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Effects of postnatal treatment with naloxone on plasma gonadotropin, prolactin, testosterone and testicular functions in male rats

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Abstract. Opioid peptides are implicated in the control of gonadotropin and prolactin secretion. The role of opioid antagonist naloxone and its effects on plasma gonadotropin, prolactin, testosterone levels and testicular hyaluronidase, acid phosphatase, [^{3}H]uridine and thymidine incorporation, RNA, DNA and protein concentrations were evaluated in rats after administration of naloxone beginning day 1 through 21 and autopsied on 45, 60 and 90 days of age. Plasma gonadotropin and testosterone levels were significantly elevated after naloxone treatment. Testicular hyaluronidase and acid phosphatase activity increased till 60 days post treatment and declined thereafter. Concentrations of RNA and protein did not change significantly but the concentration of DNA declined at 45 and 60 days of age. These results suggest that endogenous opioid peptides exert regulatory influence on gonadotropin secretion which in turn control the testicular function in the male rat.

Keywords. Opioid peptides; pituitary; plasma hormone levels; naloxone; neonatal rats.

Introduction

Narcotics and opioid peptides are known to decrease the activity of hypothalamic pituitary gonadal axis where as narcotic antagonists increase serum LH in rodents and humans (Cicero *et al.,* 1976, 1977; Miller *et al.,* 1986). It has also been shown that opioid peptides modulate luteinizing hormone (LH) secretion during sexual maturation in the rat (Blank *et al.,* 1979). Naloxone, an opioid receptor antagonist competitively inhibits the effects of opioids on LH secretion. However, treatment with morphine, naloxone and morphine plus naloxone do not modify the secretion of follicle stimulating hormone (FSH) in normal and castrated male rats (Piva *et al.,* 1986). The results with opioids and of their antagonists on FSH secretion is still not clear. Naloxone could increase LH levels or block testosterone's negative feedback control of LH by displacing the androgen from its receptors in the brain or pituitary (Cicero *et al.,* 1979). Lee and Loh (1975) have recently noted from [^{3}H] morphine incorporation studies that DNA is one of the narcotic receptors and chronic morphine treatment increases the chromatin template activity. In contrast, decrease of chromatin template activity is also noted (Hodgson *et al.,* 1971). Chronic morphine treatment in mice however, inhibited incorporation of labelled uridine and thymidine and hence RNA and DNA synthesis (Datta and Antopol, 1971). The present experiments were designed to evaluate the effects of opioid

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Abbreviations used: LH, Luteinizing hormone; FSH, follicle stimulating hormone; RIA, radioimmunoassay; EOP, endogenous opioid peptides; LHRH, luteinizing hormone releasing hormone; TSH, thyroid stimulating hormone.

antagonist naloxone treatment in neonatal rats beginning day 1 of birth. Concentration of DNA, RNA and protein, acid phosphatase and hyaluronidase enzyme activity in the testes and plasma levels of gonadotropin, prolactin and testosterone were evaluated on 45, 60 and 90 days post-treatment.

Materials and methods

Female Wistar rats with pups were maintained in the School animal house facility under a 14 h light and 10 h dark regimen. They were fed on standard rat pellets purchased locally and with drinking water *ad libitum.* Day of birth was assigned as day 1 and pups were kept with their mother till day 21 postpartum. On day 22 they were weaned, males and females were kept in separate cages till they attained the age of 45, 60 and 90 days respectively.

Naloxone (2 mg/kg) was injected subcutaneously on alternate days from day 1 till 21 of age. Controls received equal volume of saline. Body weights of the animals were recorded during the treatment period as well as on day 45, 60 and 90 respectively, before they were decapitated. Testes, seminal vesicles and ventral prostate were dissected out, kept at 4°C, cleaned and weighed to the nearest 0.2 mg on a torsion balance. Trunk blood was collected and plasma was separated by centrifugation at 4°C and kept frozen for later assay of hormones. Pituitary and plasma LH, FSH, prolactin and testosterone were measured by radioimmunoassay (RIA). RIAs were carried out, using kits obtained from NIH-NIAMDD, by a double antibody technique and the data were expressed as ng/ml of plasma with reference to RP-1 reference standard for rat-LH, FSH and prolactin. Testosterone levels were measured by steroid hormone RIA (courtesy Diagnostic System Inc, Houston, USA) and the results are expressed as ng/ml of plasma.

Testes were decapsulated and a part of the testicular tissue was incubated 2 μCi [^{3}H] thymidine (sp. activity 17,000 mCi/mmol) in Dulbecco's Eagle medium containing 40 mM HEPES (pH 7·4), 1% bovine serum albumin (BSA) and 1% glucose. Incubation was carried out at 34°C for 90 min. The reaction was stopped by placing the tissues in ice, washed with cold distilled water and processed for nucleic acid extraction by the procedure standardised in the laboratory (Mistry and Vijayan, 1985; Prasad, 1986).

An aliquot of the sample was taken and put in Bray's scintillation fluid and counted in a Beckman LS 1800-counter with 40% efficiency for tritium. The above procedure was repeated for studying the incorporation of [^{3}H] uridine (sp, activity 6,000 mCi/mmol) into RNA in the testis. Two μCi of uridine was added to the medium containing the tissue and incubation was carried out for 2 h (Mistry and Vijayan, 1985).

Assay of hyaluronidase activity

Testicular hyaluronidase activity was assayed according to the procedure of Males and Turkington (1971) and Doak and Zahler (1979) with slight modifications. Ten μl of the enzyme preparation was used and free N-acetylglucosamine (NAGA) end groups produced by hyaluronidase action were estimated as described earlier (Prasad and Vijayan, 1986, 1987).

Assay of acid phosphatase activity

Acid phosphatase activity was assayed according to the method of Henrickson and Clever (1972) and Pertulla *et al.* (1986) as standardised in the laboratory. Testicular tissue was homogenised in 0·3% Triton-X100. Acetate buffer (pH 6) containing enzyme samples were incubated with 0·1 mM cobalt chloride. After incubating for 10 min, 2 μmol of phosphatase substrate was added. Substrate was freshly prepared and the reaction terminated with 0·1 N NaOH after 1 h and the liberated *p*-nitrophenol (P N P) was estimated at 420 nm against the enzyme blank.

Estimations of DNA, RNA and protein were carried out according to standard procedures routinely adopted in the laboratory (Mistry and Vijayan, 1985; Prasad, 1986).

Statistics: Group means were compared by analysis of variance followed by Duncan's new multiple range test with $P < 0{\cdot}5$ required for significance.

Results

Pituitary LH and FSH concentrations did not differ significantly in the control rats of 45 and 60 days of age. However, naloxone treatment produced significant ($P<0{\cdot}05$) increase in both LH and FSH at 45 and 60 days of age while there was no appreciable difference in gonadotropin concentration at 90 days of age. Pituitary prolactin concentration did not show any significant change at any age studied (table 1).

Table 1. Gonadotropin and prolactin content in the pituitary gland of neonatal rats treated with naloxone.

	Age at autopsy (days)		
Treatment	45	60	90
LH (μg/pit)			
Control	146·41 ± 14·62	148·45 ± 24·81	153·61 ± 32·90
Naloxone	254·82 ± 24·71**	214·20 ± 3·80*	140·62 ± 21·32
FSH (μg/pit)			
Control	141·32 ± 15·62	138·61 ± 17·01	94·60 ± 19·41
Naloxone	184·63 ± 16·13*	196·25 ± 21·40**	106·44 ± 14·22
Prolactin (μg/pit)			
Control	4·40 ± 0·20	4·70 ± 0·30	5·40 ± 0·30
Naloxone	4·70 ± 0·80	5·40 ± 0·10	6·20 ± 0·20

Naloxone 2 mg/kg was injected on day 1 through 21 once daily on alternate days. Controls received equal volume of saline.
Values are mean ± SEM for 6–8 rats per group.
Values *$P<0{\cdot}05$, **$P<0{\cdot}01$ *vs* control.

During normal maturation in male rats, plasma FSH and LH levels did not show any significant differences from day 45 until 90. In the present study neonatal rats treated with naloxone exhibited increase in both LH ($P < 0{\cdot}05$) and FSH ($P < 0{\cdot}01$) levels in plasma of rats autopsied on day 45. There was a further increase in the

levels of LH at 60 ($P<0{\cdot}01$) and 90 days ($P<0{\cdot}001$) of age whereas increase in the levels of FSH in the plasma was constant at 60 and 90 days of age ($P<0{\cdot}01$) when compared to their respective control values (table 2).

Table 2. Plasma levels of gonadotropin, prolactin and testosterone at different ages after neonatal treatment with naloxone.

	Age at autopsy (days)		
Treatment	45	60	90
LH (ng/ml)			
Control	2·30 ± 0·20	3·00 ± 0·30	2·50 ± 0·30
Naloxone	3·70 ± 0·50*	7·00 ± 1·00**	9·18 ± 1·08***
FSH (ng/ml)			
Control	586·20 ± 38·20	672·60 ± 24·20	642·60 ± 78·40
Naloxone	984·70 ± 41·20**	1108·00 ± 102·00**	1204·40 ± 64·60**
Prolactin (ng/ml)			
Control	21·00 ± 2·10	16·80 ± 1·40	15·20 ± 3·00
Naloxone	28·20 ± 2·40	17·00 ± 1·80	18·10 ± 2·40
Testosterone (ng/ml)			
Control	1·0 ± 0·4	2·6 ± 0·6	3·2 ± 0·2
Naloxone	2·2 ± 0·2*	4·4 ± 0·4*	4·8 ± 0·4*

Naloxone 2 mg/kg was injected subcutaneously from day 1-21 on alternate days for one month in neonatal rats. Controls received equal volume of saline.
Values represent mean ± SEM for 6-8 animals.
Values *$P<0{\cdot}05$, **$P<0{\cdot}01$, ***$P<0{\cdot}001$ are significantly different from their respective control.

Plasma testosterone levels showed a gradual increase with age in control rats. Naloxone treatment however, induced significant increase ($P<0{\cdot}05$ *vs* control) in testosterone levels at all ages studied which correlated with the increase in plasma LH levels (table 2).

There was no significant difference in body weights between control and naloxone treated rats in any particular age group. Body weights however, increased with age in both control and naloxone treated rats. There was a marginal, but significant ($P<0{\cdot}05$ *vs* control) increase in the weight of the testes and seminal vesicle in rats treated with naloxone and autopsied on day 45. The weight of testes increased significantly ($P<0{\cdot}01$ *vs* control) at 60 days of age whereas the weight of the seminal vesicle and ventral prostate did not exhibit any significant difference when compared to those of the controls. At 90 days after naloxone treatment the weights of testes, seminal vesicle and ventral prostate did not differ significantly from control values though plasma testosterone levels were significantly higher in these rats (table 3).

The rate of [^{3}H] thymidine incorporation into DNA increased significantly in the testes of rats treated neonatally with naloxone and autopsied at 45 and 60 days of age ($P<0{\cdot}01$ *vs* control), whereas at 90 days of age there was no significant difference. Testicular hyaluronidase activity increased at 45 and 60 days of age while the acid phosphatase activity increased only in 45 days old rats whereas the activity of both the enzymes decreased significantly ($P<0{\cdot}01$ *vs* control)'at 90 days of age (table 4). The DNA concentration however decreased ($P<0{\cdot}01$) at 45 and 60

Table 3. Weight of the body, testes and accessory reproductive glands at different ages after neonatal treatment with naloxone.

	Age at autopsy (days)		
Treatment	45	60	90
Body weight (g)			
Control	160 ± 10	180 ± 10	200 ± 10
Naloxone	150 ± 15	195 ± 15	220 ± 10
Testis (mg)			
Control	442 ± 46	812 ± 90	913 ± 104
Naloxone	640 ± 94*	1201 ± 77**	782 ± 105
Seminal vesicle (mg)			
Control	25·00 ± 4·30	83·00 ± 19·00	253·00 ± 41·00
Naloxone	51·00 ± 12·50*	67·00 ± 10·00	268·00 ± 37·00
Ventral prostate (mg)			
Control	35·70 ± 8·80	105·70 ± 8·20	293·00 ± 58·00
Naloxone	49·50 ± 13·80	107·70 ± 5·70	245·00 ± 141·00

Naloxone 2 mg/kg was injected on day 1 through 21 once daily on alternate days. Controls received equal volume of saline. Body weight is represented in g and weight of accessory reproductive glands and testis is represented in mg.
Values are mean ± SEM for 6–8 rats per group.
Values *P<0·05, **P<0·01 *vs* control.

days of age. Concentrations of RNA and protein and [^{3}H] uridine incorporation into RNA in the testes of naloxone treated rats, however, did not exhibit any significant alteration (table 4).

Discussion

Endogenous opioid peptides (EOP) and their antagonists appear to influence the release of hormones from the pituitary mediated *via* hypothalamus or *via* hypothalamic neurotransmitters. Norepinephrine and epinephrine have been suggested as possible neurotransmitters mediating the action of opioid peptides (Meites *et al.,* 1977, 1979). Chronic administration of naloxone to neonatal rats increases pituitary as well as plasma LH and FSH levels, thus indicating an increased synthesis and release of gonadotropin under blockade of endogenous opioid inhibition. As opioid peptides and their antagonists do not have a direct action on the pituitary (Weisner *et al.,* 1984) the increased synthesis and release may be due to an alteration in the activity of luteinizing hormone releasing hormone (LHRH) caused by naloxone directly or *via* neurotransmitters. It may also be due to the continuous blocking effects of naloxone at the opioid receptors when administered chronically to neonatal rats. Days 2-7 postnatally is a highly critical stage in the development of opiate receptors in the rat brain (Simon and Hiller, 1978) and naloxone treatment during the early neonatal days could possibly modify the interaction between endogenous opioids and their binding sites. Naloxone treatment antagonizes the inhibitory tone of the opioid peptides on gonadotropin and thus there are increased levels gonadotropin in the plasma till 90 days. Sylvester *et al.* (1980) have shown simultaneous stimulation of LH and FSH

Table 4. Effect of neonatal treatment with naloxone on (i) *in vitro* [^{3}H] uridine and thymidine incorporation, (ii) activity of hyaluronidase and acid phosphatase and (iii) concentration of RNA, DNA and protein in the testes of rats at 45, 60 and 90 days of age.

	Age at autopsy (days)		
Treatment	45	60	90
Specific activity (DPM × 10^{-3}/ mg RNA) of RNA			
Control	46·61 ± 4·60	42·15 ± 6·92	40·80 ± 4·05
Naloxone	40·65 ± 4·46	45·21 ± 2·39	46·08 ± 18·11
Specific activity (DPM × 10^{-4}/ mg DNA) of DNA			
Control	46·34 ± 1·44	35·26 ± 3·24	29·60 ± 4·81
Naloxone	52·41 ± 2·16**	45·80 ± 2·82**	28·95 ± 0·99
Hyaluronidase[a]			
Control	0·61 ± 0·06	1·85 ± 0·06	1·69 ± 0·15
Naloxone	1·38 ± 0·19***	2·46 ± 0·16***	1·21 ± 0·15**
Acid phosphatase[b]			
Control	5·18 ± 0·48	5·46 ± 0·53	7·50 ± 0·83
Naloxone	7·36 ± 0·48**	6·13 ± 0·39	4·50 ± 0·43**
DNA μg/100 mg tissue			
Control	236 ± 23	355 ± 11	189 ± 19
Naloxone	138 ± 24**	249 ± 29**	168 ± 9
RNA μg/100 mg tissue			
Control	642 ± 19	563 ± 60	406 ± 63
Naloxone	661 ± 38	522 ± 70	391 ± 18
Protein mg/100 mg tissue			
Control	4·50 ± 0·14	5·20 ± 0·15	4·20 ± 0·25
Naloxone	4·20 ± 0·21	4·80 ± 0·31	4·20 ± 0·40

Naloxone 2 mg/kg was injected on day 1 through 21 once daily on alternate days. Controls received equal volume of saline.
Values are mean ± SEM for 6–8 rats per group.
Values *P<0·05, ** P<0·01, ***P< 0·001 *vs* control.
Activity of enzymes expressed as [a]μmol NAGA formed/mg/protein/h and [b]μmol PNP released/mg/protein/h.

secretion by naloxone. Presumably this effect is due to naloxone's antagonism of the actions of EOP at a common receptor in the hypothalamus or possibly at extra-hypothalamic sites in the brain. Thus EOP containing systems may play a physiologically relevant role in tonically inhibiting the release of LHRH and thus the disinhibition induced by naloxone results in a sudden discharge of LHRH.

Though there was not much difference in the concentration of RNA and protein per 100 mg tissue at 45, 60 and 90 days of age between experimental and control animals, the significant increase in the content of RNA and protein per testis and decrease in concentration of DNA at 45 and 60 days of age may possibly be due to significant increase in the weight of testes. The weights of ventral prostrate and seminal vesicles were less affected in 60 and 90-day old animals than in the 45-day old animals. Though the increase in gonadotropin levels were higher at 90 days of age than those in 60 days of age the magnitude of stimulatory effect on plasma testosterone levels was nearly equal. The failure of enhanced levels of gonadotropin

to stimulate corresponding increase in testosterone levels may possibly be due to desensitization of LH receptors in the testes. This is consistent with the known phenomena of negative regulation of receptors in the target organ of the rat testes (Catt *et al.,* 1980; Tsuruhara *et al.,* 1977).

Activity of testicular acid phosphatase and hyaluronidase enzymes have been suggested as the enzyme products of hormone induced protein synthesis during maturation of germinal epithelium (Steinberger and Steinberger, 1975; Prasad and Vijayan, 1986, 1987). The increased levels of gonadotropin and testosterone have caused an increase in the activity of both the enzymes, though the activity of acid phosphatase is not significantly different from the control values at 60 days of age.

The increase in testes weight and function of seminiferous tubules as reflected by the increased activity of the enzymes are obviously the consequence of naloxone stimulated increase in LH, FSH and testosterone levels which stimulated the enzyme activity and macromolecular synthesis. Gonadotropin and testosterone stimulated the activity of hyaluronidase and acid phosphatase enzymes in testes (Males and Turkington, 1971; Prasad and Vijayan, 1986, 1987). However, a reduction in both hyaluronidase and acid phosphatase activity at 90 days of age, inspite of the increased gonadotropin and testosterone levels, were observed. The possible mechanism underlying this is not clear and require further studies.

Opioid peptides are known to have a variety of other effects. Blockade of opioid receptors by naloxone could cause changes in thyroid function also. There is evidence to suggest that thyroid status influences gonadal sensitivity to gonadotropin (Hadley, 1984). Since thyroid stimulating hormone (TSH) release *per se* was not measured in these experiments changes in thyroid status of these rats to gonadotropin and testosterone levels could play only a minor role in the effects of naloxone. Measurement of TSH levels could reveal the role if any.

The data presented here indicates that EOP exert some regulatory role in the development of reproductive function in the adult male rat *via* controlling the synthesis and secretion of gonadotropin and testosterone. The increase in gonadotropin and testosterone levels could possibly be due to decrease in negative feedback by testosterone at hypothalamic and/or pituitary level. Blockade of opioid receptors in neonatal rats by chronic treatment with naloxone disturb the physiological levels of gonadotropin in adult rats.

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