Effects of 6-hydroxydopamine treatment of rats on the in-vitro release of LHRH and pituitary responsiveness to LHRH

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Summary. Injection of 150 µg 6-hydroxydopamine (6-OHDA) into the third ventricle of rats depleted the mean hypothalamic concentration of noradrenaline by 71% whereas the mean dopamine concentration was only reduced by an insignificant 7%. Isolated perfused pituitary glands taken from intact 6-OHDA-treated rats showed a markedly increased LH response to pulses of LHRH although there was no significant difference in the circulating levels of LH. Ovarian cyclicity was disrupted and the hypothalamic content of LHRH was significantly reduced. Hypothalamic synaptosomes prepared from intact 6-OHDA-treated animals consistently released less LHRH than did controls although the difference was not significant. Noradrenaline, dopamine and adrenaline did not significantly affect LHRH release from experimental or control synaptosome preparations. In ovariectomized rats 6-OHDA treatment did not inhibit the positive feedback effects of progesterone administration to oestrogen-primed animals, although the negative feedback effects of the priming oestrogen treatment was augmented. The results indicate that depletion of hypothalamic noradrenaline causes subtle changes in the endogenous release of LHRH and may alter the negative feedback effects of steroids on the hypothalamic–pituitary axis.

Introduction

The neurotoxin 6-hydroxydopamine (6-OHDA) has been used as a pharmacological tool to investigate the role of catecholamines in the control of gonadotrophin secretion (see Barraclough & Wise, 1982). It causes a relatively selective destruction of catecholamine-containing neurones when administered into the central nervous system, the degeneration of aminergic terminals reaching a maximum within 7–14 days (Hartman, Blehm, Kalmbach & Hartman, 1975). The central administration of 6-OHDA causes a transient reduction of LH secretion within a few hours of the injection, but serum LH concentrations then return to control levels (Kitchen, 1974) or are significantly elevated (Fenske & Wuttke, 1976). The spontaneous preovulatory LH surge in pro-oestrous rats and the progesterone-induced LH surge in oestrogen-primed ovariectomized animals are both inhibited when 6-OHDA is injected a few hours before the LH surge (Martinovic & McCann, 1977). However, ovarian cyclicity is only temporarily disrupted by 6-OHDA (Nicholson, Greeley, Humm, Youngblood & Kizer, 1978; Hancke & Wuttke, 1979), indicative of some non-specific pharmacological effects of 6-OHDA during the immediate period after treatment, with recovery occurring in time.

To evaluate more precisely the action of 6-OHDA on gonadotrophin secretion, we have investigated the effects of 6-OHDA, administered into the third ventricle, on ovarian cycles and LH secretion over a 9-day period. Pituitary responses to LHRH, the feedback effects of steroids in

ovariectomized rats, the hypothalamic content of LHRH and the release of LHRH from hypothalamic synaptosomes were then investigated 9 days after 6-OHDA treatment, since by this time near maximal depletion of catecholamines has occurred without any substantial regenerative processes (Hartman *et al.*, 1975).

Materials and Methods

Virgin female Porton Wistar rats weighing 200–250 g were housed under controlled conditions of lighting (lights on 06:00–18:00 h) and temperature (22°C) and had free access to food and water. Daily vaginal smears were examined and only animals displaying regular 4-day oestrous cycles were used for these experiments. All surgical operations were carried out under ether anaesthesia. On Day 2 of dioestrus rats were injected with 150 µg 6-OHDA (Sigma, Poole, Dorset, U.K.) dissolved in 1 µl 0.9% (w/v) NaCl containing 0.1% ascorbic acid. Controls received an equivalent volume of vehicle alone. The injections were made into the third ventricle using co-ordinates AP = 5.6, L = 0.1, H = –3.5 (De Groot, 1959), and the solutions were infused over a 1-min period using a Hamilton microsyringe. The animals were maintained for 9 days after drug treatment and vaginal smears were monitored daily. In a group of 14 animals (see Table 2), 0.5 ml blood was taken at 12:00 h by cardiac puncture under light ether anaesthesia before and on Days 2, 4, 7 and 9 after the brain injections. For all experiments, except the feedback studies, animals were decapitated between 11:00 and 12:00 h and trunk blood was collected for hormone assay. The hypothalami and pituitary glands were dissected for the appropriate experiments, as described below. For the feedback studies, rats on Day 2 of dioestrus were ovariectomized 3 weeks before the intraventricular injection of the 6-OHDA solution or vehicle.

Histological verification of the 3rd ventricular injection site could only be made in a small randomized sample of the rats used in these experiments. Frozen sections of 8 formalin-fixed brains showed that in all cases the tip of the syringe needle had extended into the third ventricle. Visual inspection of the ventral surface of the brains of 6-OHDA-treated animals also indicated correct placement of the injection since the tissue surrounding the third ventricle was darker in colour. A symmetrical pattern of tissue discoloration along the ventral surface of the third ventricle was not observed in 2/38 animals: these 2 rats were not included in the results.

In-vitro pituitary response to LHRH

The pituitary glands were rapidly dissected out and after removal of the neurointermediate lobe, the anterior lobe was bisected and both hemipituitaries placed in a single perfusion chamber and perfused with Krebs-Ringer bicarbonate (KRB) containing glucose (2 g/l) and bovine serum albumin (2.5 g/l). The system used has been described in detail by Carter & Whitehead (1981). The glands were perfused for an initial 2-h stabilization period and then serial 10-min perfusate fractions were collected for a further 2 h during which time the glands were subjected to two 5-min pulses of synthetic LHRH, 10 ng/ml (Hoechst, Middlesex, U.K.) with an interval of 1 h between the delivery of each pulse. The perfusate samples were stored at –20°C until assayed for LH.

In-vitro release of LHRH from hypothalamic synaptosomes

A block of hypothalamic tissue measuring approximately 5 × 4 × 2 mm and including the median eminence was excised. The optic chiasma and mamillary bodies were the rostral and caudal limits, and the edges of the hypothalamus were the lateral limits. The tissue was immediately placed in pre-weighed ice-cold 0.32 M-sucrose and the weight for groups of 6 treated or control hypothalami was determined (170–200 mg). Synaptosomes were prepared according to the rapid method of Dodd *et al.* (1981). In each experiment an experimental and a control gradient were run in parallel using 6 hypothalami from 6-OHDA- or vehicle-injected animals respectively.

The synaptosomal pellets were re-suspended at a concentration of 0.5 hypothalamic equivalents/ml KRB containing 2 g glucose/l and saturated with 95% O₂–5% CO₂. Samples (0.5 ml) of the suspensions were incubated at 37°C in a shaking water bath with a 15-min preincubation period being allowed before 10 µl test drug (noradrenaline, dopamine or adrenaline: Sigma), or KRB as control, were added to triplicate samples. Drug solutions were made up in KRB containing 0.1% ascorbic acid immediately before use, to give a final concentration in the synaptosome suspensions ranging from 10⁻⁴ to 10⁻⁸ M. Synaptosomes were incubated for a further 30 min, in the presence of the drugs, before being deposited by centrifugation. Supernatants were snap frozen and stored at –20°C until assayed for LHRH.

Steroid feedback in ovariectomized rats

Steroid feedback studies began 9 days after 6-OHDA administration. Animals received oestradiol benzoate (80 µg/kg: Sigma) subcutaneously at 12:00 h and progesterone (10 mg/kg: Sigma) 72 h later; both steroids were dissolved in 0.1 ml corn oil. Blood samples were taken by cardiac puncture under light ether anaesthesia immediately before each steroid injection and at 5 and 6 h after progesterone administration. Serum was collected and stored at –20°C until assayed for LH and the hypothalami were rapidly dissected as described above, placed on solid CO₂, weighed, and then stored at –70°C until extraction of LHRH or catecholamines. LHRH was extracted by the boiling method of Koch & Baram (1976) in the presence of 1 mM-bacitracin (Sigma); catecholamines were extracted using a procedure described by Welch & Welch (1969).

Assays and statistical analyses

Perfusate and serum LH concentrations were measured by radioimmunoassay (RIA) using the procedure outlined by NIH, and are expressed in terms of NIAMDD-rat LH-RP1. Inter- and intra-assay variations were 10% and 9.0% respectively, and the assay sensitivity was 10 ng/ml. LHRH was measured by a single antibody RIA (Jeffcoate, Fraser, Holland & Gunn, 1974) using synthetic LHRH (Hoechst, Frankfurt). The antiserum to LHRH was a gift of Dr H. M. Fraser (M.R.C. Unit of Reproductive Biology, Edinburgh); inter- and intra-assay variations were 10% and 6.7% respectively and assay sensitivity was > 30 pg/ml. Catecholamines were assayed according to the method of Shellenbergher & Gordon (1971) with inter- and intra-assay variations being 17% and 3.6% for noradrenaline and 23% and 10% for dopamine. For the pituitary responses to LHRH, the geometric area under the response peak and the height of the response peak were measured. The basal release of LH *in vitro* was assessed by obtaining the mean LH concentration measured in the perfusate fractions at 120, 170, 180 and 240 min.

Student's *t* test was used to analyse the statistical significance of all results except the synaptosome data for which a two-tailed *t* test was used.

Table 1. The effects of intraventricular 6-OHDA or its vehicle on hypothalamic concentrations of LHRH, noradrenaline and dopamine 9 days after treatment

	6-OHDA	Vehicle
LHRH (pg/mg tissue)	96.1 ± 15.8 (5)*	151.1 ± 20.0 (4)
Noradrenaline (ng/100 mg tissue)	81.75 ± 9.0 (4)**	283.7 ± 10.7 (6)
Dopamine (ng/100 mg tissue)	227.2 ± 21.4 (4)	246.2 ± 20.26 (5)

Values are mean ± s.e.m. for the no. of rats indicated in parentheses.

* *P* < 0.05, ** *P* < 0.005 compared with value for vehicle-treated controls (Student's *t* test).

Table 2. Serum LH concentrations in rats after intravenous injection of 6-OHDA or its vehicle

Days after injection	Serum LH (ng/ml)	
	6-OHDA	Vehicle
0	37.4 $\pm$ 5.0 (8)	29.5 $\pm$ 4.4 (6)
2	23.7 $\pm$ 6.6 (6)	14.7 $\pm$ 3.3 (4)
4	30.0 $\pm$ 7.5 (7)	36.8 $\pm$ 15.3 (5)
7	29.0 $\pm$ 8.0 (6)	28.7 $\pm$ 10.1 (5)
9	24.4 $\pm$ 4.5 (6)	17.3 $\pm$ 4.5 (6)

Values are mean $\pm$ s.e.m. for the no. of rats given in parentheses.

Text-fig. 1. In-vitro LH responses of pituitary glands from rats treated with 6-OHDA (150 μ g) or vehicle (0.9% NaCl + 0.1% ascorbic acid) in response to 5-min pulses of 10 ng LHRH/ml (arrows). Animals were treated 9 days before the experiment. Values are mean $\pm$ s.e.m. for 9 observations. There was a significant increase in the magnitude of the LH response peaks ($P < 0.005$) and the mean basal LH release ($P < 0.025$) in the 6-OHDA-treated animals compared with the paired controls.

Results

Treatment with 6-OHDA significantly reduced hypothalamic noradrenaline concentrations by 71% ($P < 0.005$) compared with controls although the dopamine concentrations fell only by 7% (Table 1). Although turnover studies may give a more direct assessment of hypothalamic adrenergic activity they were not appropriate for these experiments since it was desirable to measure hormone levels and pituitary LH responses in the same animals from which catecholamine content could be assessed. Ovarian cycles were disrupted in 90% of the treated animals ($N = 22$) and of these, 50% remained in constant oestrus while the rest exhibited irregular vaginal smear patterns throughout the 9-day period. An intraventricular injection of vehicle alone did not disrupt the typical 4-day cycle of vaginal smears in the majority of animals ($N = 18$) although in 4 of these controls the first oestrus after treatment was delayed by 24 h. All experimental tests on rats treated with 6-OHDA and in the control group were made when the animals were in dioestrus except for those animals in constant oestrus. Therefore, any marked differences in pituitary LH responses to LHRH, the hypothalamic content of LHRH and the in-vitro release of LHRH between experimental and control groups could not be attributed to changes in the hypothalamic-pituitary axis which occur during pro-oestrus. Since there was no difference in the LH responses or LHRH release between constant oestrous and dioestrous drug-treated animals all the data have been pooled.

Circulating levels of LH were not significantly altered by 6-OHDA treatment although the mean serum concentrations were higher in the experimental than in the control rats (Table 2). However, the LH response of isolated pituitary glands to pulses of LHRH was significantly enhanced ($P < 0.005$) after 6-OHDA treatment; the area under the response peak was $21.96 \pm 3.08 \text{ cm}^2$ compared with $6.95 \pm 0.63 \text{ cm}^2$ in the control rats (Text-fig. 1). There was also a significant increase ($P < 0.025$) in the mean basal LH from the pituitary glands of drug-treated animals.

The hypothalamic LHRH content of intact rats was significantly reduced ($P < 0.05$) 9 days after 6-OHDA administration, the mean LHRH concentration being 36% lower than in the controls (Table 1). In parallel, the basal release of LHRH from hypothalamic synaptosomes prepared from 6-OHDA-treated rats was lower than that from control preparations, although the differences were not significant (Table 3). There were no significant effects of catecholamines, at

Table 3. Effects of catecholamines on LHRH release from hypothalamic synaptosomes prepared from animals treated with 6-OHDA or vehicle alone 9 days earlier

Treatment	LHRH released (pg/ml)	
	6-OHDA	Vehicle
KRB (control)	87.0 ± 9.8	102.0 ± 20.2
Adrenaline		
10^{-6} M	117.3 ± 3.9	130.3 ± 12.0
10^{-4} M	98.5 ± 6.1	118.9 ± 16.1
Noradrenaline		
10^{-8} M	92.7 ± 16.6	114.7 ± 17.0
10^{-6} M	87.7 ± 15.8	98.9 ± 10.6
Dopamine		
10^{-8} M	96.0 ± 17.2	116.4 ± 12.9
10^{-6} M	84.7 ± 13.1	109.5 ± 17.9
10^{-4} M	100.8 ± 15.8	96.2 ± 15.8

Values are mean $\pm$ s.e.m. for 4 synaptosome preparations and for each individual experiment the catecholamines were tested on triplicate samples.

any of the concentrations tested, on LHRH release from control or experimental preparations although 10^{-6} M-adrenaline appeared to have a small stimulatory action.

When exogenous steroids were tested 9 days after 6-OHDA treatment of ovariectomized rats (Text-fig. 2) there was no significant difference in the blood LH levels before the priming injection of oestrogen although there was a significant difference ($P < 0.005$) in the circulating LH concentrations 72 h after the oestrogen treatment (6-OHDA treatment: 155 ± 14.8 pg LH/ml; controls 283 ± 33 pg LH/ml). However, there was no difference in the magnitude of the progesterone-induced LH surge between the experimental and control groups.

Text-fig. 2. Steroid feedback effects on serum LH in ovariectomized rats treated with intraventricular 6-OHDA or vehicle only. Values are mean $\pm$ s.e.m. for 8 observations. EB = oestradiol benzoate (80 μ g/kg) at 12:00 h on Day 1; P = progesterone (10 mg/kg) at 12:00 h on Day 3. * $P < 0.005$ compared with equivalent control value (Student's *t* test).

Discussion

The results have confirmed earlier reports that a depletion of hypothalamic noradrenaline disrupts the cyclic pattern of vaginal smears. However, there are discrepancies in the literature pertaining to the time interval between treatment and resumption of normal oestrous cyclicity, this interval varying between 1 or 2 days and up to 3 weeks (Benedetti, Sala & Otegui, 1976; Clifton & Sawyer, 1979; Hancke & Wuttke, 1979). Furthermore, there is the question of whether the disruption of cycles is due to the reduced hypothalamic content of noradrenaline or to some non-specific surgical damage (Nicholson *et al.*, 1978). In agreement with other studies (Fenske & Wuttke, 1976; Nicholson *et al.*, 1978) we found that the circulating basal concentrations of LH were not significantly altered by 6-OHDA treatment.

In contrast, the depletion of hypothalamic noradrenaline caused a very marked increase in pituitary responsiveness to LHRH, not only in those animals in which the drug had induced a state of persistent oestrus, but also in those that exhibited an irregular pattern of vaginal smears and were

tested during dioestrus. Therefore the increased responsiveness may not solely be accounted for in terms of raised steroid secretion in persistent oestrous animals but may be due to a subtle alteration in the rate of LHRH secretion which can lead to changes in the pituitary LHRH receptor mechanisms without altering LH release (Clayton, 1982). The observed decrease in the hypothalamic content of LHRH in the 6-OHDA-treated animals could be indicative of an increase in secretion of LHRH although this was not supported by the circulating concentrations of LH. However, it would be pertinent to measure the regional content of LHRH in microdissected hypothalamic tissue. In parallel to the lowered hypothalamic content of LHRH, the synaptosome preparations prepared from tissue of 6-OHDA-treated animals also showed a trend toward a lower basal release of the neurohormone compared with controls. There were no significant effects of the catecholamines on LHRH release although adrenaline (10^{-6} M) appeared to have a small stimulatory action. These findings are unlike those of Bennett, Edwardson, Holland, Jeffcoate & White (1975) who reported that dopamine concentrations between 10^{-6} and 10^{-10} M had stimulatory effects on LHRH release from synaptosomes, but do agree with their observations on the absence of response to noradrenaline.

It is difficult to evaluate the physiological significance of the synaptosome data because, like others (Bennett *et al.*, 1975; Joseph-Bravo, Charli, Palacios & Kordon, 1979), we did not stimulate LHRH release with a raised potassium (K^+) stimulus, although Warberg, Eskay, Barnea, Reynolds & Porter (1977) obtained conflicting results. Warberg (1982) has investigated the inconsistent effects of K^+ stimulation on LHRH release and has found that hypothalamic synaptosomes are able to respond to K^+ when incubated in isotonic sucrose but that they lose this capacity when incubated under more physiological conditions using saline media. The underlying reasons for this difference have not been resolved and the validity of the synaptosome model for studying peptide secretion must therefore be questioned.

The final series of experiments showed that 6-OHDA treatment did not inhibit the positive feedback effects of progesterone on LH release in oestrogen-primed animals, although the negative feedback effects of the first oestrogen treatment appeared to be augmented in the noradrenaline-depleted animals. It has been reported that 6-OHDA can block the progesterone-induced LH surge in ovariectomized rats (Martinovic & McCann, 1977; Simpkins & Kalra, 1979; Simpkins, Advis, Hodson & Meites, 1979) but in all these experiments the 6-OHDA had either been administered coincidentally with the progesterone or 24 h previously. The blockade of the surge may therefore simply reflect some non-specific pharmacological or surgical effect of the drug as discussed above. In intact rats long-term depletion of hypothalamic noradrenaline by lesions of the ventral noradrenergic tract does not modify the timing or the magnitude of the LH surge (Clifton & Sawyer, 1979). In contrast, an acute treatment with a noradrenaline synthesis inhibitor blocks the spontaneous LH surge (Clifton & Sawyer, 1979) and acute depletion of adrenaline blocks the stimulatory feedback effects of progesterone in oestrogen-primed ovariectomized animals (Crowley, Terry & Johnson, 1982).

In summary the results have confirmed other reports that 6-OHDA can severely deplete hypothalamic noradrenaline whilst leaving the dopamine content unaffected (Nicholson *et al.*, 1978; Hancke & Wuttke, 1979). They also show that the drug treatment disrupts the oestrous cycle or induces a state of persistent oestrus as reported by Benedetti *et al.* (1976). The observed increase in the pituitary responsiveness may be caused by a change in LHRH release and consequently pituitary LHRH receptors (Clayton, 1982), but whether this is linked to any change in the sensitivity of steroid feedback mechanisms, as indicated by the increase in the negative feedback effects of oestrogen in ovariectomized rats, remains to be established.

We thank Dr C. A. Wilson for assaying catecholamines; the Wellcome Trust for financial support; the NIH Pituitary Program, Bethesda, MD, U.S.A., for materials used in the LH radioimmunoassays; Dr H. M. Fraser for the LHRH antiserum; and Dr M. Bland for help with the statistical analysis.

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Received 8 July 1983