

Testosterone modulates growth hormone secretion at the hypothalamic but not at the hypophyseal level in the adult male rhesus monkey

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Abstract

We investigated a possible modulation of growth hormone (GH) secretion by testosterone by measuring the growth hormone releasing hormone (GHRH)-stimulated and *N*-methyl-D,L-aspartic acid (NMA)-induced GH secretion in adult rhesus monkeys. Intact, orchidectomized and testosterone-substituted (testosterone enanthate 125 mg/week, i.m. for 5 weeks) orchidectomized monkeys ($n=5$) were used in the study. GHRH (25 µg/kg body weight) or NMA (15 mg/kg body weight) was infused through a Teflon cannula implanted in the saphenous vein. Sequential blood samples were collected 30–60 min before and 60 min after the injection of the neurohormone or the drug at 10–20-min intervals. All bleedings were carried out under ketamine hydrochloride anaesthesia (initial dose 5 mg/kg body weight i.m., followed by 2.5 mg/kg at 30-min intervals). The plasma concentrations of GH, testosterone and oestradiol (E_2) were determined by using specific assay systems. Administration of GHRH elicited a

significant increase in GH secretion in all three groups of animals. There was no significant difference in the responsiveness of pituitary somatotrophs to exogenous GHRH challenges between intact and orchidectomized monkeys and testosterone replacement in orchidectomized animals did not significantly alter the GHRH-induced GH response. The responsiveness of hypothalamic GHRH neurones apparently did undergo a qualitative change after orchidectomy, as GH response to NMA was less in orchidectomized animals than in intact monkeys. The responsiveness of GHRH neurones to exogenous NMA was restored and even potentiated when orchidectomized monkeys were treated with testosterone. Taken together, these findings suggest that testosterone does not affect the sensitivity of the pituitary somatotrophs to GHRH but stimulates the secretion of GH by modulation of the NMDA drive to GHRH neurones.

Journal of Endocrinology (2000) **165**, 337–344

Introduction

The acceleration in linear growth during normal puberty in man has been attributed mostly to the combined physiological effects of the somatotrophic and gonadal axes (Giustina & Veldhuis 1998) because high-amplitude pulsatile release of growth hormone (GH), concomitant with an increase in testosterone concentrations, becomes evident at around this phase of development (Mauras *et al.* 1987, 1989, Martha *et al.* 1989, 1992, Wennink *et al.* 1990, Rose *et al.* 1991, Kerrigan & Rogol 1992.) The contribution of the steroids to the overall increase in body growth could be due to their independent anabolic effects on target tissues that may also be responsive to GH (or

growth factors, systemically or locally produced), or to a modulation of GH secretion (synthesis/release) itself.

Data obtained in humans with constitutional delay of puberty or hypogonadotrophic hypogonadism have indicated that androgens specifically and significantly augment the endogenous GH secretory rate by amplifying the magnitude of GH secretory episodes (Link *et al.* 1986, Ulloa-Aguirre *et al.* 1990, Keenan *et al.* 1993, Eakman *et al.* 1996). Although the action of steroids on growth through mediation by the somatotrophic axis has been indicated by a number of clinical investigations, the mechanism whereby androgens affect GH secretion is not clear (Link *et al.* 1986). It is not known whether steroids act directly on pituitary somatotrophs, hypothalamic

growth hormone releasing hormone (GHRH) neurones or both to modify GH secretion. Thus androgens may increase the responsiveness of somatotrophs to GHRH (Hassan *et al.* 1992) or induce enhanced GHRH secretion by the hypothalamic neurones (Lima *et al.* 1989).

Although evidence mainly derived from studies on the human and the rat indicates that endogenous and exogenous sex steroids modify GH secretion, few studies have evaluated the action of these hormones on GH secretion in the non-human primate (Wheeler & Styne 1988). The present study examines the effects of exogenous administration of testosterone on GH secretion in adult male rhesus monkeys. A possible modulation of GH secretion by androgens at the pituitary level has been assessed by measuring the GHRH-stimulated GH, whereas the role of testosterone in influencing the hypothalamic release of GHRH has been investigated by determining the effect of *N*-methyl-D,L-aspartate (NMA), an agonist of *N*-methyl-D-aspartate (NMDA) receptor, on plasma GH secretion in intact, orchidectomized and androgen-substituted orchidectomized monkeys. NMA, an analogue of the excitatory amino acid neurotransmitters, glutamate and aspartate, has previously been shown to stimulate the secretion of pituitary hormones via a hypothalamic release of neuropeptides (Brann 1995).

Materials and Methods

Animals

Adult intact (9.1 ± 0.46 kg) and orchidectomized (7.7 ± 0.68 kg) male rhesus monkeys ($n=5$, 5–6 years of age) were included in this study. Bilateral orchidectomies were carried out at least 2 years before initiation of the study. The study was approved by the Ethics Committee of the Quaid-i-Azam University and the animals were maintained at the Primate Facility in Islamabad.

Catheterization

Before handling, the animals were anaesthetized with ketamine hydrochloride (5 mg/kg; Ketavet, Parke-Davis, Freiburg, Germany) and while they were under sedation a Teflon cannula (Vasocan Braunule 0.8 mm/22 G, o.d., Braun, Melsungen, Germany) was inserted in the saphenous vein for blood sampling and drug or neuropeptide infusion. Ketamine hydrochloride anaesthesia (2.5 mg/kg i.m.) was given at 30-min intervals. The dose of ketamine used was not enough to induce narcosis but was sufficient to immobilize the animal.

Bledings

Sequential blood samples (~2.0 ml) were obtained at 10–20-min intervals into heparinized syringes. After with-

drawal of each sample, an equal volume of heparinized (5 IU/ml) saline was injected into the tubing. All bleedings were carried out between 1000 and 1400 h to minimize diurnal variations. Blood samples were immediately centrifuged at 3000 r.p.m. for 10 min. Plasma was separated and stored at -15°C until required for analysis.

Pharmacological agents

GHRH-44 (GEREF 50; Laboratories Serono, S.A., Madrid, Spain) and NMA (purchased from Sigma Chemical Co., St Louis, MO, USA) were dissolved in normal saline immediately before use and passed through a 0.22 μm filter unit (Millipore Corp., Bedford, MA, USA) at the time of injection. Testosterone enanthate (TE; Testoviron Depot, Schering AG, Berlin, Germany) was in oily solution.

Hormone determinations

Plasma concentrations of GH, testosterone and E_2 were determined in duplicate using specific assay systems. Plasma GH concentrations were determined using an AutoDELFLIA time-resolved fluoroimmunoassay (Wallace Oy, Turku, Finland). The minimum detection limit of this assay was 0.03 mU/l; the intra-assay coefficient of variation was 3% and the interassay coefficient of variation was 2%. Plasma concentrations of testosterone in the samples were determined using Coat-A-Count RIA kit (Diagnostic Products Corporations, Los Angeles, CA, USA). The assay can detect as little as 4 ng/dl (0.14 nmol/l) testosterone in the sample. The intra- and interassay coefficients of variation were 6% and 8% respectively. Testosterone in the sample expressed as ng/dl was divided by 100 to convert it to ng/ml. The plasma concentrations of E_2 were determined by using Estradiol Serozyme kit (Biochem ImmunoSystem Inc., Allentown, PA, USA). The sensitivity of the assay was 5 pg/ml. The intra- and interassay coefficients of variation were 6% and 9% respectively.

Experimental procedures

Adult intact, orchidectomized and testosterone-replaced (TE 125 mg/week i.m. for 5 weeks) orchidectomized male rhesus monkeys ($n=5$) were given a single i.v. injection of GHRH (25 $\mu\text{g}/\text{kg}$ body weight). Blood samples were collected in heparinized syringes at -30 , -15 , 0, 15, 30, 45 and 60 min relative to GHRH challenges at 0 min. At the end of week 5 of testosterone treatment, the animals were challenged with a single i.v. injection of NMA (15 mg/kg body weight). Blood samples were collected from 60 min before until 60 min after the injection, at 10–20-min intervals. Testosterone treatment was continued up to week 5.

Statistical analysis

Basal hormone concentrations for the three experimental groups were calculated by averaging all the concentrations obtained before GHRH or NMA treatment. To assess the GHRH- or NMA-induced stimulation of GH secretion, the area under GH curve (AUC) was computed by the trapezoid method. For calculation of AUC, the baseline GH concentration was subtracted from the subsequent values. When subtraction of the baseline value from a subsequent value resulted in a negative number, a zero value was assigned. In addition, responsiveness to GHRH or NMA was assessed by comparing plasma GH concentrations at 0 min and peak values subsequent to the injection of the neuropeptide or the neuroexcitatory amino acid. The results were analysed for statistical significance using analysis of variance and Duncan's test. A value of $P < 0.05$ was taken as significant.

Results

Basal hormone concentrations

Basal hormone concentrations in the three groups of animals are shown in Fig. 1. There was no significant difference ($P > 0.05$) between the mean basal plasma GH concentrations in intact and orchidectomized monkeys (Fig. 1). Testosterone replacement to orchidectomized monkeys for a period of 4–5 weeks resulted in a marked increase ($P < 0.05$) in plasma GH concentrations (from 14.3 to 40.3 ± 2.3 mU/l) these were also significantly different ($P < 0.05$) from the basal plasma GH concentrations (10.1 ± 1.0 mU/l) in intact animals.

Circulating concentrations of testosterone that were low or undetectable before initiation of TE treatment increased severalfold ($P < 0.05$) after treatment. In testosterone-replaced orchidectomized monkeys, mean plasma concentrations of testosterone (30.4 ± 2.2 ng/ml) at the end of the treatment period were ~6-fold greater than those of normal adult animals (5.3 ± 0.7 ng/ml). Plasma E_2 concentrations that were undetectable (< 5 pg/ml) before treatment increased progressively to 24 ± 4 pg/ml after testosterone treatment.

GHRH-stimulated GH secretion

The effect of a single i.v. injection of GHRH on plasma concentrations of GH in intact, orchidectomized and testosterone treated orchidectomized monkeys is shown in Fig. 2. The administration of GHRH elicited a significant ($P < 0.05$) increase in GH secretion within 15–30 min of the injection. The differences in GH responses to GHRH injection observed in the three treatment groups were not significant ($P > 0.05$), although the increase in plasma GH appeared to be greater in orchidectomized and testosterone-replaced monkeys than that observed in the

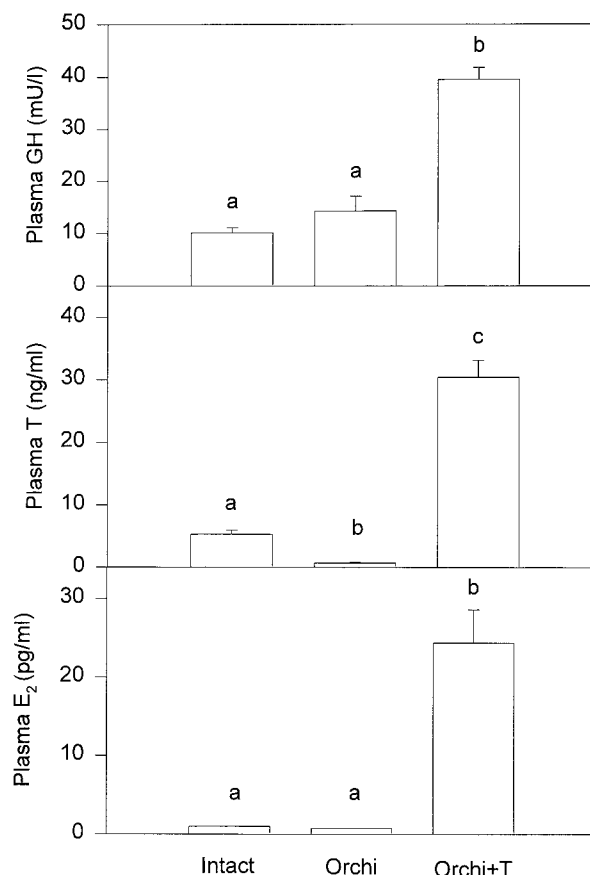


Figure 1 Basal plasma concentrations of GH (top panel), testosterone (T; middle panel) and oestradiol (bottom panel) in intact, orchidectomized (Orchi) and TE-treated orchidectomized (Orchi+T) monkeys. Different letters (a and b, a and c, b and c) indicate significant ($P < 0.05$) differences between groups.

intact animals. The values of AUC (Fig. 3) calculated for a 1-h period (0–60 min) also were not significantly different for the three treatment groups.

NMA-stimulated GH secretion

The mean GH profiles before and after a single i.v. injection of the neuroexcitatory amino acid NMA in the three treatment groups are shown in Fig. 4. In intact animals, plasma GH concentrations after NMA injection increased rapidly from 12.0 ± 3.6 mU/l before administration of the agonist to 46.7 ± 2.7 mU/l ($P < 0.05$) 10 min later. Circulating GH concentrations then declined progressively to reach values of 22.6 ± 6.7 mU/l at 60 min after injection. In orchidectomized monkeys, no significant ($P > 0.05$) increase in plasma GH concentrations was noticed after NMA administration. The mean plasma GH concentrations before and (10 min) after NMA injection were 18.7 ± 6.9 and 20.8 ± 7.2 mU/l, respectively.

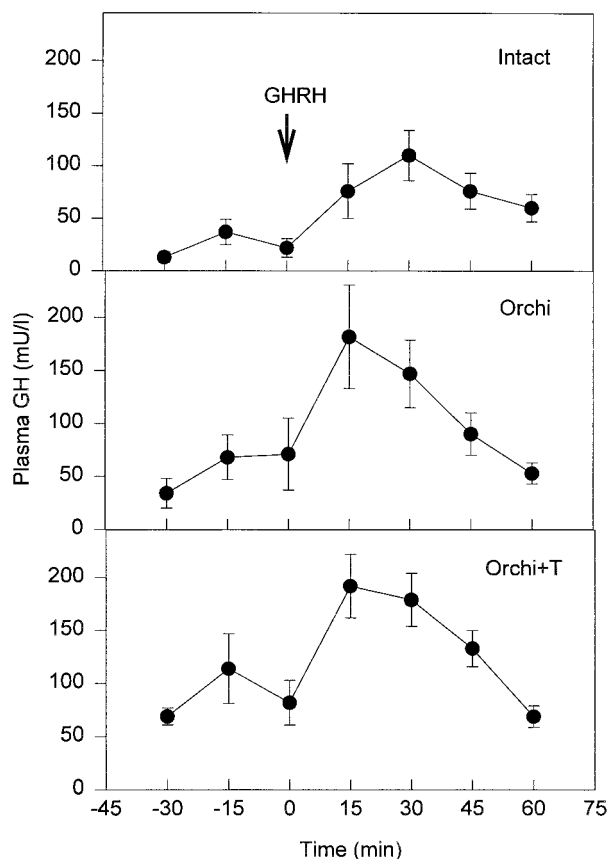


Figure 2 Plasma GH concentrations (mean \pm S.E.M.) in intact, orchidectomized (Orchi) and TE-treated orchidectomized (Orchi+T) monkeys before and after a single i.v. injection of GHRH at 0 min. A significant ($P < 0.05$) increase in plasma GH was observed within 15–30 min of the injection in all groups.

However, a significant increase in mean GH concentration over the basal value ($P < 0.002$) in response to the amino acid injection was observed in the orchidectomized monkeys treated with TE. The plasma GH concentrations before and (10 min) after NMA injection were 34.3 ± 7.6 and 94.0 ± 8.8 mU/l respectively. Intact and TE-treated orchidectomized animals had significantly greater values for AUC than the orchidectomized group of monkeys (Fig. 5). The mean GH AUCs were also greater in testosterone-replaced orchidectomized animals than in intact monkeys, but the differences were not statistically significant.

Discussion

In the present study, although no significant difference was observed in mean basal GH concentrations between intact and orchidectomized monkeys, basal concentrations of GH underwent a significant increase after testosterone

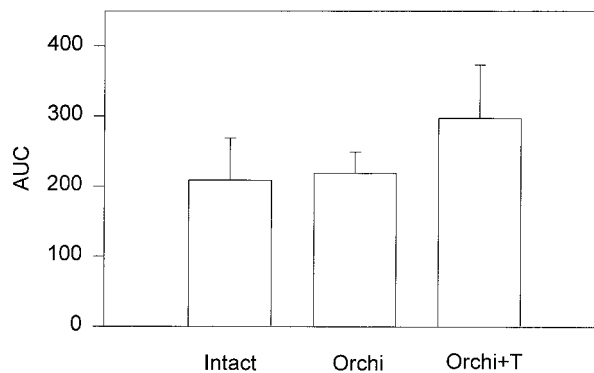


Figure 3 Response AUCs for plasma GH (mU \times h/l) during a 0–60-min period after a single i.v. injection of GHRH at 0 min in intact, orchidectomized (Orchi) and TE-treated orchidectomized (Orchi+T) monkeys. The differences between groups were not significant ($P > 0.05$).

replacement. In a recent study of boys with isolated hypogonadotropic hypogonadism, testosterone treatment for a period of 4 week significantly increased mean serum GH concentrations, GH pulse amplitude and GH pulse frequency (Giustina *et al.* 1997). Similarly, long-term testosterone replacement therapy or normalization of circulating concentrations of testosterone with human chorionic gonadotrophin in hypogonadal men increased mean serum GH concentrations and GH pulse amplitude (Liu *et al.* 1987, Weissberger & Ho 1993). Moreover, administration of testosterone for 3 months or more to boys with constitutional delayed development has been shown to increase mean serum GH concentrations and GH pulse amplitude (Link *et al.* 1986, Ulloa-Aguirre *et al.* 1990, Keenan *et al.* 1993, Eakman *et al.* 1996). The observation that in pre-pubertal boys and men peripheral testosterone concentrations are correlated positively with GH secretory mass and amplitude has led Giustina & Veldhuis (1998) to suggest that androgens promote a relatively greater ratio of GHRH/somatostatin effects on somatotrophs.

As observed in this investigation, the responsiveness of pituitary to GHRH stimulation did not change after treatment of orchidectomized animals with testosterone. Furthermore, Ross *et al.* (1987) have demonstrated that, although stilbestrol treatment of children with short stature increased basal concentrations of GH and peak concentrations during insulin hypoglycaemia, there was no effect of steroid priming on the GH response to exogenous GHRH. These results support the view that sex steroids act via the hypothalamus by increasing endogenous GHRH release. Short-term gonadal blockade in normal men (Lima *et al.* 1989), long-term testosterone treatment in boys with isolated hypogonadotropic hypogonadism (Brann 1995) or dihydrotestosterone replacement in boys with constitutional delay in growth and adolescence (Eakman *et al.* 1996) also failed to bring about a change in the responsiveness of the

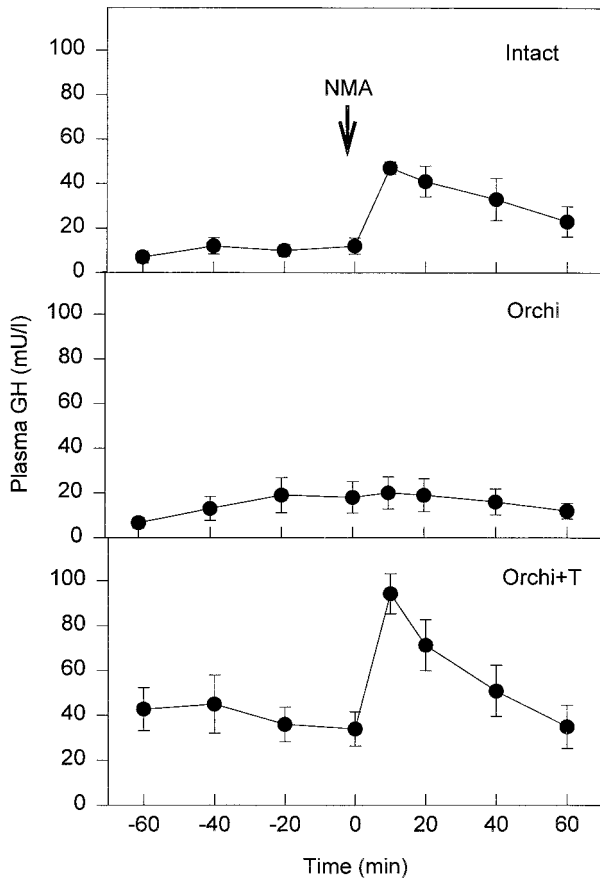


Figure 4 Plasma GH concentrations (mean \pm S.E.M.) in intact, orchidectomized (Orchi) and TE-treated orchidectomized (Orchi+T) monkeys before and after a single i.v. injection of NMA at 0 min. A significant ($P < 0.05$) increase in plasma GH was observed at 10 min after injection in intact and testosterone-replaced monkeys but not in untreated orchidectomized animals.

pituitary to exogenous GHRH. Furthermore, the responsiveness of the hormone to exogenous GHRH stimulation has been shown to be identical during the low-testosterone prepubertal stage and the high-testosterone pubertal stage (Ghigo *et al.* 1996). However, studies in rodents demonstrate that the pituitary response of male rats to GHRH decreases after orchidectomy and that replacement therapy reverses this effect (Wehrenberg *et al.* 1985). Similarly, *in vitro* studies have shown that neonatal and prepubertal orchidectomies result in decreased baseline and GHRH-stimulated release of GH in the rat (Ohlsson *et al.* 1987) and that the effect can be reversed by *in vivo* testosterone replacement therapy (Hertz *et al.* 1989). Furthermore, Ge *et al.* (1989) demonstrated that male rats release more GHRH *in vitro* than do female rats.

In this investigation, the finding that i.v. administration of the neuroexcitatory amino acid agonist NMA induced

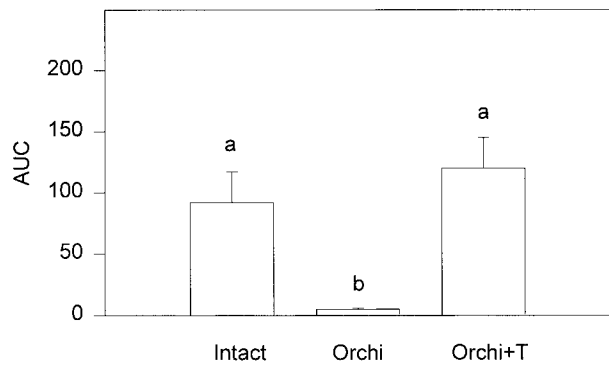


Figure 5 Response AUCs for plasma GH (mU \times h/l) during a 0–60-min period after a single i.v. injection of NMA at 0 min in intact, orchidectomized (Orchi) and TE-treated orchidectomized (Orchi+T) monkeys. Different letters (a and b) indicate significant ($P < 0.05$) differences between groups.

a prompt discharge of GH in adult intact male monkeys is not surprising. The ability of NMA to evoke a release of GH has been well documented for rats (Mason *et al.* 1983), pigs (Barb *et al.* 1992), sheep (Estienne *et al.* 1989), holstein bulls (Shahab *et al.* 1993) and monkeys (Plant *et al.* 1989, Medhamurthy *et al.* 1992). The excitatory effects of NMA on GH secretion have been shown to be exerted via the discharge of GHRH from the hypothalamus (Cocilovo *et al.* 1992). It has also been suggested that, in primates, parenterally infused NMA excites NMDA receptors on GHRH neurones located in the median eminence (Medhamurthy *et al.* 1992).

A significant finding of the present study is that the GH response of the pituitary to the i.v. administration of NMA was markedly attenuated in chronically orchidectomized monkeys as compared with intact animals, although no large differences were observed between the mean basal plasma GH concentrations of agonadal and intact animals. The inability of NMA to stimulate a significant increase in GH secretion in orchidectomized adult monkeys is contrary to earlier reports that demonstrated that NMA can elicit a significant increase in GH secretion in orchidectomized prepubertal monkeys (Plant *et al.* 1989, Medhamurthy *et al.* 1992) and in some other mammalian species (Estienne *et al.* 1989, Barb *et al.* 1992).

The inability of NMA to stimulate GH release significantly in orchidectomized adult monkeys can be ascribed neither to a reduction in the pituitary store of GH nor to an inherent decreased refractoriness of the somatotrophs to GHRH, as the pattern of GH discharge elicited by a single i.v. infusion of GHRH in intact animals was indistinguishable from that observed in untreated castrated monkeys. A number of previous reports provided evidence that the responsiveness of pituitary hormones to NMA may change as a result of an altered physiological state. Thus a lack of prolactin

(PRL) response to the stimulatory action of the neuroexcitatory amino acid has been reported in the rat during lactation (Pohl *et al.* 1989) and in the adult orchidectomized monkey (Arslan *et al.* 1991). Taking into account the present findings and the results of previous investigations, it may be speculated that the sex steroids influence the NMDA-mediated GHRH drive to somatotrophs. Furthermore, a possible intermediary role of other steroid-sensitive neuroendocrine factors in 'setting' the NMDA tone in response to the prevailing steroid environment cannot be ruled out.

In the present investigation, the effect of orchidectomy on the GH response to NMA was reversed with testosterone replacement. A similar observation has previously been reported for plasma PRL secretion, demonstrating that in chronically gonadectomized male monkeys testosterone treatment reinstates the PRL-releasing effect of NMA (Arslan *et al.* 1991). It has also been shown that steroid replacement re-establishes the luteinizing hormone response to NMA challenges in some species of mammals (Estienne *et al.* 1990, Shahab *et al.* 1993). Finally, compared with that in normal boys, the GHRH response to L-dopa stimulation is less in naturally androgen-deficient individuals with idiopathic delayed puberty (Argente *et al.* 1987, Liapi *et al.* 1988), whereas administration of oxandrolone to these patients significantly increases the L-dopa induced GHRH release (Liapi *et al.* 1988). NMA has previously been reported to stimulate the release of somatostatin in the median eminence *in vivo* (Benyassi *et al.* 1991) and from cultured hypothalamic neurones *in vitro* (Rage *et al.* 1993). Furthermore, NMDA induces a significant increase in somatostatin mRNA levels and the non-competitive NMDA antagonist MK 801 has been shown to reduce somatostatin mRNA content significantly in cultured hypothalamic neurones *in vitro* (Rage *et al.* 1994). It may, therefore, be possible that NMA stimulates both GHRH and somatostatin and the net GHRH secretion depends on the relative magnitude of the prevailing NMDA drives to this system. Furthermore, testosterone or its metabolites may be involved in modifying the relative response of somatostatin and GHRH to the neuroexcitatory amino acid.

As testosterone treatment of orchidectomized monkeys resulted in significant increases in plasma concentrations of both testosterone and E₂, it is difficult to ascertain whether the action of testosterone on GH secretion was exerted by the androgen itself or through its aromatization to E₂, or both. Endogenous and exogenous E₂ have both previously been shown to stimulate GH in man and non-human primates (Copeland *et al.* 1984, Ho *et al.* 1984, Kerrigan & Rogol 1992, Liu *et al.* 1987, Mauras *et al.* 1989, Wheeler & Styne 1988, Bethea 1991). It has been reported that serum concentrations of E₂, and not those of testosterone, correlate positively with mean serum GH concentration, GH pulse amplitude and fraction of GH secreted in

pulses in men (Ho *et al.* 1987). Furthermore, chronic administration of tamoxifen, an oestrogen receptor blocker, to normal and testosterone-treated hypogonadal men (Weissberger & Ho 1993) leads to a significant reduction in mean serum GH concentrations, GH pulse amplitude and GH burst frequency. In contrast, studies in boys with constitutional delay in growth and development treated with non-aromatizable androgens suggested that testosterone affects GH secretion by acting directly on androgen receptors: an increase in basal (Clayton *et al.* 1988) and GHRH-stimulated (Loche *et al.* 1986) GH secretion has been reported in boys with constitutional delay in growth and development treated with oxandrolone. Similarly, Ulloa-Aguirre *et al.* (1990) have demonstrated that treatment of boys with delayed growth and development with oxandrolone increases mean serum GH concentrations, GH pulse amplitude and mass of GH secreted per burst. A more recent study in boys with isolated hypogonadotropic hypogonadism treated with varying doses of testosterone has demonstrated that initial changes in GH secretion were evident even in the presence of very small increases in serum testosterone concentrations when circulating concentrations of E₂ were still undetectable (Giustina *et al.* 1997). These observations suggest that testosterone can act both directly through androgen receptors and indirectly through oestrogen receptors after its aromatization to E₂. Investigations (mainly in the rat) have revealed a differential effect of testosterone on the GH axis, resulting in a sexually dimorphic pattern of GH secretion (Weherenberg & Giustina 1992). Whether a similar testosterone-dependent trend is operative in non-human primates has yet to be determined.

It may be mentioned that dissociative anaesthetics such as phencyclidine and ketamine are known to act as non-competitive NMDA receptor antagonists (Monaghan *et al.* 1989). Ketamine in doses greater than those used in the present study has been shown to decrease circulating GH concentrations in baboons (Lehmann *et al.* 1997). In another study no significant difference was observed in plasma GH concentrations in rhesus monkeys sedated with phencyclidine compared with those in conscious animals (Wheeler & Styne 1988). As all animals in the present study were immobilized utilizing a uniform dose regimen, the relative differences between treatment groups presumably remained unaffected by use of the sedative during bleeding. Furthermore, the dose regimen of ketamine used in the present investigation failed to evoke by itself an acute change in circulating GH concentrations in intact or castrated animals.

In conclusion, the present investigation failed to demonstrate an androgen-mediated change in the responsiveness of somatotrophs to GHRH in the adult male monkey, but the findings suggest a modulation of the NMDA-dependent GH release via stimulation of hypothalamic GHRH neurones.

Acknowledgements

This study was supported by a grant from the WHO Special Programme for Research in Human Reproduction (to M A). The technical assistance of R Sandhowe is gratefully acknowledged.

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Received 1 October 1999

Accepted 22 December 1999