Ageing does not influence the ultrashort feedback control of GnRH secretion in vitro

Elio Messi, Mariarosa Zanisi, and Luciano Martini

Institute of Endocrinology, University of Milan, Milan, Italy

Abstract. Evidence indicates that long and short feedback systems are altered in the aged male rat. Data also indicate the existence of an ultrashort feedback mechanism controlling GnRH secretion. The present experiments were performed to test whether the ultrashort feedback control of GnRH is operating also in old male rats. Medial basal hypothalami of 18-month-old male rats were perfused in vitro either in the presence or in the absence of a GnRH agonistic analogue (Buserelin: [D-Ser(TBU)6,Des-Gly10]GnRH ethylamide) and stimulated with 5-min pulses of K+(for a total of six pulses) in order to test their ability to release GnRH. The hypothalamic fragment was exposed to the GnRH analogue either for a part of the experimental period (at the beginning or at the end) or for the whole duration of the perfusion. In both cases, the presence of the analogue diminished or totally abolished the responses to K+ stimulation. This is in line with the results obtained in young animals. The data suggest that the ultrashort feedback mechanism controlling GnRH release is normally functioning also in aged male rats despite the fact that other types of feedback mechanisms (long and short loop) are substantially altered.

The activity of the hypothalamic-pituitary-testicular axis is impaired in aged male rats, but the mechanisms underlying this phenomenon have not been completely clarified. Aged male rats have reduced circulating levels of testosterone (1), LH (2,3) and FSH (4) along with decreased intrapituitary stores of gonadotropins (2). Both amplitude and frequency of LH pulses (5) and testosterone pulsatility (6) are lower in old than in young rats. Moreover, old animals exhibit fewer testicular gonadotropin receptors (7) and reduced pituitary responsiveness to GnRH (3), possibly owing to a decrease of pituitary GnRH receptors (8).

A defect in hypothalamic GnRH secretion has been proposed (9) as the primary phenomenon for the degenerative changes occurring in the function of the pituitary-testicular axis in old animals. However, while deterioration and decline in the number of GnRH secreting neurons have been shown to occur with advancing age (10), hypothalamic GnRH content, as measured by biological (2) or immunological methods (11), appears to remain normal in aged rats. Furthermore, Zanisi et al. (11) have reported that age does not induce any major defect on GnRH release machinery since the hypothalamus of old rats, in vitro is able to secrete GnRH and to respond to a stimulatory agent (K+) similarly to that of young rats.

Of the various types of feedback systems regulating the hypothalamic-pituitary-gonadal axis (12), the long feedbacks are altered in old male rats, since serum LH is low, in the presence of decreased circulating levels of testosterone (2,4). Moreover, in aged male rats, castration leads to a significantly smaller rise in serum gonadotropin levels than in younger animals (5,13) and lower doses of testosterone are needed to depress serum LH levels (13,14). Little evidence is available as to whether short loop feedback systems are also altered in old rats, except for a reduced inhibitory effect of prolactin on serum prolactin levels and pulsatility in...
24-26 months old ovariectomized rats compared with younger animals (15).

Recently in vivo (16,17) and in vitro (18-20) studies have suggested the existence of a negative feedback effect exerted by GnRH on its own secretion (ultrashort loop feedback mechanism). In particular, Zanisi et al. (19) have shown that perfusion of the mediobasal hypothalamus of normal adult male rats with a GnRH analogue decreases both the basal and the K+-stimulated GnRH release in vitro. The existence of ultrashort feedback systems controlling the release of somatostatin and GHRH has been proposed by other authors (21,22).

The present experiments were designed to evaluate whether the ultrashort feedback mechanism controlling GnRH release might be altered in the hypothalamus of old male rats using a GnRH agonistic analogue (Buserelin:[D-Ser(TBU)⁶,Des-Gly⁶]GnRH ethylamide), which does not cross-react in the RIA used to measure endogenous GnRH (19).

Materials and Methods

Animals
Adult male Sprague-Dawley rats (Nossan, Correzzana, Italy), 18 months old and weighing 597±39 g, were used throughout these experiments. Animals were maintained in a temperature- and humidity-controlled animal quarters with a lighting schedule of 14 h light and 10 h darkness (light on, 06.30-20.30 h). Water and pellet food were supplied ad libitum.

Tissue preparation and perfusion procedure
Animals were killed by decapitation, the brain was chilled and the hypothalamic fragment was rapidly dissected out. The hypothalamic tissue was about 1-2 mm deep, and was limited frontally by the caudal border of the optic chiasm, laterally by the lateral sulci, and caudally by the rostral border of the mamillary bodies. Immediately after dissection, each mediobasal hypothalamus was halved and weighted: the halved mediobasal hypothalamus was placed in the perfusion chamber maintained at 37°C and containing Krebs-Ringer medium (in molar concentrations: NaCl, 0.119; KCl, 4.6-10⁻²; CaCl₂, 1.89-10⁻³; NaHCO₃, 2.5-10⁻²; Na₂HPO₄, 1.14-10⁻³; MgCl₂, 6.6-10⁻³; glucose, 1.05-10⁻²; pH 7.4). Bactracin was added to prevent peptide degradation (1.1-10⁻⁴ mol/l). The perfusion system used in these experiments has been previously described (11,19). After a 30-min stabilization period, 1 ml of the effluent was collected, every 5 min into tubes containing 10 µl HCl (1 mol/l) to acidify the samples (final pH 2). Each sample was then centrifuged and the supernatant stored at -20°C until measurement of GnRH, which was performed the next day.

Serum LH levels were measured and found to be 1.53±0.22 µg/l (1); the standard LH values in young animals of the same colony are 3.86±0.57 µg/l (1).

Experiment I

In this preliminary experiment, 1 hypothalamic fragment (2 halves) was placed in each chamber. The tissue in chamber 1 was initially perfused with Krebs-Ringer medium (for 110 min) and stimulated three times with 5-min pulses of K⁺ (0.11 mol/l) every 30 min. After this period, the control medium was replaced by Krebs-Ringer medium containing the GnRH analogue (5-10⁻⁵ mol/l; Buserelin®, Hoechst AG, FRG); after 60 min of exposure to the analogue (effluent not collected), three subsequent K⁺ stimulations were applied (5 min pulses every 30 min). The tissue in chamber 2 was perfused in a similar fashion, but the medium containing the GnRH analogue was used at the beginning of the experiment rather than at the end.

Experiment II

In the second series of experiments, single halved fragments of the mediobasal hypothalamus were placed in each chamber. The mediobasal hypothalamus in chamber 1 was exposed, for the whole duration of the experiment, to Krebs-Ringer medium, whereas the mediobasal hypothalamus in chamber 2 was continuously perfused with medium containing the GnRH analogue (5-10⁻⁵ mol/l). In both cases, after an initial period of 110 min, collection of samples was interrupted for 60 min, and then resumed for 105 min. Six pulses of K⁺ (0.11 mol/l for 5 min every 30 min) were applied to the tissue in each chamber.

Hormonal assays

The GnRH content of the perfusion effluents was determined by RIA using reagents supplied by BIODATA (Italy). All samples were run in duplicate. The assay sensitivity (98% of total binding) was 0.5 pg/tube (100 µl/sample). Inter- and intra-assay coefficients of variations were 9.4 and 6.6%, respectively. The absence of cross-reactivity between the GnRH analogue and the antiserum used in the GnRH-RIA has been previously assessed (19).

Serum LH concentrations were measured by RIA using an anti-ovine LH antiserum provided by Dr G. D. Niswender (Colorado State University, Fort Collins, CO). Values are expressed in terms of NIH-LH-S20. The assay sensitivity was 10 pg/tube (100 µl/sample), and the intra-assay coefficient of variation was 2.45%.

Statistical analysis

The results of the perfusion experiments are expressed as ng/l, and as Δ in nanograms of GnRH released. Δ represents the total amount of GnRH released during the whole secretory response to the K⁺ stimulus, minus the
amount of neuropeptide released over the same period of time under basal conditions. The data expressed in Fig. 2 have been statistically evaluated by t-test to compare basal levels at the beginning of the perifusion between mediobasal hypothalami perifused with Krebs-Ringer or with Krebs-Ringer plus the analogue. The results reported in Fig. 3 have been analysed by Repeated Measures with Grouping Factors-ANOVA, to compare the responses (Δ) between and within each group.

Statistical tests were calculated using the statistical package «SYSTAT» (Systat Inc, Evanston, IL), on computer Macintosh Plus, Apple.

Results

Experiment I

Fig. 1 shows the results of two representative experiments in which the same mediobasal hypothalamus has been alternatively perifused with Krebs-Ringer medium or with Krebs-Ringer plus the GnRH analogue. The upper panel (A) shows basal secretion and responses of the mediobasal hypothalamus to K+ stimulations during an initial perifusion with Krebs-Ringer, and subsequently with Krebs-Ringer containing the GnRH analogue. The lower panel (B) shows the basal secretion and the responses to K+ stimulations of a single mediobasal hypothalamus perifused initially with the GnRH analogue and subsequently with the Krebs-Ringer medium. It appears from panels A and B of Fig. 1 that the mediobasal hypothalamus perifused with Krebs-Ringer is able to respond to three subsequent K+ challenges with bursts of GnRH secretion, and that these responses are either markedly reduced or completely abolished when the K+ stimuli are applied during perifusion with the GnRH analogue-containing medium.

Experiment II

Fig. 2 summarizes the results obtained when single mediobasal hypothalami of old male rats are perifused, for all the duration of the experiment, either with control medium (A) or with a GnRH analogue-enriched medium (B). In these experiments, the hypothalamic fragments have been stimulated three times with K+; after a resting period of 60 min (during which time the effluent was not collected), three additional K+ stimuli have been applied. First of all, it is clear that the mediobasal hypothalamus, when perifused with Krebs-Ringer, maintains its ability to respond to K+ stimulations with quantitatively comparable bursts of GnRH for a period of over 5 h. It may also be observed that, before any K+ stimulation, the basal release of GnRH from the mediobasal hypothalamus perifused with medium containing the GnRH analogue

Fig. 1.
A and B: GnRH release in vitro from a single mediobasal hypothalamus per chamber (two different chambers) perifused with Krebs-Ringer (●) or Krebs-Ringer plus GnRH analogue (○). See text for details.
(1.46±0.8 ng/l; below the sensitivity of the assay) is significantly (p<0.001) lower than that observed for the mediobasal hypothalamus perfused with Krebs-Ringer (11.97±1.3ng/l). Moreover, the presence in the perfusion medium of the GnRH analogue strongly reduces or completely abolishes the K+-induced release of GnRH.

Fig. 3 provides the Δ of the GnRH hypersecretion induced by the K+ stimuli in old male rats either in the presence or in the absence of the analogue. In the mediobasal hypothalamus perfused with control medium it is clear that 1. all K+ pulses are followed by an increase in GnRH release; 2. after the first stimulation there is a tendency towards a decrease of GnRH secretion, but this phenomenon is not significant and there were no significant differences in the various GnRH responses (p>0.05). It is also clear from Fig. 3 that, when the hypothalamus of old male rat is perfused with the GnRH analogue, the first K+ pulse is able to induce a small increase in GnRH release, but the tissue becomes completely refractory to the K+ stimulus with progression of time of exposure to the analogue. No statistically significant differences among the Δ obtained in the presence of the analogue were detected (p>0.05). All the Δ of the group perfused with Krebs-Ringer are significantly different (p<0.05) from those obtained from the mediobasal hypothalamus perfused with the GnRH analogue.

Discussion

The results here reported confirm our previous findings (11,19) that it is possible to use one single mediobasal hypothalamus in an in vitro perfusion system to study the dynamics of GnRH secretion. The data confirm that this type of preparation remains viable for a long period of time (5 h), independently of whether the tissue has been taken from young (19) or old animals (11). Finally, the data obtained in the control mediobasal hypothalamus (perfusion with Krebs-Ringer medium) confirm our reported results (11) that the mediobasal hypothalamus of old rats is able to release GnRH spontaneously, and to respond to K+ challenges with repeated bursts of GnRH secretion. The present data suggest once more that the GnRH neuronal system is not impaired in old rats (11).

The most important finding in this series of experiments is that perfusion with a GnRH agonistic analogue inhibits the basal as well as the K+-in-
duced release of endogenous GnRH from the mediobasal hypothalamus of old rats, in a fashion similar to that previously reported for the mediobasal hypothalamus of young animals (19). Therefore, the present data suggest that the ultrashort feedback mechanism through which GnRH is able to affect its own secretion is not altered in old male rats. The present findings, while providing support for the existence of a negative ultrashort feedback mechanism controlling GnRH release, do not allow any conclusion on the mechanism of action of the GnRH analogue. The analogue might act directly on GnRH-producing cells, exerting its inhibitory effect either on the cell body or on the dendrites or neurosecretory terminals. De Paolo et al. (20) reported that a GnRH analogue different from the one used in the present experiments inhibits spontaneous and K⁺-induced GnRH release from the mediobasal hypothalamus but not from the median eminence. These results suggest that the ultrashort feedback effect of the analogues might be exerted at a site higher than the median eminence, a structure that contains only nerve terminals and axons (23).

Another possibility to be considered is that the GnRH analogue, rather than acting directly on the GnRH-producing neurons, might modify the function of other hypothalamic systems (adrenergic, serotoninergic, etc.), which in turn, act on the GnRH neurons. In this connection, it may be recalled that Andersson et al. (24) found that, in young hypophysectomized rats, administration of GnRH increases dopamine turnover in the lateral palisade zone of the median eminence and decreases noradrenaline turnover in the nucleus preopticus medialis.

An additional potential problem in the interpretation of the data is that only little information is available on the presence of specific GnRH receptors in the brain in general and in the hypothalamus in particular (25). However, it must be underlined that there are evidences to suggest that GnRH may exert direct effects on the central nervous system, suggesting either the existence of specific receptors for its activity or the possibility of non-receptorial mechanisms. For instance, it has been demonstrated that GnRH, administered either peripherally or centrally (26), potentiates the lordosis reflex in estrogen-primed rodents, and significantly enhances proceptivity in ovariectomized estrogen-treated marmosets (27). Moreover, both excitatory and inhibitory responses to locally applied

---

Fig. 3.
K⁺-induced GnRH release in vitro from single mediobasal hypothalami perifused with Krebs-Ringer (■) or Krebs-Ringer plus GnRH analogue (□). Value represent the mean ± SEM of Δ of the three experiments shown in Fig. 2.
GnRH of neurons located in the mediobasal hypothalamus, hippocampus, and midbrain central gray have been reported (28,29).

As previously reported, the turnover of dopamine is increased by administration of GnRH to young hypophysectomized male rats (24). If one accepts the view that the GnRH analogue exerts its ultrashort feedback effect through hypothalamic neurotransmitters, the present data might suggest that the effect of GnRH on hypothalamic dopaminergic activity is not lost with advancing age, even if dopamine stores, turnover and activity are decreased in the central nervous system of old animals.

Acknowledgments

Thanks are due to Dr A. Carandente, Hoechst (Milan, Italy) for the gift of Buserelin and to Dr G. D. Niswender for supplying the anti-LH-antiserum.

This work was supported by the Joint Project of the Consiglio Nazionale delle Ricerche, Italy (Grant 88.005.04) and grants from the Ministero della Pubblica Istruzione.

References

21. Lumpkin MD, Samson WK, McCann SM. Effects of intraventricular growth hormone-releasing factor on growth hormone release: further evidence for ul-


Received July 24th, 1989.
Accepted November 16th, 1989.

Dr M. Zanisi,
Institute of Endocrinology,
via G. Balzaretti 9,
1-Milan 20133,
Italy.