

## TIME-DEPENDENT EFFECTS OF STARVATION ON PITUITARY, HYPOTHALAMIC AND SERUM PROLACTIN LEVELS IN RATS: COMPARISON TO THE GALANIN EXPRESSION PATTERN

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Received: May 25, 2015; Revised: July 9, 2015; Accepted: August 8, 2015; Published online: December 8, 2015

**Abstract:** Given that both prolactin and galanin take part in the regulation of energy homeostasis and that galanin is localized within lactotrophs, this study was aimed at comparing the pituitary expression patterns of prolactin and galanin during different phases of metabolic response to starvation in adult Wistar male rats. Food was removed at the onset of the dark phase (6:00 pm) and the animals were deprived for 6, 12, 24 and 48 h. Each of the starved groups (n=6) was killed simultaneously with a group of *ad libitum*-fed rats (n=6), and the intrapituitary levels of prolactin and galanin were examined. Galanin expression in the hypothalamus and the circulating levels of prolactin were also assessed. Starvation induced a rise in the intrapituitary prolactin level ( $p < 0.001$ ), whereas the opposite trend was detected in the serum ( $p < 0.05$ ). The galanin pituitary level was initially increased (6, 12 h) ( $p < 0.05$ ), but as starvation progressed, it first reached (at 24 h) and ultimately fell below the level recorded in the *ad libitum* rats (at 48 h) ( $p < 0.05$ ). Both prolactin and galanin were elevated in the hypothalamus after 24- and 48-h starvation. The results show that the starvation-induced increase in the pituitary prolactin expression did not lead to the rise in prolactin circulating levels, but rather resulted in the elevation of the prolactin hypothalamic content. Furthermore, the results suggest that under the circumstances of disturbed energy homeostasis, galanin might be responsible for the augmented prolactin production, initially at the pituitary and subsequently at the hypothalamic level.

**Key words:** prolactin; galanin; pituitary; hypothalamus starvation; rat

### INTRODUCTION

It is established that in all animals, limitations in food resources may ultimately lead to starvation-induced mortality. Starvation denotes the biological condition wherein a postabsorptive animal, otherwise willing to eat, is unable to do so as a result of some extrinsic limitation on food resources (McCue, 2010).

Prolactin is primarily produced within the pituitary lactotroph cells and some of its functions are relevant in to maintenance of energy homeostasis. It modulates ATPase activity in the brain (Kumaran et al., 1989), activates lipoprotein lipase in adipocytes (Gualillo et al., 1999) and influences the expression

of appetite regulators, such as insulin (Nielsen et al., 1982) and leptin (Gualillo et al., 1999).

Galanin has a widespread distribution and is co-expressed with a number of transmitters and other peptides in neurons in various brain regions, including the hypothalamus (Kyrkouli et al., 1990). It has already been shown that when injected intracerebroventricularly (ICV) or microinjected into the hypothalamic paraventricular nucleus (PVN), galanin stimulates food intake in satiated rats (Dube et al., 1994). This peptide was demonstrated to regulate fat intake preference in mice (Adams et al., 2008). Additionally, it has been shown that a reliable change in

hypothalamic galanin synthesis occurs in response to food ingestion (Gundlach, 2002). Galanin is also synthesized in the pituitary (Merchenthaler et al., 1993).

Based on the facts that prolactin and galanin take part in the regulation of energy homeostasis and that galanin is localized within lactotrophs (Steel et al., 1989), our aim was to compare the expression patterns of prolactin and galanin in rats during food deprivation. We hypothesized that the expression of galanin is linked to the synthesis of prolactin during starvation in rats.

## MATERIALS AND METHODS

Experiments were performed on adult male Wistar rats, weighing  $250 \pm 20$  g, bred in the vivarium of the Belgrade University Faculty of Biology. Two rats were housed per cage under controlled temperature conditions ( $21 \pm 1^\circ\text{C}$ ) and lighting (12 h light-12 h of darkness). The food was removed at the onset of the dark phase (6 pm) and the groups of animals remained food deprived for 6, 12, 24 or 48 h ( $n=6$ ). Each of the starved groups was sacrificed simultaneously with the group of *ad libitum*-fed rats ( $n=6$ ). All the animals had free access to tap water. The experiment was performed according to the rules for animal care proposed by the Serbian Laboratory Animal Science Association, a member of the Federation of European Laboratory Animal Science, and approved by the Ethics Committee of the Faculty of Biology, University of Belgrade.

Animals were decapitated without anesthesia with a guillotine (Harvard-Apparatus, Holliston, MA). Blood was collected from the trunk. Serum prolactin was measured using an ELISA kit (IBL-79179, USA) and the values were expressed as ng/ml. The pituitary glands and brains were quickly excised. Hypothalami were removed and then frozen at  $-80^\circ\text{C}$  until further use.

### Tissue sample preparation

After decapitation, rat hypothalami were homogenized on ice with an Ultra-turrax homogenizer in

buffer (pH 7.4) containing (in mM): 150 NaCl, 10 Tris, 1 EDTA; 10% glycerol, 1% Triton X-100, protease inhibitor cocktail with additional 2 mM PMSF and 2 mM sodium orthovanadate. Homogenates were centrifuged at 600xg for 20 min at  $4^\circ\text{C}$ , and obtained supernatants were ultracentrifuged for 60 min at 100000xg. Protein concentration was determined by the BCA method.

### SDS-PAGE and Western blot

Protein lysates (1 mg per lane) were separated by 20% SDS polyacrylamide gels and transferred to polyvinylidene fluoride membranes as previously described (Vujović et al., 2011). After Ponceau S staining and destaining, the membranes were blocked for 3 h in 5% nonfat dry powder milk (Santa Cruz) in Tris-buffered saline containing 0.1% Tween 20 (TBST) and probed with antibody directly against galanin (1:3500 dilution, sc-25446), overnight at  $4^\circ\text{C}$  with agitation. After washing, membranes were incubated with anti-rabbit secondary horseradish peroxidase conjugated antibody (1:5000 dilution, sc-25446). The bound antibodies were visualized by enhanced chemiluminescence (ECL) (Amersham) and exposure to X-OMAT film. Signals were quantified by a densitometry by using Image Quant 5.2 (Molecular Dynamics) program.

### qPCR

TaqMan PCR assays were carried out using Assay-On-Demand Gene™ Expression Products (Applied Biosystems, USA) for galanin (Rn005836180\_m1; GCACCG TGCCGTAGTAGCTTAG). The gene expression assays contained primers for amplification of the target gene and the TaqMan Minor Groove Binder (MGB) probe 6-FAM dye-labeled for the quantification. Reactions were performed in a 25- $\mu\text{L}$  reaction mixture containing 1X TaqMan Universal Master Mix with AmpErase UNG, 1X Assay Mix (Applied Biosystems) and cDNA template (10 ng RNA converted to cDNA). PCR was carried out in the ABI Prism 7000 Sequence Detection System at  $50^\circ\text{C}$  for 2 min,  $95^\circ\text{C}$  for 10 min, followed by 40 cycles at  $95^\circ\text{C}$  for 15 s and  $60^\circ\text{C}$  for 1 min. The experimental threshold was calculated based

on the mean baseline fluorescence signal from cycle 3 to 15 plus 10 standard deviations. The point at which the amplification plot crosses this threshold defined as Ct, represents the cycle number at this point and is inversely proportional to the number of target copies present in the initial sample. Each sample was run in triplicate and the mean value of each Ct triplicate was used for further calculations. A reference, endogenous control, was included in each analysis to correct the differences in the inter-assay amplification efficiency and all transcripts were normalized to  $\beta$  actin (Rn01412977\_m1; TCATGTGCCAGGGTG-GTGACTTCAC) expression.

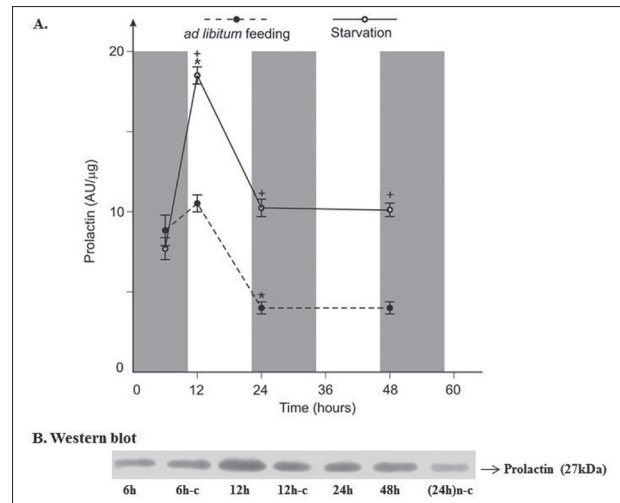
The results were analyzed by the RQ Study Add On software for 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System) with a confidence level of 95% ( $p < 0.05$ ). The relative expression of the target gene was expressed in relation to the calibrator, i.e., the control sample. Due to individual differences among animals, the sample of the control groups with the expression value close to the mean of all samples in the group and with the lowest measurement error was used as a calibrator. The final result is reported as fold change relative to the calibrator and normalized to  $\beta$  actin using the equation:  $N_{\text{sample}} = 2^{-\Delta\Delta Ct}$ .

### Statistical analysis

Values are expressed as means  $\pm$  SEM, with n values representing the number of experiments. The one-way ANOVA and Tukey's posterior multiple comparison tests were employed for comparison of the experimental groups, the level of significance being set at  $p < 0.05$ .

## RESULTS

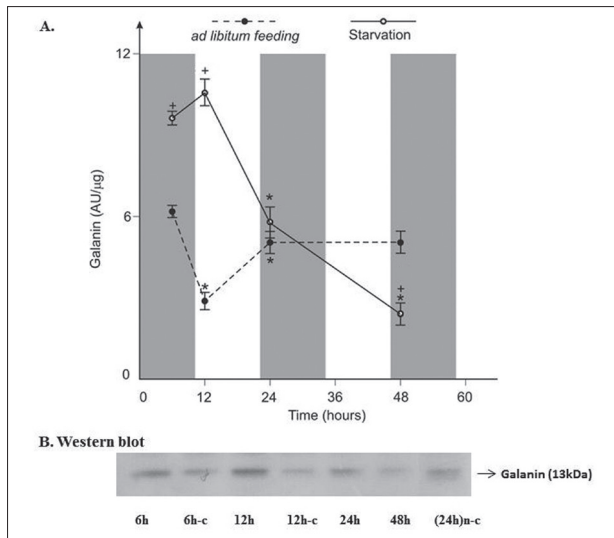
The aim of this study was to compare the starvation-induced changes in prolactin and galanin pituitary expression. We therefore first investigated the prolactin content in this endocrine gland. We established that although intrapituitary prolactin (Fig. 1.) was unchanged after 6 h of starvation, it was elevated for the remainder of the examined period in comparison to the level detected in *ad libitum*-fed rats ( $p < 0.05$ ).



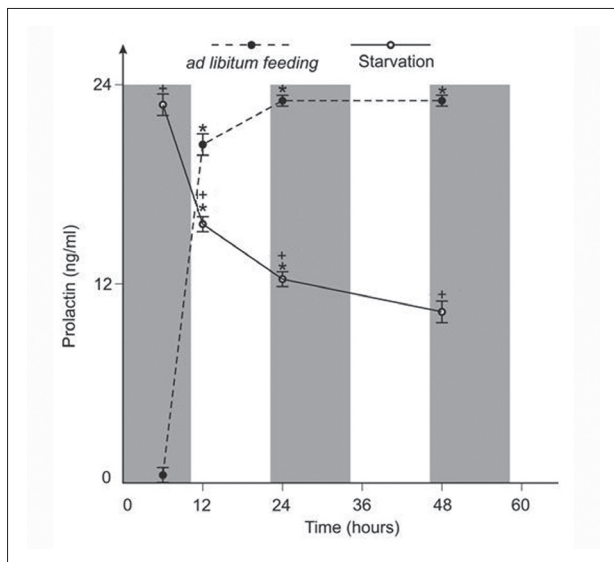
**Fig. 1.** Prolactin content in the pituitary of starved and *ad libitum*-fed rats. A. Data generated from densitometry of the separate Western blots. Y-axis is prolactin protein content expressed as an AU/ $\mu$ g and x-axis is time of starvation. Gray areas indicate dark periods; white areas indicate light periods in the vivarium. Data are presented as means  $\pm$  SEM from six measurements per point. Significant differences between starved and corresponding *ad libitum*-fed group: \* $p < 0.05$ . Significant differences between adjacent values within starved or *ad libitum*-fed group: \* $p < 0.05$ . B. Representative Western blot (6h – 6-h-starved rats; 6h-c – *ad libitum*-fed group killed simultaneously with 6-h-starved rats; 12h – 12-h-starved rats; 12-c *ad libitum*-fed group killed simultaneously with 12-h-starved rats; 24h – 24-h-starved rats; 48h – 48-h-starved rats; (24n)-c: *ad libitum*-fed group killed simultaneously with 24- and 48-h-starved rats).

However, significant reduction of prolactin pituitary content was detected between 12- and 24-h-starved rats ( $p < 0.05$ ). As regards galanin, its intrapituitary content (Fig. 2.) was increased after 6 and 12 h of starvation ( $p < 0.05$ ). It subsequently reached (after 24 h) and then finally dropped below the control level after 48 h of food deprivation ( $p < 0.05$ ). Additionally, significant reduction of intrapituitary galanin was detected between 12 and 24 h ( $p < 0.05$ ), as well as between 24- and 48-h-starved rats ( $p < 0.05$ ).

We then wanted to determine whether changes in circulating prolactin reflected the starvation-induced changes of prolactin pituitary content (Fig. 3). Prolactin circadian rhythm detected in the serum of the *ad libitum* rats was completely abolished by food deprivation. After 6-h starvation, the level of this hormone was significantly elevated in comparison to the value



**Fig. 2.** Galanin content in the pituitary of starved and *ad libitum*-fed rats. A. Data are presented as means $\pm$ SEM from six measurements per point. Significant differences between starved and corresponding *ad libitum*-fed group: \* $p$ <0.05. Significant differences between adjacent values within starved or *ad libitum*-fed group: \* $p$ <0.05. B. Representative Western blot. Abbreviations are the same as shown for Fig. 1.



**Fig. 3.** Prolactin concentration in the serum of starved and *ad libitum*-fed rats. The y-axis is prolactin protein concentration expressed as an ng/ml and x-axis is time of starvation. Gray areas indicate dark periods; white areas indicate light periods in the vivarium. Data are presented as means $\pm$ SEM from six measurements per point. Significant differences between starved and corresponding *ad libitum*-fed group: \* $p$ <0.05. Significant differences between adjacent values within starved or *ad libitum*-fed group: \* $p$ <0.05.

detected in the simultaneously sacrificed *ad libitum*-fed group ( $p$ <0.05). However, the opposite trend was observed in the rest of the examined groups (12, 24, 48 h) ( $p$ <0.05). Additionally, a significant decrease in this hormone's concentration in the blood was detected after 12 and 24 h of food deprivation ( $p$ <0.05).

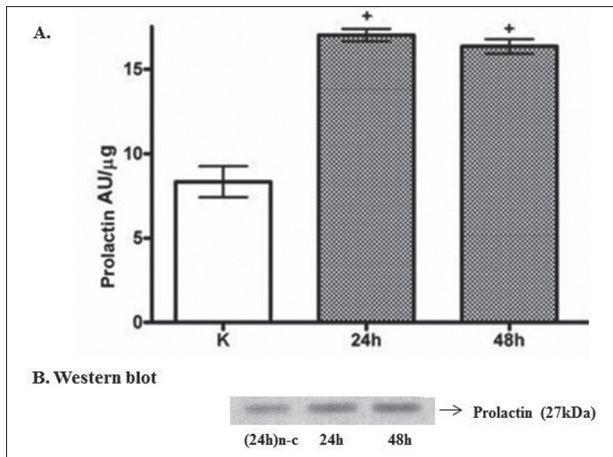
Bearing in mind that intrapituitary prolactin did not lead to an increase in the circulating prolactin level, we wanted to investigate if the same trend could be observed in starvation-induced changes between the pituitary and hypothalamic prolactin content. Our results showed that hypothalamic prolactin content (Fig. 4.) was increased after 24 and 48 h of starvation ( $p$ <0.05).

Changes in the level of hypothalamic galanin protein in starved and *ad libitum* rats are summarized in Fig. 5. Significant reduction ( $p$ <0.05) of galanin hypothalamic content was detected after 6 and 12 h of starvation, whereas the opposite trend was detected after 24 and 48 h of food deprivation ( $p$ <0.05). The level of galanin detected in the hypothalamus of 24-h-starved rats was also significantly elevated ( $p$ <0.05) when compared with 12-h-starved animals.

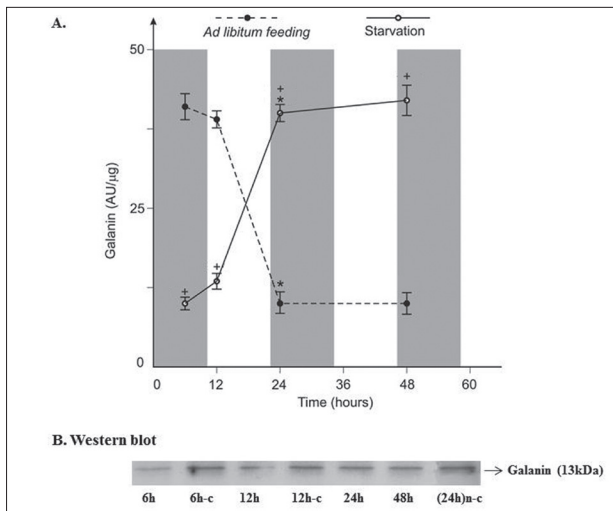
We also found that the increase in hypothalamic galanin content coincided with the increase in relative expression of galanin mRNA in the hypothalamus. (Fig. 6.). Namely, our results showed that mRNA for galanin was upregulated after 24 and 48 h of food deprivation ( $p$ <0.05).

## DISCUSSION

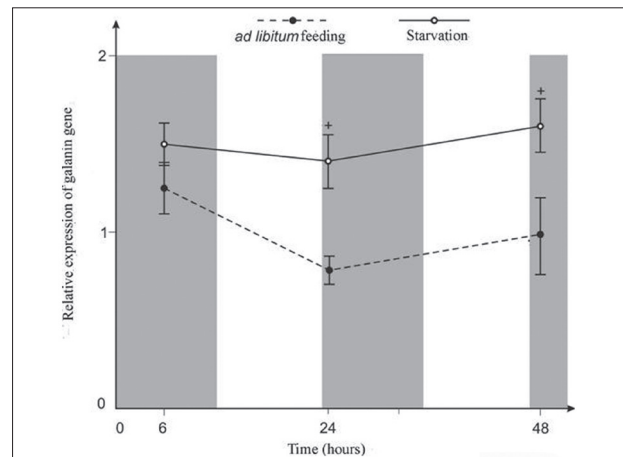
This research was designed to examine the effects of different starvation periods on the pituitary, hypothalamic and serum prolactin levels in rats. Our results confirmed that food deprivation induced upregulation of pituitary prolactin expression 12 h after food removal. The physiological relevance of boosted prolactin production in times of negative energy balance stems from the fact that this hormone helps maintain energy homeostasis on different levels. For example, prolactin activates lipoprotein lipase in adipocytes (Gualillo et al., 1999), consequently facilitating the



**Fig. 4.** Prolactin content in the hypothalamus of starved and *ad libitum*-fed rats. A. The y-axis is prolactin protein content expressed as an AU/μg and x-axis is time of starvation. Data are presented as means±SEM from six measurements per point. Significant differences between starved and corresponding *ad libitum*-fed group: \* $p < 0.05$ . B. Representative Western blot. Abbreviations are the same as shown for Fig. 1.



**Fig. 5.** Galanin content in the hypothalamus of starved and *ad libitum*-fed rats. A. Data are presented as means±SEM of six measurements per point. Significant differences between starved and corresponding *ad libitum*-fed group: \* $p < 0.05$ . Significant differences between adjacent values within starved or *ad libitum*-fed group: \* $p < 0.05$ . B. Representative Western blot. Abbreviations are the same as shown for Fig. 1.



**Fig. 6.** The relative expression of the galanin mRNA in the hypothalamus of starved and *ad libitum*-fed rats. Data are presented as means±SEM six measurements per point. Significant differences between starved and corresponding *ad libitum*-fed group: \* $p < 0.05$ .

supply of energy substrates to various tissues. We showed there was a simultaneous rise in the intrapituitary concentration of both prolactin and galanin after 12 h of food deprivation. It is well known that galanin is predominantly synthesized in the lactotroph population of the anterior pituitary (Hyde et al., 1991). Furthermore, it was demonstrated that galanin is a mitogen to the 235-1 clonal lactotroph cell line, acting via a pituitary-specific galanin receptor (Wynick et al., 1998). Even though the central galanin administration causes the increase in prolactin production in the pituitary (Murakami et al., 1995), galanin also inhibits prolactin secretion into the bloodstream (Hammond et al., 1996). These observations are correlation with our data, that the period of a starvation-induced increase (12-48 h) of prolactin level in the pituitary overlaps with the decreased concentration of this hormone in serum. Thus, our results suggest that hypophyseal galanin may affect prolactin synthesis and secretion during short-term starvation.

However, the augmented galanin level within the pituitary was only detected after 6 and 12 h of starvation whereas prolactin remained elevated even after 24 and 48 h of food deprivation. In addition to being synthesized in the lactotroph anterior pituitary cells, it has been shown that, in rats, galanin is also produced in the paraventricular hypothalamic

nucleus (Hyde et al., 1991), Therefore, we have examined the hypothalamic galanin expression once starvation reduced galanin pituitary content below the level of the adequately *ad libitum*-fed group. Our results show that the hypothalamic galanin mRNA level was upregulated after 24 and 48 h of starvation. Additionally, the amount of the peptide itself was also raised in the same brain region 24 and 48 h after food intake had been prevented. These results suggest that galanin-regulated production of prolactin might be shifted from the pituitary to the hypothalamus during a prolonged state of disturbed energy homeostasis. This is in agreement with the adopted view that the regulation of energy homeostasis involves the interplay of neuronal circuitries controlling food intake with endocrine secretions modulating the activity of the neurons making up those circuitries (Richard and Baraboi, 2004). There are indications that a stimulatory effect of hypothalamically derived galanin on prolactin expression may be carried out by vasoactive intestinal polypeptide (Koshiyama et al., 1987).

As the starvation-induced upregulation of prolactin synthesis in the pituitary did not result in an increase in prolactin circulating level, a compelling question was where was the newly synthesized prolactin redistributed. Since it has already been demonstrated that centrally administered prolactin is involved in the hypothalamic-driven stimulation of food intake (Gerardo-Gettens et al., 1989), we hypothesized it was relevant to determine the prolactin levels within this brain region under the circumstances of a starvation-induced increase in hypothalamic galanin expression. Our results confirmed that the rise in the hypothalamic galanin content coincided (24, 48 h) with the increase in prolactin levels in the same brain region. Although expression of the prolactin gene was documented in the hypothalamus (Torner et al., 2002), the increase of prolactin in the hypothalamus can also be attributed to the long recognized retrograde transport of this hormone from the pituitary (Mezey et al., 1979).

As mentioned above, the starvation-induced increase in hypothalamic prolactin levels could be accounted for by the documented action of this hormone

in food stimulation. The translocation of galanin-regulated prolactin production mechanisms from the pituitary into the hypothalamus during later stages of starvation is in agreement with the reports of prolactin interference with the signaling pathways of appetite-inhibiting leptin (Naef and Woodside, 2007). Namely, rats that had been provided with chronic infusions of prolactin (5 µg/h) into the cerebral ventricles for 10 days did not show a reduction in food intake or body weight after a central injection of 4 µg murine leptin, whereas the expected reduction in both of these parameters was seen in vehicle-infused rats (Torner et al., 2002). Additionally, prolactin was found to suppress leptin signaling through the induction of SOCS3 expression in the hypothalamus (Bjorbak et al., 2000).

In summary, the concentration of prolactin was changed by starvation in a time-dependent and tissue-specific manner. Results from this study show that the starvation-induced increase in pituitary prolactin expression did not lead to a rise in prolactin circulating levels, but rather resulted in the elevation of the prolactin hypothalamic content. Viewed in the light of already existing data, our results suggest that under the circumstances of disturbed energy homeostasis, galanin may be responsible for augmented prolactin production, initially from the pituitary and subsequently from the hypothalamic level.

**Acknowledgments:** This work was supported by the Serbian Ministry of Education, Science and Technological Development (Grants No. 173023 and No.173033). Rabbit anti-rat prolactin hormone (anti-PRL) was obtained from Dr A. F. Parlow (National Institute of Diabetes & Digestive & Kidney Diseases, National hormone and peptide program, Torrance, CA).

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