2006). In addition, we have shown that this maternal treatment affects several proteins critical to CNS homeostasis in the fetal brain including tight junction

proteins and Na⁺,K⁺-ATPase (Malaeb et al., 2009, Mehter et al., 2009, Sadowska et al., 2010). Tight junction proteins are well known to be vital constituents of the endothelial tight junction, which regulate blood-brain barrier function (Abbott et al., 2010). Connexins are increasingly recognized as important partners in the tight junctional complex (Meyer et al., 1992, Derangeon et al., 2009) and recent evidence suggests that connexins can also influence barrier function in the CNS (De Bock et al., 2014). Glucocorticoids have also been shown to affect connexins in several organ systems other than the brain (Kwiatkowski et al., 1994, Sasson and Amsterdam, 2002, Rafacho et al., 2007). Dexamethasone increased connexin-32 mRNA levels in rat hepatocyte cultures, connexin-36 mRNA and protein expression in pancreatic rat islets, and connexin-43 protein expression in human granulosa cells (Kwiatkowski et al., 1994, Sasson and Amsterdam, 2002, Rafacho et al., 2007). A recent report also indicates that dexamethasone treatment increases connexin-43 expression and the number of coupled astrocytes in primary glial cocultures of newborn rats (Hinkerohe et al., 2011). Taken together, there is sufficient evidence to consider that maternal treatment with glucocorticoids in doses, which are similar to those used to treat women in premature labor (Brownfoot et al., 2013), could also influence the expression of connexins within the fetal brain.

The purpose of current study was to examine the effects of maternal treatment with single and multiple courses of dexamethasone on connexin-32, -36, and -43 protein expression in the cerebral cortex, cerebellum, and cervical spinal cord of the preterm fetal sheep brain. Full term gestation in sheep is 145–147 days. Therefore, the neurodevelopment of the sheep fetus in the current study at 107 days of gestation, is approximately similar to that of preterm human infants between 28 to 32 weeks of gestation (McIntosh et al., 1979, Back et al., 2006, Koome et al., 2013).

EXPERIMENTAL PROCEDURES

The study was conducted after approval by the Institutional Animal Care and Use Committees of the Alpert Medical School of Brown University and Women & Infants Hospital of Rhode Island according to the National Institutes of Health Guidelines for the use of experimental animals.

Animal Preparation and Experimental Design

The samples of fetal brain in this study were obtained from animals enrolled in our previous studies (Hai et al., 2002, Kerzner et al., 2002, Stonestreet et al., 2004, Sadowska et al., 2006, Sadowska et al., 2010). The surgical and experimental procedures have been previously reported (Stonestreet et al., 1996, Sadowska et al., 2006). Surgery was performed under 1–2% halothane anesthesia on the 28 mixed breed pregnant ewes at 99 to 102 days of gestation as previously described in detail (Stonestreet et al., 1996). Briefly, polyvinyl catheters were placed into a brachial vein of the fetus for isotope administration and the thoracic aorta via the brachial artery for blood sample withdrawal for the studies designed to quantify blood-brain barrier permeability (Sadowska et al., 2006). Pregnant ewes were included in four groups as follows: (1) Single placebo course (n=6) or (2) dexamethasone (n=7) or (3) five placebo courses (n=6) or (4) five dexamethasone courses (n=9). The pregnant ewes assigned

to the single course groups were given four intramuscular injections of 6 mg of dexamethasone (Fujisawa USA, Deerfield, IL, concentration=4 mg/ml) or placebo (0.9% NaCl, 1.5 ml) over 48 hours every 12 hours. The pregnant ewes assigned to the multiple course groups were given the identical treatment once a week for five successive weeks beginning at 76–78 days of gestation as we previously described (Kerzner et al., 2002, Sadowska et al., 2006). After the pregnant sheep were given the fourth course of glucocorticoids, the fetuses and ewes were then catheterized at 99–101 days of gestation, so that the timing of surgery in the fetuses and ewes exposed to the multiple courses would be the identical to those that were exposed to the single courses. The fifth and last course was begun on the fourth day after surgery at 104–107 days of gestation.

The rationale for choosing this regimen (i.e. 6 mg of dexamethasone every 12 hours for 48 hours) was to use a schedule and doses of dexamethasone similar to those recommended to enhance fetal maturation in women at risk for preterm delivery (Consensus, 1995). In addition, pregnant women who are at continued risk for premature deliver can be exposed to several courses of glucocorticoids. The treatment of the pregnant ewes assigned to the multiple course groups was begun at 76–78 days of gestation. Although this is slightly earlier than treatment with glucocorticoids would begin in human pregnancies (22–23 weeks or 55–57% of gestation), the fetal sheep brain at 127 days of gestation (85% of gestation) is thought to be similar to the near term human brain (Barlow, 1969, Gunn et al., 1997). Hence, the fetal sheep brain is relatively more mature at full term gestation *e.g.* 147–148 days of gestation compared with the human fetal brain at full term (39–40 weeks). Consequently, the onset of administration of glucocorticoids at 52% of the ovine gestation does not differ substantially from that of the human fetus at 22–23 weeks of gestation (55–57%) of the human pregnancy. In our original studies (Sadowska et al., 2006), we selected this early time in gestation because we sought measure blood-brain barrier permeability after these antenatal treatments in preterm fetal sheep (Sadowska et al., 2006).

The ewes in single and multiple course groups stayed in the animal care facility for different durations, *e.g.* approximately two weeks in the single and six weeks in the multiple course groups. As a result, the two groups of ewes could have received slightly different diets as previously described (Gray et al., 2006) and experienced different activity levels, *i.e.* potentially more activity in the single than multiple course groups, as the single course groups remained on the farm for a longer duration than the multiple course groups before the commencement of the studies.

The fetal cerebral cortical, cerebellar, and cervical spinal cord samples were harvested and placed in liquid nitrogen 18 hours after the final dose of dexamethasone or placebo was given to the ewes at 105–108 days (70%) of gestation (Sadowska et al., 2010). The cerebral cortical samples were obtained from the parietal cortex most likely contained primarily grey matter and some white matter. The parietal cortex was examined because it represents a relatively large proportion of the fetal sheep cerebral cortex. Although the cerebral cortex contains mostly grey matter, based upon scanning and area fraction measurements (NIH Image J 1.45k, Softonic, International) of Luxol fast blue/H&E stained sections from our previous work (Petersson et al., 2002), we estimate that white matter represents approximately 27% of the parietal cortex in fetal sheep at 85% of gestation. We do not have

slides from brains of fetal sheep at similar gestational ages to those in the current study. Therefore, we can only estimate that the frozen samples of parietal cortex at 107 days (73%) of gestation could have contained approximately 20% white matter based upon the earlier gestational age when less white matter is present (Grever et al., 1996). The cerebellum contains both grey and white matter. In contrast, the cervical spinal cord contains mostly white matter. We selected these regions for analysis, as they are major regions from the rostral and hindbrain with different developmental trajectories (Barlow, 1969). The brain samples were kept at -80°C until the analysis.

Protein Extraction

Cell membrane fractions from the regional brain samples were extracted as we previously described (Sadowska et al., 2010). Total protein concentrations of the homogenates were determined with a bicinchoninic acid protein assay (BCA, Pierce, Rockford, IL) and the extracted aliquots stored at -80°C for later analysis.

Western Immunoblot Detection and Quantification of Proteins

Fifty micrograms of total protein per well was fractionated using SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) by the use of a semi-dry technique. After blocking the membranes with 10% non-fat milk for 1 h at room temperature, they were washed in Tris-buffered saline with 0.1% Tween-20 (TBST) 3 times for 10 minutes for each wash. Membranes were probed for connexin-32 (cat # 13-8200, Zymed, San Francisco, CA), connexin-36 (ca# 37-4600, Zymed), and connexin-43 (ca# 13-8300, Zymed) with primary mouse monoclonal antibodies at a dilution of 1:5,000 and for vinculin with primary mouse monoclonal antibodies (cat# MA1-83523, Thermo Scientific™ Pierce, Waltham, MA) at a dilution of 1:10000. After overnight incubation with primary antibodies at 4°C , the immunoblots were washed in TBST three times and then incubated for 1 h at room temperature with goat anti-mouse (Zymed) horseradish peroxidase conjugated secondary antibodies at a dilution of 1:10,000 for each of the proteins examined. Immunoblots were again washed in TBST 4 times. Secondary antibody binding was detected by the use of enhanced chemiluminescence (ECL plus, Western Blotting Detection reagents, Amersham Pharmacia Biotech, Inc., Piscataway, NJ) before exposing the film to autoradiography (Daigger, Vernon Hills, IL).

The experimental samples were normalized to a protein standard obtained from a brain of fetal sheep at 70% of gestation that was not part of the current study. As an example, the study samples for connexin-43 from the cerebral cortex were normalized by the use of connexin-43 protein obtained from the cerebral cortex of a single fetal sheep that had not been exposed to any of the experimental interventions. These standard samples in the current report are designated as internal control samples. As we have previously reported (Ron et al., 2005, Kim et al., 2006, Malaeb et al., 2007, Sadowska et al., 2009, Sadowska et al., 2010), these samples serve as internal controls for loading quality, sample transfer in order to normalize the regional brain densitometric values. The use of the internal control standard is unique to our laboratory and allows for the comparison of large groups of animals over several different immunoblots. The experimental protein autoradiographic densitometrical values were expressed as a ratio to the internal control protein standards,

thus facilitating normalized comparisons among the various groups and several different immunoblots. Vinculin was also used as a loading control to ensure that equal amounts of protein had been applied to each lane. However, the experimental protein bands were normalized to the internal control standards rather than to vinculin.

All immunoblots contained samples from the four experimental groups and the three internal control standard samples that were placed as the first, middle, and last samples on each immunoblot. We calculated a coefficient of variation for the internal control standard samples on each immunoblot and accepted the experimental samples as valid only when the percent coefficient of variation for the internal control samples was <20% on the immunoblot. Each immunoblot also contained molecular weight standards (Bio-Rad Laboratories, Hercules, CA). Inter-lane loading uniformity was confirmed with Coomassie blue (Sigma, St. Louis, MO) staining of the polyacrylamide gels and transfer uniformity to the polyvinylidene difluoride membranes by Ponceau S staining (Sigma, St. Louis, MO) (Tseng et al., 2005).

Rat liver was used as a positive control for connexin-32 and rat brain for connexin-36 and -43. Positive controls were included on the same immunoblot with the brain samples to determine that the proteins identified by these antibodies in the sheep brain were the same as the known protein for which these antibodies were specific. Detection of connexin-32, -36 and -43 bands at 32, 36 and 43 kDa, respectively, was dependent upon incubation with primary antibody, the omission of which resulted in the absence of the signal.

Relative differences in connexin protein expression among the cerebral cortical, cerebellar, and cervical spinal cord brain regions were also examined under basal conditions. In order to examine basal variations in protein expression among the brain regions, we measured the expression of each protein from fetuses of ewes treated with a single course of placebo. For this analysis, we normalized each protein to an internal control standard sample obtained from the cerebral cortex of a non-study fetal sheep to facilitate the comparisons of the three brain regions to a single standard on the same immunoblot.

Samples from the four groups were placed on each immunoblot to permit identical standard conditions among the tissue samples. However, in order to allow graphic clarity, to facilitate group comparisons among the brain regions, and to put the immunoblots above the bar graphs, we selected specific immunoblots that most closely represented the mean expression value for each bar graph value in each tissue.

Densitometric Analysis

We analyzed band intensities with a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) and normalized the study samples to the respective internal controls using the average of the three internal control standard values from each immunoblot. The final values were an average of the densitometry values obtained from at least two different immunoblots and shown as a ratio to the internal control standard samples. As we reported, this method correlates well with values that were normalized as ratios to β -actin (Kim et al., 2006), but our method facilitates comparisons of values derived from more than one immunoblot.

Statistical Analysis

Results were expressed as means $\pm$ SD. Two-way analysis of variance (ANOVA) was used to compare values among fetuses of the ewes exposed to the single or multiple dexamethasone or placebo courses. If there was a significant difference by ANOVA, we used the Fischer's protected least-significant difference (LSD) post hoc test to detect differences among the various groups. However, we unexpectedly found significant differences between single and multiple course placebo treated groups. Consequently, we did not compare the groups treated with the single and multiple courses of glucocorticoids by post hoc analysis. Differences were examined amongst the three brain regions for each gap junction protein by using one-way ANOVA. The expressions of connexin-32, -36, and -43 were also compared within each brain region by one-way ANOVA. When a significant difference was detected by ANOVA, specific differences among the brain regions were detected by the Fischer LSD test. A P-value of <0.05 was considered statistically significant.

RESULTS

Connexin 36

Increases in connexin-36 protein expression were detected in the cerebral cortex after maternal treatment with a single course of dexamethasone, but multiple courses of dexamethasone did not affect cerebral cortical connexin-36 expression (Fig. 1A, ANOVA, main effect for dexamethasone versus placebo treatment, $F=13.78$, $P<0.01$; main effect for course frequency, single versus multiple courses, $F=2.47$, $P=0.13$). In contrast, maternal treatment with multiple courses of dexamethasone was associated with increases in connexin-36 expression in the cerebellum, but a single course of dexamethasone did not affect its expression in the cerebellum (Fig. 1B; ANOVA, interactions for dexamethasone versus placebo treatment, and course frequency, single versus multiple courses, $F=6.87$, $P<0.02$). Unexpectedly, connexin 36 expression in the cerebellum was higher after maternal treatment with a single course of placebo compared with the multiple courses (ANOVA, main effect for course frequency, single versus multiple courses, $F=5.27$, $P<0.05$). Maternal treatment with both single and multiple courses of dexamethasone was associated with increases in connexin-36 in the fetal cervical spinal cord (Fig. 1C, ANOVA, main effect for dexamethasone versus placebo treatment, $F=13.37$, $P<0.01$; main effect for course frequency, single versus multiple courses, $F=2.15$, $P=0.16$).

Connexin 43

Maternal therapy with both single and multiple courses of dexamethasone were associated with increases in connexin 43 protein expression in the cerebral cortex (Fig. 2A, ANOVA, main effect for dexamethasone versus placebo treatment, $F=15.18$, $P<0.01$). Unexpectedly, connexin 43 expression in the cerebral cortex was also higher after maternal treatment with multiple than a single course of placebo (ANOVA, main effect for course frequency, single versus multiple courses, $F=36.34$, $P<0.01$). Maternal treatment with multiple courses of dexamethasone was associated with increases in connexin-43 expression in the cerebellum, but a single course of dexamethasone did not affect its expression in cerebellum (Fig. 2B; ANOVA, main effect for dexamethasone versus placebo treatment, $F=14.33$, $P<0.01$; main effect for course frequency, single versus multiple courses, $F=0.0$, $P=1.0$). Maternal therapy

with a single course of dexamethasone was associated with increases in connexin-43 in the fetal cervical spinal cord, but multiple courses of dexamethasone did not affect its expression in spinal cord (Fig. 2C, ANOVA, main effect for dexamethasone versus placebo treatment, $F=16.13$, $P<0.01$; main effect for course frequency, single versus multiple courses, $F=0.87$, $P=0.36$).

Connexin 32

Maternal dexamethasone treatment was not associated with increases in connexin-32 expression in the fetal cerebral cortex, cerebellum, or cervical spinal cord (Fig. 3 A–C).

In table 1, we have schematically summarized the changes in connexin protein expression in the fetuses of ewes exposed to the single and multiple dexamethasone courses compared with the fetuses of the ewes that were placebo treated.

Connexin Expression under Basal Conditions

The relative expression of connexin-32, -36 and -43 proteins in the cerebral cortex, cerebellum, and cervical spinal cord in fetuses of ewes exposed to a single course of placebo were compared in order to determine if there were any differences in gap junction protein expression among these brain regions under basal conditions (Fig. 4). Connexin-32 expression was higher in the cerebellum than in the cerebral cortex and cervical spinal cord ($F=27.80$, $P<0.01$). In contrast, connexin-36 expression was slightly, but significantly lower in the cerebellum than in the cervical spinal cord ($F=3.74$, $P<0.05$), but there were no significant differences between the cerebellum and cerebral cortex. Connexin-43 expression was higher in the cerebellum and cervical spinal cord than in the cerebral cortex ($F=32.68$, $P<0.01$). Within the cerebral cortex, connexin-43 was higher than connexin-32 and -36 ($F=11.59$, $P<0.01$, for clarity statistical designations are not indicated in Fig. 4). Within the cerebellum, connexin-43 was higher than connexin-32 and -36 and connexin-32 was higher than -36 ($F=84.29$, $P<0.01$). Within the cervical spinal cord, connexin-43 was higher than connexin-32 and -36 ($F=102.82$, $P<0.01$).

DISCUSSION

The purpose of the current study was to determine the effects of single and multiple courses of maternal glucocorticoids in doses similar to those used in the clinical setting on the protein expression of gap junctions in the brain of preterm fetal sheep. The novel findings of our study are as follows. First, maternal treatment with glucocorticoids is associated with increases in the expression of select gap junction proteins *in vivo* in the brain of fetal sheep. Second, the expression of specific gap junction proteins was differentially regulated by maternal treatment with glucocorticoids. Third, the responses of gap junction proteins in the fetal brain differ with respect to the brain region examined. Fourth, there are differences in the relative expression of the gap junction proteins among the fetal brain regions under basal conditions. Lastly, we unexpectedly found higher connexin 43 expression in the cerebral cortex but lower connexin 36 in cerebellum after exposure to maternal treatment with the multiple compared with single courses of placebo.

Fetuses at risk of premature delivery are now routinely exposed to maternal treatment with glucocorticoids and multiple courses may be given in the form of a rescue therapy when the risk of premature delivery is recurrent (Crowley, 1995, Wapner et al., 2006, Crowther and Harding, 2007, Wapner et al., 2007, Brownfoot et al., 2013). The findings of the current study are important as they demonstrate that this ubiquitous maternal therapy is associated with differential regulation of critical proteins that form the gap junction channels in the preterm brain. Maternal glucocorticoids increase connexin-36 and -43, which are predominantly expressed in neurons and astrocytes, respectively, (Dermietzel et al., 1989, Dermietzel et al., 1991, Bittman et al., 2002, Nagy et al., 2004). The increased expression of these connexin proteins could potentially facilitate the production of an increased number of gap junction channels and/or possibly hemi-channels among neurons and astrocytes in select regions of the fetal sheep brain *in vivo*.

Previous work has shown that exposure of pregnant nonhuman primates to dexamethasone at a dose of 5 mg/kg results in 30% reductions in MRI measured brain volumes in the neonates at 20 months of age (Uno et al., 1994). In addition, prenatal dexamethasone treatment of pregnant dams with 0.4 mg/kg on days 18–21 of gestation, resulted in increases in the number of cleaved caspase-3-positive neurons in the amygdala suggesting that prenatal dexamethasone induces neuronal apoptosis (Zuloaga et al., 2011). Postnatal treatment of rat pups with dexamethasone also results in apoptosis the developing brain (Bhatt et al., 2013). In contrast to these findings in nonhuman primates and pregnant dams (Zuloaga et al., 2011, Bhatt et al., 2013), we have shown that maternal treatment with a single course of antenatal glucocorticoids in the same paradigm as in the current study significantly reduces apoptosis and caspase 3 activity in the fetal sheep brain (Malaeb et al., 2009). In addition, although exposure to multiple courses of dexamethasone did not affect the number of apoptotic cells, caspase 3 activity was reduced (Malaeb et al., 2009).

The pregnant ewes in our study were given 6 mg of dexamethasone every 12 hours for 48 hours, which represented approximately 0.09 mg/kg per dose in the pregnant ewes (Stonestreet et al., 2004) compared with the 0.4 mg/kg dose in rodents (Zuloaga et al., 2011) and 5 mg/kg in nonhuman primates (Uno et al., 1994). The differences between our findings and those in the nonhuman primates and rodents are likely related to differences in the doses of dexamethasone, relative differences in the percentages of the pregnancy over which the glucocorticoids were administered, the time in gestation when the glucocorticoids were given and potentially species differences. For example, in our study the duration of exposure of the ewes represented 1.3% of the ovine pregnancy, whereas in the rodents the exposure duration represented 13.6% of the rodent gestation (Zuloaga et al., 2011). Nonetheless, even after this clinically relevant low dose exposure, we observed increases in connexin 36 and 43 expression in the fetal brain. Although theoretically the increased connexin expression could have contributed to increases in apoptosis reported after dexamethasone (Zuloaga et al., 2011, Bhatt et al., 2013), our previous findings reveal that apoptosis did not increase after our dexamethasone regimen in the fetal sheep brain (Malaeb et al., 2009). On the other hand, the glucocorticoid related enhanced connexin expression that we observed could potentially accentuate injury under adverse fetal conditions such as asphyxia (Koome et al., 2013).

Recent evidence suggests that maternal treatment with dexamethasone in doses similar to those in our study was associated with increased epileptiform activity, consistent with electrographic and clinical seizures on fetal cortical EEG recordings, and with later changes in the fetal EEG indicative of increased maturation of fetal sleep architecture (Davidson et al., 2011). The increases in cerebral cortical connexin-36 and -43 that we detected potentially reflect increases in neuronal and astrocytic gap junction channels that could facilitate increased cell-to-cell electrical communication (Eugenin et al., 2012) and have allowed for the recently reported increased epileptiform activity observed after maternal dexamethasone treatment (Davidson et al., 2011).

Maternal treatment with glucocorticoids was associated with increases in select gap junction proteins in specific regions of the fetal sheep brain. Treatment with single and multiple maternal courses of dexamethasone also had different effects on the gap junction proteins among brain regions such that connexin 36 expression was higher in the cerebral cortex after a single course, higher in cerebellum after multiple courses, but higher in spinal cord after both treatment regimens. Whereas, connexin 43 was higher in spinal cord after a single course, higher in cerebellum after multiple courses, but higher in cerebral cortex after both courses of dexamethasone. However, maternal dexamethasone treatment did not affect connexin 32 expression. Therefore, the effects of maternal glucocorticoids on connexin expression appear to differ as a function of the specific connexin, brain region, and the type of maternal treatment. We cannot be certain of the mechanism(s) by which the maternal glucocorticoids differentially affect connexins in the fetal brain regions. However, several possibilities exist. There are two types of glucocorticoid receptors in brain (Funder, 1997, De Kloet et al., 1998). The type 1 receptor is a mineralocorticoid receptor that has a high affinity for corticosterone but binds poorly to dexamethasone. In contrast, the type 2 receptor is a glucocorticoid receptor, which has a high affinity for dexamethasone but lower affinity for corticosterone (Funder, 1997, De Kloet et al., 1998). Glucocorticoid receptors are expressed in most brain regions and, when dexamethasone is administered, the glucocorticoid receptor is activated. Although we did not measure glucocorticoid receptors in our study, it remains possible that the effects of maternally administered glucocorticoids could have had variable effects on glucocorticoid receptor activation among the various brain regions because the receptor distribution are not necessarily uniform among all regions of the sheep brain (Keller-Wood et al., 2006).

Our current findings of differential regulation of connexin expression by maternal treatment with single and multiple glucocorticoid courses are also consistent with our previous work, in which apoptosis decreased in brain after single, but not multiple maternal courses (Malaeb et al., 2009) and Na^+, K^+ -ATPase activity decreased after multiple but not single courses (Mehter et al., 2009). Likewise, we previously have found differential increases in tight junction proteins with regard to the specific tight junction protein examined, brain region and with regard to the maternal glucocorticoid treatment regimen (Sadowska et al., 2010). Our findings regarding the glucocorticoid-related increases in connexin-43 expression are also consistent with work showing increased expression after dexamethasone treatment of astrocytes in primary glial cocultures from newborn rats (Hinkerohe et al., 2011). We have previously shown that connexin-43 expression increases with maturation in cerebral cortex (Sadowska et al., 2009) and, thus the maternal dexamethasone-induced increases in

connexin proteins most likely represents one mechanism by which maternal glucocorticoids could accelerate fetal brain maturation.

Connexins are also important in vascular development and the maintenance of vascular integrity (Michelle et al., 2012). In addition, connexin based gap junctions might be required to maintain the endothelial barrier function (Nagasawa et al., 2006). In this regard, we have previously shown decreases in blood-brain barrier permeability after maternal treatment with both single and multiple courses of glucocorticoids (Sadowska et al., 2006), and therefore, the increases in connexin protein expression that we detected could potentially have contributed to the decreases in barrier permeability that we reported (Sadowska et al., 2006, De Bock et al., 2014).

We also observed differences in the expression patterns of connexins among the cerebral cortex, cerebellum, and cervical spinal cord under basal conditions. Our findings are consistent with prior work suggesting that the frequency and distribution of gap junctions varied in among different regions of the developing and adult cortex, possibly reflecting differences in the cellular composition and functional organization of the regions (Nadarajah et al., 1996, Nadarajah and Parnavelas, 1999).

Finally, we unexpectedly found higher connexin-43 expression in the cerebral cortex but lower connexin-36 in cerebellum after exposure to maternal treatment with multiple courses of placebo compared with the single courses. Although we cannot be certain of the reason underlying this finding, animals in the multiple course groups remained in the animal care facility for six weeks compared to two weeks for the single course groups. They also received multiple intramuscular injections on a weekly basis. Therefore, they were potentially exposed to differing diets, activity, and stress levels. Findings from both human subjects and experimental animals suggest that prenatal stress may have long-lasting adverse effects on the developing fetal brain (Weinstock, 2001, Talge et al., 2007, Lupien et al., 2009, Charil et al., 2010). Prenatal stressors such as anxiety, restraint, or diet restrictions can affect the maternal hypothalamic-pituitary- adrenal axis (Austin et al., 2005, Kapoor et al., 2006) as well as the placenta (O'Donnell et al., 2009). Nonetheless, this *unexpected* finding emphasizes the importance of the *in utero* environment in the programming of fetal regional cellular brain development.

There are several limitations to our study. The tissue samples that we had available for this study represented residual frozen sections of brain regions from our previous studies. The cerebral cortical section that we obtained was from the intact parietal cortex and most likely contained mainly grey matter but some white matter as well. There is a paucity of white matter that can be dissected grossly from fetal sheep brain at this early gestational age. Hence, we did not have white matter remaining from our former studies (Sadowska et al., 2006). Although connexin-43 is predominantly from astrocytes and connexin-32 is mainly from oligodendrocytes, we were able to detect these connexins in the parietal cerebral cortex from the fetal sheep (Figs. 2a and 3a). In addition, even though connexin-43 is mainly in astrocytes, this connexin was relatively higher in the cerebral cortex than connexin-36, which is mainly in neurons. It would have been optimal also to examine the expression of these molecules by immunolabeling. Unfortunately, we did not have sufficient brain tissue

appropriately saved to perform the immunohistochemical analysis. Consequently, we cannot comment upon the location and specific cellular types that express the various different connexins. Another limitation of our study is that we did not perform functional outcomes for the connexins measured in our study. As described in the methods, fetal brain samples in the current study were derived from animals enrolled in our previous studies (Hai et al., 2002, Kerzner et al., 2002, Stonestreet et al., 2004, Sadowska et al., 2006, Sadowska et al., 2010) and, consequently, represented frozen samples.

CONCLUSIONS

Maternal treatment with glucocorticoids modulates the expression of molecular components of the gap junctions in the fetal brain. Gap junction proteins exhibit differential expression in the cerebral cortex, cerebellum, and cervical spinal cord under basal conditions and expressions are regulated as a function of different maternal glucocorticoids treatment regimens. Effects of dexamethasone and differences in the *in utero* environment result in changes to connexins that potentially influence the development of the fetal brain.

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- Maternal glucocorticoids increase gap junction proteins in fetal brain.
- Gap junction proteins are differentially regulated by maternal glucocorticoids.
- Single and multiple maternal glucocorticoid courses modify gap junction proteins.
- Basal gap junction protein expression differs among brain regions in fetal brain.

Fig. 1.

Connexin 36 protein expression in the cerebral cortex, cerebellum, and cervical spinal cord of ovine fetuses of ewes exposed to single and multiple courses of placebo or dexamethasone.

A: Top: Representative Western immunoblots: IC is the internal control protein and vinculin shown as a loading control; Bottom: bar graphs. Connexin 36 expression in cerebral cortex plotted as a ratio to the internal control for fetuses of single course placebo-treated ewes (n=6) and dexamethasone-treated ewes (n=7); fetuses of multiple course placebo-treated

ewes (n=6), and dexamethasone-treated ewes (n=9; ANOVA, main effect for treatment, $F=13.78$, $P<0.01$).

B: Connexin 36 expression in the cerebellum of fetuses of single course placebo-treated ewes (n=6) and dexamethasone-treated ewes (n=6); multiple course placebo-treated ewes (n=6) and dexamethasone-treated ewes (n=6; ANOVA, main effect for frequency, $F=5.27$, $P<0.03$, interactions for dexamethasone versus placebo treatment, and course frequency, single versus multiple courses, $F=6.87$, $P<0.02$).

C: Connexin 36 expression in the cervical spinal cord of fetuses of single course placebo-treated ewes (n=6) and dexamethasone treated ewes (n=6); multiple course placebo-treated ewes (n=6), and dexamethasone-treated ewes (n=6; ANOVA, main effect for treatment $F=13.37$, $P<0.01$). Open bars represent fetuses of placebo-treated ewes; closed bars represent fetuses of dexamethasone-treated ewes. Values are $M\pm SD$, * $P<0.05$.

Fig. 2.

Connexin 43 protein expression in the cerebral cortex, cerebellum, and cervical spinal cord of ovine fetuses of ewes exposed to single and multiple courses of placebo or dexamethasone.

A: Western immunoblots as for figure 2. Connexin 43 expression in cerebral cortex for fetuses of single course placebo-treated ewes (n=5) and dexamethasone-treated ewes (n=7); multiple course placebo-treated ewes (n=6), and dexamethasone-treated ewes (n=8);

ANOVA, main effect for treatment, $F=15.19$, $P < 0.01$; main effect for course frequency, $F=36.34$, $P < 0.01$).

B: Connexin 43 expression in cerebellum for fetuses of single course placebo-treated ewes ($n=6$) and dexamethasone-treated ewes ($n=6$); multiple course placebo-treated ewes ($n=6$), and dexamethasone-treated ewes ($n=6$; ANOVA, main effect for treatment, $F = 14.33$, $P < 0.01$).

C: Connexin 43 expression for fetuses of single course placebo-treated ewes ($n=6$), and dexamethasone-treated ewes ($n=6$), multiple course placebo-treated ewes ($n=6$), and dexamethasone-treated ewes ($n=6$; ANOVA, main effect for treatment, $F=16.13$, $P < 0.01$). Bars legends and statistics as for figure 2.

Fig. 3.

Connexin 32 protein expression in the cerebral cortex, cerebellum, and cervical spinal cord of ovine fetuses of ewes exposed to single and multiple courses of placebo or dexamethasone.

A: Western immunoblots as for figure 2. Connexin 32 expression in cerebral cortex for fetuses of single course placebo-treated ewes (n=6) and dexamethasone-treated ewes (n=7); multiple course placebo-treated ewes (n=6), and dexamethasone-treated ewes (n=9).

B: Connexin 32 expression in cerebellum for fetuses of single course placebo-treated ewes (n=6) and dexamethasone-treated ewes (n=6); multiple course placebo-treated ewes (n=6), and dexamethasone-treated ewes (n=6),

C: Connexin 32 expression for fetuses of single course placebo-treated ewes (n=5), and dexamethasone-treated ewes (n=5), multiple course placebo-treated ewes (n=5), and dexamethasone-treated ewes (n=6). Bars legends and statistics as for figure 2.

Fig. 4.

Protein expression of connexin-32, -36 and -43 plotted for the cerebral cortex, cerebellum, and cervical spinal cord to illustrate differences in the magnitude of expression among the brain regions. Top: Representative Western immunoblots: IC is the internal control protein and vinculin shown as a loading control. The expression is shown for connexin-32, -36 and -43 in the brain region. Bottom: Bar graphs representing the gap junction protein expression in the brain regions of fetuses of ewes exposed to the single course placebo treatment for connexin-32 (n=6, ANOVA, main effect for brain region, $F=27.80$, $P<0.01$), connexin-36 (n=6, ANOVA, main effect for brain region, $F=3.7$, $P<0.05$) and connexin 43 (n=6, ANOVA, main effect for brain Region, $F=32.68$, $P<0.01$). Values are $M \pm SD$, * $P<0.05$.

Connexin Protein Expression in the Fetuses of the Dexamethasone Treated Ewes Compared with Placebo Treated Ewes Schematically Summarized

Table 1

Gap Junction Protein	Cerebral Cortex		Cerebellum		Cervical Spinal Cord	
	Single Course	Multiple Courses	Single Course	Multiple Courses	Single Course	Multiple Courses
Connexin-32	↔	↔	↔	↔	↔	↔
Connexin-36	↑	↔	↔	↑	↑	↑
Connexin-43	↑	↑	↔	↑	↑	↔

↔ = no change compared with fetuses of placebo treated ewes from the same treatment course; ↑ = increase compared to fetuses of placebo treated ewes from the same treatment course.