Feeding Behaviour in Galanin Knockout Mice Supports a Role of Galanin in Fat Intake and Preference

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It has been widely suggested that saturated fat consumption has fuelled the current obesity epidemic. Macronutrient choices appear to be important not only as potential factors influencing obesity, but also independently as risk factors for diabetes, cardiovascular disease and cancer. The neuropeptide galanin has previously been implicated in the regulation of fat intake, although its precise role has been contested. The present study investigated mice with targeted knockout of the galanin gene (GKO). We demonstrate that, when only a high fat diet (HFD) was available, wild-type (WT) animals consumed significantly more energy than the GKO mice (89.85 ± 4.57 kJ/day versus 76.84 ± 3.55 kJ/day, P < 0.001, n = 17 versus 15). Consistent with this, WT animals gained more body weight when fed the HFD than GKO animals (3.48 ± 0.44 g versus 2.02 ± 0.62 g, P < 0.001, n = 17 versus 15). In a macronutrient choice scenario, WT mice ate almost three-fold more fat than GKO animals (0.63 ± 0.02 g versus 0.23 ± 0.01 g, P < 0.001, n = 18 versus 24). Chronic administration of galanin by mini-osmotic pumps into the lateral ventricle of GKO animals partially reversed the fat avoidance phenotype. Fat intake was significantly lower in the phosphate-buffered saline-treated GKO group compared to galanin-treated GKO animals (0.32 ± 0.01 g versus 0.38 ± 0.01 g, P < 0.005, n = 17 versus 17). These data are compatible with the hypothesis that galanin specifically regulates fat intake, and implies that an antagonist to one or more of the galanin receptor subtype(s) may be of use in the treatment of some forms of obesity.

Key words: galanin, macronutrient, obesity.

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lar nucleus (PVN) of the hypothalamus induced a dramatic increase in consumption of standard rodent chow in laboratory rats maintained with free access to food and water in the home cage, this effect was found to be dose-dependent. Time course analysis after an injection of 3 nmol of galanin into the PVN indicated an immediate onset of action, maximal over the first 30 min, with no significant difference in food intake between saline vehicle and galanin treatments on consumption of rat chow between 40 and 240 min after microinjection (16). The effect of galanin on food intake has been replicated in several other studies (16–19). Further, in rats pre-treated with leptin injected i.c.v. into the third ventricle 1 h before treatment with galanin i.c.v., it was found that leptin significantly inhibits galanin-induced consumption of chow (20), suggesting that the effects of galanin on food intake are mediated via mechanisms downstream of leptin.

The initial report linking galanin specifically to the intake of fat was provided by Tempel et al. (21) in which the authors demonstrated that injection of galanin into the PVN caused a specific and preferential increase in the consumption of the fat diet. They went on to show that, when the fat diet was removed from the animals, intake of carbohydrate was selectively increased with no change in protein intake. A second study by the same group found that although the total amount of food consumed after PVN galanin injection was similar in the early and late dark periods, the macronutrient selection patterns exhibited at these two times were different. During the early dark period, PVN galanin had a small stimulatory effect on carbohydrate, in addition to a strong enhancement of fat intake. By contrast, galanin only stimulated fat intake in the late dark period (22).

Following these reports by Tempel et al. (21) and Tempel and Leibowitz (22), Smith et al. (23) conducted a study using male Sprague-Dawley rats in a 24-h macronutrient choice paradigm in which animals injected i.c.v. with galanin were allowed to freely select from the three macronutrient diets for the following 24 h. They found that galanin treatment induced large increases in consumption of mixed nutrient diets (high in fat or carbohydrate). However in contrast to earlier reports, no specific macronutrient increases were found; instead they proposed that injection of galanin i.c.v. only served to increase the animals baseline intake with the same macronutrient profile as observed prior to the treatment (24).

In the present study, we describe a series of dietary manipulations using a mouse with a targeted knockout of the galanin gene, (25), designed to test the involvement of the galanergic system in the regulation of macronutrient intake in both single-diet and three-diet choice scenarios. We show that galanin is a key regulator of fat intake and, by proxy, of energy balance as a whole.

**Materials and methods**

**Animals**

All experiments were carried out on mice homozygous for a targeted mutation in the galanin gene as previously described (25). The galanin knockout (GKO) colony has remained inbred on the 129OlaHsd strain. Strain-, sex- and age-matched wild-type (WT) mice were employed as controls during experiments. All procedures were carried out under current United Kingdom Home Office guidelines (licence PPL: 60/2881).

Three groups of GKO and age- sex- and strain-matched WT controls were transported to the University of Aberdeen, UK, where GKO and WT animals were housed in groups of five or six in mouse stock boxes (425 x 268 x 150 mm), under a 16 : 8 h light/dark cycle (lights on 07.00 h) and an ambient temperature of 22 °C ± 2 °C. Individuals were identified by ear markings. Mice had access ad lib to standard rodent chow prior to commencement of studies (Rat and Mouse Number 1 Maintenance; Special Diets Services, BP Nutrition, Essex, UK) and water, with sawdust supplied for bedding. Prior to the start of each study, mice were allowed 2 weeks to acclimate to single housing in either a standard cage or a cage equipped with food hopper dividers, in the case of the macronutrient measurements.

**High fat diet**

Mice (17 WT and 15 GKO) were maintained on standard chow prior to the experiment for 5 days, to provide a baseline intake. After the initial 5-day baseline period, animals were provided with a 45% high fat diet (HFD) (pelleted rodent diet D12451; Research Diets, Inc., New Brunswick, NJ, USA) on day 1 of the study. Daily at 12.00 h (± 30 min) for 30 days, animals and diet were weighed (accuracy ± 0.01 g). Body mass data are expressed as difference from baseline weight.

**Macronutrient selection**

WT (n = 18) and GKO (n = 24) animals were placed on a macronutrient selection protocol for 21 days during which they were allowed to self-select from three diets consisting of high fat, high carbohydrate and high protein respectively (Appendix A and B). Macronutrient choice experiments were performed blind with respect to to which diets corresponded to which macronutrient. The food hopper of each cage was separated into three compartments of equal size using steel dividers, each compartment was then filled with approximately 10 g of one of the three diets. The location of each diet was randomised daily to remove any possible order effects. The animals were then allowed to self select for 2–3 days to become accustomed to this method of feeding. After this period, body mass (g) and intake of each of the diets (g) was measured (accuracy ± 0.01) on a daily basis at 12.00 h (± 30 min) for a period of 21 days. The bedding of each cage was searched thoroughly prior to each weighing to ensure no diet was lost. Bedding of animals on the macronutrient protocol was reduced by approximately 25% to allow ease of collection of any spilled diet from the cage. Previous quantifications of the food ground into the sawdust that is missed by such a protocol indicate an average loss of approximately 2.3% (26). There was no indication that the WT and GKO mice differed in their food spillage, although we did not rigorously quantify this effect.

**Galanin treatment of GKO animals**

GKO mice were randomly allocated to two groups (n = 17 versus 17) and placed on a macronutrient selection protocol for 44 days during which they were allowed to self-select from three diets consisting of high fat, high carbohydrate and high protein. Intake and diets were measured as above. All measurements were made blind of the actual macronutrient compositions of the diets.

On day 30, mice were implanted with miniosmotic pumps (details below) containing either phosphate-buffered saline (PBS) or recombinant galanin. Animals were anaesthetised with gaseous isoflurane for the duration of the
surgery and were dosed with 0.1 μl per 10 g body mass of buprenorphine post surgery. Implantation of the cannulae and mini-osmotic pump assembly took 25 min on average.

The surgical area was shaved and cleaned with povidone–iodine and alcohol swab. A dorsal incision of approximately 2.5 cm running laterally on the scalp was made a hole was then drilled at co-ordinates of −0.82 mm anteroposterior and ± 0.30 mm lateral from the bregma. The cannulae was then implanted at a depth of −2.5 mm below dura and connected to the pump assembly which was placed into the area of loose skin below the neck.

Miniosmotic pumps (Alzet model 2004, capacity 200 μl, release rate 0.25 μl/h, duration of pumping approximately 4 weeks; Direct Corporation, Cupertino, CA, USA) containing either recombinant galanin at a dosage of 360 pmol/μl (Sigma Aldrich, Poole, UK) dissolved in sterile PBS or PBS alone (sham procedure).

Resting metabolic rate measurements

Resting metabolic rate (RMR) of GKO (n = 24) and WT (n = 22) mice was measured 20 ± 1 °C. Animals were maintained on rodent chow diet (Rat and Mouse Number 1 Maintenance, Special Diets Services; BP Nutrition) and had not been subject to any other manipulations prior to the measurement. Immediately before and after the RMR measurements, body mass was recorded. Individual mice were not denied access to either food or water before commencement of RMR measurements. RMR was measured using an open-flow respirometry system and was conducted in the animals light phase. The protocol employed has previously been described in detail (27, 28). Briefly, mice were placed individually into a sealed perspex chamber (850 ml) located within an incubator (INL-401 N-010, Gallenkamp, Loughborough, UK), air-dried using silica gel (BDH Laboratory Supplies, Poole, UK) drawn through the system (Charles Austin Pumps Ltd, Melton Mowbray, UK) at a flow rate of 500–775 ml/min, and a 150 ml per sub-sample of the dried excurrent air was passed through the oxygen analyser (Model 1100A; Servomex Ltd, Crowborough, UK).

To optimise the precision of the derived estimates of energy expenditure, carbon dioxide was not absorbed prior to oxygen analysis (29). The output from the oxygen analyser was then recorded directly on a computer at 30-s intervals, and the ten lowest consecutive readings (equal to 5 min within the respirometry chamber) were used to estimate RMR (27), after correcting for temperature and pressure. The temperature of the incubator in which the respirometry chamber) were used to estimate RMR (27), after correcting for temperature and pressure. The temperature of the incubator in which the chamber was located was maintained at 20 ± 1 °C for the duration of the 2.5-h measurement period.

Statistical analysis

Statistical analysis was carried out using the MINITAB (version 14) statistical package (Minitab Inc., College Station, PA, USA). P < 0.05 was considered statistically significant. All data are reported as the mean ± SE. (n = sample size per group). Data was analysed using either two-way repeated measures ANOVA or via general linear modelling.

Results

High fat diet

Prior to the start of the experiment no differences were found between the two genotypes in daily intake (kJ/day) or body mass gain/loss (g/day) on standard laboratory chow during the 5-day baseline period. However, when fed a HFD (45% fat) for 30 days, energy intake was significantly greater in the WT mice, which consumed significantly more energy (kJ) than the GKO animals (89.85 ± 4.57 kJ/day versus 76.84 ± 3.55 kJ/day, P < 0.001, n = 17 versus 15, Fig. 1a). WT animals rapidly gained body weight when fed the HFD, increasing on average by 3.48 g over the 30 days from their baseline weight. By contrast, GKO animals increased by only 0.04 g from baseline. The difference in mass gain between the groups was highly significant (3.48 ± 0.44 g versus 2.02 ± 0.62 g, P < 0.001, n = 17 versus 15). Error bars represent SEM. RMR, Resting metabolic rate.

Macronutrient selection

When presented with a macronutrient choice consisting of three isocaloric diets that differed in their macronutrient composition, GKO mice differed in their macronutrient selection profile compared to WT control mice. There was a significant difference in body mass gained/lost over the duration of the protocol (Table 1).

WT animals gained mass over the duration of the protocol whereas GKO animals lost mass over the same period (0.7 ± 0.04 g versus −0.38 ± 0.04 g, P = <0.001, n = 18 versus 24). Consistent with the changes in body weight, total food intake (kcal/day) was
significantly different between groups with the WT group consuming significantly more energy than the GKO group (69.52 ± 0.74 kJ/day versus 66.29 ± 0.87 kJ/day, P = <0.001, n = 18 versus 24) (Table 1). Fat intake was significantly greater in the WT animals than in the GKO animals (0.65 ± 0.02 g versus 0.23 ± 0.01 g, P = <0.001, n = 18 versus 24) for the duration of the protocol (Table 1; Fig. 2 C).

Galanin treatment of GKO animals

During the 30 days prior to surgery, no significant differences were found in the two groups of GKO for total food intake (kJ/day) or any individual macronutrient intake (g/day) (Table 2). There was also no difference in body mass between the two groups (Table 2). After 14 days of galanin or PBS administration, fat intake was significantly lower in the PBS-treated group compared to those treated with galanin (0.32 ± 0.01 g versus 0.38 ± 0.01 g, P = <0.001, n = 17 versus 17, Table 2). Carbohydrate and protein intake (g) postoperatively were not significantly different between the two groups (Table 2). Post-surgery, there was no significant difference between the mean body mass (g) of the PBS-treated and galanin-treated groups (Table 2), although there was a significant difference in total food intake, with the PBS-treated group consuming less than the galanin-treated group (Table 2).

Resting metabolism

There was a strong effect of body mass on resting energy expenditure at 20 °C (Fig. 3). There was no significant difference in the slope of the mass effect between genotypes (genotype × body mass interaction, F = 1.81, P = 0.186, n = 22 versus 24). We adjusted the metabolic rates to account for the mass effects using ANCOVA (30). When adjusted, there was no significant difference in the RMR of the WT animals compared to that of the GKO group (1.11 ± 0.15 ml/O2/min versus 1.17 ± 0.131.11 ± 0.15 ml/O2/min, n = 22 versus 24, P = 0.353, Fig. 3).

Discussion

Current models of the neuroendocrine regulation of food intake (1, 7, 8) have indicated a critical role for a concert of signals from the alimentary tract and other peripheral tissues (such as leptin from adipose tissue and insulin from the pancreas) that have receptors in the hypothalamus. The pivotal roles of leptin and insulin interacting with both NPY/AGRP and POMC/CART neurones in the ARC, which then interact with other neurones containing melanocortin 4 receptor (MC4R) and melanocortin 3 receptor (MC3R) in the PVN, have been emphasised. However, the pathway beyond MC4R and MC3R neurones in the PVN remains obscure, as does the contribution of other mechanisms upstream of the melano-

<table>
<thead>
<tr>
<th>Variable</th>
<th>Wild-type</th>
<th>Galanin knockout</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass change (g)</td>
<td>0.70 (0.04)</td>
<td>-0.38 (0.05)</td>
<td>274.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carbohydrate intake (g)</td>
<td>1.38 (0.03)</td>
<td>1.66 (0.04)</td>
<td>99.29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Protein intake (g)</td>
<td>0.95 (0.02)</td>
<td>0.78 (0.02)</td>
<td>105.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fat intake (g)</td>
<td>0.65 (0.02)</td>
<td>0.23 (0.01)</td>
<td>1209.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total daily intake (kJ)</td>
<td>69.52 (0.74)</td>
<td>66.29 (0.87)</td>
<td>9.12</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Fat, protein and carbohydrate intake was found to be significantly different between the wild-type (WT) and galanin knockout (GKO) animals, the greatest difference was in fat intake, with the GKO animals consuming significantly less fat than the WT group. No difference in body mass between the groups was noted.

Table 1. Body Mass Gain/Loss (g/day) and Total Daily Caloric Intake (kJ) and Relevant Statistics are Presented for a Three-Choice Macronutrient Paradigm for a Period of 21 Days.

Fig. 2. Galanin knockout (GKO) and wild-type (WT) animals were presented with a three choice macronutrient paradigm for a period of 21 days: (a) carbohydrate, (b) protein and (c) fat intakes (g/day) in WT (closed circles, n = 18) and GKO mice (open circles, n = 24). Significant differences between the genotypes were observed for all three food types; the greatest difference was in fat intake, with the GKO animals consuming significantly less fat than the WT group. Error bars represent SEM.
Role of galanin in fat intake and preference

Several previous studies demonstrated that i.c.v. injection of GAL has a significant influence on intake of standard laboratory chow (16, 21, 22). The finding that GKO animals have no significant differences in either food intake or in body mass compared to WT controls maintained on a standard chow diet (31) may be explained by one of two factors. First, compensatory mechanisms may regulate food intake to maintain normal energy balance in the GKO animals. This form of genetic redundancy has been identified in several other neuropeptide systems relating to energy homeostasis (32, 33). Second, the regulation of food intake by GAL is mainly related to the regulation of specific macronutrients, which is masked when animals are fed a single complete diet. When maintained on a standard chow diet, body weights and fat pad mass of the GKO mice are not significantly different from WT controls during the first 8 weeks or in adulthood (25). We have also previously reported that the GKO mice have only very slight differences in endocrine profile with a 28% up-regulation in follicle-stimulating hormone levels being the most notable change (30). GKO animals exhibit normal leptin levels and respond in the same manner as WT mice to a fast; however, when GKO mice are chronically injected with leptin, they lose significantly more weight than WT controls, indicating the possibility of a requirement for GAL in normal leptin signalling (30).

Previous studies have reached different and sometimes opposing conclusions about the role of GAL as a factor influencing macronutrient selection. The original work on GAL and its role in macronutrient selection showed a clear preference for the intake of fat and, to a lesser extent, carbohydrate intake (16, 21, 22). Recent reports show that although GAL may not have a specific effect on fat intake, the stimulation of feeding via injected GAL is more pronounced and longer lasting in animals maintained on a HFD or in strains of rats that naturally prefer fat (33). Supporting the link with the regulation of fat intake, studies by Leibowitz and Kim (34) and also Odorizzi et al. (35) show that administration of a GAL antagonist has a strong suppressive effect on fat intake. Akabayashi et al. (36) show that repeated injections of oligonucleotides to GAL mRNA produce a reduction in fat intake coupled with a reduction in endogenous GAL levels. It is worth considering that exposure to a HFD has been demonstrated to increase GAL expression in the PVN (37). Therefore, when WT animals are placed on HFD, a positive feedback loop may be in effect leading to increased GAL expression.

Fat intake and total food intake expressed in terms of calories were found to be significantly different in GKO animals treated with intracerebroventricularly galanin compared to the animals that received saline. No other nutrients were found to be different between the groups nor was any difference in body mass noted.

### Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>PBS-treated</th>
<th>Galanin-treated</th>
<th>Group</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g) (pre-operative)</td>
<td>29.56 (0.19)</td>
<td>29.01 (0.13)</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body mass (g) (post-operative)</td>
<td>29.24 (0.19)</td>
<td>28.58 (0.15)</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate intake (g) (pre-operative)</td>
<td>1.63 (0.01)</td>
<td>1.62 (0.01)</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate intake (g) (post-operative)</td>
<td>1.62 (0.01)</td>
<td>1.67 (0.01)</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein intake (g) (pre-operative)</td>
<td>0.82 (0.01)</td>
<td>0.87 (0.01)</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein intake (g) (post-operative)</td>
<td>0.81 (0.01)</td>
<td>0.87 (0.01)</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat intake (g) (pre-operative)</td>
<td>0.30 (0.01)</td>
<td>0.32 (0.01)</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat intake (g) (post-operative)</td>
<td>0.32 (0.01)</td>
<td>0.38 (0.01)</td>
<td>61.51</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Total intake (kJ) (pre-operative)</td>
<td>76.50 (0.62)</td>
<td>76.29 (0.57)</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total intake (kJ) (post-operative)</td>
<td>75.95 (0.56)</td>
<td>79.35 (0.57)</td>
<td>16.57</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Fat intake and total food intake expressed in terms of calories were found to be significantly different in GKO animals treated with intracerebroventricularly galanin compared to the animals that received saline. No other nutrients were found to be different between the groups nor was any difference in body mass noted.
which in turn leads to increased HFD intake. This mechanism would be absent in the GKO animals and thus may partially explain the increasing difference in HFD intake over time.

The GKO mouse provides a useful model to further explore the role of galanin in the regulation of macronutrient intake. We have generated three different datasets that clearly indicate that GAL is a selective regulator of fat intake. First, we have shown that in a macronutrient choice protocol the GKO mice select significantly less fat than WT animals. Second, when fed only with a HFD, the GKO animals eat significantly less than the WT controls and, consequently, do not gain as much mass. Finally, we demonstrated that the aversion to fat in the GKO animal is due to the absence of GAL centrally because we were able to partially and significantly reverse this effect by administering exogenous GAL directly into the brain. The lack of an increase in body mass in GAL-treated animals that consume an increased amount of fat may be due to several factors; however, our data indicate that this is not mediated by changes in RMR. Other factors that may contribute the lack of difference in body mass when treated with GAL include activity and dietary efficiency, which have not been measured in GKO mice to date. Our findings therefore support the original study regarding the effects of GAL on macronutrient intake profiles (21) and are consistent with the work of Yun et al. (38) who demonstrated that repeated i.v. injections of GAL stimulates feeding to a greater extent in animals maintained on a HFD than those maintained on a low fat diet.

Another mechanism whereby GAL may exert its effects on energy balance is via an effect on energy expenditure. We found a strong effect of body mass on RMR consistent with the effects reported in other strains of mice (28, 39–41). However, there were no significant differences in the RMR between the GKO and WT mice regarding other strains of mice (28, 39–41). This work was supported by a BBSRC CASE studentship in conjunction with AstraZeneca.

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Appendix A

Table A1. Diet Compositions (Ingredients Expressed as % of Total).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Diet A (%) (high cholesterol)</th>
<th>Diet B (%) (high protein)</th>
<th>Diet C (%) (high fat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry meal, fine</td>
<td>9.83</td>
<td>38.89</td>
<td>22.9</td>
</tr>
<tr>
<td>Maize gluten, yellow</td>
<td>9.83</td>
<td>38.89</td>
<td>22.9</td>
</tr>
<tr>
<td>Broken rice, parboiled</td>
<td>37.69</td>
<td>8.56</td>
<td>22.69</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>37.69</td>
<td>8.56</td>
<td>22.69</td>
</tr>
<tr>
<td>Beet pulp fibre</td>
<td>1.7</td>
<td>1.76</td>
<td>3.04</td>
</tr>
<tr>
<td>Brewer’s yeast pure</td>
<td>2.96</td>
<td>3.06</td>
<td>5.29</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.28</td>
<td>0.29</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Also included in all diets were all vitamins and minerals required for normal animal growth. Diet C was coated with a high fat vacuum coat to achieve the necessary fat content.

Appendix B

Table A2. Approximate Macronutrient Compositions of Diets.

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>Diet A (%) (high cholesterol)</th>
<th>Diet B (%) (high protein)</th>
<th>Diet C (%) (high fat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>5.9</td>
<td>9.6</td>
<td>28.6</td>
</tr>
<tr>
<td>Protein</td>
<td>24.1</td>
<td>44.0</td>
<td>25.7</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>55.6</td>
<td>29</td>
<td>32.1</td>
</tr>
</tbody>
</table>