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Postnatal penile growth concurrent with mini-puberty predicts later sex-typed play behavior: Evidence for neurobehavioral effects of the postnatal androgen surge in typically developing boys

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ABSTRACT

The masculinizing effects of prenatal androgens on human neurobehavioral development are well established. Also, the early postnatal surge of androgens in male infants, or *mini-puberty*, has been well documented and is known to influence physiological development, including penile growth. However, neurobehavioral effects of androgen exposure during mini-puberty are largely unknown. The main aim of the current study was to evaluate possible neurobehavioral consequences of mini-puberty by relating penile growth in the early postnatal period to subsequent behavior. Using multiple linear regression, we demonstrated that penile growth between birth and three months postnatal, concurrent with mini-puberty, significantly predicted increased masculine/decreased feminine behavior assessed using the Pre-school Activities Inventory (PSAI) in 81 healthy boys at 3 to 4 years of age. When we controlled for other potential influences on masculine/feminine behavior and/or penile growth, including variance in androgen exposure prenatally and body growth postnally, the predictive value of penile growth in the early postnatal period persisted. More specifically, prenatal androgen exposure, reflected in the measurement of anogenital distance (AGD), and early postnatal androgen exposure, reflected in penile growth from birth to 3 months, were significant predictors of increased masculine/decreased feminine behavior, with each accounting for unique variance. Our findings suggest that independent associations of PSAI with AGD at birth and with penile growth during mini-puberty reflect prenatal and early postnatal androgen exposures respectively. Thus, we provide a novel and readily available approach for assessing effects of early androgen exposures, as well as novel evidence that early postnatal aes human neurobehavioral development.

Introduction

The fetal testes begin to produce testosterone (T) and other androgens by week 8 of gestation with T peaking between about weeks 8 to 24 (Reyes et al., 1974; Winter et al., 1976). There is also a surge of androgens during early postnatal development, often referred to as "mini-puberty" (Rajpert-De Meyts et al., 2013), with T peaking at about one to three months postnatal and declining to minimal levels by about 6 months postnatal, where it remains until puberty (Achermann and Hughes, 2011; Kuiri-Hänninen et al., 2011). These early periods of androgen production are necessary for normal development of the urogenital tract, including formation of the external genitalia in early embryonic development and further growth of the genitalia, including increased penile length, during later perinatal development (Main et al., 2000). At the same time, neural plasticity is high as the human brain develops through gestation and into early infancy (de Graaf-Peters and Hadders-Algra, 2006). Though these parallel processes have been characterized, their potential behavioral consequences, especially in the early postnatal period, have yet to be fully understood.

Experimental studies of rats and non-human primates link androgen exposure during perinatal development to changes in sexually dimorphic reproductive and neural morphology, as well as to changes in behaviors that show sex differences (Gorski et al., 1978; Goy et al., 1988; MacLeod et al., 2010; McCarthy et al., 2009). In humans, there is evidence that androgens during prenatal development influence later gender-related behaviors. This evidence comes mainly from studies of girls and women exposed to elevated concentrations of androgens in utero, due to the genetic condition, congenital adrenal hyperplasia (CAH). In addition to showing physical virilization of the urogenital

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tract (Merke and Bornstein, 2005), girls with CAH show increased preferences for boys' toys and activities and for boys as playmates, compared to unaffected girls (Berenbaum and Hines, 1992; Ehrhardt and Baker, 1974; Hines et al., 2004; Pasterski et al., 2005, 2007, 2011). In adulthood, women with CAH show reduced heterosexual orientation and are less female-typical in their gender identity compared to other women (Hines, 2011b; Slijper et al., 1998). Studies of individuals with other conditions causing prenatal abnormalities of androgen exposure, and studies relating prenatal androgen exposure to postnatal behavior in typically developing individuals, also support the conclusion that prenatal androgen exposure influences human neurobehavioral development (Auyeung et al., 2002; Hines, 2011b; Hines et al., 2002).

By contrast, evidence for neurobehavioral effects of early *postnatal* androgen exposure in humans is scarce. While the physiological consequences of the postnatal surge in androgens have been characterized in a large scale population study (Boas et al., 2006), and anatomical anomalies subsequent to progressive hypogonadism in the early postnatal period have been reported (Main et al., 2000), only a single study has prospectively linked variance in early postnatal androgen during mini-puberty to variance in subsequent gender-related behavior (Lamminmäki et al., 2012). Using urine samples obtained at day 7 postnatal and then monthly from the age of 1 month to 6 months, Lamminmäki et al. (2012) found that the area under the curve (AUC) for concentrations of T predicted subsequent gender-typical play behavior, assessed at age 14 months, in typically developing children. Though these results support an influence of early postnatal T exposure on the development of gender-related behavior, the authors were unable to assess effects of prenatal and early postnatal T separately. Because prenatal and early postnatal levels of T might correlate, and prenatal concentrations of T are known to relate to later gender-typical play, effects could not be attributed specifically to early postnatal T exposure.

Distinct aspects of physical development reflecting gradations in androgen exposure at different times may provide a readily available methodology for separating effects of prenatal and early postnatal androgen exposure on human behavior. Anogenital distance (AGD), which is measured as the distance from the anus to the scrotum in males and to the vagina in females (Thankamony et al., 2009), may be a marker of androgen exposure specific to early fetal development. In humans, AGD is sexually dimorphic, with boys having nearly twice the AGD of girls at birth (Lamminmäki et al., 2012; Thankamony et al., 2009). Also, AGD appears to be sensitive to changes in fetal androgens (Swan et al., 2005; Thankamony et al., 2014). For example, boys born with manifestations of testicular dysgenesis syndrome such as cryptorchidism (absence of one or both testes from the scrotum) or hypospadias (incomplete formation of the penis) have reduced AGD reflective of the degree of disruption (Thankamony et al., 2014). Also, research looking at exposure to environmental androgen disruptors, such as phthalates, has linked metabolites measured during gestation to reduced AGD in boys at 12 months of age (Swan, 2008) and prenatal exposure to phthalates has been linked to reduced masculine behavior in boys (Swan et al., 2010). Finally, epidemiological studies of healthy men have demonstrated that concurrent measurements of AGD predict sperm production, count and quality (Dean and Sharpe, 2013). While these findings suggest that AGD could be a marker of prenatal androgen exposure, which may also relate to sexually differentiated behavior in humans, no reports directly linking AGD to human behavior have been published.

By contrast, growth in penile length during the first few months postnatally could provide a measure of androgen exposure during mini-puberty (Boas et al., 2006; van den Driesche et al., 2011). Experimental evidence from rats has shown that while initial formation of the male external genitalia takes place early in embryonic development, a further period of penile growth reflects late gestational and early neonatal influences of androgens (van den Driesche et al., 2011). Similar evidence in humans comes from a large scale population study of boys, showing that serum T, measured in blood at age three months, correlated with penile development, including growth in penile length from birth to three months of age (Boas et al., 2006).

The aim of the current study was to assess for possible neurobehavioral effects of the postnatal surge in androgens, as reflected in growth in penile length in boys, prospectively, while controlling for potential confounding influences, including prenatal androgen exposure and body growth in general. Using multiple linear regression and bivariate correlation, we related changes in penile length across the first two years of life to gender-related behavior at three to four years of age in typically developing boys. We discriminated effects specific to the window of the postnatal surge in androgens, as opposed to later effects, by including sequential measurements of penile length, beginning with baseline at birth and the subsequent addition of measurements at three, 12, 18 and 24 months of age. To account for pre- versus postnatal androgen exposure, we included AGD at birth, and because other factors which contribute to body growth in early infancy may also contribute to penile growth, we included body length at birth and at 12 months to control for general body growth during the developmental period under study. Based on findings which have established the postnatal surge in T to peak at about one to three months postnatal (Boas et al., 2006; Lamminmäki et al., 2012), concurrent with rapid penile growth up to three months (Boas et al., 2006), we expected change in penile length from birth to three months postnatal to predict variance in subsequent gender-related behavior. Furthermore, given previous findings linking AGD at birth to prenatal androgen concentrations (van den Driesche et al., 2011; Welsh et al., 2008), we expected this parameter to reflect prenatal T exposure, which could be confirmed by unique predictive value in the multiple regression analysis, and controlled for in analyses investigating the behavioral consequences of androgen exposure during mini-puberty.

Method

Participants

Participants included 81 typically developing boys recruited from the larger Cambridge Baby Growth Study, an ongoing longitudinal study established to characterize hormonal, genetic, and environmental influences on infant growth and male reproductive development. Mothers were recruited from the maternity unit of Addenbrooke's Hospital, University of Cambridge, at about week 12 of gestation when they attended for routine prenatal ultrasound procedures, between 2006 and 2008. Mean maternal age was 33.24 ± 3.66 years at delivery. Mothers who agreed to participate gave written informed consent for themselves and for their children to participate in the longitudinal study. The research protocol was approved by the Cambridge Local Research Ethics Committee, and the study was conducted in accordance with the standards of Good Clinical Practice. Anthropometric measurements were initially taken either at the hospital or at the participant's home and subsequently in a dedicated follow-up research clinic. As the infants in the larger study began to turn three years old, their mothers were invited to complete a questionnaire measuring childhood gender-typed behavior. Of 102 male invitees, 83 returned the questionnaires and had complete data across all measurements. One boy showed severely compromised body growth in the first year of life and another had ratings on the questionnaire that were >3 SDs from the mean, suggesting error. These two were not included in the further analyses, so the final sample of boys for the current report was 81.

Although female infants are necessarily excluded from studies of male reproductive development, including studies of postnatal testicular activation, data for a comparable subset of females from the larger study of infant growth were included in the current report to compare the relationship between measurements of AGD and later gender-related behavior in typically developing females. Of 93 female invitees, 73 returned the questionnaires and had complete data across all measurements (mean maternal age at delivery was 34.06 ± 4.06).

Measures of clitoral length and growth in girls, although perhaps comparable to penile length and growth in boys, were not obtained, because the focus of the study was male reproductive development.

Measurements

Anthropometrics

Penile length, AGD, and body length were among the growth parameters assessed as part of the larger baby growth study. Measurements were taken at birth, and at three, 12, 18, and 24 months of age in typically developing infants. At each interval, three measurements were taken for penile length and AGD by a research nurse trained with standardized procedure, using Vernier calipers. Penile length was measured in centimeters from the lower edge of the pubic bone to the tip of the penis, excluding the foreskin and avoiding erection. AGD was measured in millimeters from the center of the anus to the junction of smooth perineal skin and rugated skin of the scrotum in boys, and from the anus to the lower vaginal opening in girls. Detailed findings with respect to AGD have been published for the larger study (Thankamony et al., 2009). Quality control exercises led by an anthropometrist yielded relative and absolute technical error measurements (TEM; Ulijaszek and Kerr, 1999) for AGD that were 9.6% and 3 mm for boys and 5.7% and 1 mm for girls. With respect to intra-observer agreement for the current subset of data, the range was $r = .91$ to $r = 1.00$ for penile length and AGD measurements, including AGD measurements in females. The mean of the three values was calculated for each parameter at each interval, and used for statistical analyses. Weight was measured in kilograms and body length in centimeters.

Gender-related behavior

Gender-related behavior was measured at three to four years of age using the Preschool Activities Inventory (PSAI) (Golombok and Rust, 1993). The PSAI is a 24-item parent-report standardized psychometric instrument that assesses children's gender-typed toy and activity preferences. There are 12 items asking about female typical activities, such as "likes to play with dolls" and "likes to play with girls," and 12 items asking about male-typical activities such as "likes to play with cars, trains or airplanes" and "likes to play with boys." Each item is scored on a 5-point Likert scale from 1 = "Not at all like my child" to 5 = "A lot like my child." Final scores were calculated using the following formula, with higher scores representing more masculine/less feminine behavior and lower scores representing more feminine/less masculine behavior:

$$\text{Score} = 48.25 + 1.1 \times (\text{sum of "male" items} - \text{sum of "female" items}).$$

Results

Sample characteristics

Table 1 shows anthropometric and behavioral data for the current sample along with that for samples of children from the larger Cambridge Baby Growth Study (Thankamony et al., 2009) and from the standardization of the PSAI (Golombok and Rust, 1993). The larger samples show expected gender-related differences and our subsample of 81 boys was similar to the larger samples of boys in terms of birth weight, birth body length, birth penile length, AGD at birth, and PSAI scores.

Correlations between PSAI scores and anthropometrics

Table 2 shows Pearson's *r* and significance values for zero-order correlations between penile/AGD length/growth and gender-related behavior (PSAI scores) using bivariate correlational analysis. The pattern of results showed that while PSAI scores correlated significantly with penile length at three, 12, and 24 months of age, the association appears to have been established largely in the period from birth to three months. Specifically, correlations for growth parameters with PSAI scores showed that penile growth from birth to three months related significantly to PSAI scores, but growth after three months did not. Thus, the correlations with penile length at 12 and 24 months appear to reflect persistence of the relationship established during the first three months postnatal. By contrast, AGD at birth, and not at later time points, showed a positive relationship with PSAI scores that approached significance, $r(79) = .20$, $p = .07$. It can be seen in the following regression analyses that the relationship between AGD at birth and PSAI scores was enhanced when body length and penile length at birth were controlled (Beta = .25, $p < .05$; see Table 3). Postnatal AGD growth was not related to PSAI scores. For girls, correlations between AGD at the 5 time points and PSAI scores were all insignificant.

Regression analysis

To further investigate potential effects of androgen exposure in mini-puberty on behavior, we conducted multiple regression analysis with PSAI as the dependent variable and predictors entered in three blocks, represented as Models 1 and 2A/2B. For completeness and

Table 1
Sample characteristics and comparisons to published data/norms.

Anthropometric data Means ± SD	Cambridge Baby Growth Study (CBGS; 2006–2008)[a]		Growth study sex difference		Current subsample	Comparisons: Subsample males with CBGS males	
	Males N = 134	Females N = 118	*p*	*d*[b]	Males N = 81	*p*	*d*[b]
Birth weight (kg)	3.60 ± 0.47	3.42 ± 0.44	<.01	0.39	3.50 ± 0.58	ns	−0.20
Birth body length (cm)	51.29 ± 2.07	50.26 ± 1.76	< .001	0.54	51.37 ± 2.51	ns	0.04
Birth AGD (mm)	19.82 ± 6.04	9.04 ± 2.24	< .001	2.53	20.07 ± 5.95	ns	0.04
Birth penile length (cm)	3.01 ± 0.35	–	–	–	2.98 ± 0.41	ns	−0.08
Pre-school Activities Inventory (PSAI)	**Standardization samples[c]**		**Standardization sample sex difference**		**Current subsample**	**Comparison: Subsample males with standardization males**	
	Males N = 1070	Females N = 1260	*p*	*d*[b]	Males N = 81	*p*	*d*[b]
Age range	18–71 M	18–71 M			36–59 M		
PSAI score	60.06 ± 10.00	40.49 ± 9.74	< .001	1.98	61.35 ± 9.63	ns	0.13

CBGS = Cambridge Baby Growth Study; AGD = anogenital distance; M = months.
[a] Data from Thankamony et al. (2009) data. Note that the larger cohort study N excludes cases from the current subsample for comparison purposes.
[b] Effect size is Cohen's *d*: $M_1 - M_2 / [(SD_1 * N_1) + (SD_2 * N_2)] / (N_1 + N_2)$ (Cohen, 1988).
[c] Data from Golombok and Rust (1993).

Table 2
Zero-order correlations for anthropometrics and Δ in anthropometrics with Pre-school Activities Inventory (PSAI) scores.

PSAI and length		Males (N = 81)				
		Birth	3 M	12 M	18 M	24 M
Penile length	*r*	−.029	**.276**	**.264**	.153	**.230**
	p	.797	**.013***	**.017***	.172	**.040***
AGD	*r*	.201	.126	−.008	.066	.097
	p	.071	.262	.941	.558	.390
PSAI and growth		**Males (N = 81)**				
		Birth to 3 M	3 M to 12 M	12 M to 18 M	18 M to 24 M	
Δ penile length	*r*	**.307**	.027	−.073	.067	
	p	**.005****	.809	.519	.553	
Δ AGD	*r*	−.176	−.164	.038	.052	
	p	.116	.145	.739	.644	
PSAI and AGD		**Females (N = 73)**				
		Birth	3 M	12 M	18 M	24 M
AGD	*r*	−.048	−.014	.065	−.027	.019
	p	.906	.906	.582	.819	.873

AGD = anogenital distance; M = months.
Δ signifies change in the parameter.
Bold type highlights significant correlations for ease of evaluation.
* $p < .05$.
** $p < .01$.

comparison, we entered subsequent predictors in the form of raw scores for penile and body length measurements in Model 2A and we entered change scores to represent penile and body growth in Model 2B. Table 3 shows significance levels along with R^2 change and F change when predictors were added, as well as effect sizes for each model (Cohen's f^2). Beta represents the effect sizes for the individual predictors. In Model 1 we included newborn measurements for AGD, penile length, and body length. The initial model allowed us to assess potential variance in PSAI scores accounted for by prenatal androgen exposure reflected in AGD at birth, and to provide a baseline for penile growth we included penile length at birth, while controlling for general body size, using body length. By adding subsequent measurements, we were able to account for additional variance in PSAI scores as a function of changes in length scores, or growth, for the specified parameters. In Model 2A, we added raw scores for penile length at the four subsequent time points (three, 12, 18, and 24 months) as well as body length at 12 months. In Model 2B we included change scores to represent growth in the periods from birth to three months, from three to 12 months, from 12 to 18 months, and from 18 to 24 months as well as change in body length from birth to 12 months. Including the body length at 12 months in Model 2A and growth in body length from birth to 12 months in Model 2B controlled for general body length/growth during the period of the postnatal testicular surge. Table 4 shows further statistics for both Models 2A and 2B, including unstandardized coefficients (B), confidence intervals for B, and collinearity statistics.

Table 3
Regression statistics using physiological markers to predict masculine/feminine behavior in boys measured using the Pre-school Activities Inventory (PSAI).

N = 81 males	R^2	R^2 change	F change		Standardized coefficients		
			F	*p*	Beta	*t*	*p*
Model 1	**.102**	**.102**	**2.87**	**.042***			
($p = .042^*$, $f^2 = 0.11$)†							
AGD birth					**.254**	**2.31**	**.024***
Penile length birth					−.041	−0.36	.721
Body length birth					−.195	−1.74	.086
Model 2A	**.242**	**.140**	**2.63**	**.031***			
($p = .009^{**}$, $f^2 = 0.32$)†							
AGD birth					**.261**	**2.46**	**.016***
Penile length birth					**−.258**	**−2.05**	**.044***
Body length birth					−.002	−0.01	.989
Penile length 3 M					**.271**	**2.05**	**.044***
Penile length 12 M					.125	1.05	.298
Penile length 18 M					−.041	−0.35	.729
Penile length 24 M					.096	0.76	.453
Body length 12 M					−.218	−1.65	.103
Model 2B	**.242**	**.140**	**2.63**	**.031***			
($p = .009^{**}$, $f^2 = 0.32$)†							
AGD birth					**.261**	**2.46**	**.016***
Penile length birth					.121	0.92	.363
Body length birth					−.210	−1.73	.087
Δ penile length birth to 3 M					**.432**	**3.00**	**.004****
Δ penile length 3 M to 12 M					.191	1.13	.264
Δ penile length 12 M to 18 M					.081	0.45	.657
Δ penile length 18 M to 24 M					.112	0.76	.453
Δ body length birth to 12 M					−.119	−1.65	.103

AGD = anogenital distance.
Bold type highlights significant relationships for ease of evaluation.
† Effect size is Cohen's $f^2 = R^2 / 1 - R^2$ (Cohen, 1988).
* $p < .05$.
** $p < .01$.

Taken together, the three regression models suggested that change in penile length during the first three months postnatal was a significant predictor of PSAI scores, independent of other relevant factors, including factors related to prenatal androgen exposure. Model 1, which included newborn measurements for AGD, penile length, and body length, was significant, $p < .05$, $f^2 = 0.11$, and, of the three predictors, only AGD at birth was significant at $p < .05$. Model 2A shows that when penile length at four subsequent time points and body length at 12 months were added to Model 1, the overall model was again significant, $p < .01$, $f^2 = 0.32$, and R^2 change was significant, $p < .05$, suggesting that adding further penile and body length measurements significantly increased the amount of variance explained. In terms of significant predictors, AGD at birth, penile length at birth, and penile length at three months each accounted for unique variance in PSAI scores ($p < .05$, Beta = .261, $p < .05$, Beta = −.258, and $p < .05$, Beta = .271, respectively). Next, Model 2B showed that when *growth* scores for penile length between birth and three months, between three and 12 months, between 12 and 18 months, and between 18 and 24 months along with body growth between birth and 12 months were added to Model 1, both AGD at birth and penile growth from birth to three months were significant predictors of PSAI scores (Beta = .261, $p < .05$ and Beta = .432, $p < .01$, respectively).

Though R^2, significance levels, and effect sizes (f^2) for Models 2A and 2B are necessarily identical, variance attributed to specific predictors varies between the two. In both models, measurements for AGD at birth are identical in accounting for significant variance in later PSAI scores; and penile length at three months in Model 2A and penile growth from birth to 3 months in Model 2B were also positively predictive. However, though penile length at birth was not a significant predictor in Model 1, nor did it correlate directly with PSAI scores (Table 2), it became significant in Model 2A with a negative relationship, Beta = −.258, $p < .05$. This appears to be due to the suppression of positive variance exerted by the inclusion of the raw score for penile length at three months. Penile length at birth positively correlated with penile length at three months ($r = .46$, $p < .001$) but did not correlate with PSAI scores ($r = -.03$, $p = .797$). In regression Model 2A, the minimal positive covariance that existed between penile length at birth and PSAI scores was removed by positive covariance with penile length at three months, itself being a positive predictor of PSAI scores (Beta = .27, $p < .05$, essentially unchanged from the zero-order correlation, $r(79) = .28$, $p < .05$). In Model 2B, the variance is shared across penile length at birth and at three months, and the overall effect is positive and significantly predictive of PSAI scores.

Finally, statistics for tolerance (all values > 0.2) and variance inflation factor (VIF; all values < 5.0) showed that the effects were not due to multi-collinearity; and zero-order correlations for penile length

Table 4
Further regression statistics for Models 2A and 2B in Table 3, including confidence intervals for B and collinearity statistics.

$R^2 = .242$ $p = .009^{**}$, $f^2 = 0.32^{\dagger}$, N = 81	Coefficients	Confidence intervals (95%) for B		Standardized coefficients	Dependent variable PSAI		Collinearity statistics	
	B	Lower bound	Upper bound	Beta	*t*	*p*	Tolerance	VIF
Predictors Model 2A								
AGD birth	4.27	0.81	7.74	**.261**	**2.46**	**.016***	0.94	1.06
Penile length birth	−6.02	−11.85	−0.18	**−.258**	**−2.05**	**.044***	0.68	1.47
Body length birth	−0.01	−1.02	1.01	−.002	−0.01	.989	0.60	1.66
Penile length 3 M	5.51	0.15	10.87	**.271**	**2.05**	**.044***	0.61	1.64
Penile length 12 M	2.23	−2.02	6.84	.125	1.05	.298	0.76	1.32
Penile length 18 M	−0.64	−4.32	3.04	−.041	−0.35	.729	0.77	1.30
Penile length 24 M	1.74	−2.85	6.33	.096	0.76	.453	0.67	1.50
Body length 12 M	−0.79	−1.75	0.17	−.218	−1.65	.103	0.61	1.63
Predictors Model 2B								
AGD birth	4.27	0.81	7.74	**.261**	**2.46**	**.016***	0.94	1.06
Penile length birth	2.83	−3.34	8.99	.121	0.92	.363	0.61	1.64
Body length birth	−0.80	−1.72	0.12	−.210	−1.73	.087	0.73	1.37
Δ penile length birth to 3 M	8.84	2.97	14.72	**.432**	**3.00**	**.004****	0.51	1.95
Δ penile length 3 M to 12 M	3.33	−2.57	9.23	.191	1.13	.264	0.37	2.69
Δ penile length 12 M to 18 M	1.10	−3.81	6.01	.081	0.45	.657	0.32	3.10
Δ penile length 18 M to 24 M	1.74	−2.85	6.33	.112	0.76	.453	0.48	2.08
Δ body length birth to 12 M	−0.79	−1.75	0.17	−.119	−1.65	.103	0.74	1.36

AGD is anogenital distance; M = months.
Bold type highlights significant relationships for ease of evaluation.
† Effect size is Cohen's $f^2 = R^2 / 1 - R^2$ (Cohen, 1988).
* $p < .05$.
** $p < .01$.

between time points confirmed this finding (see Table 5). That is, though measurements for penile length showed significant and positive relationships across all time points (except for that between measurements at birth and those at 18 months), none was higher than $r(79) = .455$, $p < .001$. This is well below the generally accepted range suggestive of a collinearity problem (i.e., $r > .80$; see Field, 2005).

Penile growth in boys with low, middle, and high PSAI scores

Finally, for purposes of graphical illustration and for completeness, we grouped participants into tertiles for PSAI scores (low, middle, and high) as the independent variable, and compared relative gains in penile length (% growth) as the dependent variable, across three consecutive growth periods, from birth to 18 months (see Fig. 1). Tertile groups for PSAI scores approximated the bottom, middle, and top 33% of scores as closely as possible, keeping participants with identical scores grouped together (no overlap between groups in range scores). Means ± SDs and sample sizes for PSAI scores in the three tertile groups were: M = 51.04 ± 5.41 and N = 28 for the low PSAI group; M = 61.69 ± 2.50 and N = 27 for the middle PSAI group; and, M = 72.11 ± 4.36 and N = 26 for the high PSAI group. Percent increase in penile length specific to each growth period was calculated as the difference between measurements at the two time points divided by length at the start of the growth period. Percent increase scores were chosen for clarity and to illustrate relative change in each growth period. 3 × 3 ANCOVA (tertile for PSAI scores × growth period), with growth in body length spanning the three growth periods (birth to 18 months) as the covariate, revealed a main effect of growth period, $F(2, 150) = 6.03$, $p < .01$, such that the most growth occurred in the period from birth to three months for all participants, $t(80) = 3.07$, $p < .01$ for "birth to 3 months" compared to "3 to 12 months," and $t(80) = 3.49$, $p < .01$ for "birth to 3 months" compared to "12 to 18 months." A PSAI tertile × growth period interaction approached significance, $F(4, 150) = 2.16$, $p = .08$; however, simple effects analysis of *a priori* predictions revealed the expected pattern. That is, within the period of greatest growth, i.e., birth to three months, penile growth was greatest for boys in the "high" PSAI tertile group compared to those in the "middle" PSAI tertile group, $t(51) = 2.06$, $p < .05$, $d = 0.61$, and compared to those in the "low" PSAI tertile group, $t(52) = 3.41$, $p < .01$, $d = 1.00$ (all two-tailed). There were no other significant group differences.

Table 5
Zero-order correlations for measurements of penile length between 5 time points.

N = 81 males		3 M	12 M	18 M	24 M
Birth	*r*	**.455*****	**.291****	.121	**.309****
	p	**.000**	**.008**	.283	**.005**
3 M	*r*	–	**.436*****	**.373*****	**.417*****
	p		**.000**	**.001**	**.000**
12 M	*r*	–	–	**.279***	**.343****
	p			**.012**	**.002**
18 M	*r*	–	–	–	**.417*****
	p				**.000**

M = months.
Bold type highlights significant relationships for ease of evaluation.
* $p \le .05$.
** $p \le .01$.
*** $p \le .001$.

Discussion

The current report makes three major contributions. First, it provides the first demonstration that early postnatal androgen elevation, or mini-puberty, contributes to human neurobehavioral sexual differentiation, independent of prenatal androgen exposure. Second, it provides the first evidence linking AGD at birth to subsequent gender-related behavior in humans. Third, it suggests that AGD at birth and penile growth from birth to three months may provide inexpensive and non-invasive measures of prenatal androgen exposure and androgen exposure during mini-puberty, respectively.

Prior research has found that penile growth from birth to three months correlates positively and significantly with serum T at age three months (Boas et al., 2006), supporting penile growth during the first three months postnatal as a bioassay for the androgen elevation sometimes referred to as mini-puberty. Our findings suggest that this bioassay may be useful for studies on the role of mini-puberty not only in physical development, but also in neurobehavioral development. In addition, our findings augment prior research which found that T measured in boys' urine samples during mini-puberty predicted later gender-related behavior (Lamminmäki et al., 2012). That study provided some support for

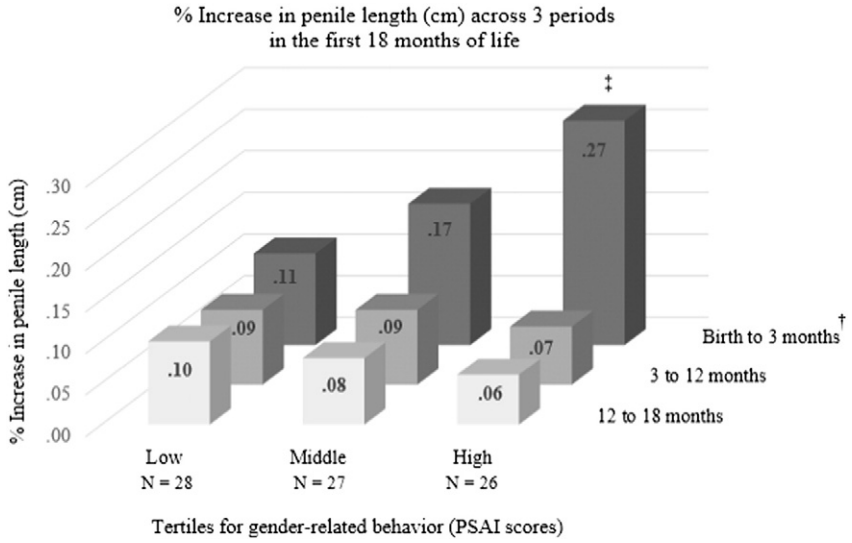

Fig. 1. Percent increase in penile length across the first 18 months of life shown as a function of gender-related behavior measured using the Pre-school Activities Inventory (PSAI). High PSAI scores are more masculine and less feminine. †*Main effect*: All PSAI tertile groups experienced the most growth in the period "birth to 3 months," $p < .01$ compared to "3 to 12 months" and compared to "12 to 18 months." ‡*Simple effects*: The "high" PSAI group showed more growth than both "middle" and "low" PSAI tertile groups, $p < .05$ and $p < .01$, respectively.

neurobehavioral effects of early postnatal androgen exposure, but, as the authors noted, androgen concentrations prenatally and postnatally may correlate. Therefore, the relationship seen previously between early postnatal T and behavior could have resulted from prenatal androgen exposure, particularly given the numerous studies associating prenatal androgen exposure with later gender-related behavior (Hines, 2011a, 2011b). By measuring AGD at birth and including it in our regression analyses, we were able to separate prenatal from early postnatal effects of androgen exposure, and found that early postnatal androgen exposure, reflected in penile growth from birth to three months postnatal, contributed to later masculine behavior, independent of prenatal exposure.

In addition, we found that AGD at birth also was an independent predictor of later gender-related behavior in boys. Although AGD has been established previously as a marker of prenatal androgen exposure in boys (Thankamony et al., 2014), no prior report has linked AGD to later gender-related behavior in humans. Some research has linked exposure to endocrine disruptors to both AGD and gender-related behavior assessed with the PSAI (Swan et al., 2005, 2010), but the potential link between AGD and later gender-typical behavior was not reported on in those studies. Thus, the present study is the first to provide this important link.

A vast literature spanning many species has demonstrated neurobehavioral effects of early androgen exposure (Hines, 2011a, 2011b). In humans, numerous studies have also shown effects of prenatal androgen exposure on later behavior, including sexual orientation, and gender identity, as well as sex-typed childhood play and other behaviors. Our current finding, that the postnatal androgen surge also contributes to children's gender-related activity preferences, encourages exploration of the possibility that it contributes to other gendered aspects of human behavior as well. Similarly, the possibility that AGD at birth also relates to other gender-related neurobehavioral outcomes, in addition to gender-related play, merits investigation.

The inexpensive and non-invasive measures of prenatal and early postnatal androgen exposure suitable for typically developing children that we report on in this manuscript could contribute to several research areas. For example, hundreds of studies have attempted to measure prenatal androgen exposure using digit ratios (2D:4D; the ratio between the second and fourth digits of the hand) (Voracek, 2011). These publications document the broad interest in finding measures of early androgen exposure that can be easily applied in typically developing populations. Finger ratios show only small sex differences, however, $d = 0.20$ for the right hand and 0.16 for the left hand (Manning et al., 2007), compared to $d = 2.40$ for birth AGD (Thankamony et al., 2009), and reports relating finger ratios to behavior have been inconsistent (Constantinescu and Hines, 2012). For example, one large on-line study of over 20,000 participants found that 2D:4D, measured by the participants themselves, related to sexual orientation as predicted in males but not in females (Collaer et al., 2007); meanwhile, a meta-analytic study (Grimbos et al., 2010), that did not include this large on-line study, suggested a different conclusion, however, finding the predicted relation in females but not in males.

Furthermore, evidence that finger ratios relate to prenatal androgen exposure in typically developing individuals is weak. Although one study reported that the magnitude of 2D:4D covaried with a polymorphic repeat (CAG) sequence in the gene coding the androgen receptor in men (Manning et al., 2003), this finding failed to replicate in two subsequent studies using larger samples (Hampson and Sankar, 2012; Hurd et al., 2011). Measures of AGD at birth and penile growth during the first three months postnatal could be more effective measures than finger ratios for studies aimed at understanding the role of early androgen exposure in human gender-related development.

Our findings could also rekindle interest in the early postnatal period as potentially important for human sexual differentiation. Prior research with rhesus macaques has been interpreted to suggest that the early postnatal androgen surge has little or no influence on neurobehavioral development in primates. Five studies have attempted to evaluate the link between androgen during early postnatal development and subsequent sex-related behavior in macaques (Eisler et al., 1993; Hurd et al., 2011; Wallen et al., 1995; Nevison et al., 1997; Brown and Dixson, 1999). The general consensus from four out of five of those studies was that the postnatal androgen surge plays little or no role in the development of sex-related behavior in primates (Hurd et al., 2011; Wallen et al., 1995; Nevison et al., 1997; Brown and Dixson, 1999). Sample sizes in these studies were small, however; Ns ranged from 4 to 10 animals per group, perhaps making it difficult to detect effects. For example, one study reported no significant treatment related differences, even though the effect size for masculine sexual behavior compared between androgen treated and untreated females was moderate to large $d = 0.66$ (Nevison et al., 1997). Another study found a similar effect size, $d = 0.62$, for masculine play comparing control males and males whose early postnatal T was suppressed (Wallen et al., 1995). Thus, negative conclusions based on studies with weak statistical power may have led

to a discounting of the importance of mini-puberty for neurobehavioral development in primates. In contrast, our results suggest that the early postnatal androgen exposure associated with mini-puberty influences human neurobehavioral sexual differentiation.

Finally, AGD was also measured in girls, but no significant relationships to later behavior were observed. These negative results in girls, in contrast to boys, may reflect reduced variance in AGD scores in girls compared to boys. An estimate of variance using the standard deviation for AGD at birth in girls was half of that in boys, SD = 0.31 compared to 0.60, respectively. Effects of prenatal androgens on AGD in typically developing girls may be too subtle to detect relations to behavioral outcomes. Our findings suggest that AGD may be more useful for studying early androgen influences on male development than on female development. This is consistent with the preponderance of androgen/AGD related publications focussing on male, but not on female, reproductive development (e.g., Dean and Sharpe, 2013; Swan et al., 2005, 2010; Thankamony et al., 2014).

Limitations

With respect to implementing our methodology in future studies, one potential limitation is that early postnatal penile growth can only be used in studies of males. Early postnatal testosterone levels are lower in girls than in boys (Lamminmäki et al., 2012) and resulting changes in development of the external genitalia are less easily measured. Although AGD at birth can be measured in both boys and girls, our results suggest that this measure may also be more useful in boys than in girls.

Nevertheless, measurement of AGD and early penile growth could provide useful information on how early androgen exposure relates to human development. For instance, future studies might evaluate whether mini-puberty is important for additional human gender-related behaviors, including psychiatric disorders that differ by sex, such as depression, autism and eating disorders (Kendler and Gardner, 2014; Mandy et al., 2012; Swanson et al., 2011). In addition, this method could be used to study interactions between early androgen exposure and other factors known to influence human gender development, such as postnatal socialization by parents or self-socialization based on the cognitive understanding of gender. Such studies have been difficult to conduct, because individuals with major androgen dysfunction are not numerous, thus precluding definitive studies of relatively rare psychiatric conditions or of interactions with other types of factors. In contrast, the physical measures used in the current study could be used in large samples of typically developing individuals.

Conclusion

AGD at birth and penile growth during the first three months postnatal independently predicted increased masculine/decreased feminine behavior in boys at three to four years of age. Our findings suggest that AGD at birth may be employed as a biomarker of prenatal androgen exposure, while penile growth during mini-puberty may reliably reflect variance in early postnatal androgen exposure. Future research could use these biomarkers in large-scale population studies to further elucidate neurobehavioral effects of perinatal androgen exposure. Such large-scale investigations could also allow for prospective assessment of other factors known to influence variance in gender-related behavior, such as socialization and cognitive development, along with their interactions with early androgen exposure.

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