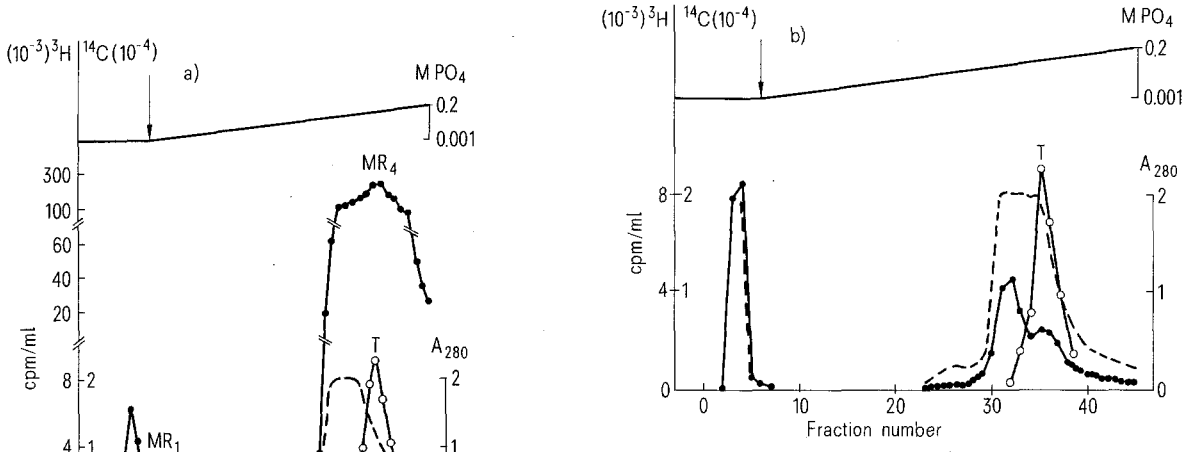

Fig. 3. Separation of renal R-5020 binders on DEAE-52 columns. 4 ml renal cytosol (a) or undiluted serum (b) was incubated with 10^{-7} M ^{3}H-R-5020; 2 ml fresh serum with 0,5 µCi ^{14}C-corticosterone was used for cochromatography as for figure 2, b, and Agarwal[2,3,6]. --------, A_{280}; ●—●, ^{3}H; ○—○, ^{14}C.

mechanism of greater significance than the agonist specific component (MR_2) that has a low capacity. The name protoreceptor seems appropriate for its multifunctional role although receptor maturation from a proreceptor can not be ruled out[8] without receptor purification. Finally, the discordance between physical separation vs mere competition establishes that the Scatchard type of analysis is not adequate by itself for the interpretation of receptor activity and may even be misleading, so that its widespread use needs some caution.

1 Present address: 15, rue de l'Ecole de Médecine, F-75270 Paris Cedex 06, France, for all correspondence. Supported in part by grants from the CNRS AI 03 1917 and UER Broussais Hôtel Dieu.
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Changes in total and polymerized tubulin of the medial basal hypothalamus and adenohypophysis of castrated or hormone-injected rats[1]

C.E. Valenti*, R.J. Lombardo, C. Libertun and D.P. Cardinali

Centro de Estudios Farmacológicos y de Principios Naturales and Instituto de Biología y Medicina Experimental, Obligado 2490, 1428 Buenos Aires (Argentina), 29 October 1979

Summary. Treatment of orchidectomized rats with LH, FSH or prolactin decreased the tubulin content of the medial basal hypothalamus (MBH), whereas FSH or prolactin augmented it in the adenohypophysis (AH). After castration, negative correlations existed between serum LH and total or polymerized MBH tubulin, whereas in the AH positive correlations were found. After estradiol-progesterone treatment of spayed rats a significant correlation was found between serum LH and the percentage of AH tubulin in the polymerized form.

In a previous study[2], we observed that orchidectomy decreased the concentration of tubulin, the protein constituting microtubules, in rat medial basal hypothalamus (MBH), whereas testosterone administration augmented it in MBH and adenohypophysis (AH); likewise estradiol treatment resulted in a marginal increase of MBH tubulin. It seemed feasible that these changes in tubulin levels could be associated with modification of transport and/or release of materials of neuroendocrine significance, and the present experiments were performed to examine further hormone-related changes of MBH and AH tubulin by determining: 1. the effects of gonadotrophins and prolactin on tubulin levels in acutely castrated rats; 2. the changes in the equilibrium between the polymerized and depolymerized forms of tubulin following acute or sustained stimulation of the hypothalamic hypophyseal axis.

Material and methods. Adult Wistar rats (180–220 g) were kept under light between 0700 and 2100 h daily and were given access to chow and water ad libitum. 3 experiments were performed. Experiment 1: to assess the effects of FSH, LH and prolactin on MBH and AH tubulin levels, acutely orchidectomized rats received 2 s.c. injections of 100 µg of FSH (NIH-FSH-S12) or LH (NIH-LH-S19) 3 h and 18 h after castration, and the animals were killed 3 h later. Prolactin (NIH-S-P12, 200 µg) was injected according to the same schedule. Controls received the vehicle alone (1 ml of saline). Experiment 2: to examine the effects of a sustained rise in gonadotrophin levels on the polymerized and depolymerized forms of MBH and AH tubulin, male rats were castrated or sham-operated under ether anesthesia at 0800 h, and were killed 96 h later. Experiment 3: to examine the changes in the polymerized and depoly-

merized forms of tubulin in MBH and AH during acute pituitary activation, rats oophorectomized 3 weeks earlier and having received 0.5 and 50 µg of estradiol for 2 consecutive days and progesterone (4 mg) at 0800 h of the 3rd day, were killed 8 h after progesterone administration. Measurement of the total tubulin levels in MBH and AH was carried out by the colchicine binding assay as described previously[2]. Quantification of the polymerized and depolymerized forms of tubulin was carried out by the procedure described by Pipeleers et al.[3], which is based on the observation that homogenization of tissue in concentrated glycerol solutions at room temperature stabilizes and protects intracellular microtubules from disassembly. Individual AH or MBH were homogenized in a microtubule-stabilizing solution containing 50% glycerol, 5% dimethylsulfoxide, 0.5 mM GTP, 0.5 mM $MgCl_2$ and 0.5 mM EGTA in 10 mM phosphate buffer, pH 6.95, at room temperature. Aliquots of the homogenate were centrifuged at 50,000 × g for 60 min at room temperature and tubulin was measured in the supernatant by the colchicine binding assay; this portion is an estimation of the depolymerized tubulin pool of the cells[3]. The pellet was resuspended in cold tubulin depolymerizing solution containing 0.25 M sucrose, 0.5 mM $MgCl_2$ and 0.5 mM GTP in 10 mM phosphate buffer, pH 6.95, and was centrifuged for 60 min at 50,000 × g at 4 °C. The final supernatant, which contains the tubulin derived from depolymerized microtubules, was assayed by the colchicine binding assay. Serum samples were analyzed for LH using a double antibody radioimmunoassay technique[4].

Results. LH, FSH or prolactin treatment depressed MBH tubulin levels of orchidectomized rats, whereas FSH and prolactin augmented it in the AH (table). 4 days after castration plasma LH levels increased significantly by 210%; at this time a significant, 29% depression of the polymerized form of MBH tubulin was detectable (data not shown). A negative correlation between serum LH and total or polymerized MBH tubulin was found in castrated rats, whereas in the AH significant correlations existed between LH and total or polymerized forms of tubulin both in control and castrated rats (figure 1). An analysis of covariance indicated a significant difference ($p < 0.01$) in the slopes for castrated and sham-operated rats when the correlation serum LH-polymerized AH tubulin was analyzed.

Following estradiol-progesterone injection of ovariectomized rats, a significant 840%-increase of serum LH was detectable in the evening (data not shown); a correlation analysis of serum LH and MBH and AH tubulin indicated significance only for the correlation between LH and the percentage of AH tubulin in the polymerized form in steroid-treated rats (figure 2). No changes were observed in soluble protein concentration after any of the foregoing treatments.

Discussion. The existence of a link between the polymerized form of tubulin and the secretory process has been suggested by studies on pancreatic islets[3], liver[3], parathyroid[5] and ovarian cells[6], lymphocytes[3] and platelets[3]. The present results indicate that in male rats a correlation exists between LH release and the amount of total or polymerized AH tubulin both in sham-operated and castrated rats. On the assumption that the gonadotrophs constitute no more than 10% of the cell population[7] it is remarkable that such a correlation was detectable in our experiments. When an acute stimulus for gonadotrophin and prolactin release was superimposed, i.e. following estradiol-progesterone administration to spayed rats, LH secretion correlated with the percentage of AH tubulin in the polymerized form. In this case not only the gonadotrophs but also the lactotrophs, which comprise a large proportion of the AH cell population[7] were stimulated in a pulse fashion, therefore

Effect of LH, FSH and prolactin on tubulin levels of the medial basal hypothalamus (MBH) and adenohypophysis (AH) of castrated male rats

Treatment	Tubulin levels (nmoles/mg protein) MBH	AH
Vehicle	1.91 ± 0.13 (7)	0.98 ± 0.13 (7)
LH	1.47 ± 0.01 (6)*	1.15 ± 0.10 (7)
FSH	1.44 ± 0.09 (7)*	1.96 ± 0.23 (7)*
Prolactin	1.44 ± 0.05 (7)*	2.13 ± 0.36 (6)*

Results are expressed as mean ± SEM (n). Asterisks represent significant differences from vehicle-injected controls. ($p < 0.05$, analysis of variance. Dunnett's test.)

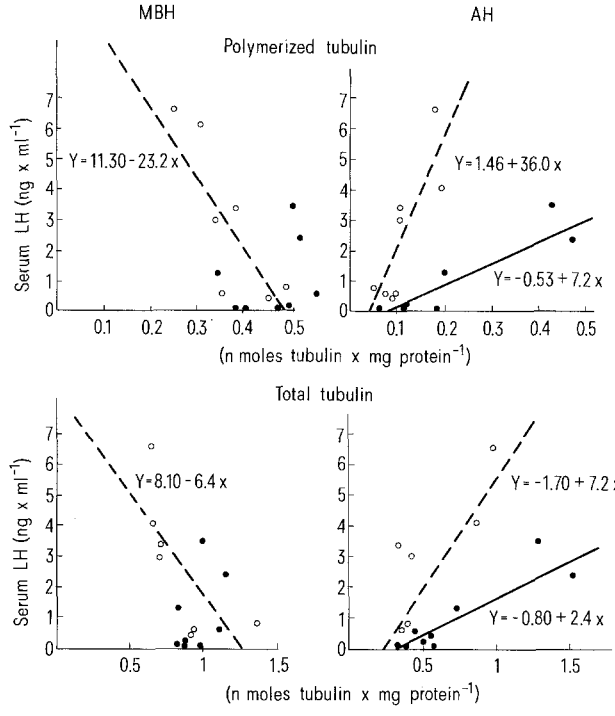

Fig. 1. Correlation between serum LH and polymerized and total tubulin in medial basal hypothalamus (MBH) and adenohypophysis (AH) of male rats castrated or sham-operated 96 h earlier. Correlation coefficients (r), slopes (b) and 95% confidence limits of b were: polymerized MBH tubulin, ♂: r = −0.84, b = −0.29 (−0.50, −0.07). Total MBH tubulin, ♂: r = −0.72, b = −0.08 (−0.17, −0.01). Polymerized AH tubulin, ♂: r = 0.91, b = 0.09 (0.05, 0.13). Polymerized AH tubulin, ♂: r = 0.84, b = 0.45 (0.11, 0.78). Total AH tubulin, ♂: r = 0.91, b = 0.03 (0.02, 0.04). Total AH tubulin, ♂: r = 0.81, b = 0.09 (0.02, 0.16).

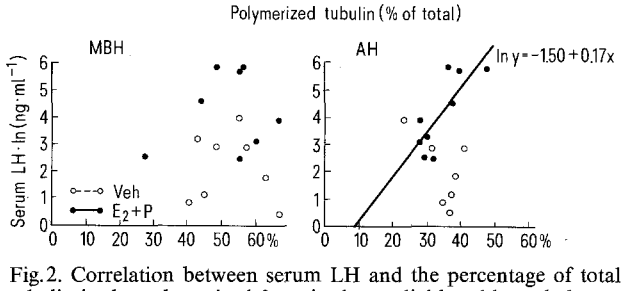

Fig. 2. Correlation between serum LH and the percentage of total tubulin in the polymerized form in the medial basal hypothalamus (MBH) and adenohypophysis (AH) of ovariectomized rats injected with estradiol progesterone (E_2-P) or vehicle. Data on serum LH are presented as natural logarithms. Correlation coefficient (r), slope (b) and 95% confidence limits of b in E_2-P injected rats were: r = 0.78, b = 0.17 (0.03, 0.31).

offering a basis for explaining why only the polymerized form of tubulin correlates with serum hormone concentration.

We have previously observed that testosterone administration augmented MBH tubulin levels[2]; this effect could be exerted directly on MBH cells, or alternatively, testosterone could act through depression of gonadotrophin release. The present results are compatible with the view that testosterone acts partly via changes in gonadotrophin secretion to affect MBH tubulin. Moreover following a 4-day exposure to high plasma gonadotrophin titres after orchidectomy, a negative correlation between serum LH and the total or polymerized forms of tubulin was detectable in MBH. It seems feasible that this mechanism could be a reflection of the short feedback loop controlling gonadotrophin release.

A final aspect of the present work deserves comment. Treatment of acutely castrated rats with FSH or prolactin affected AH tubulin levels. Therefore the results from figures 1 and 2, which can be a reflection of the changes in AH mechanisms leading to hormone release, could be considered alternatively as representing a consequence of increasing plasma hormone levels. Further experiments are needed to shed light on this point; perhaps they should await the development of methods of measuring the dynamic equilibrium between polymerized and depolymerized tubulin in cell populations which are enriched in a single AH cell type.

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PRO EXPERIMENTIS

Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method[1]

W.M. Howell and D.A. Black

Department of Biology, Samford University, Birmingham (Alabama 35209, USA), 14 January 1980

Summary. A 1-step silver-staining technique, requiring only 2 min to perform, is described for the differential staining of nucleolus organizer regions. A protective colloidal developer is used to control the reduction of the silver.

Silver-staining methods have been developed for the differential staining of nucleolus organizer regions (= Ag-NORs) of animal and plant chromosomes[2–7]. However, cytogeneticists using these techniques, or slight modifications of them, often experience one or more of the following problems: 1. silver precipitate over the slide caused by too rapid a development; 2. development time cannot be standardized because the ammoniacal-silver and formalin developer solutions are unstable, causing over- and/or under-development of Ag-NORs; 3. microscopic monitoring of Ag-NOR development is usually required; 4. uneven staining of Ag-NORs occurs across the slide, making reliable Ag-NOR counts difficult; 5. incubation in aqueous silver nitrate alone[4,6] is time-consuming, requiring from 3–24 h; and, 6. it is expensive, e.g., ammoniacal-silver has a short shelf-life and must be discarded after a few days.

Over the past 3 years our laboratory has conducted experiments trying to develop an Ag-NOR method in order to eliminate the above problems. By the use of a protective colloidal developer, in combination with aqueous silver nitrate, we have perfected a simple, 1-step, 2-min technique which abolishes staining problems associated with the current methods.

The new method requires the use of 2 solutions. A colloidal developer solution is prepared by dissolving 2 g powdered gelatin, USP into 100 ml deionized water and 1 ml pure formic acid. Constant stirring for 10 min is required in order to dissolve the gelatin. This solution is stable for 2 weeks. An aqueous silver nitrate solution is prepared by dissolving 4 g $AgNO_3$ into 8 ml deionized water. This solution is stable. Both the colloidal developer and silver solutions are stored in capped, amber-glass bottles.

For the selective staining of Ag-NORs, 2 drops of the colloidal developer and 4 drops of the aqueous silver nitrate are pipetted onto the surface of a microscope slide containing chromosome preparations. The solutions are mixed and covered with a coverglass. The slide is placed onto the surface of a slide warmer which has been stablized at 70 °C. Within 30 sec, the silver-staining mixture will turn yellow, and within 2 min, it will become golden-brown. The slide is then removed and the coverglass and staining mixture are rinsed off under running deionized water. The slide is blotted dry and may be examined immediately. The nucleolus organizer regions are stained black while the chromosome arms are stained yellow (figure). Nuclei are yellow with black nucleoli. Even-staining of metaphases occurs across the slide. The slide background is homogeneously clean, with little or no extraneous silver precipitate. It is important, however, that slides be freshly-prepared and clean. Old slides contaminated with bacteria, dust and cellular debris will give background precipitate as these things, too, will stain with silver.

If desired, trypsin-Giemsa banding methods can be performed on the silver-stained metaphases in order to identify unequivocally individual chromosomes[8].

The use of protective colloids to control silver-staining is not a new idea. Liesegang[9] developed an improved method of silver-staining of nerve tissue using gum arabic as a protective colloid to prevent the intense reduction of silver nitrate. Later, Bartelmez and Hoerr[10] used albumin as a protective colloid. Silver[11] used various experiments to explain the colloidal factors controlling silver-staining. Until now, however, cytogeneticists have not used protective colloids to control silver-staining of Ag-NORs.