Hypothalamic food intake regulation in a cancer-cachectic mouse model

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Received: 9 May 2013 / Accepted: 3 September 2013 / Published online: 13 November 2013
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Abstract

Background Appetite is frequently affected in cancer patients leading to anorexia and consequently insufficient food intake. In this study, we report on hypothalamic gene expression profile of a cancer-cachectic mouse model with increased food intake. In this model, mice bearing C26 tumour have an increased food intake subsequently to the loss of body weight. We hypothesise that in this model, appetite-regulating systems in the hypothalamus, which apparently fail in anorexia, are still able to adapt adequately to changes in energy balance. Therefore, studying changes that occur on appetite regulators in the hypothalamus might reveal targets for treatment of cancer-induced eating disorders. By applying transcriptomics, many appetite-regulating systems in the hypothalamus could be taken into account, providing an overview of changes that occur in the hypothalamus during tumour growth.

Methods C26-colon adenocarcinoma cells were subcutaneously inoculated in 6 weeks old male CDF1 mice. Body weight and food intake were measured three times a week. On day 20, hypothalamus was dissected and used for transcriptomics using Affymetrix chips.

Results Food intake increased significantly in cachectic tumour-bearing mice (TB), synchronously to the loss of body weight. Hypothalamic gene expression of orexigenic neuropeptides NPY and AgRP was higher, whereas expression of anorexigenic genes CCK and POMC were lower in TB compared to controls. In addition, serotonin and dopamine signalling pathways were found to be significantly altered in TB mice. Serotonin levels in brain showed to be lower in TB mice compared to control mice, while dopamine levels did not change. Moreover, serotonin levels inversely correlated with food intake.

Conclusions Transcriptomic analysis of the hypothalamus of cachectic TB mice with an increased food intake showed changes in NPY, AgRP and serotonin signalling. Serotonin levels in the brain showed to correlate with changes in food intake. Further research has to reveal whether targeting these systems will be a good strategy to avoid the development of cancer-induced eating disorders.

Keywords Cancer · Hypothalamus · Appetite · Serotonin · Transcriptomics · Anorexia
1 Introduction

Anorexia affects 60–80 % of all patients with cancer and considerably contributes to disease-related malnutrition and cachexia, which in turn strongly affect patient’s morbidity, mortality and quality of life [1].

Anorexia is often linked to cachexia, a complex metabolic syndrome associated with underlying illness which is characterised by progressive loss of muscle (muscle wasting) with or without loss of fat mass resulting in weight loss [2]. Although anorexia and cachexia are likely to be initiated by similar pathologies, several lines of evidence suggest that both conditions progress via distinct mechanisms. However, the presence of cachexia makes it difficult to disentangle the primary underlying mechanisms of cancer anorexia since this might be due to tumour growth, cachexia progression or other disease-related mechanisms.

Cancer anorexia is generally considered to be a multifactorial condition. Contributing to its complexity is the observation that evolution has developed powerful physiological mechanisms favouring food intake. It has been shown that upon shifting the balance to anorexia, pathways can become redundant when they are not functioning properly. This is for example shown by data obtained from studying knockout animals for well-known food intake regulators, the NPY knockout mouse [3], the AgRP knockout mouse [4] or the ghrelin knockout mouse [5]. These mice display regular food intake and body weight regulation despite the loss of a significant key modulator in appetite regulation. The difficulties encountered in studying cancer anorexia inspired us to approach the problem from a different angle. Cancer-induced anorexia is suggested to be predominantly caused by the inability of the hypothalamus to respond adequately to pivotal peripheral signals involved in appetite regulation [6]. This hypothalamic resistance to peripheral neuroendocrine signals is believed to be due to the increase in pro-inflammatory cytokines resulting from tumour growth [6]. In this study, we report on hypothalamic gene expression profiles in a cancer-cachectic model with increased food intake. In this model, appetite-regulating systems, which apparently fail in anorexia, are still able to adapt adequately to changes in energy balance. By applying transcriptomics, many appetite-regulating systems in the hypothalamus could be taken into account. Here, we provide an overview of changes that occur in the hypothalamus during tumour growth which could be important in the development of cancer-induced eating disorders.

2 Materials and methods

2.1 Tumour model

Male CDF1 (BALB/cx DBA/2) mice aged 6 to 7 weeks were obtained from Harlan Nederland (Horst, The Netherlands). Animals were individually housed 1 week before start of the experiment in a climate-controlled room (12:12 dark–light cycle; 21 °C ± 1 °C).

Mice were placed on a standard ad libitum diet (AIN93M, research Diet Services, The Netherlands) and had free access to water.

Murine C26 adenocarcinoma cells were cultured and suspended as described previously [7]. Under general anaesthesia (isoflurane/N2O/O2), tumour cells in 0.2 ml HBSS were inoculated subcutaneously into the right inguinal flank. Controls were sham-injected with 0.2 ml HBSS.

All experimental procedures were approved by the Animal Ethical Committee (DEC, Bilthoven, The Netherlands) and complied with the principles of good laboratory animal care.

2.2 Experimental design

On day 0, tumour cells were injected. BW, food intake and tumour size were measured three times a week. Tumour size was determined by measuring the length and width of the tumour with a calliper. On day 20, body composition was determined by DEXA (Lunar, PIXImus). Subsequently, blood was collected by cardiac puncture. After sacrifice, brain, hypothalamus, organs and lower leg skeletal muscles were weighted and frozen at −80 °C. Two studies were performed with similar settings: study A was a pilot study to optimise experimental conditions and was followed by study B. Table 1 shows the number of tumour cells used for inoculation in the different groups that were included in the two studies.

2.3 Blood plasma amino acids and cytokines

Amino acids were measured by using HPLC with orthophthalaldehyde as derivatization reagent and L-norvaline as internal standard (Sigma Aldrich). The method was adapted from van Eijk et al. [8].

Cytokines were measured using a mouse cytokine 10-plex bead immunoassay (Biosource, Etten-Leur, The Netherlands). Prostaglandin E2 was measured using an enzyme-immunoassay kit (Oxford Biomedical Research, Oxford, MI, USA).

2.4 Serotonin and dopamine levels

Hypothalamic samples were used for microarray experiments, while remaining brain parts were used to determine serotonin and dopamine levels. Brains were homogenized in 1 ml containing 40 mM Tris, 1 mM EDTA, 5 mM EGTA, 0.50 % Triton X-100 and PhosSTOP phosphatase inhibitor (Roche Nederland, The Netherlands). Citric acid (1 %) was added to prevent serotonin oxidation. Serotonin and dopamine levels were measured using enzyme-immunoassay kits (BAE-5900, BAE-5300, LDN, Nordhorn, Germany).
2.5 Statistics

Data was analysed by statistical analysis of variance followed by a post hoc Tukey’s multiple comparison/Bonferroni test or by a Student’s t test. Differences were considered significant at a two-tailed $P<0.05$. Statistical analyses were performed using Graphpad Prism 5. For statistical analysis of microarray data, see microarray section (below).

2.6 Microarray studies

Total RNA from the hypothalamus was isolated by using RNeasy Lipid tissue kit (Qiagen, Venlo, The Netherlands). RNA concentrations were measured by absorbance at 260 nm (Nanodrop). RNA quality was checked using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) according to the manufacturer’s protocol. For each mouse, total RNA (100 ng) was labelled using the Ambion WT expression kit (Life Technologies, Bleiswijk, The Netherlands). Microarray

| Study A | Study B |
|---------|---------|
| C: control, sham injected ($n=4$) | C: control, sham injected ($n=6$) |
| TB-0.5: tumour-bearing, $0.5\times10^6$ C26-cells ($n=4$) | TB: tumour-bearing, $1\times10^6$ C26-cells ($n=9$) |
| TB-1: tumour-bearing, $1\times10^6$ C26-cells ($n=3$) | |

Table 1 Groups included in study A and study B. Pilot study A was performed prior to study B. In study A, mice were injected with different amounts of C26-colon adenocarcinoma cells, while study B comprised of one tumour-bearing group and one control group.

Fig. 1 Effect of tumour inoculation on food intake, tumour size, tumour weight, body weight and body composition in studies A and B. a Time course of change in food intake of TB mice in study A. b Tumour weight at day 20 in study A. c Tumour width and length were measured twice a week with a calliper and used to calculate tumour volume. d Time course of change in food intake of TB mice in study B. e Time course of change in body weight in study B. f Body composition determined by DEXA scan in study B. *$P<0.05$ (significantly different from C). Data is expressed as mean ± SEM. C sham-injected control, TB-0.5 injected with $0.5\times10^6$ tumour cells, TB-1 injected with $1\times10^6$ tumour cells and TB injected with $1\times10^6$ tumour cells.
Experiments were performed by using Affymetrix Mouse Gene ST 1.0 (study A) and 1.1 (study B). For both studies A and B, samples were pooled for each group. Also, individual samples from study B were included in a subsequent microarray experiment to confirm the findings on appetite regulators and canonical pathways. In this microarray experiment, four control samples and five samples from tumour-bearing mice were included in this experiment; however, one control sample gave various spots on the array and was therefore excluded from analysis.

| ID    | Study A | Study B | P-value | Gene | Description         |
|-------|---------|---------|---------|------|---------------------|
|       | TB-0.5  | TB-1    | pools   | mean |                     |
| 109648|         |         | 0.000   | Npy  | neuropeptide Y      |
| 11604 |         |         | 0.001   | Agrp | agouti related protein |
| 14599 |         |         | 0.028   | Gh   | growth hormone      |
| 208188|         |         | 0.007   | Ghsr | growth hormone secr. R |
| 110312|         |         | 0.001   | Pmch | pro-melanin-concentrating hormone |
| 207911|         |         | 0.017   | Mchr1| melanin-concentrating hormone R1 |
| 14427 |         |         | 0.011   | Galr1| galanin R1          |
| 12801 |         |         | 0.032   | Cnr1 | cannabinoid R1 [brain] |
| 14419 |         |         | 0.075   | Gal  | galanin              |
| 14601 |         |         | 0.107   | Ghrh | growth hormone releasing hormone |
| 18387 |         |         | 0.113   | Oprk1| opioid R, kappa 1   |
| 14429 |         |         | 0.120   | Galr3| galanin R3          |
| 14428 |         |         |         | Galr2| galanin R2          |
| 66177 |         |         | 0.136   | Ubi5 | ubiquitin-like 5    |
| 230777|         |         | 0.137   | Hcrt1| hypocretin [orexin] R1 |
| 381073|         |         | 0.138   | Npw  | neuropeptide W       |
| 18167 |         |         | 0.191   | Npy2r| neuropeptide Y R Y2  |
| 18386 |         |         | 0.228   | Oprd1| opioid R, delta 1   |
| 587285|         |         | 0.364   | Hcrt2| hypocretin [orexin] R2 |
| 15171 |         |         | 0.442   | Hcrt  | hypocretin           |
| 227717|         |         | 0.451   | Qrtp | pyroglutamylated RFamide peptide |
| 58991 |         |         | 0.681   | Ghrl | ghrelin              |
| 18390 |         |         | 0.887   | Oprm1| opioid R, mu 1      |
| 18166 |         |         |         | Npy1r| neuropeptide Y R Y1  |
| 18168 |         |         |         | Npy5r| neuropeptide Y R Y5  |

| ID    | Study A | Study B | P-value | Gene | Description         |
|-------|---------|---------|---------|------|---------------------|
|       | TB-0.5  | TB-1    | pools   | mean |                     |
| 18976 |         |         | 0.037   | Pmoc | pro-opiomelanocortin-alpha |
| 12424 |         |         | 0.013   | Cck  | cholecystokinin      |
| 27220 |         |         | 0.098   | Cartp| CART prepropeptide   |
| 12921 |         |         | 0.097   | Crhr1| Corticotropin-releasing hormone R1 |
| 14652 |         |         | 0.611   | Gipr1| glucagon-like peptide 1 R |
| 14526 |         |         |         | Gcg  | glucagon             |
| 14527 |         |         |         | Ggr  | glucagon R           |
| 16847 |         |         | 0.393   | Lepr | leptin R             |
| 16846 |         |         |         | Lep  | leptin               |
| 54598 |         |         | 0.678   | Calc1| calcitonin R-like   |
| 232836|         |         | 0.885   | Galp  | galanin-like peptide |
| 53322 |         |         | 0.859   | Nucb2| nucleobindin 2      |
| 14829 |         |         | 0.823   | Gspr | gastrin releasing peptide R |
| 12922 |         |         | 0.742   | Cnhr2| corticotropin releasing hormone R2 |
| 68039 |         |         | 0.513   | Nmb  | neurenomedin B       |
| 12425 |         |         | 0.408   | Cckar | cholecystokinin AR    |
| 217212|         |         |         | Pyy  | peptide YY           |
| 54615 |         |         | 0.176   | Npff | neuropeptide YY-amide precursor |
| 12311 |         |         | 0.026   | Calc1| calcitonin R         |
| 17202 |         |         | 0.053   | Mc4r | melanocortin 4 R     |
| 18101 |         |         | 0.085   | Nmbr | neurenomedin B R     |
| 67405 |         |         | 0.077   | Nts  | neurotensin           |
| 12209 |         |         | 0.110   | Bts3 | bombesin-like R 3    |
| 18429 |         |         | 0.001   | Oxt  | oxytocin              |
| 83428 |         |         | 0.002   | Ucn3 | urocortin 3           |
| 22044 |         |         | 0.000   | Trh  | Thyrotropin-releasing hormone |
| 20604 |         |         | 0.013   | Sst  | somatostatin          |
tumour cells. In study B, TB mice were injected with $1 \times 10^6$ tumour cells

The heat map in Fig. 2 shows fold changes of orexigenic and anorexigenic gene expressions. Orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) expression were found to be significantly higher by 1.9 and 1.6-fold, respectively, in TB mice. Orexigenic ghrelin expression was comparable between TB mice and controls. However, expression of the growth hormone-secretagogue receptor (GHSR), which mediates ghrelin signalling, showed to be slightly higher by 1.2-fold. In addition, growth hormone (GH) expression, which also acts via GHSR and stimulates food intake, showed to be highly upregulated in TB mice. Expression of anorexigenic somatostatin showed to be 1.2-fold higher in TB mice compared to controls. Somatostatin is a strong negative feedback regulator of GH, suggesting that its upregulation could be a result of increased GH expression.

3.2 Microarray analysis of the hypothalamus

The heat map in Fig. 2 shows fold changes of orexigenic and anorexigenic gene expressions. Orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) expression were found to be significantly higher by 1.9 and 1.6-fold, respectively, in TB mice. Orexigenic ghrelin expression was comparable between TB mice and controls. However, expression of the growth hormone-secretagogue receptor (GHSR), which mediates ghrelin signalling, showed to be slightly higher by 1.2-fold. In addition, growth hormone (GH) expression, which also acts via GHSR and stimulates food intake, showed to be highly upregulated in TB mice. Expression of anorexigenic somatostatin showed to be 1.2-fold higher in TB mice compared to controls. Somatostatin is a strong negative feedback regulator of GH, suggesting that its upregulation could be a result of increased GH expression.

3 Results

3.1 Body weight and food intake

In study A, tumour size and tumour weight did not increase correspondingly to the number of tumour cells injected (Fig. 1b, c). However, carcass weight, epididymal fat pad weight and skeletal muscle weight decreased proportionally to the number of tumour cells injected, suggesting that body wasting increases with tumour load despite the weight of the tumour being similar (Supplementary table S1). Food intake in all tumour-bearing animals was found to increase after 15 days. At day 19, tumour-bearing (TB) mice in TB-0.5 and TB-1 groups ate approximately 45% more than the controls. An increase of food intake in TB mice was again noticed in subsequent study B (Fig. 1a, d). In this study, food intake of TB mice was 40% higher than controls at day 19. On day 13, after tumour inoculation, TB mice started to lose body weight (BW). Synchronously to the decline in body weight, an increase in food intake in TB mice was measured, suggesting compensatory eating by TB mice in order to cope with loss of BW. The loss of lean mass, fat mass and skeletal muscle weight in TB mice in study B was comparable with that of study A, showing that the level and severity of cachexia developed in TB animals was similar in both studies (Supplementary table S1).
Pathway analysis using Ingenuity Systems showed that the serotonin (5-HT) receptor signalling pathway was significantly altered ($P < 0.05$) in the hypothalamic tissues of TB mice (Supplementary figure 1). Expression of genes involved in both 5-HT synthesis and 5-HT degradation showed to be lower in TB mice than in controls, pointing towards a compensatory mechanism regulating expression of these enzymes.

Pathway analysis further showed that besides 5-HT signalling, also dopamine (DA) signalling was altered (Supplementary figure 1). Several genes involved in 5-HT signalling are also of importance in dopamine signalling. Changes in these shared genes between the 5-HT and DA pathways are therefore likely to have an effect on both neurotransmitters. Expression of *gch1*, *qdp*, and *ddc*, which are involved in the synthesis of both 5-HT and DA, were strongly downregulated. Also, transporter *vmat*, which is important in transporting 5-HT and DA into the neuronal synapse, showed to be 1.7-fold lower in TB mice compared to controls. Tryptophan hydroxylase (*tph*) and tyrosine hydroxylase (*th*), rate-limiting enzymes in the synthesis of 5-HT or DA, respectively, were also strongly downregulated. In addition, SERT and DAT, re-uptake transporters of 5-HT and DA, respectively, in order to terminate activation in the synaptic cleft showed to be more than twofold lower in TB mice. This indicates that besides shared genes between the 5-HT and DA pathways, also genes specifically involved in either DA or 5-HT synthesis, were altered.

Figure 3a shows an overview of genes involved in 5-HT and DA signalling and their fold changes. To determine the effects of these changes on gene expression, 5-HT and DA levels were measured. Serotonin levels showed to be significantly lower in the TB mice, whereas DA levels showed not to be different in TB mice compared to control animals (Fig. 3c–e). Since both DA and 5-HT have been discussed for their role in food intake and feeding behaviour, correlation between these neurotransmitters and food intake were studied. Serotonin levels were found to correlate with food intake in both C and TB mice, while this correlation could not be made for DA and food intake (Fig. 3d–f).

### 3.4 Plasma amino acid levels and immune parameters

In study B, levels of various amino acids in plasma were measured (Supplementary table S3). TRP levels relative to branched-chain amino acids (BCAA) is often used as a predictor for 5-HT status in the brain. Surprisingly, TRP/BCAA ratios showed to be significantly higher in TB animals compared to controls (Fig. 4a).

To assess tumour-driven inflammatory response, PGE$_2$, TNFα and IL-6 were measured in blood plasma (Fig. 4b–d). TNFα levels showed to decrease, while pro-inflammatory mediators IL-6 and PGE$_2$ showed to be significantly elevated.
A specific characteristic for this model is that in this particular setting, food intake of TB mice does not change and is not different from that of healthy controls. However, in the meantime, various research groups have reported a strong decrease in food intake in mice injected with these C26 cancer cells [19, 20], suggesting that changes in morphology of the cell line, variation in the strain of mice and differences in number of tumour cells used for inoculation might lead to these discrepancies in findings on food intake. It has already been reported that C26-induced cachexia and anorexia can vary according to the inoculation site [21] and origin of C26 cells [22], as well as the use of solid tumour fragments or cell suspensions for inoculation can cause variation [23]. Also, adaptation of C26 cells to in vitro culture conditions can cause mutations in the cell line leading to changes in cell characteristics, sensitivity to chemotherapy, metastatic potential and tumour-induced cachexia in mice, suggesting that C26 cells can differentiate to different variants and change tumour characteristics despite being derived from the same source [24]. Subsequently, the extent and type of inflammatory response that is induced by tumour growth might play a role in the severity of cachexia and anorexia. Differences in tumour-driven inflammation, might therefore explain differences between various cancer models. To confirm tumour-induced inflammation in our model, various cytokines and PGE2 levels in blood plasma were measured. IL-6 and PGE2 showed to be elevated in TB mice, which also has been reported previously [25]. However, in contrast to previous results, TNFα levels in blood plasma were not elevated in TB mice compared to control mice.

Elevated concentrations of TNFα are reported to decrease food intake [26], suggesting that the absence of TNFα-mediated inflammation might play a role in compensating feeding behaviour in this model. All together, we would like to propose the hypothesis that although the “C26 model” is referred to as such, in fact the model is heterogeneous with many varieties. Small differences in experimental settings and spontaneous mutations in the cell line used might lead to great changes in characteristics of the model.

In the present study, pathway analysis indicates serotonin (5-HT) and dopamine (DA) signalling to be altered in TB mice compared to controls. DA and 5-HT are both important neurotransmitters involved in eating behaviour. The signalling pathways of 5-HT, DA and DA metabolites norepinephrine and epinephrine are closely linked by shared synthesising enzymes and transporters. Therefore, it is very likely that changes in these shared genes will propose these comprehensive effects. Since both pathways were predicted to be altered, we measured 5-HT and DA levels in whole brain homogenates. Serotonin levels were found to be significantly lower in TB mice compared to control. This might be caused by decreased TPH and SERT expression, which have been directly correlated to lowered 5-HT levels in other studies [27, 28]. However, DA levels in TB mice showed not to be different from levels in controls, suggesting that effects on expression of shared genes are of greater impact on 5-HT synthesis than on DA synthesis. In addition, in relation to changes in food intake in TB mice, only 5-HT levels showed to inversely correlate with food intake whereas DA levels did not. A limitation of the present setup is that gene pathway analysis was based on hypothalamic transcripts whereas analysis of 5-HT and DA levels took place in homogenates of remaining brain material. Therefore, levels of these neurotransmitters reflect an indication and local differences in the various regions of the brain in both DA and 5-HT cannot be ruled out.
that the decrease in DA in the hypothalamus was a consequence of the presence of insulin resistance in the muscle might distort amino acid profiles in blood and subsequently higher brain protein metabolism in the body and is dependent on the physical status of the subject. In the case of severe cachexia, it could be that large metabolic alterations in muscle and the presence of insulin resistance in the muscle might distort amino acid profiles in blood in order to predict brain 5-HT levels via TRP ratios adequately.

In the present study, various appetite regulators were studied for their role in the observed increased food intake in TB mice. AgRP and NPY expressions were highly upregulated in TB mice. Central infusion of AgRP in cachectic C26 tumour-bearing mice results in an increase in food intake, which supports our findings. However, increased expression of NPY and its relation to potentiate feeding in this study is more difficult to interpret, as messenger NPY has been reported to correlate with NPY in the hypothalamus in cancer cachectic conditions. Several studies have shown that in cachectic and anorectic TB mice and rats, messenger NPY is also elevated. However, translation of messenger NPY or transport of NPY to NPY terminals showed not to correspond to mRNA changes shown by measurements of NPY levels and immunohistochemistry. Serotonin has been discussed to play a role in this imbalance between messenger NPY and NPY signalling in feeding behaviour in cancer anorexia. Inhibition of 5-HT signalling showed to increase NPY levels, while induction of 5-HT signalling reduced NPY levels in rats.

All together, this suggests that 5-HT signalling can interfere with NPY synthesis or transport. Therefore, it could be that in the current study, decreased 5-HT levels and lower 5-HT signalling might preserve NPY signalling.

In this study, we report on the transcriptomic analysis of a cancer-cachectic model with an increased food intake. In this model, appetite-regulating systems, of which failure might contribute to anorexia, are able to adapt properly to changes in energy balance. We showed that alterations in NPY, AgRP and serotonin signalling are likely to explain compensatory eating behaviour of mice bearing a C26 tumour. Therefore, targeting these systems might offer promising strategies to avoid the development of cancer-induced anorexia.

Acknowledgments We thank Nicole Buurman, Gerrit de Vrij and Angeline Visscher for their technical support. The work presented in this manuscript was funded by the European Union Seventh Framework Program (FP7/2007-2013) under grant agreement no. 266408 (Full4Health). The authors confirm that they comply with the principles of ethical publishing in the Journal of Cachexia, Sarcopenia, and Muscle 2010;1:7–8 (von Haehling S, Morley JE, Coats AJ and Anker SD).
Conflict of interest  M.V  Boekschoten,  M.  Müller,  J.M  Argilè,  A.  Laviano  and  R.F  Witkamp  declare  that  they  have  no  conflict  of  interest.  J.T  Dwarkasing  is  a  guest  employee  and  M.van  Dijk,  F.J  Dijk,  J.  Faber  and  K.van  Norren  are  employees  of  Nutricia  Research,  a  medical  nutrition  company.

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