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# Studies of the effects of subacute treatment with N-(cyclopropylmethyl)-19-isopentylnororvinol (M320) on timing of parturition in the rat

[1]R.G. Evans, *G.E. Rice, & J.E. Olley

Departments of Pharmacology and *Physiology, Monash University, Clayton, Victoria, Australia 3168

1 Administration of $10\,\mu g\,kg^{-1}$ of the long lasting potent $\kappa$- and weaker $\mu$-opioid agonist N-(cyclopropylmethyl)-19-isopentylnororvinol (M320) twice daily from day 20 of gestation prolonged the internal gestation period of the rat and retarded the development of the offspring in the perinatal period.

2 The capacities of myometrial, placental and cervical tissues to produce prostaglandin $E_2$ ($PGE_2$) were not affected by M320 treatment.

3 During the period in which parturition normally occurred in saline-treated rats, foetal pituitary levels of immunoreactive oxytocin (ir-OXY) but not immunoreactive arginine-vasopressin (ir-AVP) were greater in M320- compared to saline-treated animals. Following the completion of parturition, foetal pituitary ir-OXY and ir-AVP levels continued to rise in saline-treated rats, but fell dramatically in rats treated subacutely with M320.

4 These data indicate that subacute treatment with M320 may inhibit foetal oxytocin release at term. This foetal OXY release may be a stimulus for the initiation of labour.

## Introduction

Induction of labour results from a complex cascade of biochemical events thought to be initiated by the foetus, at a time when it is sufficiently mature to survive extra-uterine life (for review see, Rice *et al.*, 1987a). In most species, this process involves: (i) generation of one or more signals from the foetus; (ii) translation of these signals into biochemical events which affect biosynthesis and release of steroids (e.g. progesterone and oestrogen), peptides (e.g. oxytocin) and prostaglandins (e.g. prostaglandin $E_2$, $PGE_2$); (iii) increased sensitivity of the myometrium to stimulatory agonists and enhanced contractile activity; and (iv) changes in cervical morphology. Factors which perturb one or more of the steps in this cascade of events may affect timing of the onset of labour in rats; for example, prostaglandin synthase inhibitors (Chester *et al.*, 1972; Strauss *et al.*, 1975) and oxytocin antisera (Schriefer *et al.*, 1980; 1982).

Subacute administration of $\mu$- and $\kappa$-opioid agonists to pregnant rats causes prolonged internal gestation (Evans *et al.*, 1987a). The mechanism underlying this effect of opioids, however, remains equivocal. We therefore examined the effects of the long-acting $\kappa$- and $\mu$-opiate receptor agonist, N-(cyclopropylmethyl)-19-isopentylnororvinol (M320) (Boura & Fitzgerald, 1966; Abrahams *et al.*, 1986) on aspects of peptide and prostaglandin synthesis and/or release in parturient rats. Specifically, we assessed the effects of M320 on: (i) the capacity of uterine (placental and myometrial) and cervical tissues to synthesize $PGE_2$ *in vitro*, and (ii) foetal oxytocin (OXY) and arginine-vasopressin (AVP) release, as assessed from foetal and neonatal pituitary immunoreactive (ir)-OXY and ir-AVP content during the perinatal period. A preliminary account of some of these findings was presented to the Tenth Congress of The International Union of Pharmacology (Evans *et al.*, 1987b).

## Methods

### *Animals*

Virgin female Long Evans rats (180–250 g) were housed in groups of 5 with free access to food (Clark King GR2) and water. Rooms were illuminated from 06 h 00 min to 18 h 00 min and maintained at 20°C.

[1] Author for correspondence at present address: Baker Medical Research Institute, P.O. Box 348, Prahran, Victoria, Australia, 3181.

Pregnant rats were obtained by placing them, individually, with an experienced male breeder in cages with wire grid floors. The day that one or more vaginal plugs were found in the bottom of the cage in the morning was designated as day zero of gestation. In all studies, treatment consisted of subcutaneous administration of either saline or 10 $\mu$g kg$^{-1}$ of M320 at 09 h 00 min and 17 h 00 min each day from day 20 gestation. Treatment continued until parturition commenced. Animals were observed continuously during the lighted period, so that timing of the start of parturition could be accurately assessed. Animals were divided into 4 categories according to their reproductive state. They were categorized as being either pregnant/non-parturient, pre-parturient, parturient or post-parturient. Pregnant/non-parturient rats were defined as those pregnant animals displaying no signs of impending birth. Pre-parturient rats were defined as those animals having dilated or bloody vaginal openings but which had not yet given birth. Parturient dams were those which had given birth to one or more but not all of their litter, while post-parturient dams were defined as those which had expelled their final pup within the last hour.

*The capacities of myometrial, placental and cervical tissues to synthesize $PGE_2$: effects of subacute administration of M320*

The capacities of myometrial, placental and cervical tissues to synthesize $PGE_2$ were assessed using a method similar to that described by Jeremy & Dandona (1986). Parturient animals were decapitated and the uterine horns, placentae and cervix were removed and placed in cold (4°C) sterile saline. The uterus and cervix were opened by longitudinal incision after removal of the adhering vasculature and connective tissue. The endometrium was removed by scraping with two passes of a glass slide. The uterine horns were cut longitudinally into two strips and then laterally into 2 mm segments. Placentae and the cervix were cut into 2 × 2 mm explants. Individual tissues were placed in 50 ml Medium 199 (M199) (Gibco Laboratories), pregassed with 95% $O_2$: 5% $CO_2$ and containing 100 units ml$^{-1}$ penicillin and 100 $\mu$g ml$^{-1}$ streptomycin. After incubation at 37°C for 1 h in a shaking water bath (20 orbits min$^{-1}$) under an atmosphere of 95% $O_2$:5% $CO_2$, the tissues were then placed in sterile multi-well culture trays (Linbro, Flow Laboratories Inc.; 50 mg wet weight per well) and incubated in 3 ml M199 for 1 h (control) and subsequently in M199 containing 10 $\mu$mol l$^{-1}$ arachidonic acid (AA-stimulated) for 1 h. The medium from each incubation period was collected and processed for $PGE_2$ assay.

*Foetal and neonatal pituitary levels of oxytocin and arginine-vasopressin: effects of subacute administration of M320*

Foetuses ($n = 5$ per dam) were removed and decapitated, and their pituitary glands excised, after laparotomy of pregnant/non-parturient dams anaesthetized with pentobarbitone sodium (100 mg kg$^{-1}$ i.p.) at 10 h 30 min on days 20, 21 and 22 of gestation, and of anaesthetized pre-parturient animals. Neonatal pituitary glands ($n = 4$–5 per litter) were collected either within 1 h of the completion of parturition (post-parturient) or at 16 h 00 min on day 23 of gestation. Because of the small size of foetal and neonatal pituitary glands, a small portion of the skull, immediately underneath them was included in the dissection in order to ensure that the entire gland was collected in the sample. Samples were stored in 0.5 ml of 0.1 M HCl at −20°C until extraction and assay.

*Radioimmunoassays*

Prostaglandin $E_2$ was assayed according to the method described by Rice *et al.* (1987b). The OXY and AVP content of pituitary samples were assayed according to methods previously described (Rice & Thorburn, 1985; Woods & Johnston, 1983) excepting that samples were extracted by use of Sep Pak cartridges (C18, Waters Associates, MA, U.S.A.). In addition, for OXY radioimmunoassay, antibody bound [$^{125}$I]-OXY was separated from free [$^{125}$I]-OXY by addition of 2 ml of cold (4°C) ethanol rather than anti-rabbit serum. Similar standard curves were, however, obtained with both methods.

OXY and AVP were extracted from the tissue samples by a modification of the method of Walsh & Niall (1980). The tissue samples, in 0.5 ml of 0.1 M HCl, were placed in 10 ml polythene tubes containing trifluoroacetic acid/formic acid/NaCl/1.0 M HCl (15:5:1:80 vol/vol/w/vol) at 0°C. The samples were then homogenized for 30 s with a Teflon pestle before being centrifuged at 18,000 r.p.m. for 20 min at 4°C (Sorval SS-3).

The Sep Pak cartridges were primed by washing sequentially with 3 ml acetonitrile/trifluoroacetic acid/water (80:0.1:19.9 vol/vol/vol) and 10 ml water/trifluoroacetic acid (99.9:0.1 vol/vol). The supernatant of each homogenized tissue sample was mixed with 3 ml of 0.1 M phosphate buffer, pH 7.4, and loaded during 1 min on to individual Sep Pak cartridges.

After washing the cartridges with 10 ml water/trifluoroacetic acid, the peptides were eluted with 3 ml acetonitrile/trifluoroacetic acid/water and dried at 37°C under compressed air. Dried samples were stored at −20°C until assay. The recoveries of syn-

thetic OXY and AVP were 84 ± 5% (d.f. = 12) and 95 ± 12% (d.f. = 16) respectively, being linear over the range from 6–192 pg per sample.

Pituitary ir-OXY and ir-AVP levels were not corrected for recovery. The curves for OXY and AVP constructed from the assay of serial dilutions of pituitary glands were parallel to the standard curves for the assays, indicating that extraction procedure did not modify the nature of the OXY and AVP immunoreactivity in the samples.

### *Materials*

The following drugs were used: N-(cyclopropylmethyl)-19-isopentylnororvinol HCl (M320), (Reckitt and Colman); sodium pentobarbitone (Ceva Chemicals, Hornsby, Aust.), streptomycin, (Gibco Laboratories, Ohio, U.S.A.); penicillin (Flow Laboratories Inc., Virginia, U.S.A.); and arachidonic acid, (PL Biochemicals, Pharmacia, Uppsala, Sweden). Sterile, multi-well culture trays (Linbro) were obtained from Flow Laboratories Inc. (Virginia, U.S.A.) while M199 was obtained from Gibco Laboratories (Ohio, U.S.A.).

For *in vivo* studies, M320 was calculated as the base, dissolved in 0.9% w/v NaCl and injected subcutaneously in a volume of $1\,ml\,kg^{-1}$. Pentobarbitone sodium was obtained as Nembutal ($60\,mg\,ml^{-1}$) and injected intraperitoneally in a volume of $1.67\,ml\,kg^{-1}$.

### *Statistics*

All results are expressed as the mean ± s.e. mean. Statistical significance ($P < 0.05$) was assessed by either Student's unpaired $t$ test or two way Analysis of Variance. Analysis of radioimmunoassays employed a computer programme for the fitting of a standard curve and the subsequent interpolation of the immunoreactivity of unknown samples.

## Results

### *Internal gestation period and neonatal weight: effects of subacute administration of M320*

The mean internal gestation period of the saline-treated animals was 22.00 ± 0.09 days ($n = 21$). Administration of M320 ($10\,\mu g\,kg^{-1}$ s.c.), twice daily from day 20 of gestation, was accompanied by a significantly prolonged internal gestation period of 22.97 ± 0.11 days ($n = 26$) (Student's unpaired $t$ test), together with retardation in the development in the rat pups. At birth, pups treated *in-utero* with saline weighed 5.63 ± 0.12 g ($n = 25$). Those treated with M320 did not weigh more (5.84 ± 0.06 g, $n = 25$) even though they were older. Furthermore, by 16 h 00 min on day 23 of gestation, the mean weight of the saline-treated neonates (6.44 ± 0.10 g, $n = 20$) was significantly greater than the M320-treated neonates (5.79 ± 0.08 g, $n = 16$) (Student's unpaired $t$ test).

### *The capacities of myometrial, placental and cervical tissues to synthesize $PGE_2$: effects of subacute administration of M320*

Synthesis of ir-$PGE_2$ by myometrium during a 240 min incubation in M199 is shown in Figure 1. Myometrial ir-$PGE_2$ synthesis was maximal immediately following dissection. A stable rate of synthesis, however, was achieved after 45 min of incubation. In all subsequent incubations tissues were, therefore, pre-incubated for 60 min before the rate of prostaglandin synthesis was assessed.

The inclusion of $10\,\mu mol\,l^{-1}$ AA in the incubation medium stimulated ir-$PGE_2$ synthesis, by an average of 300%, in all tissues from both saline- and M320-treated animals. The effects of M320 on immunoreactive $PGE_2$ (ir-$PGE_2$) synthesis by myometrium, placenta and cervix obtained from parturient rats are presented in Table 1. Although M320 treatment significantly delayed onset of parturition, neither the basal (control) nor AA-stimulated synthesis of ir-$PGE_2$ was reduced (Student's unpaired $t$ test; two way analysis of variance).

### *Foetal and neonatal pituitary levels of OXY and AVP: effects of subacute administration of M320*

At 10 h 30 min on day 20 of gestation, the foetal pituitary levels of ir-OXY (0.079 ± 0.021 ng per gland, $n = 20$) were approximately 25 times less than the levels of ir-AVP (1.96 ± 0.26 ng per gland, $n = 20$;

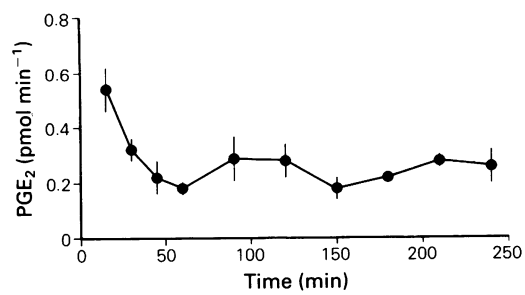


**Figure 1** Synthesis of immunoreactive prostaglandin $E_2$ (ir-$PGE_2$) in the absence of arachidonic acid by myometrium following tissue dissection and incubation at 37°C. Each point is the mean of three observations; vertical lines represent s.e. mean.

**Table 1** The capacities of myometrial, cervical and placental tissues to produce prostaglandin $E_2$ ($PGE_2$): comparison of tissues from parturient rats pretreated subacutely with M320 or saline

| | | $PGE_2$ (fmol mg$^{-1}$ h$^{-1}$) | | |
|---|---|---|---|---|
| *Pretreatment* | *AA* (μM) | *Myometrium* | *Cervix* | *Placenta* |
| Saline | 0 | 85 ± 20 (5) | 24 ± 5 (3) | 19 ± 4 (3) |
| M320 | 0 | 126 ± 21 (7) | 75 ± 30 (5) | 33 ± 7 (3) |
| Saline | 10 | 225 ± 6 (3) | 108 ± 10 (3) | 64 ± 10 (3) |
| M320 | 10 | 278 ± 32 (3) | 142 ± 73 (3) | 53 ± 5 (3) |

Tissue production of immunoreactive-prostaglandin $E_2$ (ir-$PGE_2$) in the absence and presence of archidonic acid (AA) is expressed as mean ± s.e.mean.

Figure 2) Pituitary levels of ir-OXY and ir-AVP in foetuses of saline-treated rats rose during late gestation and continued to rise after birth. In contrast, the pituitary ir-OXY and ir-AVP levels of M320-treated neonates fell dramatically after birth. During the period before birth, the rate of increase in foetal pituitary levels of ir-AVP was similar in saline- and M320-treated animals. M320-treatment did not affect the pre-partum increase in foetal pituitary ir-OXY until days 21 and 22 of gestation, that is, the period in which parturition normally occurs in saline-treated rats. At this stage, foetal ir-OXY was greater in M320- compared with saline-treated animals (Student's unpaired $t$ test).

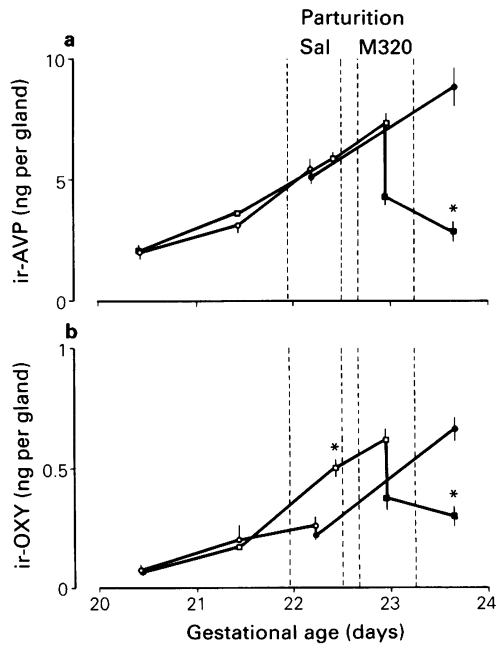


**Figure 2** The effects of subacute treatment *in-utero* with saline (○, ●) and M320 (□, ■) on foetal (open symbols) and neonatal (closed symbols) pituitary levels of immunoreactive arginine-vasopressin (ir-AVP, a) and immunoreactive-oxytocin (ir-OXY, b). Each point is the mean of 19–25 measurements; a vertical line represents s.e. mean. M320-treated animals significantly (*$P < 0.05$, Student's unpaired $t$ test) different from those treated with saline. Also shown (between broken parallel lines) are the 95% confidence intervals for the mean internal gestation period of saline- ($n = 9$) and M320- ($n = 10$) treated dams from whose litters samples were taken either at the initiation of, or within 1 h of, completion of parturition.

## Discussion

M320 is a potent and long acting κ- and μ-opiate receptor agonist (Abrahams *et al.*, 1986). Previous studies have shown that subacute administration of M320 during late gestation prolongs duration of the internal gestation period of the rat (Evans *et al.*, 1987a). Repeated administration of this compound, therefore, appears to disrupt the physiological processes associated with the onset of parturition. The present study has confirmed the earlier findings of Evans *et al.* (1987a).

The physiological processes involved in the onset of parturition in the rat are complex and poorly understood. A number of factors of importance can, however, be identified. The role of prostaglandins in the initiation of parturition is well known (Chester *et al.*, 1972; Aiken, 1972; Strauss *et al.*, 1975; Kuriyama & Suzuki, 1976; Elger, 1979; Alexandrova & Soloff, 1980; Hurst & Peplow, 1986; Chan, 1987). The effects of M320-treatment on the capacities of uterine, placental and cervical tissues to synthesize $PGE_2$ were therefore studied.

Initial studies demonstrated that ir-$PGE_2$ production was maximal immediately after dissection. A stable rate of basal $PGE_2$ production was, however, attained after 45 min and continued for at least another 195 min. Ir-$PGE_2$ production was assessed both in the absence and presence of AA (10 μmol l$^{-1}$). Ir-$PGE_2$ production in the absence of exogenous AA indicates basal synthesis and the availability of endogenous AA. Ir-$PGE_2$ synthesis in

the presence of exogenous AA is indicative of stimulated prostaglandin synthesis and, therefore, an indicator of prostaglandin synthase activity. Treatment with M320 did not reduce the abilities of uterine, placental or cervical tissue to produce ir-$PGE_2$, nor did it reduce the prostaglandin synthase activities of these tissues. These data indicate that the ability of subacute M320 treatment to delay parturition is not due to inhibition of $PGE_2$ production by the uterus, placenta or cervix.

Considerable evidence supports the hypothesis that OXY of foetal but not maternal origin is involved in the initiation of parturition in the rat (Fuchs & Poblete, 1970; Kumaresan *et al.*, 1971; Schriefer *et al.*, 1980; 1982; Boer *et al.*, 1980). Schriefer *et al.* (1982) demonstrated that the administration of antibodies raised against OXY to foetuses delayed the onset of parturition in rats. No effect was observed after its administration to dams. Opioids inhibit OXY release in mature rats (Clarke *et al.*, 1979), indicating that inhibition of foetal OXY release could contribute to prolonged internal gestation period after M320-treatment. The effects of subacute administration of M320 on foetal and neonatal pituitary ir-OXY and ir-AVP content during the perinatal period were therefore investigated. Changes in tissue hormone levels, while reflecting the balance of synthesis, degradation and release, provide an estimate of comparative hormone release.

The levels of ir-OXY and ir-AVP in the pituitary glands of saline-treated animals continued to rise after birth whereas the levels in M320-treated animals dropped dramatically after parturition. It is conceivable that this phenomenon could result from release of OXY and AVP as part of a generalized withdrawal syndrome in the M320-treated neonate. Massive release of OXY is certainly a characteristic of precipitated withdrawal in morphine-dependent mature rats (Russell, 1984). Alternatively this profound depletion of neurohypophysial peptide stores could reflect a toxic action of subacute administration of M320, due both to its powerful central depressant activity (Boura & Fitzgerald, 1966) and its disruptive effect on maternal behaviour (Evans *et al.*, unpublished results).

The other effect that was observed was more subtle but of far greater importance in regard to the possible mechanism(s) of action of subacute M320-treatment in delaying the onset of parturition. During the period immediately before delivery of the saline-treated pups (on days 21 and 22 of gestation) foetal pituitary ir-OXY but not ir-AVP was significantly greater in animals treated with M320 compared with the saline-treated controls. These data provide further evidence that foetal OXY is released prior to parturition in the rat and indicate that this release is opioid-sensitive. Mechanisms for opioid inhibition of neurohypophysial hormone release may, therefore, be present very early in the development of the animal. These data may also suggest a role for the foetal neurohypophysis in the effect of M320 on the timing of parturition in rats, since other studies have demonstrated that OXY of foetal but not maternal origin is a major factor in the timing of parturition in the rat (Boer *et al.*, 1980; Schriefer *et al.*, 1982). Furthermore, subacute administration of M320 is not associated with reduced maternal OXY release at term (Evans *et al.*, 1987a; Evans *et al.*, unpublished results), indicating that maternal OXY release at term (Evans *et al.*, 1987a), indicating that maternal OXY does not play a role in the effects of M320 on the timing of parturition. On the other hand, the present study has not provided any direct evidence linking the effect of M320-treatment on foetal pituitary ir-OXY levels with its effect on internal gestation period, and the possibility that other factors are involved cannot be discounted. Specifically, retardation of development in the offspring of M320-treated dams in the perinatal period and the powerful central depressant activity of this opioid (Boura & Fitzgerald, 1966) may be involved.

It is also possible, however, that M320-treatment is followed by increased synthesis rather than reduced release of foetal neurohypophysial OXY but not AVP. This hypothesis requires further investigation, both by measurement of neurohypophysial hormone levels in foetal blood and analysis of oxytocin-messenger-RNA activity around the time of parturition, possibly by the use of the technique of hybridization histochemistry (Clements *et al.*, 1985).

The present study has confirmed the observation of Evans *et al.* (1987a) that subacute administration of the potent and long-lasting opioid M320 prolongs the internal gestation period of rats. This effect does not appear to involve reduced ability of uterine, placental or cervical tissue to produce $PGE_2$ but could possibly be mediated via inhibition of a foetal signal for the initiation of parturition; the release of OXY.

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