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Placental and embryonic tissues exhibit aromatase activity in the viviparous lizard *Niveoscincus metallicus*

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ABSTRACT

Aromatase is a key regulator of circulating testosterone (T) and 17-β-oestradiol (E_2), two steroids which are critical to the development, maintenance and function of reproductive tissues. The role of aromatase in sexual differentiation in oviparous (egg-laying) reptiles is well understood, yet has never been explored in viviparous (live-bearing) reptiles. As a first step towards understanding the functions of aromatase during gestation in viviparous reptiles, we measured aromatase activity in maternal and embryonic tissues at three stages of gestation in the viviparous skink, *Niveoscincus metallicus*. Maternal ovaries and adrenals maintained high aromatase activity throughout gestation. During the early phases of embryonic development, placental aromatase activity was comparable to that in maternal ovaries, but declined significantly at progressive stages of gestation. Aromatase activity in the developing brains and gonads of embryos was comparable with measurements in oviparous reptiles. Aromatase activity in the developing brains peaked mid development, and declined to low levels in late stage embryos. Aromatase activity in the embryonic gonads was low at embryonic stage 29–34, but increased significantly at mid-development and then remained high in late stage embryos. We conclude that ovarian estrogen synthesis is supplemented by placental aromatase activity and that maternal adrenals provide an auxiliary source of sex steroid. The pattern of change in aromatase activity in embryonic brains and gonads suggests that brain aromatase is important during sexual differentiation, and that embryonic gonads are increasingly steroidogenic as development progresses. Our data indicate vital roles of aromatase in gestation and development in viviparous lizards.

1. Introduction

The aromatase enzyme complex comprises of cytochrome P450 aromatase and nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase. The aromatase enzyme complex, hitherto referred to as aromatase, catalyses the conversion of androstenedione (A_4) and testosterone (T) to estrone (E_1) and 17-β-oestradiol (E_2) respectively (Conley and Hinshelwood, 2001; Simpson et al., 2002; Payne and Hales, 2004). Both the components and the functions of aromatase are highly conserved across vertebrate species (Conley and Hinshelwood, 2001; Simpson et al., 2002; Payne and Hales, 2004), highlighting the ubiquitous nature of estrogen synthesis in all vertebrates, and the pivotal role of aromatase in vertebrate reproductive function.

Aromatase is fundamental to embryonic development for several reasons. Aromatase activity influences circulating ratios of T and E_2 (Conley and Hinshelwood, 2001; Simpson et al., 2002; Payne and Hales, 2004), therefore, aromatase plays a fundamental role in sexual differentiation of phenotype. Testosterone and E_2 are critical to differentiation of the reproductive system and the expression of phenotypic diversity in sexually dimorphic traits (Clark and Galef, 1995; Wilson and Davies, 2007; Ramsey and Crews, 2009). Although there are both species-specific and sex specific variations in aromatase activity during embryonic development, aromatase is expressed in the developing brains and gonads of male and female vertebrates (Krohmer and Baum, 1989; Weniger, 1990, 1993; Willingham et al., 2000; Blázquez et al., 2008).

Many studies have concluded that an external source of hormones including T and E_2, is required to initiate development of the embryonic gonads (Conley et al., 1997; Janzen et al., 1998; Paitz and Bowden, 2009). In amniotes (mammals, birds and reptiles), parity mode influences the source of these external hormones. In oviparous (egg-laying) species, the egg yolk is the major source of steroids of maternal origin that are deposited in yolk during vitellogenesis (Schwabl, 1993; Lovern and Wade, 2003; Paitz and Bowden, 2009): the chorioallantoic membrane

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(CAM), which is now known to be steroidogenic (Albergotti et al., 2009; Cruze et al., 2012, 2013), provides an additional source. In eutherian mammals, the amnion and chorion fuse to form the placenta which allows transport of maternal hormones throughout gestation (Pasqualini, 2005) and steroid hormones are also synthesised by the placenta (Leiser and Kaufmann, 1994; Strauss et al., 1996).

The embryos of oviparous reptiles and eutherian mammals are clearly exposed to maternal hormones via very different routes (yolk versus placenta), but what do we know of those viviparous reptiles that utilise both a placenta and a yolk? Viviparity has evolved more than 100 times in reptiles and approximately 30% of all reptilian species are viviparous (Blackburn, 1982, 1985, 1993). Due to the number of independent origins of viviparity, there is considerable variation in the form and complexity of the chorioallantoic placenta, therefore, four main placental types have been defined in viviparous reptiles (Blackburn, 1993, 2000; Stewart and Thompson, 2000; Thompson and Speake, 2006). Type I species are predominantly lecithotrophic: these species have shell-less yolky eggs that are retained in the oviduct and the very simple placenta allows for water and respiratory gas exchange (Thompson and Speake, 2006). At the other end of the spectrum, Type IV species are highly placentrophic: a complex placenta provides the majority of the nourishment to developing embryos while a microlecithal yolk provides minimal nourishment to embryos (for review see Stewart and Thompson, 2000; Thompson and Speake, 2006; Stewart, 2013). The intermediate Types II and III fall along a spectrum of placental complexity and degree of placentotrophy (Stewart and Thompson, 2000; Thompson and Speake, 2006).

To date, there is scant information on the steroidogenic capacity of any reptilian placentae. While it has been demonstrated that the delivery of nutrients varies with placental type, the capacity for synthesis of hormones may not. The demonstration of steroidogenesis by the CAM of the oviparous reptiles *Alligator mississippiensis* and *Pseudemys nelsoni*, as well as the bird *Gallus gallus*, indicates that such steroidogenic function of the extraembryonic membranes is a conserved attribute of all amniotes (Albergotti et al., 2009; Cruze et al., 2012, 2013). This idea is further supported by progesterone production by representative reptilian species with three of the four placental types (Girling and Jones, 2003; Guarino et al., 1998; Painter and Moore 2005). However, the presence of placental aromatase, and, therefore, placental capacity to synthesise oestrogens, has not been confirmed in any viviparous reptile.

This study therefore aims to identify key sites and activity levels of aromatase during gestation in the reproductive tissues of the viviparous lizard, *Niveoscincus metallicus*. This species provides an excellent model for a first investigation of the activity and putative functions of aromatase in viviparous lizards. The reproductive physiology of *N. metallicus* is well understood (Jones and Swain, 1996, 2006; Swain and Jones, 1997; Jones et al., 1998) and the moderately complex Type II placenta has been extensively studied (Stewart and Thompson, 1994, 2000, 2003; Jones and Swain, 1996; Jones et al., 1998; Stewart, 2013). We hypothesise that aromatase is present in placental tissue of *N. metallicus*, and that aromatase activity increases toward the later phases of gestation because placental development progresses with embryonic development (Stewart and Thompson, 2003). We hypothesise that aromatase in the developing embryonic brains will peak during the mid-phases of embryonic development around the time of sexual differentiation. We also suggest that aromatase activity in embryonic gonads will increase from early to late development, because the steroidogenic potential of the gonads in early development in other reptilian species is very low (Girling and Jones, 2006; Milnes et al., 2002; Smith and Joss, 1994; Willingham et al., 2000).

2. Methods

We measured aromatase activity in selected maternal and embryonic tissues of *N. metallicus*. *N. metallicus* is a small lizard with snout-vent length (SVL) of $\leqslant$65 mm and body mass 2–3 g. *N. metallicus* has a type II reproductive cycle as defined by Taylor (1985). Vitellogenesis is initiated in autumn and completed after spring emergence (September): ovulation typically takes place in mid spring ($\sim$October in Tasmania) with gestation typically lasting $\sim$3 months. Females give birth to between one and six young. We collected adult female lizards at three stages during gestation: 'early' (embryonic stages 29–34, $n = 9$); 'mid' (embryonic stages 35–37, $n = 10$) or 'late' (embryonic stages 38–40+, $n = 11$) gestation, as defined by Dufaure and Hubert (1961). In *N. metallicus*, an additional stage of embryonic development (stage 40+) has been defined: at this stage, embryos have internalised hemipenes, no yolk remaining and development is $\geqslant$90% complete (Swain and Jones, 1997). We collected gestating females when we anticipated embryos would have reached the aforementioned stages; however, accurate staging of embryos is not possible in viviparous lizards until dissection, and thus we have unequal numbers of females (and embryos) for each stage.

2.1. Animal and tissue collection

Pregnant female *N. metallicus* were collected by mealworm fishing or noosing in and around the Sandy Bay campus of the University of Tasmania: 42°54′24.9″S, 147°19′21.89″E and Old Farm Rd: 42°53′38.33″S, 147°19′21.29″E in greater Hobart, Tasmania, Australia. The adult female lizards were collected from October through till December 2009 and 2010. Lizards were co-housed overnight in cages 200 × 600 mm with pureed fruit as a food source and water *ad libitum*.

Adult female lizards were weighed and measured (SVL) prior to humane sacrifice with an IP bolus injection of sodium pentobarbital, at a dose of 500 ng/g diluted 1:100 in saline solution. At dissection, litter size was recorded. Both ovaries and adrenal glands and samples of skeletal muscle tissue from a hind limb were removed and submerged in ice-cooled RPMI-1640 incubation medium (Sigma–Aldrich, Australia) while the dissections were completed. Maternal ovaries served as a 'positive control' tissue as we anticipated high aromatase activity in this primary steroidogenic tissue. Similarly, skeletal muscle tissue was included as a 'negative control' as we expected limited capacity for steroidogenesis in this tissue. Embryos were dissected free from the yolk and placental tissue. All embryos within each litter were utilised. Placental tissue (early: $n = 23$, mid: $n = 35$ and late: $n = 37$) was rinsed and placed in ice cooled RPMI-1640 incubation medium. The embryos were carefully dissected under a stereomicroscope. The small size of *N. metallicus* prevents isolation of the developing Adrenal–Kidney–Gonad complex (AKG) until after stage 37 so the AKG complex of embryos up to stage 36 (early: $n = 32$, mid: $n = 34$, late: $n = 33$) was sampled by removing the entire torso of the embryos (Girling and Jones, 2003). Similarly, the entire head of embryos at or before stage 36 was used to assess aromatase activity in the brain (early: $n = 31$, mid: $n = 35$, late: $n = 37$). The AKG and the brain of embryos were dissected out in embryos post stage 37. The wet weight of maternal ovaries, skeletal muscle tissue and placentae were recorded. For maternal adrenal glands, embryonic heads, brains, trunks and AKGs tissue weight was not recorded, as an accurate weight could not be obtained for maternal adrenals and embryonic AKGs. Embryonic trunks and heads contain non-steroidogenic tissue, thus the weight of such samples is not biologically meaningful and was also not included in the analysis. All tissues were finely minced prior to incubation with a fine pair of forceps.