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Research Report

Cell death in the central division of the medial preoptic nucleus of male and female lamb fetuses

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ABSTRACT

The medial preoptic area of the adult sheep contains an ovine sexually dimorphic nucleus (oSDN) that is larger and has more neurons in males than in females. In the lamb fetus, the nascent oSDN occupies the central division of the medial preoptic nucleus (MPNc) and consists of a cluster of cells that is organized by the action of testosterone during gestational days 60–90 of a 147 day term pregnancy. The current study sought to determine whether programmed cell death contributes to the emergence of the oSDN. Male and female lamb fetuses were euthanized at different ages spanning the period during which the oSDN is organized. The expression of the pro- and anti-apoptotic genes *bcl-2* and *bax*, respectively, was measured by quantitative RT-PCR to assess possible sex differences in neuron vulnerability to programmed cell death. The appearance of DNA-fragmentation was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and used to estimate the occurrence of apoptotic cell death. We found that *bcl-2* and *bax* mRNA expression in the medial preoptic area of the developing lamb fetus decreased during the last half of the 147-day gestation. The ratio of bcl-2/bax gene expression was highest at gestational day 85 but was equivalent between males and females. TUNEL staining in the MPNc was very low and although it decreased significantly with age, it was not significantly different between sexes. These results using two different methods to assess cell death indicate that a sex difference in the incidence of cell death is not a primary mechanism leading to sexual differentiation of the oSDN.

Abbreviations: Bax, Bcl-2-associated X protein; Bcl-2, B cell lymphoma 2; CYP 19, cytochrome P450 aromatase; DAPI 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MPNc, central component of the medial preoptic nucleus; MPOA, medial preoptic area; oSDN, ovine sexually dimorphic nucleus; RT-PCR, reverse transcriptase-polymerase chain reaction; SDN-POA, sexually dimorphic preoptic nucleus; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

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1. Introduction

A sexually dimorphic nucleus (SDN) exists in the central division of the medial preoptic nucleus (MPNc) of sheep and can be identified as a distinctive cluster of cells that express cytochrome P450 aromatase (CYP 19) mRNA (Roselli et al., 2004). This nucleus, which is referred to as the ovine sexually dimorphic nucleus (oSDN), is larger and contains more neurons in males than in females and has been associated with gender-typical sexual partner preferences in sheep (Roselli et al., 2004). The oSDN volume becomes sexually dimorphic in fetal life by gestational day 135 of a 147 day term sheep pregnancy (Roselli et al., 2007). The sex difference appears to be due to the organizational effects of testosterone since treatment of females with testosterone from gestational days 60–90 eliminates the sex difference in the oSDN while testosterone manipulations in adulthood do not (Roselli et al., 2011; Roselli et al., 2009). However, the cellular mechanism whereby testosterone controls the development of the oSDN is not yet established. The current study sought to determine whether programmed cell death contributes to the emergence of sex differences in the oSDN.

Several mammals possess sexually dimorphic preoptic nuclei (SDN-POA) including: rats, gerbils, guinea pigs, hyenas, ferrets, monkeys, and humans (Allen et al., 1989; Byne, 1998; Commins and Yahr, 1984; Fenstemaker et al., 1999; Gorski et al., 1978, 1980; Hines et al., 1995; Swaab and Fliers, 1985; Tobet et al., 1986). Of these, the rat SDN-POA is the best studied. The SDN-POA is 3–5 times larger in male than in female rats and depends on the actions of estrogenic compounds derived by the aromatization of testosterone (Dohler et al., 1984). The hormone-induced masculinization of the SDN-POA is due, at least in part, to differential cell death during postnatal development (Davis et al., 1996; Dodson and Gorski, 1993). Female rats have a higher rate of cell death than males between postnatal days 7 and 10 and by the second week of life have fewer cells in the SDN-POA than do males (Chung et al., 2000; Davis et al., 1996; Dodson and Gorski, 1993; Tsukahara et al., 2006). Treating female rat pups with testosterone or estradiol around the time of birth reduces postnatal apoptosis in the SDN-POA (Arai et al., 1996; Chung et al., 2000; Davis et al., 1996; Yang et al., 2004), and masculinizes its volume in adulthood.

Short-lived changes in cell and nuclear morphology that presage cell death are dramatic and defining characteristics of apoptosis. They are accompanied by DNA fragmentation and can be quantified by labeling the terminal end of nucleic acids using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Gavrieli et al., 1992). Apoptosis in the developing brain is regulated by proteins of the B cell lymphoma 2 (Bcl-2) family (Merry and Korsmeyer, 1997). Bcl-2 is anti-apoptotic and promotes cell survival, whereas Bcl-2-associated X protein (Bax) promotes apoptosis by forming heterodimers with Bcl-2 in vivo to neutralize its actions (White et al., 1998). The ratio of Bcl-2 to Bax determines the relative vulnerability of cells to succumb to apoptosis (Oltval et al., 1993). Higher levels of Bcl-2 and lower levels of Bax protein are present in the SDN-POA of male rats than of female rats during the postnatal critical period (Tsukahara et al., 2006). Moreover, treatment with estradiol not only masculinizes the volume of the SDN-POA, but also reverses the sex difference in the Bcl-2/Bax ratio (Tsukahara et al., 2008). The precise cellular mediators promoting neuronal survival in males are not yet known, although several proteins have been implicated including NMDA receptor signaling molecules and neurotrophic proteins such as RNA binding motif protein 3 and alpha-tubulin (Hsu et al., 2005, 2001; Tsukahara, 2009; Tsukahara et al., 2008).

Despite strong evidence of a role for apoptosis in the rat model, modulation of cell death does not appear to contribute to the formation of the SDN in the male ferret (Park et al., 1998). More recently, the role of cell death as a universal mechanism underlying brain sex differences in rodents has been called into question. The use of genetically modified mice that either overexpress Bcl-2 or lack expression of Bax demonstrate that apoptotic mechanisms do not generate sex differences in all neuronal phenotypes within specific nuclei (De Vries et al., 2008; Semaan et al., 2010; Zup et al., 2003). For example, these genetic manipulations did not alter the sex difference in the number of vasopressinergic neurons in the sexually dimorphic bed n. of the stria terminalis (De Vries et al., 2008). Thus, it remains an open question to what extent apoptosis/cell survival is involved in the determination of brain sex differences in other species such as the sheep.

In contrast to most models of brain sexual differentiation, the sheep is a long-gestation species in which brain sexual differentiation occurs largely prior to birth over an extended period of fetal growth and thus more closely mimics human brain development. The current study found that a reduction in programmed cell death during the critical period does not contribute to the emergence of the male-typical oSDN in sheep.

2. Results

2.1. *Relative expression of pro- and anti-apoptotic genes*

Analysis of bcl-2 mRNA expression by 2-way ANOVA revealed significant main effects of age ($F[3,23]=19.6$; $P<0.001$) and sex ($F[1,23]=25.4$; $P<0.001$. The expression of bcl-2 mRNA was greater in females than in males and decreased significantly with age in both sexes across the four fetal ages studied (Fig. 1A). Bax mRNA expression exhibited a significant main effect of age ($F[3,23]=73.3$; $P<0.001$) and a significant age × sex interaction ($F[3,23]=3.6$; $P<0.05$). Bax expression was significantly higher at gestational day 65 than at all other ages and significantly lower at gestational day 85 than at all other ages (Fig. 1B). Although no main effect of sex was observed ($F[1,23]=0.3$; $P>0.05$), post hoc analysis revealed that bax mRNA was greater in males than in females at gestational day 65 accounting for the interaction. Fig. 1C shows the ratio of bcl-2 to bax expression across the fetal ages studied. There were no sex differences in bcl-2 to bax ratio ($F[1,23]=2.5$; $P>0.05$), but there was a main effect of age on the ratio bcl-2 to bax ($F[2,23]=49.9$; $P<0.001$). In both sexes, the ratio was significantly higher at gestational day 85 than at all other ages ($P<0.05$).