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ONTOGENY OF TIGHT JUNCTION PROTEIN EXPRESSION IN THE OVINE CEREBRAL CORTEX DURING DEVELOPMENT

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Abstract—Tight junctions of the blood–brain barrier are composed of transmembrane and associated cytoplasmic proteins. The transmembrane claudin proteins form the primary seal between endothelial cells and junctional adhesion molecules (JAMs) regulate tight junction formation. We have previously shown that claudin-1, claudin-5, zonula occludens (ZO)-1, and ZO-2 exhibit differential developmental regulation from 60% of gestation up to maturity in adult sheep. The purpose of the current study was to examine developmental changes in claudin-3, -12, and JAM-A protein expression in cerebral cortices of fetuses at 60%, 80%, and 90% gestation, and in newborn and adult sheep. We also examined correlations between changes in endogenous cortisol levels and tight junction protein expression in cerebral cortices of the fetuses. Claudin-3, -12 and JAM-A expressions were determined by Western immunoblot. Claudin-3 and -12 were lower ($P < 0.01$) at 60%, 80%, 90% and in newborns than in adults, and JAM-A was lower in adults than in fetuses at 80% and 90% gestation. Claudin-3 expression demonstrated a direct correlation with increasing plasma cortisol levels ($r = 0.60$, $n = 15$, $P < 0.02$) in the fetuses. We conclude that: claudin-3, -12 and JAM-A are expressed as early as 60% of gestation in ovine cerebral cortices, exhibit differential developmental regulation, and that increasing endogenous glucocorticoids modulate claudin-3 expression in the fetus.
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Key words: blood–brain barrier, claudin, development, sheep, tight junction proteins.

INTRODUCTION

The blood–brain barrier is a regulated interface between the peripheral circulation and the central nervous system (CNS) (Hawkins and Davis, 2005). The blood–brain barrier develops during fetal life (Stonestreet et al., 1996; Nico et al., 1999; Virgintino et al., 2004; Daneman, 2012) and consists of a complex cellular system of highly specialized endothelial cells connected by intercellular tight junctions (Brightman and Reese, 1969; Engelhardt, 2003). The endothelial cells of the blood–brain barrier are supported and regulated by pericytes embedded in the vascular basement membrane, perivascular microglial cells, astrocytes and neurons, which together form the neurovascular unit (Virgintino et al., 2004; Abbott et al., 2010; Luissint et al., 2012). In addition to the blood–brain barrier, there are three other main barriers in the brain: blood–cerebral spinal fluid (CSF) barrier, meningeal barrier, and fetal CSF–brain barrier (Ek et al., 2012). Similar to the cellular structure of the cerebrovascular endothelial cells of the blood–brain barrier, the ependymal cells of the choroid plexus of the blood–CSF barrier and the outer cells of the arachnoid membrane of the meningeal barrier are connected by tight junction proteins. The blood–brain barrier along with the blood–CSF barrier, meningeal barrier, and fetal CSF–brain barrier maintains the homeostasis of the CNS by limiting the passive diffusion of polar substances from the blood-to-brain and maintaining optimal ionic composition necessary for synaptic signaling function (Betz and Goldstein, 1986; Betz, 1992; Begley and Brightman, 2003; Wolburg et al., 2009; Abbott et al., 2010; Ek et al., 2012; Luissint et al., 2012).

Tight junctions mediate the adhesion between adjacent cells and limit the free passage of molecules through the paracellular pathway (Mullier et al., 2010; Abbott and Friedman, 2012). Previous studies in humans and other species demonstrate that an adult-like pattern of the endothelial tight junctions is present by midgestation, and the paracellular pathway is already limited early in brain development (Stonestreet et al., 1996; Virgintino et al., 2004; Ek et al., 2006).

Tight junctions are complex molecular structures composed of transmembrane and associated cytoplasmic proteins. The transmembrane proteins including occludins, the claudin family, and junctional adhesion molecules (JAMs) are connected to the associated cytoplasmic proteins, zonula occludens (ZO)-1, ZO-2 and ZO-3, etc., which stabilize the tight junctions by connecting them to the cell structure and actin (Hawkins and Davis, 2005; Abbott et al., 2006). More recently, claudin-3 and -12 have been identified to contribute to the integrity of the blood–brain barrier

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Abbreviations: ANOVA, analysis of variance; CNS, central nervous system; CSF, cerebral spinal fluid; EDTA, ethylenediaminetetraacetic acid; IOD, integrated optical density; JAMs, junctional adhesion molecules; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with 0.1% Tween 20; ZO, zonula occludens.

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(Wolburg et al., 2003; Neuhaus et al., 2008; Shin et al., 2008; Milatz et al., 2010; Haseloff et al., 2015). They have been shown to participate in tight junction formation, to establish a primary seal between the brain microvascular endothelial cells, and to contribute to the high transendothelial electrical resistance (Abbott et al., 2006; Schrade et al., 2012; Haseloff et al., 2015). In addition, JAM-A is involved in the formation and maintenance of tight junctions (Abbott et al., 2006) and modulates monocyte transmigration through the blood–brain barrier (Aurrand-Lions et al., 2002).

The ovine fetus has been widely used to investigate the development of the brain (Gunn et al., 1997; Stonestreet et al., 2000; Back et al., 2006) because the neurodevelopment of the immature ovine brain is similar to that of premature infants with respect to the time course of neurogenesis, onset of cerebral sulcation, and detection of cortical auditory evoked potentials (Barlow, 1969; Cook et al., 1987; Back et al., 2006). The sheep brain at 80–85% gestation is generally thought to be similar to that of a near-term newborn infant (Barlow, 1969; Back et al., 2006).

We have previously shown ontogenic decreases in blood–brain barrier permeability with development from 60% of gestation up to maturity in adult sheep, and that endogenous increases in cortisol concentrations are associated with decreases in blood–brain barrier permeability during normal fetal development (Stonestreet et al., 2000). We also previously reported differential developmental regulation of occludin, claudin-1 and -5 and ZO-1 and ZO-2 in the sheep cerebral cortex (Duncan et al., 2009).

The hypothalamic–pituitary–adrenocortical matures during fetal development and endogenous cortisol concentrations increase markedly in the latter part of part of gestation (Wintour, 1984). Glucocorticoids have been shown to have an important role in modulating blood–brain barrier function during development and affecting the expression of some tight junction proteins during normal and pathological conditions (Stonestreet et al., 2000, 2003; Malaeb et al., 2007; Duncan et al., 2009).

The purpose of the current study was to extend our previous work to examine ontogenic changes in some of the more recently identified tight junction proteins including claudin-3, claudin-12, and the tight junction associated protein, JAM-A, in the cerebral cortices of fetuses at 60%, 80%, and 90% gestation and in newborn and adult sheep. We also examined associations between endogenous increases in plasma cortisol concentrations during gestation and changes in the expression of these tight junction proteins during fetal development.

EXPERIMENTAL PROCEDURES

Animal preparation and experimental design

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

The plasma and cerebral cortical tissue samples for the present study were obtained from placebo treated control subjects in our former studies (Stonestreet et al., 2000; Sysyn et al., 2001; Ron et al., 2005; Sadowska et al., 2006). The surgical procedures and physiological measures were performed as previously described in detail (Stonestreet et al., 2000; Sysyn et al., 2001; Sadowska et al., 2006). Briefly, under 1–2% halothane anesthesia, polyvinyl catheters were placed into a brachial vein of the fetuses and newborns for isotope administration and the thoracic aorta via the brachial artery for blood sample withdrawal for the previous studies, which were designed to quantify blood–brain barrier permeability (Stonestreet et al., 2000; Sysyn et al., 2001; Sadowska et al., 2006). The fetal sheep at 60% were 87–90 days (n = 4–7), 80% were 118–120 days (n = 3–5), and 90% were 135–138 days of gestation (n = 5), newborn lambs were 4–6 days (n = 6) and adult sheep were 3 years (n = 3) of age at time of study. Full-term gestation in fetal sheep is 147 days.

Western immunoblot detection and quantification of claudin-3, -12, and JAM-A

Samples from the cerebral cortex were extracted in Triton/Deoxycholate/sodium dodecyl sulfate (SDS) (100 mM NaCl, 1% Triton X, 0.5 sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 1 mM benzamidine) with 1% of complete protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The total protein concentrations of the homogenates were determined with a bicinchoninic acid protein assay (BCA, Pierce, Rockford, IL, USA).

Fifty micrograms of total protein per well was fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride membranes (0.2 μm, Bio-Rad Laboratories, Hercules, CA, USA) using a semi-dry technique. Membranes were blocked with 10% nonfat milk (Bio-Rad) for 1 h at room temperature and then washed in Tris-buffered saline with 0.1% Tween 20 (TBST) three times for 10 min per wash. Membranes were probed with primary rabbit polyclonal antibodies for claudin-3 (Zymed, San Francisco, CA, USA), claudin-12 (Bios, Woburn, MA, USA) and for JAM-A (Zymed) at the dilution of 1:5000. Vinculin was probed with primary mouse monoclonal antibody (Thermo Scientific™ Pierce, Waltham, MA, USA) at a dilution of 1:10,000. Membranes were incubated with primary antibodies overnight in 4 °C. After three washes in TBST, the membranes were incubated for 1 h at room temperature with goat anti-rabbit (San Antonio, TX) for claudin-3, -12 and JAM-A or goat anti-mouse (Life Technologies, Grand Island, NY, USA) for vinculin at the dilution 1:10,000 for all of the examined proteins. After four additional washes, binding of the secondary antibody was detected with enhanced chemiluminescence (ECL Prime, Western Blotting Detection reagents, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) before an exposure to autoradiography film (Phenix, Candler, NC, USA).

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