A 'window of time' during which testosterone determines the opiateergic control of LH release in the adult male rat

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Summary. Male rats castrated before puberty (when 26 days of age) showed a progressively decreasing susceptibility to the inhibitory effects of morphine (5 mg/kg) upon LH secretion for up to 28 days after gonadectomy (~100%, 40% and 10% inhibition at 5, 12 and 28 days after castration), but thereafter morphine again caused ~50% reduction in serum LH values; the minimum inhibition found at 28 days after castration (age 54 days) occurred at the time at which male rats normally reach puberty. When rats were castrated at 59 days of age, morphine maximally suppressed serum LH concentrations (to <70%) 2 and 5 days after castration, but had no effect thereafter.

In prepubertal castrates, testosterone replacement between Days 26 and 50 of life resulted in responses to morphine similar to those found in rats castrated after puberty, i.e. serum LH levels were not reduced. Morphine significantly reduced LH levels in prepubertal castrates given testosterone after 60 days of age.

Treatment with morphine consistently elevated serum prolactin concentrations (>100%) in castrated rats of all ages, regardless of the time elapsed after gonadectomy.

These results indicate (1) a transient fall in the inhibitory opiateergic tone upon LH secretion as the normal age of puberty approaches, (2) that the ability of opiates to alter LH release in adulthood may depend upon testicular steroids secreted during the peripubertal period, and (3) that the LH responses do not reflect general changes in the neuroendocrine response to opiates after castration since the prolactin response to morphine remains intact in rats castrated before and after puberty.

Introduction

Several lines of evidence indicate that endogenous opioids interact with gonadal steroids to modulate the secretion of luteinizing hormone-releasing hormone (LHRH) and, in turn, that of luteinizing hormone (LH) from the pituitary (Cicero *et al.*, 1979; Kalra & Kalra, 1984; Millan & Herz, 1985). Male rats deprived of sex steroids by pre- or post-pubertal gonadectomy have been reported to become 'subsensitive' or 'tolerant' to the LH-suppressive effects of opiates (Cicero *et al.*, 1982; Bhanot & Wilkinson, 1984) and also to the LH-releasing effects of the opiate antagonist naloxone (Bhanot & Wilkinson, 1984; Petraglia *et al.*, 1984). We have confirmed these observations in in-vitro studies on the release of LHRH from hypothalamic slices taken from post-pubertally castrated male rats (Nikolarakis *et al.*, 1986), but in in-vivo experiments we found that pre-pubertally castrated rats still responded to the opiate morphine when it was administered beyond the normal age of puberty (O. F. X. Almeida, R. Schulz & A. Herz, unpublished observations). While this finding conflicts with that of Bhanot & Wilkinson (1984), the latter authors studied their animals only up to 45 days of age when the pubertal process would not yet have been completed. Bhanot & Wilkinson (1983) had previously shown an age-related rightward shift in the opiate-LH dose-response curve of young rats and suggested that a decrease in opioid tone may be part of the neural mechanism underlying the onset of puberty. It would therefore seem that sex steroids released during puberty may determine

LHRH neurone responsiveness to opiateergic inhibition in adulthood. In the present experiments, we studied the changes in the ability of morphine to suppress LH in pre- and post-pubertally castrated rats over several weeks after gonadectomy. To determine whether there is a 'critical period' for the action of gonadal secretions, pre-pubertally castrated rats which received implants of testosterone at various ages up to the normal age of puberty were also tested with morphine. To test whether the LH responses reflected general changes in neuroendocrine responsiveness to opiates, we measured the prolactin responses because, in rats, the serum concentrations of this hormone are normally inversely related to those of LH and the two hormones are oppositely regulated by opiates (Meites, 1984; Millan & Herz, 1985).

Materials and Methods

Male Sprague-Dawley rats were obtained from a commercial supplier at least 1 week before experimental manipulations; juveniles were purchased with their mothers and weaned at 23 days of age. Castrations were performed under light ether anaesthesia on Day 26 or 59 of life. The animals were housed in groups of 6–8 per cage in a light- and temperature-controlled room (lights on from 06:00 to 18:00 h; 21°C) and allowed free access to food and water.

Experiment 1. At various intervals after castration, groups of 6 pre- and post-pubertally castrated rats were injected subcutaneously with saline (9 g NaCl/l) or morphine hydrochloride (5 mg/kg, a dose previously found to produce maximal inhibition of LH secretion in intact and gonadectomized adult rats of both sexes). The animals were decapitated 30 min later and serum samples were stored at -22°C until measurement of LH and prolactin concentrations.

Experiment 2. Rats were castrated at 26 days of age and were implanted with Silastic capsules containing testosterone at the following ages: 26 days (5 mm capsules on Days 26 and 34; 10 mm on Days 42 and 50), 42 days (10 mm on Days 42 and 50), 50 days (10 mm on Day 50) and 60 days (10 mm on Day 60); one group received empty capsules on Day 26 at the time of castration. The capsules consisted of Silastic tubing (Dow Corning, Midland, MI, U.S.A.; Cat. No. 602-285) filled with crystalline testosterone (Sigma, München, F.R.G.), cut into lengths of 5 or 10 mm and sealed at either end with silicon glue (Dow Corning). They were soaked in 0.9% (w/v) saline for 1 day before subcutaneous implantation. The rats bore the capsules until they were challenged with 5 mg morphine/kg (s.c.) at 66 days of age. Trunk blood was collected, after decapitation, 30 min after the morphine injection, and the serum was assayed for LH.

Experiment 3. Since the results from Exp. 2 might have been confounded by the fact that the testosterone implants remained *in situ* during the tests with morphine, rats that had been castrated on Day 26 of age were given testosterone implants (10 mm) at 45 days of age, and carried them until they were 57 days old. On Day 66 of age (see Exp. 2), the LH responses were tested as described above.

Hormone and data analyses. LH and prolactin were measured using reagents provided by the National Institutes of Health Pituitary Agency, Baltimore, MD, U.S.A. The reference preparations used were RP-2 and RP-3 for LH and prolactin respectively. The LH assay had a sensitivity of 0.1 ng/ml and the prolactin assay a sensitivity of 0.05 ng/ml. Intra- and inter-assay coefficients of variation were <10% and <15%, respectively, for both hormones. The data were analysed for significant differences by Student's *t* test after analysis of variance.

Results

Experiment 1

LH. Serum concentrations of LH were significantly suppressed by morphine for up to 12 days after castration in rats that were gonadectomized at 26 days of age. However, when these rats were tested with morphine between 19 and 33 days after castration (age 45–59 days), LH values were not significantly reduced. The inhibitory effects of the opiate upon LH secretion were once again observed beyond 40 days after castration. The results are shown in Fig. 1(a) and are summarized in Fig. 2.

Rats that were castrated at 59 days of age, after puberty, responded with a 70–80% decrease in serum LH concentrations for up to 5 days after castration when challenged with morphine. However, after 12 days of castration, opiate administration resulted in only a 20% inhibition of LH secretion and by 28 days the inhibition was <10% as shown in Fig. 1(b) and Fig. 2.

Fig. 1. Serum LH concentrations in male rats at various ages after castration on (a) Day 26 and (b) Day 59 of age and after morphine treatment. Values are mean $\pm$ s.e.m. for 6–8 rats/group.

Fig. 2. Degree of morphine inhibition of serum LH concentrations in male rats castrated before (26 days) or after (59 days) puberty. Mean values from 6–8 rats per group are shown.

Prolactin. Morphine significantly elevated serum prolactin concentrations in the pre-pubertally castrated rats at all times tested; the increases paralleled the age-related increases in prolactin concentrations found in saline-treated animals aged 28–54 days (2–28 days after castration), as depicted in Fig. 3.

As in the prepubertally castrated rats, morphine significantly increased the blood concentration of prolactin of the rats castrated as adults (59 days), irrespective of the time elapsed since gonadectomy (Fig. 3). The elevated concentrations of prolactin found 2 days after castration most probably reflect post-surgical stress.

Experiments 2 and 3: testosterone replacement in prepubertally castrated males

Prepubertally castrated rats that were given testosterone implants at the time of castration (age 26 days) or up to 24 days after castration (age 50 days) did not have reduced serum LH values after

Fig. 3. Morphine-induced increases in serum prolactin concentrations of male rats castrated (a) before (26 days) or (b) after (59 days) of puberty. Values are mean $\pm$ s.e.m. for 6–8 rats/group.

Fig. 4. Serum LH concentrations after saline or morphine treatment of rats castrated on Day 26 of age and either given empty Silastic capsules (histogram 1) or testosterone-filled capsules at the time of castration (histogram 2) or at various intervals thereafter (histograms 3–6). Capsules remained *in situ* until the time of morphine testing on Day 66 of age, except for the rats represented in histogram 6. Values are mean $\pm$ s.e.m. for 8–12 rats/group. Asterisks indicate significant differences between saline- and morphine-treated groups ($P < 0.05$).

morphine given at 5 mg/kg on Day 66 of age (Fig. 4). In contrast, castrates that received testosterone implants 34 days after castration (age 60 days) showed significant reductions in serum LH values when challenged with morphine on Day 66 (Fig. 4). This result resembled that seen in prepubertally castrated rats that were not treated with testosterone (Fig. 4). When testosterone was implanted at 45 days of age and the implants were removed on Day 57, serum LH concentrations were not significantly suppressed by morphine (Fig. 4).

Discussion

In common with other investigators (Cicero *et al.*, 1982; Bhanot & Wilkinson, 1984), we found that castration of adult male rats leads to a significant reduction of the inhibitory effects of opiates, such as

morphine, upon LH secretion. Subsensitivity to opiates was also found to develop in rats castrated when 28 days old, for up to 33 days (when they were 59 days old, the age at which puberty is complete in intact rats: Swerdloff *et al.*, 1971). Bhanot & Wilkinson (1984), studying males castrated as juveniles (24 days of age), also found this decline in responsiveness to opiates, up to the age of 45 days. The results obtained in the present study are an important extension to Bhanot & Wilkinson's (1984) findings: they show that there is a partial reinstatement of LH susceptibility to opiate suppression beyond Day 60 of age in prepubertally castrated males, i.e. the reduction of response is only a transient event.

The state of opiate subsensitivity that we observed was not generalized to the whole neuro-endocrine system since rats that were castrated as juveniles or adults showed normal prolactin responses to morphine. Bhanot & Wilkinson (1984) made similar observations.

The loss of LH responsiveness to opiates could be taken to reflect changes in opioidergic transmission (e.g. loss of receptors, uncoupling of receptor binding sites from the subsequent transducing system or changes in release rates of endogenous opioids). Several groups have attempted to measure changes in opiate receptor density after gonadectomy. Contrary to what might be expected, Hahn & Fishman (1979, 1985) reported an increase in whole brain opiate binding sites; however, others have failed to detect any significant changes (Wilkinson *et al.*, 1981; Cicero *et al.*, 1982; Diez & Roberts, 1982). Nevertheless, since the adaptation seems to be selective for the LHRH system, better methods for receptor estimation in discrete brain regions may need to be applied to resolve this issue.

If the loss of responsiveness to morphine resulted from an adaptation to increased levels of endogenous opioid peptide secretion, a heightened response to the opiate antagonist naloxone would be expected (see Almeida *et al.*, 1986, for discussion). However, many in-vivo studies have shown that the LH-stimulating effects of naloxone are diminished in the absence of gonadal steroids, indicating a reduced opioidergic tone (e.g. Bhanot & Wilkinson, 1984; Petraglia *et al.*, 1984; Leadem & Kalra, 1985). Furthermore, in in-vitro experiments, naloxone cannot induce LHRH release from hypothalami taken from male rats castrated as adults (Nikolarakis *et al.*, 1986). An increased opioidergic tone therefore does not satisfactorily account for the reduced responsiveness to opiate agonists in castrates. Nevertheless, it has been found that naloxone evokes a greater LH response in pubertal and adult males than in prepubertal males. This suggests an increase in the endogenous opioidergic tone as sexual maturation proceeds (Blank *et al.*, 1979; Ieiri *et al.*, 1979; Schulz *et al.*, 1982), and the finding that the opiate agonist FK33-824 becomes less potent at inhibiting LH as rats develop into adults (Bhanot & Wilkinson, 1983) may be partly explained in terms of increased competition for binding sites by endogenous ligands.

The exact mechanism by which castration induces subsensitivity to opiates is not clear. The experiments reported here, however, indicate that testosterone plays a major role in determining the opiate responsiveness of adult male rats. Male rats that are deprived of testosterone during the time when the neural mechanisms governing LH release are maturing (Days 45–57 of age) display only a transient subsensitivity to opiates; sensitivity is partly restored once the normal age of sexual maturation has passed. On the other hand, males that are exposed to the androgen during this period of development remain unresponsive to the LH-suppressing effects of opiates. The actions of testosterone during this short 'window of time' around the peri-pubertal period are probably distinct from those seen at other times. For example, in contrast to what is reported here for testosterone replacement after the 60th day of age, testosterone can eventually restore opiate responsiveness after long-term post-pubertal castration (Cicero *et al.*, 1980; Nikolarakis *et al.*, 1986).

Gonadal steroids have two influences upon central nervous system function, namely 'organizational' and 'activational' (MacClusky & Naftolin, 1981; Toran-Allerand, 1984; Södersten, 1984). The former effects are structural, involving the organization of neural circuits, whereas the latter effects relate to patterns of neural responses to given stimuli which may be altered or lost in the absence of the relevant hormone. In the rat, the organizing influence of steroid hormones is believed to occur during the late prenatal to neonatal (Day 5) period, whereas their activating influence is exerted throughout life (MacClusky & Naftolin, 1981). It is therefore not surprising that post-

pubertal castration only results in the loss of the activating functions which can be restored upon steroid replacement.

According to the above view, castration after 5 days of age would also be expected to produce only a loss of steroid-activating effects, but the results are more complicated. Up to about the age at which puberty would normally have occurred, the absence of the gonads seems to have rendered our rats subsensitive to morphine. Our finding, that testosterone replacement for just a few critical days in the prepubertal castrate produces an adult-like response later in life, could be advanced as an argument that the steroid plays an organizational, rather than activational, role. Although the data of Bhanot & Wilkinson (1983) may at first seem to counter this argument, it should be noted that the age-related declines in opiate responsiveness reported by them were not dependent upon a particular steroid (it occurred in males and females alike). Secondly, they observed subsensitivity 48 h after gonadectomy, but it seems that subsensitivity due to 'inactivation' takes up to 1 week to develop, at least in adult male rats (Cicero *et al.*, 1980; Nikolarakis *et al.*, 1986).

In summary, our experiments demonstrate a critical role for testosterone in determining the opiate control of LH release in the adult male rat. Since prepubertally castrated males resemble female rats which continue to respond to opiate agonists several weeks after ovariectomy (Pfeiffer *et al.*, 1985), we infer that androgens released during puberty in the male rat provide a degree of 'maleness' to the gonadotrophin-releasing centre of the brain. In addition, although we cannot provide supporting morphological evidence, our data indicate that a further period during which testosterone can induce 'organizational' changes in the LHRH-LH releasing system occurs at puberty. A study of the motor neurones involved in male copulatory functions has revealed that synaptic reorganization can be induced by androgen deprivation and replacement well after puberty (Kurz *et al.*, 1986). These pieces of evidence make it necessary to review present ideas on the time when steroids lose their influence over motor neurone, behavioural and neuroendocrine functions.

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