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Role of Neuromedin S in Plasma Testosterone and Cortisol Concentrations in Male Adult Rhesus Monkey (*Macaca mulatta*)

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Abstract.- Neuromedin S (NMS), a 36 amino acid peptide structurally related to neuromedin U, identified in rat brain as ligand for the G protein-coupled receptor FM4/TGR-1, also termed neuromedin U receptor type-2 (NMU2R). Its central expression is restricted to the suprachiasmatic nucleus, and involved in the regulation of dark light rhythms and suppression of food intake. Stimulatory role of NMS on hypothalamic pituitary gonadal axis (HPG) is reported in rodents. Yet the potential contribution of NMS in the control of reproductive axis in higher primates remains unexplored. In the present study, the effect of peripheral administration of NMS on testosterone and cortisol secretion in the adult male rhesus monkey was evaluated. Four adult male Rhesus monkeys were used for this study. Three different doses of NMS (20nmol, 40nmol and 60nmol) were injected through a teflon cannula implanted in saphenous vein. Blood samples were collected 45 min before and 120 min after NMS administration at 15 min intervals. The plasma concentration of testosterone and cortisol were determined by using specific assay systems. The NMS induced testosterone secretion was significantly (P<0.05) increased at 60 and 40nmol compared to 20nmol. The highest response was observed at a dose of 60nmol. A dose dependent stimulatory role of NMS was noticed in these animals. NMS administration showed a significant (P<0.05) decrease in cortisol secretion at 40nmol and 60nmol doses while non significant (P>0.05) difference was observed at 20nmol. NMS has stimulatory role in testosterone secretion whereas its effect was inhibitory on cortisol secretion. In conclusion, the present study suggests the involvement of neuromedin S in the regulation of hypothalamic pituitary gonadal and adrenal axis in non human primates.

Key words: Neuromedin S, steroidogenesis, G protein-coupled receptor, Rhesus monkey, plasma testosterone, cortisol

INTRODUCTION

Neuromedin S (NMS) is a new member of a peptide family which is highly expressed in the suprachiasmatic nucleus (SCN) of the hypothalamus. Neuromedin S binds with higher affinity to FM-4/TGR-1, the receptor which may mediate the genuine physiological actions of this neuropeptide (Mori *et al.*, 2005). In CNS, the expression of FM-4/TGR-1 receptor is the highest in the hypothalamus, especially in the paraventricular nuclei (PVN) and SCN. The paraventricular expression argues for a putative role of the receptor in the regulation of the HPA axis and feeding (Guan *et al.*, 2001), while the SCN receptors may govern the sleep/wake cycle and the circadian rhythm of temperature, motor phenomena and hypothalamic hormone *e.g.* gonadotropin releasing hormone and CRH secretion (Nakahara *et al.*, 2004). Outside the hypothalamus, the receptor is found in abundance in the hippocampus, the amygdala, the thalamus and the cerebellum (Raddatz *et al.*, 2000), which suggests its putative role in the regulation of behavior, emotions and motor phenomena. Moreover, the distribution of neuromedin S itself raises the possibility that, like other neuromedins (especially neuromedin U), it may play a role in the regulation of the above mentioned hypothalamic functions and is highly expressed in SCN (Mori *et al.*, 2005). Neuromedin S expression is markedly higher than that of neuromedin U in the hypothalamus (Rucinski *et al.*, 2007).

The SCN is the site of the master circadian pacemaker in mammals, which governs the circadian rhythm of behavioral and physiological processes. The pacemaker generates the circadian rhythm by an auto regulatory transcription/ translation feedback loop composed of clock-gene families of transcription factors, and is entrained to the 24-h daily cycle by periodic environmental factors, such as light/dark cycle and temperature, which are typical examples of photic and nonphotic signals, respectively (Lowrey and Takahashi, 2000; Reppert and Weaver, 2001, 2002).

Central administration of NMS acutely elicits LH secretion in female rats at different

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physiological and experimental conditions, along with exaggerated LH responses in females at estrus and under metabolic stress by food deprivation. The activation of paraventricular CRH secretion and pro-opiomelanocortin release from the arcuate nucleus appear to play crucial roles (Vigo *et al.*, 2006).

NMS associated with SCN is a potent activator of the HPA axis, evoking marked cortisol and ACTH responses. These effects appear to depend on CRH release and activation of the CRHR1 pathway (Miklos *et al.*, 2007). There are evidences available on SCN control of cortisol secretion as stimulatory as well as inhibitory. The evening rise in ACTH is abolished by SCN ablation in adrenalectomized animals supplemented with cortisol pellets (Cascio *et al.*, 1987). Also, the increases in ACTH and cortisol secretion upon AVP antagonist infusion are most pronounced during the second half of the light period, consistent with the stimulatory activity appearing during this period (Kalsbeek *et al.*, 1996b).

The main objective of the present study was to show the effect of intravenous injection of NMS on testosterone and cortisol secretion in primates. The distribution of NMS in the hypothalamic region mainly in the SCN and PVN raises the possibility like that of other neuromidins, that it may play a role in the regulation of GnRH and CRH secretion. Therefore, the present study was carried out to examine the possible role of NMS in plasma testosterone and cortisol secretion in adult male rhesus monkey.

MATERIALS AND METHODS

Animals

Four adult male rhesus monkeys (*Macaca mulatta*), 6–8 years old, weighing7–9 kg were used in this study. The animals were kept in separate cages, and maintained under standard colony conditions at the Primate Facility of the Quaid-i-Azam University, Islamabad. The animals were fed daily with fresh fruits (0900–0930 h), boiled eggs at 1100 h and bread at 1300–1330 h. Water was also available there. To reduce the effects of stress on blood sampling, animals were habituated to chair restraint for a month before start of the experiments. The time duration of restraint was increased gradually, upto a daily period of 3 hours was attained. The animals were sedated with ketamine hydrochloride (Rotexmedica, Trittau, Germany 5 mg/kg body weight i.m.) for placement in and removal from the restraining chair. All experiments were approved by the Departmental Committee for Care and Use of Animals.

Catheterization

For continuous blood sampling and i.v. administration of NMS, the animals were anesthetized with ketamine hydrochloride (10 mg/kg body weight, i.m.) and a teflon cannula (Vasocan Branule, 0.8 mm/22 G O.D, B. Braun Melsungen AG, Belgium) was inserted in the saphenous vein. The distal end of the cannula was attached to a syringe via a butterfly tube (length 300 mm, volume 0.29 ml, 20 GX3/4″, JMS, Singapore). Experiment was started after the complete recovery of animals from sedation.

Pharmacological agents

Ketamine hydrochloride, (Rotexmedica, Trittau, Germany) and heparin (Sinochem Ningbo, China) were purchased locally. Neuromedin S was purchased from Anaspec, USA. All the doses of NMS were prepared in normal saline (0.9% NaCl).

Experimental protocol

Blood samplings (2.0 ml each after every 15 min) were started 45 min prior to NMS administration and 120 min after NMS administration. NMS was administered quickly after taking the sample at 0 min. Following each sample, an equal volume of heparinized (5 IU/ml) saline was injected. All blood samples were obtained between 10:00am - 01:00 pm to minimize diurnal variation. Blood samples were immediately centrifuged at 3000 rpm for 10 min. Plasma was separated and stored at -15°C until analyzed for testosterone and cortisol concentration. Three different doses of NMS *i.e.* 20, 40 and 60 nmol were used. The experiment was conducted in three steps with a gap of two weeks in each step and only one of three doses was given at each step.

Enzyme immunoassay (EIA) for testosterone

Plasma testosterone concentrations were determined by using EIA kits purchased from Amgenix International, USA. All the samples were used in this assay for determination of testosterone concentrations. The assay was performed according to the manufacturer's instructions.

Radioimmunoassay (RIA) of cortisol

Plasma cortisol concentrations were determined by using solid phase competitive RIAs. The cortisol RIA kit was purchased from Immunotech Marselle Cedex 9, France. The RIAs was performed as per manufacturer's instructions.

Statistical analysis

All data presented are Mean±SEM. Student's `t' test was employed to determine differences between pre- and post-treatment testosterone and cortisol values. Statistical significance was set at $P \leq 0.05$.

RESULTS

Plasma testosterone level

After single intravenous injection of 20nmol of NMS, increase and decrease in testosterone concentration was observed rhythmically at different time periods. But this change was found statistically non-significant ($P>0.05$) (Table I). A non significant decrease in testosterone concentration was noticed in the mean pre treatment samples as compared to mean post treatment samples (Fig. 1A).

After the injection of 40nmol of NMS, an increase in concentration of testosterone was observed at different time intervals and maximum raise was found after 75 minutes, which was statistically significant ($P<0.05$) (Table I). The comparison between the mean pre and post treatment showed a significant ($P<0.05$) increase in testosterone concentration after treatment (Fig. 1B).

After the injection of 60nmol of NMS the testosterone concentration increased significantly ($P<0.05$) at various time intervals (Table I). The mean comparison between pre and post treatment also showed a significant ($P<0.05$) increase in testosterone levels after treatment (Fig. 1C).

Table I.- Plasma testosterone concentrations (ng/ml) after *i.v.* administration of 20 nmol, 40 nmol and 60 nmol of Neuromedin S.

Time (min)	Plasma testosterone concentration (ng/ml) after neuromedin S administration		
	20 nmol	40 nmol	60 nmol
-45	0.80±0.27*	1.10±0.23	1.06±0.45
-30	0.78±0.17	1.22±0.23	1.13±0.46
-15	0.74±0.27	1.17±0.23	0.99±0.44
0	0.85±0.30	1.29±0.23	1.13±0.47
15	0.53±0.22	1.70±0.19	1.06±0.37
30	0.84±0.14	1.57±0.19	1.58±0.43
45	0.49±0.13	1.28±0.08	2.05±0.43
60	0.85±0.14	1.92±0.65	1.81±0.61
75	0.74±0.05	2.15±0.40 a	1.36±0.40
90	0.68±0.08	1.54±0.21	2.04±0.45 a
105	0.44±0.28	1.90±0.36	2.24±0.55 a
120	0.67±0.05	1.37±0.44	1.73±0.62

*Mean±SEM; a, $P<0.05$ vs. 0 min. sample.

Plasma cortisol level

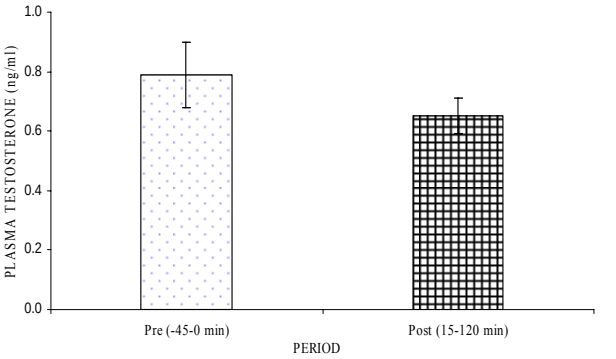
For cortisol concentrations the samples, 15 minutes before and 90 minutes after NMS administration were used. Following 20nmol of NMS *i.v.* administration, a decrease in mean cortisol concentration was observed (Table II). The comparison between mean pre and post treatment cortisol concentration showed no significant difference ($P>0.05$) (Fig. 2A).

The administration of 40nmol of NMS significantly ($P<0.05$) decreased post cortisol concentration as compared to pre treatment (Table II, Fig. 2B). A significant ($P<0.05$) decrease in mean plasma cortisol concentration after administration of 60nmol of NMS was also observed (Table II, Fig. 2C).

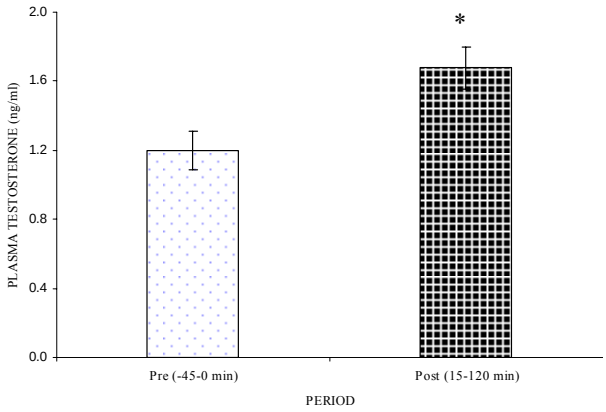
DISCUSSION

Reproduction and nutrition are tightly linked to each other. NMS is a neuropeptide, expressed at the SCN within the brain, which is involved in the control of circadian rhythms and has anorexigenic activity (Mori *et al.*, 2005; Ida *et al.*, 2005). In rodents the endocrine actions of NMS have been noted but not investigated in higher primates so far.

In the current study, therefore, we examined effect of peripheral administration of NMS on testosterone and cortisol secretion in the adult male



A



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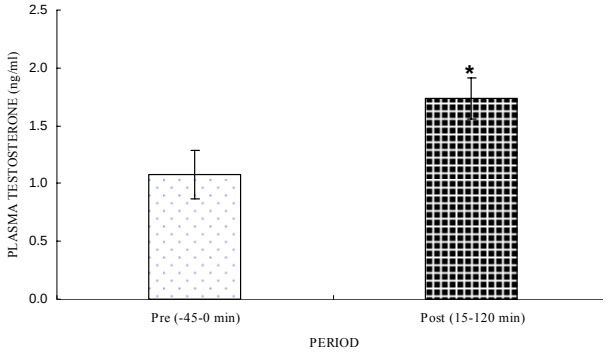

C

Fig. 1. Comparison of Mean±SEM plasma testosterone concentrations in pre and post NMS *i.v.* administration (A, 20 nmol; B, 40 nmol; C, 60 nmol) in adult male rhesus monkeys (n=4). [*=P< 0. 05.]

rhesus monkeys. The present study suggests that neuromedin S has a stimulatory effect on testosterone concentration at 60nmol and 40nmol (P<0.05) compared to 20nmol. On cortisol concentration, NMS showed inhibitory effect at 60 nmol and 40 nmol (P<0.05) and no change was noticed at 20nmol.

Table II.- Plasma cortisol concentrations (ng/ml) after *i.v.* administration of 20 nmol, 40 nmol and 60 nmol of Neuromedin S.

Time (min)	Plasma cortisol concentration (ng/ml) after Neuromedin S administration		
	20 nmol	40 nmol	60 nmol
-15	0.86±0.15*	1.14±0.28	1.03±0.10
0	0.78±0.07	1.27±0.33	1.06±0.10
15	0.75±0.13	0.99±0.15	0.93±0.08
30	0.82±0.12	0.85±0.11	0.85±0.05
45	0.75±0.13	0.74±0.10	0.99±0.10
60	0.71±0.10	0.69±0.09	0.78±0.11
75	0.67±0.09	0.64±0.04	0.88±0.12
90	0.67±0.13	0.65±0.06	0.69±0.10

*Mean±SEM.

This observation is supported by the findings of Vigo *et al*. (2006) in rodents, where they observed the same result with LH, but in contradiction with the finding of Miklos *et al*. (2007), as their study demonstrates that NMS has stimulatory effect on cortisol concentration.

The ability of NMS to influence testosterone secretion was not totally unpredicted, as Vigo *et al*. (2006) has reported stimulatory effect on HPG axis in rodents and similarly the actions of NMU, which operates through the same NMU2R centrally, having opposite role to NMS on LH release had inhibitory effect in ovariectomized female rats (Quan *et al.*, 2003, 2004).

The neuroendocrine circuitry responsible for such a positive effect of NMS on testosterone secretion remains to be investigated. However, considering that NMS is able to modulate neuropeptide expression at the ARC (Ida *et al.*, 2005), which is a major center for the integrated control of energy balance and reproduction with abundant expression of NMU2R; it is plausible that the central mechanism, whereby NMS stimulating testosterone secretion, involves the activation of ARC pathways. Potential candidates for such an intermediary action, such as kisspeptin and galanin-like peptide, which are prominently expressed at the ARC (Gottsch *et al.*, 2004; Tena-Sempere, 2006) may have role in HPG axis stimulation.

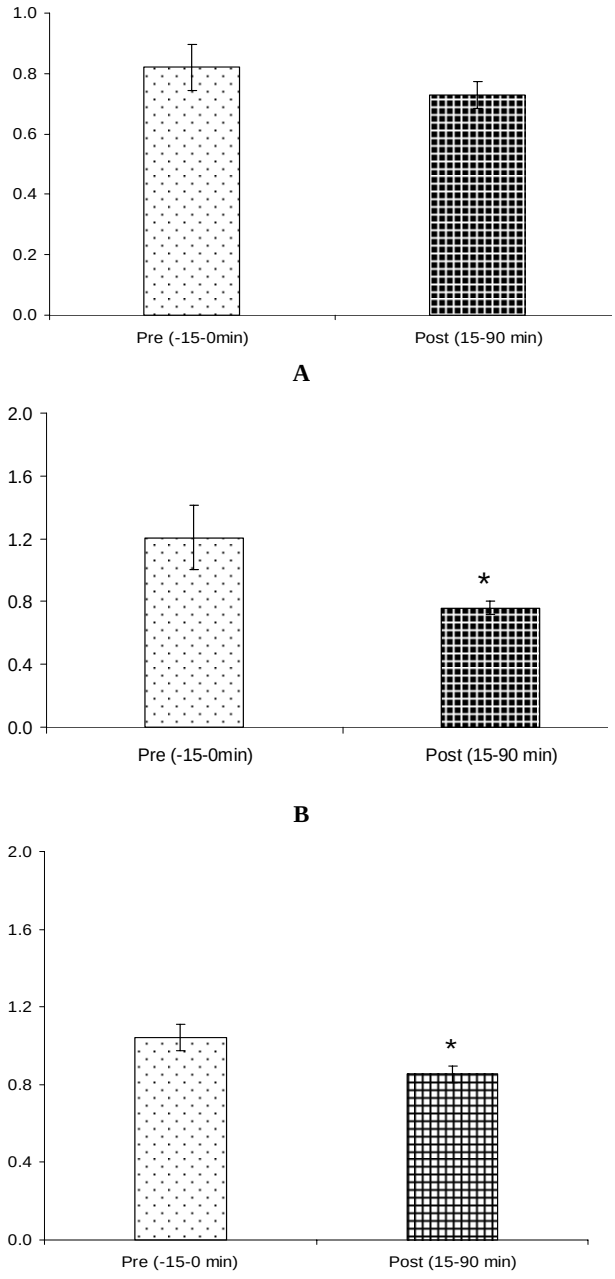

Fig. 2. Comparison of Mean±SEM plasma cortisol concentrations in pre and post NMS *i.v.* administration (A, 20 nmol; B, 40 nmol; C, 60 nmol) in adult male rhesus monkeys (n=4). [*=P< 0. 05.]

Vigo *et al*. (2006) also reported that stimulatory effects of NMS on LH secretion were detected after suppression of gonadotropin secretion by short-term fasting, with exaggerated responses *vs.* control females at diestrus. Enhanced LH secretory responses to different stimuli (such as kisspeptin and galanin like peptide) have been detected in underfed animals (Castellano *et al.*, 2005; 2006). In any event, this observation evidences that NMS is able to counteract the inhibitory effect of energy insufficiency on the gonadotropic axis, thus reinforcing the potential role of this neuropeptide in the joint regulation of reproductive function and energy balance.

The central effects of NMS on gonadotropin secretion, reported in Vigo *et al*. (2006) study, do not exclude additional actions of this neuropeptide at other levels of the HPG axis. In this regard, expression of NMU1R and NMU2R has been very recently observed in mouse pituitary, which suggest its possible action at the pituitary level (Vigo *et al.*, 2006). Expression of mRNA of NMS in the testes (Mori *et al.*, 2005) make a possible action of this neuropeptide at gonadal level but no study has been reported yet.

Miklós *et al.* (2007) showed that NMS stimulate basal cortisol secretion, but the current study in primates demonstrates that cortisol concentration decreases after NMS administration. A comprehensive study is required to evaluate the reason for this decrease in cortisol after NMS administration.

It has been well established that the circadian rhythm is generated by an endogenous pacemaker generated in the SCN, and that precise rhythmic oscillation of this pacemaker is coordinated by various neurochemical substances (Lowrey and Takahashi, 2000; Reppert and Weaver, 2001, 2002). The data obtained in this study regarding testosterone concentration suggest that NMS function as a regulator of the testosterone concentration. Present data and previous studies (Mori *et al.*, 2005) suggest that NMS shifted phase of the testosterone concentration, and increase the amplitude and the period of pulses of testosterone dose dependently. Maximum amplitude and pulses were observed at the 60nmol dose of NMS.

The specific distribution pattern of NMS containing neurons (Mori *et al.*, 2005; Rucinski *et al.*, 2007) raises the possibility that this neuropeptide is one of the regulators of circadian

endocrine rhythms. NMS expression is specifically high in the SCN (Mori *et al.*, 2005), the master circadian pacemaker in mammals (Lowrey and Takahashi, 2000). Not only the distribution itself, but also the light/dark cycle dependent expression of NMS supports this hypothesis (Mori *et al.*, 2005). The present experiments and previous results regarding feeding (Ida *et al.*, 2005) and LH secretion (Vigo *et al.*, 2006) suggest that NMS released from the SCN may function as a pacemaker of these processes. NMS has stimulatory role in testosterone secretion and its effect was inhibitory on cortisol secretion. In conclusion, the present study suggests the involvement of neuromedin S in testosterone and cortisol secretion in monkeys. Further studies are required to explore its exact mechanism of action.

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