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PUBERTY-RELATED INCREASE IN EPISODIC LHRH RELEASE FROM RAT HYPOTHALAMUS *IN VITRO*[1]

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ABSTRACT: The retrochiasmatic hypothalamus (RCH) was removed from brains of male rats between 12 and 50 days of age, and immediately studied *in vitro*. The release of LHRH from the RCH was evaluated by periodic (7.5-min) collections of culture medium and subsequent RIA. With synthetic LHRH in the experimental system, the mean (± 1 SD) recovery was 94 ± 7 % with a variation coefficient of 14 ± 3 %. An increase in LHRH release was considered to be significant when it exceeded 6 pg/7.5 min. Biological viability of RCH *in vitro* was assessed by an increased release of LHRH in response to the depolarizing effect of veratridine. As age increased, from 12 to 50 days, the hypothalamic LHRH content steadily increased. However, a significant increase in veratridine - induced release of LHRH occurred only at 23 days and thereafter. At various ages, single hypothalami were studied during a mean 112-min period to evaluate the spontaneous release of LHRH. In all age groups, the *in vitro* LHRH release occurred in pulses. However, mean pulse frequency increased significantly with age: in 12- and 17-day-old rats, 0.3 pulse/112 min was observed; at 23,25 and 27 days, this frequency varied between 1.8 and 3.0 pulses/112 min. At 50 days of age, the observed frequency was within the same range. We conclude that the RCH obtained from rats of various ages may retain *in vitro* its capacity to release LHRH episodically and that the frequency of these episodic pulses markedly increases with age to the time of the onset of puberty in male rats.

In adults of several species, the pulsatile nature of LHRH release has been demonstrated *in vivo* (1,2). The measurements of plasma gonadotropins have suggested that an accelerated frequency of LHRH pulses may be the cause of pituitary activation at puberty: in immature as well as in adult monkeys with lesioned hypothalami, the injection of synthetic LHRH at an appropriate frequency induces or restores a pubertal pattern of gonadotropin secretion (3). In man, the pulses of LH in plasma may occur more frequently at puberty (4).

In the adult rat, LH secretion (5) and, more recently, LHRH secretion (6) have been shown to be pulsatile. LH pulses have also been observed in immature rats (7). However, the ontogenesis of LHRH release from the hypothalamus has not been directly studied, since the *in vivo* evaluation is very difficult. The pulsatile release of LH has been shown to be not or hardly affected by complete hypothalamic deafferentation *in vivo* (8,9). Therefore, we have assumed that the hypothalamus may retain its capacity to generate pulses *in vitro*. In a preliminary report, we have shown that the *in vitro* release of LHRH from the adult rat hypothalamus occurred episodically (10). We have further used our experimental system to study age-related variations in LHRH release from the hypothalamus.

MATERIALS AND METHODS

Male Wistar rats (Iffa Credo, Brussels, Belgium) were 12, 17, 19, 21, 23, 25, 27 and 50 days old at the time of the experiments. After decapitation at around 1300 h, the retrochiasmatic hypothalamus (RCH) was dissected. This fragment was delimited by coronal sections rostrally at the caudal border of the optic chiasma and caudally at the back of the mammillary bodies, by sagittal cuts along the lateral hypothalamic sulci, and by a frontal cut 3 mm deep. The fragment was immediately transferred to an incubator. The rest of the brain was fixed in 4 % formol. Sagittal sections stained with luxol blue and cresyl violet were examined under the light microscope (11). This confirmed the removal of the median eminence, the arcuate and ventromedial nuclei, as well as of the largest part of the mammillary bodies and of the anterior and suprachiasmatic nuclei.

The *in vitro* system consisted of an incubator with 12 polystyrene chambers, each containing one hypothalamus, in a water-saturated atmosphere of 95 % O_2, 5% CO_2 (vol/vol), at 37.5 C. The culture medium used (0.5 ml/chamber) was Dulbecco's Modified Eagle's Medium (DMEM, Flow, Mac Lean, VA) which contains 25 mM glucose and 1.25 mM Ca^{++}; this Ca^{++} concentration has been shown to be in the optimal range for the hypothalamic LHRH release by depolarization with 15-56 mM K^+ (6,12-15). At the end of every 7.5-min period, the medium (0.5 ml) was sampled from each chamber and collected in tubes containing 0.01 ml of a bacitracin solution (Sigma, St Louis, MO), that were placed in ice and subsequently frozen. A final concentration of 20 μM bacitracin was maintained to prevent degradation by hypothalamic peptidases (12). At the same time, 0.5 ml of fresh medium was removed from a container in the incubator and slowly reinjected into each chamber. After incubation of hypothalamic fragments *in vitro*, their LHRH content was extracted and assayed (17).

A highly specific LHRH RIA was performed in duplicate at 4 C on 0.1 ml of medium with RR-5 antiserum (16) at a final dilution of $1:10^5$. After a 18-h preincubation, [^{125}I]-LHRH (New England Nuclear, Boston, MA) was added for 24 h. Separation of the bound hormone was obtained with a second antibody coupled to cellulose (Institut National des Radioéléments, Fleurus, Belgium). The standard curves were prepared in DMEM supplemented with bacitracin. Non-specific binding was < 5 % of the total radioactivity. The interassay coefficient of variation was 18 %. The sensitivity varied between 0.5 and 1 pg/tube; the limit of detection was thus 5 pg/7.5 min. Synthetic LHRH and the immunoreactivity released from the hypothalamus *in vitro* induced a parallel displacement of radioiodinated LHRH.

The recovery of synthetic LHRH from the experimental system was evaluated at different concentrations (25 to 175 pg/0.5 ml) that were studied for 15 successive 7.5-min periods. The mean (± 1 SD) recovery was found to be 94 ± 7 % and the coefficient of variation 14 ± 3 %. LHRH release was considered to be unequivocally increased ($P < 0.05$) when it was more than 4 times this coefficient of variation as applied to the highest value of mean basal LHRH release (10.5 pg/7.5 min). Therefore, an increase in LHRH release was considered significant when it exceeded 6 pg/7.5 min.

In all age groups, the viability of hypothalamic tissue *in vitro* was assessed with a depolarizing

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agent, veratridine (Sigma, St Louis, MO) (15,18,19), added at a final concentration of 20 μM for 3 consecutive 7.5-min periods. In the presence of veratridine, the maximal increment of LHRH release over the pretreatment level was calculated. When this value was undetectable, it was considered to be at the limit of sensitivity, i.e., 5 pg/7.5 min. The effect of veratridine was compared at the beginning and before the end of an experiment. The frequency of LHRH pulses was calculated on a 112-min basis, this being the mean study period for the hypothalami. From the individual n of pulses observed from each RCH, the mean frequency (± 1 SD) was determined in each age group. Statistical analysis of age-related differences in LHRH content and release was performed with Student's *t* test.

RESULTS AND DISCUSSION

Since pulsatility of LH secretion has been shown to be maintained *in vivo* after deafferentation of the RCH but not after isolation of the mediobasal hypothalamus (9), we decided to study the RCH *in vitro*. This fragment is likely to contain axons and terminals originating from perikarya of LHRH neurons; most of these are located in the preoptic area (20) and

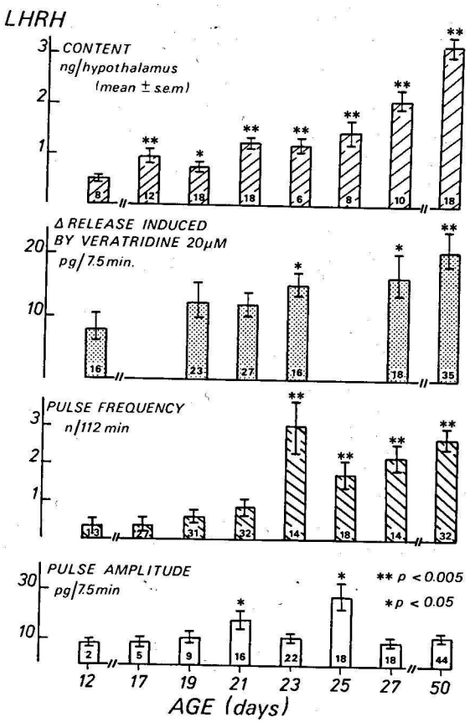

Figure 1. Age-related variations in the LHRH content of the RCH, the veratridine-induced release of LHRH, the frequency and the amplitude of pulses during spontaneous LHRH release *in vitro*. Below each column are indicated n of RCH studied, n of veratridine stimulations, n of h studied and n of pulses observed ; asterisks indicate values significantly different from those in 12-day-old rats.

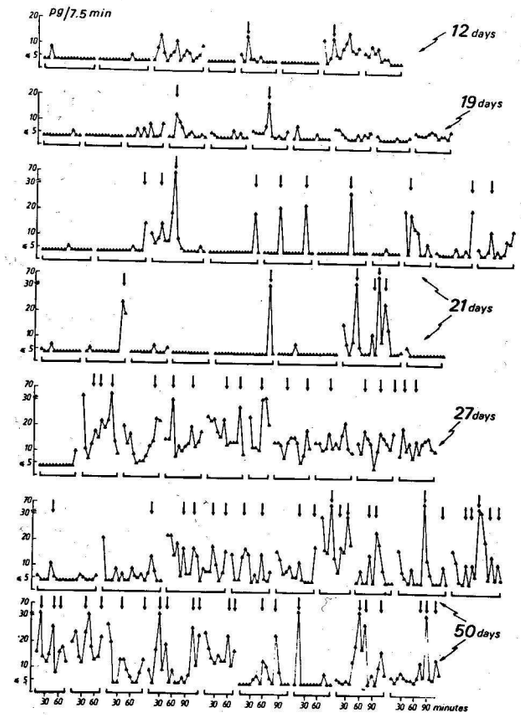

Figure 2. Spontaneous LHRH release *in vitro* from single retrochiasmatic hypothalami of male rats is shown as a function of time. Arrows indicate unequivocal pulses. Horizontal bars represent the study period for one RCH.

probably not present in the RCH fragment (21).

The viability of hypothalamic tissue *in vitro* was assessed by the increased LHRH release in response to the depolarizing effect of veratridine. Most investigators have tested the biological responsiveness of hypothalamus *in vitro* with high K^+ concentrations - 15 to 60 mM - (13,14,22-24). Since these resulted in hypertonicity and required control experiments, the use of veratridine was preferred (15,19). Veratridine 's effect was similar at the beginning and at the end of an experiment. The increment of LHRH release induced by veratridine increased with age (Fig. 1). It was significantly higher in 23-day-old rats and thereafter, with respect to 12-day-old animals, while tissue content of LHRH began increasing at 17 days (Fig. 1).

We then studied the variations in spontaneous LHRH release at 7.5-min intervals during the *in vitro* incubation of single unagitated RCHs (Fig. 2). These experimental conditions were chosen in order to evaluate the pulsatile nature of the LHRH release. Thus we took into account the short half-life of LHRH - around 5 min - (25) and the frequency of episodic LH discharges (7-9). Individual LHRH pulses might be masked by sampling at 30-min intervals (13,22,26) and by studying 3 to 10 whole or fragmented hypothalami per chamber (14,15,22,23,26) with the use of Dubnoff agitators (13,24,26).

As shown in Figure 2, unequivocal pulses of LHRH

release were rarely observed between 12 and 19 days of age. In these rats, the mean frequency of LHRH pulses was, respectively, 0.3 and 0.6 pulses/112 min (Fig. 1). No significant increase in pulse frequency was seen in 21-day-old rats. From 23 days onwards, the LHRH pulses were significantly more frequent and varied from 1.8 to 3.0/112 min. No significant changes were observed among 23-,25-,27- and 50-day-old rats. Wide variations in pulse amplitude occurred during the incubation of each RCH (Fig. 2). However, mean amplitude was found to be the highest in 21- and 25-day-old rats (Fig. 1). The frequency of LHRH discharges *in vitro* was greater than that expected from the LH pulsatility in intact immature rats (8). This could be the consequence of the hypothalamic disconnection from the rest of the brain. In immature rats, hypothalamic deafferentation results in precocious puberty (27). An acceleration of LH pulsatility has not been documented in these animals. Nevertheless, our observations might suggest that such a precocious puberty could be related to an increased frequency of the pulsatile release of LHRH.

Between 17 and 27 days of age, gradual changes in LHRH secretion are likely to occur (at an individually variable rate) before sexual maturation becomes manifest from the rapid increase in testis weight. We have previously found (28) that the hypothalami of 21-day-old rats released more LHRH *in vitro* than those of 50-day-old rats. The former had however, almost twice as much hypothalamic LHRH (2.08 ± 0.42 ng) as similar rats in this study.

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