Suppression of oocyte release in rats by local administration of the noradrenergic neurotoxin DSP4

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The presence of noradrenergic neuronal innervation in the ovaries and cyclic alterations in ovarian noradrenaline suggest a role for such innervation in oocyte release. The current experiments evaluated the relationship between ovulation and alterations in ovarian concentrations of noradrenaline induced by unilateral, intrabursal administration of the specific noradrenergic neurotoxin DSP4. Intrabursal injections of DSP4 (0–10 μ moles per ovary) given at 19:00 h at pro-oestrus induced a prompt, dose-related reduction in ovarian noradrenaline on the injected and non-injected sides. Although this result suggests that injected material was reaching the contralateral ovary, ovulation was suppressed only on the injected side. This suppression was persistent, and lasted through at least the next two cycles following either unilateral or bilateral treatment. The reductions in noradrenaline could be mostly, if not entirely, attenuated by prior administration of desipramine which blocks re-uptake of noradrenaline, while the ipsilateral ovulatory effects remained unchanged. Although it has been reported that DSP4 binds the opiate receptor, intrabursal co-administration of the antagonist naloxone was ineffective in altering ovulatory suppression. These results suggest that while decreases in ovarian noradrenaline in response to local exposure to a noradrenergic neurotoxin may accompany a reduction in oocyte release or a block in ovulation, the anti-ovulatory effect of DSP4 is independent of the changes in noradrenaline concentrations and may be due to some other ovarian response.

Introduction

It has been known for some time that regulation of the cyclic pattern of ovulation in female mammals involves the participation of noradrenergic neurotransmitter mechanisms. In the hypothalamus, such neurotransmitter activity is a critical component of the sequence of events leading to the generation of the ovulatory surge of LH. Perturbations in this hypothalamic activity can suppress the midcycle increase in LH and block ovulation (Everett and Sawyer, 1950; Kalra and McCann, 1974; Goldman *et al.*, 1994). However, noradrenergic neuronal innervation is also present in the ovaries, suggesting that an alteration in noradrenergic activity by systemic xenobiotic exposure could affect the process of ovulation, not only by suppressing the LH surge, but also through a direct influence on the ovaries.

In mammals, noradrenergic sympathetic input reaches the ovary via the superior ovarian nerve and the nerves of the ovarian plexus that accompany the ovarian artery. In addition to this vascular innervation, catecholaminergic terminals have been identified histochemically within the stroma and follicular wall (Jacobowitz and Wallach, 1967). There is evidence that this innervation is involved in the process of follicular maturation and ovulation, although the precise nature of this participation has not been completely characterized. In prepubertal rats, peripheral noradrenergic depletion by multiple intraperitoneal injections of the adrenergic neurone blocking agent guanethidine delays vaginal opening and increases the number of oocytes detected at first ovulation (Flores *et al.*, 1990). A morphometric analysis of the ovaries from juveniles receiving guanethidine for 3 weeks beginning on day 7 after birth revealed a retardation of follicular development (Lara *et al.*, 1990). These experiments also showed that gonadotrophin stimulation of progesterone and oestrogen secretion *in vitro* was also reduced. Complementary work in adult rats demonstrated that bilateral injections of the adrenergic neurotoxicant 6-hydroxydopamine under the ovarian bursa induced a chemical sympathectomy that caused an initial disruption to the oestrous cycle and an increase in the appearance of large atretic follicles (Curry *et al.*, 1984).

The purpose of the present study was to extend further the evidence for participation of such adrenergic mechanisms in the ovulatory process and to characterize those changes in the concentration of ovarian noradrenaline that could contribute to identifiable alterations in oocyte release. Normally cyclic, adult rats were given unilateral intrabursal injections of the noradrenergic neurotoxin *N*-(2-chloro-ethyl)-*N*-ethyl-2-bromobenzylamine (DSP4). This tertiary haloalkylamine

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derivative is both effective as a long-lasting inhibitor of the re-uptake of noradrenaline (Ross, 1973; Dudley *et al.*, 1990) and can block the synthesis of noradrenaline by suppressing the activity of dopamine- β -hydroxylase (Ross, 1976). However, unlike 6-hydroxydopamine, these effects do not appear to be accompanied by nonspecific cellular damage (Dudley *et al.*, 1990) or to cause alterations in dopamine (Ross, 1976; Lookingland *et al.*, 1986), the neurotransmitter that also serves as a metabolic precursor to noradrenaline. Preliminary work in our laboratory showed that DSP4, given either systemically as a single dose or by intrabursal injection, could induce a prompt and marked decline in ovarian noradrenaline, suggesting its suitability for investigations of this nature.

Materials and Methods

Animals

Ninety-day-old, female Long-Evans hooded rats were obtained from Charles River Laboratories (Raleigh, NC) and maintained under a photoperiod of 14 h light:10 h dark (midnight corresponding to the midpoint of darkness) at $22 \pm 2^\circ\text{C}$ with food and water available *ad libitum*. Oestrous cycles were monitored daily by vaginal lavage, and only those animals between 4 and 5 months of age exhibiting at least three consecutive 4 day cycles immediately before experimentation were used.

Experiment 1

Experiment 1 established the effects of intrabursal DSP4 administration on ovarian concentrations of noradrenaline and determined whether any such alterations, if present, were confined to the site of injection or were additionally detectable elsewhere within the reproductive system. This is particularly important since studies using unilateral intrabursal injections have often used the uninjected or vehicle-injected ovary as a control (see Viggiano *et al.*, 1989; Bicsak *et al.*, 1991), even though there is evidence in rats for a small slit-like bursal opening to the peritoneal cavity located near the fimbriated tip of the oviduct (Alden, 1942). Although this foramen was reported to be functionally closed before ovulation (Alden, 1942), its presence may explain contralateral alterations observed following such intrabursal administration (Reich *et al.*, 1983).

The animals were anaesthetized with Brevital[®] (Eli Lilly, IN; 60 mg kg^{-1}) and the dorsolateral flanks shaved. Ovaries were exteriorized through a small incision made in the dorsolateral flank, taking care not to damage the suspensory ligament. Various amounts (0, 2.5 or $10 \mu\text{moles}$ in $10 \mu\text{l}$ sterile water) of DSP4 (Sigma Chemical Co., St Louis, MO) were then unilaterally injected beneath the ovarian fat pads into randomly chosen left or right bursa using a $50 \mu\text{l}$ gas-tight syringe (Hamilton Co., Reno, NV) with an attached 30 gauge needle. The doses chosen were based upon preliminary experiments with the compound. With the needle in place, a small region of the surrounding bursa was gently pinched to the shaft with fine serrated forceps during both the injection itself and withdrawal of the needle. The injection site was then closed with a drop of

rapidly sealing cyanomethacrylate surgical adhesive (Nexabond S/C[®]; Tri-Point Medical L.P., Raleigh, NC). In preliminary studies this treatment prevented back leakage of a solution of trypan blue. The ovary was carefully returned to the peritoneal cavity, and the abdominal wall and overlying skin were sutured.

A possible suppressive effect of DSP4 on the midcycle surge of LH from the pituitary into the circulation (either directly via passage out from the bursal cavity or indirectly via an alteration in hormonal feedback to the brain or pituitary) was precluded by giving intrabursal injections at 19:00 h on the day of vaginal pro-oestrus, just after the appearance of the surge (which under the above photoperiod begins between 15:00 and 17:00 h (Goldman and Cooper, 1993)). Groups of animals were also given (19:00 h) i.p. or intracardiac injections to compare the effects of systemic exposure to DSP4 on ovulation and concentrations of noradrenaline in tissue. Between 14:00 and 15:00 h on the day of oestrus, the animals were killed by decapitation in an adjoining room within 15 s of removal from their home cages. Both ovaries were removed, trimmed of fat, and frozen (-55°C) until used.

Any possible effects of such intrabursal administration on hypothalamic noradrenaline were also evaluated, although the responsiveness of this area to DSP4 is minimal (Grzanna *et al.*, 1989; Zaczek *et al.*, 1990), and any such effects, if present, would have had no impact on the hypothalamic-pituitary mechanisms triggering the earlier ovulatory surge of LH. For this evaluation, rats received intrabursal injections ($10 \mu\text{moles}$ per ovary) as above and were killed 1, 3, 7 or 19 h later. Anterior and posterior hypothalamic regions were dissected and frozen as described previously (Goldman *et al.*, 1994). Seven hours after the injection, portions of the somatosensory cortex were also taken for analysis, since cerebrocortical noradrenaline concentrations are affected markedly by systemic DSP4 administration (Jonsson *et al.*, 1981; Lookingland *et al.*, 1986).

Noradrenaline determinations. Tissue concentrations of noradrenaline were determined by HPLC. Upon thawing, the ovaries were weighed and quickly diced and sonicated over ice as a 1:11 dilution in cold HPLC mobile phase (pH 3.1) consisting of 115 mmol dibasic sodium phosphate l^{-1} (Fisher Chemical, Fairlawn, NJ), 0.19 mmol EDTA l^{-1} (Mallinckrodt, Paris, KY), 3 mmol 1-heptanesulfonic acid l^{-1} , sodium salt (Eastman Kodak, Rochester, NY), and 9% (v/v) acetonitrile (Burdick & Jackson, Muskegon, MI) in HPLC grade water previously filtered and deionized through a Milli-Q system (Millipore, Marlborough, MA). Hypothalamic fragments were also diluted and sonicated as above. After removing a $10 \mu\text{l}$ aliquot for total protein analysis, all samples were then centrifuged (10 min, 8500 g , 4°C) before supernatants were drawn off and filtered (Gelman Acrodisc LC13, $0.2 \mu\text{m}$, Ann Arbor, MI). Hypothalamic samples were subsequently diluted 1:2.5 for HPLC injection, while ovarian supernatants were used without further dilution. The HPLC-electrochemical detection system used was as described by Goldman *et al.* (1994), and a flow rate of 1.5 ml min^{-1} was used for all separations. Noradrenaline, dopamine, adrenaline and 3,4-dihydroxy-phenylacetic acid (DOPAC) standards for calibration were obtained from Sigma Chemicals.

Total protein for each sample was measured by the bicinchoninic acid assay (Smith *et al.*, 1985). Materials were obtained from Pierce Chemical (Rockford, IL), and the assay was conducted on a Thermomax microplate reader (Molecular Devices, Menlo Park, CA). Preliminary standardization showed no interference in this procedure by catecholamines at the detected hypothalamic concentrations.

Experiment 2

Experiment 2 was designed to establish dose- and time-response relationships for the influence of DSP4 on ovarian noradrenaline and also to evaluate the impact of exposure to DSP4 on oocyte release at pro-oestrus.

Regularly cyclic females were given unilateral intrabursal injections as above at pro-oestrus, in doses ranging from 0 to 10 μ moles per ovary (in 10 μ l sterile water). Rats were killed by decapitation on the following day (14:00–15:00 h, oestrus), and ovaries were removed and trimmed of fat before they were frozen for subsequent noradrenaline determinations. Noradrenaline assays were also conducted on previously frozen ovaries taken from those rats described in Expt 1 that were killed at 1, 3, or 7 h after injection, with the 7 h sample selected to fall during the approximate period of ovulation (see, Plas-Roser *et al.*, 1985). Other rats received intrabursal injections (unilateral, 19:00 h pro-oestrus) of 10 μ moles DSP4 per ovary and were examined for evidence of ovulation on vaginal oestrus on either of the next two cycles (that is, 5 or 9 days later) to evaluate a more persistent effect of administration. Other females were bilaterally injected with 0 or 2.5 μ moles DSP4 per ovary and evaluated for oocyte release 1 or 9 days later.

Oocytes were collected (14:00–15:00 h) and quantified according to procedures described by Perreault and Mattson (1993). Briefly, the oviduct was dissected from the ovary and a cut made at its junction with the uterus. Under a dissecting microscope, the ampulla was flushed with 200 μ l of Dulbecco's PBS (Gibco, Gaithersburg, MD) containing 0.1% (w/v) BSA (fraction V, Sigma Chemical) using a 1 ml syringe with attached 30 gauge blunt needle inserted into the infundibulum. The cumulus mass was extruded from the cut end of the oviduct, and individual cumulus-encased oocytes were counted.

Experiment 3

The relationship between DSP4-induced shifts in ovarian noradrenaline and alterations in ovulation were investigated further by pretreating rats at pro-oestrus with i.p. injections (20 mg kg⁻¹ body mass) of the noradrenaline reuptake inhibitor desipramine (desmethylinipramine HCl; Sigma Chemical Co.) 1 h before (18:00 h) the unilateral intrabursal injection of DSP4 (0, 1.25 or 10 μ moles per ovary). Such prior administration interferes with the access of DSP4 to the cell and prevents, at least partially, the depletion of noradrenaline (Ross, 1976; Jonsson *et al.*, 1982). These animals were killed as before (14:00–15:00 h, oestrus). Ovaries were taken for determinations of noradrenaline, and the oviducts were examined for evidence of ovulation.

In addition to its effect on the uptake of noradrenaline, DSP4 competes for opiate-binding sites in the brain (Wilkinson *et al.*,

1985). While an opioid role in ovarian function is unclear, ovaries of a variety of species, including rats, contain immunoreactive opioid peptides and their precursors (Lolait *et al.*, 1985; Douglass *et al.*, 1987). Consequently, we attempted to evaluate the ovulatory effect of co-administration of the opiate-receptor antagonist naloxone with DSP4 injected intrabursally.

At 19:00 h on the day of pro-oestrus, regularly cyclic females were given unilateral, intrabursal, injections of 2.5 μ moles DSP4, 8 μ moles naloxone (Sigma Chemical Co.) or 2.5 μ moles DSP4 plus 8 μ moles naloxone. Ten microlitres of sterile, distilled water was used as the injection vehicle, and the procedure was as detailed for Expt 1. Between 14:00 and 15:00 h on the following day, the oviducts were flushed as above and examined for evidence of ovulation.

Statistical analyses

Differences in noradrenaline concentrations and numbers of oocytes among dose groups were analysed for statistical significance by analysis of variance (ANOVA) under the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS, Cary, NC) package. Significant main effects of treatment ($P < 0.05$) were subjected to further analysis using Tukey's test for multiple comparisons. For evaluation of treatment effects on the incidence of ovulation, dose groups were compared against controls using the Fisher exact test of probability.

Results

Experiment 1

The ovarian concentrations of noradrenaline 18–19 h after the pro-oestrous injection of 2.5 or 10 μ moles DSP4 are shown in Table 1. A marked fall in the concentrations of noradrenaline in injected and non-injected ovaries occurred. Although the mean magnitude of the decrease for the injected side in the 2.5 μ moles group (approximately 13% of the vehicle-injected controls) was somewhat larger than that for the non-injected ovary (approximately 28% of controls), the difference was not statistically significant. Administration of DSP4 by i.p. or intracardiac injection (Table 1) also resulted in a large fall in noradrenaline concentrations, although the fall was not as large as those caused by the intrabursal injections of DSP4.

Samples of anterior and posterior hypothalamic tissue taken 0, 1, 3, 7 or 19 h after injections showed no changes in the concentrations of noradrenaline in response to the intrabursal administration of 10 μ moles DSP4 per ovary, a normal pH dose, shown in pilot experiments to cause the maximum depletion in ovarian noradrenaline by 19 h. Over this period, mean values ($\pm$ SEM) were 20.7 ± 0.9 and 15.3 ± 0.4 ng mg⁻¹ protein for control anterior and posterior regions, respectively, and 21.4 ± 1.1 and 15.0 ± 0.4 ng mg⁻¹ protein for these two regions in animals receiving the 10 μ mole dose. However, measures of cerebrocortical noradrenaline from tissue samples obtained 7 h after the injection did reveal a small but statistically significant ($P < 0.05$) fall (2.1 ± 0.4 ng mg⁻¹ protein versus control concentrations of 3.1 ± 0.1 ng mg⁻¹).

Table 1. Alterations in ovarian concentrations of noradrenaline in rats 19 h after unilateral intrabursal or systemic administration of DSP4

Dose of DSP4 ^a	Route	Ovary	n	Ovarian noradrenaline (ng mg ⁻¹ protein)
0	Intrabursal	Injected	6	3.79 ± 0.40
		Non-injected	6	3.93 ± 0.66
2.5	Intrabursal	Injected	5	0.57 ± 0.07*
		Non-injected	5	1.04 ± 0.08*
10	Intrabursal	Injected	7	0.58 ± 0.11*
		Non-injected	7	0.64 ± 0.09*
2.5	Intraperitoneal	Left and right combined	6	1.70 ± 0.34*
10	Intraperitoneal	Left and right combined	5	1.82 ± 0.36*
2.5	Intracardial	Left and right combined	6	1.40 ± 0.29*

^aDoses for intrabursal treatments are in μ moles per ovary, while those for intraperitoneal and intracardial routes are in μ moles per rat. * $P < 0.01$ for comparisons against the 0 dose treatment for that ovary (intraperitoneal and intracardial treatments were compared against values for the intrabursal control ovaries).

Fig. 1. Dose–response effect of unilateral intrabursal DSP4 administration on ovarian noradrenaline concentrations in rats. All rats were injected at 19:00 h at pro-oestrus, and injected and non-injected ovaries were removed 19 h later. Group sizes are indicated at the base of each treatment category. (■) Injected ovaries; (□) non-injected ovaries. * $P < 0.01$ for comparisons against control for that ovary; † $P < 0.01$ for comparisons against the non-injected ovary within that dose group.

Experiment 2

The effects of increasing doses of intrabursally administered DSP4 on ovarian noradrenaline are shown for injected and non-injected ovaries (Fig. 1). A clear, dose-related decline was seen for both ovaries at 19 h, with treatments as low as 0.16 μ moles per ovary causing a significant depletion on the injected side. Differences between the two ovaries (with one exception, evident up to a dose of 1.25 μ moles per ovary) were no longer present as noradrenaline concentrations decreased further in response to 2.5, 5 and 10 μ moles DSP4. The fall in

ovarian noradrenaline over this period in response to 10 μ moles DSP4 (the dose which at 19 h induced maximum depletion of noradrenaline) is shown (Fig. 2). Both ovaries showed relatively rapid depletions, such that by 3 h values were approximately 66% of 0 h values.

The number of oocytes and the percentage of ovaries showing evidence of ovulation (release of either identifiable cumulus-encased oocytes or cumulus only) also showed a dose-related decline in response to 0.62 and 10 μ moles DSP4 per ovary (Fig. 3). However, this effect was observed only for

Fig. 2. Changes in ovarian concentrations of noradrenaline (mean $\pm$ SEM) after unilateral intrabursal administration of 10 μ moles DSP4 per ovary. Animals were injected at 19:00 h (arrow) in pro-oestrus. (a) Injected ovary; (b) non-injected ovary. The shaded areas represent the dark portion of the photoperiod. Group sizes are given in (a) at each time point. (—●—) Vehicle controls; (---○---) DSP4-treated. (▲) Combined values for pairs of ovaries in non-injected animals at time 0 h. * $P < 0.01$ for comparisons against controls at that time point.

the injected ovaries; the non-injected side released a full complement of oocytes throughout the dose range. At the 10 μ moles DSP4 dose, this block persisted through at least the next two ovulatory cycles (Table 2), with these rats showing progressive evidence of a compensatory increase in oocyte release on the contralateral side. This persistence was also evident after bilateral injections of 2.5 μ moles DSP4 per ovary (Table 3), and the reduction in the number of oocytes collected on the day after DSP4 administration did not show any evidence of further decline by the third oestrous day. With two exceptions (that were excluded from the results), the bilaterally injected DSP4 animals continued to display normal patterns of vaginal cytology (as did the controls). Those animals that showed evidence of ovulation, when examined at 1 or 9 days after treatment, generally ovulated from only one ovary (that is, five of six rats had oocytes only on a single side).

Experiment 3

The marked depletions in noradrenaline seen in the injected ovaries for both the 1.25 and 10 μ moles DSP4 groups were at least partially prevented by prior administration of desipramine (Table 4). In the case of the 1.25 μ moles DSP4 group, pretreatment with desipramine represents a 37% difference in ovarian noradrenaline (obtained by comparing a 60% decrease in desipramine plus DSP4 with the marked decline to 23% for DSP4 exposure only). In contrast, the non-injected ovaries for this same dose of DSP4 displayed a relatively small improve-

ment (ovarian noradrenaline values were 69% that of controls after pretreatment with desipramine compared with 56% in its absence). Treatment with 10 μ moles DSP4 per ovary caused reductions to values that for both ovaries at 19 h were 23% of control values. Prior administration of desipramine resulted in values for noradrenaline that were 44% and 78% of control values for injected and non-injected ovaries, respectively. In the injected ovaries, this reduction in the effect of DSP4 on ovarian noradrenaline in response to desipramine was not associated with a corresponding increase in the number of oocytes released or in the percentage of rats ovulating (Table 4). Although a small improvement in the 1.25 μ moles DSP4 group was seen in the ratio of ovulating ovaries to the total number examined for that group (in parentheses on the right-hand side of the table), it was not statistically significant.

Co-injections of the opiate receptor antagonist naloxone did not cause any detectable alterations in the effect of 2.5 μ moles DSP4 on the incidence of ovulation or the number of oocytes released (Table 5). Data obtained following the intrabursal administration of naloxone alone were comparable to those from vehicle-injected controls.

Discussion

The present study was designed to investigate alterations in ovarian concentrations of noradrenaline caused by local, late-pro-oestrous injections of the noradrenergic neurotoxin DSP4

Fig. 3. Alterations in ovulation induced in rats after a unilateral intrabursal injection of DSP4. All animals were injected in pro-oestrus and killed 19 h later at oestrus. Mean numbers of oocytes for ovulating rats only are represented for (■) injected and (□) non-injected ovaries; the numbers of ovulating females/group size are listed above each treatment condition. **P* < 0.01 for comparisons against the control treatment for that ovary; †*P* < 0.05 for comparisons against the non-injected ovary within that dose group.

Table 2. Oocyte release in cyclic rats after intrabursal injection of 10 μmoles DSP4 per ovary

Time of oocyte retrieval (days after injection)	Injected ovary	Non-injected ovary
1	— (0/6)	7.8 ± 1.4 (6/6)
5	1 (1/6)	10.0 ± 0.9 (6/6)
9	— (0/5)	11.6 ± 0.7 (5/5)

Mean number of oocytes (± SEM) represent only individual ovaries showing evidence of ovulation. The numbers in parentheses represent the number of ovaries ovulating divided by the total number of rats within that group displaying 4 day cycles after treatment.

and the relationship of such changes to oocyte release. Unilateral intrabursal injections were found to induce dose-related depletions of noradrenaline in the injected ovary and in areas peripheral to the site of administration. Sealing the injection site with a veterinary surgical adhesive did not prevent reductions in noradrenaline values in the contralateral ovary or in cerebrocortical noradrenaline. These results indicate that DSP4 was not confined to the sub-bursal space, and are consistent with the similar magnitude of alterations in ovarian noradrenaline seen in groups receiving DSP4 by intrabursal or systemic (that is, i.p. or intracardial) routes of administration. It is also in agreement with the extra-ovarian distribution of an intrabursal injection of [³H]thymidine (J. M. Goldman, unpublished observations) and with previously reported biochemical

Table 3. Persistence of the effects of a bilateral pro-oestrous injection of DSP4 on ovulation in cyclic rats

Dose of DSP4 (μmoles per ovary)	Time of oocyte retrieval (days after injection)	Mean number of oocytes	Number of oocytes from left ovary or right ovary	
0	1	14.5 ± 0.6 (4/4)	7	7
			6	7
			10	6
0	9	14.4 ± 0.5 (5/5)	8	7
			5	8
			9	7
2.5	1	4.2 ± 0.6 (4/8)	8	6
			7	7
			4	0
2.5	9	3.0 ± 2.0 (2/6)	4	2
			3	0
			0	4
2.5	9	3.0 ± 2.0 (2/6)	4	0
			0	1
			0	1

Mean numbers of oocytes (± SEM) represent only ovaries showing evidence of ovulation. The numbers in parentheses represent the number of rats ovulating divided by the total number of rats within that group displaying 4 day cycles after treatment.

alterations for a non-injected side following intrabursal administration (Reich *et al.*, 1983). While the effect could be attributable to the direct escape of DSP4 from the bursal space into the

Table 4. Effects of desipramine on the suppression by DSP4 of ovarian noradrenaline and oocyte release in rats

Treatment ^a	Ovarian noradrenaline (ng mg ⁻¹ protein)		Number of oocytes	
	Injected ovary	Non-injected ovary	Injected ovary	Non-injected ovary
Control	3.79 ± 0.40	3.93 ± 0.66	6.7 ± 0.7 (6/6)	7.5 ± 0.5 (6/6)
DSP4 (1.25 µmoles per ovary)	0.86 ± 0.08**	2.19 ± 0.20**	2.8 ± 1.2** (5/10)	6.8 ± 0.4 (10/10)
Desipramine + 1.25 µmoles DSP4	2.28 ± 0.23**†	2.72 ± 0.20*	2.7 ± 1.2** (7/8)	7.1 ± 0.7 (8/8)
DSP4 (10 µmoles per ovary)	0.89 ± 0.19**	0.92 ± 0.16**	— (0/6)	7.8 ± 1.4 (6/6)
Desipramine + 10 µmoles DSP4	1.67 ± 0.19**	3.06 ± 0.36†	1 (1/6)	6.3 ± 0.7 (6/6)

^aIntrabursal injections of DSP4 or water vehicle were given at 19:00 h on pro-oestrus. Desipramine (20 mg kg⁻¹ i.p.) was administered 1 h earlier. All rats were examined for evidence of ovulation 19 h later on the day of oestrus. In the DSP4 groups not receiving desipramine, i.p. saline-pretreated animals given subsequent intrabursal DSP4 were combined with animals receiving DSP4 only. *n* = 6–10 animals per group. The numbers in parentheses indicate the number of ovaries showing evidence of ovulation divided by the total number examined in that group. **P* < 0.05, ***P* < 0.01 compared with controls for that ovary. †*P* < 0.01 when compared to DSP4-injected ovary for that dose.

Table 5. Influence on ovulation of intrabursal co-injections of DSP4 and naloxone in rats

Treatment ^a (dose per ovary)	Injected ovaries ovulating/ total animals treated	Mean number of oocytes released (± SEM)	
		Injected ovary ^b	Non-injected ovary
2.5 µmoles DSP4	3/6	2.0 ± 1.2 ^{†‡}	7.2 ± 0.5
2.5 µmoles DSP4 plus 8 µmoles naloxone	2/6*	2.0 ± 1.4 ^{†‡}	7.3 ± 0.9
8 µmoles naloxone	6/6	8.0 ± 1.1	8.3 ± 1.0

^aAll animals were injected at 19:00 h on the day of pro-oestrus and examined on the following day (13:00–14:00 h) for evidence of ovulation. ^bGroup numbers of oocytes for the injected ovary represent means for ovulating ovaries only. **P* < 0.05 for comparison to ovulation frequency of naloxone-injected ovary. †*P* < 0.01 for difference between injected and non-injected ovaries within treatment group. ‡*P* < 0.01 for comparison with mean oocyte number of naloxone-injected ovary.

circulation, the bursal foramen (thought to be functionally sealed before ovulation in rats (Alden, 1942)) may allow the i.p. passage of more material than was previously suspected. However, regardless of the route, the results clearly indicate that, in rats, the contralateral ovary should not be used as a control. The dose-related patterns of noradrenaline decline in the individual ovaries did, however, allow informative comparisons to be made between the amounts of noradrenaline depletion and the incidence of ovulation on each side, relationships explored in Expt 2.

While the presence of ovarian noradrenaline has been known for some time, a local noradrenergic role in ovulation has been the subject of some debate. Ovarian noradrenaline changes during the oestrous cycle (Bahr and Ben-Jonathan, 1981; Arbogast *et al.*, 1987), suggesting a local functional role. There is an abundant neuronal supply to the theca externa of follicles, and a few fibres (possibly perivascular in nature) have been detected in the theca interna and corpora lutea (Stefenson *et al.*, 1981; Sporrang *et al.*, 1985); the luteal fibres are probably perivascular in nature. It is also reasonable that secreted noradrenaline reaches more interior regions of the follicle via diffusion through the basement membrane.

There has been some speculation that secretion of noradrenaline within the follicular wall, triggered by the LH

surge, causes an increase in capillary permeability before ovulation (Dmowski, 1990), or that such release may be involved in the ovulatory contraction of the smooth muscle of the wall (Wallis *et al.*, 1986). Kannisto *et al.* (1985) observed that administration of noradrenaline or the β_2 -receptor agonist terbutaline to gonadotrophin-primed, immature rats increased the number of corpora lutea, which was used as an index of ovulation. In addition, bilateral intrabursal injections of the α -adrenergic receptor antagonists phenoxybenzamine (Wallis *et al.*, 1977) or phentolamine (Kannisto *et al.*, 1985) reduced the number of corpora lutea in similarly primed immature females. Intrabursal DSP4, on the other hand, caused no alterations in the number of corpora lutea (J. M. Goldman, M. B. Parrish and T. E. Stoker, unpublished data), even though the number of oviductal oocytes was reduced, implying the formation of luteinized, unruptured follicles on the injected side. Other research has shown that transection of the superior ovarian nerve with its large bundle of noradrenergic fibres caused a marked decrease in ovarian noradrenaline, but failed to alter first ovulation in young rats (Aguado and Ojeda, 1984).

Differences in the nature of the pharmacological/surgical treatment, and age and cyclic status of the animals and the endpoint measures taken make it difficult to reconcile the

difference in the above findings with those in the current study. Nevertheless, the present data indicate that while intrabursal administration of DSP4 can induce a persistent ovulatory suppression, the effect is not directly associated with depletion in noradrenaline concentrations. A comparable pattern of noradrenaline decrease in the contralateral ovary was not followed by any detectable effects on the percentage of ovaries presenting evidence of ovulation or on the number of oocytes released. Prevention of the considerable depletion of noradrenaline by prior injections of desipramine did not significantly alter the block to ovulation. In addition, no alterations in the DSP4-induced suppression of ovulation were detected following intrabursal co-administration of the opiate receptor antagonist naloxone, in spite of reports that at least some effects of DSP4 may be attributable to such receptor binding (Jacobson *et al.*, 1985; Wilkinson *et al.*, 1985).

The discrepancy between oocyte release from the injected and non-injected ovaries, coincident with a comparable depletion in noradrenaline concentrations may indicate a distinction between the influence of DSP4 on metabolism of noradrenaline (in both ovaries) and some effect on noradrenergic input (of greater impact at the site of injection). This had previously been suggested to explain similar disparities between noradrenaline concentrations and DSP4 toxicity in the central nervous system (Gibson, 1987). One possibility is that the ovarian neurotoxic effects of DSP4 at the site of injection involve an immune-mediated mechanism, something that has been described for guanethidine noradrenergic neurotoxicity (Manning *et al.*, 1983). Whether some compound-induced cytotoxic alterations did in fact occur over the 6–10 h between injection and the time that ovulation typically would have taken place is not known, although routine histological evaluation of the ovaries 19 h after intrabursal administration failed to reveal any indication of follicular cytotoxicity (data not shown). Whatever mechanism is involved, the effect appears to be persistent, and the degree of ovulatory suppression was the same for each injection condition studied (that is, 10 μ moles unilateral or 2.5 μ moles bilateral), regardless of whether ovulation was evaluated on the ensuing oestrus or two cycles later.

It appears, therefore, that the observed ovulatory effects in response to DSP4 or its aziridinium derivative are attributable to as yet undefined forms of pharmacological treatment. Conversely, the results indicate that alterations in the concentration of ovarian noradrenaline, in response to a noradrenergic toxicant, do not necessarily provide an accurate reflection of the impact of the treatment on the functional integrity of the ovulatory process.

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