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Long-term under-masculinization in male rabbits due to maternal stress is reversed by prenatal administration of testosterone

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ABSTRACT

It is well established that in mammals prenatal exposure to exogenous testosterone has a masculinizing effect on female morphology and behavior. Fewer studies, however, have been conducted in males on this subject, and the results are controversial. In the present study, we investigated the long-term effect of administering extra prenatal testosterone (testosterone proprionate; TP) on adult male domestic rabbits' morphology and behavior using two different control groups, non-treated and vehicle injected mothers. Unexpectedly, administering the vehicle alone had a clear under-masculinizing effect on all morphological and behavioral measures; lower body mass, smaller anogenital distance and smaller chin glands, lower chin-marking activity and greater timidity. Administration of TP counteracted this effect in a dose-dependent manner such that animals exposed to the highest dose prenatally showed values on the morphological and behavioral measures equivalent to but not greater than the non-treated control group. We conclude (1) that additional testosterone beyond what male fetuses produce in utero does not result in increased masculinization, and thus, that male fetuses are less susceptible prenatally to hormonal effects than females, and (2) that presumably stress-related effects of administering the vehicle alone resulted in under-masculinization, which could be recovered by the prenatal administration of TP. These results may partly account for the contradictory findings of previous studies, and indicate the importance of including both non-treated and sham- (vehicle) treated controls in future experiments.

1. Introduction

In mammals, differences among same-sex littermates in morphology and behavior are not only due to genetic factors, even when present from birth (Lewejohann et al., 2011; Phoenix et al., 1959). For example, it is well known that the sexual differentiation of individual offspring in mammals is affected in utero by the amounts of steroid hormones, they themselves produce or that reach them from other sources (Gerall et al., 1992). Among these hormones testosterone (T), or its metabolites, play a major role in the prenatal masculinization process in a wide variety of mammals, influencing many aspects of an individual's morphology as well as areas of the brain involved in the regulation of sexual behavior (Morris et al., 2004). Male fetuses start to produce T earlier and in larger amounts than females, and this is essential for the masculinization process, which typically occurs during a species-specific sensitive period (Dean and Sharpe, 2013; Harris and Levine, 1965; MacLusky and Naftolin, 1981; Ward and Weisz, 1980).

In some species, T can readily cross the fetal membranes, meaning that in litter-bearing mammals hormones may reach individual young from adjacent fetuses (Clemens et al., 1978; vom Saal, 1989). As a consequence, the intrauterine position of a fetus relative to the number and sex of neighboring littermates can affect the probability of it being exposed to additional, exogenous amounts of T (Even et al., 1992; vom Saal and Dhar, 1992).

Experimental manipulation of the fetal hormonal environment has also been shown to influence sexual differentiation. One of the most commonly used morphological biomarkers of early masculinizing effects of T is anogenital distance (AGD) which exhibits sex-related variation in several rodent species (Clark and Galef, 1995; Drickamer, 1996), lagomorphs (Bánszegi et al., 2009) and

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in humans (Salazar-Martinez et al., 2004). In all these species, males have longer AGD than females. Male rodents exposed to anti-androgens during prenatal life are permanently under-masculinized in their morphology and physiology (Clemens et al., 1978; Macleod et al., 2010; Perakis and Stylianopoulou, 1986; vom Saal, 1978; Welsh et al., 2008), while exposure to exogenous androgens permanently masculinizes females (Gandelman et al., 1979; Goldfoot and Van Der Werff Ten Bosch, 1975; Rhees et al., 1997; vom Saal, 1979; Wolf et al., 2002). Nevertheless, in males, relatively few studies have been conducted examining the developmental effects of the early administration of exogenous T and the results are somewhat contradictory. Whereas in some mammalian studies, prenatal treatment with T had no effect on male AGD or weight at birth or adulthood (Dela Cruz and Pereira, 2012; Keisler et al., 1991; vom Saal, 1979), in other studies it had (Gill and Hosking, 1995; Juárez et al., 1995; Manikkam et al., 2004). Furthermore and somewhat paradoxically, in rats prenatal treatment resulted in no effect on or decreased AGD (Dela Cruz and Pereira, 2012; Hotchkiss et al., 2007; Tehrani et al., 2013; Wolf et al., 2002) and in delayed puberty, but increased aggression, altered sexual preference and increased vocalization (Henley et al., 2010; Hotchkiss et al., 2007; Juárez et al., 1995; Kršková and Talarovičová, 2005).

In the European rabbit *Oryctolagus cuniculus*, the extensively researched representative of a rather different mammalian taxonomic group to the rodents (Order Lagomorpha), the fetal gonads begin to differentiate around gestational day (GD) 14 (Allen, 1904; Veyssiere et al., 1976) and male fetuses start to produce T. The T level increases between GD 18 and 19 and reaches a maximum at GD 20–21, which is the critical sensitive period for sexual differentiation (masculinization programming window) in this species (Ivanova, 1981). From GD 21–25 plasma T level vary little (Veyssiére et al., 1980), but increase from GD 28 until birth (GD 30–31), reaching the values observed in newborns (Veyssiere et al., 1975).

As in other mammals, females which were exposed to exogenous T from their male neighbors in utero show evidence of masculinization. They have large AGD and show elevated chin-marking activity as adults (Bánszegi et al., 2009). Administration of T to pregnant females also has clear masculinizing effects on their daughters, increasing their AGD and their chin-marking activity as adults compared to the daughters of non-injected control females (Bánszegi et al., 2010). In rabbits, serum levels of T are positively correlated with postnatal growth (Hudson et al., 2011) and are positively associated with agonistic behavior and social dominance in adults (von Holst, 1998). Dominance, in turn, is positively correlated with chin gland size and chin-marking activity (Arteaga et al., 2008; Black-Cleworth and Verberne, 1975; Farabollini, 1987; Girolami et al., 1998; Mykytowycz, 1965), and administration of T increases chin-marking in males whereas castration reduces or eliminates it (Briganti et al., 2003; Chirino et al., 1993; González-Mariscal et al., 1993; Martínez-Gómez et al., 1997).

Rabbits are often used as experimental subjects in studies of prenatal effects on prenatal development because they are polytocous and they have hemochorial placentation (as in rat, house mouse and humans) (Faber and Hart, 1967; Flexner and Pohl, 1941; Osol and Mandala, 2009). Although, effects of prenatal T administration have been established in female rabbits (Bánszegi et al., 2009, 2010), no studies have been made on the effect of prenatal T administration on males. Given the scarce and somewhat contradictory reports on the developmental effects on mammalian (mainly rodent) males of prenatal exposure to exogenous T, it was our aim in the present study to investigate the long-term effects of administering T to pregnant rabbits on the morphology and behavior of their sons, using established indicators of masculinization such as increased AGD, increased chin gland size and increased chin-marking activity.

Recently, it has been show that other, non-social behaviors such as boldness can also be influenced by levels of androgens; experimentally elevating levels of T resulted in increased boldness in African striped mouse males (Raynaud and Schradin, 2014). Since our group has long experience measuring boldness in rabbits, we tested for long-term effects in the prenatally TP-exposed males on this parameter also (Csatádi et al., 2005; Dúcs et al., 2009; Pongrácz and Altbäcker, 1999).

2. Materials and methods

2.1. Animals

We used multiparous, chinchilla breed domestic rabbits (*O. cuniculus*) and their offspring, raised at the Biological Research Station of Eötvös University, Göd, Hungary. Animals were kept separately in standard wire mesh rabbit cages (45 × 55 × 65 cm) with continuous access to pelleted rabbit food (Galgavit Rt., Hungary) and water. The trays beneath the cages were cleaned daily. Temperature was maintained between 18 and 21 °C and the daily light:dark cycle was set at 14:10 h. with lights on at 8 am.

2.2. Procedure

Experimental animals were kept and treated in accordance with the European Community's Council Directive of 24 November 1986 (86/609/EEC) and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA.

Females were brought to the buck's cage for mating, and then returned to their own cages. The ratio of successful matings in this induced ovulator was close to 100%, but if the pair did not mate within 5 min, the female was removed and paired with another male. Hence, the exact date of fertilization was known. The day of mating was considered GD 0. On day 28 of pregnancy an opaque plastic nest box (30 × 30 × 40 cm) was fitted to the female's cage and hay was provided for nest building. Litter size ranged from 6 to 9 pups (Table 1). Following parturition (around GD 31 in the rabbit), the sex of pups was determined by external genital inspection and they were marked in their ears with ink for individual identification. The nest box was then closed and the mothers were allowed to nurse once each day between 09:00 and 10:00 h (Schlolaut et al., 2013; Venge, 1963; Zarrow et al., 1965) following the natural pattern of nursing behavior of this species (Hudson and Distel, 1989; Rödel et al., 2012). Pups were weaned on postnatal day 28, when they were fitted with a numbered ear tag and individually housed in separate cages under the standard conditions described above.

To test the effect of prenatal exposure to additional T on the sexual development of males, the does were re-mated after weaning the first set of pups, and those that were again pregnant were

Table 1
General characteristics of first (baseline) and second (experimental) litters at birth.

	1st litter	2nd litter	*F*	*p*
Litter size (#)	6.97 ± 1.95	7.54 ± 2.13	1.95	0.17
Litter mass (g)	371.51 ± 94.32	417.55 ± 88.57	3.55	0.07
Sex ratio (%)	55.87 ± 17.68	49.05 ± 15.88	2.59	0.12

Means ± SD are reported. Results of statistical tests represent Repeat*Group values derived from a repeated measures General Linear Model (see Section 2.3 for details).