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# RELATIONSHIPS BETWEEN ANDROGENS, SEROTONIN GENE EXPRESSION AND INNERVATION IN MALE MACAQUES ☆

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**Abstract—Androgen administration to castrated individuals was purported to decrease activity in the serotonin system. However, we found that androgen administration to castrated male macaques increased fenfluramine-induced serotonin release as reflected by increased prolactin secretion. In this study, we sought to define the effects of androgens and aromatase inhibition on serotonin-related gene expression in the dorsal raphe, as well as serotonergic innervation of the LC. Male Japanese macaques (*Macaca fuscata*) were castrated for 5–7 months and then treated for 3 months with (1) placebo, (2) testosterone (T), (3) dihydrotestosterone (DHT; non-aromatizable androgen) and ATD (steroidal aromatase inhibitor), or (4) Flutamide (FLUT; androgen antagonist) and ATD ($n$ = 5/group). This study reports the expression of serotonin-related genes: tryptophan hydroxylase 2 (TPH2), serotonin reuptake transporter (SERT) and the serotonin 1A autoreceptor (5HT1A) using digoxigenin-ISH and image analysis. To examine the production of serotonin and the serotonergic innervation of a target area underlying arousal and vigilance, we measured the serotonin axon density entering the LC with ICC and image analysis. TPH2 and SERT expression were significantly elevated in T- and DHT + ATD-treated groups over placebo- and FLUT + ATD-treated groups in the dorsal raphe ($p < 0.007$). There was no difference in 5HT1A expression between the groups. There was a significant decrease in the pixel area of serotonin axons and in the number of varicosities in the LC across the treatment groups with T > placebo > DHT + ATD = FLUT + ATD treatments. Comparatively, T- and DHT + ATD-treated groups had elevated TPH2 and SERT gene expression, but the DHT + ATD group had markedly suppressed serotonin axon density relative to the T-treated group. Further comparison with previously published data indicated that TPH2 and SERT expression reflected yawning and basal prolactin secretion. The serotonin axon density in the LC agreed with the area under the fenfluramine-stimulated prolactin curve, providing a morphological basis for the pharmacological results. This suggested that androgen activity increased TPH2 and SERT gene expression but, aromatase activity, and neural production of estradiol (E), may subserve axonal serotonin and determination of the compartment acted upon by fenfluramine. In summary, androgens stimulated serotonin-related gene expression, but aromatase inhibition dissociated gene expression from the serotonin innervation of the LC terminal field and fenfluramine-stimulated prolactin secretion.** 

**Key words: TPH2, SERT, 5HT1A, androgen, serotonin, aromatase.**

## INTRODUCTION

Previous studies with humans, macaques and rodents have measured various endpoints and inferred that androgens decrease CNS serotonin function (Brown and LInnoila, 1990; Virkkunen et al., 1995; Coccaro et al., 1997; Clark and Henderson, 2003). Furthermore, low CNS serotonin was thought to play a causal role in androgen-induced aggressive behavior in humans, non-human primates, foxes and rodents (Martinez-Conde et al., 1985; Brown and LInnoila, 1990; Popova et al., 1991; Higley et al., 1996; Clark and Henderson, 2003; Popova, 2006). However, a few studies contradicted this dogma. For example, depletion of serotonin with para-chlorophenylalanine (PCPA) in rats did not increase aggression unless androgens were present (Kubala et al., 2008). Also in rodents, low doses of anabolic steroids increased serotonin activity (Thiblin et al., 1999) and T increased serotonin content in the frontal cortex and hypothalamus (Kubala et al., 2008). One study found elevated T during the mating season of rhesus macaques

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*Abbreviations:* 5HIAA, serotonin metabolite; 5HT1A, serotonin 1A autoreceptor; ANOVA, analysis of variance; AR, androgen receptor; ATD, aromatase inhibitor; CSF, cerebrospinal fluid; DHT, dihydrotestosterone; ER, estrogen receptor; FLUT, flutamide; IHC, immunohistochemistry; ISH, *in situ* hybridization; KPBS, potassium phosphate-buffered saline; LC, locus ceruleus; MAO-A, monoamine-oxidase A; me5, mesencephalic 5 tract; NE, norepinephrine; NGS, normal goat serum; SERT, serotonin reuptake transporter; TPH2, tryptophan hydroxylase 2.

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that was associated with elevation of a serotonin metabolite, 5HIAA, and elevated aggression (Mehlman et al., 1997). We hypothesized that the effect of testosterone (T) on serotonin is more complex than often suggested from studies of one serotonergic endpoint.

Other data suggest that serotonin is reduced in castrated or androgen-deprived men. Fenfluramine-induced prolactin secretion involves serotonin release with reuptake block; and castration reduced fenfluramine-induced prolactin secretion compared to normal men (Foresta et al., 1987). An increased incidence of depression and anxiety were observed in men with hypogonadism, and serotonin supports these functions (Ponholzer et al., 2009; Giltay et al., 2010). Also, castration increased serotonin 1A autoreceptor (5HT1A) expression in the rat raphe, an indirect indication of lower serotonin release from dendrites (Zhang et al., 1999). Therefore, although many studies link elevated androgens and reduced serotonin, other studies point to reduced serotonin in the absence of androgens.

The serotonin system has different compartments that can show independent or coordinated regulation. The most accessible and commonly described compartments *in vivo* are (1) serotonin metabolites, which are dependent upon enzymatic degradation, (2) serotonin per se in different brain regions, which is dependent upon serotonin transport to terminal fields, and (3) fenfluramine-induced prolactin secretion, which is dependent upon a poorly defined releasable pool of serotonin, that in turn stimulates prolactin secretion. Moreover, testosterone (T) is metabolized to active ligands of the androgen receptor (AR) and the estrogen receptor (ER). In females, we showed that estradiol (E) increases tryptophan hydroxylase 2 (TPH2) gene and protein expression, and increases serotonin reuptake transporter (SERT) binding, both thought to be markers of increased serotonin neurotransmission (Lu et al., 2003; Sanchez et al., 2005).

The direct regulation of serotonin at the level of gene expression by androgens needed clarification in male primates. We previously showed that androgen administration did not correlate with fenfluramine-stimulated serotonin release and prolactin secretion if aromatase was inhibited (Bethea et al., 2013b). This study continues our previous observations with direct measurements of serotonin-related gene expression in the dorsal raphe, and analysis of serotonin transport to a pivotal target field, the locus ceruleus (LC).

The midbrain LC contains norepinephrine (NE) producing neurons. In macaques, it increases arousal, vigilance and attention to external stimuli (Grant et al., 1988; Aston-Jones et al., 1991b, 1996; Rajkowski et al., 1994). The majority of input to the LC comes from the autonomic nervous system (Aston-Jones et al., 1991a). The LC also has serotonergic innervation, and the source of the serotonin neurons has been ascribed to the local pericoerulear area (Aston-Jones et al., 1991c; Miller et al., 2011). Others have suggested that as much as 50% of the serotonergic innervation of the LC originates in the dorsal raphe of rats (Kaehler et al., 1999; Kim et al., 2004). In general, serotonin appears to be inhibitory of LC activity (Aston-Jones et al., 1991a), but there is a complex interplay with other excitatory amino acids (Charlety et al., 1993) and corticotrophin releasing factor (CRF) (Valentino et al., 1993, 2001; Jedema and Grace, 2004; Reyes et al., 2005; Curtis et al., 2012), as well as different modes of NE discharge (Aston-Jones and Cohen, 2005).

In order to examine serotonin gene expression and innervation under different conditions we used 2 different androgens (testosterone, T, and non-aromatizable dihydrotestosterone, DHT), an androgen antagonist (flutamide, FLUT), and an aromatase inhibitor (ATD). The objectives were to (1) remove endogenous androgens, but allow de novo neurosteroid production to proceed in castrated animals; (2) to restore androgen activity with T, which is converted to metabolites, DHT and 17β-estradiol (E); (3) to restore only androgen activity and eliminate all E activity by using the non-metabolizable androgen, DHT, together with aromatase block by ATD; and (4) to block all androgen activity and de novo E synthesis in the brain with FLUT + ATD. In this manner, we hoped to differentiate effects mediated by androgen versus estrogen activity (summary in Fig. 8).

We determined the expression of three genes related to serotonin neurotransmission: TPH2, SERT and 5HT1A. In addition, we measured the density of serotonin axons innervating the LC. Correlations between serotonin-related gene expression, serotonin axon density (this study), yawning, fenfluramine-induced prolactin, and basal prolactin secretion (previous study) were sought. We show that androgens *increased* serotonin-related gene expression. However, serotonin axonal transport was altered by aromatase inhibition.

## EXPERIMENTAL PROCEDURES

This experiment was approved by the IACUC of the Oregon National Primate Research Center and conducted in accordance with the 2011 Eight Edition of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Male Japanese macaques (*Macaca fuscata*) were utilized for study.

### Troop

The Japanese macaques were born and raised in a 2-acre outdoor corral at ONPRC with approximately 300 individuals. The troop has been the subject of extensive behavioral studies since it arrived at ONPRC in 1965 (Eaton, 1974; Eaton et al., 1990). The troop composition is relatively stable and the age structure is comparable to that of a natural troop (Maruhashi, 1982). Like other macaque species, the hierarchical organization of the troop is along matriarchal lineages. The matriarchal lines and dominance hierarchies within the troop are well documented, and have remained stable for the past 40 years. In the wild, males normally leave the natal troop and so their dominance is less a function of their mother's status and more a function of their age, size and social skills. Males cannot leave our troop on their own so in that respect, there are more males than a natural troop