The effect of decrease in body temperature with Nembutal on meiosis and ovulation after induction by gonadotrophin-releasing hormone and luteinizing hormone in adult rats

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Summary. Sodium pentobarbital (Nembutal) is often used to block the pro-oestrous luteinizing hormone (LH) surge in rats. Nembutal is also known to lower body temperature. This study was designed to investigate whether Nembutal affected the time course of meiosis and timing of ovulation induced by exogenous hormones, and whether the possible effects of Nembutal on these processes were related to temperature. Gonadotrophin-releasing hormone (GnRH), the GnRH-analogue Ovalyse, or rat luteinizing hormone (LH) were administered to trigger resumption of meiosis and ovulation; Nembutal (35 mg kg⁻¹ body weight) or saline was given 10 or 60 min later. Plasma profiles of LH were measured and Graafian follicles were studied histologically for meiotic progress and ovulation. Nembutal suppressed the spontaneous surge of LH at pro-oestrus and caused a long-lasting decrease in body temperature. If 1000 ng GnRH was given 2 h before the pro-oestrous LH surge, most of the oocytes had extruded a polar body 10 h later and most follicles had ovulated 14 h later. Nembutal given 1 h after GnRH delayed extrusion of the polar body and ovulation by about 2 h. Nembutal caused a similar delay in ovulation when it was administered after 100 ng of Ovalyse, and it also delayed meiosis when given after 1000 ng of LH. This effect of Nembutal was prevented if body temperature was maintained at 37°C. The delaying effect of Nembutal on meiosis and ovulation induced by exogenous GnRH or LH is related to a long-lasting decrease in body temperature.

Keywords: Nembutal; temperature; meiosis; ovulation; rat

Introduction

Resumption of meiosis and ovulation is triggered by a surge of luteinizing hormone (LH) on the day of pro-oestrus in mammals. The LH surge has a diurnal rhythm (Everett & Sawyer, 1950) and begins about 2 h after the middle of the light period in rats (Everett *et al.*, 1949). Ovulation occurs about 14 h after the onset of the spontaneous LH surge (Sridaran *et al.*, 1979). Sodium pentobarbital (Nembutal) administered before the presumed surge of LH on pro-oestrus suppresses this LH secretion (Everett & Sawyer, 1950; Daane & Parlow, 1971) and postpones ovulation by 1 day (Everett & Sawyer, 1950; van der Schoot, 1980). Nembutal-treated pro-oestrous rats may therefore be used as a model to study the temporal aspects of meiosis, ovulation and luteinization. However, the dose of Nembutal used to block spontaneous ovulation causes a considerable and long-lasting reduction of body temperature (Janocko & Mycek, 1986). It is possible that decreased body temperature retards various biochemical processes; thus meiotic progress and ovulation induced by exogenous gonadotrophins may be retarded in rats anaesthetized with Nembutal. This study investigated how far Nembutal interferes with progression of meiosis and ovulation induced by

exogenous LH or gonadotrophin-releasing hormone (GnRH). We also examined whether temporal aspects of meiosis and ovulation in rats anaesthetized with Nembutal remained normal when a decrease in body temperature of the rats was prevented.

Materials and Methods

Animals and treatments

The trials used virgin female F_1 hybrid rats of two Wistar substrains (U-inbred males and R-inbred females), aged 3–5 months and weighing 220–240 g. Rats were housed individually under controlled conditions of temperature ($21 \pm 1^\circ\text{C}$) and photoperiod (14 h light:10 h dark; lights on at 02:00 h). Cyclicity of oestrus was monitored daily by vaginal lavage and observation of oestrous behaviour, for which a male was briefly allowed in each cage. Only rats with three consecutive 5 day cycles were used. The rats that were used for blood sampling were supplied with a permanent indwelling cannula of the jugular vein (Mattheij & van Pijkeren, 1977). Cyclicity of oestrus was monitored in these rats from the day after implantation of the cannula.

Body temperature was measured in pro-oestrous rats by a deep rectal thermometer, using a Keith Ley electrode type A thermometer. The thermometer was pushed gently into the rectum to a depth of 4 cm and kept in place for 1 min for each measurement. Because the rats were used to daily handling, the method was also used with conscious rats. Temperature was measured in nine untreated rats at intervals of one hour between 09:00 and 17:00 h, and in nine Nembutal-treated rats at intervals of 15 min. The ambient temperature at which a decrease in body temperature could be prevented was assessed by injecting groups of six rats with Nembutal and subsequently placing them in a climatic chamber at 31, 32, 33 or 34.5°C , respectively; body temperature was measured before and at intervals of 2 h for 8 h afterwards. The climatic chamber was equipped for biochemical work; temperature fluctuations around a set temperature were less than 0.5°C .

Materials

GnRH was purchased from Calbiochem (LHRF, Lot 286031). Ovalyse (des- $^{10}\text{GnRH}$ -ethylamide) was a gift from Upjohn, Ede, The Netherlands. The LH used was rat LH obtained from the NIDDK (r-LH-16; AFP-6833C) (Bethesda, MD). The hormones were dissolved in saline containing 1% bovine serum albumin (Sigma RIA-grade). Hormone injections were given intravenously (i.v.). Nembutal (sodium pentobarbital) was obtained from Sanofi, Maassluis, The Netherlands. In all experiments, Nembutal was given intraperitoneally (i.p.) at a dose of 35 mg kg^{-1} body weight, in a volume of 0.12–0.14 ml. Controls received a similar amount of saline i.p.

Radioimmunoassay

Blood samples, 0.25 ml, were collected in heparinized tubes on ice. After centrifugation plasma samples of 0.1 ml were diluted with 2 volumes of phosphate-saline buffer (pH 7.5) and stored at -25°C . LH was measured by radioimmunoassay (RIA), using materials provided by NIDDK. r-LH-16 was labelled with ^{125}I ; anti-rLH-S10 was used as first antibody; rLH RP2 was used as LH standard. The second antibody was donkey-anti-rabbit (Sac-cel, Wellcome Reagents, Beckenham, England). The variation within and between assays in relative standard deviation were 7.4% and 10.9%, respectively. The detection level of the assay was 0.1 ng per tube at 90% B/B₀ level.

Histology

Ovaries were fixed in Bouin's solution and embedded in paraffin wax. Serial sections (10 μm) were stained with Harris' haematoxylin (Gurr, GB) and eosin, and mounted in DePeX (Gurr, GB). In both ovaries of each rat, healthy follicles exceeding 600 μm and number of ruptured follicles were counted. Ruptured follicles could easily be distinguished from corpora lutea, because the former were compact structures containing numerous small dark-staining nuclei. Large follicles with signs of atresia were excluded. For assessment of meiotic progress, metaphase I, anaphase and telophase were considered together as 'MAT' stage. Because the first polar body disappears rapidly (Tsafirri & Kraicer, 1972), we could divide metaphase-II oocytes into two categories: metaphase II with visible polar body, i.e. within 2 h of reaching telophase; and metaphase II without polar body, i.e. more than 2 h after extrusion.

Experiments

In Expt 1, the timing and magnitude of the pro-oestrous surge of LH were measured at intervals of one hour between 10:00 and 16:00 h ($n = 9$); the suppressive effect of Nembutal on this surge was verified ($n = 9$). In Expts

Table 1. Procedure for expts 2–4

	Time of hormone injection	S (saline) or N (Nembutal)	<i>n</i>	Temp. (°C)	Blood samples from	Histology, interval after hormone (h)
Experiment 2 (1000 ng GnRH)	10:00 h Pro.	S	7	21	10:00–16:00 h	—
		S	15	21	—	10, 12, 14
		N	7	21	10:00–16:00 h	—
		N	17	21	—	10, 12, 14
Experiment 3 (100 ng Ovalyse)	16:00 h di-3.	S	12	21	—	12, 14, 16
	10:00 h Pro.	S	9	21	10:00–14:00 h	—
		S	12	21	—	12, 14, 16
		N	9	21	10:00–14:00 h	—
		N	16	21	—	12, 14, 16, 18
		N	12	33	—	12, 14, 16
Experiment 4 (1000 ng LH)	00:00 h Pro.	S	9	21	—	10
		N	9	21	—	10
		N	9	33	—	10

Pro.: pro-oestrus; di-3.: day 3 of dioestrus; Temp.: temperature of rat room; *n*: number of rats tested; —: not performed; GnRH: gonadotrophin-releasing hormone; LH: luteinizing hormone.

2–4, we studied the effect of Nembutal on time course of meiosis and on time of ovulation induced by exogenous hormones. The experimental outline of these experiments is summarized (Table 1).

In Expt 2, GnRH, 1000 ng, was injected; this dose previously induced normal ovulation in 5-day cyclic rats when given on day 3 of dioestrus (Mattheij *et al.*, 1986). In Expt 3, 100 ng of Ovalyse, a potent LH-releasing GnRH analogue, was injected. In Expt 4, 1000 ng of rat LH was injected; in a pilot study, 500 ng of LH triggered meiotic resumption in less than 20% of the oocytes and 1000 ng induced meiosis in more than 60% of the oocytes. In Expts 2 and 3, Nembutal was given 60 min after GnRH or Ovalyse, whereas in Expt 4 Nembutal was given 10 min after LH. In Expt 2, blood was taken every 20 min during the first hour and later at intervals of one hour. In Expt 3, blood was taken every half hour. Total reduction of blood volume in any rat was less 20%; we therefore did not return the removed blood cells suspended in saline.

Statistical analysis

Plasma LH profiles were evaluated by split-plot analysis of variance (Gill & Hafs, 1971). Two-sample comparisons of paired and independent LH concentrations were made by Student's *t* test (Snedecor & Cochran, 1989). This test was also used to evaluate the temperature data. The histological data were analysed, using Mann–Whitney's U test (Snedecor & Cochran, 1989). Values of *P* < 0.05 were considered significant. All data were expressed as means with SEM.

Results

Effect of Nembutal on body temperature

After administration of Nembutal, body temperature decreased rapidly, reaching a nadir of (28.5 ± 0.5)°C between 2 and 3 h after injection of Nembutal (Fig. 1a). The Nembutal-induced fall of temperature was partly or completely prevented at higher ambient temperatures (Fig. 1b); at a room temperature of 33°C, the rats maintained normal body temperature. Ambient temperature was therefore kept at 33°C to maintain normal body temperature after Nembutal injection in parts of Expts 3 and 4.

Experiment 1

From 12:00 h onwards, a significant increase in LH concentration was observed (*P* < 0.05); Nembutal administered at 10:00 h completely prevented this surge (Fig. 2).

Fig. 1. Effect of Nembutal given at 09:00 h in pro-oestrus on body temperature. (a) (●) controls; (○) Nembutal-treated rats kept at a room temperature of $21 \pm 1^\circ\text{C}$. Both groups contained nine rats. (b) Effect of increased ambient temperature on body temperature changes after injection of Nembutal. After injection of Nembutal the rats were kept at an ambient temperature of (▲---▲) 34.5°C , (▲—▲) 33°C , (△---△) 32°C and (△—△) 31°C ; six rats were tested at each temperature. Data are means and SEM.

Experiment 2

On the day of pro-oestrus, 1000 ng of GnRH at 10:00 h caused a transient rise in plasma LH and did not interfere with the LH surge beginning at about 12:00 h (Fig. 2). Nembutal given 1 h after GnRH prevented the surge of LH. Nembutal delayed the meiotic process by about 2 h, and delayed ovulation (Table 2).

Experiment 3

Injection of 100 ng of Ovalyse at 10:00 h during pro-oestrus caused a significantly greater rise in LH concentration (Fig. 3) than did 1000 ng of GnRH ($P < 0.001$). Administration of Nembutal

Fig. 2. Plasma profiles of luteinizing hormone (LH) during pro-oestrus. (●) the LH surge ($n = 9$); (○) the suppressive effect of Nembutal given at 11:00 h on this surge ($n = 9$); (▲) the effect of 1000 ng gonadotrophin-releasing hormone (GnRH) given at 10:00 h in pro-oestrus on release of LH ($n = 7$) and (△) summarizes the effect of GnRH at 10:00 h followed by Nembutal at 11:00 h on release of LH ($n = 7$). Data are means and SEM.

Table 2. Effect of Nembutal on meiosis and ovulation, induced by 1000 ng gonadotrophin-releasing hormone (GnRH). GnRH was given at 10:00 h during pro-oestrus and followed by saline or Nembutal at 11:00 h

Interval between GnRH and section Number of rats	Saline			Nembutal		
	10 h	12 h	14 h	10 h	12 h	14 h
	5	5	5	6	6	6
Follicle stages:						
MAT	0	0	0	$7.9 \pm 2.2^*$	2.5 ± 1.6	0
Metaphase II plus PB	11.2 ± 0.6	1.6 ± 0.7	0.2 ± 0.2	5.2 ± 2.4	$8.0 \pm 1.8^*$	0.2 ± 0.2
Metaphase II, no PB	2.0 ± 0.5	8.8 ± 1.6	2.4 ± 0.5	0*	$2.8 \pm 0.9^*$	$11.8 \pm 0.5^*$
Ruptured follicles	0	1.8 ± 0.7	11.6 ± 0.7	0	0*	$1.0 \pm 0.4^*$

MAT comprises metaphase I, anaphase and telophase. PB: polar body; *significant effect compared to the same follicular stage of corresponding control group. Values given are mean $\pm$ SEM.

did not interfere with the ongoing Ovalyse-induced release of LH (Fig. 3). In the samples taken at 13:00–14:00 h in Ovalyse-Nembutal rats, LH concentration was lower than in the corresponding samples of the Ovalyse-saline group ($P < 0.01$), indicating that Nembutal suppressed the spontaneous LH surge. Ovulation rates after injection of Ovalyse on day 3 of dioestrus at 16:00 h and on pro-oestrus at 09:00 h were similar (Table 3). Nembutal had an ovulation-delaying effect of about 2 h at 21°C, but not when body temperature of the rats was kept at 37°C.

Experiment 4

During pro-oestrus 1000 ng of LH given at 00:00 h triggered meiotic resumption in 60–80% of the large follicles. At 10 h after LH, these oocytes had all reached metaphase II. Nembutal given

Fig. 3. Plasma profiles of luteinizing hormone (LH) (●) after injection of 100 ng Ovalyse at 10:00 h in pro-oestrus; (○) the effect of 100 ng Ovalyse at 10:00 h, followed by Nembutal at 11:00 h pro-oestrus. Both groups contained nine rats. Data are means and SEM.

Table 3. Ovulation rate expressed as ruptured follicles, induced by 100 ng of Ovalyse

Interval between Ovalyse and section	Saline		Nembutal	
	A	B	C	D
12 h	2.5 ± 0.6	2.3 ± 0.6	0	0
14 h	8.7 ± 1.8	9.2 ± 1.4	0.8 ± 0.5*	10.0 ± 0.4
16 h	12.6 ± 0.8	12.8 ± 0.9	9.3 ± 1.1	13.2 ± 0.6
18 h	ND	ND	11.0 ± 0.7	ND

Ovalyse given at (A) 16:00 h on day 3 of dioestrus followed by saline at 17:00 h; (B) 10:00 h pro-oestrus, followed by saline at 11:00 h; (C, D) 10:00 h during pro-oestrus followed by Nembutal at 11:00 h. (D) experiment was performed at 33°C ambient temperature. Groups of four rats were killed at 12, 14, 16 and 18 h, respectively, after injection of Ovalyse. Values given are mean ± SEM. ND: not determined. *Significant effect compared with value at 14 h of groups B and D.

10 min after LH retarded meiotic progress significantly at an ambient temperature of 21°C, but not at an ambient temperature of 33°C.

Discussion

These results corroborate the suggestion that Nembutal inhibits secretion of GnRH, but has no direct effect on release of LH (Blake & Sawyer, 1972; Fink *et al.*, 1976). Nembutal also retarded

meiotic progress. Vermeiden & Zeilmaker (1974) found that metaphase II was reached sooner after injection of pituitary extract in hypophysectomized rats than after injection of hCG in Nembutal-treated rats; this was attributed to different properties of the injected materials. An effect of Nembutal on the rate of meiosis may have been unnoticed, because in their study the interval between hCG and Nembutal varied.

We found that in unanaesthetized rats, most Graafian follicles had ovulated 14–16 h after administration of the stimulating factor; ovulation was retarded when Nembutal was given subsequent to the stimulation. Sridaran *et al.* (1979) did not report a delaying effect of phenobarbital on ovulation. However, their trials were at a room temperature 4°C higher, whereas the interval between LH and phenobarbital was at least 3 h. It is also conceivable that phenobarbital has less effect on body temperature than Nembutal.

Holsinger & Everett (1972) found that ovulation occurred in a restricted period of the night, regardless of the time of stimulation by gonadotrophin. This observation was not confirmed by Sridaran *et al.* (1979). In this study, ovulation was complete 14–16 h after injection of Ovalyse, regardless of whether ovulation was induced at 16:00 h on day 3 of dioestrus or at 10:00 h in pro-oestrus. Also oocyte maturation occurred at a similar rate, regardless of whether it was induced at 00:00 or 10:00 h in pro-oestrus; this confirms an observation by Vermeiden & Zeilmaker (1974).

Nembutal is known for its hypothermic effect. The Nembutal dose that is generally used to block GnRH release and that consequently prevents spontaneous ovulation causes a profound reduction of body temperature (Ishikawa & Suzuki, 1986; Simpkins & Katovich, 1987; Wixson *et al.*, 1987). Simpkins & Katovich (1987) kept their rats at an ambient temperature of 26°C after injection of Nembutal and found a decrease in body temperature to 30–32°C. We measured a drop to below 30°C, when the temperature of the room was 21°C; and the fall of body temperature could be prevented at increased ambient temperature. The data indicate that Nembutal impairs temperature regulation rather than changing the hypothalamic set-point temperature.

Nembutal has an activity like GABA (γ -amino-butyric acid) (Roberts, 1987). GnRH neuronal activity is inhibited by GABA (Weiner *et al.*, 1988). The anaesthetizing and temperature-lowering effects of Nembutal are assumed also to be caused by GABA-like effects on the central nervous system (Chwey *et al.*, 1987). Sites binding GABA are also found in granulosa cells (Schaeffer & Hsueh, 1982; Hsueh *et al.*, 1984). Local administration of GABA has been reported to affect ovarian blood flow (Erdö *et al.*, 1985). It is unlikely that the delaying effect of Nembutal on meiosis and ovulation are caused by GABA-like effects of the drug in the ovary, because this retardation was not observed when a decrease in body temperature was prevented. A decrease in body temperature may slow down biochemical reactions including processes involved in meiosis and ovulation. Since a delay of these processes was prevented by raising ambient temperature, we conclude that these delaying effects of Nembutal were mediated entirely by an effect of Nembutal on body temperature.

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