Hypothalamic GnRH-like bioactivity and immunoreactivity in prepubertal and adult male rats

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ABSTRACT

In prepubertal (21-days old) and adult (50-days old) male rats, the immunological and biological properties of GnRH-like material extracted from the hypothalamus were studied. At both ages, hypothalamic material and synthetic GnRH resulted in a parallel inhibition of the binding of labelled GnRH to 2 different anti-GnRH antisera (As I and As II). Using both antisera, a similar amount of immunoreactivity was measured in several extracts from 50-day hypothalami. In contrast, hypothalamic extracts obtained at 21 days contained a greater immunoreactivity using As II than using As I. This discrepancy was only observed with the hypothalamic content whereas the immunoreactivity released in vitro was similar with the two antisera at both ages studied. Filtration of hypothalamic extracts on biogel P2 revealed two immunoreactive fractions, the major one being eluted as the synthetic decapeptide and showing a similar immunoreactivity using both antisera. A high molecular weight fraction was proportionally predominant in 21-day extracts and showed a greater immunoreactivity using As II than using As I. The biopotency of the hypothalamic extracts upon rat pituitary cells in vitro was similar at the two ages but around 30 times higher than the bioactivity expected for the immunoreactivity. We conclude that the heterogeneous physicochemical and immunological nature of hypothalamic GnRH is different before and after sexual maturation in the male rat, whereas the bioactivity, although much greater than expected, is similar at both ages.

INTRODUCTION

After the sequence of Gonadotrophin-Releasing Hormone (GnRH) had been elucidated in ovine (Burgus et al, 1971) and porcine (Matsuo et al, 1971) hypothalamic extracts, several authors attempted to determine the nature of GnRH in the hypothalamus of rat and man. From chromatographic studies, it was concluded to the identity between hypothalamic and synthetic GnRH (Jeffcoate et al, 1974 ; Mortimer et al, 1976 ; Bourguignon et al, 1979 ; King et Millar, 1980 ; Samson et al, 1980) although some authors had detected an heterogeneous immunoreactive material (Fawcett et al, 1975). Gautron et al. (1981) provided an explanation for this discrepancy, the presence of a high molecular weight fraction of GnRH being observed or not according to the specificity of the anti-GnRH antibodies used. More recent studies suggested that this fraction was consistent with the existence of a Pro-GnRH or precursor of the decapeptide (Curtis and Fink, 1983).
During growth and sexual maturation in the rat, several authors demonstrated an increase in immunoreactive hypothalamic GnRH, up to the age of 2 months (Araki et al, 1975 ; Chiappa and Fink, 1977). In addition, the distribution of immunoreactive GnRH within the hypothalamus has been found to change with age, the most striking increase being located in the mediobasal hypothalamus (Araki et al,

1975 ; Watanabe, 1980). Since hypothalamic GnRH of adult rats was known to be heterogeneous and in variable amounts according to the antibody used, we aimed to investigate the possible influence of age and maturity upon the nature of hypothalamic GnRH. Therefore, we compared the physicochemical, immunological and biological properties of GnRH-like material extracted from hypothalamus of prepubertal and adult male rats.

MATERIAL AND METHODS

- Rats

Male rats of the Wistar strain were studied. They were aged 21 and 50 days. At 21 days of age, rats were just before the pubertal increase in testicular growth whereas, at 50 days, testicular weight increased less rapidly (Bourguignon et al, 1984). Arbitrarily, these animals were respectively called prepubertal and adults.

- GnRH-like material contained and released from the hypothalamus.

The techniques to remove the hypothalamus from the brain and to extract GnRH-like material have been described previously (Bourguignon et al, 1984). Briefly, retrochiasmatic hypothalamus was dissected and immediately immersed in 1 ml acetic acid 2N, pH 2.3, cooled on ice. After homogeneization, the extracts were frozen until assayed, after neutralization. The mean (± 1 SD) recovery of synthetic GnRH extracted with brain cortex was 73 ± 4 %. GnRH released was measured in unextracted medium Dulbecco's Modified Eagle's Medium,DMEM) collected during in vitro superfusion of rat hypothalamus, according to a technique described elsewhere (Bourguignon and Franchimont, 1984).

- Radioimmunoassays of GnRH and immunological studies.

The radioimmunoassay of GnRH was performed in duplicate using a double antibody method as previously described (Bourguignon et al, 1974, 1979, 1984). The sensitivity was around 1 pg/tube. Two anti-GnRH antibodies were used. Antiserum I was the RR-5 antiserum highly specific for the decapeptide (Copeland et al, 1979), generously provided by Dr A. Root. Antiserum II was prepared in the laboratory and resulted in a significant cross-reactivity in the presence of C-terminal fragments of GnRH, especially the 2-10 nonapeptide (Bourguignon et al, 1979).

- Physicochemical studies

The extracts from 10 hypothalami of 21-day-old rats and 14 hypothalami of 50-day-old rats were studied by filtration at 4°C on Biogel P2 (Biorad^{R}), 28 x 1 cm and 80 x 1,6 cm respectively. The elution medium was phosphate buffered saline 0.05 M, pH 7.5 enriched with gelatin 1 g/l.

- Biological studies

Extracts were prepared from 54 hypothalami of 21-day-old rats, 30 hypothalami of 50-day-old rats and brain cortex from the same animals. Several dilutions of the extracts (1/20 to 1/10.000) or several concentrations of synthetic GnRH (0.3 to 100 nM) were added for 5 h to dishes (4/each concentration) with pituitary cells in monolayer culture, prepared according to the technique of Hopkins and Farquhar (1973) modified by Demoulin et al. (1979). Thereafter, culture medium was frozen until the radioimmunoassays of rat FSH (rFSH) and rat LH (rLH) were performed (Franchimont et al, 1975) using reagents kindly provided by Dr A.F. Parlow from the pituitary agency of NIAMDD. The results were expressed with reference to the International Reference Preparation IRP-1.

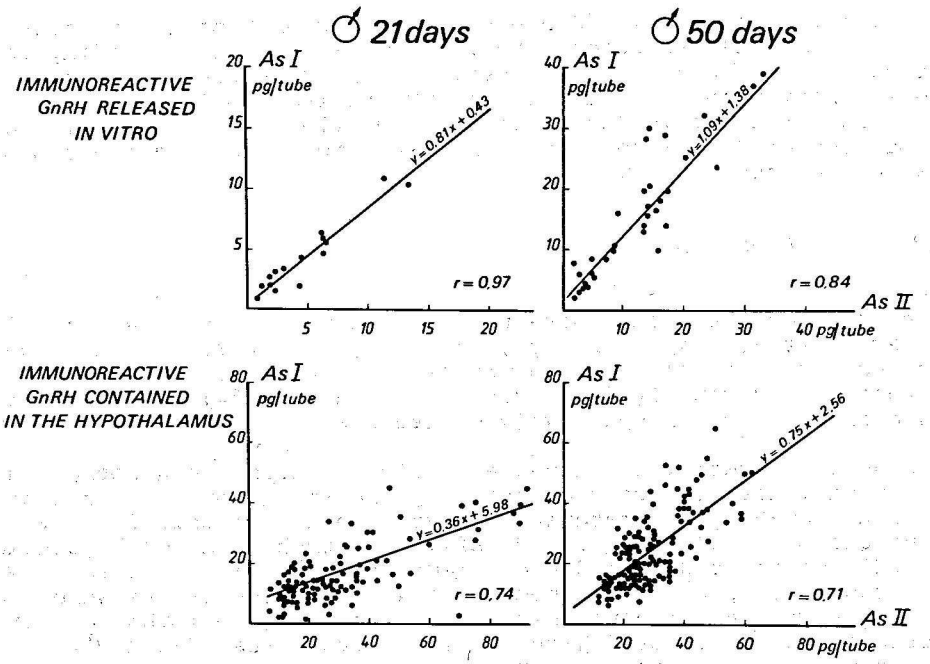

Figure 1. Linear correlations between the amounts of immunoreactive GnRH detected using Antiserum I and Antiserum II in culture medium of superfused hypothalami (upper panel) and in hypothalamic extracts (lower panel) obtained in rats aged 21 days (left panel) and 50 days (right panel).

RESULTS

- Immunological studies

Several dilutions of extracts prepared from prepubertal and adult hypothalami resulted in an inhibition of the binding of labelled GnRH to Antisera I and II. These inhibition curves were parallel to those obtained with the synthetic decapeptide whereas no immunoreactivity could be detected in brain cortex. However, as shown in figure 1, immunoreactive GnRH contained in the hypothalamus was found to be heterogeneous in relation to the antibody used, higher amounts being detected using antiserum II than using antiserum I. The slopes of the regression lines were lower than 1, especially when extracts of prepubertal hypothalami were studied. At 21 days, the slope was highly significantly lower ($p < 0.001$) than that observed at 50 days. In contrast, the amounts of immunoreactive GnRH released in vitro at both ages were similar using the 2 different antisera, as indicated by the slopes found to be around 1.

- Physicochemical studies

As shown in figure 2, filtration of hypothalamic extracts on biogel P2 resulted in two fractions observed at both ages. A major fraction was eluted in the same volume as GnRH and showed a similar immunoreactivity with both antisera. The other fraction was eluted in the external volume of the column, suggesting its apparently high molecular weight. In addition, this fraction exhibited a greater immunoreactivity for antiserum II than for antiserum I and appeared to be predominant in the prepubertal extract when compared to that obtained from adult hypothalami.

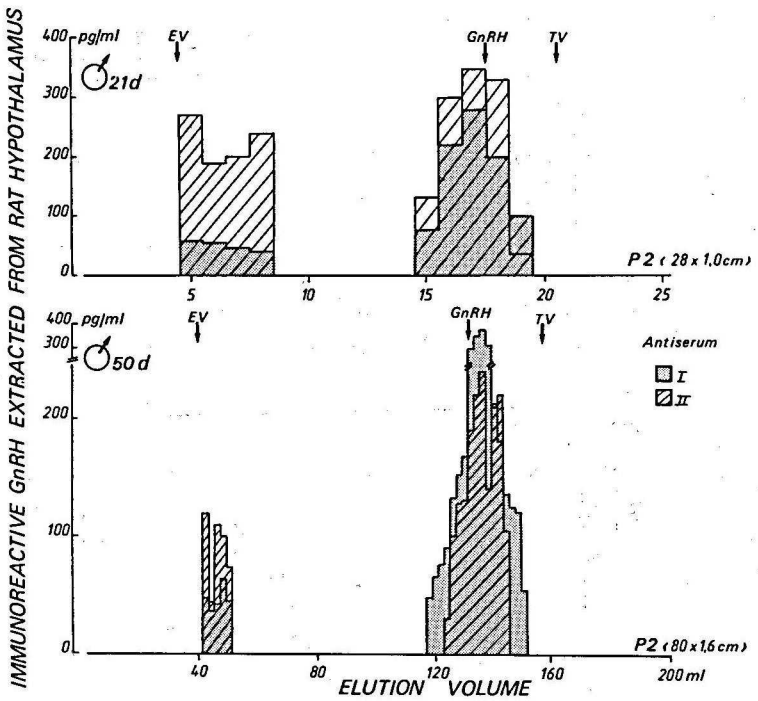

Figure 2. Gel filtration of hypothalamic extracts from prepubertal (upper panel) and adult (lower panel) male rats. The RIA of GnRH was performed in the fractions eluted using 2 antisera. Arrows indicate elution volume of reference preparations. GnRH : synthetic decapeptide ; EV : external volume ; TV : total volume

- Biological studies

At both ages, the release of FSH and LH from pituitary cell cultures was not affected by hypothalamic extracts used at concentrations $\leq 1/4.000$ whereas the maximal stimulation of FSH and LH release was observed in the presence of concentration $\geq 1/100$. Similar concentrations of brain cortex extract ($\geq 1/100$) did not induce any response except a slight but significant increase in LH release ($p < 0.01$) using extracts obtained in 21-day-old rats.

In figure 3 are represented the in vitro responses of LH and FSH to a 5 h challenge using hypothalamic extracts and synthetic GnRH. Results are expressed as percentages of the maximal response in relation to concentrations of immunoreactive GnRH as determined using antiserum I. Typical dose-response curves were obtained with hypothalamic extracts of prepubertal and adult rats, without any significant difference between these 2 ages. However, the bioactivity of the hypothalamic extracts was 30 times higher than that of synthetic GnRH, according to the half maximal effective doses for the two different materials.

DISCUSSION

Our data provide evidence for the heterogeneity of immunoreactive GnRH contained in the hypothalamus of prepubertal male rats. The chromatographic pattern of hypothalamic extracts from prepubertal rats was not different from that previously observed in adult animals (Gautron et al, 1981). However, we demonstrated that the immunoreactive characters of hypothalamic GnRH studied with two

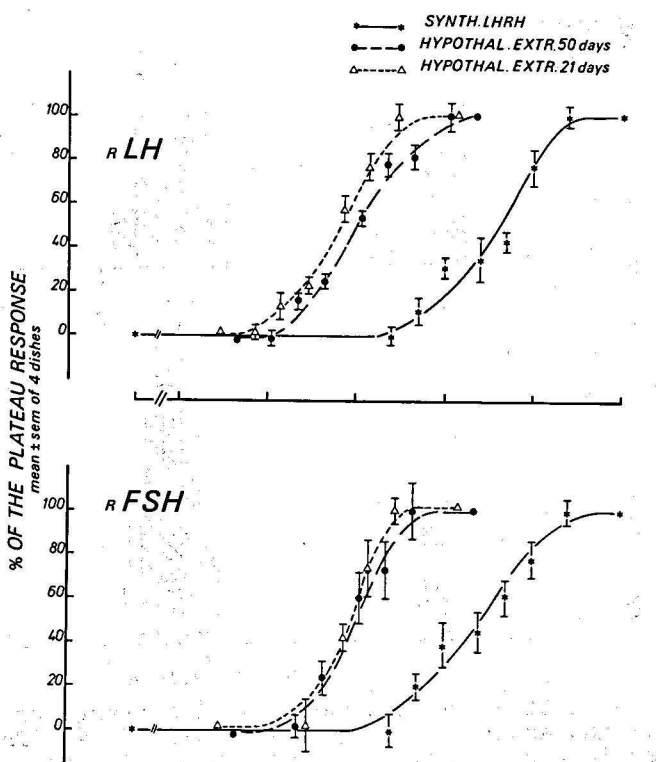

Figure 3. Release of rat gonadotrophins (as a percentage of the maximal release) after incubation of pituitary cell cultures for 5 h in the presence of several concentrations of synthetic GnRH or hypothalamic extracts obtained in rats aged 21 and 50 days.

antibodies, were different at 21 and 50 days of age. According to our observations, the high molecular weight fraction of GnRH appeared to be a major constituant in the prepubertal hypothalamus when compared to that of adult rats. This might suggest ontogenetic variations in the nature of hypothalamic GnRH. We also showed that the biopotency of hypothalamic GnRH-like material was similar at 21 and 50 days. Finally, we showed that the age-related variations in the immunoreactive nature of GnRH contained in the hypothalamus did not affect the nature of the material released which was consistent with the decapeptide at both ages studied. The heterogeneity of hypothalamic immunoreactive GnRH has been suggested from the discordances between immunocytochemical studies in relation to the anti-GnRH antibody used (Sternberger et al, 1981). Evidence for a pro-GnRH precursor was also provided by the increased amounts of the decapeptide after incubation of hypothalami in the presence of proteolytic enzymes (Millar et al, 1977). Several factors may account for the discrepant data obtained in chromatographic studies : the medium used to extract hypothalamic GnRH, the hypothalamic areas included in the dissected fragment, the chromatographic conditions and, finally, the anti-GnRH antibody used. Recently, the aminoacid sequence of the high molecular weight

fraction has been identified from cloned complementary DNA prepared using the human placental messenger RNA for the pro-GnRH (Seeburg and Aldeman, 1984). This Pro-GnRH seems to be homologous with the hypothalamic precursor and contains a prolactin inhibiting peptide (Philipps et al, 1985).

So far, very little is known about the ontogenesis of the hypothalamic Pro-GnRH. Our data suggest some possible variations in the nature of the hypothalamic material between prepubertal and adult rats. These age-related variations in immunoreactive GnRH are a reason to be highly careful in the interpretation of hypothalamic GnRH content measured at different ages and to use a highly specific antiserum to the decapeptide. In addition, the presence of a precursor may be taken into account to explain the very potent bioactivity of the hypothalamic material with respect to its immunoreactivity, as shown in this work and by others (Nett and Niswender, 1976 ; Kao et al, 1977 ; Yu et al, 1979 ; Lumpkin et al, 1980 ; Samson et al, 1980). High potassium concentrations were suggested to be responsible for the increased biopotency of hypothalamic extracts (Kao et al, 1977 ; Zolman and Valenta,1981). This hypothesis is not supported by our data since, at the 1/100 dilution resulting in the maximal biological effect, the hypothalamic extract contained 1.8 mEq K /l, a concentration similar to that of brain cortex extract which exhibited no biological activity. A contamination of the hypothalamic extract by pituitary gonadotrophins is unlikely ; it has been shown to represent around 1 % of the gonadotrophins released in vivo after administration of hypothalamic extracts (Gay et al, 1970). This was confirmed by the absence of immunoreactive FSH and LH measurable in our extracts. The study of pro-GnRH biopotency and of its variations with age would help to our understanding of hypothalamic maturation in relation to puberty.

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