



Regulation of NUCB2/nesfatin-1 production in rat's stomach and adipose tissue is dependent on age, testosterone levels and lactating status

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ABSTRACT

Nesfatin-1, which is derived from the NEFA/nucleobindin 2 (NUCB2) precursor, was recently identified as an anorexigenic peptide that is produced in several tissues including the hypothalamus. Currently, no data exist regarding the regulation of NUCB2/nesfatin-1 production in peripheral tissues, such as gastric mucosa and adipose tissue, through different periods of development. The aim of the present work was to study the variations on circulating levels, mRNA expression and tissue content in gastric mucosa and adipose tissue of NUCB2/nesfatin-1 with age and specially in two clue periods of maturation, weaning and puberty. The weaning period affected NUCB2/nesfatin-1 production in gastric tissue. The testosterone changes associated with the initiation of puberty regulated NUCB2/nesfatin-1 production via adipose tissue and gastric NUCB2/nesfatin-1 production. In conclusion, the production of NUCB2/nesfatin-1 by the stomach and adipose tissue fluctuates with age to regulate energy homeostasis during different states of development.

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1. Introduction

In 2006, nesfatin-1 was identified as a novel satiety molecule that regulates food intake. This peptide is formed from the N-terminal end of its 396 aa precursor protein NUCB2.¹ The prohormone convertases PC-1/3 and PC-2 have been proposed to cleave NUCB2 into three fragments, namely nesfatin-1 (aa 1–82), nesfatin-2 (aa 85–163) and nesfatin-3 (aa 166–396) (Oh et al., 2006). At the moment, the physiological functions of segments 2 and 3 are unknown.

In rat brain, nesfatin-1 has been identified in the hypothalamic nuclei involved in the regulation of food intake (Oh et al., 2006) as well as in CSF.² Protein expression of NUCB2/nesfatin-1 colocalize with several neuropeptides implicated in energy balance (urocortin-1, melanin-concentrating hormone, proopiomelanocortin, amphetamine-regulated transcript, α -melanocyte-stimulating hormone, vasopressin and neuropeptide Y) (Foo et al., 2008; Fort et al., 2008; Kohno et al., 2008). NUCB2/nesfatin-1 immunoreactivity has also been shown in other brain areas related to stress and in autonomic regulatory centers (Brailoiu et al., 2007; Goebel et al., 2009). Intracerebroventricular injection of nesfatin-1 and/or NUCB2 reduces dark-phase feeding in a dose-dependent manner and results in the loss of total body fat and weight upon chronic administration (Oh et al., 2006). In the brain, nesfatin-1 causes anorexia by a leptin-independent mechanism through the melanocortin pathway

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¹ NUCB2: NEFA/nucleobindin 2.

² CSF: cerebrospinal fluid.

(Maejima et al., 2009). NUCB2/nesfatin-1 expression is regulated by nutritional status, because during fasting, the NUCB2 protein and mRNA levels decrease in the PVN,³ but these levels are recovered after refeeding (Garcia-Galiano et al., 2010; Kohno et al., 2008; Oh et al., 2006). Other actions of nesfatin-1 include the regulation of anxiety, sleeping, gastric emptying, gastric acid secretion, glucose metabolism and insulin secretion (Stengel and Taché, 2013).

Nesfatin-1 crosses the blood–brain barrier in both directions through a non-saturable mechanism (Pan et al., 2007). Intraperitoneal nesfatin-1 administration also reduces food intake in mice (Shimizu et al., 2009), suggesting that peripheral nesfatin-1 can also play a role in central regulation. NUCB2/nesfatin-1 expression has been described in the gastrointestinal tract, pancreas, pituitary gland, heart, adipose tissue and testis (Garcia-Galiano et al., 2012; Ramanjaneya et al., 2010; Stengel and Taché, 2013). In the stomach, NUCB2/nesfatin-1 is synthesized in ghrelin-containing X/A-like cells of the oxyntic mucosa. Gastric NUCB2 is down-regulated by fasting and returned to basal levels with refeeding. NUCB2 mRNA levels in the gastric mucosa were tenfold higher than in the brain (Stengel et al., 2009). These observations indicate that gastric mucosa may be an important source of plasma NUCB2/nesfatin-1 and related this protein to ghrelin, the gastric hormone with widely reported effect on feeding behavior (Hansson et al., 2014). Another potential source of nesfatin-1 is adipose tissue. In humans, there are controversial data about the correlation between BMI and circulating NUCB2/nesfatin-1 concentrations (Ramanjaneya et al., 2010; Tsuchiya et al., 2010). NUCB2/nesfatin-1 protein expression in adipose tissue was increased in mice that were fed a high-fat diet and decreased under food deprivation. Furthermore, secretion of nesfatin-1 is increased during the differentiation of 3T3-L1 preadipocytes into adipocytes (Ramanjaneya et al., 2010).

It is known that neuroendocrine peptide levels, for example ghrelin levels, change for an organism to adapt to the metabolic requirements of different stages of maturation (Al-Massadi et al., 2010). It was shown that the NUCB2/nesfatin-1 levels vary in gastroenteropancreatic tissues and serum with age in males, suggesting a potential role in the developmental physiology of rats during growth (Mohan and Unniappan, 2012). Nesfatin-1 is indispensable for the onset of normal puberty in female rats (Garcia-Galiano et al., 2010). Furthermore, gonadotropic hormones regulate NUCB2/nesfatin-1 expression in the rat testis, and testicular testosterone secretion is regulated by nesfatin-1 (Garcia-Galiano et al., 2012). These results indicate a possible interaction between nesfatin-1 and sex hormones during puberty or adulthood. Another important metabolic event is the end of the breastfeeding period, when gut morphology suffers alterations due to the severe dietary change. Nesfatin-1 has been detected in human breast milk (Aydin, 2010) and might be involved in regulating energy homeostasis during the weaning stage.

The aims of this article are to determine (1) the gastric and adipose NUCB2/nesfatin-1 expression in different stages of development, (2) the contribution of the nesfatin-1 secretion of these tissues to plasma nesfatin-1 levels, and (3) the role of testosterone and breastfeeding in NUCB2/nesfatin-1 regulation.

2. Materials and methods

2.1. Ethics statement

The authors of this paper declare that all of the procedures carried out with animal models in this study were performed under 15005AE/10/FUN01/FIS02/LSC1 according to the institutional

guidelines and the European Union standards for the care and use of experimental animals (Real Decreto 1201/2005, October 10th, regarding the animals used for the protection of research animals). The procedures were approved by Conselleria de Medio Rural, Government of Galicia and the Animal Care Committee of Santiago de Compostela University (Santiago de Compostela, Spain). Animal experimentation was designed by LM Seoane, with diploma type C, which was expedited by Conselleria de Medio Rural, Government of Galicia, Spain.

2.2. Animal and experimental design

Male and female Sprague–Dawley rats were housed in air-conditioned rooms (22–24 °C) under a controlled light/dark cycle (12 hours light, 12 hours darkness) with free access to food and water.

The surgical procedures were performed under anesthesia from an ip⁴ injection of a mixture of ketamine–xylazine (ketamine 100 mg/kg body weight + xylazine 15 mg/kg body weight). The animals were euthanatized by decapitation at 2, 4, 6 and 8 weeks of age in the age-associated experiments, 4 weeks of age in the breastfeeding experiments and 8 weeks of age in the testosterone effect in vivo experiment. The trunk blood was collected in tubes containing EDTA-2Na (1 mg/ml) and aprotinin (500 U/ml) and immediately centrifuged. The plasma was stored at –80 °C. The stomach and white adipose tissue from subcutaneous location were surgically excised.

2.2.1. Age-associated variations in NUCB2/nesfatin-1 levels

To investigate whether the production of NUCB2/nesfatin-1 differs during development, rats of 2, 4, 6 and 8 weeks of age were used (n = 10). The NUCB2/nesfatin-1 plasma concentrations were tested using ELISA. The variations in the NUCB2 mRNA expression were measured in the rat gastric mucosa and subcutaneous adipose tissue by real-time PCR. Immunoblotting was performed to test the NUCB2 protein levels in the gastric mucosa, subcutaneous adipose tissue and hypothalamus.

The plasma testosterone levels were previously characterized (Al-Massadi et al., 2010).

2.2.2. Breastfeeding effect on NUCB2/nesfatin-1

For an assessment of the weaning effect on NUCB2/nesfatin-1 regulation, a model of DW⁵ was developed by preventing the pups from eating solid food from day 21 (3 weeks of age) until day 28 (4 weeks of age), leaving the pups with the mother for this period (n = 10). The corresponding controls (4 weeks) were previously weaned at 3 weeks of age (n = 10). The body weight and plasma testosterone of these pups were previously recorded (Al-Massadi et al., 2010). Plasma NUCB2/nesfatin-1 concentrations were tested by ELISA. The gastric content and subcutaneous adipose tissue content of the NUCB2 protein were measured by western blot; the NUCB2 mRNA expression was determined in the gastric mucosa and adipose tissue by real time-PCR.

2.2.3. Effects of in vivo testosterone on NUCB2/nesfatin1 production

To study the role of testosterone on NUCB2/nesfatin-1 regulation, adult animals (8 weeks old) were bilaterally gonadectomized or sham operated under ketamine–xylazine anesthesia. After 1 week, one group of gonadectomized animals was implanted subcutaneously with a Silastic tubing (i.d. 1.98 mm, o.d. 3.18 mm, and length 20 mm) cannula containing testosterone propionate (Flucka; Sigma

³ PVN: paraventricular nucleus.

⁴ ip: intraperitoneal.

⁵ DW: delayed weaning.

Aldrich; Steinheim, Germany) ($n = 8-10$). After 3 days, the rats were killed by euthanasia. In these animal models, the circulating NUCB2/nesfatin-1 levels and the variations in the NUCB2 mRNA and protein content both in the gastric mucosa and adipose subcutaneous tissue were measured by ELISA, real time PCR and western blot respectively.

2.3. Immunoblotting

Whole tissue proteins were prepared by homogenization using a Tissue Lyser II (Qiagen, Tokyo, Japan) as described (Senin et al., 2013). Western blots were performed using independent samples from different rats from each group. Fifteen micrograms of gastric tissue, 25 μ g of adipose tissue and 30 μ g of hypothalamus proteins were run on 10% SDS-PAGE gels and electroblotted onto nitrocellulose membranes. The membranes were probed successively with primary antibodies and peroxide-conjugated secondary antibodies (Thermo Scientific, Pierce, Rockford, IL, USA). Specific antigen-antibody binding was visualized using a chemiluminescence method according to the manufacturer's instructions (Pierce ECL Western Blotting Substrate, Thermo Scientific). Primary anti-nesfatin-1 (1-82) was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, USA) and anti-GAPDH from Ambion (Life Technologies, Paisley, UK). Equal loading was confirmed by GAPDH detection in all tissue western blots. The data are expressed as percentages normalized by the housekeeping protein level (arbitrary units) for tissue.

2.4. RNA isolation and real-time quantitative RT-PCR

Total RNA was isolated from the stomach mucosa and adipose tissue using TRIzol (Invitrogen, CA, USA) according to the manufacturer's recommendations (Seoane et al., 2007). Quantitative real-time PCR was performed using a StepOne Plus Instrument (Applied Biosystems) with specific Taqman qRT-PCR primers and probes. For analyses, the NUCB2 gene expression levels (Assay ID: Rn01510621_m1) were normalized to the mRNA expression levels of the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Assay ID: Rn01527840_m1, TaqMan: Applied Biosystems) and are expressed relative to the average value of the control group.

2.5. Biochemical analysis

NUCB2/Nesfatin-1 levels were determined via ELISA using reagents kits and methods provided by Phoenix Pharmaceuticals, Inc. (Burlingame, USA). The assay sensitivity limit was 0.1 ng/ml and the range is 0.1–1000 ng/ml. The results are expressed as ng/ml of NUCB2/nesfatin-1.

2.6. Statistical analysis

Statistical analyses were performed using the SPSS version 20.0 software statistical package (SPSS, Chicago, IL). When necessary, data were log transformed to achieve a satisfactory fit to the normal distribution or variance homogeneity. Data were analyzed using one-way ANOVA and then the Student–Newman–Keuls (SNK) post-hoc test for multiple comparisons (normally distributed data) or Kruskal–Wallis test followed by Mann–Whitney U test (non-normally distributed data). The relation between variables (testosterone correlation with NUCB2/nesfatin-1) was analyzed using Spearman's rank correlation. A P value <0.05 was considered significant. The statistical significance is indicated as follows: * $p < 0.05$ and ** $p < 0.01$. Data are presented as the mean \pm SEM.

3. Results

3.1. Age-associated variations in NUCB2/nesfatin-1 levels

The circulating NUCB2/nesfatin-1 levels dramatically increased with age in male rats (3.43 ± 0.3 ng/ml at 2 weeks vs 5.64 ± 0.42 ng/ml at 4 weeks, 11.46 ± 2.63 ng/ml at 6 weeks and 10.82 ± 2.13 ng/ml at 8 weeks; ** $p \leq 0.01$) (Fig. 1). In female rats, circulating NUCB2/nesfatin-1 levels were not modified by age (Supplementary Fig. S1). The NUCB2 protein content in gastric mucosa, as measured by western blot, increased at 4 weeks of age (100 ± 3.70 at 2 weeks, 128.27 ± 6.5 at 4 weeks vs 83.69 ± 3.73 at 6 weeks and $67.01 \pm 4.51\%$ at 8 weeks; ** $p \leq 0.01$ vs 2 weeks; *** $p \leq 0.01$ vs 4 weeks; # $p \leq 0.05$ vs 4 weeks) (Fig. 2A). Accordingly to previous data in the bibliography by using the same antibody, NUCB2 detection in gastric mucosa by western blot image showed a double band at 50 kDa (Goebel et al., 2011; Li et al., 2012; Riva et al., 2011; Stengel et al., 2009). Measurements of the gastric NUCB2/nesfatin-1 expression showed that the mRNA levels of NUCB2 were significantly higher just after the weaning, i.e., at 4 weeks of age (1.00 ± 0.05 at 2 weeks, 1.41 ± 0.10 at 4 weeks vs 1.29 ± 0.13 at 6 weeks and 1.14 ± 0.09 at 8 weeks arbitrary units; * $p \leq 0.05$ vs 2-weeks-old) (Fig. 2B).

Subcutaneous adipose tissue was also evaluated in the different groups for the NUCB2 mRNA expression and protein content. The NUCB2 content in the subcutaneous fat was markedly decreased at 6 weeks of age (100 ± 1.82 at 2 weeks, 74.24 ± 12.96 4 weeks vs 46.15 ± 12.96 at 6 weeks and $40.75 \pm 13.61\%$ at 8 weeks; ** $p \leq 0.01$) (Fig. 3A). Different from that found in gastric mucosa, the NUCB2 detection by western blot in adipose tissue presented one only band at 50 kDa as previously described in the bibliography for this tissue with the same antibody (Osaki et al., 2012; Ramanjaneya et al., 2010; Tagaya et al., 2012). Conversely to protein expression the NUCB2 mRNA expression peak was at 6 weeks of age (2 weeks old: 1.01 ± 0.06 , 4 weeks old: 1.31 ± 0.17 , 6 weeks old: 1.50 ± 0.14 and 8 weeks old: 0.98 ± 0.06 arbitrary units; ** $p < 0.01$) (Fig. 3B).

The western blot study of the NUCB2 content in the hypothalamus showed no differences in expression with age (Supplementary Fig. S2).

3.2. Breastfeeding effect on NUCB2/nesfatin-1 secretion

Circulating NUCB2/nesfatin-1 levels were unchanged by delayed weaning (Fig. 4A). Accordingly, delayed weaning did not show changes in the NUCB2 content in the gastric mucosa (Fig. 4B) or mRNA levels (Fig. 4C).

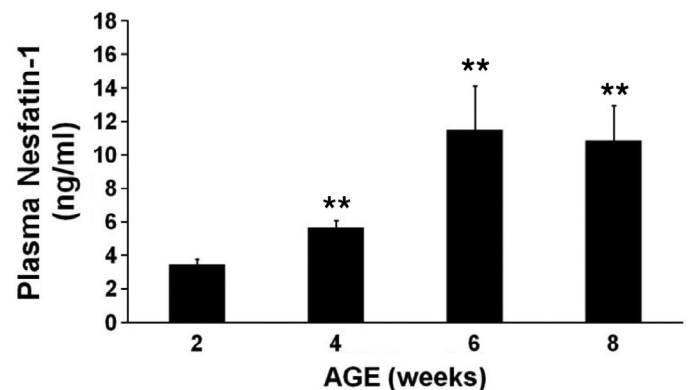


Fig. 1. Plasmatic NUCB2/nesfatin-1 concentration in rats of different ages (2–8 weeks). Values are expressed as mean \pm SEM. Error bars indicate SEM. $n = 10$; ** $p \leq 0.01$ vs 2-week-old.

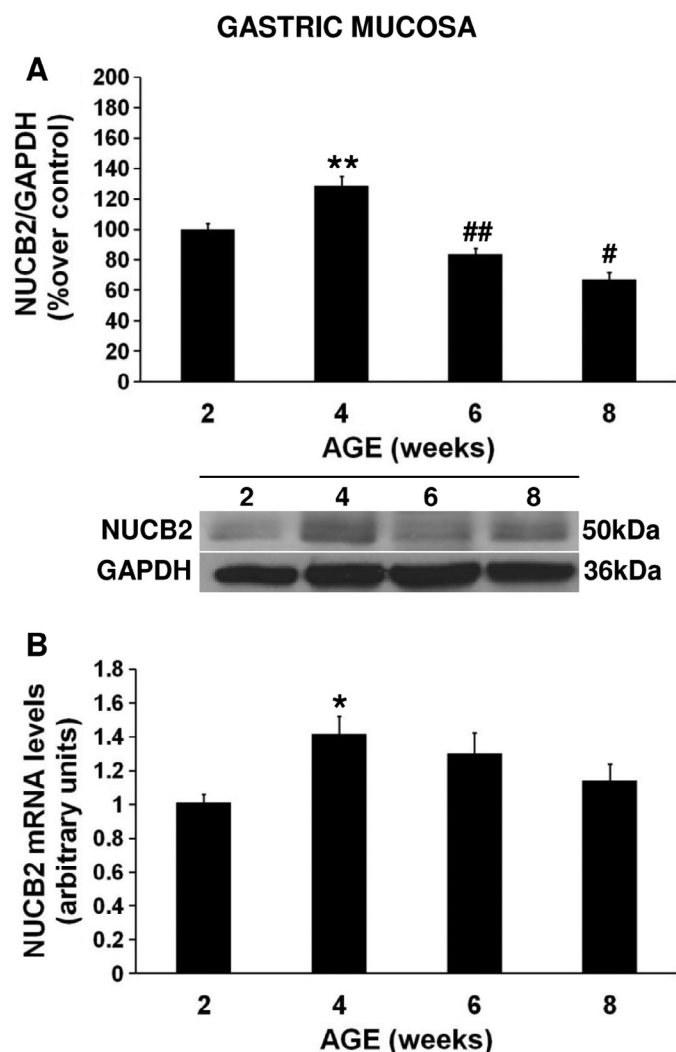


Fig. 2. (A) NUCB2/GAPDH protein content in gastric mucosa from males of different ages (2–8 weeks) and representative western blot. ** $p \leq 0.01$ vs 2-week-old; # $p \leq 0.05$, ## $p \leq 0.01$ vs 4-week-old. $n = 10$. (B) NUCB2 mRNA expression in gastric mucosa measured by quantitative real-time RT-PCR and standardized by HPRT1 mRNA levels in animals of different ages (2–8 weeks), * $p \leq 0.05$ vs 2-week-old. $n = 6–10$. Values are presented as mean \pm SEM. Error bars indicate SEM.

In addition, there were no differences in the subcutaneous adipose tissue NUCB2 protein content or NUCB2 mRNA levels (Fig. 4D and E).

3.3. Effects of testosterone on NUCB2/nesfatin-1 production

The plasma NUCB2/nesfatin-1 levels increased in the gonadectomized animals treated exogenously with testosterone (control (C): 6.67 ± 0.67 ng/ml, gonadectomy (GNX): 7.05 ± 0.60 ng/ml and gonadectomy+testosterone (GNX+T): 11.24 ± 0.79 ng/ml; $p < 0.01$) (Fig. 5A).

Immunoblotting studies showed that testosterone treatment to gonadectomized rats decreased gastric NUCB2 protein content (C: 100 ± 2.01 , GNX: 99 ± 10.08 and GNX+T: $70 \pm 6.47\%$; $p < 0.01$) (Fig. 5B). Real-time PCR measurements of the mRNA levels of NUCB2 in the gastric mucosa showed that gonadectomy decreased gastric NUCB2 mRNA levels, remaining lower after testosterone treatment (C: 1.04 ± 0.11 , GNX: 0.73 ± 0.08 and GNX+T: 0.65 ± 0.05 arbitrary units; $p < 0.05$) (Fig. 5C).

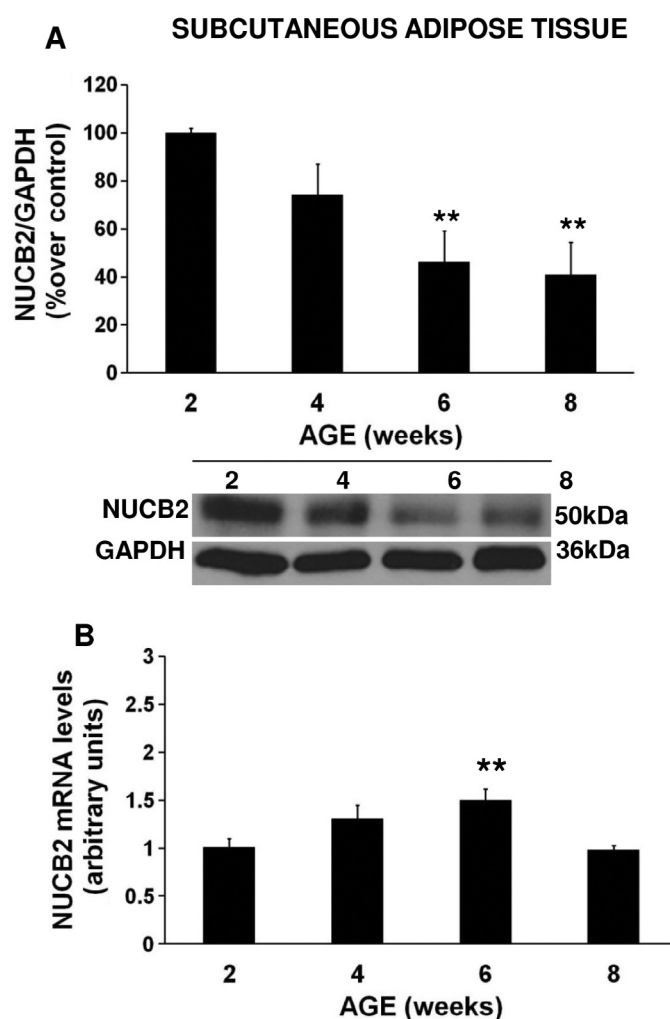


Fig. 3. (A) NUCB2/GAPDH protein content in subcutaneous adipose tissue from males of different ages (2–8 weeks) and representative western blot. ** $p \leq 0.01$. $n = 6–10$. (B) NUCB2 mRNA expression in subcutaneous adipose tissue measured by quantitative real-time RT-PCR and standardized by HPRT1 mRNA levels in males of different ages (2–8 weeks). $n = 6–10$, ** $p \leq 0.01$. Values are presented as mean \pm SEM. Error bars indicate SEM.

The drop in testosterone levels induced by surgical gonadectomy provoked a decrease in the NUCB2 protein storage in the subcutaneous adipose tissue, which was reverted after the testosterone replacement (C: 100 ± 2.01 , GNX: 78 ± 10.9 ; GNX+T: $103 \pm 18.5\%$; $p < 0.05$) (Fig. 5D). The NUCB2 mRNA levels in the subcutaneous adipose tissue also decreased in males after gonadectomy and were restored to control levels after testosterone replacement (C: 1.033 ± 0.09 , GNX: 0.75 ± 0.05 , GNX+T: 1.00 ± 0.13 arbitrary units; not significant) (Fig. 5E).

In addition, it was found that circulating nesfatin-1 levels were correlated positively with plasma testosterone concentration at the different ages ($r = 0.674$, $p = 0.000$ obtained by Spearman's rank correlation) (Supplementary Fig. S3).

4. Discussion

The present study is focused on the regulation of circulating NUCB2/nesfatin-1 levels during postnatal life and its production in peripheral tissues. The data in the present paper showed that age, testosterone and dietary modifications regulate the production of NUCB2/nesfatin-1 from both the stomach and subcutaneous adipose

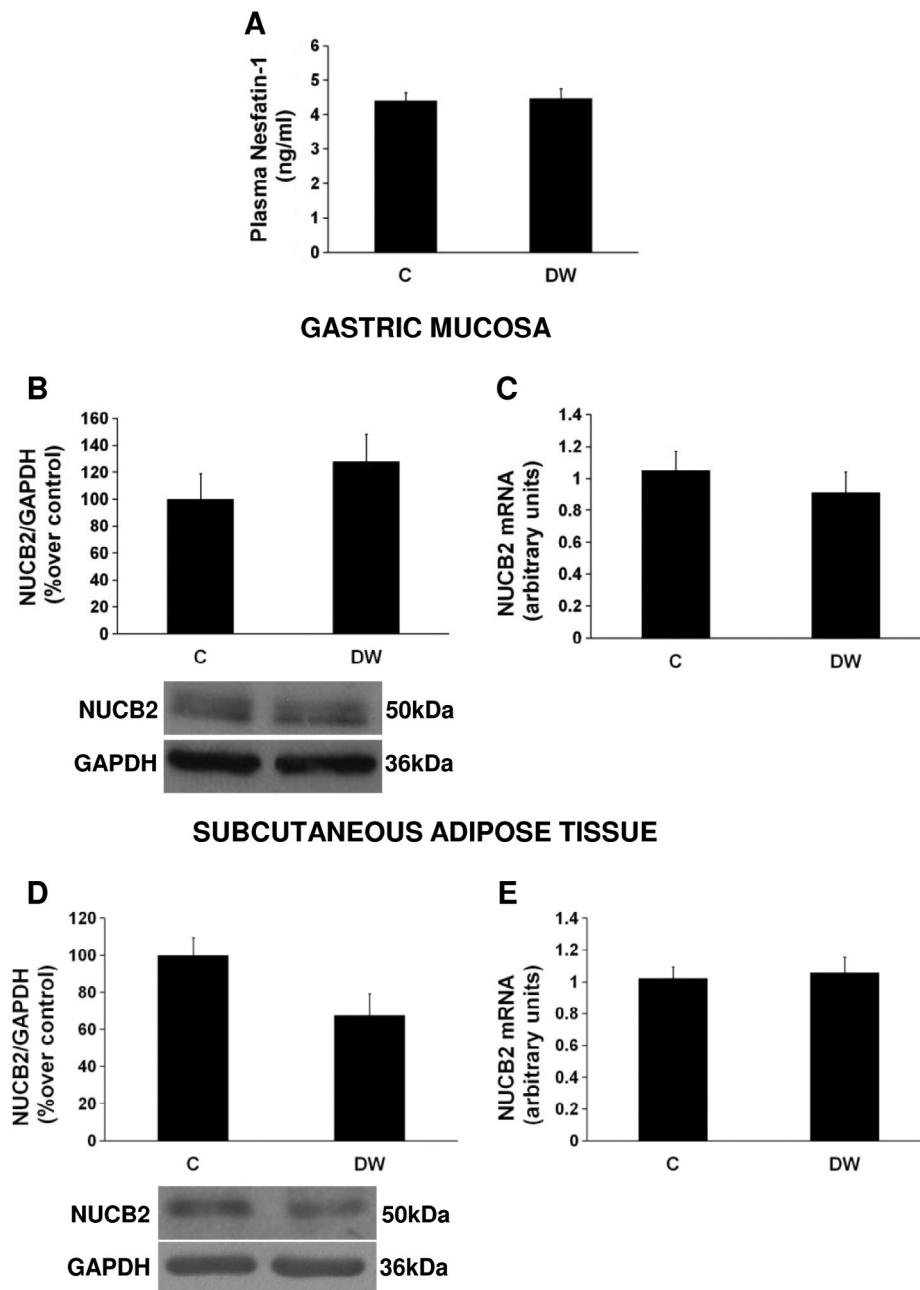


Fig. 4. (A) Plasma NUCB2/nesfatin-1 levels in 4-week-old male rats. (B) NUCB2/GAPDH content and representative western blot in gastric mucosa and (D) subcutaneous adipose tissue. (C) NUCB2 mRNA expression measured by quantitative real-time RT-PCR and standardized by HPRT1 mRNA levels in gastric mucosa and (E) subcutaneous adipose tissue. C: control; DW: delayed weaning. Values are presented as the mean \pm SEM. Error bars indicate SEM. $n = 10$.

tissue. The most relevant findings in this paper were the following. First, NUCB2/nesfatin-1 circulating levels increase with age in males from the postnatal period to adulthood. Second, the hormonal modifications associated with puberty, i.e., variations in testosterone levels, regulate the production of NUCB2/nesfatin-1 by gastric and/or subcutaneous adipose tissue. Third, weaning-associated dietary modifications affect NUCB2/nesfatin-1 production in the stomach. All together suggest those tissues, stomach and adipose tissue, might contribute to modulate circulating NUCB2/nesfatin-1 levels.

Measurements of the circulating NUCB2/nesfatin-1 levels in different stages of development showed that circulating NUCB2/nesfatin-1 levels increased at 4 weeks of age with a peak in the

concentration at 6 weeks of age and continued stay elevated at 8 weeks (Fig. 1).

A previous study by Mohan et al. in 2012 suggests that NUCB2/nesfatin-1 has a potential age- and tissue-specific role in the developmental physiology of growing rats (Mohan and Unniappan, 2012). However, until now, there was no any study that analyzed the NUCB2/nesfatin-1 production from subcutaneous adipose and gastric tissues throughout development. The present data suggest that two periods of maturation are relevant for the regulation of nesfatin-1 levels. The first stage occurs at 4 weeks of age, when the circulating levels of nesfatin-1 increased significantly (Fig. 1), immediately after the weaning, which is the transition from breastfeeding to solid intake and is associated with severe

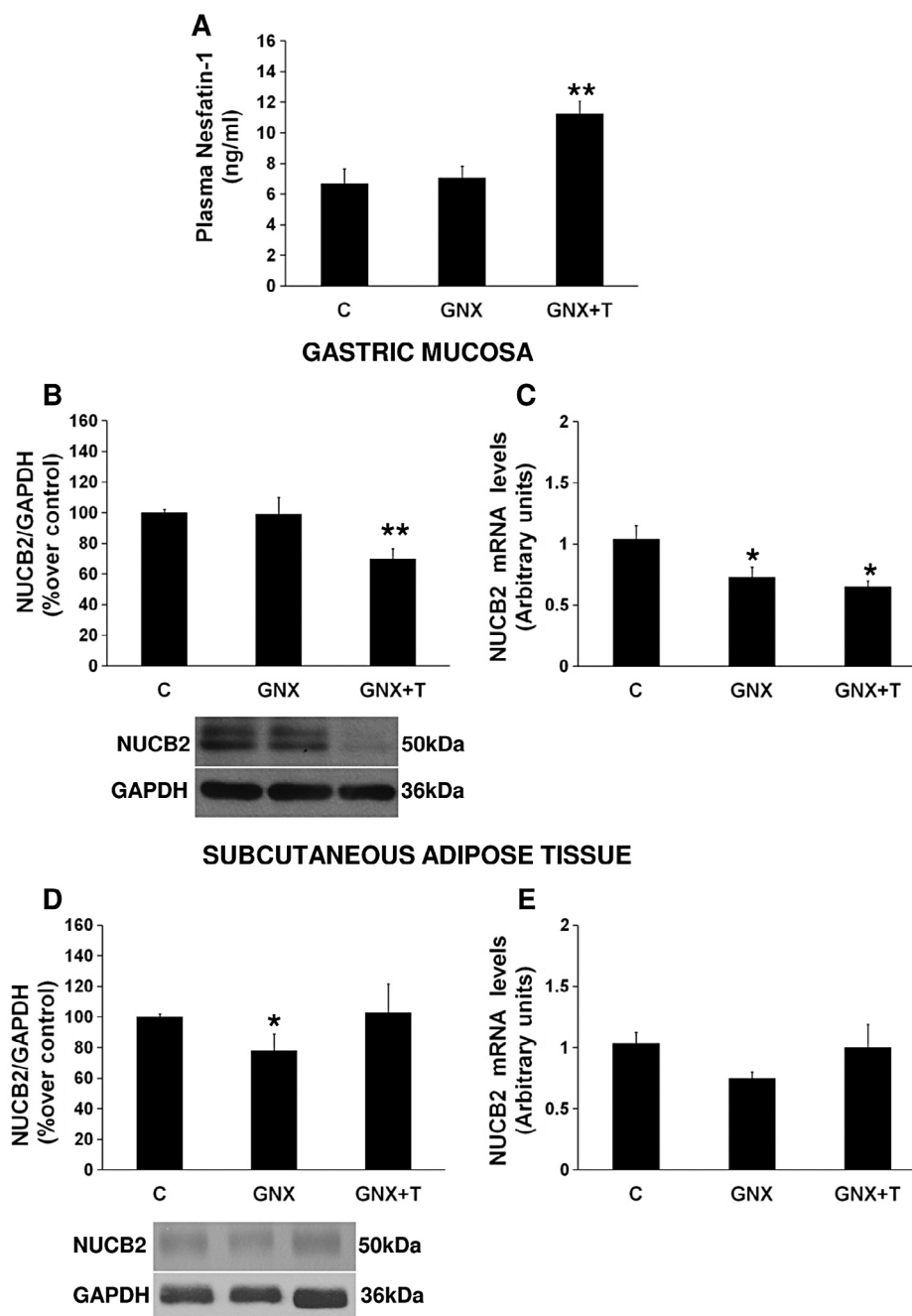


Fig. 5. (A) Plasma NUCB2/nesfatin-1 levels from male rats. (B) NUCB2/GAPDH content and representative western blot of the gastric mucosa and (D) subcutaneous adipose tissue from male rats under different conditions (control: 8-week-old rats; GNX: 8-week-old rats subjected to surgical gonadectomy; GNX+T: 8-week-old rats treated with testosterone propionate and previously subjected to surgical gonadectomy). (C) NUCB2 mRNA expression measured by quantitative real-time RT-PCR and standardized by HPRT1 mRNA levels in gastric mucosa and (E) subcutaneous adipose tissue. * $p < 0.05$, ** $p \leq 0.01$, $n = 6-10$. Values are expressed as mean \pm SEM. Error bars indicate SEM.

modifications in stomach morphology and secretions, as previously shown for ghrelin (Al-Massadi et al., 2010). It was shown that the NUCB2/nesfatin-1 content in gastric mucosa increases just after weaning at 4 weeks of age (Fig. 2A); this increase was furthered by the elevated NUCB2 mRNA expression found in gastric mucosa (Fig. 2B). At this point it should be mentioned that in the western blot studies we did not detect the mature nesfatin-1 peptide in gastric mucosa or adipose tissue, accordingly to previously published data (Garcia-Galiano et al., 2010; Stengel et al., 2009) which might be indicating the existence of postsecretory cleavage involved in the regulation of NUCB2. Taking into account that the used nesfatin

antibodies present cross reactivity with NUCB2, throughout the paper we refer to it as NUCB2/nesfatin-1.

Considering that the stomach is an important source of nesfatin-1, with the expression of this peptide is 10-fold higher in this organ than in the brain (Stengel et al., 2009), these results suggest for the first time the involvement of the stomach in the fluctuations of circulating NUCB2/nesfatin-1 levels found at 4 weeks of age (Fig. 1) coinciding with the weaning.

These data fit well with the previous data showing an age-associated variation in the distribution of cells for NUCB2/nesfatin-1 in the stomach during the perinatal period, possibly related to the

weaning period when the diet changed from milk to rat chow (Mohan and Unniappan, 2012). Thus, the change from breastfeeding to solid intake only affects NUCB2/nesfatin-1 production in the stomach but not in adipose tissue. It was previously proved that hypothalamic NUCB2 mRNA and protein are regulated by nutritional status. Furthermore, data exist about a decrease in hypothalamic NUCB2 in food deprived animals, and a restoration of these levels after re-feeding (Kohn et al., 2008; Oh et al., 2006). In addition, different stressors, hormones and nutrients have shown to regulate hypothalamic NUCB2 expression (Stengel et al., 2013). Interestingly, some gut peptides affecting food intake regulate NUCB2/nesfatin-1 hypothalamic production as cholecystokinin (Noetzel et al., 2009) and ghrelin (Inhoff et al., 2008). Thus it is possible that the changes in NUCB2/nesfatin-1 in gastric mucosa and adipose tissue with age might be mediated by nutritional status effects on gastrointestinal derived-hormones that regulate NUCB2/nesfatin-1 production.

However, in the delay weaning model, plasmatic NUCB2/nesfatin-1 production was not affected, probably due to the fact that this animals showed lower levels of testosterone than their age-matched controls (Al-Massadi et al., 2010), indicating a prominent role of testosterone on NUCB2/nesfatin-1 regulation. Supporting this, the present data showed a peak in the circulating nesfatin-1 levels at 6 weeks of age (Fig. 1), which coincide in males with the increase in testosterone levels (Al-Massadi et al., 2010) characteristic of puberty. In 2010, Ramanjaneya et al. reported for the first time that nesfatin-1 is produced by subcutaneous adipose tissue in a higher degree than in other adipose depots and regulated by the metabolic status (Ramanjaneya et al., 2010) which led us to consider the possibility of a role for this tissue in the regulation of circulating NUCB2/nesfatin-1 levels with age. Accordingly, the present study showed the rate of NUCB2/nesfatin-1 production in the subcutaneous adipose tissue that was also increased at 6 weeks of age coinciding with the initio of puberty (Fig. 3A and B). This modulation possibly occurs to meet the demands of the organism during this specific period of maturation mediated by the variations in the circulating levels of testosterone, which may act in the stomach/adipose tissue to regulate NUCB2/nesfatin-1 production. To explore this possibility, the testosterone variations in puberty were reproduced in the laboratory by the chronic administration of testosterone to adult animals that were previously subjected to surgical gonadectomy. The testosterone treatment increased the plasmatic NUCB2/nesfatin-1 levels (Fig. 5A). Moreover, the lack of testosterone induced 1 week after surgical gonadectomy was reflected by a decrease in the amount of NUCB2 protein content and mRNA levels in gastric mucosa (Fig. 5B and C), although replacement with testosterone for 3 days were not enough to revert this drop in gastric NUCB2/nesfatin-1 production. Accordingly, the blockade of the testosterone by surgical gonadectomy decreased the rate of NUCB2/nesfatin-1 production also in the subcutaneous adipose tissue and this effect was reverted after testosterone treatment (Fig. 5D and E). Altogether, the data showed that testosterone not only affects gastric nesfatin-1 secretion but also has a role in adipose tissue NUCB2/nesfatin-1 production in males.

It is widely known that body fat influences the onset and progression of puberty (Bohler et al., 2010), and the present data suggest that NUCB2/nesfatin-1 production in adipose tissue might be involved in the onset of puberty and sex maturation, as was previously shown for other adipokines, such as leptin (Carro et al., 1997). Accordingly, in rat, mice and human males, NUCB2/nesfatin-1 expression in testes, along with the protein content, gradually increases during the transition from puberty to adult age (Garcia-Galiano et al., 2012). Together, the data indicate that NUCB2/nesfatin-1 production in peripheral organs might be designed to meet the demands of the organism during puberty and thus links metabolism to reproductive function. Moreover, these findings explain the previously

mentioned lack of effect of delayed weaning on gastric and adipose NUCB2/nesfatin-1 production in males because delayed weaning animals exhibit lower testosterone levels and this factor might mask the effect of the breastfeeding.

The data from the present work are consistent with previous data from Li et al, which showed that the plasma nesfatin-1/adipose tissue ratio is severely decreased in old mice (Li et al., 2014) since at the light of our findings, the decreased production of nesfatin-1 by adipose tissue suggested by Li et al. in elderly might be related to the decrease in sex steroids during old age. This fact is tremendously interesting because a beneficial effect on glucose metabolism is attributed to nesfatin-1 (Feijoo-Bandin et al., 2013; Su et al., 2010) and the impairment in glucose metabolism and cardiovascular-associated diseases that are characteristic of old age might be partly due to the decreased NUCB2/nesfatin-1 production by adipose tissue. Moreover, several lines of evidence show that NUCB2/nesfatin-1 levels are negatively correlated with type II diabetes (Foo et al., 2008; Li et al., 2010; Zhang et al., 2012).

In summary, the sex hormone testosterone is the most potent regulator of circulating NUCB2/nesfatin-1 levels; testosterone stimulates NUCB2/nesfatin-1 production at both the gastric and adipose levels. However, weaning, a physiological process that is connected to morphological changes in the stomach affects only gastric NUCB2/nesfatin-1 secretion.

Futures studies should be performed to determine whether a nesfatin-1 deficiency with age characterized by a drop in sex hormones levels might be responsible for the impairment in glucose metabolism and cardiovascular-associated pathologies, the incidence of which increases with age.

5. Short conclusion

The stomach and the adipose tissue participate in the regulation of circulating NUCB2/nesfatin-1 levels to allow an organism to adapt to the different requirements in each stage of development.

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Appendix: Supplementary material

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