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Ontogeny of connexin 32 and 43 expression in the cerebral cortices of ovine fetuses, newborns, and adults

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Abstract

Gap junctions are specialized membrane structures that mediate intercellular communication and facilitate passage of ions and small molecules between adjacent cells. Connexins comprise a multigene family of transmembrane proteins that form gap junctions. Connexin-32 and connexin-43 are among the most abundant connexins in brain and are highly expressed during development. Connexin-32 is expressed primarily in oligodendrocytes and connexin-43 in astrocytes in adult brain. However, both connexins are expressed in neurons during development. We examined the effects of ontogeny on connexin-32 and connexin-43 protein abundance in cerebral cortices of sheep during development. Western immunoblot was used to measure connexin-32 and connexin-43 expression in cerebral cortices of fetuses at 60%, 80%, and 90% of gestation, in newborn lambs and adult sheep. Values were expressed as ratios to a single adult control cerebral cortical sample. Connexin-32 abundance was higher ($P<0.05$) in cerebral cortices of fetuses at 60% of gestation (3.0±0.68, mean ±SD), than in those at 90% of gestation (1.7±0.3), in newborn (1.8±0.55), and adult sheep (0.84 ±0.19), respectively. In contrast, connexin-43 abundance was higher ($P<0.05$) in cerebral cortices of fetuses at 90% of gestation (0.44±0.17), newborn (0.69±0.12) and adult sheep (1.14±0.13), than in those at 60% of gestation (0.05±0.01). We conclude that (1) connexin-32 and connexin-43 protein are expressed early in fetal life and throughout development, (2) each connexin displays a unique pattern of change with development, (3) connexin-43 exhibited ontogenic increases in protein abundance, whereas, connexin-32 exhibited reciprocal decreases in abundance late in fetal development, in newborn and adult sheep.

Keywords

Brain; Connexin; Gap junction; Development; Gestation; Maturation; Sheep

1. Introduction

Gap junctions are specialized membrane structures that mediate cell-to-cell communication via channels that link the cytoplasmic compartments of cells and allow for exchange of ions, small organic metabolites, and second messengers of up to 1 kDa in size between adjacent cells (Bennett et al., 1991; Bruzzone and Dermietzel, 2006; Nagy and Rash, 2000). They are composed of a multigene family of transmembrane intercellular channel-forming membrane

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proteins termed connexins (Herve et al., 2007). Each gap junction channel consists of a pair of connexons each of which is comprised of six connexin proteins (Bennett et al., 1991).

In the mature central nervous system, neurons and glial cells are coupled by gap junctions, which mediate the propagation of electrical signals (Rozental et al., 2000) and intercellular propagation of calcium waves (Bruzzone and Dermietzel, 2006). In the rodent, connexin-32 and connexin-43 represent two of the most ubiquitous connexin isoforms in the brain (Kumar and Gilula, 1996).

Intercellular communication by gap junctions is thought to be widespread in the developing central nervous system (Guthrie and Gilula, 1989; Nadarajah et al., 1997) and to play a role in a variety of developmental events such as cell proliferation, migration, regional differentiation, axon growth and guidance, neuronal circuit and synapse formation (Bruzzone and Dermietzel, 2006; Nadarajah et al., 1997). Intercellular pathways of communication through gap junctions may be the likely route for exchange signals between networks of cells during the critical stages of cortical development (Bittman et al., 1997; Bruzzone and Dermietzel, 2006).

Connexin-32 and connexin-43 are highly expressed in the brain during development (Bruzzone and Dermietzel, 2006; Elias and Kriegstein, 2008). Connexin-32 is mainly expressed in oligodendrocytes (Dermietzel et al., 1989). Connexin-43 is the predominant gap junction protein found in astrocytes (Dermietzel et al., 1991). In addition, both of these connexins can be found in neurons (Bruzzone and Dermietzel, 2006; Talhouk et al., 2008) during development. Connexin-32 and connexin-43 have been shown to exhibit specific temporal patterns of expression during development in the rodent brain (Nadarajah et al., 1997).

Others and we have extensively used the ovine fetus to investigate many aspects of the central nervous system development (Back et al., 2006; Gunn et al., 1997; Riddle et al., 2006; Stonestreet et al., 2000). The neurodevelopment of the immature ovine brain is similar to that of the premature infant with respect to the completion of neurogenesis, onset of cerebral sulcation, and detection of the cortical component of the auditory and somatosensory evoked potentials (Back et al., 2006; Barlow, 1969; Bernhard et al., 1967; Cook et al., 1987). It is important to point out that the sheep brain at 80% to 85% of gestation is generally thought to be similar to the newborn infant at term (Back et al., 2006; Gunn et al., 1997).

Although connexins are very important in central nervous system development (Bruzzone and Dermietzel, 2006), information is not available regarding the presence of connexins in the brain of a precocial species such as sheep. Given the above considerations, the purpose of the present study was to examine expression pattern of connexin-32 and connexin-43 in the developing ovine cerebral cortex.

2. Results

The fetal, newborn and adult sheep from which the cerebral cortical samples for connexin-32 and connexin-43 protein abundance were obtained had pH, arterial blood gases and mean arterial blood pressures within the physiological range for our laboratory in each age group (Stonestreet et al., 1999; Stonestreet et al., 2000; Sysyn et al., 2001), and similar to values reported from other laboratories (Unno et al., 1998). Connexin-32 and connexin-43 proteins were detectable as early as 60% of gestation in ovine fetal cerebral cortex.

Connexin-32 abundance shown relative to adult control values demonstrated gradual steady decreases with development and was lower (ANOVA, main effects; F=11.58, P<0.05) in the fetuses at 90% of gestation, in the newborn lambs and the adult sheep than in the fetuses at 60% of gestation (Fig. 1). In contrast, connexin-43 abundance shown relative to adult control

values demonstrated steady increases and was higher (F=46.34, P<0.05) in the fetuses at 90% of gestation, in the newborn lambs and in the adult sheep than in the fetuses at 60% of gestation.

3. Discussion

The purpose of the present study was to examine the ontogeny of connexin-32 and connexin-43 protein abundance in the cerebral cortices of sheep during development. We examined this brain region because we had frozen cerebral cortical samples available from the our previous studies (Stonestreet et al., 1999; Stonestreet et al., 2000; Sysyn et al., 2001). Connexin-32 and connexin-43 protein abundance have not been previously measured in the ovine brain. The novel findings of our study are the following. (1) Connexin-32 and connexin-43 proteins were expressed in the cerebral cortex early in fetal life and throughout ovine development. (2) Each connexin demonstrated a unique pattern change with development. (3) Connexin-43 demonstrated steady increases in protein abundance from 60% of gestation through the neonatal period up to maturity in adult sheep, whereas the changes in connexin-32 were reciprocal with decreases in connexin-32 abundance late in fetal development, in newborn and adult sheep.

In rodents, Nadarajah et. al demonstrated that connexin-43 was detected at embryonic day (E) 12, increased to E 16, then decreased on E 19 and increased steadily after birth from postnatal day zero until postnatal day 28 (Nadarajah et al., 1997). On the other hand, connexin-32 was not detected in the rodent fetal brain at any prenatal age, was first detected on postnatal day 7, and increased until postnatal day 21 (Nadarajah et al., 1997). These changes in connexin 32 appear to coincide with the transition to mature oligodendroglia, with the start of cortical myelination (Back et al., 2002; Back et al., 2001). In contrast to these findings in rodents, we observed steady gradual decreases in connexin-32, and reciprocal increases in connexin-43 protein expression with development from 60% of gestation through the neonatal period up to maturity in adult sheep. We suggest that the differences between our findings in sheep and those in the rodent brain result from maturational differences in brain development among species (Dobbing and Sands, 1979). A large proportion of brain development in the sheep occurs before birth and, similar to the human, the sheep exhibits two distinct phases of brain growth (Dobbing and Sands, 1979; McIntosh et al., 1979). The first phase occurs between 40 and 80 days of gestation and is thought to represent neuronal multiplication and the second phase occurs between 95 and 130 days of gestation and represents neuralgia multiplication and myelination (Barlow, 1969; Dobbing and Sands, 1979; McIntosh et al., 1979). In contrast, the majority of the rodent brain growth occurs after birth (Dobbing and Sands, 1979).

Connexin-43 is highly expressed in neurons and radial glia during development but is largely restricted to astrocytes in the adult brain (Elias and Kriegstein, 2008; Nadarajah et al., 1997), whereas connexin-32 is mainly expressed in oligodendrocytes in the adult brain (Dermietzel et al., 1989), but also can be found in neurons (Talhouk et al., 2008). The increase in connexin-43 expression with development in the sheep could reflect an increase in the number of astrocytes and/or an increase in the number of connexin-43 containing gap junctions. The decrease in connexin-32 expression with development was an unexpected finding and could reflect a decrease in the number of connexin-32 containing gap junctions, and/or changes in connexin-32 containing gap junctions with oligodendrocyte maturation (Back et al., 2006).

The expression of the connexins is highly dependent on the number and nature of the underlying cell types (Nagy et al., 2004). As neurons are found essentially exclusively in the grey matter ribbon, and oligodendrocyte cell bodies are found in the underlying white matter, the precise area of cerebral cortex and the proportion of white matter sampled at each age could have influenced the type and amount of connexin expressed in our different age groups (Back et al., 2006). However, an equivalent sample of brain was obtained from each age group from the

same anatomical cerebral cortical region. In view of this, it is likely that the adult sheep cerebral cortical sample contained more, rather than less, white matter relative to the fetal sheep at 60% of gestation. Back et. al have recently shown that there is a regional heterogeneity in the oligodendrocyte lineage maturation between 60% and 80% of the ovine gestation. Although at this time in gestation we did not observe significant differences in the abundance of connexin-32, it remains possible that the changes in connexin-32 that we observed could reflect connexin-related changes with oligodendrocyte lineage maturation at the later times in gestation.

We had limited frozen tissue samples available from our previous studies, hence we were not able to do immunostaining for protein localization or examine specific cell types. Although we did not examine immunostained sections, previous work in rodents has shown that relative expression of connexins reflected closely the developmental profile of these antigens observed in immunostained sections (Nadarajah et al., 1997). Nonetheless, based upon our Western immunoblot analysis of a single brain region, we cannot be certain of the cell types that were represented by the changes in connexin-32 and connexin-43 expression that we observed with ovine cerebral cortex development. We also only had frozen cerebral cortex available from our previous studies (Stonestreet et al., 1999; Stonestreet et al., 2000; Sysyn et al., 2001) and, consequently, were not able to examine other brain regions.

The increases in connexin-43 and decreases in connexin-32 expression could also reflect changes in the quantity of gap junctions within a given cell type with development (Nagy and Rash, 2000). Nonetheless, the pattern of connexin-32 and connexin-43 expression that we observed in the sheep during development were distinctly different from those observed in the rodent (Elias and Kriegstein, 2008; Leung et al., 2002;Nadarajah et al., 1997). The increases in connexin-43 abundance that we observed within the sheep brain are consistent with increases in connexin-43 abundance in the adrenal glands, which are derived from neural crest cells, between 100 and 130 days of gestation in ovine fetuses (McDonald et al., 2003). Our findings provide comparative results for a species that has been widely used in physiological studies, suggest that there could be a species-related dependency in the gap junction protein expression, and could call into question extrapolation of results from rodents to other species that are more mature at birth, such as sheep and humans (Ron et al., 2005).

4. Experimental procedure

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

4.1. Animal preparation

The cerebral cortical samples for this experiment were obtained from sheep in our previous studies (Stonestreet et al., 1999; Stonestreet et al., 2000; Sysyn et al., 2001). As described in detail, surgery was performed under 1–2% halothane anesthesia in pregnant ewes at 60% (84–86 days, n=5), 80% (116–120 days, n=3), and 90% (126–129 days, n=7) of gestation for the purpose of measuring blood–brain barrier permeability (Stonestreet et al., 1999; Stonestreet et al., 2000). Fetal catheters were placed for the blood–brain barrier studies in the brachial vein and in the thoracic aorta via a brachial artery. An amniotic fluid catheter was placed for pressure monitoring and to correct fetal arterial blood pressures. After recovery from surgery, the ewes at each gestational age were given 1.5 ml of placebo (0.9% NaCl) by intramuscular injection every 12 h for a total of four doses over 48 h. In the 2-day-old lambs (n=6), catheters were placed under 0.5–1% isoflurane anesthesia as previously described (Sysyn et al., 2001). Twenty-four hours after surgery, the lambs received four intramuscular injections of placebo given 12 h apart on days 3 and 4 of age. Cerebral cortical samples also were obtained from

adult non-pregnant sheep (*n*=3) exposed to the same treatment protocol as described for the ewes above. The sheep in the present study were the placebo treated control sheep for glucocorticoid treated animals as reported in our previous studies (Stonestreet et al., 1999; Stonestreet et al., 2000; Sysyn et al., 2001). Eighteen hours after the last placebo injection was given, in each age group examined, the entire frontal lobe from a single hemisphere was removed via a coronal section immediately rostral to the anterior limit of the corpus callosum and snap frozen in liquid nitrogen. A single identical section was taken in each sheep in each age group. A single one-gram sample from the rostral portion of the frontal cerebral cortical sample was used for the tissue homogenate for Western blot analyses.

4.2. Experimental protocol and methodology

The cerebral cortical crude total membrane fractions were separated from soluble proteins by homogenization and differential centrifugation (Jorgensen, 1974) using the following homogenization buffer: 5% sorbitol, 5 mM histidine brought to pH 7.5 with 5 mM imidazole, 0.5 mM Na_2EDTA and proteolytic enzyme inhibitors: 10 μg of aprotinin, 1 μg of leupeptin, and 25 μg of 4-aminoethyl-benzensulfonyl fluoride per ml of extraction buffer. Protein concentrations of the homogenates were determined by a bicinchoninic acid protein assay (BCA, Pierce, Rockford, IL). Extracted samples were aliquoted and stored at −80 °C.

Cerebral cortical samples were extracted and protein concentration determined as described above. Fifty micrograms of total protein per well were fractionated by 12% SDS-polyacrylamide gel electrophoresis, and transferred onto PVDF membranes (Polyvinylidene diflouride, 0.2 μm, Bio-Rad Laboratories, Hercules, CA) using a semi-dry technique. The membranes were blocked with 5% nonfat milk for 1 h in room temperature and then washed in TBST (Tris-buffered saline with 0.1% of Tween-20) three times for 10min per wash. The immunoblots were then incubated in primary antibody overnight at 4 °C. Membranes were probed for connexin-32 with monoclonal mouse anti-connexin-32 and for connexinx-43 with monoclonal mouse anti-connexin-43 (Zymed, South San Francisco, CA) at a dilution of 1:3000 for connexin-32 and at 1:5000 for connexin-43. After incubation with primary antibody the immunoblots were washed in TBST three times for 10 min per wash. Then they were incubated for 1 h at room temperature with donkey anti-mouse horseradish peroxidase conjugated secondary antibody (Affinity Bioreagents, Golden, CO) at a dilution of 1:6000 for connexin-32 and 1:10,000 for connexin-43. The immunoblots were again washed in TBST four times for 10 min per wash. Binding of the secondary antibody was detected with enhanced chemiluminescence ECL-Plus Western blotting detection reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) before exposure to Hyperfilm ECL (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

All experimental samples were normalized to a reference connexin-32 or connexin-43 protein standard that had been obtained from a homogenate pool from the cerebral cortex of one adult sheep. The adult control cerebral cortical brain samples were included as the first, middle, and last samples on each immunoblot. The adult control cerebral cortical samples served as an internal control and to normalize the cerebral cortical densitometry values so that the values could be compared among the different immunoblots performed on different days. The mean value of the three adult cerebral cortical control samples on each immunoblot was used to normalize the values of the cerebral cortical samples from the different age groups. As we have previously described, these samples served as adult control reference standards for quality control for loading, transfer, verification of potential internal variability across the gel, and for normalization of the cerebral cortical densitometric values to permit accurate comparisons against a single control standard among the different immunoblots and groups (Kim et al., 2006). For the purpose of this report, we refer to the internal control reference standards from the adult sheep cerebral cortex hereafter as the adult control samples. We calculated a

coefficient of variation for the adult control samples on each immunoblot. The values for the experimental samples in this study were accepted as valid only if the percent coefficient of variation for the adult control samples on each immunoblot was less than 20%. The final values represented an average of the densitometry values obtained from the different immunoblots. We have previously shown that this method correlates well with values that have been normalized as ratios to β-actin (Kim et al., 2006). Uniformity in inter-lane loading was confirmed by Coomassie blue staining of the polyacrylamide gels, and uniformity of transfer to the PVDF membrane confirmed by Ponceau S. (Tseng et al., 2005).

Rat liver and rat heart were used as positive controls for connexin-32 and connexin-43, respectively. The anti-connexin-32 monoclonal antibodies accurately identified rat liver, ovine fetal and adult cerebral cortical connexin-32 and the anti-connexin-43 monoclonal mouse antibody accurately identified the rat heart and ovine fetal and adult cerebral cortical connexin-43. Western immunoblots preformed without the primary antibodies were used to rule out non-specific binding. Detection of the connexin-32 and connexin-43 bands at 32 and 43 kDa, respectively, were dependent on incubation with primary antibody, omission of which resulted in the absence of this signal.

4.3. Densitometric analysis

Band intensities were analyzed with a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD). The experimental densitometry values were normalized to the average of the three adult control cerebral cortical samples on each immunoblot. We used the mean value of the three adult control cerebral cortical samples on each immunoblot to normalize experimental values. Samples from the different age groups were analyzed on at least three Western immunoblots. The final values represented an average of the densitometry values obtained from the different immunoblots. We have used a similar normalization technique to examine the ontogeny and effects of corticosteroid pretreatment on aquaporin water channels in the ovine cerebral cortex (Ron et al., 2005), effects of postnatal steroids on Na^+/K^+-ATPase activity and $alpha_1$-and $beta_1$-subunit protein expression in the cerebral cortex and renal cortex of newborn lambs (Kim et al., 2006) and on effect of maternal treatment with corticosteroids on tight junction protein expression in the cerebral cortex of the ovine fetus with and without exposure to *in utero* brain ischemia (Malaeb et al., 2007).

4.4. Statistical analysis

All results were expressed as means±SD. Analysis of variance (ANOVA) was used to determine the effect of maturation on connexin-32 and connexin-43 protein expression in the cerebral cortices of the sheep. When a significant difference was shown by ANOVA, the Fischer least significant difference test was used to detect differences among age groups. $P<0.05$ was considered statistically significant.

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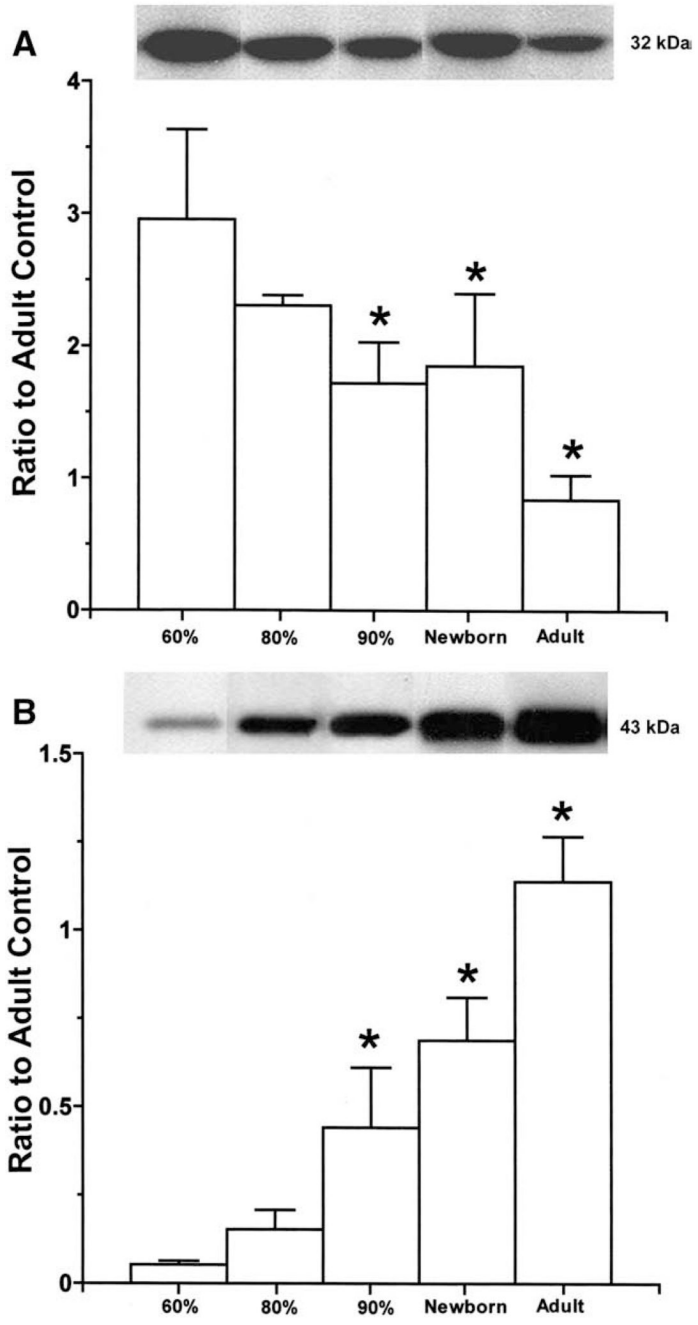

Fig. 1.

(A) Representative Western immunoblot and bar graphs of connexin-32 expression in the cerebral cortex of the ovine fetuses at 60% (*n*=5), 80% (*n*=3) and 90% of gestation (*n*=7), newborn lambs (*n*=5) and adult sheep (*n*=3). **P*<0.05 versus 60% of gestation. (B) Representative Western immunoblot and bar graphs of connexin-43 protein expression in the cerebral cortex of the ovine fetuses at 60% (*n*=5), 80% (*n*=3) and 90% of gestation (*n*=7), newborn lambs (*n*=6) and adult sheep (*n*=3). **P*<0.05 versus 60% of gestation.