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# Prolactin and the dietary protein/carbohydrate ratio regulate the expression of SNAT2 amino acid transporter in the mammary gland during lactation

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ABSTRACT

The sodium coupled neutral amino acid transporter 2 (SNAT2/SAT2/ATA2) is expressed in the mammary gland (MG) and plays an important role in the uptake of alanine and glutamine which are the most abundant amino acids transported into this tissue during lactation. Thus, the aim of this study was to assess the amount and localization of SNAT2 before delivery and during lactation in rat MG, and to evaluate whether prolactin and the dietary protein/carbohydrate ratio might influence SNAT2 expression in the MG, liver and adipose tissue during lactation. Our results showed that SNAT2 protein abundance in the MG increased during lactation and this increase was maintained along this period, while 24 h after weaning it tended to decrease. To study the effect of prolactin on SNAT2 expression, we incubated MG explants or T47D cells transfected with the SNAT2 promoter with prolactin, and we observed in both studies an increase in the SNAT2 expression or promoter activity. Consumption of a high-protein/low carbohydrate diet increased prolactin concentration, with a concomitant increase in SNAT2 expression not only in the MG during lactation, but also in the liver and adipose tissue. There was a correlation between SNAT2 expression and serum prolactin levels depending on the amount of dietary protein/carbohydrate ratio consumed. These findings suggest that prolactin actively supports lactation providing amino acids to the gland through SNAT2 for the synthesis of milk proteins.



## 1. Introduction

Lactation is a physiological process necessary to provide all nutrients to the newborns. This process is fundamental for all mammals, and it requires large amounts of building block substrates for the synthesis of proteins, triglycerides and lactose among others for the milk synthesis. Thus, circulating amino acids from the mother are actively taken up for protein synthesis. It has been evidenced that during the lactation period the most abundant amino acids transported into the mammary gland are alanine and glutamine [1] and are particularly important in regulating the amino acid intracellular pool. Specifically, glutamine is an efflux substrate for other amino acid transporters like the amino acid heteroexchanger system L (LAT1), facilitating the uptake of branched-chain amino acids, particularly leucine that activates the TOR pathway involved in protein synthesis [2,3].

These amino acids, alanine and glutamine, as well as other small neutral amino acids are mainly transported across membranes through System A which is comprised by three subtypes known as SNATs (sodium-coupled neutral amino acid transporters) 1, 2 and 4 [4]. However, SNAT2, which represents the classical characteristics of System A, is widely expressed in mammalian cells and is $Na^+$-dependent [5–7]. Additionally, SNAT2 is regulated by environmental conditions, proliferative stimuli, developmental changes, and hormonal signals [8].

Several studies showed that the mammary glands possess characteristics of System A activity [9,10], determined by using the non-metabolizable analog MeAIB. In addition, SNAT2 in the mammary gland shows a unique characteristic of this transport system observed in other cell types called adaptive regulation [11].

*Abbreviations:* BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CRE, cAMP response element; DAB, diaminobenzidine; ECL, enhanced chemiluminescence; HPRT1, hypoxanthine phosphoribosyl transferase 1; HPR, horseradish peroxidase; JaK2, janus kinase 2; MAPK, mitogen-activated protein kinase; MeAIB, methylaminoisobutyric acid; MG, mammary gland; MMLV, Moloney murine leukemia virus; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PVDF, polyvinylidene difluoride; RIA, radioimmunoassay; RIPA, radio-immunoprecipitation assay; RT-PCR, real time polymerase chain reaction; SNAT2, sodium-coupled neutral amino acid transporter 2; STAT5, signal transducer and activator of transcription 5; TBS, Tris-buffered saline

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Several hormones play an important role in the regulation of SNAT2 gene expression in the mammary gland. There is evidence during gestation of an increase in the SNAT2 mRNA abundance in this tissue that is associated with concomitant changes in serum estradiol levels [12]. Recently, it has been demonstrated that upregulation of SNAT2 gene expression during gestation is mediated by the estrogen receptor α bound to the coactivator glyceraldehyde 3-phosphate dehydrogenase that specifically binds to an estrogen response element found in the SNAT2 gene promoter [13]. Interestingly, we have evidence that after gestation, there is a second increase of SNAT2 mRNA that reaches its maximal in the peak of lactation in the mammary gland [12]. There is preliminary evidence that suggests that prolactin could be responsible for the maintenance and regulation of this amino acid transporter during lactation, however this has not been established.

Prolactin is a single polypeptide with a molecular weight of 23 kDa that is produced in the pituitary gland [14]. This hormone is secreted with a pulsatile pattern during this physiological stage [15]. Prolactin has two receptor isoforms, a long- and a short-isoform [16]. Interestingly, the mammary gland during lactation mainly expresses the long-isoform which activates several signaling pathways, particularly those involving JAK2/STAT5, PI3K/PKB and MAPK [14]. It is known that prolactin is involved in the activation of the expression of several genes that include the β-casein gene to synthesize one of the most abundant proteins in milk *via* the STAT5 transcription factor [17]. Therefore, in order to sustain an elevated rate of milk protein synthesis, the supply of large amounts of amino acids is necessary. There is evidence that the expression of several amino acid transporters is up-regulated during lactation, including SNAT2 [18–20].

Interestingly, It has been demonstrated that incubation of mammary gland explants with prolactin increases the uptake of amino acids transported *via* System A [21]. However, there are no studies that have established whether prolactin in fact increases the expression of SNAT2 in the mammary gland.

Moreover, it has been demonstrated that the circulating levels of prolactin are dependent on the amount of dietary protein consumed. The lower the amount of protein, the lower the concentration of serum prolactin [22]. As a result there is a decrease in milk production that is reflected in the growth pattern of the pups. It is important to study whether the changes in the dietary protein/carbohydrate ratio may affect the expression of SNAT2 not only in the mammary gland but also in other tissues such as the adipose tissue and liver. It has been demonstrated that the prolactin receptor is present in these tissues among others [23–27]. However, there is no knowledge whether during lactation the SNAT2 gene is regulated in a similar fashion in these tissues compared with the mammary gland.

Therefore, the aim of the present work was to demonstrate if there are changes in SNAT2 protein abundance before delivery and during lactation, to study if prolactin was able to increase the expression and promoter activity of SNAT2 in mammary gland explants and T47D cells and finally, to establish whether the dietary protein/carbohydrate ratio may influence serum prolactin levels and SNAT2 gene expression in the mammary gland, liver and adipose tissue.

## 2. Materials and methods

The experimental design and procedures of this study were reviewed and approved by the Animal Care Committee of the Instituto Nacional de Ciencias Médicas y Nutrición, México, in accordance with the international guidelines for the use of animals in research.

### 2.1. Animals

This study was divided into two independent studies in order to achieve the objectives described.

#### 2.1.1. Study 1

This study was designed to determine the change in SNAT2 gene expression during late gestation and lactation. Female Wistar rats weighing 200–250 g were obtained from the animal research facility at the Instituto Nacional de Ciencias Médicas y Nutrición. The animals were housed in individual stainless steel cages at 21 °C with a 12:12 h light–dark cycle. The animals were allowed free access to water and chow diet. Gestational age was determined by vaginal smear to detect spermatozoa. Mammary gland explants were obtained as previously reported [11] from pregnant rats at day 20, lactating rats at days 5, 12, and 18, or weaning (rats 21 days postpartum separated from their pups for 24 h). After normal pregnancy and delivery, the litter size was adjusted to 8 pups/dam. Five rats per group were used.

#### 2.1.2. Study 2

This study was designed to determine if dietary protein modifies SNAT2 protein abundance during late gestation and lactation. The rat strain, the maintenance thereof, and the gestational age determination were the same as study 1. The animals were allowed free access to water and to a low-protein/high-carbohydrate (10/73%), normal-protein/normal-carbohydrate (20/63%) or high-protein/low-carbohydrate (30/53%) diets, according to the American Institute of Nutrition (AIN93) lab rodent diet recommendations [28]. The adipose tissue, liver and mammary gland were obtained from pregnant rats on day 20, or rats that had been lactating for 5 and 12 days. After delivery, the litter size was adjusted to 8 pups/dam. Five rats per group were used. The food intake of the dams was recorded daily, and the dams and pups were weighed every other day.

### 2.2. Quantitative real-time PCR

Total RNA was extracted from the mammary gland by the guanidinium thiocyanate–cesium chloride method as previously described [12]. The RNA concentration was measured using a NanoDrop spectrophotometer 1000 (ND-1000; Thermo Scientific, Wilmington, De, USA). RNA integrity was corroborated by visualizing the 28S and 18S ribosomal subunits in a 1% (w/v) agarose gel and quality was assessed with the 260/280 nm ratio absorbance of 2.0. RNA (3 μg) was reverse-transcribed to cDNA by the use of Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). For the real-time PCR analyses, 300 ng of cDNA was used in a final reaction volume of 10 μl per reaction. Predesigned TaqMan assay (Applied Biosystems, Foster City, CA, USA) probes for sodium-coupled neutral amino acid transporter SNAT2 (Rn00710421_m1) and HPRT1 (hypoxanthine phosphoribosyl transferase 1) (Rn01527840_m1) were used. RT-PCR was performed using the following PCR amplification conditions: denaturation for 5 min at 95 °C, annealing for 1 min at 56.2 °C, extension for 1.30 min at 72 °C for 34 cycles; and final extension for 7 min at 72 °C. The amplification and detection of specific products was performed with the ABI PRISM 7000 (Applied Biosystems). The mRNA level of the SNAT2 was normalized to the HPRT1 gene. HPRT1 was used as a housekeeping gene since cyclophilin or β-actin showed great variation among samples. The relative amount of mRNA was calculated using the comparative CT method (User Bulletin no. 2; PE Applied Biosystems).

### 2.3. Western blot

Proteins were extracted from the mammary gland, liver and adipose tissue using RIPA (radio-immunoprecipitation assay) lysis buffer containing the following: 50 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, 1% NP40, 0.25% Na-deoxycholate, and 1 mmol/l PMSF. We added a 1× Roche mini complete protease mixture. The protein concentration was measured in duplicate using the Lowry method. Before being loaded, the samples were prepared by mixing 40 μg of protein with Laemmli buffer in a 1:1 ratio and heated at 80 °C for 5 min.

The proteins were separated by electrophoresis on a polyacrylamide gel (8% v/v), and transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham GE Healthcare). For gel electrophoresis and semi-dry transfer, we used a Tris/glycine buffer. After transfer, the membranes were blocked with 5% (w/v) non-fat milk blocking solution with 1 × Tris-buffered saline (TBS) and 0.1% Tween 20 for 1 h. The membranes were incubated overnight at 4 °C with the different primary antibodies as follows: SNAT2 (Santa Cruz Biotechnology, sc-166366, 1:800), and β-actin (Santa Cruz Biotechnology, sc-1615, 1:1000), which were diluted in TBS, 5% (w/v) non-fat milk and 0.1% Tween 20. We used different secondary HRP (horseradish peroxidase) conjugated antibodies as follows: anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005, 1:3500) and anti-goat IgG-HRP (Santa Cruz Biotechnology, sc-2768, 1:3500) diluted in TBS, 5% (w/v) non-fat milk and 0.1% Tween 20. The chemiluminescence produced was measured using the Amersham Enhanced Chemiluminescence (ECL) detection reagents by exposure to X-ray film.

### 2.4. Immunohistochemistry

For the immunohistochemistry examination, rat mammary gland was obtained from pregnant rats at day 20, or lactating rats at 5 and 12 days. Before staining, tissue sections were immersed in 10% (v/v) hydrogen peroxide ($H_2O_2$)/methanol for 15 min, washed in two changes of distilled water (2 × 5 min) and deparaffinized through xylene and different percentages of ethanol (100, 95, and 70% twice). Sections were rinsed in phosphate-buffered saline (PBS), pH 7.4, and blocked for 30 min in 3% (v/v) bovine serum albumin (BSA) in PBS. The primary antibody used was mouse polyclonal affinity-purified IgG anti-SNAT2 or 0.1 MPBS instead of primary antibodies for negative controls. Primary antibodies were incubated overnight for 18 h at 4 °C. Conventional immunohistochemistry was performed using a biotinylated goat anti-mouse secondary antibody. After extensive wash using PBS, the color reaction was developed with diaminobenzidine (DAB; Sigma-Aldrich) in PBS and 30% (v/v) $H_2O_2$ for about 10 min. Slides were washed with deionized water, air-dried, and counterstained.

### 2.5. Preparation of mammary tissue explants

Mammary tissue explants were prepared as described previously [11]. Briefly, rats were anesthetized and the mammary gland was immediately removed and placed at 37 °C in 30 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, equilibrated with 95% $O_2$–5% $CO_2$ and contained the following (in mmol/l): 141 NaCl, 5.6 KCl, 3.0 $CaCl_2$, 1.4 $KH_2PO_4$, 1.4 $MgSO_4$, 24.6 $NaHCO_3$, and 11 glucose. After the removal of the connective tissue, the mammary tissue was diced into 2- to 5-mg explants. The tissue explants were rinsed repeatedly with buffer at 37 °C prior to assay. The explants were incubated in the presence of 0, 25, 250 or 2500 ng/ml of prolactin for 1 h. Subsequently, the mammary tissue was used to determine the SNAT2 gene expression by quantitative real-time PCR and western blot techniques as mentioned above.

### 2.6. Transfection and reporter gene assays

Human T47D mammary gland ductal carcinoma cells that express the prolactin receptor were grown in RPMI (Roswell Park Memorial Institute) medium supplemented with 5% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% $CO_2$ incubator. The p-SNAT2 promoter (−1872 to +265 bp) construct was inserted in the pGL3 basic vector (Promega, Fitchburg, WI, USA), which contained a luciferase reporter gene. T47D cells (150,000 cells/well) were plated in 12-well plates in RPMI medium, supplemented with 5% fetal bovine serum (FBS) 24 h before transfection. Transient transfection on Human T47D cells was performed with 0.25 μg of the p-SNAT2 luciferase reporter gene construct and 0.01 μg of pRL-TK Renilla expression vector (Promega, Fitchburg, WI, USA), used as an internal control to normalize the transfection efficiency, using the PolyFect (Qiagen) reagent according to the manufacturer's instructions. After transfection, the cells were incubated with different concentrations of prolactin (2, 20, 200, 2000 or 20,000 ng/ml) or without prolactin referred as control. Luciferase activity was measured as relative light units with a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). Each transfection was performed in triplicate, and three independent experiments were performed for each construct.

### 2.7. Serum prolactin quantification

Prolactin determinations were done in duplicate by specific radioimmunoassay (RIA) using reagents and protocols provided by the National Hormone and Peptide Program (Torrance, CA 90509, USA). The results are expressed in terms of the international reference standard NIDDK-rPRL-RP-3. The coefficients of variation intra- and interassay were 8.7 and 14.1, respectively. All samples were analyzed together to eliminate interassay variability.

### 2.8. Statistical analysis

The results are reported as the means ± SEM and gene expression data were tested using a 1-way and 2-way ANOVA, and significant differences among groups were analyzed by Bonferroni adjustments. The linear regression method was used to analyze correlations. Differences were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Protein expression of SNAT2 in the mammary gland during gestation and lactation

The western blot analysis revealed that SNAT2 protein abundance in the mammary gland increased during lactation. From delivery until day 5 of lactation, there was an increase of about 11.7-fold, and this was maintained until the peak of lactation (days 12 and 18). After 24-h weaning SNAT2 protein tended to decrease however it did not reach statistical significance (Fig. 1A, B). To determine the cellular localization of SNAT2, immunohistochemistry was performed in the mammary gland from pregnant, lactating and weaning rats. The mammary gland from rats after delivery showed a poor SNAT2 immunostaining (Fig. 1C). In contrast, at days 5 and 12 of lactation (Fig. 1D, E respectively) a strong SNAT2 immunostaining was seen in alveolar cells, while during weaning a decrease of immunoreactivity was observed (Fig. 1F). These results well correlated with the expression of SNAT2 protein studied with western blot (Fig. 1A, B).

### 3.2. Effect of prolactin on the expression of SNAT2

Prolactin stimulated SNAT2 mRNA and protein abundance in mammary gland explants. Incubation of explants with 25 ng/ml for 6 h significantly stimulated SNAT2 concentration by approximately 24%, and with 250 ng/ml the increment in the protein abundance was about 49%. Further increase in the concentration of prolactin did not have an additional stimulation in the SNAT2 protein concentration (Fig. 2A).

We then performed a time course analysis to assess the effect of prolactin on the expression of SNAT2 in mammary gland explants. For this study we added 250 ng/ml of prolactin to the explants. Our data clearly showed that prolactin was able to significantly stimulate SNAT2 expression in mammary gland explants, and this response initiated at about 6 h of incubation reaching the maximal response after 8 h of incubation by approximately 4.5-fold. Then, after 10 h of incubation we observed a sharp decrease in the expression of this transporter (Fig. 2B).

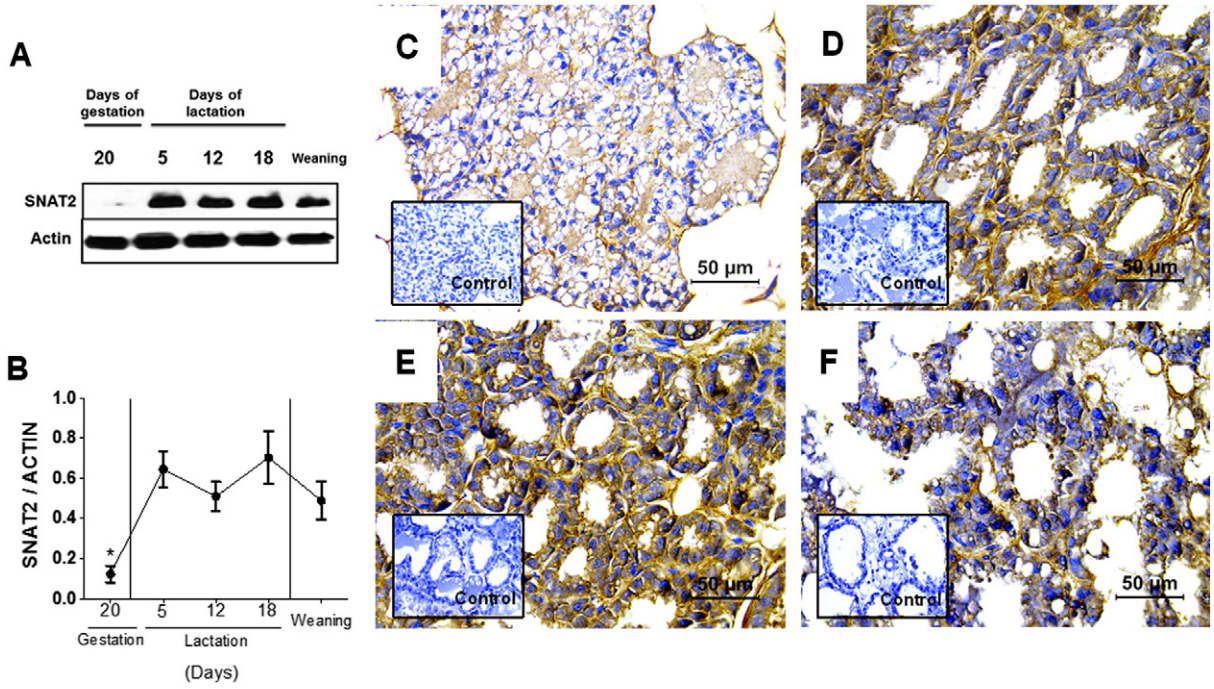


**Fig. 1.** SNAT2 protein abundance in rat mammary gland. A) A representative immunoblot of SNAT2 and actin as constitutive protein of gestation day 20; lactation days 5, 12, and 18; and weaning. B) Western blot densitometric analysis of SNAT2/actin. Values are the mean ± SEM of three different blots. n = 5. Representative immunohistochemistry of SNAT2 detection in different physiological phases of the mammary gland. C) Day 20 of gestation shows almost negative SNAT2 detection D) at day 5 and E) day 12 of lactation there is a strong SNAT2 immunostaining in lobular epithelial cells. F) During weaning there is a mild SNAT2 immunostaining (all micrograph scale bars: 50 µm).

In addition, luciferase reporter gene assays in T47D cells using the SNAT2 promoter region showed a significant increase in the luciferase activity after incubating with 20, 200, 2000 and 20,000 ng/ml compared to the control without prolactin stimulus. The maximum effect was observed with 200 ng/ml concentration having a two-fold increase in the SNAT2 promoter activity (Fig. 2C). These results demonstrate that prolactin is able to activate SNAT2 transcription *in vitro*.

### *3.3. Effect of the dietary protein/carbohydrate ratio in the diet on the expression of SNAT2*

To determine whether the supply of the amino acid/carbohydrate ratio, provided by the consumption of different amounts of dietary protein, could modify SNAT2 expression after delivery and during lactation in different tissues (mammary gland, liver and white adipose tissue), we measured the expression of the SNAT2 protein abundance in these tissues from rats fed with a low-protein/high-carbohydrate (10/73%), or a normal-protein/normal-carbohydrate (20/63%) or a high-protein/low-carbohydrate (30/53%) diet. We observed that SNAT2 was expressed in the mammary gland, as well as in the white adipose tissue and liver as has been previously reported [29]. In the mammary gland we found the same SNAT2 protein abundance just before delivery independently of the amount of dietary protein/carbohydrate ratio consumed. Interestingly, during the lactation period SNAT2 protein abundance increased as the dietary protein/carbohydrate ratio increased, particularly on days 5 and 12 of lactation (Fig. 3A).

In the liver and adipose tissue we observed a different expression pattern depending on the dietary protein/carbohydrate ratio before delivery. Rats fed with a high-protein/low-carbohydrate (30/53%) diet showed a significant increase in the SNAT2 protein abundance, in contrast with the results observed in the mammary gland. However, during lactation, SNAT2 protein expression followed a similar trend than the observed in the mammary gland with a significant increase of the SNAT2 protein depending on the dietary protein/carbohydrate ratio (Fig. 3B, C).

### *3.4. Prolactin concentration during lactation increased as the dietary protein/carbohydrate ratio increased and correlated with SNAT2 expression*

To determine whether the increase in SNAT2 expression was associated with an increase in prolactin concentration as a consequence of the dietary protein/carbohydrate ratio, we measured serum prolactin concentration during lactation. We observed that serum prolactin levels increased as dietary protein/carbohydrate ratio increased during lactation (Fig. 3D). Interestingly, we found a significant correlation between SNAT2 protein concentration and serum prolactin levels ($r^2 = 0.9854$, 0.8935 and 0.9177 in the mammary gland, adipose tissue and liver respectively) on day 12 of lactation (Fig. 4A–C).

## 4. Discussion

Amino acids are essential nutrients for protein synthesis and other metabolic functions. Particularly, during the lactation period there is a sharp increase in the amino acid requirements to support the milk protein synthesis in the mammary gland. Amino acids are captured into the cells by amino acid transporters, which play an important role in this physiological period to supply the amount of amino acids required. In our study we showed that the expression of SNAT2, one of the most abundant amino acid transporters, was regulated in the mammary gland by prolactin stimuli as well as by the dietary protein/carbohydrate ratio during lactation.

We observed an increase of the SNAT2 protein abundance in the mammary gland on day 5 of lactation. It is known that prolactin rapidly rises in the first days of lactation as we also demonstrated by measuring the serum levels of this hormone; therefore prolactin could contribute to the up-regulation of SNAT2 protein abundance. In fact, we also showed that prolactin can regulate the expression of this transporter in mammary gland explants by incubating the tissue in the presence of physiological levels reached by this hormone during the lactation period. It is important to discuss that in this study, we decided to use mammary gland explants from lactating rats because they are mainly

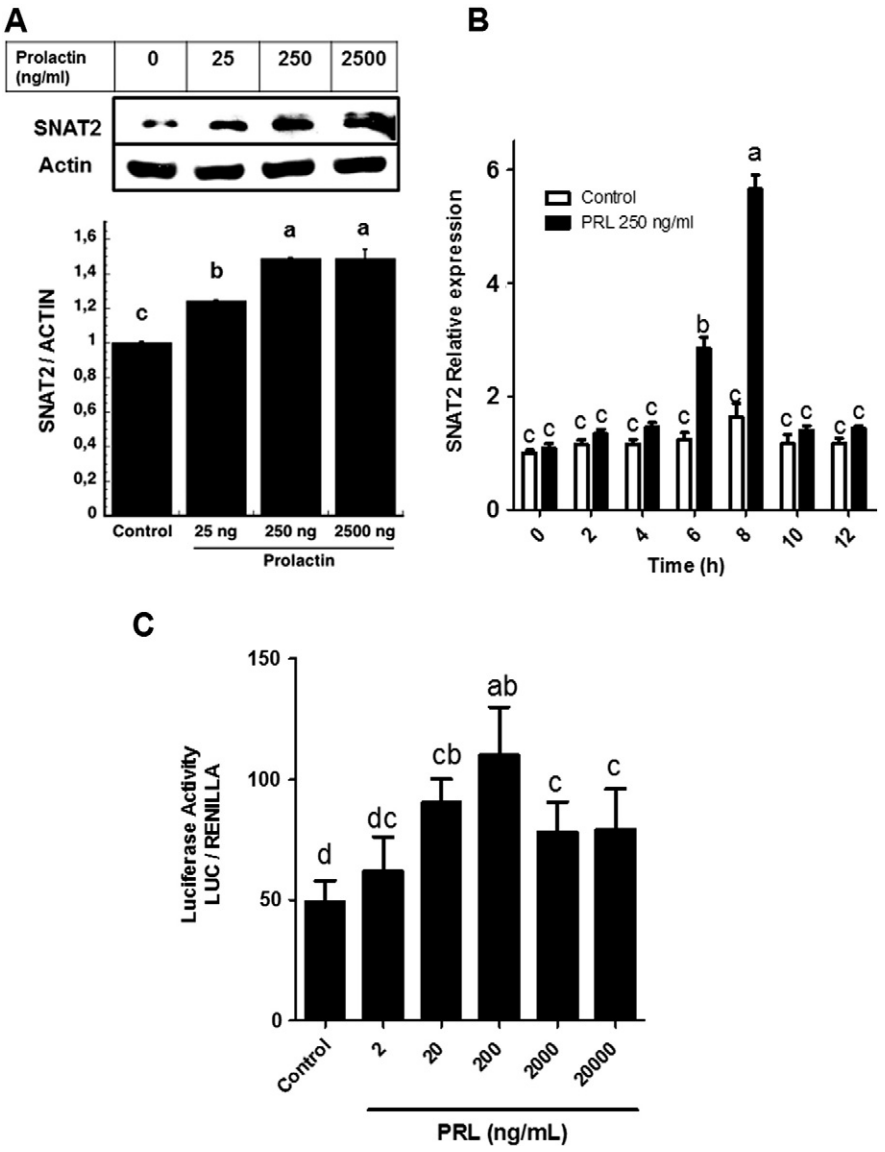


**Fig. 2.** Effect of prolactin in SNAT2 expression in rat mammary gland and T47D cells. A) A representative immunoblot of SNAT2 and actin, as constitutive protein, of mammary gland explants at day 12 of lactation incubated with 25, 250 and 2500 ng/ml of prolactin and western blot densitometric analysis of SNAT2/actin of mammary gland explant lactation day 12 in the presence of different concentrations of prolactin. B) Time course (0, 2, 4, 6, 8, 10, 12 h) of SNAT2 relative mRNA levels in rat mammary gland explants incubated with 250 ng/ml of prolactin. Values are the mean ± SEM of three different blots. n = 5. C) Effect of prolactin (PRL) on SNAT2 promoter activity in T47D mammary ductal carcinoma cells co-transfected with SNAT2 promoter–reporter construct and pRL-TK (Renilla expression vector for normalization), and incubated in the presence of different concentrations (2, 20, 200, 2000 or 20,000 ng/ml) of PRL or without stimulus (control). The results are the mean ± SEM of 3 independent experiments. Different letters differ: a > b > c > d. $P < 0.05$.

conformed by epithelial cells, in comparison to non-lactating mammary glands which have a considerable amount of adipose tissue. However, the effect of exogenous prolactin in this experiment could be misinterpreted by using already lactating dams that have been stimulated *in vivo* by the endogenous increase of this hormone. To clarify the effect of prolactin in SNAT2 expression we performed additional *in vitro* studies in T47D cells using the promoter region of this gene, and incubating the cells in the presence or absence of prolactin. Our results demonstrated that physiological levels during lactation of this hormone were able to significantly increase SNAT2 promoter activity in T47D cell line.

Viña and colleagues reported arteriovenous changes of amino acids across the mammary gland, and that prolactin can regulate the amino acid uptake by this tissue. Interestingly they observed a decrease in the uptake of the SNAT2 substrates, specially alanine and glutamine by adding the prolactin inhibitor bromocriptine [1]. Our data as well as those observed by other authors suggest that the activity of SNAT2 could be stimulated by prolactin during the first days of lactation in the mammary gland.

Interestingly, the SNAT2 protein abundance in the mammary gland on day 12 of lactation, which corresponds to the maximal peak of milk production in the rat, was dependent on the dietary protein/carbohydrate ratio consumed. It has been explored whether the amount of dietary protein could affect the levels of circulating prolactin. Moretto et al. clearly demonstrated that in fact when rats consumed a low protein diet, the concentration of serum prolactin was significantly reduced [22]. Our results also demonstrated that the dietary protein/carbohydrate ratio influences the serum prolactin concentration. This may imply that the reduction of prolactin decreases SNAT2 expression or that the dietary protein/carbohydrate ratio can directly modulate the expression of this gene possibly *via* cAMP, as has been reported in the liver [30]. Further studies are needed to dissect this mechanism.

Interestingly, the mechanisms involved in the regulation of SNAT2 expression in the mammary gland during gestation and lactation are

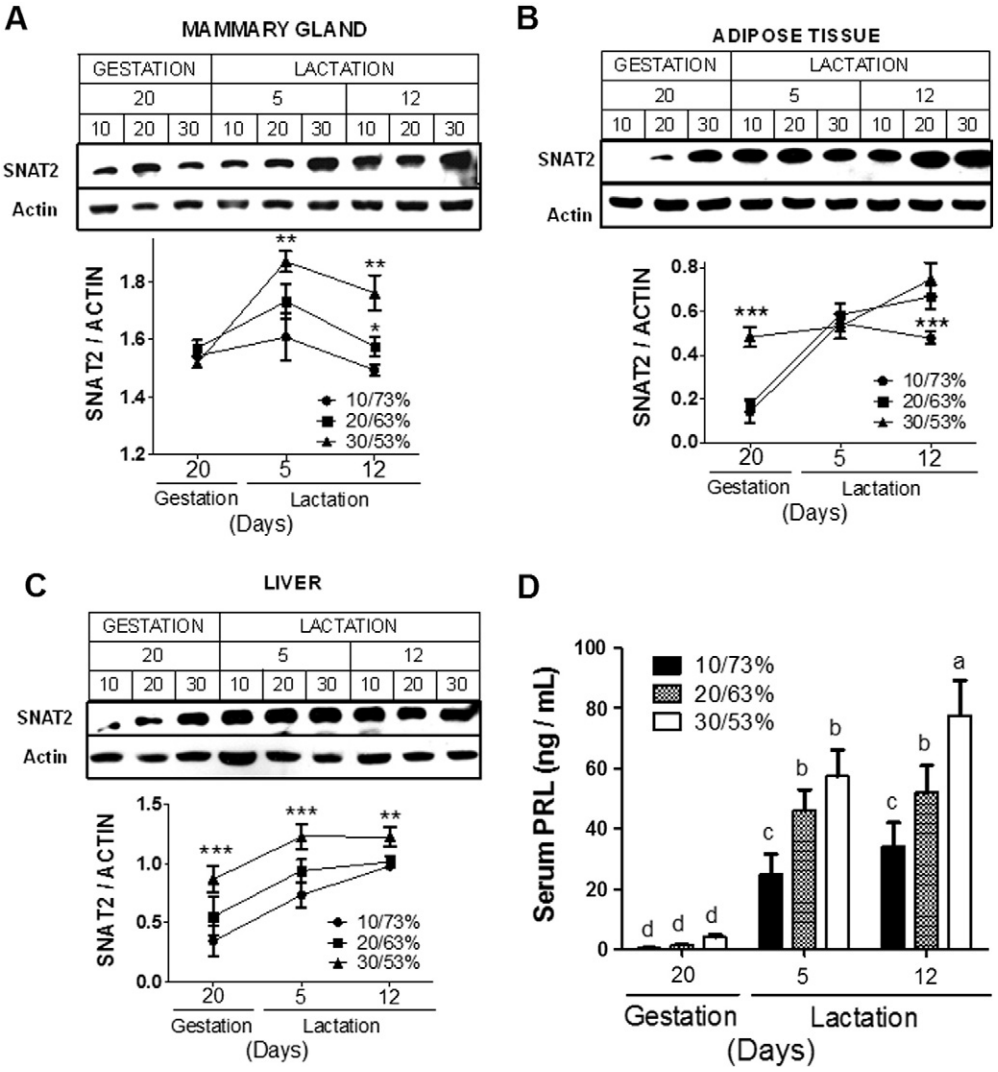


**Fig. 3.** Effect of dietary protein/carbohydrate ratio in SNAT2 protein abundance and serum prolactin levels. A representative immunoblot and western blot densitometric analysis of SNAT2/Actin in rat A) mammary gland, B) adipose tissue and C) liver; and D) prolactin serum levels during gestation day 20 and lactation days 5 and 12 of rats fed a low-protein/high-carbohydrate (10/73%), normal-protein/normal-carbohydrate (20/63%), or high-protein/low-carbohydrate (30/53%) diet. Values are the mean ± SEM of three different blots. n = 5. *P < 0.05, **P < 0.01, ***P < 0.001. Different letters differ: a > b > c > d. P < 0.05.

not systemic. The SNAT2 protein abundance followed a different pattern of expression in the liver and adipose tissue with respect to the mammary gland during the last day of gestation. Before delivery, the expression of this transporter was not modified in the mammary gland due to the dietary protein/carbohydrate ratio consumed, whereas during lactation there was an increase in SNAT2 expression that was dependent on this ratio. It is known, that during lactation rats increase their food intake by 300% [31,32]. Therefore, the supply of amino acids is significantly increased when rats consumed a high protein diet as has been previously reported [33]. It has been demonstrated that an increase in the amount of dietary protein, stimulates the serum glucagon levels, which in turn upregulates the expression of SNAT2 *via* PKA in the liver, since the SNAT2 promoter gene contains a cAMP response element (CRE) at −48 bp [30].

On the other hand, serum prolactin levels, modulated by the dietary protein/carbohydrate ratio, can directly regulate the expression of this transporter by activating its classical pathway mediated by the prolactin receptor not only in the mammary gland but also in other tissues such as the liver and adipose tissue. The long form of the prolactin receptor is expressed in the liver and adipose tissue [34,35], and prolactin plays important roles in these organs during lactation. Moreno-Carranza and colleagues demonstrated that prolactin promotes hepatic cell proliferation and survival, especially in neonates and lactating female rodents [36]. In these stages, SNAT2 amino acid transporter regulated by this hormone could be involved in this process. In the adipose tissue it is known that prolactin receptor is increased during lactation and differentiation, and that prolactin favors the adipogenesis process [37]. During the lactation period prolactin decreases lipogenesis and glucose uptake in the adipose tissue favoring the contribution of energy substrates to the mammary gland [38]. Further studies are needed to identify possible prolactin response elements in the SNAT2 gene to elucidate the molecular mechanism by which this hormone regulates its expression during lactation. *In silico* analysis revealed that the SNAT2 promoter contains a putative STAT3 response element that could be involved in the induction of this gene. We are conducting studies to understand the possible molecular mechanism by which prolactin stimulates the transcription of SNAT2 and its implication in several tissues during the lactation period.

## 5. Conclusion

The mammary gland during the lactation period requires an active uptake of amino acids to support the newborn needs. Our results suggest that prolactin actively supports lactation by providing amino

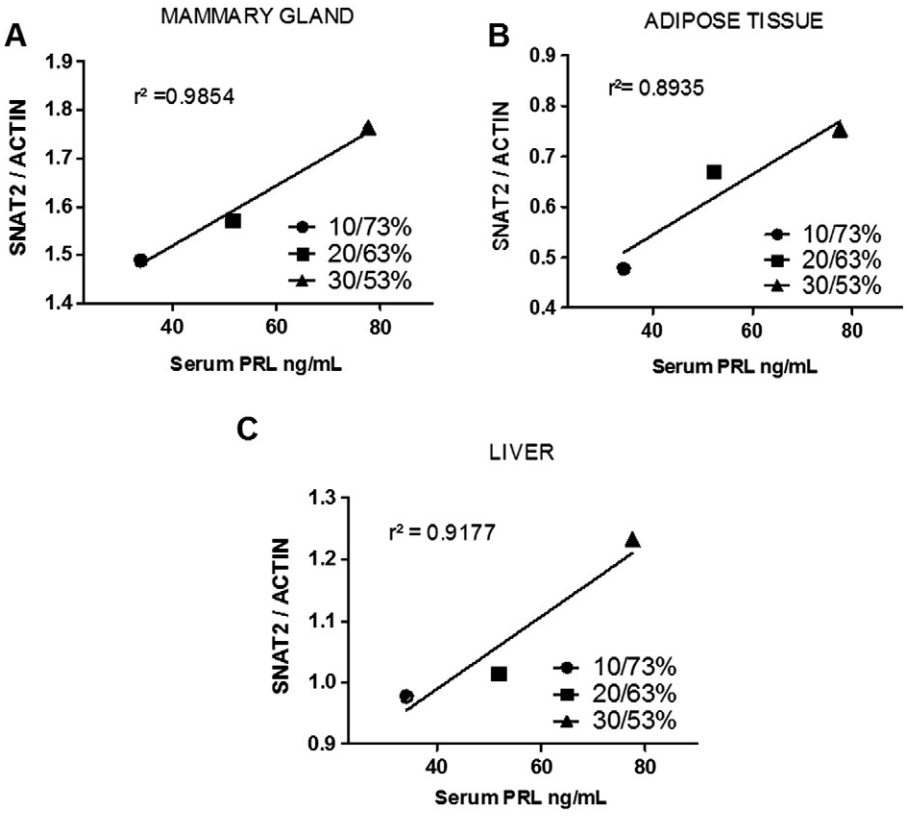


**Fig. 4.** Correlation between SNAT2 protein abundance and serum prolactin levels. Linear regression of rat A) mammary gland, B) adipose tissue and C) liver SNAT2 protein concentration and serum prolactin levels during lactation day 12 in rats fed a low-protein/high-carbohydrate (10/73%), normal-protein/normal-carbohydrate (20/63%), or high-protein/low-carbohydrate (30/53%) diet.

acids into the mammary epithelium through the activation of the expression of amino acid transporters, particularly SNAT2, for the synthesis of milk proteins. The dietary protein/carbohydrate ratio consumed also influenced SNAT2 abundance and correlated with serum prolactin levels not only in the mammary gland, but also in the liver and adipose tissue indicating a new way of regulation of this amino acid transporter expression.

## Author contributions

ART and NT design research. LAVV, AMLB, JCLC and OG performed research. LAVV, AMLB, RHP and ART analyzed and interpreted the data. LAVV, AMLB, RHP and ART wrote the manuscript. All authors revised critically the manuscript and approved the final version to be submitted.

## Disclosure statement

All authors declare no conflict of interest.

## Transparency document

The Transparency document associated with this article can be found, in the online version.

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