cells. In the rostral part of the AP, which contains corticotroph (ACTH), thyrotroph (TSH) and prolactin (PRL) cells[14,15], ir fibers were encountered in digitations of the NH and were characterized by an accumulation of small clear vesicles facing the basement membrane (fig., B). In addition, ir profiles were also observed in close apposition to the AH cells, in particular PRL cells (fig., B). In some cases, an accumulation of small clear vesicles, facing a slight thickening of the fiber membrane, suggested a synaptic-like contact (fig., B). However, no really clear picture of membrane differentiation could be observed. The proximal part of the AP contains gonadotrophs (GTH) and growth hormone (GH) cells[18,19] which are easily differentiated at the electron microscopic level on the basis of their ultrastructural aspect. Both cell types were found in close apposition to type B ir fibers (fig., C) and synaptic-like contacts were sometimes encountered. In the NIL, ir fibers were present in the NH and in apposition to the two cell types of the pars intermedia, MSH-secreting cells and calcium-sensitive cells[20].

Discussion. The immunochemical and immunocytochemical controls described suggest that ir structures contain GABA. The results provide direct evidence that GABAergic terminals of CNS origin are present in the AP, establish direct synaptic-like contacts with the secretory cells, and thus, most likely, influence their activity. To date, except for a role in ACTH secretion[21], little is known about the effects of GABA on AP hormone secretion in teleosts. In mammals, it has been shown that GABA acts, either directly or via the hypothalamus, on the secretion of all AP hormones[1-4]. In support of a hypophysiotrophic role of GABA in the AP of mammals, the mediator itself[1], high-affinity receptors for GABA[22,23] and GABA-transaminase activity[1] have been found at the level of the AP, while GAD activity could be detected only in the NIL[1]. GAD-ir fibers in the external layer of the median eminence[8,10], and the presence of measurable amounts of GABA in the portal blood[24,25] were also reported. Nevertheless, except for a direct inhibitory effect at the level of the AP on PRL release[1,4], the site of action of GABA on other AP functions, either hypothalamic or hypophyseal, remains unclear. On the other hand, a function of GABA has been demonstrated in the IL of mammals where GAD-ir fibers were found in direct contact with the MSH cells[7,9]. At this level, GABA has been shown to modulate hormone output[6] by affecting the electrophysiological properties of the cells[5]. Although its functional significance remains to be established, the direct GABAergic innervation of all cell types in the pituitary of teleosts suggest that GABA is involved in the regulation of some, if not all, anterior pituitary functions.

Acknowledgment. This work was supported by the CNRS and the Foundation for Medical Research (A.C.).

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0014-4754/87/0300-03$1.50 + 0.20/0

Hormonal facilitation in the release of sperm from the spermatheca of the red-spotted newt

M.P. Hardy[1] and J.N. Dent

Department of Biology, Gilmer Hall, University of Virginia, Charlottesville (Virginia 22901, USA), 6 May 1986

Summary. Several neurotransmitters and hormones with potential to trigger a simultaneous contraction of the oviducts and the spermathecal myoepithelium were examined. Saline (0.05 ml), or 0.05 ml saline plus acetylcholine (9 mg), norepinephrine (50 µg), arginine-vasotocin (25 units), prostaglandin $F_{2\alpha}$(3 µg) were injected into the spermathecal region of female newts (n = 24 per group). The numbers of sperm present in the cloacae of prostaglandin-injected animals (107 ± 30 SEM) were significantly greater than the numbers detected in saline (27 ± 5 SEM) and in uninjected (14 ± 3 SEM) controls. Smaller and less consistent increases in the numbers of sperm were detected in the vasotocin- and norepinephrine-injected groups. Study of sections from ovulating female newts failed to produce evidence that pressure from the passage of ova through the posterior portion of the oviduct forced sperm from the spermatheca. Observations indicate an active role for the spermathecal myoepithelium in the discharge of stored sperm and of a role for prostaglandin $F_{2\alpha}$ in triggering that discharge.

Key words. Spermatheca; newt spermatheca; sperm storage; sperm discharge from storage; hormonal action in sperm discharge; prostaglandin and sperm discharge.

Fertilization occurs internally in most species of urodele amphibians. After insemination sperm are stored in the spermatheca, a set of glandular diverticula in the cloaca of the female, for prolonged periods. It has been proposed[3] that storage of sperm enables the female to select times and locations for egg-laying that are best suited to the survival of progeny.

In the red-spotted newt the spermatheca is composed of a group of 20 to 40, convoluted, flask-shaped tubules that open from the dorsal wall of the cloaca, two to three millimeters anterior to the vent[4]. In them sperm may be stored in a fertilizable state for up to at least six months prior to oviposition[3]. A mechanism for synchronizing the meeting of sperm and ova in the cloaca appears to be required.

Kingsbury[4] first reported that a muscular sheath surrounds each spermathecal tubule and proposed that sperm are discharged into the cloaca as a result of muscular contractions. Boisseau and Joly[2] have suggested, however, that mechanisms other than spermathecal contraction may assist in the release of sperm in some urodele species. They propose that during its passage through the posterior position of the oviduct, the egg compresses the spermatheca, forcing stored sperm from it; or that sperm may be stimulated to swim actively out of the spermatheca.

In the present study, observations on effects of stimulants of smooth muscle contraction and on temporal relations between the movements of eggs and sperm in ovulating females indicate that spermathecal contraction is the primary mechanism by which sperm are released from storage in the female newt.

Materials and methods. Female red-spotted newts (*Notophthalmus viridescens viridescens,* Rafinesque) in breeding conformation were collected from ponds in central Virginia and were stored at 4 °C. Animals that weighed three grams or more were selected for experimental use.

Female newts were kept in aerated gravel-bottomed aquaria that had been amply furnished with *Anachris,* at 22 °C under a light cycle of 16 L:8 D. Those conditions simulated the natural pond environment during late May to early June when egg-laying of the red-spotted newt is at its peak. Ovulation was induced by i.p. injection on alternate days of 200 U of human chorionic gonadotropin in 0.05 ml of saline.

Egg-laying behavior commenced about one week after the first injection. Eggs of the red-spotted newt are laid singly and are wrapped in the leaves of aquatic plants. The exact moment of extrusion is concealed, however, because in the egg-laying posture the female clasps the foliage tightly against her vent. In our study, the durations of individual acts of egg-deposition were measured from the time the laying posture was assumed until the female released the *Anachris* foliage and swam away. The mean period of deposition was found to be six minutes (SEM = ± 3).

With the intent of obtaining sections of the cloaca during the passage of the ovum through the posterior region of the oviduct, eight ovipositing animals were removed from the *Anachris* and were rapidly frozen 6 min after they had assumed the egg-laying posture. Those animals were fixed for 24 h at 22 °C in Gregg and Puckett's fixative[8]. Fixed material was sectioned at 6 µm and stained according to the polychrome procedure of Shoobridge[9].

Through the side of the cloacal mound and into the spermathecal region of each of eight female newts, 0.05 ml of 20% Holtfreter's saline solution or 0.05 ml of saline plus 9 mg acetylcholine chloride, 50 µg of norepinephrine, 25 U of arginine-vasotocin or 3 µg of prostaglandin $F_{2\alpha}$ were injected. The doses were selected for maximal potency for stimulation of muscular contraction[5]. The animals were put into aged tap water and were frozen quickly one hour later by immersion in Freon 12 cooled with liquid nitrogen. The experiment was repeated three times and the data were pooled to give a sample size of 24 in each group. The frozen animals were fixed in Bouin's solution for 24 h at 22 °C, whereupon the cloacal regions were removed and prepared for histological examination. The numbers of sperm present in the cloacae were tabulated. Wide variation within the groups was observed and attributed to variation among female newts in the numbers of sperm sorted within the spermatheca. Rank transformations of the raw data were obtained to approximate more closely the assumptions of analysis of variance[6]. The groups were compared by analysis of variance followed by Dunn-Sidak's method of multiple comparisons[7].

Results. Discharge of sperm in response to neuromuscular stimulants. Upon the injection of stimulants of muscular contraction (fig. 1) only prostaglandin $F_{2\alpha}$ elicited a statistically significant increase in the number of sperm present within the cloacal lumen (107 ± 30 sperm) when compared to both uninjected (14 ± 3) and saline-injected (27 ± 5) control animals ($p < 0.01$). Numbers for animals injected with norepinephrine (55 ± 10) and arginine-vasotocin (61 ± 13) differed significantly from those of uninjected controls (at $p < 0.05$ and $p < 0.01$, respectively) but neither was significantly different from those of saline-injected controls. Acetylcholine apparently had no effect on the number of sperm released (20 ± 14) from the spermatheca.

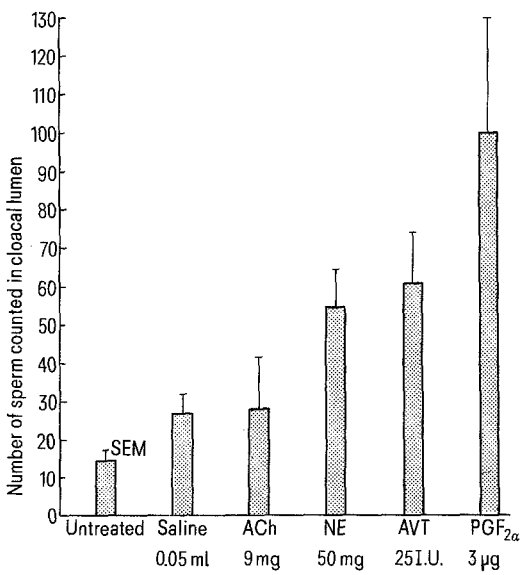

Figure 1. Mean numbers of sperm accumulated within the cloacae of female newts in response to stimulants of smooth muscle contraction. ACh = acetylcholine, NE = norepinephrine, AVT = arginine-vasotocin, $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$.

In control and acetylcholine-injected animals sperm were found occasionally in the central and ventral regions of the cloacal lumen, but rarely in the vicinity of spermathecal orifices (fig. 2). In animals injected with norepinephrine, arginine-vasotocin, or prostaglandin, however, sperm were frequently observed within or near the openings of spermathecal tubules.

Release of sperm during the passage of ova through the oviduct. In one of the animals fixed during oviposition, the egg was being extruded from the vent at the time of fixation, but in seven others the egg was either in the terminal portion of the oviduct or in the oviducal papilla. In two specimens, including one in which the egg extended from the vent, no sperm were found in the cloaca. Those animals had apparently not been inseminated, since in neither were sperm detected in the spermatheca.

Passage of the egg through the oviducal papilla into the cloaca did not appear to compress the expanded distal portions of spermathecal tubules (fig. 2). A mean of 65 sperm (SD = ± 21) was observed in the cloacae of the animals of this series. Those sperm were nearly all located within or near the openings of spermathecal tubules, as were those observed in the cloacae following the injection of neuromuscular stimulants.

Discussion. Fox[10] suggested that stored sperm are released into the oviducts of snakes when an egg in its passage through the oviduct exerts pressure on the walls of the spermatic receptacles. Grigg[11] tested this mechanism in the hen. Sperm were extruded

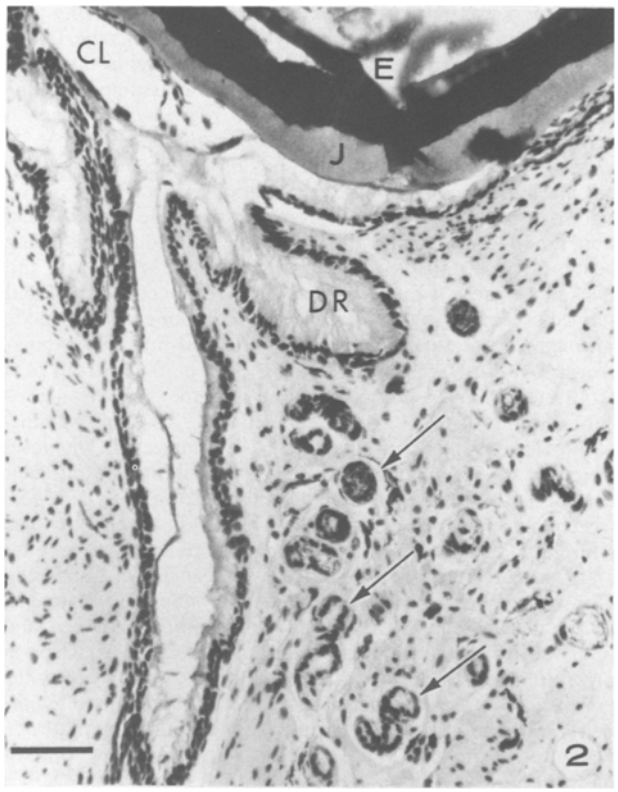

Figure 2. Section of the spermathecal region of an ovulating female newt. An egg (E) with surrounding jelly layer (J) has reached the cloacal lumen (CL) at the level of the dorsal recess (DR) of the cloaca and is in a position to exert pressure on spermathecal tubules (arrows); however, the tubules show no sign of collapse. Bar = 100 µm.

from the infundibular sperm glands after artificial ova were passed through the anterior oviduct. He proposed that the egg stretches the walls of the oviduct during its passage in vivo, compressing the sperm glands and forcing the discharge of sperm. In contrast, we found that in ovulating newts, sperm were apparently expelled from the spermatheca before the egg moved into the cloacal region of the urogenital tract. Similarly, in *S. Salamandra,* Joly and Boisseau[12] demonstrated that sperm are released from the spermatheca and move into the posterior portion of the oviduct before the arrival of the egg. Further evidence against the involvement of passive compression of the spermatheca in the discharge of sperm comes from our observation that the spermathecal tubules were not collapsed in specimens in which an egg had entered the oviducal papilla, even though while in the papilla the egg would seem to have been poised ideally to exert maximal pressure against the spermatheca. Finally, pressure brought about by the injection of 0.05 ml of saline into the spermathecal region failed to elicit a significant increase in the number of cloacal sperm compared to uninjected control animals.

Sperm are quiescent within the spermatheca of the newt[13]. It is possible that sperm are activated by a secretory infusion of substances into the spermathecal lumina just prior to oviposition and that they then swim out into the cloaca. Increased secretory activity is characteristic of the spermatic receptacles of the snake and of the hen during ovulation[14,15]. Dent[16] noted, however, that the spermathecae of ovulating female newts did not exhibit greatly increased secretory activity and Hardy (unpublished observations) observed that spermathecal sperm remain quiescent in ovulating animals. We suggest that the sperm are activated to motility after their expulsion into the cloaca by the consequent exposure to the fluid milieu of that chamber, probably by a lowering of ambient osmolality, by exposure to a diffusable component of the egg jelly coat, or possibly, some combination of the two[13].

Dent[16] presented ultrastructural observations leading to the conclusion that contraction of the spermathecal myoepithelium expels sperm into the cloaca of the ovulating female red-spotted newt. He suggested that such contractions may derive phylogenetically from the original glandular function of the spermatheca. Myoepithelial cells also ensheath the spermathecae of *S. salamandra*[2] and of the dwarf salamander[17].

The present data support further the proposition that sperm are discharged by active contractions of the spermatheca of the red-spotted newt.

Acetylcholine, arginine-vasotocin, and norepinephrine have each been shown to stimulate contractions of the urodele oviduct[18]. The data presented in figure 1 indicate that acetylcholine probably does not elicit contractions of the spermatheca. Stimulation by arginine-vasotocin and norephinephrine of spermathecal discharge is suggested, although those agents did not increase the numbers of cloacal sperm significantly over the numbers obtained by injection of saline alone. In contrast, prostaglandin $F_{2\alpha}$ elicited a clear and substantial increase in the number of cloacal sperm. We infer that synchronization of the discharges of eggs and of sperm into the cloaca of the newt is probably mediated by prostaglandin $F_{2\alpha}$.

It is of interest that the numbers of sperm released in response to neuromuscular stimulants were of similar orders of magnitude to the numbers in the cloacae of ovipositing female newts.

The small numbers of sperm in the cloacae of the uninjected control animals did not appear to have been expelled recently from the spermatheca because they were found in or near the openings of the spermatheca in only two of 24 specimens. Those sperm may have persisted in the cloaca after having failed to enter the spermatheca following earlier insemination, but that is improbable since all animals continued to void solid waste through the cloaca during storage prior to use. It seems more likely that small numbers of stored sperm are continually lost from the spermatheca by spontaneous leakage.

Acknowledgements. This research was supported in part by USPHS Grant # 5-R01-HD16386 and by Developmental Training Grant # 5-T32-HD07192-05 from the U.S. NIH.

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