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ABSTRACT—Hormonally induced changes in synaptic inputs to Mauthner cells (M cells) were examined in adult male red-bellied newts, *Cynops pyrrhogaster*. Hypophysectomized, castrated newts were treated with saline, prolactin, testosterone propionate or both prolactin and testosterone propionate every other day for three weeks. The medulla oblongata containing M cells was examined at ultrastructural level. In the hypophysectomized, castrated newts treated with prolactin, testosterone propionate or both prolactin and testosterone propionate, the mean number of synapses onto somata of M cells were significantly larger than that in the hypophysectomized, castrated animals treated with saline. There were no differences in the parameter among the prolactin-, testosterone propionate- and both prolactin and testosterone propionate-treated animals. These findings suggest that prolactin and androgen are critical for maintaining reorganization of synaptic inputs to somata of M cells in the adult male newts.

INTRODUCTION

Sexually active male urodeles perform a series of reproductive behavior (Salthe and Mecham, 1974; Arnold, 1976, 1977; Halliday 1977; Moore, 1987). In the Genus *Cynops* and *Triturus*, a sexually active male vibrates his tail vigorously in front of a female partner at an early stage of courtship behavior. Around the reproductive period, tail height of sexually developed male newts increases markedly. The enhancement of tail movement and well-developed tail fin appear to result in generating a water flow efficiently for carrying sex attractants from a male to a female (Salthe, 1967). Grant (1966) stated that prolactin is a crucial factor in eliciting courtship behavior in male newts. In fact, prolactin has been shown to promote the growth of the tail fin of various species of newts (Tuchmann-Duplessis, 1949; Vellano *et al.*, 1970; Singhas and Dent, 1975; Kikuyama *et al.*, 1986). Moreover, a combination of prolactin and androgen has been demonstrated to elicit the tail movement (Kikuyama *et al.*, 1980; Malacarne *et al.*, 1982; Toyoda *et al.*, 1993) and development of abdominal glands of the cloaca (Kikuyama *et al.*, 1975; Norris *et al.*, 1989) which secrete female-attracting substance(s) (Toyoda *et al.*, 1994). Recently, a decapeptide, sodefrin that has a potent female-attracting activity was isolated from the abdominal glands of male red-bellied newts (Kikuyama *et al.*, 1995). Synthesis and release of the attractant is also facilitated by treatment with a combination of prolactin and androgen (Yamamoto *et al.*, per-

sonal communication). The fact that blood levels of prolactin (Matsuda *et al.*, 1990) and androgen (Lofts, 1974; Tanaka and Takikawa, 1983) are much higher in males in the breeding season than those in the non-breeding season supports the hypothesis that these hormones could participate in performance of courtship behavior in male newts.

Mauthner cells (M cells) in urodeles, anuran larvae as well as in fish are located at the level of the VIII cranial nerve root in the medulla oblongata (Stefanelli, 1951; Korn *et al.*, 1990; Will, 1991). In urodeles an M cell has two main dendritic trees; a medioventral and a lateral one (Will, 1991). The latter is clearly separated into a slender dorsolateral and a broader lateral branch. The M cells receive inputs which originate from the vestibular nuclei, lateral line organs, trigeminal nucleus and mesencephalon. Numerous electrophysiological, neuroanatomical and behavioral studies have revealed that the M cells are involved in the tail movement (Korn *et al.*, 1990). Since a combination of prolactin and androgen have been shown to fully enhance the tail movement during male courtship behavior (Toyoda *et al.*, 1993), these hormones may exert a stimulatory influence on neuronal functions of the M cells in sexually active male newts. Matsumoto *et al.* (1995) pointed out that treatment of hypophysectomized male newts with both prolactin and gonadotropin increases size of nuclei and cell bodies of the M cells. The fact suggests that the M cells of adult male newts retain a great deal of plasticity in response to the hormonal environment. Hormonal regulation of somatic membranes of the M cells implies that the synaptic input to the M cells is concomitantly altered. In the present study, as one step to clarify hormonal regulation of synaptic organiza-

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tion of the M cells, we performed quantitative electron microscopic analysis on M cells in hypophysectomized, castrated male adult red-bellied newts with or without hormonal manipulation.

MATERIALS AND METHODS

Adult male red-bellied newts, *Cynops pyrrhogaster*, weighing 6-7 g, were obtained from a commercial dealer. They were kept in the laboratory for approximately three weeks before sacrifice under conditioned photoperiod (12 hr light/12 hr dark) and temperature (23 ± 1°C) , and fed daily with *Tubifex* worms.

Twenty four male newts were used, 6 per group. They were castrated under anesthesia with 0.01% MS222 (Sigma). One month later, hypophysis of each castrated animal was removed through a small incision made in the sphenoid bone beneath the hypophyseal region under anesthesia with 0.01% MS222. The animals were treated with physiological saline, ovine prolactin (PRL, 2 IU, Sigma) , testosterone propionate (TP, 10 μg, Sigma) or both PRL (2 IU) and TP (10 μg) every other day for three weeks after hypophysectomy. The day following the last injection, they were perfused transcardially with a mixture of 1% paraformaldehyde and 1% glutaraldehyde in 100 ml 0.1 M phosphate buffer (PB, pH 7.4) under anesthesia with 0.01% MS222. The brain stem in each animal was removed, placed in the same fixative for 5 hr and rinsed in 0.1 M PB for 24 hr at 4°C. The brain stems were cut transversely with a Vibratome (Lancer Instruments, St. Louis) into 200 μm sections. After a short rinse in 0.1 M PB, the sections were postfixed in 1% OsO_4 in 0.1 M PB for 2 hr at 4°C. Then, the sections were dehydrated in graded ethanols and embedded in epoxy resin. Thick sections (1-2 μm in thickness) of the brain stems were stained with toluidine blue to identify M cells containing nucleus. Adjacent ultrathin sections were stained with uranyl acetate and lead citrate and examined under a JEOL 1200EX electron microscope. Fixation of one animal treated with both PRL and TP was so bad that 5 ones were examined.

For each animal, electron micrographs were taken of the perimeter of an M cell body through the plane of the nucleus (final magnification of 10,400X). We analyzed the amount of synaptic afferent contact onto the somatic membrane. Neuronal contacts were classified into two types. (1) The contact of an axon terminal containing synaptic vesicles which possessed thickening of the pre- and postsynaptic membranes was classified as a synaptic contact (Fig. 3). (2) The contact of an axon terminal without membrane specialization was classified as a synaptoid contact (Fig. 3). The perimeters of somata and the covering lengths of synaptic contacts and synaptoid contacts were measured with a digitizing tablet and microcomputer. The percent covering of neuronal contacts (total length of neuronal contact/perimeter of soma), the frequency of neuronal contacts (number of neuronal contacts/μm of somatic membrane) and the size of neuronal contacts (total length of neuronal contacts/number of neuronal contacts) were calculated for each type of neuronal contacts. Since in the present study attention was focused on the analysis of cell bodies of the M cells, analysis of main dendritic trees was not carried out.

Tail heights were recorded on both the first day of hormone treatment and day of sacrifice.

Statistical analysis was made by ANOVA and Duncan's multiple range test.

RESULTS

The M cells were located at the boundary between the white and gray matters of the medulla oblongata at the level of the VIII cranial nerve root (Fig. 1). Their cell bodies were oriented obliquely or nearly horizontally at the border of the white and gray matters. They were large and pale cells with prominent nucleus. In the cytoplasm, various cytoplasmic organelles such as rough endoplasmic reticulum, mitochondria, Golgi apparatus and lysosomes were observed to be lined up along the main cell axis (Fig. 2). Although myelinated fibers were observed in the proximity of M cells, the neuropil immediately adjacent to the cells was fully occupied by unmyelinated axons, dendrites and glial cells. Most of the neuronal contacts on somatic membranes consisted of synaptic and synaptoid contacts which contained a number of spherical synaptic vesicles (about 50 nm in diameter) and mitochondria (Fig. 3). Occasionally, a small number of large granular vesicles (about 100 nm in diameter) were found to coexist with spherical vesicles in the axon terminals. The incidence of nerve endings containing flat vesicles (Fig. 4) and club endings (Fig. 5) was very low. The cytoplasm of the club endings was filled with neurofilaments and scattered neurotubules running parallel to the long axis of the fibers and contained spherical synaptic vesicles and mitochondria.

In the hypophysectomized, castrated newts treated with PRL, TP or both PRL and TP, the mean length of neuronal perimeters of M cells (F=4.53; Saline *vs.* PRL or TP, p<0.05; Saline *vs.* PRL + TP, p<0.01; Table 1) and mean number of synapses onto somata of M cells (F=3.71, p<0.05, Table 2) were significantly larger than those in the hypophysectomized, castrated animals treated with saline. These parameters were not different among the PRL-, TP- and both PRL and TP-treated animals. There were no significant differences in the percent covering of synaptic contacts (F=2.29) and frequency of synaptic contacts (F=0.23) among the groups examined (Table 2). The mean size of synaptic contacts in the TP- or both PRL and TP-treated newts (F=4.41, p<0.01) was significantly larger than that in the saline-treated ones. The parameter was not significantly different between the saline- and PRL-treated groups, and between the PRL- and TP- or both PRL and TP-treated groups. As for synaptoid contacts, all of the measures were not significantly different among the groups examined (number of synaptoid contacts, F=2.11; percent covering of synaptoid contacts, F=0.26; frequency of synaptoid contacts, F=0.14; size of synaptoid contacts, F=1.86; Table 3). The number of axon terminals containing flat vesicles was very small and not significantly different among the groups examined (F=0.51, Table 4). The incidence of club endings was quite low in all of the groups. Only one club ending was observed in one out of 6 animals in the saline- or TP-treated group. Three and two club endings (one/each animal) were observed in 3 out of 6 animals in the PRL-treated animals and in 2 out of 5 animals in the both PRL and TP-treated animals, respectively.

There was significant difference in mean percent increase in tail height between the saline- or TP-treated groups and PRL- or both PRL and TP-treated groups (F=7.97, Table 5). The mean percent increase in tail height in the PRL- and both PRL and TP-treated animals was significantly larger than that in the saline- (p<0.01) or TP-treated ones (p<0.05). The value in the TP-treated newts was not significantly different from
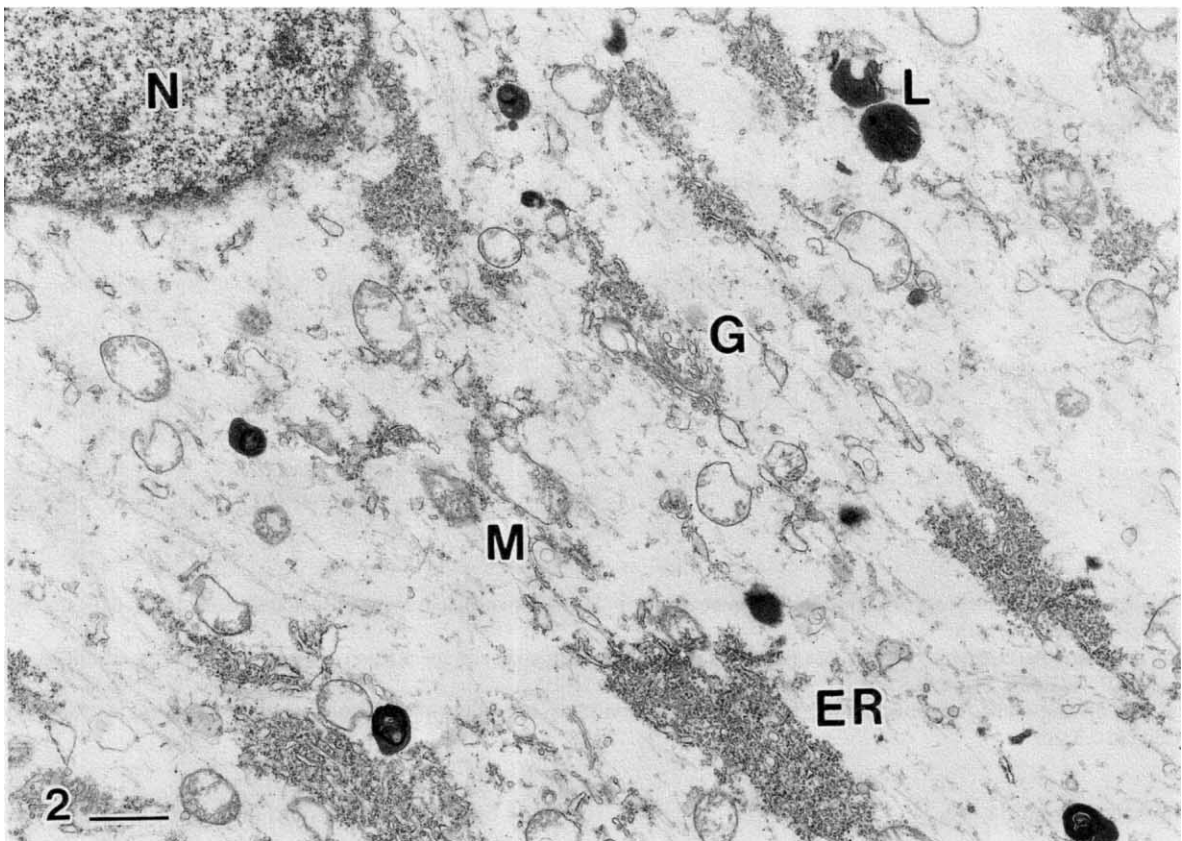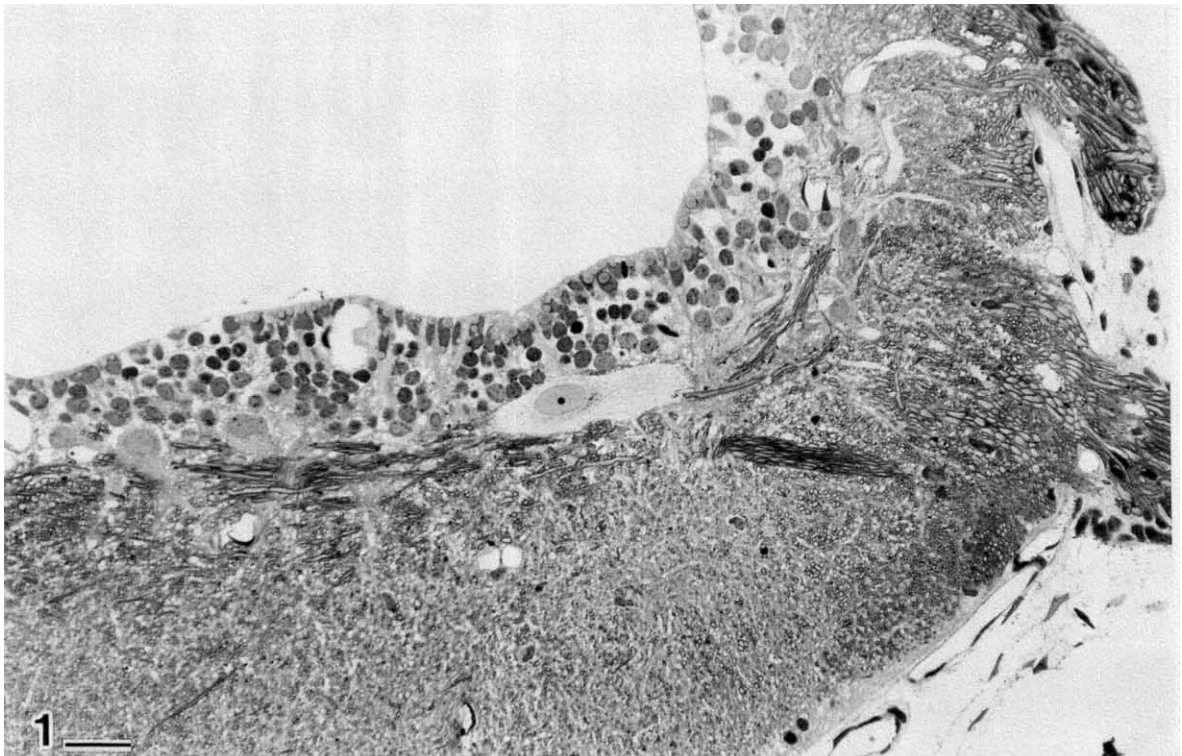
Fig. 1. An M cell of a hypophysectomized, castrated newt treated with PRL and TP is located at the boundary between the white and gray matters of the medulla oblongata at the level of the VIII cranial nerve root. The cell body is oriented obliquely at the border of the white and gray matters. Stained with toluidine blue. X170. Scale; 50 μm.

Fig. 2. A cell body of an M cell in a hypophysectomized, castrated newt treated with PRL and TP. Rough endoplasmic reticulum (ER) is well developed, and mitochondria (M), the Golgi apparatus (G) and lysosomes (L) are prominent. N; nucleus. X10,400. Scale; 1 μm.

Fig. 3. Synaptic (arrow) and synaptoid (arrowhead) contacts onto an M cell soma (S) in a hypophysectomized, castrated newt treated with PRL and TP. X19,200. Scale; 0.5 μm.

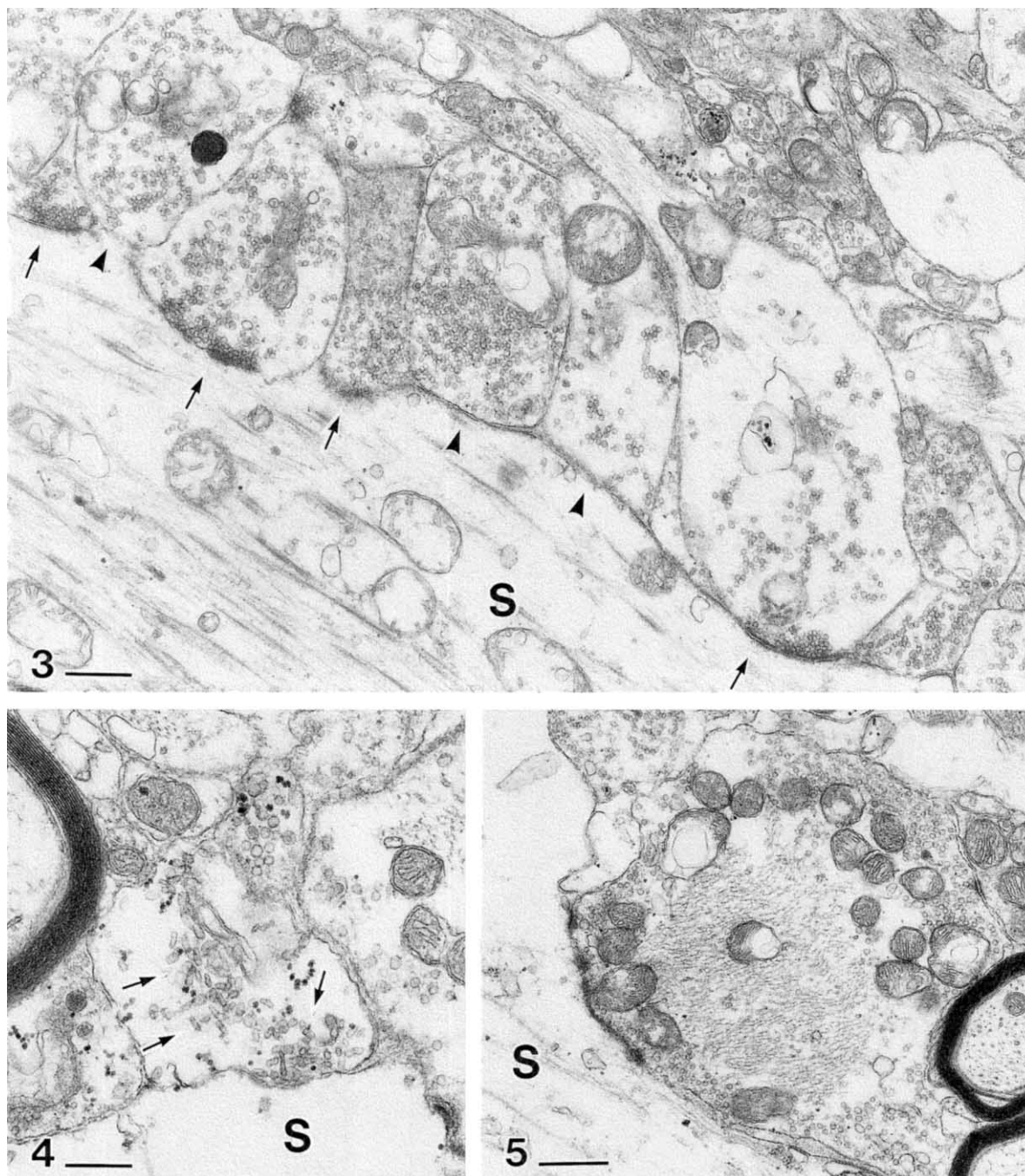**Fig. 4.** An axon terminal containing flat vesicles (arrow) makes contact with an M cell soma (S) in a hypophysectomized, castrated newt treated with PRL and TP. X32,000. Scale; 0.3 μm.

Fig, 5. A club ending makes synaptic contact with an M cell soma (S) in a hypophysectomized, castrated newt treated with PRL and TP. X19,200. Scale; 0.5 μm.

that in the saline-treated ones.

DISCUSSION

The present study is first demonstration of hormonal alteration of synaptic input to the M cells in urodeles. We demonstrated that treatment of hypophysectomized, castrated male newts with prolactin, androgen or both prolactin and androgen induces significant increase in the number of synaptic contacts onto M cells of adult male newts. A combination of prolactin and gonadotropin has previously been reported to increase size of nuclei and cell bodies of the M cells of adult male newts (Matsumoto *et al.*, 1995). Together with the previous study, these findings suggest that in adult newts, the

Table 1. Effect of hormones on length of the perimeter of somata of the M cells in control and experimental animals

Group	Number of newts	Perimeter of M cells (μm)
Saline	6	306 ± 44
Prolactin (PRL)	6	583 ± 91[a]
Testosterone propionate (TP)	6	542 ± 66[a]
PRL + TP	5	670 ± 83[b]

Means ± SEM are given. Comparison with Saline; [a]p<0.05, [b]p<0.01.

Table 2. Effect of hormones on number of synaptic contacts, percent covering of synaptic contacts, frequency of synaptic contacts and size of synaptic contacts on somata of the M cells in control and experimental animals

Group	Number of newts	Number of synaptic contacts	Percent covering of synaptic contacts (%)	Frequency of synaptic contacts	Size of synaptic contacts (μm)
Saline	6	100 ± 14	48.4 ± 2.3	0.34 ± 0.03	1.49 ± 0.06
Prolactin (PRL)	6	217 ± 46[a]	56.7 ± 5.3	0.36 ± 0.04	1.66 ± 0.04
Testosterone propionate (TP)	6	183 ± 21[a]	58.5 ± 1.7	0.34 ± 0.02	1.77 ± 0.06[b]
PRL + TP	5	218 ± 22[a]	57.2 ± 2.3	0.33 ± 0.03	1.76 ± 0.08[b]

Means ± SEM are given. Comparison with Saline; [a]p<0.05, [b]p<0.01.

Table 3. Effect of hormones on number of synaptoid contacts, percent covering of synaptoid contacts, frequency of synaptoid contacts and size of synaptoid contacts on somata of the M cells in control and experimental animals

Group	Number of newts	Number of synaptoid contacts	Percent covering of synaptoid contacts (%)	Frequency of synaptoid contacts	Size of synaptoid contacts (μm)
Saline	6	70.2 ± 8.8	27.8 ± 2.9	0.24 ± 0.02	1.17 ± 0.04
Prolactin (PRL)	6	115.0 ± 25.2	23.7 ± 2.3	0.19 ± 0.02	1.35 ± 0.07
Testosterone propionate (TP)	6	114.2 ± 14.0	27.6 ± 0.6	0.21 ± 0.00	1.31 ± 0.04
PRL + TP	5	132.6 ± 15.9	25.9 ± 1.6	0.20 ± 0.01	1.31 ± 0.09

Means ± SEM are given.

Table 4. Effect of hormones on number of axon terminals containing flat vesicles on somata of the M cells in control and experimental animals

Group	Number of newts	Number of axon terminals containing flat vesicles
Saline	6	4.00 ± 0.82
Prolactin (PRL)	6	4.50 ± 1.59
Testosterone propionate (TP)	6	5.00 ± 1.37
PRL + TP	5	6.60 ± 2.25

Means ± SEM are given.

Table 5. Effect of hormones on percent increase in tail height in control and experimental animals

Group	Number of newts	Percent increase in tail height (%)
Saline	6	–2.17 ± 1.37
Prolactin (PRL)	6	6.18 ± 1.54[a]
Testosterone propionate (TP)	6	0.13 ± 1.63
PRL + TP	6	7.13 ± 1.87[a]

Means ± SEM are given. [a]Comparison with Saline; p<0.01, comparison with TP; p<0.05.

M cells can retain a great deal of morphological and synaptic plasticity which is regulated under the influence of hormonal environment. Circulating levels of prolactin (Matsuda et al., 1990) and androgen (Lofts, 1974; Tanaka and Takikawa, 1983) in male newts have been reported to elevate around the period when they show a courtship behavior and treatment with both prolactin and androgen markedly enhances male courtship behavior (Toyoda et al., 1993). There seems to be little doubt, therefore, that changes in the synaptic input to the M

cells and changes in the morphology of the M cells themselves must result in important changes in the function of the M cell system.

Injection of antiserum against newt prolactin to male newts which had been exhibiting courtship behavior in the field diminishes both incidence and frequency of the behavior, indicating the involvement of endogenous prolactin in the expression of courtship behavior (Toyoda et al., 1996). Hypophysectomy induces a cessation of tail movement during court-

ship behavior in male newts (Kikuyama *et al.*, 1980; Malacarne *et al.*, 1982). Although treatment of hypophysectomized male newts with prolactin partially restores courtship activity (Kikuyama *et al.*, 1980; Malacarne *et al.*, 1982), replacement with both prolactin and gonadotropin sufficiently sustains the behavior (Kikuyama *et al.*, 1980). On the other hand, sexual behavior in male newts is depressed by castration and partially restored by replacement with androgen (Andreoletti *et al.*, 1983; Moore and Zoeller, 1979). From these observations, it is suggested that prolactin participates synergistically with androgen in eliciting the behavior. Recently, Toyoda *et al.* (1993) pointed out that treatment of hypophysectomized, castrated males with both prolactin and androgen sufficiently facilitates the behavior.

Prolactin or androgen alone can induce male courtship behavior although the incidence and frequency of behavior are low (Moore and Zoeller, 1979; Kikuyama *et al.*, 1980; Malacarne *et al.*, 1982; Andreoletti *et al.*, 1983; Toyoda *et al.*, 1993). In the present study, differences were not detected in the number of synaptic contacts onto somata of the M cells among the prolactin-, androgen- and both prolactin and androgen- treated male newts. It is not likely, therefore, that neural connections of the M cell somata is different among the three groups. Prolactin but not androgen is known to enhance the development of tail fin (Tuchmann-Duplessis, 1949; Vellano *et al.*, 1970; Singhas and Dent, 1975; Kikuyama *et al.*, 1986), whereas prolactin is synergistic with androgen on the functional development of the abdominal glands (Kikuyama *et al.*, 1975; Norris *et al.*, 1989; Toyoda *et al.*, 1994). Malacarne and Giacoma (1980) stated that lesion of the preoptic area abolishes the courtship behavior in male newt, *Triturus cristatus*. Moreover, androgen receptor-containing neurons exist in several regions of the brain including the preoptic area in male newt, *Taricha granulosa* (Davis and Moore, 1996). The evidence suggests that the preoptic area plays a important role for courtship behavior. Thus, it is plausible that neural circuits driving the courtship behavior become functional when both the central nervous system and peripheral organs involved in the behavior are activated by a combination of prolactin and androgen. Although afferents to the M cell somata consist of one of the important neural components, prolactin- or androgen-induced neural connectivity to M cells itself seems to be insufficient to fully evoke the courtship behavior.

Recent studies have provided evidence for hormonally-induced structural alterations in neural circuitry driving reproductive neuroendocrine and behavioral functions in adulthood (Matsumoto, 1992). For example, in adult male rodents, androgen has been reported to regulate somatic size (Breedlove and Arnold, 1981), dendritic length (Kurz *et al.*, 1986) and afferent inputs to the androgen-sensitive motoneurons of the spinal nucleus of the bulbocavernosus (Matsumoto *et al.*, 1988), which have an important role in male copulatory behavior. Estrogen has been shown to induce synaptogenesis in completely deafferented (Matsumoto and Arai, 1979) or intact hypothalamic arcuate nucleus (Garcia-Segura *et al.*, 1986) and in the hypothalamic ventromedial nucleus (Carrer and

Aoki, 1982) in female rats. All of these regions have been found to accumulate sex steroids (Pfaff and Keiner, 1973; Breedlove and Arnold, 1980). The evidence indicates that brain regions containing sex steroid-accumulating neurons in adult animals possess a considerable plasticity in response to sex steroids, and that sex steroids have the potential to stimulate the growth of neuronal processes and remodel neural circuits in the adult brain. The results of the present study indicate that prolactin and androgen substantially contribute to the reorganization of the neural circuits of adult M cells. It has not yet been clarified whether the M cells of male newts contain prolactin and/or androgen receptors. To understand how prolactin or androgen influence the synaptic organization of M cells, it will be important to determine the sites of hormone actions.

Previous studies have suggested which kinds of neurotransmitter may be contained in afferents to M cells in teleost. Electrophysiological studies revealed that iontophoretical application of glycine or γ-aminobutyric acid (GABA) induces membrane conductance fluctuations in M cells (Faber and Korn, 1980). The findings suggest that the M cells receives glycinergic and GABAergic afferent inputs. Immunoreactive GABA fibers have been observed closely surrounding the M cells (Lee *et al.*, 1993). Furthermore, the presence of glycine receptors (Triller *et al.*, 1990) and GABA$_A$ receptors (Sur *et al.*, 1995) in the M cells has been reported. In addition, axon terminals containing immunoreactive serotonin (Mintz *et al.*, 1989; Gotow *et al.*, 1990) and somatostatin colocalized with GABA or glutamate (Sur *et al.*, 1994) are known to make synaptic contact with M cells. In urodeles, however, it has not yet known which kinds of neurotransmitter are contained in afferents to the M cells. The present study indicates that synaptic inputs to the M cells can be regulated by prolactin, androgen or both and that hormonally induced remodeling of neural connectivity of the M cells seems to be functional. Further studies are needed to determine the chemical identity of neuronal inputs to the M cells which are affected by these hormones.

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