Persistent changes in somatostatin and neuropeptide Y mRNA levels but not in growth hormone-releasing hormone mRNA levels in adult rats after intrauterine growth retardation

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Abstract

A reduction in the availability of oxygen and nutrients across the placenta in the last trimester of pregnancy may lead to intrauterine growth retardation (IUGR) which, in turn, may cause a persistent postnatal growth failure. However, it is unknown whether this persistent growth retardation is centrally mediated through alterations in the components of the growth hormone (GH)-axis. We tested the hypothesis that alterations in the development of the central components of the GH-axis contribute to the persistent growth failure observed after experimentally induced IUGR or early postnatal food restriction (FR) in the rat. Using semi-quantitative in situ hybridization, we compared somatostatin (SS), GH-releasing hormone (GHRH) and neuropeptide Y (NPY) mRNA levels in adult rats experimentally subjected to IUGR or FR. We report that IUGR increased the expression of SS mRNA in the periventricular nucleus (PeN) of adult male and female rats by 128% and 153% respectively, did not alter the expression of GHRH mRNA in the arcuate nucleus (ARC) and decreased the NPY mRNA expression in the ARC by 73% in males and 61% in females, whereas in the FR group no changes in the expression of these mRNAs were observed. These data show that the timing of malnutrition or the presence of the placenta is important for the long-term alterations since the effects only occurred in the prenatally induced growth retardation and not in the early postnatally induced growth retardation group.


Introduction

Fetal growth is critically dependent on the availability of oxygen and nutrients from the mother across the placenta (Gluckman & Harding 1997, Godfrey 1998, Prada & Tsang 1998, Wollmann 1998, Dwyer et al. 1999). Interference with this substrate supply may lead to intrauterine growth retardation (IUGR). The fetus adapts to its limited substrate supply by decreasing the rate of cell division especially in tissues with periods of rapid cell proliferation, although the fetus demonstrates a compensatory redistribution of arterial blood flow to the brain (‘brain-sparing effect’) at the expense of other organs (liver, pancreas, kidneys) (Rizzo & Arduini 1991, Sadig et al. 1999). During these critical periods, cell multiplication slows down as a direct effect of undernutrition or through altered bioavailability of growth factors (Widdowson & McCance 1963, 1974). This phenomenon may alter the expression of the fetal genome, which may be related to lifelong consequences on a range of physiological processes. This is supported by epidemiological research showing that low birth-weight is associated with a number of diseases in adulthood, such as cardiovascular diseases, non-insulin-dependent diabetes mellitus and hypertension (Barker et al. 1993, Barker 1998).

Animal studies show that experimentally induced IUGR due to a limited supply of substrates results in postnatal growth failure without any signs of catch-up growth (Wigglesworth 1964, Gluckman & Harding 1997) whereby the structure and function of different organ systems are affected as well (Langley & Jackson 1994, Harding 1995, Gluckman et al. 1996, Bauer et al. 1998, Rajakumar et al. 1998).

Postnatal growth is largely influenced by growth hormone (GH) release from the pituitary. The pulsatile secretion of GH indirectly exerts effects on growth through insulin-like growth factor-I (IGF-I) (Gluckman et al. 1991). The pulsatile GH secretion from the pituitary is mainly controlled by the coordinated release of two hypothalamic hormones, somatostatin (SS) and growth
hormone-releasing hormone (GHRH). GHRH is released from neurons in the arcuate nucleus (ARC) and it stimulates the synthesis and secretion of GH. SS is released from neurons in the periventricular nucleus (PeN) and inhibits the secretion of GH (Tannenbaum 1991, Giustina & Veldhuis 1998). Neuropeptide Y (NPY) also plays a role in the regulation of GH release. NPY is released from neurons in the ARC and stimulates the secretion of SS thereby indirectly inhibiting GH secretion (Chan et al. 1996b). In addition, NPY is involved in the control of food intake (Clark et al. 1984, Kalra et al. 1999).

We postulated that malnutrition in a developmental phase will result in changes in the programming of the central control of growth, which may contribute to the postnatal growth failure. To investigate this hypothesis, we performed semi-quantitative in situ hybridizations to determine alterations in SS, GHRH and NPY mRNA levels in individual neurons in the PeN and ARC of adult male and female rats following prenatal or early postnatal experimentally induced growth retardation. To compare the effects of pre- versus postnatal malnutrition on alterations in SS, GHRH and NPY gene expression in adulthood, we used two models of growth restriction – experimentally induced IUGR and early postnatal food restriction (FR).

Materials and Methods

Animals and surgery

Timed pregnant Wistar rats were obtained from Harlan CPB (Rijswijk, The Netherlands) and housed under a constant light-darkness cycle (lights on: 0600 h; lights off: 1800 h) in a temperature controlled room (22 ± 1 °C).

For experimental induction of IUGR, the method of Wigglesworth (1964) was used. Pregnant females (gestational day 17) were anaesthetized with a mixture of Aescoket (100 mg/ml) and xylazine (20 mg/ml) (diluted 4:1; 1 ml/kg body weight i.m.). The mesometrium of one uterine horn was exposed through a midline incision in the abdominal wall and the uterine artery was ligated near the cervical end of the arterial arcade. The uterine artery of the other horn was ligated in a similar manner and the abdomen was closed. After surgery, females were allowed to recover and to give birth. Pups were considered IUGR if their weights at postnatal day 2 were below minus 2 standard deviations (weight <5.3 g) of mean weight of the litter size to 20 pups per mother (male/female ratio 1:1) from postnatal day 1 onwards (Widdowson & McCance 1963).

For each experimental group, the mothers had free access to rat chow and tap water. The pups remained with their mother until weaning at day 25. After weaning, the rats were housed in groups of 2–3 animals per cage with free access to food and tap water. All procedures were approved by the Institutional Animal Ethics Committee.

Experimental design

Levels of SS mRNA in the PeN, and GHRH and NPY mRNA in the ARC were compared among the groups of IUGR, FR and control rats at postnatal day 72–74 (adulthood) in both males and females. Each group consisted of 6 animals except for the female group in the SS measurements which contained 5 animals.

Tissue preparation

Rats were killed between 0900 h and 1100 h. The brains were rapidly removed and frozen in dry-ice-cooled 2-methylbutane. Brains were stored at −80 °C until further processing. Coronal cryostat sections were cut at 20 µm and thaw-mounted on 2% 3-aminopropylethoxysilane-coated slides. Consecutive brain sections were cut starting rostrally at the PeN (−0.26 mm) and continuing caudally to the end of the ARC (−3.60 mm) (Paxinos & Watson 1986). Four sets of slides were collected; every fourth slide was placed into a given set. Slides were stored at −80 °C until in situ hybridization was performed. One set (32 sections per animal) was used per in situ hybridization.

Preparation of 35S-labelled cRNA probes

Riboprobes complementary to the coding region of preproSS mRNA were generated by in vitro transcription of the 395 bp cDNA fragment of rat preproSS cloned into pSP65 vector (Rogers et al. 1987). Antisense cRNA probes were made by linearizing this plasmid by HindIII and transcribing with T7 RNA polymerase (Promega, Madison, WI, USA) and transcribing with SP6 RNA polymerase (Promega, Madison, WI, USA) in the presence of 35% [35S]UTP (NEN Life Sciences, Boston, MA, USA).

GHRH cRNA riboprobes were generated by in vitro transcription of the 217 bp cDNA fragment of rat GHRH cloned into the pGEM4 vector (Robert Steiner, Seattle, WA, USA). Antisense cRNA probes were made by linearizing with EcoRI and transcribing with T7 RNA polymerase in the presence of 35% [35S]UTP. Sense cRNA probes were made by linearizing with HindIII and transcribing with SP6 RNA polymerase in the presence of 35% [35S]UTP.

NPY cRNA riboprobes were generated by in vitro transcription of the 511 bp cDNA fragment of rat preproNPY ligated into the EcoRI site of the Bluescribe M13(−) vector (Robert Steiner). Antisense cRNA probes

were made by linearizing with FspI and transcribing with T3 RNA polymerase in the presence of 35% [35S]UTP. Sense cRNA probes were made by linearizing with SmaI and transcribing with T7 RNA polymerase in the presence of 35% [35S]UTP.

Transcription was terminated by the addition of RQ1 DNase (Promega). The cRNA probe was precipitated with 1/10 vol 3 M sodium acetate (pH 5.2) and 2.5 vol ethanol at −20 °C. Labelled precipitated riboprobes were resuspended in 100 mM dithiothreitol.

In situ hybridization

The specificity of the binding of the preproSS cRNA was determined by control experiments (Rogers et al. 1987). To demonstrate the specificity of the binding of the GHRH and preproNPY cRNA probes, sense cRNA probes were used in a test in situ hybridization. The control hybridizations to the mRNA strand with the sense probes resulted in an absence of photographic clusters of grains over cells, proving that the antisense probes bind to their specific sequences. Different concentrations of antisense cRNA probes (range 10 000–160 000 d.p.m./µl) were used to determine optimal probe concentration. The optimal probe concentration for the SS, GHRH and NPY antisense probes appeared to be 40 000 d.p.m./µl.

Six in situ hybridization assays were performed. The tissues from female animals for each of the three probes were run in three separate assays and the tissues from male animals for each probe were run in the other three. Sections were air dried at room temperature and fixed for 20 min in freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4), rinsed three times for 5 min in PBS and rinsed briefly in water. After acetylation for 10 min with 0.25% acetic anhydride in 1% triethanolamine and two washing steps in PBS, sections were rinsed in 2× standard saline citrate (SSC) for at least 5 min. The slides were prehybridized for 2 h at 37 °C in hybridization buffer containing 50% formamide, 10% dextran sulphate, 1× Denhardt’s solution, 0.2× SSC, 500 µg/ml denatured salmon sperm DNA (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 250 µg/ml tRNA (Sigma-Aldrich, St Louis, MO, USA). After prehybridization, the denatured antisense SS, GHRH or NPY cRNA probes at a final concentration of 2.5×10⁶ d.p.m./slide were applied. Sections were covered with paraffin and incubated overnight in moist chambers at 50 °C (for SS and GHRH) or 65 °C (for NPY). The following day the sections were rinsed in 1× SSC and washed twice under high stringency in 0.1× SSC at 60 °C (for SS and GHRH) or 64 °C (for NPY), followed by treatment with 30 µg/ml RNase A in RNase buffer (10 mM Tris, pH 8.0; 1 mM EDTA; 50 mM NaCl) at 37 °C for 30 min. Finally, the sections were washed twice in 0.1× SSC at room temperature and air dried.

Autoradiography

Hybridized slides were dipped in emulsion (Ilford K5) that had been diluted 1:1 in 2% glycerol solution and heated to 45 °C in a water bath. Dipped slides were allowed to air dry for 1 h. Slides were placed in light-tight boxes and exposed at 4 °C for 5 days for the SS in situ hybridizations, 6 days for the NPY in situ hybridizations and 7 days for the GHRH in situ hybridizations.Slides were developed, counterstained with nuclear fast red and dehydrated through a series of alcohol followed by application of coverslips.

Image analysis

Prior to image analysis of the in situ hybridizations, age- and sex-matched slides were assigned a random three-letter code, alphabetized, and read in random order with an automated image processing by an operator unaware of the animal’s experimental group.

The system used for image analysis of the SS in situ hybridizations consisted of a PixelGrabber Video acquisition board (Perceptics, Knoxville, TN, USA) attached to a MacIntosh fx computer. Video images were obtained by a Dage model 65 camera (Dage, Inc., Michigan City, IN, USA) attached to a Zeiss Axioskop (Zeiss, New York, NY, USA) equipped with a × 40 objective and side-illumination.

For the GHRH and NPY in situ hybridizations, the image analysis system consisted of a PixelGrabber Video acquisition board attached to a MacIntosh IICi computer. Video images were obtained by a COHU model 4913 CCD camera (Cohu, San Diego, CA, USA) attached to a Zeiss Axioskop equipped with a × 40 oil immersion objective and a halogen light source. Each analysed image was an average of 32 video frames.

In each section, the number of grains over each individual cell in the PeN or ARC was determined by use of the UW Grain Counting Analysis Program (D Clifton, Seattle, WA, USA). The total number of labelled cells in the PeN (bregma −0.26 mm to −1.80 mm) or ARC (bregma −2.12 mm to −4.30 mm) (Paxinos & Watson 1986) on both sides of the third ventricle was counted. Cells were considered to express specific binding of the labelled probes when the number of silver grains overlying the cytoplasm exceeded five times the background levels.

The number of grains per cell is referred to as the mRNA signal representing semi-quantitative message levels allowing comparison of relative differences in mRNA expression levels between groups. To verify the specificity of the observed changes, the number of grains in NPY mRNA expressing neurons in the cortex were analysed. In six sections from each animal, 15 cells (total 90 cells) in the perirhinal cortex were analysed at the level of bregma −2.56 mm to −3.14 mm (Paxinos & Watson 1986).
**Table 1** Mean weight of adult male and female IUGR, FR and control (CON) rats. Values are given as mean weight in grams ± S.E.M.

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<th>CON</th>
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<th>IUGR</th>
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<tr>
<td>Males</td>
<td>335 ± 6·3</td>
<td>259 ± 7·7*</td>
<td>280 ± 9*</td>
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<tr>
<td>Females</td>
<td>204 ± 1·7</td>
<td>186 ± 3·6*</td>
<td>190 ± 5·9*</td>
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*Significantly different compared with controls (P<0·05).

**Statistical analysis**

For the different experimental groups, mean and S.E.M. grains per cell expressing the specific miRNAs was calculated. The effects of IUGR and FR on SS, GHRH and NPY mRNA expression and on the number of SS-, GHRH- or NPY-containing cells and on body weight were assessed by a one-way ANOVA followed by Fisher’s protected least significant difference test. Differences were considered statistically significant when P<0·05.

**Results**

Growth retardation induced either prenatally by ligation of the uterine artery (IUGR) or early postnatally by food restriction (FR) resulted in a persistent postnatal growth failure into adulthood reflected in lower body weights compared with controls (Table 1).

The number of cells containing SS mRNA in the PeN and GHRH or NPY mRNA in the ARC in IUGR and FR animals were not significantly different from their controls (Table 2).

SS mRNA levels in the PeN were significantly increased in IUGR males (P=0·002) and females (P=0·002) to 128% (IUGR males: 302 ± 10 grains/cell; CON males: 236 ± 7 grains/cell) and 153% (IUGR females: 273 ± 18 grains/cell; CON females: 179 ± 9 grains/cell) respectively compared with controls (Fig. 1A, B). In contrast, the SS mRNA expression in FR males (FR: 220 ± 17 grains/cell; CON: 236 ± 7 grains/cell) and FR females (FR: 205 ± 10 grains/cell; CON: 179 ± 9 grains/cell) were not different from controls (Fig. 1A, B and Fig. 2A, B, C).

GHRH mRNA expression in the ARC was not altered in IUGR males (IUGR: 137 ± 8 grains/cell; CON: 147 ± 11 grains/cell) and IUGR females (IUGR: 169 ± 23 grains/cell; CON: 174 ± 4 grains/cell) compared with their controls. This was also observed in the FR males (FR: 148 ± 12 grains/cell; CON: 147 ± 11 grains/cell) and FR females (FR: 169 ± 16 grains/cell; CON: 174 ± 4 grains/cell), where no difference was found in GHRH mRNA levels compared with the controls (Fig. 1C, D and Fig. 2D, E, F).

NPY mRNA levels in the ARC were significantly decreased in IUGR males (P=0·0001) and females (P=0·0001) to 73% (IUGR males: 101 ± 5 grains/cell; CON males: 138 ± 2 grains/cell) and 61% (IUGR females: 89 ± 5 grains/cell; CON females: 145 ± 8 grains/cell) respectively compared with their controls. NPY mRNA expression was not different in FR males (FR: 135 ± 4 grains/cell; CON: 138 ± 2 grains/cell) and FR females (FR: 149 ± 6 grains/cell; CON: 145 ± 8 grains/cell) compared with controls (Fig. 1E, F and Fig. 2G, H, I). NPY mRNA levels in the cortex were not different among the various groups (Table 3) indicating that the effects of perinatal malnutrition on NPY gene expression are confined to the NPY neurons that participate in the regulation of GH secretion.

**Discussion**

During prenatal and early postnatal development, when a system still has plasticity, a suboptimal substrate supply may result in a changed programming of the various organs, affecting growth of the organ as well as its function (Lucas 1994). Early malnutrition injures the developing brain in rat and men. Depending on the time period, either the cell number or cell size is mainly affected (Winick 1969). Underdevelopment of the brain may involve neuro-endocrine systems including the central control of growth. Substrate limitation in utero can be followed by complete or incomplete catch-up growth depending on the length and severity of the malnutrition. There is evidence that the persistent growth failure after malnutrition is the result of failure to grow of the target organs themselves, but it can also be due to changes in the growth regulating mechanisms (Gluckman & Harding 1997, Albertsson-Wikland et al. 1998). In children born with a low birth weight who have a persistent postnatal growth failure, a decreased growth hormone secretion

**Table 2** Number of counted SS-, GHRH- or NPY-containing neurons of adult male and female IUGR, FR and control (CON) rats. Values are given as mean number of cells ± S.E.M.

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<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS cells</td>
<td>298 ± 56</td>
<td>279 ± 87</td>
<td>126 ± 53</td>
</tr>
<tr>
<td>GHRH cells</td>
<td>48 ± 10</td>
<td>41 ± 11</td>
<td>76 ± 27</td>
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<tr>
<td>NPY cells</td>
<td>76 ± 18</td>
<td>93 ± 26</td>
<td>81 ± 26</td>
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<th>CON</th>
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<tr>
<td>Females</td>
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</tr>
<tr>
<td>SS cells</td>
<td>151 ± 45</td>
<td>217 ± 56</td>
<td>106 ± 25</td>
</tr>
<tr>
<td>GHRH cells</td>
<td>61 ± 18</td>
<td>59 ± 21</td>
<td>27 ± 9</td>
</tr>
<tr>
<td>NPY cells</td>
<td>69 ± 13</td>
<td>75 ± 15</td>
<td>133 ± 31</td>
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pattern as well as decreased IGF-I levels have been described (de Waal et al. 1994).

In the present study, rats subjected to prenatal or early postnatal malnutrition showed a growth failure that persisted into adulthood. After restoration of the nutrient restriction, an adequate food intake did not elicit any catch-up growth in either model.

Postnatal growth is regulated by various hypothalamic neurotransmitters including GHRH, SS and NPY which, in turn, control GH release (Tannenbaum 1991, Giustina & Veldhuis 1998). The question arises whether an affected central regulation is involved in the persistent growth failure of our models, initiated by early malnutrition. Therefore, we investigated the number and function of

Figure 1 SS mRNA signal levels in the PeN (A and B) and GHRH (C and D) and NPY (E and F) mRNA levels in the ARC of the hypothalamus of male and female IUGR, FR and control (CON) rats at the adult stage. Values are given as mean grains/cell ± S.E.M.*P<0.05, compared with controls.
neurons secreting GHRH, SS and NPY by measuring mRNA levels.

We did not find any difference in the number of hypothalamic cells containing GHRH, SS and NPY, which indicates that the early malnutrition we applied in both models did not affect the number of neurons responsible for the control of GH secretion. Since we measured mRNA levels, we do not know about a possible

Figure 2 Photomicrographs of neurons of adult male IUGR, FR and control rats under darkfield illumination after in situ hybridization for SS, GHRH, and NPY. Silver grains from the radioactive labelled probes appear as white dots. Clusters of silver grains mark cells containing SS, GHRH, or NPY mRNA. Panels A, B, and C show the SS mRNA silver grain distribution in the PeN of control (A), FR (B), and IUGR (C) rats. Panels D, E, and F show the GHRH grain clusters in the ARC of control (D), FR (E), and IUGR (F) rats. Panels G, H, and I show clusters of NPY mRNA-expressing neurons in the ARC of control (G), FR (H) and IUGR (I) rats.

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<tr>
<td></td>
<td>CON</td>
<td>FR</td>
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<tr>
<td>ARC</td>
<td>138 ± 2</td>
<td>135 ± 4</td>
</tr>
<tr>
<td>Cortex</td>
<td>201 ± 8</td>
<td>214 ± 6</td>
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*Significantly different compared with controls (P ≤ 0.05).
diminished outgrowth of dendrites, which may explain a changed input in the neuronal network. From our study, we may conclude that although the brain is still plastic, an insult of malnutrition applied either during late gestation or directly postnatally does not influence the number of neurons secreting GHRH, SS or NPY, which are located in the mediobasal hypothalamus.

With respect to the functional capacity of these three different neurons, we observed unchanged SS mRNA levels in the periventricular SS secreting cells after early postnatal malnutrition in adult rats in contrast to rats exposed to intrauterine malnutrition in which we observed increased levels of SS mRNA. Therefore, it appears that the last days of pregnancy in contrast to the early postnatal period are critical for the function of SS neurons. In the present study, we investigated the effects of early malnutrition in the adult rat. In earlier studies we described increased SS mRNA levels at the acute phase of the postnatal period (Huizinga et al. 2000).

After the restoration of nutrient restriction in the FR group, SS gene expression normalized suggesting that postnatal food restriction appears to result in increased SS activity at that time, presumably as a temporary adaptation to the acute situation. However, the long term consequences of increased SS gene activity after intrauterine malnutrition, as we observe in the adult rat, may be explained by a changed ‘programming’ of the SS neurons, which is only possible when these cells do not have a fixed functional capacity (Barker 1998).

SS is the GH inhibiting neurotransmitter, whereas GHRH is responsible for the synthesis and release of GH. In both malnutrition models we found no change in GHRH gene expression in the adult rat, which suggests that a definite fixed activation and organization of these neurons already exists from late gestation onwards. In contrast to the late effects of early malnutrition, acute malnutrition is still able to modulate the function of the adult GHRH neuron. Bruno and co-workers (1990) observed increased GHRH mRNA levels in adult rats in an unchanged GH outcome, indicate that only the setpoint of the GH control has been changed. The decreased NPY activity appears to be compensated for by increased SS action with a similar final output on GH release.

In spite of these observed hypothalamic changes, we cannot explain the postnatal growth failure observed after early malnutrition via its effect on the GH axis. For the intrauterine malnourished group we cannot exclude the possibility that decreased NPY activity results in hypophagia which interferes with growth. Sensitivity to growth factors will also be fixed during the developmental phase and will therefore be modified by a stimulus in this period. We suggest that the cause of the growth failure after a period of malnutrition may be located at the level of the growth regulating factors such as IGF-binding proteins (IGFBPs), which regulate the biological activity of IGF-I. This is supported by the finding...
that maternal undernutrition leads to increased levels of IGFBP-1 and IGFBP-2, thereby restricting the availability of IGF-I (Woodall et al. 1996). In addition, the response of the target organ itself may be involved via a diminished availability of receptors or by the postreceptor response. These speculations will be the subject for further studies.

We conclude that intrauterine malnutrition initiates a changed ‘programming’ of the interactions of the growth hormone controlling system especially with regard to the activity of SS and NPY neurons, which is not found in the early postnatal food-restricted animals. These findings indicate that nutritional stress during a critical developmental window may induce potentially irreversible alterations in organ functions.

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