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ABSTRACTS

SECOND SYMPOSIUM ON THE AMPHIBIA : ENDOCRINOLOGY OF AMPHIBIANS WITH FOCUS ON CELLULAR AND MOLECULAR ASPECTS (*)

Leuven, Belgium, 14-17th September 1990

ULTRASTRUCTURE AND ARRANGEMENT OF CHROMAFFIN CELLS IN THE ADRENAL GLAND OF URODELES, F. Accordi, Dipartimento di Biologia Animale e dell'Uomo, Università « la Sapienza », viale dell'Università, 32, 00185 Roma (Italy).

Different conditions in the arrangement of the adrenal gland are observed in Amphibians : in Gymnophiona and Urodeles the gland consists of islets scattered on the ventral surface of the kidneys, in Anurans the islets are grouped to form a streak. In Urodeles the amount, size and position of the islets vary consistently within different families and even within a genus (1). In order to investigate whether the infraordinal variation is also extended to the fine structure of the gland, further observations were performed in Urodeles. The adrenal glands of 13 species belonging to 6 different families were studied, the ultrastructural characteristics of chromaffin cells and their relationships with interrenal cells were examined. In all the observed species the interrenal cells are always grouped, whereas the chromaffin cells appear either grouped or isolated ; as regards the relationships between chromaffin and steroidogenic cells, various degrees of aggregation are observed in different species. In primitive Urodeles (Sirenidae and Proteidae) the chromaffin cells are isolated or in small groups, mostly separated from interrenal cells and often in contact with renal cells. In Neourodeles the chromaffin cells may be found either grouped or isolated, and located generally at the periphery of the groups of steroidogenic cells, but they may also be found isolated near renal tubules or blood vessels. This double localization usually occurs in Amphiumidae and Ambystomidae, whereas in Salamandridae and Plethodontidae the chromaffin cells appear generally grouped and intermingled with steroidogenic cells.

The different relationships between chromaffin and steroidogenic cells in Urodele families may be related to their phyletic position : conditions of great dispersion occur in primitive urodeles (Sirenidae and Proteidae). An intermediate condition is observed in those families (Amphiumidae and Ambistomidae) which are considered (2, 3) ; at a lower level of organization within the Neourodeles, whereas the gland of advanced Urodeles (Salamandridae and Plethodontidae) shows a higher degree of aggregation.

As regards the cytological characteristics of the chromaffin cells of Urodeles, variable features were observed in the shape and electrondensity of chromaffin granules. In Anurans and higher Vertebrates these characteristics are homogeneous within the same cell and permit the distinction between adrenaline — and noradrenaline — cells ; only in Neourodeles can

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two types of chromaffin cells be clearly identified, whereas in primitive Urodeles this distinction is slight, since shape and electrondensity of granules vary even within the same cell.

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(3) A. MORESCALCHI (1975), *Evolutionary Biology* **8**, 339-387.

STAGE DEPENDENT REGULATION OF INTERRENAL ACTIVITY IN *XENOPUS LAEVIS* TADPOLES, S. ALBRECHT, W. KLOAS and W. HANKE, Zoological Institute II, University of Karlsruhe, Kaiserstr. 12, D-7500 Karlsruhe (F.R.G.).

The importance of the interrenal gland for osmoregulation and metamorphosis in Amphibia is well established. The cytological and biochemical pattern shows a peak in interrenal cell activity of *Xenopus laevis* tadpoles during the climax of metamorphosis (1, 2). However, data of the mainly occuring steroids coticosterone and aldosterone in the earliest larval stages are not available. The aim of the present study was to investigate the occurrence and the changes of corticosterone and aldosterone levels from egg to juvenile animals. Furthermore, we determined *in vivo* the regulatory influences of ANF, A II, AVT and ACTH on the interrenal secretion at stage 50/51 (according to the Normal Table by NIEUWKOOP and FABER). In comparison to a possible regulatory influence of ANF and A II we studied the localization of binding sites for ANF and A II in the kidneys of *Xenopus laevis* tadpoles at stages 49/50, 51/52 and 55/56 by *in vitro* autoradiography (ARG).

Adult males and females of *Xenopus laevis* twice received an injection of 6 mg human chorionic gonadotropin within two days, which is necessary for breeding. For determinations of stage-dependent corticosterone and aldosterone levels, we sampled eggs and tadpoles. Tadpoles were taken one day after hatching (stage 43/44) and in two-stage steps from 45/46 up to 65/66. The regulatory influences of ANF, A II, AVT and ACTH were studied in normal and hypophysectomized tadpoles (stage 50/51), which received a single injection of 0.1 nM hormone in 10 µl frog Ringer's solution. Control animals were treated with pure frog Ringer. The tadpoles were killed one hour after injection. The contents of corticosterone and aldosterone in homogenized eggs and larvae were determined by radioimmunoassay after extraction by dichloromethane. Eggs and larvae up to stage 47/48 had to be pooled, the steroid contents were determined (in ng steroid/g body weight). ARG for ANF and A II binding sites was performed as described elsewhere (3). The steroid determinations during development show neither corticosterone nor aldosterone in eggs and tadpoles until stage 43. In animals of stage 43/44, a low corticosterone content (1.58 ng/g body weight) was measured, but still no aldosterone. A fast increase of both steroid levels could be observed between stage 43/44 to stage 47/48 (7.70 ng corticosterone and 1.70 ng aldosterone/g body weight) which remain high for aldosterone until stage 51/52 and for corticosterone until stage 57/58. Both aldosterone and corticosterone decreased gradually from stage 57/58 onward but showed a peak at stage 61/62.

The *in vivo* treatment of *Xenopus laevis* tadpoles with ACTH and AVT resulted in an increase of both steroids in normal and hypophysectomized tadpoles. The aldosterone level was relatively more elevated than corticosterone. Val^5-A II had no effect, while ANF caused an insignificant decrease of both steroids in hypophysectomized animals.

ARG results indicate that binding sites for ANF and A II exist in glomeruli and interrenals of the kidneys in all investigated stages, while A II binding sites could be found only in glomeruli and not in interrenal tissue.

Our results demonstrate that interrenal activity develops fast, about one day after hatching (43/44). The high increase of both corticosteroids within a day (stages 43/44 to 45/46) and relatively high constant levels during the period of stages 47/48 to 53/54 suggests that corticosteroids are very important for development and osmoregulation. The gradual decrease of corticosterone and aldosterone, except the peak of both corticosteroids during mid-climax (stages 61/62), to normal levels of juveniles demonstrate the involvement in metamorphosis as observed by LELOUP-HATEY *et al.* (2).

The results of *in vivo* treatment of larvae agree with findings in adult animals (KLOAS, unpublished) where only ACTH and AVT cause an increase of both steroids, while A II is ineffective, and ANF effects may be covered by endogenous factors.

The ANF- and A II binding sites in lavae show the same pattern as in adult animals. Therefore, it can be concluded that in larvae and in adults of *Xenopus laevis* the regulation of interrenal activity is quite similar.

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(3) W. KLOAS, E. FUCHS, W. HANKE (1990), in W. Hanke (ed.) *Biology and physiology of the Amphibians*, Fortschritte der Zoologie, **38**, 229-240.

INFLUENCE OF PHOTOPERIOD AND MELATONIN ON DAILY OSCILLATION OF T_4 ACTION ON GROWTH AND METAMORPHOSIS OF *RANA PEREZI* TADPOLES, A.L. ALONSO-GÓMEZ, M.J. DELGADO, J.L. DOMINGUEZ, B. GANCEDO and M. ALONSO-BEDATE, Dept. Biología Animal II (Fisiología Animal), Fac. Biología Univ. Complutense, 28040 Madrid (Spain).

A circadian rhythm of T_4 action on anuran (*Rana pipiens, Xenopus laevis*) metamorphosis has been described, thus T_4 is more efficient as metamorphic inducer during the light than during the dark phase of the photocycle. Melatonin production is mainly regulated by photoperiod with maximal values appearing at nighttime in almost all of the vertebrate species studied.

The present study was undertaken to investigate the influence of melatonin on day-night changes in tissue responsiveness to T_4 in pre- and prometamorphic larvae of *Rana perezi*. Tadpoles were maintained under two different photoperiodic conditions (24L and 12L:12D), T_4 (1×10^{-7} M) and melatonin (5×10^{-4} M) treatments were carried out by immersion of the tadpoles for one hour twice during the daily photocycle : in the middle of the light phase and early in the dark phase (in 24L group, the time of hormone administration corresponds to subjective day and night). At the end of the experimental period (two weeks) total lenght and metamorphic stage of development were determined. Photoperiodic conditions used in this study do not affect rate growth and metamorphosis in premetamorphic larvae, T_4 stimulated metamorphic development, independently of time of administration and light cycle. In tadpoles maintained under 12L:12D photoperiod, T_4 during the light phase delayed prometamorphic growth compared to dark administration. Constant light did not allow this effect. Melatonin treatment did not interfere with T_4 action in any case.

Results obtained in the present study support a different responsiveness of tadpole tissues to T_4 administration. This response could be due to an activity change of the enzymes implied

in T4 metabolism, or to a circadian oscillation of the thyroid hormone receptors appearance. In this sense, a circadian rhythm in the type II thyroxine 5'-deiodinase activity in mammalian pineal gland has been described, together with a parallelism in melatonin production. To our knowledge there is no study concerning this possible circadian rhythm in Amphibia. If this cyclic activity existed, a high T4-T3 conversion during the light period would explain the different T4 action, depending on the time of administration, and the lack of effect observed under 24L conditions would be a consequence of the hypothetical disappearance of the deiodinase rhythm. Many more experiments are necessary to demonstrate this possibility.

OSMOREGULATORY DISTURBANCES ASSOCIATED WITH STRESS IN *XENOPUS LAEVIS* : EFFECTS ON PLASMA CORTICOSTEROIDS, P.H.M. BALM, B.G. JENKS and F. LEBOULENGER (*), Dept. of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen (The Netherlands), and (*) Lab. Endocrinol. Mol. UA CNRS 650, University of Rouen, 76134 Mont-Saint-Aignan (France).

In all vertebrate classes, corticosteroids regulate a wide variety of adaptational processes associated with stress. Peptides derived from the prohormone POMC have been established as important regulatory factors in the control of adrenal function. ACTH, secreted by POMC cells in the pituitary pars distalis, has by far recieved most of the attention, and relatively few experiments have focused on the possibility that products from the second pituitary cell type, the intermediate lobe MSH cell, could also be involved in the regulation of corticosteroidogenesis. This is surprising because, especially in the lower vertebrates, the pars intermedia is relatively large and circulating levels of αMSH are usually much higher than those of ACTH. Moreover, previous studies in our laboratory have shown that the pars intermedia of teleosts is involved in the regulation of the adrenals during stress. This was based on the finding that the teleostean pars intermedia releases factor(s) with substantial corticotropic activity.

The present study investigates the acclimatization to stressors, and the possible involvement of intermediate lobe POMC products therein, by the aquatic amphibian *Xenopus laevis*. This species provides an appropriate model for these studies, since, the activity of the pituitary MSH cells can be experimentally manipulated by changing the background color. The cells become highly activated when the background becomes black, which results in severalfold higher plasma αMSH levels. To test the effects of this modulation of MSH cell activity on plasma corticosteroids and the capacity of the animals to adapt to stressors, we challenged both black- and white adapted *Xenopus* either with a handling protocol or by lowering the environmental pH. The results show that black adapted animals, which possess high plasma αMSH levels, are more effective in coping with the experimental challenges, as indicated by measured values for several blood constituents. The role of corticosteroids, and the possible involvement of POMC derived factors, during acclimatization to the stressors in *Xenopus laevis* will be discussed.

HORMONAL CONTROL OF *XENOPUS* OOCYTE MATURATION : ROLE OF NON-STEROIDAL FACTOR, D. BOUJARD, F. CHESNEL, A. BOURRY, G. BONNEC and J. JOLY, Laboratoire de Biologie de la Reproduction URA 256 CNRS, Université de Rennes I, 35042 Rennes Cedex (France).

Resumption of meiosis in *Xenopus* oocytes, blocked at the diplotene stage of prophase I, can be induced by steroids (a great variety of non estrogenic steroid) or by proteins (IGF ; insulin ...). These two kinds of inducers act, at least partially, *via* different pathways since insulin-induced maturation can be specifically inhibited (See review of SMITH (1989), *Dev.* **107**). Whatever the inducer is, there is a great variation in the timing of Germinal Vesicle Break Down (GVBD) according to the females. This results, in part, from physiological conditions, since injection into females of low dose of gonodotrophins (« priming » dose) before oocytes denudation reduces dramatically the time of progesterone induced maturation (1). Addition of insulin may also reduce the time of progesterone-induced maturation (2), but great variations are observed according to the experiments.

To understand the cause of these differences, we have studied the effect of insulin, progesterone and both together on maturation of oocytes issued from various unstimulated females. Our results demonstrate the existence of at least two kinds of oocytes : the first category is characterized by a significant synergistic action of insulin on progesterone-induced maturation. In this case, the GVBD 50 (time at which 50 % of oocytes exhibit GVBD) is of 528 mn ± 30 with progesterone alone, of 533 mn ± 26 with insulin alone and of 438 mn ± 25 with progesterone and insulin. In the second category, where no significant synergistic action is observed, progesterone and insulin alone induce a GVBD 50 as short as both together in the first category (respectively 446 mn ± 43 ; 453 mn ± 28 ; 423 mn ± 41). Taken together, these results seem to indicate that in the oocytes of the second category some events have already occured prior to treatment. Such events should be responsible for obtaining a GVBD short time after exposure, whatever the kind of inducer used. In contrast, this « short » pathway leading to GVBD, requires the presence of the two inducers in the first category.

In addition, using the first category of oocytes, we have undertaken to demonstrate the existence, in post vitellogenic follicles of a peptidic substance which can, *in vivo*, act on oocytes as insulin does *in vitro*. Our results showed that a non steroidal factor, secreted by follicular cells, can significantly reduce the appearance time of progesterone-induced maturation. This factor is inactivated by high temperature, suggesting its proteic nature. Moreover, treatments of follicles with cyanoketone (an inhibitor of steroidogenesis), suppress the synthethis and/or secretion of this factor. All thse results strongly suggest that the different pathways leading to GVBD and meiosis have physiological importance. They also indicate that, according to the physiological stage of the female, there is a great variability in the pathway leading to MPF activation.

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***IN VITRO* HORMONAL CONTROL OF VITELLOGENIN SYNTHESIS IN *RANA ESCULENTA* LIVER**, O. CARNEVALI [1], G. MOSCONI [1], K. YAMAMOTO [2], T. KOBAYASHI [2], S. KIKUYAMA [2] and A. POLZONETTI-MAGNI [1], 1. Dept. of Cellular Biology, Camerino (Italy); 2. Dept. of Biology School of Education Nishi Waseda, Tokyo (Japan).

Vitellogenin (VTG), a very complex protein, is known as a precursor of yolk proteins : phosvitin and lipovitellin, in all oviparous vertebrates so far studied.

In amphibians as in other oviparous vertebrates, vitellogenin synthesis is a hormone-dependent process and 17β-estradiol (E_2) seems to be the factor mainly responsible for the synthesis and release in the blood circulation.

Previous results evidenced the pituitary involvement in liver VTG synthesis and secretion in *Rana esculenta.*

In vitro experiments carried out on male and female liver, homologous pituitary (HP) and E_2, both stimulated VTG synthesis although the pattern of VTG secretion, assayed by ELISA, showed some differences concerning the induction time and the rate of VTG secretion. The estradiol response was obtained after 3 days incubation, while maximal HP response came after 4-5 incubation days, and the HP response rate was higher in comparison with E_2 response.

In addition, during the refractory period (July) VTG synthesis in male and female liver was induced by HP only.

In order to identify the pituitary hormones involved in this process, mammalian FSH, LH, PRL and GH were used.

In October experiments, the incubation of *Rana esculenta* male and female liver with FSH and LH failed to induce VTG synthesis and release.

In November experiments (winter stasis), the liver in both sexes was unresponsive to incubation with PRL, while GH was able to stimulate VTG synthesis in the liver.

Moreover, *Rana esculenta* liver positively responded to *Rana catesbeiana* pituitary homogenate; in fact *Rana catesbeiana* pituitary induced the same effects as homologous pituitary on both male and female *Rana esculenta* liver.

Since at the moment *Rana esculenta* pituitary hormones are not available, we are going to experiment using hormones purified from the *Rana catesbeiana* pituitary.

HORMONAL CONTROL OF THE HARDERIAN GLAND OF *RANA ESCULENTA*, G. CHIEFFI, G. CHIEFFI BACCARI, L. DI MATTEO, M. D'ISTRIA, C. MARMORINO, S. MINUCCI and B. VARRIALE, Napels (Italy).

The Harderian gland (H.G.) is the only orbital gland in anuran amphibia whose function is to lubricate the eyeball. It is located at the medial corner of the orbita and in *Rana esculenta* is an oval acinar gland. The seromucoid secretion of this essentially nonmitotic secretory tissue is either merocrine or apocrine and varies during the year.

Little is known about the regulation of the secretory activity of the H.G. in amphibians. In our laboratory we designed some experiments with the green frog, *Rana esculenta*, keeping in mind that the H.G. displays a seasonal activity : the secretory activity is highest during July-August, drops to the lowest in September, and from October onwards resumes slowly.

Both exogenous and endogenous factors have been investigated as possible regulators of the H.G. secretory activity. With regard to endogenous factors, the following endocrine manipulations were carried out.

1) *Pinealectomy*. The presence of melatonin in H.G. and pineal tissue extracts and in plasma was radioimmunologically demonstrated. However, the melatonin content in H.G. and pineal was ten times lower than in plasma (80pg/ml), suggesting that the major source of melatonin in this frog is very likely the retina, as show in *R. perezi* and *R. tigrina regulosa*. A more detailed analysis carried out in *Rana esculenta* kept in continuous darkness for at least two weeks, showed, in a six time point assay, a daily rhythm in plasma melatonin but not in pineal or H.G. Whether H.G. melatonin is synthesized in situ is still to be investigated. Two month pinealectomized frogs (spring animals) were kept for two weeks under different photoperiods (24L:OD ; OL:24D ; 12L:12D). No effect was observed in the H.G. morphology. these data seem to exclude either a regulatory function of the pineal on the H.G. or a possible role of melatonin within the frog H.G. because of its low concentration in the gland which is unaffected by photoperiod manipulations.

2) *Hypophysectomy*. Hypophysectomy carried out during February, when the acinar cells start to elongate and accumulate secretory material, induces a further increase in the height of glandular cells which appear full of secretory granules. Hypophysectomy affected also the electrophoretic pattern of water soluble proteins preserving a protein fraction in the range of 480 kD, which disappears in the captive sham-operated controls. Replacement therapy with crude homogenate of homologous pituitary restored the morphological and biochemical pattern of intact animals.

Among pituitary hormones tested in hypophysectomized animals, bTSH and oxytocin show an inhibitory effect, while prolactin does not modify the stimulatory effect of hypophysectomy on the secretion of the glandular cells in both male and female frogs. oACTH and oLH administration provoke a reduction of secretion in the female, while they have no effect in the male. We have not been able to carry out similar experiments in summer months owing to the high mortality (over 90 %) following hypophysectomy, naturally on account of their already exhausted (postreproductive) physiological condition. Winter 1989-90 has been remarkably mild and temperatures reached 22° C. Consequently, the H.G. secretory activity was at its highest. Hypophysectomy performed in these animals was successful and provoked opposite results when compared to the previous ones : acinar cell height decreased and the protein fraction of 480 kd disappeared. Replacement therapy restored the control protein pattern.

3) *Sex hormones*. Before testing the possible sex hormone influence on the H.G. of the frog, *R. esculenta*, we first looked into the presence of sex hormone receptors. We found not only a large number of cytosolic and nuclear androgen receptors, but also reported a parallel annual profile between plasma androgen concentration and receptor number sites. Interesting was the finding of large numbers of androgen receptors in both sexes, which is not surprising if we consider that circulating androgens in the female are as high as in the male. No estrogen receptor was detected in the frog H.G. in either sex. The lack of a sex dimorphism in the frog H.G. might be related to the similar pattern of circulating androgens in both sexes, while in the golden hamster the clear sex dimorphism correlates well with the low levels of androgens in the female compared to those in the male.

Notwithstanding the presence of androgen receptors in the frog H.G., castration carried out in some, but not all, periods of the year, has no effect on the morphology of the gland. However, the influence of testosterone on the H.G. was observed at biochemical level. In fact testosterone treatment induced a marked increase of some protein fractions, which proved to be mostly glycoproteins and one of them has a MW of 480 kD, the same MW as the protein

fraction which is influenced by hypophysectomy (see above). CPA treatment, given alone or in combination with testosterone, prevent these changes.

The role of testosterone on the secretory activity of the frog H.G. was further studied by *in vitro* incorporation of ^{3}H-Uridine in presence or absence of the steroid. Since the hormonal treatment resulted in a preferential uptake in the poly (A) + fraction, it is supposed that testosterone can trigger the specific biosynthesis of some mRNAs.

FOOD INTAKE INHIBITION AND METAMORPHOSIS STIMULATION BY *SHEEP* CORTICOTROPHIN-RELEASING HORMONE (CRF) ADMINISTRATION IN *RANA PEREZI*, I. Corpas, B. Gancedo, A.L. Alonso Gomez, M.J. Delgado and M. Alonso-Bedate, Depto Fisiologia animal, Facultad de Biologia, Universidad Complutense, 28040 Madrid (Spain).

One of the most spectacular events in animal development is the process of metamorphosis. During premetamorphosis tadpoles grow Prometamorphosis comprises the developmental changes that occur between emergence of the hind limbs and emergence of the fore limbs. During the climax normal feeding is prevented and decreases fecal excretion. Biochemical, physiological, morphological and behavioral changes occur at climax, that prepare a long-tailed herbivorous, aquatic larva to become a tailess, carnivorous, terrestrial organism.

That the thyroid hormones are responsible for these events has been known for a long time. An interesting problem regarding the control of the thyroid system in amphibians development is the identity of the hypothalamic factor or factors which control TSH release by the pituitary gland. Recently, studies by Denver (1) in *Rana catesbeiana* adults suggest that ovine CRF produces a greater output of TSH than do hGnRH or TRH. On the other hand some studies in mammals indicate that CRF is an important and physiologically relevant feeding inhibitor. In the present investigation we pretend to study the effects of sheep CRF on metamorphosis events and food intake responses in *Rana perezi* tadpoles.

Tadpoles obtained by spontaneous breeding in June were kept in darkness at 10 ± 2° C temperature. They were fed with boiled spinach once a week in order to maintain the minimum metabolic requirements.

One week before the beginning of the experiments, tadpoles were acclimatized to the experimental conditions; 12L:12D photoperiod and 24 ± 2° C temperature. Spinach was weighed every day before and after tadpoles were fed and the excreta were evaluated. Three times during the course of the experiment tadpoles were anaesthetized with MS-222 (1:10000) and measured. The experiment was finished when some of the tadpoles reached metamorphic climax. Tadpoles were anaesthetized weighed and measured and then used for histological study of the thyroid gland.

Sheep CRF (Sigma) was dissolved in a minimum amount of Holtfreter solution and stored at − 30° C. For treatment, tadpoles were daily injected through the opercular opening into the dorsal part of the body near the eyes. Treatment was performed in October-November and tadpoles, eight per group, received 2µl of Holtfreter solution (controls) or 2µl of Holtfreter containing 1µg CRF per injection (total number of injections was 17).

The results obtained in the present study demonstrate that sheep CRF significantly stimulates hindlimb growth, cloacal tail piece disappearance and increases the number of thyroid follicles. Also we observed a significant decrease of food intake and fecal excretion and tadpoles were significantly smaller than controls. It is suggested that in amphibian

development a CRF-like factor, depending on the level reached at one particular stage, stimulates thyroid hormones, and consequently metamorphic events like hind limb growth, cloacal tailpiece disappearance and directly affects some hypothalamic nucleus which decreases appetite during the climax. Some experiments using hypophysectomized tadpoles are now under way in our laboratory.

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LOCALIZATION OF INSULIN IN THE PANCREAS OF *XENOPUS LAEVIS*, AND CHARACTERIZATION OF ITS LIVER PLASMA MEMBRANE INSULINE RECEPTOR, B. J. COWAN, R. A. FOTY and R. A. LIVERSAGE, Department of Zoology, University of Toronto, Ontario (Canada), M5S 1A1.

Insulin is known to play an essential role in sustaining growth and differentiation in adult urodele appendage regenerates. Amputation of a *Xenopus* forelimb results in the regeneration of a spikeshaped appendage unlike the perfect epimorphic regenerate in urodeles. As tissue regeneration in *Xenopus* is a cell proliferation event, our aim is to examine the role of insulin as a growth-promoting hormone in *Xenopus* forelimb regeneration. We are attempting to determine whether a regenerate ; (i) is a target organ for insulin, and if so (ii) does the insulin receptor capacity and affinity change during the regeneration process. Our initial research involving liver cells was performed in order that we might devise methods for *Xenopus laevis* forelimb regeneration studies. The methods include : aldehyde-fuchsin staining of the pancreas as well as PAP immunochemistry of adjacent sections . competitive inhibition and Scatchard analysis of partially purified liver cell membranes ; cross-competition assays to determine receptor specificity ; as well as affinity photolabeling of receptors to determine structure (and function). Results demonstrate the presence of aldehyde-fuchsin positive pacreatic islets, scattered throughout highly vascularized acinar tissue. PAP immunostaining utilizing a mammalian antibody shows corresponding clusters of insulin-positive cells, indicative of the site of insulin production. Homologous antigen immunoabsorbance and pre-immune serum controls were PAP negative, whereas pre-absorption of insulin antibody with heterologous antigens, including guinea pig insulin, insulin-like growth factor 1 (IGF-1), and glucagon results in PAP-positive staining, thus confirming the specificity of the antibody. Competitive inhibition and Scatchard analysis describes a two binding site receptor model ; a low affinity ($0.16 nM^{-1}$), high capacity (3.2 ± 0.9 picomoles/mg) binding site and a high affinity ($2.7 nM^{-1}$), low capacity (0.5 ± 0.3 picomoles/mg) binding site. Cross competition experiments show that radioactive insulin is more efficiently displaced by excess cold insulin than by guinea pig insulin and IGF-1. Glucagon does not cross react. This suggests that the receptor is specific. Photoaffinity labeling and immunoprecipitation demonstrate the presence of a receptor subunit with an apparent molecular weight of approximately 130 Kd. Presumably, this is the alpha (insulin binding) subunit of the receptor, under reduced conditions. ^{32}P-ATP labeling demonstrates the presence of a receptor subunit approximately 95 Kd, which undergoes insulin-stimulated phosphorylation. Currently, we are attempting to determine whether changes in receptor numbers occur at various stages of limb regeneration, and to examine the effects of blocking insulin action at the regeneration site using antibody blocking techniques. (Supported by NSERC of Canada Grant A-1208 to R.A.L.).

INFLUENCE OF THIOURACIL AND THYROID HORMONES ON TESTICULAR FUNCTION IN *RANA PEREZI*, J.L. DOMINGUEZ, I. CORPAS, M.J. DELGADO and M. ALONSO-BEDATE, Depto Fisiología Animal, Facultad de Biología, Universidad Complutense, 28040 Madrid (Spain).

A possible correlation between the gonadal and thyroidal axis has been proposed by SARKAR and RAO (1), who showed that chemical and surgical thyroidectomy prevent ovulation in *Rana cyanophlyctis*. It has been suggested that estradiol-17β silastic implants in female *Rana ridibunda* depress thyroid hormone concentrations in plasma and the in vitro 5'-monodeiodination activity of kidney homogenates (2). Thyroid hormones (THs) appear to be necessary for estrogen-induced synthesis of vitellogenin by the liver of *Xenopus laevis* (3).

The data about THs being required for male reproduction are very scarce. The objective of the present study was to determine how manipulation of the thyroidal status (thyroid hormones or thiouracil administration) modify testicular function as assessed by gonadosomatic index (GSI), plasma testosterone levels or testes responsiveness to gonadotrophins (hCG) in *Rana perezi*.

Adult male frogs (*Rana perezi* SEOANE, 1885) were captured in Galicia (NW of Spain) and sent to Madrid in March and May. They were maintained in plastic tanks with tap dechlorinated water in a room with natural photoperiod and temperature for one week after their arrival. The experiments were performed in a room with controlled photoperiod (12L:12D in March ; 14L:10D in May, lights on at 8:00 a.m.) and temperature (20 ± 2° C in March and 23 ± 2° C in May). Calliphora larvae were available three days a week and both — March and May frogs — were eating regularly.

Experiment 1 : Effects of thiouracil, human chorionic gonadotropin (hCG) or both on GSI and plasma testosterone levels. This experiment was performed in March. Frogs were divided into four groups consisting of eight frogs. Each group received two injections in the ventral lymphatic sac every second day. The first injection was given at 9:00 a.m. and the second one at about 30 minutes later. The first group (Control) was injected with 0.1 ml frog Ringer (FR) followed by 0.1 ml FR. The second was injected with 0.1 ml FR followed by 100 IU hCG/0.1 ml. The third was injected with 5μg 2-thiouracil/0.1 ml followed by 0.1 ml FR. Finally, the fourth group was injected with 5μg 2-thiouracil/0.1 ml followed by 100 IU hCG/0.1 ml. The treatment consisted of a total of six injections. Then, animals were anaesthetized with tricaine methanesulfonate (MS-222, Sandoz) and blood was collected directly from the heart, it was centrifuged and stored at −20° C until assayed for testosterone. Testes were taken to evaluate GSI (testes weight/body weight × 100). Testosterone was evaluated as described previously.

Experiment 2 : Effects of T_3 and T_4 on plasma testosterone levels and GSI. This experiment was performed in May. THs from Sigma were dissolved in a minimum amount of NaOH and then diluted in distilled water. THs were finally added to two liters of dechlorinated water and frogs were so treated by immersion. Concentration of NaOH was also taken into account in controls. The final concentration of NaOH in the tanks resulted to be 10^{-8} g/ml. Animals were divided into 3 groups of eight animals each. The first group was immersed in tap water containing 10^{-8} NaOH g/ml. The second group was immersed in 50 ng T_3/ml. The last group was immersed in 50 ng T_4 ng/ml. The water was renewed daily. After one month of treatment, animals were anaesthetized with MS-222 and blood was collected, stored and assayed for testosterone by RIA. Testes were weighed and GSI was calculated. The results obtained in the present work suggest that a long-term treatment with T_3, T_4 or thiouracil does not influence testosterone plasma levels and GSI and does not modify the

testicular response to gonadotropin administration at least in these experimental conditions in *Rana perezi.*

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ADHESION AND RECOGNITION MOLECULES DURING OOGENESIS IN THE AMPHIBIAN *TRITURUS CRISTATUS* CARNIFEX, C. Falugi [1], A. Contini [2], G. Faraldi [1], S. Fasulo [2], G. Tagliafierro [1], G. Zaccone [2], 1. Ist. Anatomia Comparata, Viale Benedetto XV 5, 16132 Genova ; 2. Dip. Biol. Animale ed Ecol. Marina, S. Agata, 98166 Messina (Italy).

Cell-cell and cell-environment communications play an important role in regulating differentiation. Communication is mediated by hormonal or, generally, chemico-physical signals, that must undergo changes at the passage through membranes to the intracellular medium, where they display their role. In this process membrane receptors are involved, such as glycocalix, for signal recognition and cell adhesion (1). During oogenesis, surface changes take place, which are needed for the reception of different signals ; they are also involved in the regulation of cell movement and shape, by interaction with the extracellular matrix (2). In this work, a study of cell surface changes was carried out on gonads of adult female specimens of the amphibian *Triturus cristatus carnifex*, by use of either FITC or HRP-labelled lectins : concanavalin A (ConA, from Sigma, USA), with affinity to carbohydrate residues of mannose and glucose ; wheat germ agglutinin (WGA, from Kem-en-tec, DK), with affinity to N-acetyl-D-glucosamine and sialic acid ; peanut agglutinin (PNA), with affinity to galactose-1-3-galactose-N-acetylgalactosamine ; soy bean agglutinin (SBA), which binds N-acetylgalactosamine and D-galactose ; *Griffonia somplicifolia* I (GS I), which binds α-D-galactose. Furthermore, we investigated the presence of fibronectin in the extracellular matrix, by use of an anti-human fibronectin antiboby (Behring, D). We found that WGA affinity sites are present in different locations in oocytes at different maturation stages. Small oocytes (20-40 μm) are weakly labelled in the cortical region, while 50-80 μm oocytes present binding sites in the cortical region and in the cytoplasm, in the form of rings, concentric to the surface ; maturing oocytes were mainly labelled in the zona pellucida. ConA binding was present in all the oocytes in the follicular envelopes, and in the cytoplasm, but not in the zona pellucida, as it was shown for mouse ovarian oocytes (3) ; SBA-binding sites are localized in the egg central region, mainly around yolk granules ; no binding was found either with PNA or with GS I. FN-like immunoreactivity was present on the outer face of the zona pellucida and in intercellular matrix of ovular envelopes close to the big oocytes. Probably, the presence of FN around big oocytes is linked to the behaviour of such oocytes, that must move towards the ovarian surface, to be released, as it was shown for migrating germ cells in embryos of amphibians (2). These data provide a first bulk of information for distinct components of the amphibian ovary and their developmental behaviour.

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HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDY ON THE SPERMATOGENESIS OF *TRITURUS CRISTATUS CARNIFEX*, C. Falugi, G. Faraldi, G. Tagliafierro, Istituto di Anatomia Comparata dell'Universita di Genova, Viale Benedetto XV, 5, I-16132, Genova (Italy).

A developmental role for neurotransmitter systems localized in non-neuromuscular cells has been generally hypothesized (1, 2) ; in particular, these systems can be involved in regulating morphogenetic movements and cell differentiation (3). Since spermatogenesis is characterized by evident changes in cell shape, we have investigated if neurotransmitters are present and active during such a morphogenetic process. Our study was carried out on specimens of the amphibian *Triturus cristatus carnifex*, obtained from De Rosa (Napels). Cholinesterase and acetycholine esterase (ChE) activities have been detected on testicular cells by a direct thiocholine method, and catecholamines by the FIF method ; indirect immunofluorescence reactions have been carried out by the use of antibodies anti-ChE (Dako, DK), anti- ChAT (Seralab, UK), anti-ACh (Biosys, F), and anti-5 HT (Inr, USA).Furthermore, the presence of acetylcholine receptors or their precursors has been investigated by the use of bungarotoxin (BuTx), labelled with FITC (control by curare pretreatment), and its glycoprotein nature by use of lectins (fluorescent ConA, WGA and PNA) ; lectins were also employed to test cellular adhesiveness.

In the sperm lineage cells, the cholinergic system, including BuTx-binding sites, was found to be present mainly during sperm maturation. This system is implicated in regulation of intracellular cation changes (1), and consequently, in the rearrangements of the cytoskeleton. In mature sperms, the cholinergic system is still active in the sperm head, around the nucleus, and in the flagellum, probably with other functions, as it was shown for sea urchin sperms (4). Catecholamines, that are known to stimulate second messengers production in sea urchin eggs (5) have been found in cells undergoing early differentiation, while 5HT, implicated in the regulation of microfilament contraction during egg and blastomere cleavage (6), is present at every stage of spermatogenesis, but has not been found in maturing and mature sperms ; ConA and WGA binding sites are also present around the nuclei of maturing and mature sperms, showing the nature of oligosaccharidic residues (mannose/glucose and sialic acid/N-acetyl-D-glucosamine), which together with the BuTx binding, suggests the presence of proteins, similar to the α-subunit of the ACh receptor. Such report is in favour of a role for neurotransmitter systems in the control of sperm differentiation, through modulation of intracellular dynamics.

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IMMUNOCYTOCHEMICAL LOCALIZATION OF NEUROPEPTIDES AND SEROTONIN IN THE GUT AND PANCREAS OF THE ITALIAN PLETHODONTID SALAMANDER, G. FARALDI, G. TAGLIAFIERRO, Istituto di Anatomia comparata, viale Benedetto XV, 5, Universita di Genova, Genova (Italy).

The distribution and localization of gut endocrine cells and nerve fibres have been studied rather extensively in the anuran amphibians, but there is relatively little information on urodeles which represent the most ancient group of this taxon (1-5). For this reason we investigated by immonocytochemical methods, the presence of some neuropeptides and serotonin in the gut and pancreas of the italian cave salamander *Hydromantes (Speleomantes) ambrosii* LANZA (1954).

Immonoreactivity was detedcted by indirect immunofluorescence and peroxidase-antiperoxidase (PAP) methods. Dewaxed sections were incubated overnight at room temperature using different rabbit antisera against several mammalian gut and pancreatic peptides. Controls were performed by absorption of the primary antiserum with its corresponding antigen. A positive reaction followed the use of antisera such as : bombesin (1:400, CRB, UK); vasoactive intestinal polypeptide (VIP) (1/200, CRB, UK); peptide histidineisoleucine (PHI) (1/200, CRB, UK); substance P (1:250, CRB, UK); glucagon (1:200, Dako, DK); somatostatin (1:200, Dako, DK); insulin (1:200, Dako, DK); pancreatic polypeptide (PP) (1:200, Dako, DK); serotonin (5HT) (1:500, INC, USA).

Immunoreactive endocrine cells and nerve fibres can be detected in the gastrointestinal tract. Bombesin-, substance P-, and PHI-like immunoreactivities (IR) are localized in both endocrine cells and nerve elements, while somatostatin-, glucagon-, 5HT-like IR are seen only in endocrine cells, and VIP-like immunoreactivity only in nerve elements. Immunoreactive endocrine cells are located along the whole gastrointestinal tract; generally they are flask-shaped and exhibit a long, narrow process reaching the lumen, and a few basal processes running beneath neighbouring cells. However bombesin-like containing cells appear to be of a closed type. All endocrine cells show an enlarged nucleus but a restricted cytoplasmic area. Immunoreactive nerve fibres seem to be particularly abundant in the lamina propria beneath the epithelium, and around the smooth muscle fibres (muscularis mucosae, muscular layers and blood vessel wall). VIP, PHI, and bombesin immunopositive neurons can be detected in the submucous and muscular layers. Pancreatic endocrine cells are immunostained by insulin, glucagon, somatostatin, and PP antisera. Apparently they do not organize endocrine islets. Glucagon- and somatostatin-like immunoreactive cells are individually scattered while the fewer insulin- and PP-Like immunoreactive cells constitute cord-like formations near blood vessels.

Thus the morphological and distributive patterns of immunoreactive endocrine cells and nerve elements appear to be rather primitive even if some more advanced characters, such as concentration of bombesin-containing cells in the stomach, and the presence of VIP immunoreactivity in nerve elements only, can be found in the italian cave salamander.

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COEXISTENCE OF DIFFERENT NEUROACTIVE SUBSTANCES IN THE HYPOTHALAMUS OF AMPHIBIA, A. FASOLO [1], F. FRANZONI [1], B. MULATERO [1], C. ANDREONE [1], H. VAUDRY [2] and F. VANDESANDE [3], 1. Dip. Biologia Animale, Via Accademia Albertina, 17, Universita di Torino, 10123 Torino (I.); 2. Lab. Endocrinologie Moleculaire, Université Rouen (F.); 3. Zoological Institute, Universiteit Leuven (Belgium).

The coexistence of different neuroactive substances in the same neuron represent probably a significant way to hamper heterogeneity and signalling complexity of the nervous tissues.

Nevertheless extensive comparative studies on the coexistence of neuroactive substances are scanty, even if they might throw some light on the evolutionary mechanisms and functional significance of such coexistence.

In the present paper the distribution of many different neuroactive substances, including neuropeptides, biogenic amines, aminoacidic transmitters, is reviewed, focusing on the coexistence in the hypothalamo-hypophysial systels of Amphibia. As experimental models the green frog, *Rana esculenta* (and *Rana ridibunda*) and the crested newt (*Triturus carnifex*) were used.

In general, the large majority of the neuroactive substances appeared localized in separate neurons, but relevant coexistence phenomena were described, e.g. for different tachykinins, for SP-like and enkephalin-like immunoreactivities.

The innervation of the hypothalamo-hypophysial complex, and in particular of the pars intermedia, is a good example of coexistence, since in the frog dopamine, GABA and NPY seemingly are co-occurring in a small subset of nerve fibers.

On the whole such a study can help to redraw the mapping for neuroactive substances. It prompts as well some intriguing comparative problems, concerning stability versus plasticity of the basic neurochemical pattern in vertebrates.

THE DISTRIBUTION OF THE ATRIAL NATRIURETIC FACTOR IN THE CENTRAL NERVOUS SYSTEM OF THE CRESTED NEWT, *TRITURUS CARNIFEX* LAUR., M. F. FRANZONI, R. TAVOLARO*, A. FROVA and M. CANONACO*, Dipartimento di Biologia Animale dell'Universita di Torino, Via Accademia Albertina, 17, I-10123 Torino; *Dipartimento di Biologia Cellulare and *Dipartimento di Ecologia dell'Universita della Calabria, I-87036, Arcavacata di Rende, Cosenza (Italy).

The first evidence of the presence of an atrial natriuretic factor (ANF)-like immunoreactivity in the central nervous system of a non-mammalian vertebrate has been provided by NETCHITAILO *et al.* (1) in the frog *Rana ridibunda*.

Because of the interest in the Urodele brain as a simple model for increasingly complex vertebrate neural organization we have studied immunohistochemically the neuronal systems containing ANF and related peptides in the brain of the crested newt, *Triturus carnifex* Laur.

Using antibodies a-alpha ANF (1-28), a-porcine BNP (brain natriuretic polipeptide), a-alpha rat ANP (8-33), a rich innervation by ANF-containing nerve processes has been shown throughout all the brain stem of the newt (and also in the hypothalamo-hypophysial complex). The ANF-immunopositive neurons appeared clustered in two main paired groups extending from the preoptic recess of the preoptic area toward the basal telencephalic regions where they fused on the median line

In respect to the organization of the ANF-containing neurons described in the frog, the pattern of distribution of neurons in the newt seemed by large simplified and in some way primitive.

As we previously reported (2), the preoptic area and preoptic recess of the newt contain abundant dopaminergic neurons (labelled by the immunopositivity for the tyrosine hydroxylase, the rate limiting enzyme in the catecholamine metabolic pathway) together with a thick dopaminergic innervation.

As biochemical assessments (3) have indicated that in the mammalians the ANF may interact importantly with dopaminergic mechanisms, we have analysed, applying immunohistochemical double sequential or simultaneous methods, the interrelationship between ANF-containing neurons and dopaminergic ones in the preoptic area of the newt.

Our results have shown the absence of co-localization of ANF and tyrosine hydroxylase in the same neuron. However close relationships between dopaminergic fibres/terminals and ANF-like containing neurons have been observed.

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SUBCELLULAR RESPONSE OF AMPHIBIAN THYROTROPHIN- AND GH-PRODUCING CELLS TO SEVERAL HYPOTHALAMIC FACTORS, F. GRACIA-NAVARRO, M.M. MALAGÓN, A. RUIZ-NAVARRO, J. CASTAÑO, S. GARCÍA-NAVARRO, R. TORRONTERAS, Department of Cell Biology, Fac. Sciences, Univ. of Córdoba, Avda. San Alberto Magno s/n. E-14004 Córdoba (Spain).

The amphibian pituitary has been the subject of numerous endocrinological studies. Nowadays, the availability of purified antisera against some amphibian hypophysial hormones for RIA and the development of indirect methods (*i.e.* measure of T3 or T4) have considerably enlarged the knowledge about the dynamics of the amphibian pituitary under different experimental conditions. One of the most interesting features of the amphibian pituitary recently reported is the fact that one cell type may specifically respond to more than one hypothalamic releasing peptide, which is not so common in higher vertebrates, especially in mammals.

We have investigated the multiple response of some of these amphibian cell types to the *in vivo* administration of several hypothalamic factors from a morphological point of view. This includes the evaluation of the changes suffered by the cytoplasmic organelles that indicate the hormonal content of the cells (volume and numerical densities of the secretory granules) and the activity of the biosynthetic machinery (volume density of the rough endoplasmic reticulum and Golgi complex). The evaluation was carried out on micrographs corresponding to cells previously identified immunocytochemically in order to assess the specificity of the measure. Two methodological methods were applied, the point-counting method and area analysis (1), using an image analyser (IBAS Kontron). Statistical differences between morphometric data were established by nested analysis of variance and a U-test.

With this methodological approach, we have studied the time course response of *Rana perezi* TSH cells to daily administration of thyrotrophin-releasing hormone (TRH) and corticotrophin-releasing factor (CRF), as well as that of GH cells to TRH and growth hormone-releasing hormone (GHRH).

Thyrotropes. The administration of 7.5 μg/20 g bw synthetic TRH induced a short-term response in TSH cells. The tripeptide evoked significant decreases of the Vv and Nv of the SG of TSH cells indicating stimulated release of hormone, which has been also suggested to occur in other amphibian species (2, 3). The dose used also activated TSH cells to stimulate synthesis since the relative cytoplasmic volume (Vv) occupied by the RER and GC was significantly increased compared to control animals.

These ultrastructural modifications indicating the activation of TSH cells were less intense when ovine CRF was administered (1μg/20 g bw), which only induced loss of secretory granules. The ability of CRF to stimulate the hypophysis-thyroidal axis has been recently reported in other amphibian species (3, 4, 5) as well as in a reptile (6). In such species, TRH is also capable of enhancing the release of thyrotrophin which supports the dual control of the secretion of this hormone in these groups of vertebrates.

After a long-lasting treatment, TRH-stimulated TSH cells tended to recover the control values of the stereological parameters while in the case of CRF-injected animals the response of TSH cells was significantly increased.

These results indicate that each peptide induces a different response on TSH cells. TRH causes a short-term response while the effects of CRF are more noticeable after a long-term treatment.

In both experimental treatments, the degranulation of TSH cells always affected small and medium secretory granules, which probably represent new synthesized and stored thyrotrophin respectively (7).

Somatotropes. With respect to GH cells, they showed significant signs of activation after TRH (7.5 μg/20 g bw) or GHRH (1 μg/20 g bw) injection, featuring a diminution of SG and increased development of the organella related to synthesis. This response was much more marked after several days of GHRH treatment while it was considerably reduced in the case of long-term TRH administration. In both experimental treatments, the loss of hormonal content seemed to affect the newly synthesized and stored pools of hormone.

The stimulation of GH release by TRH and GHRH has been also demonstrated in birds (8), in which both peptides are considered to participate in the physiological control of GH. In mammals, TRH stimulates GH secretion in some circumstances such as hypothyroidism (9), acromegaly (10), during the neonatal period (11), and in the case of the bovine pituitary *in vivo* and *in vitro* (12, 13).

Similarly to what occurs in TSH cells, GH cells seem to be differentially affected by the two peptides, GHRH being more effective than TRH in stimulating this cell type.

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DEVELOPMENT SEQUENCES IN THE MORPHOGENESIS OF THE ADRENAL GLAND IN ANURAN AMPHIBIANS, A. Grassi Milano and M. Antonietta Bracci, Dip. Biologia Animale e dell'Uomo, Università di Roma « La Sapienza », Viale dell'Università 32, 00185 Roma (Italy).

Among anurans, morphology of the adrenal gland is subject to evolutionary variation according to the systematic position of the species, so that different types of adrenal gland may be distinguished in different anuran suborders. The position of the adrenal gland on the ventral renal surface of the adult anuran is the most evident differential characteristic between archaeobatrachians and neobatrachians as regards the morphology of this gland : it is medial in the former, lateral in the latter (1, 2). Analysis of the morphogenesis of these evolutionary variations may indicate how they originate.

During ontogenesis the position of the adrenal blastema varies. At the beginning of its differentiation it occupies a dorsal position between the dorsal aorta, the caval vein and the dorsal renal surfaces. Later the blastema gradually rotates from the dorsal to the ventral surface of the kidney. In neobatrachians the shift continues towards the lateral part of this surface.

The research on the stages of this process in archaeobatrachian and neobatrachian anurans, may allow us to determine whether adrenal gland development displays chronological differences in anuran species correlated to their systematic position, and whether the position of the adrenal gland in adults is attained by means of chronological changes of the development. For these purposes, we compare the variations in the position of adrenal gland during metamorphosis of four anuran species, the archaeobatrachians *Xenopus laevis* and *Discoglossus pictus* and the neobatrachians *Rana esculenta* and *Bufo bufo*.

Morphometric analysis of the rotation process demonstrates that it is characterized by numerous statistically significant chronological differences. Some of them are intragroup differences, *i.e.* within archaeobatrachians or neobatrachians, but most of them are observed among species belonging to both suborders and are related to their sytematic position. In particular, progression towards the ventral position is slower in archaeobatrachians than in neobatrachians. On the other hand, as the adult position on the ventral surface of the kidney is nearer to the medial renal margin in archaeobatrachians than in neobatrachians, at the end of the metamorphosis the gland of the former is closer to the definitive position. Morphogenesis proceeds after the end of the metamorphosis until this arrangement is reached. It is achieved in four months in *Discoglossus pictus*, whereas it proceeds for more than one year in *Rana esculenta*. Evolutionary modifications determined by chronological variations of morphogenesis are including among the cases of heterochronic development (3). Modification of the adrenal structure towards a more advanced arrangement is therefore achieved by means of a heterochronic modification of the morphogenesis.

Most of the dorso-ventral displacement occurs during the metamorphic climax. According to numerous authors (4-7) in many anuran species synthesis and plasmatic concentration

of aldosterone and corticosterone surge at the climax. It is therefore possible that the high levels of adrenal hormones enhance the accelerated rotation during this period.

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CONTIGUITY BETWEEN CHROMAFFIN AND STEROIDOGENIC CELLS IN ADRENAL GLAND OF AMPHIBIA, E. Grassi Milano, S. Contristano and H. Manelli, Dip. Biologia Animale e dell'Uomo, Università di Roma « La Sapienza », Viale dell'Università 32, 00185 Roma (Italy).

Analysis of the evolution of the adrenal gland of Vertebrates demonstrates that a plesiomorphic adrenal gland is a diffused organ, dispersed inside the kidney, with aminergic (chromaffin) and steroidogenic cells scarcely or not at all in contact. In the following evolution the adrenal gland tends to form a discrete and compact organ, external to the kidney, in which aminergic and steroidogenic cells strictly associate, frequently with precise localization. This is the apomorphic type of gland (1). The adrenal gland of Amphibia constitutes an intermediate stage in the evolution of this organ. In the « amphibian type » arrangement, both tissues, steroidogenic and aminergic, emerge from the kidney and associate, although in different degrees. Inside this general structural type, we can recognise a primitive subtype, found in apoda, many urodeles and archaeobatrachian Anura, and a more advanced one, in some neourodeles and in all neobatrachian Anura (2-4). It was proposed (5) that the contiguity between the two types of cell is lower in the primitive subtype. This hypothesis is now verified by morphometric analysis, comparing neourodele *Hydromantes supramontis* with neobatrachian *Rana esculenta*. Preliminary data are also collected on the paedogenetic urodeles *Amphiuma tridactylum* (neourodele) and *Siren lacertina* (primitive Urodele) and on the archaeobatrachian *Discoglossus pictus*.

A quantitative approach, integrated by statistical analysis, demonstrates that in *Hydromantes supramontis* only 50.7 % of aminergic chromaffin cells (CC) are in contact with the steroidogenic cells (SC), whereas this value is 98.5 % in *Rana esculenta*. The difference is highly significant. The cellular volume in anurans is less than in Urodeles, so that in the surface unity the SC + CC are 1.92 in *H. supramontis* and 5.95 in *R. esculenta*, thus improving the contact between the cells. Furthermore the ration CC/CS in the surface unity is 1/4 in *H. supramontis* and 1/1.78 in *R. esculenta*. In *Amphiuma tridactylum* the percentage of mingling is lower than in *H. supramontis*. In *Siren lacertina* the CC are in general isolated from the SC. In *Discoglossus pictus* the degree of mingling is intermediate between the values found in *R. esculenta* and in *H. supramontis*.

The adrenal gland of urodeles then displays a lesser degree of anatomical integration between aminergic and steroidogenic cells with respect to the anurans.

Since reciprocal control mechanisms between the two tissues have been demonstrated (6-8), a high degree of contiguity between CC and SC may improve the functionality of the gland. It is possible that the more complex organization of the anuran adrenal gland, promot-

ing more integrated secretion activities, may explain the wider distribution of the anurans with respect to the urodeles.

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IMMUNOCYTOCHEMICAL AND ULTRASTRUCTURAL STUDY OF *RANA DALMATINA* PARS DISTALIS DURING LARVAL DEVELOPMENT, A. GUASTALLA, E. CAMPANTICO, K. YAMAMOTO, T. KOBAYASHI and S. KIKUYAMA, Università di Torino, Dipartimento di Biologia animale, Via Accademia Albertina 17, 10123 Torino (Italy).

Cytodifferentiation and immunocharacteristics of prolactin (PRL) and growth hormone (GH) secreting cells in the adenohypophysial primordium of *Rana dalmatina* tadpoles at developmental stages 28 to 45 (1) were studied at the electron microscopic level.

Single and double simultaneous indirect immunogold techniques were applied to ultrathin sagittal sections of Epon-Araldite embedded tadpole heads. Rabbit anti-*Rana catesbeiana* PRL and rabbit anti-*Rana catesbeiana* GH were employed in the single method ; rabbit anti-*Rana catesbeiana* PRL and monkey anti-rat GH were used in the double simultaneous technique as primary antisera.

The intensity of the immunoreaction on the pituitary cells was very strong with the antisera anti-*Rana catesbeiana* PRL and GH and virtually all of the immunogold was confined to secretory granules ; it was much weaker and exhibited considerable non-specific binding with anti-rat GH.

At earlier developmental stages, a few hours after hatching, wide intercellular spaces are present in the hypophysial primordium ; the cells contain in their sparse cytoplasm numerous vitelline platelets, lipid droplets and melanine granules. At stage 28 are already identifiable some cells with few membrane-bound secretory granules, 70-90 nm in diameter, immunoreactive with anti-*Rana catesbeiana* PRL, and some others with few secretory granules, 80-110 nm in diameter, immunoreactive with anti-*Rana catesbeiana* GH.

With the progress of the development intercellular spaces become narrower, cytoplasmic inclusions undergo a progressive reduction and immunoreactive cells increase in number. The differentiation in both cell types is shown by an increase in the cytoplasmic volume and in number, size and immunoreactivity of secretory granules. Neither PRL nor GH cells, however, exhibit ultrastructural features specific enough to allow their identification on pure morphological bases, at least up to the end of premetamorphosis.

Neither comparison of adjacent ultrathin sections stained with single labeling technique, nor results of double simultaneous labeling showed coexistence of PRL and GH within the same cell.

Double simultaneous immunogold technique was only applied at development stages 38 and 45, when pituitary cells had reached a higher degree of differentiation. The affinity for

the amphibian hormone of the monkey anti-rat GH employed by us together with the rabbit anti-*Rana catesbeiana* PRL was indeed noticeably lower, probably because raised in different species.

The present findings reject the hypothesis of the existence of « somato-mammotrophic » cells since the earliest developmental stages of hypophysial primordium in *Rana dalmatina* larvae suggested by us in a previous study performed at the light microscope employing indirect immunofluorescence and unlabeled antibody enzyme methods (2).

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REGULATION OF PITUITARY FUNCTION IN AMPHIBIA : EFFECTS OF HYPOTHALAMIC PEPTIDES AND NEUROTRANSMITTERS, T.R. HALL, Biovet Unit, Ciba-Geigy S.A., Centre de Recherches Agricoles, 1566 St. Aubin FR (Switserland).

In common with other vertebrate species, the amphibian hypothalamus contains a set of factors that modulate the secretion of hormones from the pituitary gland, these factors including various peptides and amines. The interactions between neurotransmitters, releasing hormones and pituitary function has been studied for more than three decades, but only during the past few years, with the isolation of the pituitary hormones and subsequent development of specific radioimmunoassays, has a more profound understanding of amphibian neuroendocrinology been made possible. The purpose of the present review is to summarize these findings and to highlight neglected areas of research.

Early studies revealed that acid extracts of hypothalami from several anurans (*Rana, Xenopus* and *Bufo* spp.) have been shown to possess prolactin (PRL), GH and LH-stimulating activity *in vitro*, in common with most other vertebrate species (except that mammalian and some teleost hypothalamic extracts inhibit PRL release). The hypophysiotrophic peptides first isolated from mammalian hypothalami, namely thyrotrophin releasing hormone (TRH), luteinizing hormone releasing hormone (LHRH) and somatostatin (SRIH), are biologically active in Amphibia, though sometimes with surprising effects. Using immunological techniques, HPLC, etc., peptides identical or very similar to these have been found in amphibian brain. Other peptides isolated from mammalian brian or intestinal tract have also been found in Amphibia. In addition, amphibian species are an extremely rich source of neuroactive peptides that have subsequently been demonstrated in the mammalian CNS. Unfortunately, although the activities of these peptides have been extensively studied in mammals, little is known of their functions in frogs.

The prolactin-stimulating actions of TRH were first demonstrated, using relatively crude techniques, in 1975, and confirmed by RIA a number of times. The prolactin-releasing activity of hypothalamic extract is not due solely to its content of TRH, but also to the presence of other factors, separated by chromatography and HPLC, but otherwise not identified. Vasoactive intestinal peptide and the related peptide histidine isoleucine also stimulate prolactin release in bullfrogs, as they do in mammals and birds. Whether other prolactin-releasing agents are present has not yet been determined. Prolactin release-inhibiting agents also exist. Certainly dopamine, as in other vertebrate groups, inhibits secretion of prolactin. An intriguing possibility that GAP, a peptide formed on processing pro-LHRH and that is prolactin-inhibiting in mammals, is a vertebrate prolactin release-inhibiting hormone, has not yet been tested.

GH release is under the control of both releasing and inhibiting peptides. TRH has been shown to release GH *in vitro*, though no physiological role can yet be ascribed. The GH-releasing hormone has not been isolated, though it is likely to be similar to the releasing peptide found in mammalian hypothalami. The inhibitory peptide is SRIH or another similar peptide. In mammals, SRIH exists in two forms, including a larger (28 amino acids) that is not merely a precursor of SRIH-14, but a peptide with its own physiological actions and control. In the vertebrates, a number of SRIH peptides exist, but their roles are not fully understood, and it is not clear whether they are separate entities or artifacts of preparation.

In addition to its effects on prolactin, TRH can stimulate TSH release, as measured by increased thyroid activity, in ranid and at least one urodele (*Ambystoma*) species. TRH also stimulates MSH release from frog neurointermediate lobe, a response antagonized by meuropeptide Y (NPY). Clearly, TRH has an important multifunctional role in the amphibian hypothalamus in conjunction with other influences. LHRH also activates the thyroid axis in ranids and *Ambystoma*, indicating a functional correlation between the gonadal and thyroidal axes.

Serotonin, a neurotransmitter implicated in the stimulatory control of prolactin secretion in mammals and birds, apparently has the same role in the bullfrog. Co-incubations of pituitaries and hypothalami in the presence of serotonin produces a stimulation of prolactin secretion. Moreover, during metamorphosis, when prolactin levels rise, there is a parallel increase in brain serotonin turnover. In addition, monoamine oxidase type A (MAO-A), the enzyme responsible for serotonin inactivation, also increases during this period. Larval Amphibia only have MAO-A, the B from appearing during the transition to a terrestrial lifestyle, when other monoamine transmitters may become more important in brain function. Serotonin may also stimulate GH secretion. The mechanism through which serotonin acts on the pituitary is not known, though it may involve stimulation of release of TRH, and possibly other releasing peptides.

One unsolved problem is the neuroendocrine regulation of metamorphosis and the adaptations of this system on shifting to a terrestrial habitat. Clearly this adaptation involves changes in expression of key enzymes, thereby altering the levels of neurotransmitters which regulate the secretion of the regulatory peptides. This model may hold the key to understanding the physiological adaptations during hatch in birds and during parturition in mammals, both of which involve a relatively rapid shift from an aquatic environment.

THE PHYLOGENY OF THE INFLUENCE OF NONAPEPTIDES OF THE NEUROPHYPOPHYSIS ON THE INTERRENAL GLAND, W. Hanke and W. Kloas, Zoologisches Institut, Universität, D7500 Karlsruhe (F.R.G.).

Nonapeptides of the neurohypophysis are known as hormones with vasoconstrictor and antidiuretic activity in mammals. It has also been shown that vasopressin (AVP) stimulates the turnover of phosphatidylinositol. In connection with this AVP increases aldosterone synthesis in mammals. The extent of this response was found by some authors to be lower than the response on angiotensin II, the most potent stimulator of aldosterone production in mammals. Other authors reported a similar response on AVP than on angiotensin II. The most of the recent papers suggest that the physiological effect of nonapeptides on interrenal secretion is due to a paracrine action of these hormones because they are distributed in cells of the adrenal gland.

Experiments with interrenal *in vitro* preparations of Amphibia (different species) showed that the tissue responds quite strongly to arginine vasotocin (AVT). This was already reported

much earlier than the effect was found in mammalian preparations (1). The response was stronger and obtained with lower doses than that of angiotensin II. This observation is now further characterized (2) and confirmed by others (3).

This effect was not seen in teleost fish. Investigations with interrenal preparations of tilapia did not respond to different and quite high doses of AVT, isotocin or angiotensin II and its analogues. Interrenals of carps did not react on AVT but showed some changes of the secretion rate after high doses of angiotensin II (0.5 µM given fot 60 min). This is quite high compared with Amphibia where 10 nM (given for 5 min) are already effective. Mammalian glomerulosa cells respond already to 0.1 till 1 nM. The difference between tilapia and carp might be due to the adaptational type. The tissue of the stenohaline carp seems to be more sensitive than that of the euryhaline tilapia.

Among the Amphibia, there are different types of adaptation for the environment. Tissue from neotene axolotl responds quite clearly to AVT. 10 nM given for 5 min are already effective. The response to angiotensin II was not clear. 50 or 100 nM were necessary to induce a moderate answer.

The evolution of the regulatory capacity of nonapeptides compared to angiotensin II can be summarized. Teleost fish do mostly not respond to both types of stimulators, except the response of the interrenals of the stenohaline carp to high doses of angiotensin II. Urodelean Amphibia, like axolotl, show a good response to AVT, but angiotensin II has only a moderate action. Frogs and clawed toads react very well to AVT or angiotensin II but the latter is needed in higher doses. Mammalian glomerulosa cells are generally stimulated by lower doses of angiotensin II than AVT.

An extended analysis of the response of an *in vitro* preparation of the interrenals of the clawed toad to AVT has shown that 0.1 nM AVT given for 5 min caused already a clear stimulation of corticosterone and aldosterone release. This is the same range of concentration of two other powerful stimulators, ACTH and urotensin II. The increase of corticosterone and aldosterone secretion after AVT was also seen *in vivo* when the normal concentration in the serum was increased more than 4 fold. It needed 12 hrs to adjust the elevated levels to normal values. The relative increase of the serum values of aldosterone was much higher than that of corticosterone. The C/A ratio dropped from 10-12 to 5. This suggests that the terminal pathway of aldosterone biosynthesis was stronger activated than the primary steps. There is a clear dose dependent response *in vitro* from 0.1 to 50 nM added for 5 min to the incubation medium. In the case of 50 nM, aldosterone release is about 12 fold and corticosterone release about 2.5 fold of the normal secretion rate.

The judgement of the effectiveness of different nonapeptides resulted in the following rank of power : AVT > mesotocin = oxytocin. A similar stimulation of the terminal pathway was induced by phospholipase C which causes the formation of inositolphosphates and diacylglycerol. This suggests that AVT stronger affects the conversion rate of corticosterone to aldosterone.

A special focus of the study was to find the type of receptors being involved in this AVT response. Two types of AVP receptors are discussed in mammals : The V_1 type which is the vascular (vasopressoric) type and the V_2 type which is the renal (antidiuretic) type. It was clearly shown that a V_1-antagonist did not prevent the AVT reponse and a V_2-agonist was ineffective. This indicates that a different type of receptor must exist in this tissue. This is in accordance with some results in mammals where also other types of receptors are suggested.

Further experiments demonstrated clearly the dependency of the stimulation by AVT on extracellular Ca^{++}. A mediation of the AVT effect by the inositol triphosphate system is most probable.

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AMPLEXUS INDUCES LH SURGE IN MALE TOADS *BUFO JAPONICUS*, S. ISHII and M. ITOH, Department of Biology, School of Education, Nishi-Waseda 1-6-1, Tokuo 169 (Japan).

In our recent work, we found in a wild population of *Bufo japonicus* that a rapid increase in the plasma LH level, which is similar to the LH surge in females of higher vertebrates, takes place in males as well as in females at the height of the breeding activity. Furthermore, we showed that the mean plasma LH level in males in amplexus is higher than that in males not in amplexus. These results in combination with our classic knowledge of the Galimani test strongly suggest that the amplexus induces the LH surge followed by the spermiation in male toads.

In order to test this hypothesis, we conducted the following three series of experiments. In the first series, adult male and female toads that have just started the breeding migration but not yet in amplexus were captured from a wild population, and males were kept in plastic boxes with the same number of females or without females. The plasma LH level was monitored for each male toad at about 6 hour intervals over 48 hours by collecting blood samples from the heart without dissection and by determining LH in plasma by means of the radioimmunoassay developed by ourselves. As soon as males were put together with females in the box, they formed the amplexus that lasted for 48 hours. About ten-fold elevation in the plasma LH level was observed in males kept with females. The elevation lasted for 27 hours between 6 and 33 hours after they were put together. In contrast, no significant change in the plasma LH level was detectable in males kept without females throughout the period of 48 hours.

In the second series, solitary males and females were collected near or in the pond one to two weeks later, and similar experiments were conducted. Again, the LH surge was observed in males kept with females but not in males kept alone. In this case, however, the elevation of the LH level started as soon as males were put together with females and lasted for 12 hours. Thereafter, the level declined gradually over 36 hours to the initial level.

In the last series experiments, we attempted to know whether the amplexus itself or just the presence of females is the stimulus to induce the LH surge. We gave a dummy of the female to each male of the experimental group kept in a box. Males of two control groups were kept in boxes with and without females, respectively. The dummy was a block (10 × 6 × 1.5 cm) of « konyaku », which is Japanese food made from the root of a plant (*Amorphophallus conjak*). It has a milky colour and is as elastic as jelly. Experimental males clasped dummies immediately, and the amplexus with the dummy lasted for 12 hours. During this period, the LH surge was observed in the experimental group (as high as that in the control group with females). No significant increase in the plasma LH was detectable in the control group without females.

We concluded from results of these experiments that in males of *Bufo japonicus* the amplexus, or clasping the female for a certain period, forms the stimulus to induce LH surge which in turn results in the spermiation. In the natural condition, the proportion of male individuals is extremely high during the breeding season in this species. Accordingly, spermiation should take place only in successful males in synchronization with ovulation of the female in amplexus. In order to adapt to this condition, the above mentioned neuroendocrine reflex in association with behavior may be constructed in *Bufo* during the course of its evolution.

PERIPHERAL CONVERSION OF T_4 TO T_3 IN THE FROG. INFLUENCE OF THE PITUITARY ? G. JACOBS and E.R. KÜHN, Laboratory of Comparative Endocrinology, Catholic University of Leuven, Naamsestraat 61, B-3000 Leuven (Belgium).

The occurrence of thyoxine (T_4) 5' deiodinase (5'D) activity in amphibians was first demonstrated in anuran tadpoles by Leloup and coworkers. The T_4 to triiodothyronine (T_3) converting system which operates to a small extent at late premetamorphic and early prometamorphic stages in *Xenopus*, reaches maximal activity at midclimax (1). This peripheral conversion of T_4 to T_3 seems to play an essential role in thyroid hormone stimulation of metamorphosis, thus indicating that T_3 is the major metamorphic hormone in anurans (1). The existence of T_4 5'D activity has also been demonstrated *in vivo* in some adult anurans and urodeles (1-3).

Information about the location of 5'D activity in developing and adult frogs has been provided by *in vitro* studies. The enzyme system was detected in gut and skin from metamorphosing and adult *Rana catesbeiana* frogs as well as in the regressing tadpole tail, but could not be localized in other organs, such as liver, kidney, and heart from both larval and adult animals (4). Experiments performed in our laboratory also revealed the presence of T_4 to T_3 conversion in skin homogenates prepared from *Rana ridibunda* frogs (5).

In the present study we examined the T_3-generating capacity of *R. ridibunda* kidneys under different incubation conditions. Conversion of T_4 to T_3, which was demonstrated in the 6000-g supernatant fraction of kidney homogenates, was influenced by substrate and homogenate concentrations and by temperature and duration if incubation. Heating destroyed the T_4 5'D activity. Addition of dithiothreitol (DTT), as exogenous thiolcofactor, to the reaction mixture appeared to be essential to quantitate T_3 production, which even increased markedly in the copresence of EDTA. The DTT + EDTA-stimulated enzyme could partly be inhibited by propylthiouracil (PTU) (25-45 %). When comparing the PTU- and DTT-concentrations, used in this study, with data from the literature, we should have obtained almost complete inhibition if only a type I-enzyme had been responsible for the T_4 5'D activity. Therefore it is suggested that in the frog kidney at least a less PTU-sensitive deiodinase is present. T_4 5'D activity in frog liver was mostly low or undetectable.

Growth hormone (GH) stimulates the peripheral convension of T_4 to T_3 on several vertebrates, such as the eel (6), the chick embryo, adult chicken, rat and dwarf goat (7).

In the present study possible effects of ovine GH (oGH) and bullfrog GH (bGH) (gift from Prof. Dr. S. Kikuyama (Japan)) were investigated in the frog, *R. ridibunda*. An intravenous injection of respectively 1 and 10 μg oGH given on two consecutive days (in October) did not alter plasma T_3 or T_4 concentrations significantly, nor could a change be noted in the renal T_4 5'D activity 5 hours after the second injection. In a second experiment (October) frogs were injected subcutaneously with saline, 1, or 10 μg oGH during 6 days. In the animals which had received the high GH dose, the plasma T_3 and T_4 levels were slightly

increased ($P<0.05$) 5 hours after the last injection. However the T_4 5'D activity in the kidneys remained unchanged. Subsequently, 1.5 µg bGH was administered intravenously on two consecutive days (June), but neither the plasma T_3 and T_4 concentrations, nor T_4 5'D activity were influenced.

It is too soon to conclude that GH would not be involved in the regulation of peripheral deiodination activity in the frog. On the other hand, it seems reasonable to assume that the T_4 5' deiodination system already functions maximally in the adult frog and cannot be stimulated supplementary by exogenous GH. In that case, hypophysectomy experiments combined with GH and/or T_4 injections could give an indication. In order to support this hypothesis, pars distalis- and sham-ectomized frogs were injected intravenously with 10 µg oGH (on 2 consecutive days) starting 4 days after the operation (June). At that time, basal T_4 levels were 2.5 fold decreased, while basal T_3 levels were not different from those in the sham frogs. Unexpectedly, a sharp elevation of plasma T_4 was observed in both groups of animals 2 and 4 hours after oGH-injection ($P<0.0001$). The plasma T_3 level was increased only in the sham frogs. Since the oGH we used did not contain HPLC-detectable TSH-impurities, oGH seems to exert a thyrotropic action in the frog. Therefore, the use of homologous GH appears to be more appropriate for these studies.

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MULTIDISCIPLINARY ANALYSIS OF NEUROENDOCRINE INTEGRATION IN THE PARS INTERMEDIA OF THE AMPHIBIAN, *XENOPUS LAEVIS*, B.G. Jenks, H.P. de Koning, E.P.T.C. de Rijk, T.A.Y. Ayoubi, P.M.T. Deen, I.D. van Zoest, G.J.M. Martens, H.J. Leenders and E.W. Roubos, Department Animal Physiology, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen (The Netherlands).

Melanotroph cells of the amphibian pars intermedia secrete α-melanophore stimulating hormone (αMSH), a peptide that regulates pigment dispersion in dermal melanophores during environmental (background) adaptations. Secretion from these cells is regulated by multiple factors, including neurotransmitters (e.g. dopamine, GABA) and neuropeptides (e.g. TRH, CRH, NPY). In our laboratory the mechanisms of neuroendocrine integration in the pars intermedia of the toad, *Xenopus laevis* are being investigated, using morphological, biochemical and molecular biological techniques. Given below is a summary of recent findings :

1) *Melanotroph Cell Recruitment* : Analysis of the activity of intermediate lobe melanotrophs from animals adapted to different backgrounds (white, black and shades of grey) reveals that the melanotrophs function as a heterogeneous cell population in meeting

the increasing demand for αMSH ; there is recruitment of progressively more melanotrophs from the inactive to the active state with increasing blackness of background. The proportion of active cells correlates positively with both the level of circulating αMSH and the degree of pigment dispersion in the melanophores of the animals. We are currently examining the underlying mechanisms for progressive recruitment ; initial studies indicate that there are differences among the melanotrophs in their threshold to respond to secretagogues.

2) *Intracellular Mechanisms of Integration* : Most secretagogues act directly on the melanotroph cell to regulate MSH release. The mechanisms of transmembrane signaling by the secretagogues are now under investigation. Two regulatory mechanisms are negatively coupled to the adenylate cyclase system, namely those activated by dopamine D_2 receptors and $GABA_b$ receptors. Such activation leads to an inhibition of αMSH secretion. CRH is positively coupled to adenylate cyclase and it stimulates secretion. TRH also stimulates secretion, an action which is associated with production of inositol phosphates in the melanotrophs. At least one ionotropic mechanism is present, namely that functioning through the $GABA_a$ receptor. Activation of this receptor leads to an influx of chloride and, consequently, an inhibition of αMSH secretion.

3) *Cellular Mechanisms of Integration* : Recent findings indicate the existence of an indirect mechanism in the regulation of MSH secretion, namely that exerted by neuropeptide Y (NPY). The evidence for this comes from *in vitro* superfusion experiments ; NPY inhibits αMSH secretion from the intact pars intermedia but has no effect on isolated melanotroph cells. There are two possible mechanisms for an indirect action of a secretagogue in the pars intermedia : it could function either presynaptically (*i.e.* stimulate release of inhibitory neurotransmitters from nerve terminals within the tissue) or act via a non-endocrine cell-type such as folliculostellate cells, which make intimate contact with melanotroph cells. In fact, the latter appears to be the case for NPY because : i) NPY inhibits αMSH secretion in tissue in which functional nerve terminals have been eliminated, ii) NPY containing neurons have been found to make synaptic contact with stellate cells and, iii) specific binding sites for NPY have been localized on stellate cells. Possibily, NPY induces release of an αMSH-release-inhibiting factor from the stellate cell.

4) *Morphological Basis for Regulation* : Within the neurointermediate lobe fibers are present that are immunopositive for each of the established MSH-secretagogues. In some cases the fibers are restricted to the neural lobe (*e.g.* those containing CRH and TRH), indicating a diffuse neurohormonal action on the melanotrophs. In other cases, the immunopositive fibers are found throughout the pars intermedia (*e.g.* GABA and NPY). At the ultrastructural level, the pars intermedia possesses a rich network of fiber varicosities, some of which make synaptic contacts with melanotrophs and folliculo-stellate cells. NPY and GABA have been found to be colocalized within these varicosities, NPY occuring primarily in dense core vesicles and GABA in electron-lucent vesicles.

5) *Different Secretory Pathways* : Evidence has been obtained for the functioning of different secretory pathways within melanotroph cells. One pathway involves newly synthesized peptides (secreted within 6h of their biosynthesis) and the other involves secretion of mature secretory material. The newly synthesized pathway secretes primarily the acetylated form of αMSH while desacetyl-αMSH is the main form secreted from the mature secretory pathway. The peptide content of these secretory pathways with respect to other proopiomelanocortin (POMC) derived peptides is now being studied, as is the possibility that the pathways could be independently regulated ; the latter situation would greatly enhance the potential of melanotrophs to generate diverse biological signals.

6) *Regulation of POMC Gene Expression* : The level of POMC mRNA is high in the pars intermedia of animals adapted to black background and low in white-adapted animals. These

differences are reflected in the levels of POMC biosynthesis found in the pars intermedia. In transferring black adapted animals to a white background the levels of both POMC mRNA and POMC biosynthesis fall only very gradually, taking several days to reach the level of white-adapted animals. These results could indicate either a slow inhibition of POMC gene transcription or a very stable POMC mRNA. That the latter might well be of importance is indicated by a kinetic analysis showing that the half-life of POMC mRNA is over 30 h. The effects of various secretagogues on POMC gene transcription are currently under investigation.

7) *Coordinate Gene Expression* : Differential hybridization techniques are being used to find genes that are co-expressed with the POMC gene during physiological adaptations. Such genes could code for proteins essential for the secretory function of the melanotroph cell, such as enzymes for the processing of POMC or proteins necessary for the proper functioning of the secretory machinery. One co-expressed product, namely the 7B2 protein, has been characterized. This 25 kDa protein is highly conserved during vertebrate evolution, is associated with secretory granules within the melanotroph and the 18 kDa processed product of the protein is co-secreted with POMC-derived peptides. At present, the possible role of 7B2 in the secretory process is being investigated.

EFFECTS OF CORTISOL ON CUTANEOUS WATER PERMEABILITY IN TOADS, C. B. JØRGENSEN, Zoophysiological Laboratory A, August Krogh Institute, 13 Universetetsparken, DK-2100 Copenhagen 0 (Denmark).

The increase in cutaneous water permeability in frogs and toads in response to dehydration is widely assumed to depend upon secretion of arginine vasotocin from the pars nervosa of the hypophysis. However, elimination of pars nervosa function only slightly affected the response to dehydration in the toad *Bufo bufo* (1). Moreover, effects may result from interference with pars distalis functions, particulary corticotropic function that arise as side effects of the operations (2). Therefore effects were studied of treatment with a corticosteroid, cortisol, on the cutaneous water permeability in toads, as well as its response to dehydration.

Cortisol pellets implanted under the skin of toads kept in water substantially enhanced the water influx, which approached a plateau 2-3 times higher than the initial within a week or more. Also, the cortisol implants affected the time constants of normalization of the cutaneous water permeability in rehydrating dehydrated toads. Thus, the mean halftime of decline in water influx increased from 100 ± 31 (S.D.) min (n = 15) to 190 ± 64 min (n = 9). The difference was highly significant, $P < 0.001$. By contrast, there was no demonstrable effect of the cortisol implants on the initial water influx response to dehydration.

The effects of implanting cortisol pellets in water-acclimated toads was similar to the effect of acclimation of the toads to a simulated terrestric environment, a terrarium with free access to water. The level of interrenal activity may therefore be one factor in the regulation of the cutaneous water permeability, by controling opening and/or number of epidermal water pores (3).

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CARBOHYDRATES IN AMPHIBIAN CUTANEOUS GLANDS : A LECTIN HISTOCHEMICAL STUDY , J.C. KALTENBACH, K.E. NYTCH and C.H. POTTER, Department of Biological Sciences, Mount Holyoke College, South Hadley, MA 01075 (U.S.A.).

In recent years considerable interest has focused on the chemical composition of exocrine glands in frog skin. Immunohistochemical staining has revealed the presence of a number of peptides in granular, serous glands, but not in mucous glands. For example, we have demonstrated such a distribution for the immunoreactive tripeptide, TRH. In addition, we have shown opposite glandular sites for polysaccharides (PAS technique), and acid mucopolysaccharides (alcain blue, toluidine blue), i.e., the absence of such components from serous glands, and their presence in mucous glands (1). The present study was undertaken to further characterize the carbohydrate components of frog cutaneous glands by means of peroxidase-conjugated lectins with affinities for specific sugar residues.

Pieces of dorsal skin from adult frogs (*Rana catesbeiana, Rana pipiens* and *Xenopus laevis*) and from *R. catesbeiana* tadpoles in representative developmental stages were fixed in Bouin's solution in preparation for light microscopy. Two peroxidase-labeled lectins were used : 1) soy bean agglutinin (SBA) from the soy bean *Glycine max*, with main specificity for N-acetyl-D-galactosamine and 2) wheat germ agglutinin (WGA) from the wheat species *Triticum vulgaris*, with specificity for N-acetyl-D-glucosamine and sialic acid. Sections were treated with hydrogen peroxide to block endogenous peroxidase, incubated with peroxidase-conjugated lectin, followed by H_2O_2-diaminobenzidine (H_2O_2-DAB) solution. Controls were performed 1) to assure specificity of the lectin binding (incubation with peroxidase-lectin solution containing N-acetyl-D-galactosamine, inhibitor for SBA, and N-acetyl-D-glucosamine and sialic acid, inhibitors for WGA) and 2) to check endogenous peroxidase activity (incubation of non-treated sections with H_2O_2-DAB).

SBA lectin conjugate stained all serous glands, as well as the outer epidermis. Stain was not visible in mucous glands, basal epidermis, or dermal connective tissue. Reactions were similar in the skin of the three species of frogs studied. Moreover, the same general pattern was apparent in the skin of developing tadpoles (*R. catesbeiana*) as soon as gland development became evident. Both types of controls inhibited the staining reactions. On the other hand, WGA lectin conjugate gave strong staining reactions in both serous and mucous glands, as well as in the epidermis and connective tissue. In the controls, staining was only inhibited by a peroxidase lectin solution containing N-acetyl-glucosamine, but was completely blocked by the addition of both the glucosamine and salic acid. H_2O_2-DAB controls were also negative. Our results suggest that serous, but not mucous, glands contain N-acetyl-galactosamine, while both types of glands contain sialic acid.

SBA and other lectins have been shown to bind to keratinocytes in the epidermis of *Rana perezi* and *Rana ridibunda* (2, 3). Our results confirm those findings. Moreover, to our knowledge the present study represents the first application of lectin staining to frog cutaneous glands. Our observations provide new information on the composition of stored secretions in these glands. Further studies to localize a variety of sugar moieties in the epidermis and cutaneous glands, which release their contents onto the epidermal surface, will serve as a basis for understanding the functions of the amphibian skin. This research was supported in part by a grant from the Research Corporation.

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COMPARISON OF BINDING SITES FOR ATRIAL NATRIURETIC FACTOR (ANF) AND VAL^5-ANGIOTENSIN II (A II) IN DIFFERENT AMPHIBIAN SPECIES,
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The osmomineral regulation of vertebrates is largely under endocrine control. Integration and coordination of the complex osmoregulatory processes are controlled by adrenal steroids and catecholamines, posterior pituitary peptides, the renin-angiotensin-system (RAS) and atrial natriuretic factor (ANF), by influencing the kidney and the central nervous system (CNS). Furthermore, the adrenal secretion is influenced by other hormones. In mammals, it is well known that the physiological effects of ANF and A II in kidney, adrenal and CNS directly oppose one another (1). The occurrence of these peptides in amphibians leads to the question whether a control of osmomineral regulation, similar to that found in mammals, does exist. Therefore we investigated the distribution and the properties of binding sites for ANF and A II in the kidneys, adrenal tissue and the CNS of different amphibian species by quantitative *in vitro* autoradiography to see if the binding sites are colocalized, which may imply an opposite function. The amphibian kidney is strongly connected to the adrenal tissue which is located on the ventromedian side. Earlier studies showed that in *Xenopus laevis* the binding sites of ANF and A II are colocalized only in the glomeruli of the kidney but not in the adrenal tissue (2). To see, if this finding could be used as a general model for Amphibia we studied the aquatic urodele *Ambystoma mexicanum*, the secondary aquatic anuran *Xenopus laevis* and the semiterrestrial anuran *Rana temporaria*.

Quantitative *in vitro* autoradiography : frozen sections were thaw-mounted and incubated either with ^{125}I-rANF (99-126) or ^{125}I-Val^5-A II as described elsewhere (2). Localization and quantification of binding sites were determined by computerized microdensitometry.

Specific ANF-binding sites were found in all three amphibian species but with a different distribution and quantity, respectively. In the CNS of *Xenopus laevis*, the highest density and quantity of ANF-binding sites occur in the *nuclei* (*n.*) *habenulares* and the *pars nervosa* of the pituitary, but also in the thalamic area and the *n. interpeduncularis*. A moderate number of binding sites is determined in the *bulbus olfactorius, pallium, n. accumbens septi, striatum*, lateral forebrain bundle, *tectum* and *n. infundibularis ventralis*. *Rana temporaria* shows a similar pattern, but the density of binding sites is lower than in *Xenopus*. In *Ambystoma mexicanum*, the highest density is found in the *pars nervosa*, but density and quantity of all binding sites are less than in *Rana remporaria*.

In the kidneys of all three species, ANF-binding sites are found in glomeruli and additionally in tubules of *Ambystoma mexicanum*. The diffusely distributed adrenal gland of *Xenopus laevis* shows certainly binding sites in the interrenal tissue and probably in chromaffin cells, too. In *Rana temporaria*, the tissue surrounding the adrenal gland has ANF-binding sites like some of the adrenal cells. In the adrenal tissue of *Ambystoma mexicanum* we could not detect ANF-binding sites.

The localization of specific A II-binding sites was successful only in *Xenopus laevis* and *Rana temporaria*, while in *Ambystoma mexicanum*, we observed a lack of A II-binding sites in all investigated organs.

In the CNS of *Rana temporaria* A II binding sites of high density are located in the *pars nervosa* and the area of the *amygdala*. A lower number and density of binding sites are found in the interventricular organ and the *striatum* as well as in the *tectum*. In *Xenopus laevis*, a similar pattern exists, but with much lower density and quantity. Only in the *pars nervosa* and the area of the *amygdala*, distinct binding sites could be observed and a diffuse area around the interventricular organ.

The glomeruli of both anurans contain A II binding sites, but *Rana temporaria* has a higher binding capacity than *Xenopus laevis*. The adrenal tissue of *Xenopus laevis* lacks A II binding sites, while the adrenal gland of *Rana temporaria* shows a similar pattern to ANF-binding sites.

CONCLUSIONS : Our results clearly show that a general model of osmoregulation in Amphibia does not exist. Three different species show three different patterns of binding sites for ANF and A II. Opposite physiological effects of ANF and A II could exist in the anuran glomeruli by affecting the glomerular filtration rate and in the anuran *pars nervosa* by influencing the secretion of AVT and/or mesotocin. Furthermore, in *Rana temporaria* a physiological antagonism of both peptides might act on the adrenal tissue. The lack of A II-binding sites in the organs of *Ambystoma mexicanum* and their occurence in *Xenopus laevis* and, more pronounced, in *Rana temporaria* implicates that the importance of the renin-angiotensin-system might have been developed during the metamorphosis from aquatic to terrestrial life style, while ANF seems to be a very conservative hormone playing an important role for osmoregulation in all amphibian species.

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VASOTOCIN ACTS AS A LOCAL REGULATOR OF CORTICOSTEROID SECRETION IN AMPHIBIANS, A. Larcher(1), C. Delarue(1), F. Vandesande(2), G. Pelletier(3) and H. Vaudry(1), (1) Laboratory of Molecular Endocrinology, CNRS URA 650, University of Rouen, Mont-Saint-Aignan (France) ; (2) Zoological Institute, Naamsestraat 59, Leuven (Belgique) ; (3) Laboratory of Molecular Endocrinology, Laval University, Québec (Canada).

The neurohypophysial neuropeptides vasopressin (AVP) and oxytocin (OXT) have been identified in various organs other than the brain, such as testis, placenta and ovary. The occurence of AVP- and OXT-like peptides has recently been shown in both adrenal cortex and medulla of various mammalian species. In addition, AVT has been shown to stimulate steroid secretion in the rat and bovine adrenal cortex. In amphibians, the peculiar structure of the adrenal (interrenal) gland which is composed of intermingled chromaffin and steroid-producing cells, favours interactions between the two types of cells. In fact, we have recently shown that in frogs, various neuroendocrine factors produced by chromaffin cells, such as vasoactive intestinal peptide, atrial natriurectic factor, dopamine and serotonin are involved in the regulation of adrenocortical cells.

The aim of the present study was to investigate the presence of arginine-vasotocin (AVT), the amphibian couterpart of mammalian AVP, in the frog adrenal gland and to examine the possible role of this neuropeptide in the control of corticosteroid production. We have applied the indirect immunofluorescence technique to examine the occurrence of AVT in the frog interrenal gland. Labeling of consecutive sections with antisera against AVT, tyrosine

hydroxylase (TH) and phenylethanolamine-N-methyl-transferase (PNMT), revealed that an AVT-like peptide was localized in both adrenaline- and noradrenaline- producing cells. In contrast no labeling of frog adrenal slices was observed using antibodies against mesotocin (MT). At the ultrastructural level, the immunogold technique revealed that the AVT-immunoreactive peptide is sequestered in chromaffin granules with various electron densities. Filtration of frog adrenal tissue extracts on Sep-Pak C-18 cartridges showed that the elution profile of AVT-like peptide was similar to that of synthetic AVT. The role of AVT in the regulation of frog adrenocortical cells was studied *in vitro* using a perifusion system technique. Graded doses of AVT (10^{-10} M to 10^{-7} M) induced a dose-dependent stimulation of both corticosterone and aldosterone production. Half-maximum stimulation was obtained with a concentration of 4×10^{-10} M. All other neurohypophysial peptides were able to elicit corticosteroid production but the potencies of OXT, AVP and MT were approximately 100, 400 and 1500 times lower than that of AVT. AVT was also capable of stimulating steroid secretion from acutely dispersed frog interrenal cells. These data show that AVT acts directly on adrenocortical cells to stimulate corticosteroid release. Iterative doses of AVT (10^{-9} M) given at 120 min intervals induced identical peaks of corticosterone and aldosterone. In contrast, prolonged administration (4 h) of AVT (10^{-9} M) induced a transient increase of corticosteroid secretion followed by gradual decline of steroid output suggesting the existence of a rapid desensitization phenomenon.

The mechanism of action of AVT has also been investigated in our model. The cyclooxygenase inhibitor indomethacin (5×10^{-6} M) which totally blocks angiotensin II (A II) — or acetylcholine (ACh) — induced corticosteroid secretion did not affect the response of the interrenal gland to AVT. Similarly the production of PGE_2, which was stimulated by A II or ACh, was not affected by AVT. These results indicate that the mechanism of action of AVT on corticosteroid secretion, in contrast to that of A II or ACh, is not mediated through prostaglandin synthesis. To investigate the type of receptor involved in the stimulatory action of AVT, interrenal slices were stimulated with synthetic AVT in absence or presence of various antagonists. The effect of AVT (5×10^{-10} M) was blocked by both the antidiuretic antagonist V2 ([d(CH_2)5,D-Phe^2,Ile^4,Ala^9-NH_2] AVP ; 10^{-6} M) and by the oxytocinergic antagonist ([d(Ch_2)5,Tyr(OMe)^2,Orn^8] vasotocin ; 10^{-6} M). In contrast vasopressor antagonists of type 1 such as [Asu^{1,6},Arg^8)-vasopressin] did not affect the response of the interrenal gland to AVT.

Since the anatomical organization of the frog interrenal gland favours cross-talks between corticosteroidogenic cells and chromaffin tissue, our data strongly support the concept that AVT produced by chromaffin cells may act as a local regulator (paracrine factor) of corticosteroid secretion.

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THE ROLE OF PARS DISTALIS/PARS INTERMEDIA AND THE ADRENAL IN MOULTING AND COLOUR OF THE SKIN, STRESS AND OSMOREGULATION IN TOADS, L. Olesen Larsen, Zoophysiological Laboratory A, August Krogh Institute, Universitetsparken 13, DK 2100 Copenhagen (Denmark).

Examples of interrelationships between the pituitary gland, the adrenal, skin moulting and osmoregulation will be given. I intend to provide a framework at the level of the integrated organism, in which details with regard to molecular and cellular aspects can and should be fitted. Moulting of the skin in *Bufo bufo* is especially suitable as a sensible and

easily observed criterion of function of the hypothalamic-pituitary-interrenal axis. Results obtained by experimental manipulation at all levels have been reviewed (1-5). The corticotrophin releasing hormone seems to be related to pars nervosa hormones, and corticosteroids are necessary for moulting ; all tested corticosteroids are able to elicit a moult in toads deprived of pars distalis.

Moulting of the skin, colour of the skin and stress factors. At the first symposium, I reported results obtained in crowded and non-crowded (single) *Bufo bufo* (6). Crowded toads kept on a black background showed a markedly lower melanophore index (MI) (2.0 versus 4.0) indicating increased sympathetic activity. Average interval between moults was the same in the 3 groups, indicating similar levels and patterns of circulating corticosteroids (3-5). However, the two crowded groups included more toads with irregular moulting intervals than the uncrowded group. Similar results were obtained recently with 3 groups of 5 toads each : 1. Nearly undisturbed, 2. Length, weight and MI recorded frequently, 3. As 2, but force-fed with amounts of mealworms equal to those eaten by 1 and 2. Average MI was 4.3, 3.9 and 3.6 respectively. Average interval between moults was the same in the 3 groups. Irregularity of moulting intervals increased with increasing level of disturbance. So crowding and manipulation had similar effects on the 3 parameters. Possible interpretations will be discussed.

Salinity and moulting interval. In 3 groups of *Bufo viridis* kept in tap water, 230 or 400 mOsm NaCl, moulting intervals were 4.3, 5.7 and 6.7 days (Uri KATZ, thesis, Hebrew University of Jerusalem, 1973). Possible interpretations will be discussed.

Pituitary-interrenal axis. In a recent review (7) I collected evidence for the role of interrenal steroids in sexual maturation in lampreys and other vertebrates. Here I also discussed the possibility that pars intermedia of the pituitary gland may stimulate interrenal secretion, by means of a POMC-derivative. In this connection data published by RODRIGUEZ and coworkers (8-10) become of interest. They demonstrated that when the pars distalis of *Bufo arenarum* was extirpated, interrenal cells underwent atrophy, but 60 and 90 days after the operation, they looked normal or even more stimulated, at a time when pars-distalis-like cells appeared in the pars intermedia. In spring similar cells appear spontaneously. In this light our first report on moulting in *B. bufo* becomes of interest. JØRGENSEN and NIELSEN (11), reported that toads with extirpated median eminence did not moult spontaneously, but when lysine-vasopressin was injected, moults were induced (in 12 out of 12 toads). When also the pars distalis was extirpated, lysine-vasopressin had no effect (in 0 out of 18 toads). However, injections of Insipidin (extract of neurointermadiate lobes) induced moults (in 11 out of 12 toads). We postulated that « pars distalis activity » was present as an impurity in Insipidin, but it may also be a POMC-derivative from the pars intermedia. Similar results, indicating corticotropic activity in neurointermediate lobes, were obtained with whale or toad preparations (11).

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SKELETAL GROWTH IN THE TOAD, *BUFO BUFO*, TREATED WITH BOVINE OR HUMAN GROWTH HORMONE, L. OLESEN LARSEN and ANDERSEN, Zoophysiological Laboratory A, August Krogh Institute, Universitetsparken 13, DK Copenhagen (Denmark) and Section for Biochemistry, Agricultural University of Norway, P.O. Box 36, N-1432 Aas-NLH (Norway).

Two unpublished studies showed that growth in length (reflecting skeletal growth) took place in small toads fed with carbohydrate (sucrose or starch) or lipid (pork fat). This indicates that skeletal growth and net protein synthesis can be separated. A separation of growth in body mass and in body length occured in long term studies of feeding and growth patterns (1, 2). It was therefore decided to test whether growth hormone (GH) in toads not fed at all could induce skeletal growth. It was further decided to compare bovine GH (b-GH) with human GH (h-GH), which has been shown to stimulate growth in crocodiles (ANDERSEN *et al.*, to be submitted).

Four groups of toads with body lengths ranging from 49 to 63 mm were subjected to (a) no food and injections of saline, (b) feeding ad libitum on mealworms, (c) no food and injections of 10 μg b-GH 5 days per week, (d) no food and injections of 10 μg h-GH 5 days per week. In group 1 one toad did not grow, the other 4 grew 1-2.5 mm during the first 2 weeks ; then growth stopped. In group 2 all 5 toads grew, and after 5 weeks growth had only stopped in one ; the range of increase in body length was at that time 7-16 mm. Group 3 grew for ca 3 weeks, range 2.5-6 mm ; 4 toads grew more than any starved control. Group 4 grew for ca 2 weeks, range 0.5-3.5 mm ; 3 toads grew more than any starved control.

It can be concluded that despite lack of food, control toads showed skeletal growth, further stimulated by GH, b-GH being slightly more effective than h-GH. Skeletal growth must be based on mobilization of small amounts of endogenous protein for matrix and probably on mobilization of calcium salts present in endolymphatic sacs in the brain and along the spinal cord (3).

In toads fed live food, GH only prolongs the period in which growth occurs, but does not cause supranormal growth rate (*Bufo boreas* (4) ; *Bufo bufo* (5). In these studies it was not possible to decide whether GH primarily acts at the level of anabolic processes and secondarily on the level of food intake (appetite) or vice versa. In the present study GH certainly stimulated anabolic processes in the skeleton.

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NEW INSIGHT INTO THE REGULATION OF FROG ANDRENOCORTICAL FUNCTION, F. LEBOULENGER, M. MORRA, F. HOMO-DELARCHE*, P. NETCHITAÏLO and H. VAUDRY, Lab. Endocrinologie Moléculaire, CNRS URA 650, Université de Rouen, 76134 Mont-St-Aignan (France) ; * Lab. Immunologie Clinique, INSERM U25-CNRM LA 122, Hôpital Necker, 75743 Paris (France).

In amphibians, the morphological organization of the interrenal gland, where paraneurons and steroidogenic cells are tightly intermingled, favours functional interactions between the two tissues. Steroidogenic cells are also under the influence of nerve afferences supplying the interrenal gland. In previous investigations, we have shown by immunohistochemical and biochemical methods that frog interrenal chromaffin cells contain proenkephalin A-derived peptides and vasoactive intestinal peptide (VIP). Using the perifusion technique, we have demonstrated that VIP stimulates corticosteroid production by frog interrenal slices whereas enkephalins exert no effect. More recently, we have shown that chromaffin granules also store serotonin (5-HT) and arginin-vasotocin (AVT) which both stimulate corticosteroid output in a dose-dependent manner. We have previously shown that acetylcholine (ACh) exerts a dose-dependent stimulatory action on corticosteroid production and that atrial natriuretic peptide (ANP), which is present in splanchnic nerve inputs, strongly reduces ACTH- and A II-evoked corticosteroid secretion. Several of these putative signals may interact with each other at the adrenocortical cell level. When infused simultaneously, VIP and 5-HT exert synergistic effects on corticosterone and aldosterone productions whereas concomitant administration of VIP and ACh induces stimulations of corticosterone and aldosterone release which are strictly additive. In contrast, simultaneous infusion of ACh and 5-HT causes a total blockage of the stimulatory effect of 5-HT. ACh exerts its inhibitory action upon 5-HT-induced steroidogenesis through muscarinic receptors since muscarine also blocks the steroidogenic action of 5-HT whereas nicotine does not. Recently, we have investigated a possible role for catecholamines in the regulation of corticosteroid secretion in the frog. Using the perifusion technique we found that exogenous dopamine (DA), for concentrations ranging from 5×10^{-8} to 10^{-3} M, caused a dose-dependent inhibition of corticosterone and aldosterone secretion by frog interrenal slices. Noradrenaline and adrenaline also inhibited corticosteroid release but were respectively 100 and 2000 times less potent than DA in our model. HPLC analysis combined with electrochemical detection revealed that DA is contained in substantial amounts in frog interrenal extracts (24.1 ± 3.7 ng/mg wet tissue) and that it is released by perifused interrenal slices (42 ± 5 pg/min per gland). The inhibitory effect of DA was reproducible and did not induce any desensitization phenomenon. DA also inhibited corticosteroid production by dispersed interrenal cells, indicating that DA exerts its effect directly on adrenocortical cells. Prolonged infusion of DA which induced a sustained inhibition of basal corticosteroid production did not alter the stimulatory effect exerted by ACTH or 5-HT but reduced by 50 % the angiotensin II-evoked stimulation of corticosteroid secretion. DA induced also an important reduction of prostaglandin (PGE_2 and PGI_2) release which preceded inhibition of corticosteroid output by 20 min, indicating that DA might affect arachidonic acid metabolism. Using [^{3}H] myoinositol or [^{3}H] arachidonic acid-prelabeled interrenal slices we verified that DA actually inhibits the formation of inositol phosphates (IP, IP^2 and IP^3) as well as that of diacylglycerol and arachidonic acid. These results indicate that in amphibians DA might directly depress corticosteroidogenesis via receptors coupled to phospholipid turnover by inducing inhibition of inositol phosphate formation. Immunohistochemical studies using antibodies directed against substance P (SP), a member of the tachykinin family, showed that frog interrenal gland is innervated by an abundant network of immunoreactive fibers. These fibers do not derive from splanchnic nerve since bilateral transection of this nerve or total lesion of celiac ganglion resulted within 15 days in

an increase in SP-like immunoreactivity in the fibers. Using the perifusion technique, we found that exogenous SP, for doses ranging from 10^{-8} to 5×10^{-5} M, induced a dose-dependent stimulation of corticosterone and aldosterone productions by frog interrenal slices. Corticosteroid secretion was also enhanced by a series of tachykinin-related peptides although SP was the most potent peptide of the tachykinin family; especially neurokinin A was 10 times less potent that SP in stimulating frog corticosteroid production. Repeated or prolonged infusion of SP induced a rapid and prolonged desensitization phenomenon, characterized by an attenuation of the steroidogenic response of the interrenal slices. Whether SP stimulates directly or not adrenocortical cells remains unknown since this effect was not found using dispersed interrenal cells. SP also enhanced prostaglandin (PGE_2 and PGI_2) release in the perifusate medium and this effect preceded by 10-15 min the increase in corticosteroid output, suggesting that SP may stimulate steroidogenesis by activating arachidonic acid metabolism. Characterization and identification of the tachykinin-related peptide present in the frog interrenal gland is in progress. Taken together our previous and recent results support the concept of a neuroendocrine control of corticosteroidogenesis in amphibians, involving locally produced paracrine and neuronal signals. This regulatory system might be activated during neurogenic stress. Supported by a grant from DRET (n° 87-135).

IDENTIFICATION OF AN UNKNOWN GASTRIN-34 (1-10)-LIKE PEPTIDE IN THE HYPOTHALAMO-HYPOPHYSEAL SYSTEM OF THE FROG, G. MAREELS and F. VANDESANDE, Laboratory for Neuroendocrinology, Zoological Institute, Naamsestraat 59, B-3000 Leuven (Belgium).

An immunocytochemical study with an antiserum raised against the NH_2-terminal sequence (Pyr-Leu-Gly-Pro-Gln-Gly-Pro-Pro-His-Leu) of human gastrin-34 on brain slices of the frog *Rana temporaria* showed immunoreactive neurons in the nucleus preopticus pars magnocellularis. This neurons projected through the hypothalamo-hypophyseal tractus immunoreactive fibres to the median eminence and to the pars nervosa of the pituitary.

When such brain slices were stained with an anti-CCK-8 antiserum, recognizing the COOH-terminal of both CCK and gastrin, the immunoreactivity in the pars nervosa could not be found. So this reaction is due to the presence of an — up to now — unknown peptide related to gastrin-34 (1-10).

To identify this unknown peptide we developed large amounts of specific monoclonal antibodies in ascites tumor. After purification of the ascites fluid by Protein-A Sepharose affinity chromagraphy, the antibodies can be coupled on a CNBr-activated Sepharose-4B gel.

The peptide isolation starts by homogenization, extraction and centrifugation of a hundred frog pituitaries and this supernatant can be loaded on the immunoaffinity column. Reversed phase HPLC will be used for further purification of the eluent. The last and most exciting step will be the determination of the amino acid sequence using gasphase sequenator.

URODELE PROLACTIN : PURIFICATION AND RADIOIMMUNOASSAY, K. MATSUDA (2), K. YAMAMOTO (1), S. TANAKA (2) and S. KIKUYAMA (1), (1) Department of Biology, School of Education, Waseda University, Tokyo 169 (Japan); (2) Institute of Endocrinology, Gunma University, Maebashi 371 (Japan).

A urodele prolactin (PRL) was purified from the pituitary glands of the newt (*Cynops pyrrhogaster*) by subjecting the acid acetone extract to anion-exchange high-performance liquid chromatography (HPLC) (Mono-Q), gel filtration HPLC (Superose-12), and reverse-phase HPLC (TSK-gel ODS). The newt PRL had a molecular weight of 23 KD as determined by SDS-PAGE. The isoelectric point of the newt PRL was 4.7. Its amino acid composition is in good agreement with the results of amino acid analysis of PRLs of anurans and mammals. The amino acid analysis also revealed that newt PRL possesses six half-cystines as in anurans and mammals, whereas teleost PRLs have four.

Antiserum against newt PRL was raised in a rabbit. Employing the antiserum and newt PRL, a specific and sensitive homologous radioimmunoassay for newt PRL was developed, several dilutions of plasma and pituitary homogenate of newts yielded dose-response curves that paralleled the standard curve. Plasma from hypophysectomized newts showed the least amount of crossreaction. Pituitary homogenates of other species of urodeles gave inhibition curves that parallel the standard curve, whereas purified PRLs of anurans gave inhibition curves that did not parallel the standard. Mammalian PRLs showed no inhibition of binding. The radioimmunoassay was applied to the determination of plasma PRL levels in newt captured in every month of the year. In the adult newts of both sexes, plasma PRL levels were relatively low after the breeding season (early spring) and during summer and early autumn. In the male, the levels rose markedly in March, while in the female, the levels became high in February and November.

PRL secreted prior to and during the breeding season may contribute to the development of the cloacal glands, tail fin, and Mauthner's neuron in the male and of the oviduct in the female, as suggested by the results of earlier experiments in which mammalian PRLs were exclusively used.

SEXUAL DIFFERENCES AS ADAPTATION TO THE DIFFERENT GENDER ROLES IN *XENOPUS LAEVIS*, S. MERKLE, GSF-Institut für Säugetiergenetik, Ingolstädter Landstr. 1, 8042 Neuherberg (F.R.G.).

Sexual dimorphism is a well-described phenomenon in amphibians. One of the most striking, obvious differences is the body size of males and females. An extensive study showed that in 90 % of all anuran species studied, females are larger than males (1). Moreover, sex-related differences were found in physiological parameters such as plasma concentration of calcium and lipids and were associated with differences in gametogenesis (2). However, the higher body weight loss and higher nitrogen excretion rates observed in females compared to males as seen in starving *Xenopus laevis* have not yet been explained (3). In order to test the hypothesis that this feature is a consequence of a higher turnover rate due to a higher motor activity and/or basal metabolic rate of females, fed adult animals of both sexes were investigated for oxygen consumption, motor activity, food consumption and several other physiological parameters in plasma and tissues. They were acclimated to 20° C with a light/dark cycle of 12/12.

As expected, female animals exhibited an approximately 35 % higher total oxygen consumption and motor activity increased by similar magnitude. Consequently, the difference

between the sexes in total metabolic rate observed in *Xenopus* seem to be mostly due to different motor activities. However, the difference in metabolic rate persisted even when the oxygen consumption at the same level of activity was compared. The calculated difference at an activity level of zero, for instance, was approximately 30 %, indicating that in addition, females have a higher basal metabolic rate. Furthermore, females ingested about 30 % more than males. The higher food intake and motor activity in females compared to males seem to be associated with a higher body growth and a higher substrate requirement for gametogenesis in this sex. As a consequence, in the natural environment *Xenopus* females have to be more active predators than males. This more extensive foraging behaviour would provide a good explanation for the higher motor activity of females which on one hand represents a caloric expenditure adding to the food requirement for growth and gamete production, and on the other hand increases the total metabolic rate compared with male animals.

Despite the considerably higher food and dietary protein intake of female *Xenopus*, the wasted nitrogen was not significantly increased in females compared to males. The time course of excretion of urea and ammonia during an observation period of 1 week was similar in both sexes except for the excretion of urea during the 7th day after feeding, which in males considerably exceeded urea excretion of females by about 100 %. Thus, the results suggest a relatively lower deamination rate and a higher storage rate of ingested proteins in females compared with males as well as sex-dependent differences in the course of digestion or/and the course of protein catabolism. Similarily, the sex-related differences observed in the activity of hepatic enzymes glucose-6-phosphate dehydrogenase, fructose-1,6-diphosphatase and phosphoenolpyruvate carboxykinase might be interpreted. The decreased activity levels of these enzymes in liver of females possibly reflect a relatively lower rate of lipogenesis and gluconeogenesis in this sex. The higher fat body-somatic index and glycogen concentration in liver of males, however, rather seem to reflect the absence of an additional storage organ analogous to the ovaries.

Finally, the differences found in the concentration of lipids and calcium in plasma confirm previous findings. In contrast, the higher aldosterone concentration in plasma of females is not well understood but may also be related to vitellogenesis since it is thought that corticoids act together with oestrogens in regulating the synthesis and secretion of vitellogenin in the liver of *Xenopus* (4).

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SEX DETERMINATION IN OVIPARE ANIMAL SPECIES, P. QUERINJEAN, C. BLUM, C. GILLES-GODFRIN and Ph. HERMAN, a.s.b.l. BIOCLUB ®, avenue des Combattants, 24 B-1340 Ottignies-LLN (Belgique).

Vitellogenin (Vg) is known as a female characteristic protein, the synthesis of which is governed by œstrogen regulation. In oviparous animal species from helminth up to reptiles, this protein is detectable in some conditions in the plasma by electrophoretic and/or immunological techniques.

Massive production of *Xenopus* Vg was obtained by 4 to 6 injections of 17-β-œstradiol of male animals. After treatment, the dorsal lymph sac was filled with several ml of a greenish

liquid containing more than 5 g/100 ml of proteins, of which up to 95 % was Vg. The fluid could be easily recovered with a syringe.

Antigen (Vg) was prepared by conventional EDTA-$MgCl_2$ precipitation or by immunoaffinity chromatography. Specific rabbit polyclonal and LOU rat monoclonal antibodies were prepared. Detection of *Xenopus* Vg was performed by agarose electrophoresis, ELISA or immunofixation. Plasma samples from treated and untreated male animals were chosen as positive and negative controls.

The different techniques were used to sex some fish species, i.e. *Oncorhynchus mykiss* (rainbow trout) and *Sarotherodon nilauticus* (tilapia). Altough the presence of Vg could be identified by simple agarose electrophoresis, no cross reactivity of the anti-*Xenopus* Vg could be obtained. But Vg identification was obtained for most trout specimens as early as 12 months old, thus well before sexual maturation and possible anatomic sexing.

Fish sexing is thus feasible on young animals and appropriate antisera may help to detect Vg in several physiological fluids without killing the animal.

IMMUNOHISTOCHEMICAL LOCALIZATION OF NEUROPEPTIDES IN THE CHROMAFFIN TISSUE OF ANURANS, M. REINECKE[1], H. SEGNER[2] and W. KLOAS[2], [1] Department of Anatomy, University of Zürich, Zürich (CH); [2] Department of Zoology II, University of Karlsruhe, Kaiserstr. 12, 7500 Karlsruhe (FRG).

The chromaffin cells of the adrenal glands of all vertebrates do not only produce catecholamines. Additionally, they contain other neurotransmitters and regulatory peptides. A participation of these peptides in the regulation of steroid secretion by the adrenocortical cells in lower vertebrates, which possess intermingled chromaffin and interrenal tissue, is discussed (1).

The aim of the present study is to extend our knowledge of the occurrence of regulatory peptides in chromaffin cells of amphibia by a comparative investigation of three anuran species : *Rana esculenta, Caldula pulchra* and *Bufo marinus*. Adrenal tissue from individuals of these species has been fixed in Bouin's solution without acetic acid, and embedded in paraffin. Serial sections have been stained for peptides using the biotin avidin technique (2). Single sections have been stained by double immunofluorescence for coexistence of peptides. To identify chromaffin cells, antisera against the marker enzymes dopamine-beta-hydroxylase (DBH) and tyrosin-hydroxylase (TH) have been employed.

Pronounced interspecific differences with respect to the presence of neuropeptides in chromaffin cells were found. The atrial natriuretic factor (ANF) was present in cells of the adrenal organ of all three species. ANF-immunoreactivity (IR) was obtained only using antisera against the 28 AA carboxy-terminal sequence of mammalian ANF, which represents the circulating form of ANF. The finding that antisera against this part of the ANF molecule show specific cross-reactivity with adrenal cells as they did also with atriocytes of the anurans investigated suggests that this sequence has been highly conserved during evolution. For brain natriuretic peptide (BNP), positive immunoreactivity was obtained only in the adrenal organ of *Caldula pulchra*. BNP-IR was located in cells other than those showing ANF-IR.

Recently, LIHRMANN *et al.* (3), provided evidence of ANF-positive nerve fibers in the adrenal tissue of *Rana ridibunda*. Moreover, these authors showed that ANF decreased the ACTH-stimulated secretion of corticosterone and aldosterone by interrenals. KLOAS *et al.* (4) demonstrated the presence of specific binding sites for ANF in the adrenal organ of *Xenopus laevis*. In addition, *in vitro* ANF inhibited the basal secretion of aldosterone by the interrenal

cells of *Xenopus laevis* as well as the ACTH-stimulated secretion of aldosterone and corticosterone. In mammals, specific ANF receptors in adrenals and ANF effects on corticosterone secretion are reported, too (5).

Comparing the localization of ANF-IR and DBH-IR, three populations of cells could be distinguished :

a) cells being positive for DBH only
b) cells being positive for ANF only
c) cells being positive both for ANF and DBH.

Congruent results arised comparing the distribution of TH-IR and ANF-IR.

Using double immunofluorescence technique, the coexistence of the following peptides in the chromaffin tissue of the investigated anuran species could be demonstrated :

— ANF/neuropeptide Y (NPY)
— ANF/Leu-enkephalin (ENK)
— Leu-ENK/Met-ENK-Arg-Phe
— Calcitonin-gene-related peptide (CGRP)/substance P.

Coexistence of CGRP/substance P was also found in nerve fibers. In nerve fibers of *Caldula pulchra*, additionally, NPY and ANF showed coexistence.

The results complete earlier data of DELARUE *et al.* (1990) who showed that adrenochromaffin cells of *Rana ridibunda* contain, in addition to catecholamines, neurotransmitters such as serotonin and neuropeptides such as vasoactive intestinal peptide, met-ENK, and met-ENK-Arg-Phe. Further studies must now establish the importance of these peptides for the regulation of the adrenal organ.

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IMMUNE REGULATION DURING ANURAN METAMORPHOSIS, Laurens N. RUBEN, Richard H. CLOTHIER and Michael BALLS, Biology Department, Reed College, Portland, OR. 97202 (USA) and Department of Human Morphology, Queen's Medical Centre, University of Nottingham, NG 7 2UH (UK).

The period of anuran metamorphosis offers investigators a unique vertebrate paradigm. The adult cells that arise within the immunocompetent larval body are immunologically incompatible with those of the larva. Thus, the animal should undergo immunologic self-destruction during this transition period. Unresponsiveness to these modified-self cells is required in order to avoid this. At the same time, protection against environmental pathogens must be provided. We have been exploring the regulation of these two distinctly different demands being made on the immune system during this period with *Xenopus laevis*, the South African clawed toad.

The most obvious potential regulator of immunity during metamorphosis is the extremely high corticosteroid serum titer. It has long been known that the glucocorticoids can serve as powerful immune inhibitors by effecting the viability and function of thymus-derived lymphocytes (T cells) which individually serve as effector or regulatory immunocytes. We have shown that glucocorticoid receptors increase in immunocytes during metamorphosis and that all regulatory and effector functions of T cells become impaired. Because injection of the human cytokines, interleukin-1, interleukin-1 (IL-2) or IL-2, was able to restore T cell function, we have suggested that the corticosteroids inhibit IL-1 production by antigen-presenting cells, and consequently, IL-2 production by amplifying T cells. No immunological impact can be achieved by T cells in the absence of clonal expansion and IL−2 is required for T cell clonal expansion.

But, if T-lymphocyte functions are inhibited, how can the animal protect itself from environmental pathogens? Immune responses to all proteinaceous and cellular immunogens require regulatory T cell intervention for amplification and for suppression. We have tested a number of possible mechanisms with regard to how antobody producing (B) cells may provide protection from pathogens in an internal environment of compromised T cell function, *e.g.* 1. is the antibody producing cell population enhanced, either in number or responsivity during metamorphosis, 2. is the amplifying T cell population corticosteroid resistant, 3. is the high thyroxine titer of metamorphosis responsible for increasing antibody producing cell function, and 4. with impaired suppressor function, is T cell enhancement necessary? None of these were supported by our data.

However, during this transition period, the two immune systems, larval and adult, with the capability of reacting to each other, co-exist within the metamorphic animal. A reasonable outcome of this interaction could be the secretion of a cytokine capable of bypassing corticosteroid inhibition of the IL−2 sensitive T-amplifier cells, by affecting antibody producing cells directly. We have recently identified an enhancing factor that is released by metamorphic, but not by adult splenic cellular suspensions, after a period between 12 hrs. to 3 days *in vitro*, in a fetal calf serum-free medium. The factor will amplify *in vitro* immune responses in adult spleen cell suspensions and it appears to enhance B cell function directly.

We also have recent HPLC evidence that the metamorphic spleen is temporarily lacking in norepinephrine (NE), a neurotransmitter with the capacity to regulate immunity in adults of this species. Our current functional studies with adults suggest that NE will stimulate T cell activities, but inhibit B cell functions. Thus, the absence of this reagent represents an ideal solution to the immunological problems faced by the metamorphic anuran which must be unable to respond to modified-self antigens, a T cell function, but fully able to protect against xenogeneic environmental pathogens, largely a B cell function.

We propose that there are at least three ways that immunity is regulated during metamorphosis that will allow for survival during this transition period; a period that not only exposes the larve to new adult differentiation antigens, but will expose the immature adult to a new range of environmental pathogens, as a consequence of evolving from an aquatic to a terrestrial habitat. They are : a high corticosteroid level, a reduced norepinephrine level, and the *in vivo* production of a cytokine.

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PROLACTIN mRNA LEVELS IN THE BULLFROG PITUITARY GLAND, N. TAKAHASHI, D. UCHIDA, K. YOSHIHAMA, K. YAMAMOTO, K. WAKABAYASHI*, Y. KATO* and S. KIKUYAMA, Department of Biology, School of Education, Waseda University, Tokyo 169 (Japan), *Institute of Endocrinology, Gunma University, Maebashi 371 (Japan).

In amphibians, prolactin (PRL) is known to act as an antimetamorphic hormone. Accordingly, it has been hypothesized that PRL levels are high during pre- and pro-metamorphosis and decline at early climax to facilitate metamorphic changes by thyroid hormone. However, PRL levels do not necessarily decline at metamorphic climax, but rise during late climax period when the animals are completing metamorphosis. Moreover it has also been known that the prolactin release is enhanced by a hypothalamic stimulation rather than a release from inhibition by the hypothalamus. Recently, we have obtained a cDNA clone coding the full length of bullfrog PRL. Using the PRL cDNA as a probe, PRL mRNA levels in the pituitary gland of bullfrog tadpoles of various developmental stages were measured. Effect of a hypothalamic factor (fPRF) having a potent PRL-releasing activity on the pituitary mRNA levels was also studied. Cytoplasmic RNA was isolated from the pituitary gland using the guanidium isothiocyanate/cesium chloride density gradient ultracentrifugation method. Dot-blot hybridization of cytoplasmic RNA was carried out according to the method of WHITE and BANCROFT (1982). Hybridization with chicken actin cDNA was used to correct for the variability in the amount of RNA blotted onto each dot. In the prometamorphic tadpoles (stage 18) the pituitary levels of PRL mRNA were relatively low. At advanced climax stages, the PRL mRNA levels were significantly elevated. At stage 23, the mRNA levels reached 300 % of those at stage 18. At stage 24, the levels remained considerably high. At the end of metamorphosis (stage 25), a decline of mRNA levels was observed. We have previously reported that both plasma and pituitary PRL levels are relatively low during pre- and prometamorphosis and rise as metamorphosis progesses, reaching maximum at stage 24. These results indicate that the pituitary PRL cell function is enhanced at advanced climax stages. When the bullfrog pituitary glands were incubated in the presence of fPRF (40 ng/ml), a significant elevation of both PRL levels in the medium and PRL mRNA levels in the pituitary was observed. It was concluded that fPRF stimulates both release and synthesis of PRL.

MECHANISMS OF ACTION OF DOPAMINE ON FROG MELANOTROPH CELLS, M.C. TONON, L. DESRUES, J. VALENTIJN, L. CAZIN and H. VAUDRY, Groupe de Recherche en Endocrinologie Moléculaire, CNRS URA 650, Unité Affiliée à l'INSERM, Université de Rouen, 76134 Mont-Saint-Aignan (France).

The intermediate lobe of the pituitary is a remarkable model of neuroendocrine communication. The pars intermedia is composed of a major population of endocrine cells, called melanotrophs, which synthesize the multifunctional precursor protein proopiomelanocortin (POMC). Specific proteolytic cleavage of POMC gives rise to a number of biologically active peptides such as alpha-melanocyte-stimulating hormone (αMSH) and β-endorphin. Pituitary melanotrophs receive direct innervation by hypothalamic fibers which release neurohormones in the immediate vicinity of the endocrine cells. In amphibians, the intermediate lobe of the pituitary is innervated by different populations of nerve fibers containing either classical neurotransmitters (mainly dopamine and GABA) or various neuropeptides including TRH, NPY, mesotocin and CRF. The spontaneous secretory activity of the pituitary melanotrophs is extremely high and inhibitory factors such as dopamine, GABA or NPY are thought to

play a pivotal role in the physiological control of hormonal secretion. The present study will focus on the mechanism of action of dopamine on frog pituitary melanotrophs. In perifused neurointermediate lobes (NIL), dopamine (10^{-10} to 10^{-6} M) was responsible for a dose-related inhibition of α-MSH secretion. The effect of dopamine was rapid and reversible. Both apomorphine and bromo-2-ergocryptine, two D_2 agonists, mimicked the inhibitory effect of dopamine on melanotropin release. In addition, dopamine antagonists such as haloperidol and pimozide blocked dopamine-induced inhibition of α-MSH release. In contrast, α-flupentixol, a specific D_1 antagonist, was devoid of effect on the response of NIL to dopamine, suggesting that dopamine modulates melanotroph activity through activation of a D_2-receptor subtype. In order to investigate the mechanism of action of dopamine on melanotropin secretion we have examined the effect of the neurotransmitter on the turnover of inositol phospholipids. Apomorphine (10^{-6} M) inhibited the incorporation of ^3H inositol into phospholipids. The inhibitory effect was significant at 3 hours and reached a maximum after 7 hours of incubation. In prelabelled NIL, dopamine decreased the rate of formation of all inositol phosphates, suggesting that dopamine might act through inhibition of the phospholipase C. Using the patch-clamp technique, on frog melanotrophs in primary culture, dopamine (10^{-6} M) was found to inhibit the generation of spontaneous action potentials by hyperpolarizing the cell. The hyperpolarisation was due to the activation of a voltage-dependent potassium outward current. In addition two distinct voltage-dependent calcium currents, namely a nifedipine-sensitive L-current and a nifedipine-insensitive N-Current, as well as a voltage-activated sodium current were reduced by dopamine. The effect of dopamine on spontaneous firing was blocked by the D_2-antagonist sulpiride, and unaffected by the D_1-antagonist SKF-83566.

Taken together, these data demonstrate that the frog pars intermedia, which is composed of a homogenous population of endocrine cells is a well suited model to investigate the mechanisms of action of dopamine. The data show that stimulation of dopamine D_2 receptors in this model causes marked electrophysiological effects and inhibition of polyphosphoinositid turnover. Studies are in progress to determine whether these cellular events are involved in the secretory response of the pituitary melanotrophs to dopamine.

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IMMUNE STATUS DURING NATURAL METAMORPHOSIS IN *RANA TEMPORARIA* DISTRIBUTION OF LEUCOCYTE TYPES IN CIRCULATION AND IN AREAS OF TAIL RESORPTION, A. Ph. Ussing (1 & 2) and P. Rosenkilde (1), 1) Zoophysiological Laboratory A, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen ; 2) Immunological Laboratory, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen.

Amphibian metamorphosis is a developmental period, during which immunity, and its counterpart, tolerance, towards new « self » and « non-self » antigens are established. « New », adult, and « old », larval, tissue antigens are present concomitantly in the individual during metamorphosis, thus composing an animal with « chimeric » composition. Since tolerance can be induced more readily during metamorphic development, the metamorphosis as an immunologically privileged period has been proposed. Does this exclude the possibility of autoimmune reactions, or are adult immunocytes actually involved in destruction of larval tissues ?

In the present work, we studied *Rana temporaria* tadpoles, metamorphosing individuals, and froglets. We studied blood cell pattern with a special emphasis on changes in immunocompetent cells during metamorphosis. Imprints of tail tissue from late metamorphic stages were analysed. Results : during metamorphic climax, blood lymphocyte count is low. With completion of tail resorption, lymphocytes become abundant in circulation. In tail resorption areas, an occasional eosinophilic granulocyte is seen, other granulocytes and lymphocytes are rare. Macrophages are abundant during late metamorphic stages, where they appear to be engaged in phagocytosis of striated muscle fibers. We suggest that the immune system is not directly involved in destruction of larval tissue. The role of macrophages is thought to be scavenging of cells already in the process of deterioration, and not a specific event in an immunological reaction.

STUDY OF THE THYROID FUNCTION IN RELATION TO THE OVARIAN OOCYTE DEVELOPMENT IN FEMALE *DICROGLOSSUS OCCIPITALIS*,
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A thyroidal-gonadal interrelationship has been suggested for most vertebrate classes. In the class of Amphibia, an effect could be demonstrated of a prolonged administration of estradiol-17β (E_2) or testosterone on the thyroidal axis of male and female adult frogs. However, little or no information was available regarding thyroid function in relation to the ovarian oocyte development and changes in sex hormone levels of female frogs under normal physiological conditions. The well known annual cyclicity of the thyroidal and the gonadal axis as well as the strong effect of external factors (e.g. photoperiod-temperature) on both axes in adult frogs, must be considered because their influence on both axes together could lead to a false interpretation of a thyroidal-gonadal interaction. In order to avoid these problems female giant swamp frogs (*Dicroglossus occipitalis*) were collected during a short time period in an equatorial region (Kinsangani, Zaïre) where the climatic factors are nearly constant throughout the year. Based on the developmental stage of the oocytes in the ovary, all the frogs could be subdivided in six different groups (stage I to V : going from the least developed to the full grown oocytes ; stage VI : postovulation). At a given time of the year, all the stages are represented in different frogs. Two experiments were set up in which thyroid and sex hormone concentrations, peripheral monodeiodination of T_4 (thyroxine) into T_3 (triiodothyronine) and some morphological gonadal parameters (GSI : gonadosomatic index = weight of the gonads/body weight in percent ; oviduct weight in percent of the body weight) were studied in giant swamp frogs, subdivided in the six different groups.

Going from stage I to V a progressive increase of the GSI and the oviduct weight was seen, together with a decline of these morphological parameters in stage VI. Considering the plasma E_2 concentration the same pattern is observed in the two experiments : an increase from stage I to V, followed by a drop in stage VI. However, the increase of plasma E_2 occurs faster and preceeds the weight gain of the ovary and the oviduct. This illustrates the stimulatory role of the estrogens on oviduct growth and on the process of vitellogenesis, resulting finally in an important weight gain of the oocytes. The plasma testosterone concentration is low in stage I, II, III and VI, but reaches a high level in stage IV and V. Looking at the E_2 levels in the ovaries, it was noticed that the total E_2 content increases progressively from stage I to V and declines in stage VI. However, when the E_2 concentration per gram ovary was considered, no differences could be detected between the distinct stages.

Quite different results were obtained at the level of the thyroidal axis. In neither of the two experiments was a significant difference noticed between the plasma T_4 concentrations obtained for the distinct stages. In the second experiment nearly equal plasma T_3 values were found in the six different groups. In the same experiment a slightly higher T_3 and T_4 concentration was found in the thyroids of the animals belonging to stage II and IV but this could not be related to the hormonal or morphological changes taking place at the level of the gonadal axis. In the kidney and skin homogenates, obtained from the frogs of the second experiment, the *in vitro* 5'-monodeiodination activity was estimated by measuring the produced T_3 which is derived from conversion of extra added T_4. Neither in kidney or skin homogenates the measured T_3 values were different in the six distinct groups.

Our results indicate that ovarian egg development, together with the hormonal changes at the gonadal level, are not connected with fundamental changes at the level of the thyroidal axis.

IDENTIFICATION AND CHARACTERIZATION OF AN ALDOSTERONE RECEPTOR IN THE TAIL EPIDERMIS OF BULLFROG TADPOLES, K. YAMAMOTO and S. KIKUYAMA, Department of Biology, School of Education, Waseda University, Nishiwaseda, Shinjuku-ku, Tokio 169 (Japan).

Corticoids such as aldosterone and corticosterone have been known to potentiate the action of thyroid hormone on the tadpole tail by augmenting the binding capacity of thyroid hormone (1, 2). Plasma corticoid levels rise during early climax (3-5) and administration of an inhibitor of corticoid synthesis retarded metamorphosis (6). These observations strongly suggest that endogenous corticoids are involved in metamorphosis. Accordingly, it is probable that corticoid receptors exist in the tadpole tail. There is one report concerning binding of glucocorticoid by tadpole tissue (7). Experiments were conducted to demonstrate the presence of aldosterone receptor in the tadpole tail. The temperature-dependent association experiments show that the specific binding of [^{3}H]aldosterone to the tail cytosol is thermolabile. The optimum assay conditions required for reaching equilibrium were defined as being 0° C and 3-hr incubation. Separation of bound and free hormone was performed by using a hydroxylapatite method. Specific binding of aldosterone was observed in the tail epidermis but not in the tail mesenchyme. Sucrose density gradient analysis of crude cytosol revealed a specific peak of radioactivity in 8S area. Saturation analysis revealed that specific binding of [^{3}H]aldosterone to the epidermal cytosol reached maximum between 20 and 40 nM. Scatchard plot analysis for the cytosol of the tail epidermis from tadpoles of stage XVIII yielded a straight line with a dissociation constant (Kd) of 8.1 ± 0.3 nM and the maximum number of binding sites (NBS_{max}) of 54.2 ± 2.5 fmol/mg protein. The dissociation of specifically bound [^{3}H]aldosterone from the tail epidermal cytosol obeyed first-order kinetics. The rate of dissociation of aldosterone from epidermal cytosol displayed a $t_{1/2}$ of 122 ± 2 min. The calculation of a molecular weight and a Stokes radius of aldosterone receptor gave values of 143 KDa and 4.54 nm, respectively. Steroid-binding specificity revealed a significant displacement of the [^{3}H]aldosterone by both radioinert aldosterone and corticosterone and, to a lesser extent, by cortisol, whereas 17β-estradiol and testosterone com-

peted very poorly. The number of binding sites was somewhat reduced as metamorphosis progressed with no appreciable changes in the Kd value.

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