INFLUENCES OF THE LIMBIC STRUCTURE ON BIOSYNTHESIS OF OVARIAN STEROIDS IN RABBITS

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Many investigators have demonstrated that alterations in ovarian steroid distributions and turnover rates can alter the functioning of the hypothalamo-pituitary-gonad axis, either by the discharge mechanism of ovulating hormone from the adenohypophysis or by the negative feedback control of the steroid hormones liberated from the ovaries to the central nervous system¹⁻⁸⁾.

It has been demonstrated that electrical stimulation of the hypothalamus and the amygdaloid complex will induce ovulation, accompanied by an increase in the output of progesterin from the ovary in rabbits and rats^{9,10)}. However, little work has been done on the effect of electrical stimulation of the hippocampus upon the induction of ovulation. KAWAKAMI et al.¹¹⁾ obtained evidence that electrical stimulation of the hippocampus induces ovulation as well as an increase in ovarian progesterone synthesis and output of progesterone from the ovary. These results may suggest that the hippocampus, as well as the amygdala, participates in the regulatory mechanism of ovarian function through the hypothalamo-pituitary system.

This investigation was designed to determine the effect of stimulating and lesioning of different parts of the hypothalamus and limbic structure upon ovulation and also the *in vitro* synthesis of various steroids in the ovary. The effect of implantation of progesterone into the limbic structure upon the biosynthesis of ovarian steroids was also determined in order to elucidate the feedback mechanism of steroids to the hippocampus.

MATERIALS AND METHODS

Sexually mature female New Zealand white rabbits (2.5-3.2 kg) were fed an artificial diet supplemented periodically with fresh greens and carrots and were kept under natural lighting conditions. They were isolated in individual cages for at least three weeks before being used. Stimulation, recording and lesioning were accomplished by

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means of permanent electrodes stereotaxically placed in the hypothalamus, limbic system and hippocampus with the aid of a rabbit brain atlas¹⁰.

The electrode assembly consisted of a 9-place miniature socket connected to bipolar concentric stainless steel electrodes. The electrodes were fixed directly to the skull with acrylic resin and secured, in addition, by 4 stainless steel screws fixed in the skull. Stimulation consisted of monophasic rectangular pulses delivered unilaterally with a Sanei stimulator (ES-103-Y) and isolation unit for 30 min., 60 sec. on and 60 sec. off, 190 to 210 μ A, at 0.1 msec. duration, 60 cps. Current flow was monitored with a radio-pulse transformer and oscilloscope, and the EEG activity of the brain was recorded during both stimulation and inter-stimulation periods on a Sanei electroencephalograph (EG-908) to detect whether or not an EEG seizure pattern was induced by the stimulation. Lesions in various sites of the brain were made by electrocoagulation with radio frequency waves between a lesioning electrode tip as anode and an indifferent plate electrode placed in contact with the abdomen. The small lesions of the periventricular arcuate nucleus and the pituitary gland were produced electrolytically with a direct current of 3 mA for 30 seconds. Stimulation experiments were made after a three-week period permitting recovery from cerebral edema caused by lesioning. The implanted rabbits were primed with estradiol benzoate in oil (0.1 mg s.c.) for two days prior to stimulation to ensure an estrous state after the three week recovery period. Electrical stimulation was delivered in unrestrained, unanesthetized rabbits.

Ten percent progesterone in cholesterol was gently melted by using a coil of nichrome wire as a heat source. It was tapped into the bore of 26 gauge stainless steel tubing with care taken to insure that some of the droplet was drawn into the barrel. The outside of the tubing was carefully scraped and cleaned with ether under a dissecting microscope. The amount of hormone at the tip was 50-80 μ g. The tubes were fixed in the skull with the aid of dental cement. Plain tubes with rounded tips were used as control implants.

The ovary was removed from the body 3 weeks after the implantation in order to determine the incorporation of acetate-1-C¹⁴ into ovarian steroids in ovarian homogenates. Following removal of the implanted tubing, the solid progesterone was verified to be on the tips of the steel tubing. The location of the implants electrode and lesion was determined histologically.

A laparotomy was performed after local anesthesia of the abdominal muscle with one percent novocaine and one side of ovary was removed from the body at 45 min. after the stimulation, in order to determine the incorporative rate of acetate-1-C¹⁴ into ovarian steroids with ovarian homogenates. Further, the ovary of the other side was observed to check ruptured ovarian follicles at a re-laparotomy under pentobarbital anesthesia some 48 hours later.

Incubation of the ovarian homogenates was carried out in 50 ml Erlenmeyer flasks. 100 mg of the ovarian homogenates, 5 ml of Krebs-Ringer phosphate buffer of pH 7.2, and 10 μ moles (5 μ c) of sodium acetate-1-C¹⁴ (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) were added to each flask. The flasks were closed and shaken for 60 min. at 37°C in a Taiyo incubator (MI). After incubation, each fraction of ovarian steroids in the tissue homogenates and medium was separated by the method of KAWAKAMI et al.¹¹ and the total radioactivity in each of these fractions was measured by the method of ROBERTS et al.¹². Namely, 1 mg of each of the carrier steroids listed in TABLE 1 was added to incubation flask and the mixture was extracted with ether. The residue of the ether extract was partitioned between ligroin (boiling range 65-110°C) and aqueous methanol. The aqueous methanolic phase containing the polar lipid fraction was evaporated and the steroids were extracted from the water phase with benzene. The phenolic steroid fraction was separated from the neutral fractions by shaking the ben-

zene solution with 1N sodium hydroxide. The neutral fraction was further purified on a silicic acid column chromatography eluting the steroids with benzene containing increasing proportions of ethyl acetate. The neutral steroids were then separated by silicic acid thin layer chromatography in the ethyl acetate/cyclohexane system. The phenolic steroid fraction was separated by silicic acid chromatography using the cyclohexane/ethyl acetate/ethanol system. Each fraction of the steroids was eluted from the thin layer, and was acetylated by pyridine and acetic anhydride. Each fraction of the acetylated steroids was further purified by gas-liquid chromatography (SE-30, phase, 245°C). Total radioactivity in each of these separated steroid fractions was measured in a liquid scintillation spectrometer by using 10 ml of a scintillator system composed of 0.5% PBD (phenylbiphenyloxiazol) and 0.01% POPOP (1, 4-bis-2(5-phenyloxazolyl)-benzene) in toluen.

RESULTS

The present experiment provides two-fold results.

1. The effect of limbic stimulation on steroid biosynthetic patterns in the ovaries with reference to ovulation induction.
2. The feedback effect of progesterone implants in the limbic system on steroid biosynthetic patterns in the ovaries.

Stimulation Experiments

A. Hippocampus.

It was already reported that ovulation was induced by stimulating certain portions of the limbic structure^{9,17}.

In the present experiment ovulation could also be induced by alvear stimulation in 23 out of 27 cases (FIG. 1A). In these ovulation-positive cases, the incorporation of acetate-1-C¹⁴ into progestin was remarkably accelerated. Among them the acceleration of C¹⁴-17-OH-progesterone and C¹⁴-androstedione formation was most relevant. The rate of 20 α -OH- Δ^4 -pregnen-3-one labelling was considerably increased. Acceleration of C¹⁴-progesterone formation was also significant, though not so great as in other steroids. On the other hand, it is to be noted that the incorporation of acetate-1-C¹⁴ into progestin was also found to be accelerated, though to a far less extent, in the remaining ovulation-negative case. In this case, the incorporation of acetate-1-C¹⁴ into the progesterone, androstedione and 20 α -OH- Δ^4 -pregnen-3-one showed a marked increase.

The rate of 17-OH-progesterone labelling was also significantly increased in ovulation-negative cases, but the rate of this increment was very slight as compared with the ovulation-positive cases. The increase of androstedione labelling was also considerably smaller in ovulation-negative cases than in ovulation-positive cases. The incorporation of acetate-1-C¹⁴ into estradiol and estrone was only slightly increased both in ovulation negative and positive cases (TABLE 1).

In none of 8 cases in which electrical stimulation was applied to the *cornu ammonis* of the hippocampus was ovulation produced (FIG. 1A). Biosynthesis

FIG. 1. Transverse reconstruction of rabbit hippocampus (A) and amygdala (B) indicating the extent of area which resulted in ovulation when stimulated. Note: Closed circles indicate the main portions where stimulation caused ovulation. Crosses represent the area where ovulation failed to occur on stimulation. Abbreviations: Alv, alveus; C. AM, cornu ammonis; F. DE, Fascia dentata; SUB. C. Am, subiculum cornu ammonis; N. AM, nucleus amygdale; F. I, fimbria fornix; CH, chiasma nervorum opticus; GL. P, globus pallidus; TR. OLF, tractus olfactorius; TR. OP, tractus opticus; Pu, putamen; Z. PR. S, zona praesubicularis; A. PR. S, area, prae-subicularis; VIII, ventriculus III.

TABLE 1.
Effects of electrical stimulation in hippocampus on ovulation and on biosynthesis of ovarian steroids in rabbit ovary.

Site of stimulation		Ovulation	No. of rabbits	Acetate-1-C ¹⁴ incorporation into ovarian steroids					
				Proges- terone	20 α -OH- Δ^4 - pregnen- 3-one	17-OH- proges- terone	Andros- tedione	Estra- diol	Estrone
Control		—	5	929 $\pm$ 14 (100%)	302 $\pm$ 11 (100%)	233 $\pm$ 10 (100%)	139 $\pm$ 7 (100%)	224 $\pm$ 7 (100%)	27 $\pm$ 1 (100%)
DHPC	Alveus	+	23	1490 $\pm$ 47** (160)	491 $\pm$ 32** (163)	439 $\pm$ 13** (188)	258 $\pm$ 21** (186)	247 $\pm$ 9** (110)	30 $\pm$ 5 (111)
		—	4	1243 $\pm$ 30** (134)	401 $\pm$ 27** (133)	275 $\pm$ 11** (118)	188 $\pm$ 19* (135)	240 $\pm$ 11* (105)	27 $\pm$ 2 (100)
	Cornus ammonis	—	8	1214 $\pm$ 39** (131)	379 $\pm$ 19** (125)	306 $\pm$ 14** (131)	168 $\pm$ 16** (121)	229 $\pm$ 9 (102)	26 $\pm$ 4 (96)
	Fascia dentata	—	9	1226 $\pm$ 41** (132)	359 $\pm$ 25** (119)	331 $\pm$ 22** (142)	179 $\pm$ 20** (129)	236 $\pm$ 17* (105)	27 $\pm$ 4 (100)

Note: Each value represents the mean of dpm per 1 hr per mg of ovarian protein-N $\pm$ SD. with the percentage taking the control (no stimulation) value as 100 in parentheses.

Abbreviations: DHPC, dorsal hippocampus; +, cases in which stimulation caused ovulation; —, cases in which stimulation failed to induce ovulation; * and **, respectively indicate significant difference from the control mean at 5 and 1 per cent level of confidence (t-test.)

of progesterone and 17-OH-progesterone was much accelerated by stimulation. Androstenedione and 20 α -OH- Δ^4 -pregnen-3-one biosynthesis was also, though to a somewhat less degree, accelerated. On the other hand, biosynthesis of estradiol and estrone was not affected by stimulation (TABLE 1).

Stimulation of the *fascia dentata* didn't induce ovulation in any of 9 cases (FIG. 1A). In these cases however, biosynthesis of 17-OH-progesterone and progesterone were considerably accelerated by stimulation. Estradiol and estrone biosynthesis were little affected (TABLE 1, FIG. 1A).

Summarizing the above results, ovulation was induced by stimulating the *alveus* and not the *cornus ammonis* or *fascia dentata* of the hippocampus. However biosynthesis of progestin was accelerated by hippocampal stimulation even without inducing ovulation. With respect to each fraction of progestin, progesterone formation was equally well accelerated by stimulating the *alveus* (ovulation-negative cases), *cornus ammonis* and the *fascia dentata* even though ovulation was not induced.

Biosynthesis of 20 α -OH- Δ^4 -pregnen-3-one was quite well accelerated by stimulating the *alveus* (ovulation-negative cases). An accelerating effect was also found by stimulation of *cornus ammonis* and *fascia dentata*, but to a far

less degree.

Formation of 17-OH-progesterone was most remarkably accelerated by stimulating the *fascia dentata*. Then stimulation of the *cornus ammonis* also accelerated it. Alvear stimulation (ovulation-negative cases) was far less effective than stimulation of other hippocampal portions in accelerating 17-OH-progesterone biosynthesis.

Androstedione biosynthesis was accelerated by alvear stimulation. Stimulating the *fascia dentata* also accelerated the biosynthesis of androstedione. Stimulation of the *fascia dentata* revealed some accelerating effect. On the other hand, stimulation of the *cornus ammonis* was largely ineffective. Estradiol and estrone formation was somewhat accelerated by alvear stimulation but little affected by stimulating the *cornus ammonis* or the *fascia dentata* (TABLE 1).

These results indicate that the electrical stimulation of the hippocampus considerably accelerates progestin formation in the ovaries regardless of whether or not it is accompanied by ovulation. On the other hand, it is also indicated that different portions of the hippocampus had different effects, when stimulated, on ovulation and on biosynthesis of various ovarian steroids.

B. Amygdala.

Stimulation of the intermediate or medial nuclei of the amygdala was in

TABLE 2.
Effects of electrical stimulation in amygdala on ovulation and on biosynthesis of ovarian steroids in rabbit ovary.

Site of stimulation		Ovulation	No. of rabbits	Acetate-1-C ¹⁴ incorporation into ovarian steroids					
				Proges- terone	20 α -OH- Δ^4 - pregnen- 3-one	17-OH- proges- terone	Andros- tedione	Estradiol	Es- trone
Control		—	5	917 $\pm$ 26 (100%)	305 $\pm$ 18 (100%)	239 $\pm$ 18 (100%)	131 $\pm$ 10 (100%)	229 $\pm$ 9 (100%)	25 $\pm$ 2 (100%)
AMYG	Inter- mediate principal nucleus	+	8	1360 $\pm$ 39** (148)	439 $\pm$ 31** (144)	388 $\pm$ 24** (162)	220 $\pm$ 15** (168)	265 $\pm$ 13** (120)	27 $\pm$ 4 (106)
	Medial nucleus	+	5	1351 $\pm$ 43** (147)	445 $\pm$ 23** (146)	380 $\pm$ 11** (159)	217 $\pm$ 7** (166)	260 $\pm$ 14** (118)	29 $\pm$ 5 (116)
	Medial principal nucleus	—	5	1136 $\pm$ 18** (124)	334 $\pm$ 26 (110)	293 $\pm$ 17** (123)	150 $\pm$ 8** (115)	239 $\pm$ 20* (109)	27 $\pm$ 5 (108)
	Lateral nucleus	—	5	1134 $\pm$ 29** (124)	330 $\pm$ 19* (108)	296 $\pm$ 13** (124)	154 $\pm$ 5** (118)	237 $\pm$ 16** (108)	26 $\pm$ 3 (104)

Note: Each value represents the mean of dpm per 1 hr per mg of ovarian protein-N $\pm$ SD. with the percentage taking the control (no stimulation) value as 100 in parentheses.

Abbreviation: AMYG, amygdala.

all cases accompanied by ovulation, but ovulation never occurred after stimulation of the medial principal or lateral nuclei (FIG. 1B). Biosynthesis of ovarian steroids was accelerated not only in ovulation-positive but in ovulation-negative portions, though in the latter, the accelerating effect of stimulation was less remarkable. When ovulation-positive portions were stimulated, the formation of 17-OH-progesterone and androstenedione was most markedly accelerated. Also progesterone and 20α -OH- Δ^4 -pregnen-3-one biosynthesis was considerably accelerated. Biosynthesis of estradiol and estrone was also accelerated but only to a limited extent. On the other hand, when ovulation-negative portions were stimulated, the biosynthesis of progesterone and 17-OH-progesterone was the most facilitated. Formation of androstenedione and 20α -OH- Δ^4 -pregnen-3-one was slightly facilitated. Biosynthesis of estradiol and estrone was little influenced by stimulation (TABLE 2). As described above, the regional differences within the amygdala with regards to the effect of stimulation, were observed.

C. Hypothalamus.

In 6 out of 12 cases in which the periventricular arcuate nucleus was stimulated ovulation was induced. In the other 6 cases stimulation was not

TABLE 3.
Effects of electrical stimulation in hypothalamus or central grey matter on ovulation and on biosynthesis of ovarian steroids in rabbit ovary.

Site of stimulation	Ovulation	No. of rabbits	Acetate-1- C^{14} incorporation into ovarian steroids					
			Progesterone	20α -OH- Δ^4 -pregnen-3-one	17-OH-progesterone	Androstenedione	Estradiol	Estrone
Control	—	5	931 ± 23 (100%)	305 ± 14 (100%)	230 ± 15 (100%)	142 ± 10 (100%)	226 ± 14 (100%)	26 ± 3 (100%)
ARC	+	6	$1340 \pm 47^{**}$ (144)	$434 \pm 3^{**}$ (142)	$338 \pm 11^{**}$ (147)	$206 \pm 7^{**}$ (145)	232 ± 14 (103)	28 ± 2 (108)
	—	6	$1137 \pm 38^{**}$ (122)	$348 \pm 27^*$ (114)	$279 \pm 10^{**}$ (121)	$171 \pm 13^{**}$ (120)	227 ± 11 (100)	26 ± 3 (100)
PMA	—	5	927 ± 20 (100)	310 ± 23 (102)	$249 \pm 10^*$ (108)	133 ± 16 (94)	228 ± 12 (101)	16 ± 1 (100)
SO	$\pm$	5	$1238 \pm 30^{**}$ (134)	$403 \pm 22^{**}$ (132)	321 ± 19 (140)	$220 \pm 11^{**}$ (155)	$242 \pm 10^*$ (107)	27 ± 2 (104)
	—	4	$972 \pm 21^*$ (104)	321 ± 15 (105)	$252 \pm 17^{**}$ (110)	$154 \pm 6^*$ (108)	228 ± 16 (101)	$23 \pm 1^*$ (92)
CG	—	5	918 ± 39 (99)	297 ± 28 (97)	232 ± 12 (101)	137 ± 9 (96)	226 ± 11 (100)	25 ± 3 (96)

Note: Each value represents the mean of dpm per 1 hr per mg of ovarian protein-N $\pm$ SD. with the percentage taking the control (no stimulation) value as 100 in parentheses.

accompanied by ovulation. In both ovulation-positive and ovulation-negative cases, biosynthesis of progesterone, 20α -OH- Δ^4 -pregnen-3-one, 17-OH-progesterone and androstenedione was accelerated, but in the latter acceleration was much smaller than in the former. Formation of estradiol and estrone was not affected by stimulation of the periventricular arcuate nucleus regardless of whether ovulation resulted or not (TABLE 3).

Ovulation was induced in none of 9 cases in which the supraoptic nucleus was stimulated. But in 5 of them large hemorrhagic follicles were observed. In such cases, biosynthesis of progesterone, 20α -OH- Δ^4 -pregnen-3-one, 17-OH-progesterone and androstenedione was notably accelerated. Above all, acceleration of androstenedione biosynthesis was great. Formation of estradiol and estrone, on the contrary was not influenced. On the other hand, in the 4 cases in which neither ovulation nor large hemorrhagic follicles were observed, biosynthesis of ovarian steroids did not seem to be affected by stimulation (TABLE 3).

D. Some other regions.

When the premammillary area was stimulated, ovulation occurred in none

TABLE 4.
Effects of electrical stimulation in several brain sites, LH administration or copulation on biosynthesis of ovarian steroids in ovulated rabbits.

Site of stimulation		Ovulation	Acetate-1-C ¹⁴ incorporation into ovarian steroids					
			Progesterone	20 α -OH- Δ^4 -pregnen-3-one	17-OH-progesterone	Androstenedione	Estradiol	Estrone
Control		—	100%	100%	100%	100%	100%	100%
DHPC (Alveus)		+	160	163	188	186	110	111
AMYG	Intermediate principal nucleus	+	148	144	162	168	120	108
	Medial nucleus	+	147	146	159	166	118	116
ARC		+	144	142	147	145	103	108
SO		±	134	132	148	155	101	104
LH		+	291	308	319	299	138	148
Copulation		+	303	361	346	304	140	148

Note: The results are expressed in percentage, taking the control (no stimulation) value as 100 per cent.

The ovaries were removed from the rabbits 60 minutes after administration of LH (the total dose of the 0.3 units given as a single intravenous injection). LH preparation (Lot No. R 377279) was supplied by Armour Veterinary Laboratories, U.S.A.

of 5 cases. In 5 cases was no ovulation observed as a result of stimulation. The biosynthetic activity of ovarian steroids examined was little affected by stimulation (TABLE 3).

In the case of the central grey matter, stimulation induced no ovulation nor caused any change in the biosynthesis of ovarian steroids (TABLE 3).

TABLE 4 summarizes the change in biosynthesis of various ovarian steroids by stimulating different brain areas of only ovulation positive cases (including the cases in which only large hemorrhagic follicles appeared). In addition the effect of mating and LH administration on ovarian biosynthetic activity is shown for comparison. The accelerating effect of stimulation, though not so large as that of mating and LH injection, is evident. Also, by stimulating any of these brain areas, estradiol or estrone biosynthesis was little affected in contrast to the considerable biosynthesis acceleration caused by mating or LH administration. It is to be noted that when the periventricular arcuate nucleus was stimulated or in the cases of mating or LH injection, acceleration of progesterone, 20α -OH- Δ^4 -pregnen-3-one, 17-OH-progesterone, androstenedione, estradiol and estrone synthesis was observed to approximately the same extent in each case. On the other hand in the cases of hippocampal and amygdalar stimu-

TABLE 5.
Effects of electrical stimulation in several brain sites on biosynthesis
of ovarian steroids in non-ovulated rabbits.

Site of stimulation		Ovulation	Acetate-1-C ¹⁴ incorporation into ovarian steroids					
			Proges-terone	20α -OH- Δ^4 -pregnen-3-one	17-OH-proges-terone	Andros-tedione	Estra-diols	Estrone
Control		—	100%	100%	100%	100%	100%	100%
DHPC	Alveus	—	134	133	118	135	105	100
	Cornu ammonis	—	131	125	131	121	102	96
	Fascia dentata	—	132	119	142	129	105	100
AMYG	Medial principal nucleus	—	124	110	123	115	109	108
	Lateral nucleus	—	124	108	124	118	108	104
ARC		—	122	114	121	120	100	100
SO		—	104	105	110	108	101	92
PMA		—	100	102	108	94	101	100
CG		—	99	97	101	96	100	96

Note: The results are expressed in percentage, taking the control (no stimulation) value as 100 per cent.

lation acceleration of progesterone and 20α -OH- Δ^4 -pregnen-3-one formation was considerably smaller than the other two.

TABLE 5 summarizes the change in biosynthesis of various ovarian steroids by stimulating different brain areas of only ovulation-negative cases. To compare with the results shown in TABLE 4, acceleration of biosynthesis seems much smaller in the ovulation-negative cases. It is to be noted that acceleration of 20α -OH- Δ^4 -pregnen-3-one formation is considerably lower than other steroids in the ovulation-negative cases except in the *alveus* and *cornu ammonis* of the hippocampus. In the cases in which ovulation was not induced by hippocampal or amygdala stimulation, the biosynthetic pattern is also characterized by lack of selective acceleration of 17-OH-progesterone and androstenedione biosynthesis as was observed in ovulation-positive cases.

Lesioning Experiments.

In an attempt to determine the pathway through which the activated impulses of the hippocampus stimulate the hypothalamus and consequently induce the acceleration of steroid synthesis in the ovary, the stria terminalis, the periventricular arcuate nucleus and the fornix, were destroyed (FIG. 2).

When the stria terminalis was bilaterally damaged, the electrical stimulation of the dorsal hippocampus brought about large hemorrhagic follicles in the ovaries. The incorporation of acetate-1- C^{14} into the various progestins showed an increase in these rabbits, whereas there was no effect in the bio-

FIG. 2. Reconstruction of lesions on coronal section of the stria terminalis (A) and the dorsal fornix and periventricular arcuate nucleus (B). Abbreviations: : Inclusive area destroyed by lesions. : Area common to all lesions.

synthesis of estradiol and estrone. These findings may indicate that the effect of hippocampal stimulation on the biosynthesis of ovarian progestin persists even after the stria terminalis was damaged.

On the other hand, in the case of the periventricular arcuate nucleus lesions, hippocampal stimulation was ineffective on the biosynthesis of various steroids in the ovary. These findings suggest that the effect of hippocampal stimulation upon the ovary is exerted through the periventricular arcuate nucleus.

Bilateral massive lesions of the dorsal fornix including its adjacent areas failed to produce ovulation in response to the electrical stimulation of the hippocampus. In this case, synthesis of various progestins was lower than that in the controls. These results may suggest that any effect of hippocampal stimulation upon biosynthesis of ovarian progestin is blocked by the damage of the fornix. These would in turn mean that the effect of hippocampal stimulation upon the ovary is exerted through the fornix.

TABLE 6.

Effects of hippocampal stimulation on ovulation and on biosynthesis of rabbit ovary after lesioning the stria terminalis, periventricular arcuate nucleus or fornix.

Site of lesion	Stimulation	No. of rabbits	Acetate-1-C ¹⁴ incorporation into ovarian steroids					
			Proges- terone	20 α -OH- Δ^4 - pregnen- 3-one	17-OH- proges- terone	Andros- tedione	Estradiol	Estrone
—	—	5	935 $\pm$ 35 (100%)	308 $\pm$ 21 (100%)	240 $\pm$ 11 (100%)	142 $\pm$ 7 (100%)	236 $\pm$ 16 (100%)	24 $\pm$ 4 (100%)
—	—	5	1220 $\pm$ 45** (130)	375 $\pm$ 18** (122)	322 $\pm$ 15** (134)	174 $\pm$ 14** (123)	219 $\pm$ 15 (93)	24 $\pm$ 2 (100)
Stria terminalis	—	6	916 $\pm$ 28 (98)	290 $\pm$ 17 (94)	226 $\pm$ 18 (94)	130 $\pm$ 12 (92)	224 $\pm$ 16 (95)	26 $\pm$ 3 (108)
	+	6	1149 $\pm$ 39** (123)	371 $\pm$ 20** (120)	282 $\pm$ 16** (118)	167 $\pm$ 7** (118)	221 $\pm$ 17 (94)	25 $\pm$ 2 (104)
ARC	—	5	926 $\pm$ 29 (99)	290 $\pm$ 16 (94)	228 $\pm$ 20 (95)	133 $\pm$ 16 (94)	218 $\pm$ 8* (92)	24 $\pm$ 3 (100)
	+	5	928 $\pm$ 41 (99)	293 $\pm$ 22 (95)	224 $\pm$ 16 (93)	135 $\pm$ 14 (95)	217 $\pm$ 10* (92)	25 $\pm$ 1 (104)
Fornix	—	6	829 $\pm$ 34** (89)	274 $\pm$ 16** (89)	209 $\pm$ 7** (87)	124 $\pm$ 6** (87)	213 $\pm$ 11** (90)	24 $\pm$ 1 (100)
	+	6	839 $\pm$ 27** (90)	287 $\pm$ 13* (93)	215 $\pm$ 12** (90)	133 $\pm$ 11 (94)	227 $\pm$ 9 (96)	26 $\pm$ 3 (108)
Pituitary gland	—	5	805 $\pm$ 40** (86)	260 $\pm$ 8** (84)	201 $\pm$ 6** (84)	112 $\pm$ 9** (79)	208 $\pm$ 5** (88)	23 $\pm$ 2 (96)

Note: Each value represents the mean of dpm per 1 hr per mg of ovarian protein-N $\pm$ SD. with the percentage taking the control (no stimulation) value as 100 in parentheses.

Therefore, the marked acceleration of ovarian function induced by electrical stimulation of the hippocampus may chiefly result from a direct stimulation of the periventricular arcuate nucleus through the pathway from the activated hippocampus to the periventricular arcuate nucleus (TABLE 6).

Implantation Experiment.

In order to determine the direct action of progestin on the limbic structures and hypothalamus with respect to feedback mechanisms between the brain and ovarian steroids, a minute amount of solid progesterone propionate was implanted into the hippocampus, amygdala and supraoptic nucleus (FIG. 3).

FIG. 3. Frontal section through bilateral hippocampus (A) and amygdala (B) of the rabbit. The main locations of progesterone implants and their effects on incorporation of acetate-1-C¹⁴ into ovarian steroids. Note: Full circle, increase in C¹⁴ incorporation; open circle, no effect upon steroids.

Minute amounts of crystal progesterone implanted in the hippocampus (CA₁, CA₂ or CA₄) caused elevation of acetate-1-C¹⁴ incorporation into androstenedione, progesterone and 20 α -OH- Δ^4 -pregnen-3-one but biosynthesis of 17-OH-progesterone, estradiol and estrone was little affected (TABLE 7 and FIG. 3A).

Implants in the amygdala (medial nucleus and basomedial nucleus), on the other hand, caused little change in ovarian biosynthesis of progesterone, 20 α -OH- Δ^4 -pregnen-3-one, androstenedione, estradiol and estrone. However there was some decreasing tendency in biosynthesis of 17-OH-progesterone (TABLE 7 and FIG. 3B).

Implants in the supraoptic region or the anterior hypothalamic area produced change in none of the ovarian steroids examined in this experiment (TABLE 7).

These results may suggest the existence of a functional circuit between

TABLE 7.
Effects of implantation of progesterone into limbic structure or hypothalamus
on biosynthesis of ovarian steroids in rabbit ovary.

Site of implantation	No. of rabbits	Acetate-1-C ¹⁴ incorporation into ovarian steroids					
		Proges- terone	20 α -OH- Δ^4 - pregnen- 3-one	17-OH- proges- terone	Andros- tedione	Estradiol	Estrone
Control	5	794 $\pm$ 24 (100%)	250 $\pm$ 11 (100%)	202 $\pm$ 9 (100%)	119 $\pm$ 7 (100%)	219 $\pm$ 11 (100%)	27 $\pm$ 1 (100%)
DHPC	7	929 $\pm$ 36** (123)	305 $\pm$ 13** (122)	218 $\pm$ 12* (108)	159 $\pm$ 11** (134)	218 $\pm$ 12 (99)	25 $\pm$ 3 (93)
AMYG	7	792 $\pm$ 27 (100)	252 $\pm$ 17 (101)	189 $\pm$ 14 (94)	111 $\pm$ 6 (93)	228 $\pm$ 9 (104)	27 $\pm$ 3 (100)
SO+AHA	5	807 $\pm$ 30 (102)	253 $\pm$ 9 (101)	203 $\pm$ 13 (100)	125 $\pm$ 7 (105)	220 $\pm$ 11 (100)	26 $\pm$ 2 (96)

Note: Each value represents the mean of dpm per 1 hr per mg of ovarian protein-N $\pm$ SD. with the percentage taking the control (no stimulation) value as 100 in parentheses. No animal was primed with estradiol benzoate in oil.

the hippocampus and the ovary having positive feedback control of the ovarian progesterone formation. But direct action of progesterone on the amygdala, supraoptic region or anterior hypothalamic area is uncertain.

DISCUSSION

It was proven that in the hippocampus the *alveus* was the only portion in which stimulation was effective in inducing ovulation. The *alveus* mainly consists of axons of the pyramidal cells. In other words, it is an entrance for the afferent fibers toward hippocampus. In addition here are occasionally found Golgi type II cell and basket cells, which form inhibitory synapses with the pyramidal axons¹⁴⁾. Therefore it is hard to find the reason why only the *alveus*, and not the other hippocampal portions, is effective in inducing ovulation from the present anatomical knowledge.

It is an already established fact that ovarian function is in some way or other regulated by the brain via the pituitary body. HAYWARD et al.⁹⁾ had obtained the evidence that electrical stimulation of the hypothalamus or the amygdala could induce ovulation and facilitate 20 α -OH- Δ^4 -pregnen-3-one output from the ovary like copulation or LH administration. Further KAWAKAMI et al.¹¹⁾ found that hippocampal stimulation was also effective in inducing ovulation and progesterone output from the ovary. In this case, it was pointed out that incorporation of acetate-1-C¹⁴ into progesterone by ovarian homogenates increased parallel to the increase in progesterone output from the ovary.

The present study revealed that electrical stimulation of the hippocampus, amygdala or the hypothalamus exerted discrete effects on biosynthesis of various steroid hormones in the ovarian tissue. It was also confirmed by lesion experiment that hippocampal stimulation exerted influences on ovarian function not through secondary excitation of the amygdala, but by acting on the hypothalamo-pituitary system via the fornix. It is noteworthy that the biosynthetic pattern under the influence of hippocampal or amygdalar stimulation was quite different from that observed after hypothalamic stimulation, copulation or LH administration. Thus it is possible to assume that there are some regional difference in the brain control of ovarian function.

HAYWARD⁹⁾ has pointed out the significance of the relationship between ovulation and the output of 20α -OH- Δ^4 -pregnen-3-one from the ovary. The present result showed that when brain stimulation was not accompanied by ovulation the biosynthesis of 20α -OH- Δ^4 -pregnen-3-one was not significantly increased. It is also to be noted that the biosynthetic pattern of ovarian steroids in ovulation-positive case by brain stimulation is quite different from that observed on copulation-induced-ovulation or LH administration. This finding provides a ground for the possibility of the control of biosynthetic patterns in the ovaries by some unknown factors other than LH release from the pituitary body. Therefore it may be concluded, at least that the mechanism regulating ovulation and biosynthesis of ovarian steroids are more complicated than considered at present.

As to the influence of the brain activity on ovarian steroids, the pituitary body and the hypothalamus have been considered the only places which specifically receive information concerning the change in ovarian steroids. In addition, the amygdala has been considered as an associated structure which, according to the change in sex-hormonal condition, regulates the functioning of pituitary-ovarian system. However in a recent series of experiments, KAWAKAMI et al.¹⁵⁾ suggested that the hippocampus was a focus for the positive feedback mechanism for progesterone control, and the amygdala was a focus for negative feedback on the basis of the following results: (1) the electrical activity of the hippocampus was elevated but amygdala activity was lowered after progesterone administration or copulation; (2) also the electrical activity of the hippocampus and the amygdala in rabbits at the various stages of the estrous cycle fluctuated inversely and in the former was elevated at the estrous stage whereas the latter was lowered^{16,18)}.

In this experiment progesterone implants in the hippocampus were found to facilitate biosynthesis of each fraction of progestin in the ovary, which supports the view just described.

However progesterone implantation in the anterior hypothalamus or amygdala proved almost ineffective in producing change in biosynthetic pattern of ovarian steroids. Thus precise and detailed examination is required, further,

concerning the feedback mechanism for regulation of ovarian steroids.

SUMMARY

In order to elucidate the role played by the limbic structure for the regulation of pituitary-ovarian functioning, electrical stimulation was applied to the limbic structure and the hypothalamus through chronically implanted electrodes, and its influence on biosynthesis of various ovarian steroids was studied in the mature New Zealand white rabbit.

The implanted rabbits were primed with estradiol benzoate in oil (0.1 mg s.c.) for 2 days prior to stimulation to ensure an estrous state. Electrical stimulation, consisting of monophasic square wave pulses, was delivered unilaterally for 30 min., 60 sec. on and 60 sec. off, 190–210 μ A, at 0.1 msec. duration, 60 cps. The experimental results as follows.

1. Ovulation was induced in 23 out of 27 cases by stimulating the *alveus* of the hippocampus. Facilitation of biosynthesis of various progestin fractions accompanied ovulation. Stimulation delivered to the *cornu ammonis* and the *fascia dentata* failed to induce ovulation in all of 17 cases. In these cases biosynthesis of progestin fractions was facilitated. Estrogen biosynthesis was little affected by brain stimulation regardless of whether ovulation was induced or not.
2. Ovulation was induced by stimulating the intermediate and medial nuclei of the amygdala in all of 13 cases. Progestin biosynthesis was markedly facilitated in these cases. Estrogen formation was slightly increased. Stimulation applied to the medial principal and lateral nuclei was ineffective in inducing ovulation, but progestin biosynthesis was considerably facilitated. Estrogen formation was not affected.
3. Stimulation of the periventricular arcuate nucleus of the hypothalamus was accompanied by ovulation and by facilitation of progestin biosynthesis. Supra-optic stimulation induced ovulation and facilitation of progestin formation in 5 out of 9 cases. Stimulation delivered to the central grey was ineffective in progestin formation.
4. Ovulation was induced by LH administration or copulation. Biosynthesis of progestin was remarkably facilitated, while estrogen was moderately increased.
5. The influence of hippocampal or amygdalar stimulation on the progestin biosynthetic pattern was quite different from that of copulation, LH administration or stimulation of the periventricular arcuate nucleus.
6. The effects of hippocampal stimulation on ovarian functions were cancelled by destroying the fornix or periventricular arcuate nucleus but not by lesioning the stria terminalis.
7. Progesterone implants in the hippocampus facilitated progestin biosynthesis

in the ovary. Thus hippocampus can be regarded as a focus concerned with positive feedback regulation of progesterone release.

8. Progesterone implants in the amygdala or supraoptic region or anterior hypothalamic area were almost ineffective for the steroid biosynthetic pattern of the ovary.

9. Thus the limbic system, above all the hippocampus, plays a significant role in the brain-pituitary-ovarian system.

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