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Intratesticular action of kisspeptin in rhesus monkey (*Macaca mulatta*)

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Summary

Kisspeptin-Kiss1R signalling in mammals has been implicated as an integral part of the reproductive cascade. Kisspeptinergic neurons upstream of GnRH neurons are involved in the activation of the hypothalamic GnRH pulse generator during pubertal onset. Thus, the major research focus has been on the central effects of kisspeptin. The demonstration of the presence of KissR expression in human testes suggests additional unknown actions of kisspeptin-KISS1R signalling at the distal component of the male reproductive axis. Here we explored the impact of kisspeptin at the testis in the adult male rhesus monkey. We employed the clamped monkey model to assess the intratesticular actions of kisspeptin. Plasma testosterone and LH levels were monitored in four adult male monkeys. The peripheral administration of human kisspeptin-10 (50 μg, iv bolus) caused a single LH pulse, which was followed by a robust increase in plasma testosterone levels sustained for at least 180 min. This response was abolished when kisspeptin was administered to GnRH receptor antagonist (acyline) pre-treated animals. However, kisspeptin administration significantly ($P < 0.005$) elevated hCG-stimulated testosterone levels in acyline pre-treated monkeys when compared with saline+ hCG treatment. These results revealed a novel peripheral facet of kisspeptin signalling.

Introduction

In primates, the hormonal cascade responsible for proper gonadal activity is comprised of hypothalamic decapeptide GnRH, pituitary gonadotrophins and gonadal steroids. The physiological interplay of these hormones in a very precise manner results in the successful production of gametes and reproductive cyclicity. In male primates, GnRH pulses activate the pituitary gland to release LH in a pulsatile fashion activating the LH receptor on the Leydig cell to elicit the release of testosterone. The pulsatile fashion of LH release is physiologically important as constant high serum levels of LH downregulate the LH receptor on the Leydig cell resulting in diminished testicular androgenic response. A single LH pulse will give rise to a corresponding testosterone peak. This LH-testosterone loop shows a diurnal rhythm in adult male rhesus monkeys (Schlatt *et al.*, 2008). Rhesus monkeys show seasonality in terms of reproductive efficiency. Levels of testosterone and testicular functionality are greatly reduced in the nonbreeding season as compared with the breeding season (Wickings & Nieschlag, 1980), revealing a hypothalamic influence of the photoperiod on the GnRH pulse generator.

Recently, a new hypothalamic neuropeptide named kisspeptin is proposed to be an integral part of this hormonal cascade responsible for normal reproductive cyclicity (Messager *et al.*, 2005; Shahab *et al.*, 2005). Kisspeptin has been shown to activate GnRH neurons at the critical time of puberty by acting on a G-Protein-coupled receptor named Kiss1R (KISS1R) (Seminara *et al.*, 2003), which is shown to be localised on the GnRH neurons (Pompolo *et al.*, 2006). Kisspeptin is released from kisspeptinergic neurons present mainly in the arcuate nucleus of hypothalamus in male nonhuman primates (Ramaswamy *et al.*, 2008). The coupling of kisspeptin with Kiss1R results in an increase in GnRH neuronal firing rate and an activation of pulse generator activity. As a consequence, the decapeptide is released into the pituitary portal circulation and acts on GnRH receptors present on gonadotropes causing the

release of pituitary gonadotrophin LH in a pulsatile fashion, followed by subsequent testosterone pulses and thus the activation of hypothalamic–pituitary–gonadal (HPG) axis (Plant *et al.*, 2006). In the male primate, rigorous testicular activity in terms of somatic and germ cell activity is thereby initiated (Arslan *et al.*, 1993). High levels of testosterone downregulate hypothalamic kisspeptin gene expression and lower testosterone levels act as positive feedback control mechanism (Shibata *et al.*, 2007).

In adult male monkeys (*Macaca mulatta*), the intravenous (iv) administration of kisspeptin given as a continuous infusion resulted in an increase in circulating LH levels, which peaked after 2–4 h of initial treatment and then followed by a decline (Ramaswamy *et al.*, 2007). The interesting revelation made in this study was the disproportional relationship between circulating testosterone and LH, where testosterone levels were constantly high for a given concentration of LH during iv infusion of kisspeptin thus raising the possibility of an endocrine aspect of kisspeptin-Kiss1R signalling within the primate testis.

The present study was conducted to examine the testicular androgenic response after kisspeptin infusion in the male rhesus monkey. The endocrine model we used to assess this possible testicular role of kisspeptin, which must be independent from LH influence, was that of a pituitary gonadotrophin-clamped monkey model achieved by pre-treatment of acyline, a GnRH receptor antagonist (Shahab *et al.*, 2005).

Material and methods

Monkeys

Four, 4 to 6-year-old, intact male rhesus monkeys (*M. mulatta*), weighing 6.0–8.0 kg were used in this study. Animals were housed in individual cages, under standard colony conditions, fed with monkey diet at 1300–1330 h daily and supplemented with fresh fruits in the morning in the animal house facility of Quaid-i-Azam University, Islamabad. Water was available *ad libitum*. The monkeys were trained for chair restraint prior to the experiments to sample these animals without sedation or anaesthesia. Under ketamine sedation (0.5 ml, im), animals were placed in primate chairs. After recovery from sedation, the animals were allowed to sit on the chair for gradually increasing periods of time. The animals were habituated to chair restraint over several months. All experiments were approved by the Departmental Committee for Care and Use of Laboratory Animals and were performed in accordance with regulations of the Ethics Committee of the university.

Catheterisation

To permit sequential withdrawal of the blood samples and iv administration of drugs, the animals were anaesthetised with ketamine hydrochloride (Ketamax, Rotexmedica, Trittau, Germany, 0.5 ml, im), and a teflon cannula (Vasocan Branule, B. Braun Melsungen AG, Belgium; 0.8 mm/22G O.D) was inserted in the sephanous vein before initiation of sampling, and the animals were restrained to the chair. The free end of the cannula was attached to a syringe via a butterfly tubing (20G diameter and 300 mm length). Blood sampling and infusion of treatments were carried out when the animals had fully regained consciousness.

Pharmacological agents

Human kisspeptin-10 (112-121) (Phoenix pharmaceuticals, Belmont, CA, USA) and GnRH receptor antagonist acyline (Bioqual, Rockville, MD, USA) were kindly provided by Dr. Tony M. Plant. hCG (Pregnyl®, N.V Organon Oss Holland, the Netherlands) and heparin (Rotexmedica) were purchased locally. Working solutions of kisspeptin and hCG were made in normal saline while acyline was dissolved in 5% aqueous mannitol. Ketamine hydrochloride (Ketamax, Rotexmedica) was purchased locally.

Dosage

Based upon previously reported centrally effective iv kisspeptin doses (10, 30 and 50 μg) in adult intact male rhesus monkeys (Ramaswamy *et al.*, 2007; Wahab *et al.*, 2008, 2011), the highest dose (50 μg) of kisspeptin-10 was selected for exploring a possible direct testicular action in the present study. In one of our previous studies, this dose of kisspeptin-10 was also found to be peripherally effective as it caused significant enhancement of plasma adiponectin levels in adult intact monkeys (Wahab *et al.*, 2010).

In vivo experimental design and blood sampling

To examine our hypothesis of an intratesticular action of kisspeptin-10 iv bolus in male monkeys, we used pituitary gonadotrophin-clamped monkey model. The primary site for kisspeptin action is at hypothalamic GnRH neurons expressing Kiss1R, this receptor coupling increase the GnRH pulse activity and subsequently increases the gonadotrophin levels in circulation. To block this central physiological effect of kisspeptin administration for this experiment, chemical hypophysectomy was achieved by treating the animals with a potent GnRH receptor antagonist, acyline (Herbst *et al.*, 2002; Shahab *et al.*, 2005).

Acyline competes with GnRH to bind with GnRH receptors at gonadotropes and blocking a secretory response from pituitary in response to GnRH and/or kisspeptin bolus.

The different sets of *in vivo* experiments along with the treatment and pre-treatments in this study are shown in Table.1. Same group of animals were used in each of these experiments. Animals were treated with acyline 24 and 12 h before the experiment 1(c), 2(a) and 2(b) in the Table 1. The morning (24 h prior) and evening (12 h prior) treatment was given subcutaneously with two different doses (60 μg kg^{-1} and 120 μg kg^{-1} BW, sc, 9:00 am and 9:00 pm respectively). Blood sampling for all experiments started between 0900 and 0930 h, and samples (2.5–3 ml) were obtained at 30-min intervals. First sample (−30 min) was collected 30 min before the treatment (vehicle/ kisspeptin/ hCG/ hCG + kisspeptin). Samples were taken in heparinised syringes and immediately transferred into culture tubes kept on ice. After completion of the blood sampling, these culture tubes were centrifuged at 800 ***g*** for 30 min and plasma was extracted and stored at −15 °C until assayed. After taking every sample, approximately 3 ml of normal saline containing 5 IU of heparin was injected to compensate the lost blood volume to prevent hypovolumic shock to the animals.

Effect of kisspeptin-10 on plasma testosterone and plasma LH in normal adult intact male monkeys

Impact of peripheral administration of kisspeptin-10 (50 μg) on basal testosterone and LH was monitored in normal adult intact male monkeys ($n = 4$). Kisspeptin-10 or normal saline (vehicle control, 1 ml) was administered (iv) immediately after taking 0-min sample.

Effect of kisspeptin-10 on plasma testosterone in acyline pre-treated adult intact male monkeys

Impact of kisspeptin-10 administration (50 μg) on plasma testosterone was monitored in pituitary-clamped monkey by the help of acyline pre-treatment (12 and 24 h prior). Kisspeptin-10 (50 μg) was administered (iv) immediately after taking 0-min sample, and hCG was administered (iv) at 240-min sample.

Effect of kisspeptin-10 on plasma testosterone and plasma LH in acyline pre-treated hCG challenged adult intact male monkeys

Impact of peripheral iv administration of kisspeptin-10 (50 μg) on hCG-stimulated testosterone secretion was determined in acyline pre-treated adult monkeys ($n = 4$). Acyline pre-treatment was given 12 and 24 h before the start of the experiment to assure the complete suppression of HPG axis. hCG (50 IU) and saline were given immediately after taking 0-min sample; and in the second set of experiment, hCG (50 IU) and kisspeptin-10 (50 μg) were administered after taking 0-min sample.

Plasma LH and testosterone measurement by specific RIA

Plasma LH concentrations were determined by double-antibody radioimmunoassay (RIA) with reagents supplied by NHPP (National Hormone & Peptide Program). The standard preparation used was rec-moLH-RP-1, tracer was prepared from AFP-6936A and antiserum was AFP 342994. The tubes were counted in a gamma counter from LKB (Gamma Master 1277) for 1 min. The sensitivity of the assay was 0.2 ng ml^{-1}, and the intra- and inter-assay coefficients of variants were 8.8% and 5.8% respectively.

Plasma testosterone concentrations were determined using specific solid-phase competitive radioimmunoassay. The testosterone kits were purchased from immunotech (Prague, Czech Republic). The assays were carried out according to the instructions given by the manufacturer. Tubes for testosterone were incubated at 37 °C. Tubes were then carefully decanted and placed in a Beckman gamma counter (Gamma5500) for counting inbound radioactivity. The counting time for each tube was 1 min. The sensitivity of the assay was 0.025 ng ml^{-1}, and intra- and inter-assay coefficients of variants were 14.8% and 15% respectively.

Table 1 Details of experiments and subsequent treatments

Experiment #	Administration (iv)	Pre-treatment	Animal number
1.			
(a) Vehicle control	Normal saline (1 ml)	None	Four Male monkeys
(b) Kisspeptin	Kisspeptin-10 (50 μg)	None	Four Male monkeys
(c) Kisspeptin	Kisspeptin-10 (50 μg) hCG (50 IU) at 240 min sample	Acyline (sc) 12 h and 24 h prior sampling	Three Male monkeys
2.			
(a) hCG + Saline	hCG (50 IU) + Normal saline	Acyline (sc) 12 h and 24 h prior sampling	Four Male monkeys
(b) hCG + Kisspeptin	hCG (50 IU) + Kisspeptin-10 (50 μg)	Acyline (sc) 12 h and 24 h prior sampling	Four Male monkeys

Statistics

Student's *t*-test was employed using Microsoft Excel to determine differences between means of hCG and hCG + kisspeptin treatment values. Statistical significance was set at $P < 0.01$.

Results

Impact of peripheral administration of kisspeptin-10 on plasma testosterone and LH in normal adult intact male monkeys:

Peripheral administration of 50 μg kisspeptin-10 in acyline untreated group induced a potent increase in plasma testosterone levels (Fig. 1a). Within 30 min after peripheral administration of kisspeptin-10, a single LH pulse was observed in all the animals. Subsequently, plasma testosterone level increased 2-fold and remained elevated for the next 2–3 h, while vehicle administration which served as a negative control did not influence the LH or testosterone levels (Fig. 1b). The animals were not showing any LH pulsatile activity during the sampling hours (Fig. 1b), mainly because of the quiescent hypothalamic-pituitary axis in the morning hours in adult animals.

Impact of peripheral administration of kisspeptin-10 on plasma testosterone in Acyline pre-treated adult intact male monkeys:

In acyline pre-treated group, the kisspeptin treatment in all the animals did not induce an increment in the plasma testosterone levels (Fig 1c). The testicular tissue was responsive in terms of testosterone synthesis in the acyline pre-treated group as hCG administration caused a sudden increase in plasma testosterone levels in all animals (Fig. 1c).

Impact of peripheral administration of hCG and hCG + kisspeptin-10 plasma testosterone and LH in acyline pre-treated monkeys:

Administration of hCG (50 IU) + saline in acyline pre-treated monkeys caused a moderate but sustained increase in plasma testosterone levels (Fig 2a). Administration of kisspeptin-10 (50 μg) + hCG (50 IU) significantly ($P < 0.01$) amplified plasma testosterone levels as compared to the hCG induced increase in saline + hCG treatment in all the individual animals without effecting plasma LH values (Fig 2b,c). Pituitary gonadotrophin clamping was successful by acyline treatment was obvious as kisspeptin (50 μg) treatment could not induce a LH peak in the animals (Fig 2b).

Discussion

The loci of kisspeptin action on levels other than the hypothalamus have not been systematically explored in primates. Our study aimed at assessing actions of kisspeptin at the testicular level in terms of testosterone production in the adult male rhesus monkey, a representative higher primate. To investigate the intratesticular action of kisspeptin without the influence of pituitary gonadotropic drive, we used the pituitary gonadotropic-clamped monkey model via pre-treatment with acyline, a GnRH receptor antagonist. As the hypothalamic/pituitary influence on the testis is blocked in the acyline pre-treated animals, seasonality does not play a role in our experiments. Despite the blockade of central influences at the hypothalamic–pituitary level, it was previously shown that seasonality does not affect the ability of the rhesus monkey testis to respond towards LH and the capacity of Leydig cells to secrete testosterone (Wickings *et al.*, 1981; Higashi *et al.*, 1984).

In the unclamped animals, a single bolus of kisspeptin resulted in testosterone discharge, which is preceded by a single LH pulse. Although baseline pre-treatment testosterone levels differed, the testicular testosterone response towards the kisspeptin bolus was present in all four animals and showed a similar amplitude (16 ng ml^{-1}) with comparable kinetics with the maximum testosterone level reached one hour after kisspeptin treatment. Regardless of basal testosterone levels, our observation of an equivalent and analogous testosterone response in terms of time and intensity towards a given dose of kisspeptin (50 μg) is quite remarkable.

In clamped monkeys, the lack of testosterone response towards a kisspeptin-10 (50 μg) bolus affirmed the absence of a pituitary responsiveness towards GnRH, but also negated our hypothesis that kisspeptin might have a direct endocrine action on the testis in terms of testosterone release. However, that the testicular tissue was still highly responsive is confirmed by an hCG bolus resulting in a testosterone discharge.

The significant amplification of the hCG-induced testosterone release by cotreatment with kisspeptin in clamped monkeys indicates a novel indirect action of kisspeptin on testosterone release. The amplified testosterone secretion was evident in all four animals, and the response was remarkably sustained for almost 2 h. This observation reveals a thus far unknown intratesticular effect of kisspeptin and indicates a functional kisspeptin receptor signalling cascade in the primate testis. The potential mechanism through which kisspeptin enhances stimulated testosterone would likely involve a direct action on Leydig cells or an indirect action via Sertoli cells or germ cells. Such notions are supported by

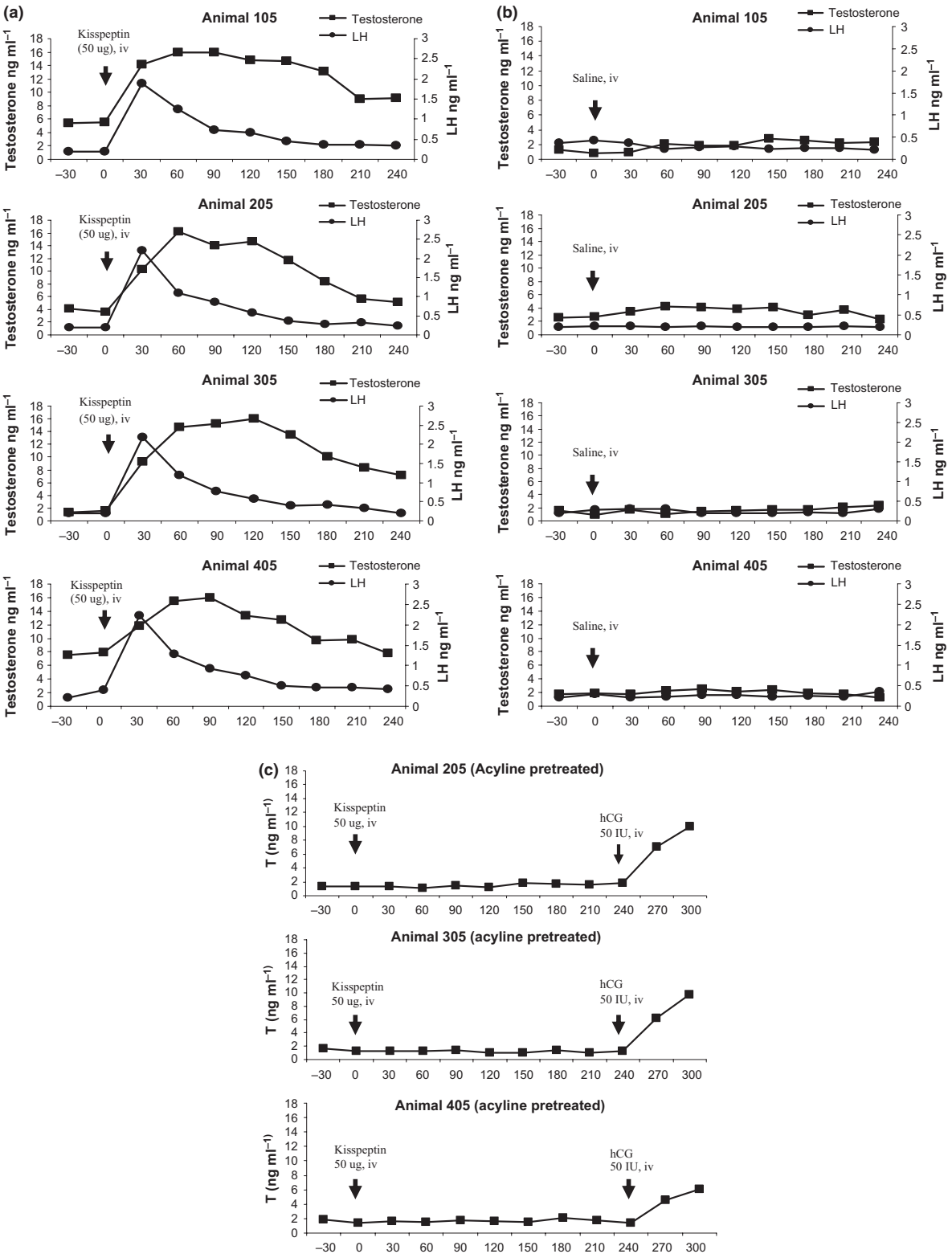

Fig. 1 (a) Individual plasma LH and Testosterone concentration in intact rhesus monkeys before and after iv administration of kisspeptin (arrow). (b) Individual plasma LH and testosterone concentration in intact rhesus monkeys before and after iv administration of saline (arrow). (c) Individual plasma testosterone concentration of acyline pre-treated intact monkeys before and after iv administration of kisspeptin (arrow).

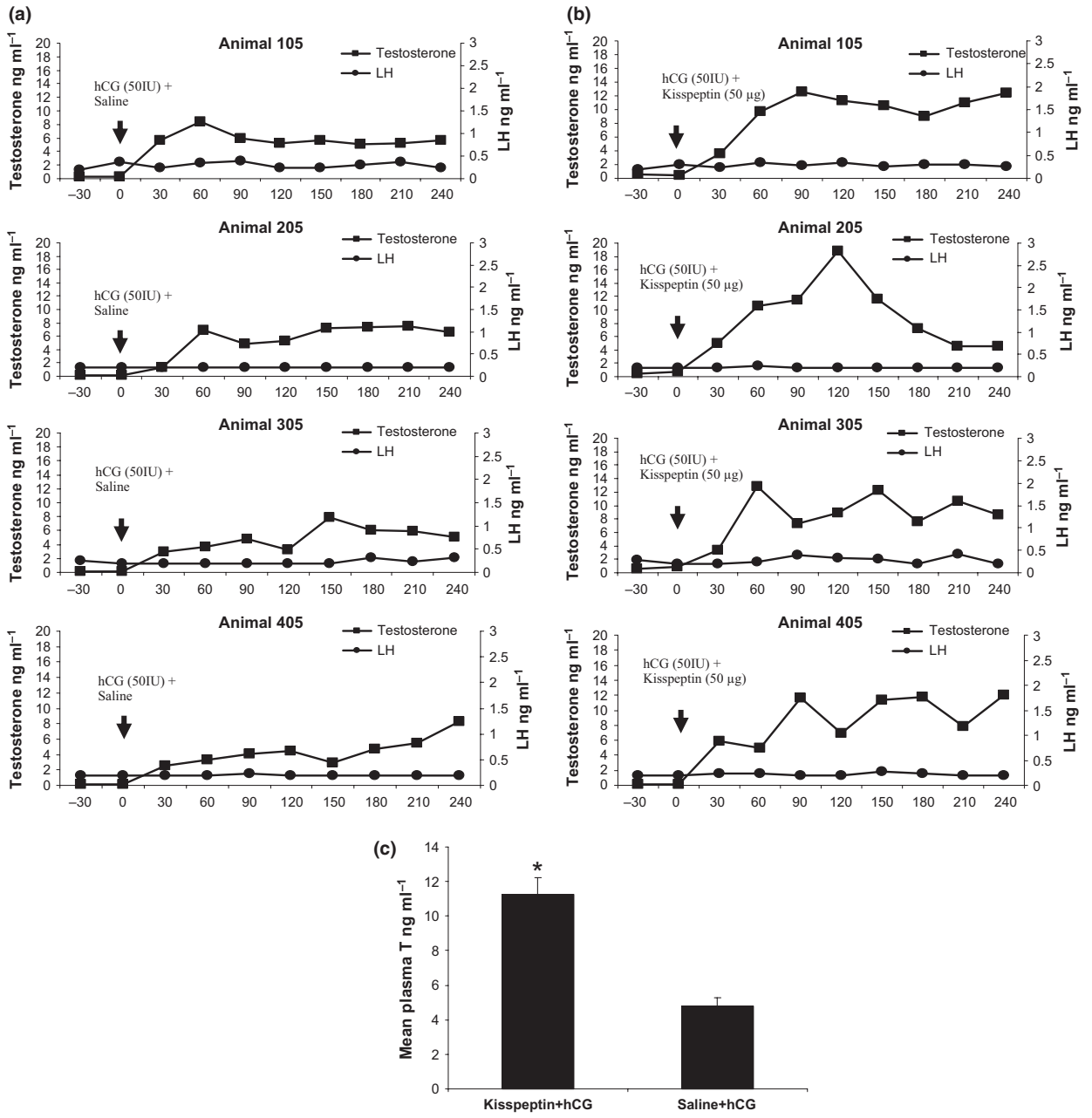

Fig. 2 (a) Individual plasma LH and testosterone concentration of acyline pre-treated intact monkeys before and after iv administration of saline + hCG (arrow). (b) Individual plasma LH and testosterone concentration of acyline pre-treated intact monkeys before and after iv administration of kisspeptin + hCG (arrow). (c) Comparison of the mean plasma testosterone values for 90–150 min time points (*) after kisspeptin + hCG treatment and saline + hCG treatment. Mean plasma T concentration was significantly higher (*t*-test, $*P < 0.005$) in kisspeptin + hCG treatment as compared to saline + hCG treatment.

presence of a certain level of paracrine signalling in the primates (Schlatt *et al.*, 1997) and that steroidogenesis can be enhanced by local secretory factors from the cells of the tubular compartment (Sharpe, 1990; Saez, 1994). Specifically, Sertoli cells have been shown *in vitro* to secrete factors, which increase Leydig cell steroidogenesis, and this effect is augmented in the presence of LH or hCG (Papadopoulos *et al.*, 1987; Papadopoulos, 1991). Further studies on the testicular localisation of KISSR are needed to clarify the site of action of kisspeptin in the primate testis and the subsequent pathway through which kisspeptin enhances the responsiveness of Leydig cells

towards LH/hCG. It is likely that kisspeptin through a direct or indirect action leads to enhancement of sensitivity of LH receptors on the Leydig cells.

Previous kisspeptin studies on primates have either utilised castrated animals (Shahab *et al.*, 2005; Seminara *et al.*, 2006) or the terminal signal for the HPG axis in males, that is, testosterone has not been assessed with regards to a direct testicular action of kisspeptin administration in higher primates (Plant *et al.*, 2006). The observation of disproportional relationship between circulating testosterone and LH during continuous infusion of kisspeptin, when testosterone levels were constantly high for a given concentration of LH by Ramaswamy *et al.*, 2007; indicated a potential peripheral action of kisspeptin in the testis. However, the animals in this study were not clamped and were exposed to a continuous kisspeptin level, which is known to downregulate KissR (Seminara *et al.*, 2006). We assume that the intratesticular action of kisspeptin can only be assessed using a clamped model and bolus injections of kisspeptin as performed in the present study. Our finding has implications for men as *KiSS-1* and *KissR* transcripts were shown to be present in the human testis (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). Studies in humans are needed to explore the intratesticular actions of kisspeptin during pubertal growth and senescence.

Our results demonstrate that kisspeptin exerts an intratesticular action in primate testes. This intratesticular kisspeptin action accelerates an increased steroidogenic response towards LH/hCG stimulation. Our results also demonstrate that kisspeptin does not affect the basal androgen production in male rhesus monkeys. The molecular and cellular mechanisms through which kisspeptin enhances Leydig cell response to LH/hCG need to be investigated. Immunocytochemical and cell culture approaches will help to further explain the peculiar pathway through which kisspeptin modulates Leydig cell steroidogenesis in the primate testis.

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