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Perspective

Dysfunctional oleoylethanolamide signaling in a mouse model of Prader-Willi syndrome

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ABSTRACT

Prader-Willi syndrome (PWS), the leading genetic cause of obesity, is characterized by a striking hyperphagic behavior that can lead to obesity, type-2 diabetes, cardiovascular disease and death. The molecular mechanism underlying impaired satiety in PWS is unknown. Oleoylethanolamide (OEA) is a lipid mediator involved in the control of feeding, body weight and energy metabolism. OEA produced by small-intestinal enterocytes during dietary fat digestion activates type-α peroxisome proliferator-activated receptors (PPAR-α) to trigger an afferent signal that causes satiety. Emerging evidence from genetic and human laboratory studies suggests that deficits in OEA-mediated signaling might be implicated in human obesity. In the present study, we investigated whether OEA contributes to feeding dysregulation in *Magel2*$^{m+/p-}$ (*Magel2 KO*) mice, an animal model of PWS. Fasted/refed male *Magel2 KO* mice eat more than do their wild-type littermates and become overweight with age. Meal pattern analyses show that hyperphagia in *Magel2 KO* is due to increased meal size and meal duration rather than to lengthening of the intermeal interval, which is suggestive of a defect in mechanisms underlying satiation. Food-dependent OEA accumulation in jejunum and fasting OEA levels in plasma are significantly greater in *Magel2 KO* mice than in wild-type controls. Together, these findings indicate that deletion of the *Magel2* gene is accompanied by marked changes in OEA signaling. Importantly, intraperitoneal administration of OEA (10 mg/kg) significantly reduces food intake in fasted/refed *Magel2 KO* mice, pointing to a possible use of this natural compound to control hunger in PWS.

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1. Introduction

Prader-Willi syndrome (PWS) is the leading genetic cause of obesity. Children with PWS develop a striking hyperphagic behavior that leads, if left unchecked, to morbid obesity, type-2 diabetes, cardiovascular disease and premature death. Mice carrying a deletion of the *Magel2* gene (*Magel2 KO* mice), which is frequently deleted or mutated in individuals affected by the disease, also display a deficit in the ability to regulate food intake [1–3], but the molecular mechanism underlying this dysregulation is unknown.

Oleoylethanolamide (OEA) is a lipid messenger that controls feeding, body weight and lipid metabolism [4–7]. Unlike peptide gastrointestinal hormones (e.g. ghrelin and cholecystokinin), which are stored in and released from enteroendocrine cells, OEA is generated on-demand by small intestinal enterocytes during the digestion of dietary fats [8,9]. The biochemical pathway responsible for OEA formation and deactivation has been elucidated [6]. Its first step is the transfer of a fatty acid, oleic acid, from phosphatidylcholine (PC) to phosphatidylethanolamine (PE). This reaction is catalyzed by the *N*-acyl transferase PLA2G4E [10] and produces various forms of *N*-acyl-phosphatidylethanolamine (NAPE), including the OEA precursor *N*-oleoyl-phosphatidylethanolamine (NOPE).

Abbreviations: PWS, Prader–Willi syndrome; OEA, oleoylethanolamide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; NAT, *N*-acyl transferase; NAPE, N-acyl-phosphatidylethanolamine; NOPE, N-oleoyl-phosphatidylethanolamine; NAPE-PLD, N-acylphosphatidylethanolamine-selective phospholipase D; FAAH, fatty acid amide hydrolase; NAAA, *N*-acylethanolamine acid amidase; PPAR-α, peroxisome proliferator activated receptor-α; PEA, palmitoylethanolamide; FAE, fatty acid ethanolamide; LC–MS, liquid chromatography-mass spectrometry; SIM, selected ion monitoring; ANOVA, analysis of variance.

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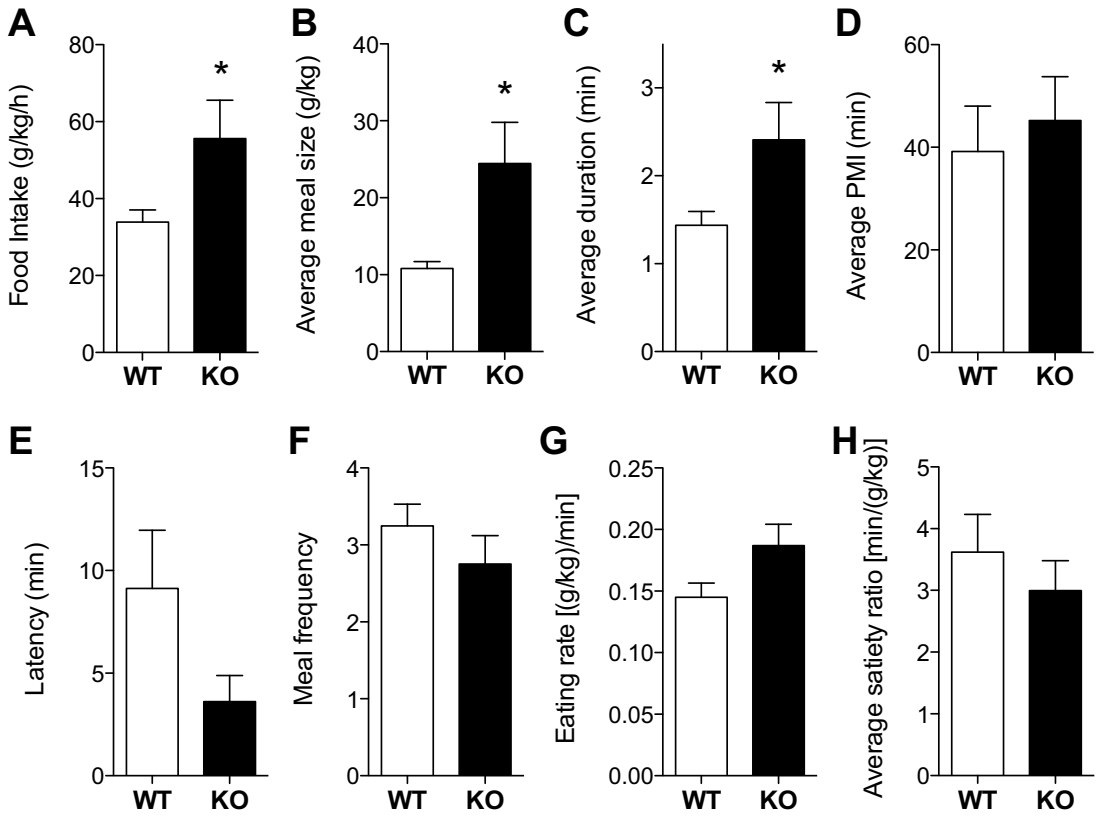

Fig. 1. Satiation deficits in fasted/re-fed *Magel2 KO* mice. (A) Food intake, (B) average meal size, (C) meal duration, (D) post-meal interval (PMI), (E) latency to feed, (F) meal frequency, (G) eating rate, and (H) satiety ratio in male *Magel2 KO* mice and their wild-type (WT) littermates. Meal patterns were measured after 24-h food deprivation. Results are expressed as mean ± SEM.*, $P < 0.05$ by unpaired Student's *t*-test (n = 12 per group).

Next, NOPE is hydrolyzed by *N*-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD) to generate OEA [11]. The biological actions of OEA are terminated by enzyme-mediated hydrolysis, which can be catalyzed by either of two intracellular amidases: fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine acid amidase (NAAA) [12–15].

OEA formation in the small intestine is primarily controlled by the animal's feeding status [8,16]. Studies have shown that intake of dietary fat is necessary and sufficient to trigger OEA production, which primarily occurs in duodenal and jejunal enterocytes. Experiments in which individual nutrients were infused separately into the rat duodenum have shown that dietary fat, rather than sugar or protein, is a potent stimulus for jejunal OEA production [9]. Additional investigations have demonstrated that enterocytes lining the lumen of the proximal gut internalize food-derived oleic acid and use it to produce NOPE and OEA [9].

OEA is a potent agonist of type-α peroxisome proliferator activated receptor (PPAR) and activation of this receptor mediates the compound's ability to cause satiety [16–20]. Accordingly, OEA-dependent hypophagia is abolished by PPAR-α deletion, is reproduced by administration of PPAR-α agonists, and is accompanied by changes in the expression of PPAR-α target genes [5]. Furthermore, the concentrations reached by the compound in the jejunum after feeding (300–400 nM) are sufficient to fully activate PPAR-α [8,21,22]. In addition to causing satiety, OEA also stimulates lipolysis in white adipose cells and hepatocytes, increases ketone body production, and enhances fatty acid oxidation in skeletal muscle cells through PPAR-α activation [23,24].

In the current study, we examined whether deficits in OEA-mediated satiety signaling might contribute to food intake dysregulation in *Magel2 KO* mice. Our results indicate that deletion of the *Magel2* gene mice interferes with OEA signaling and that administration of exogenous OEA normalizes feeding behavior in hyperphagic *Magel2 KO* mice.

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the Animal Care and Use Committee of the University of California, Irvine. Male *Magel2*$^{m+/p-}$ (C57BL/6-Magel2tm1Stw/J, *Magel2 KO*) mice and control wild-type mice obtained from the same colony were purchased from Jackson Laboratory (Bar Harbor, ME). The heterozygous *Magel2 KO* mice harbor a maternally inherited imprinted wild-type allele and a paternally inherited *Magel2-lacZ* knock-in allele that was constructed by gene-targeted replacement of the *Magel2* open reading frame with a *LacZ* reporter cassette [25,26]. Upon arrival, animals were acclimated for at least 1 week in our animal facility (temperature, 22 °C; humidity, 30–60%), with a controlled 12 h light/12 h dark cycle (on at 6:30 a.m., off at 6:30 p.m.). Animals were provided with *ad libitum* access to water and food (regular chow, 2020X, Harlan, Madison, WI), except for experiments that involved food deprivation.

2.2. Experimental design for food deprivation and re-feeding studies

Food intake was recorded using an automated monitoring system (Scipro, New York, NY), as described previously [17]. The system consists of 20 cages equipped with food baskets connected to weight sensors. Animals were housed individually in wired-

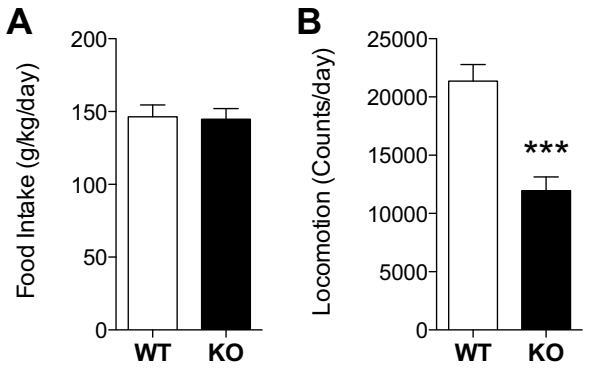

Fig. 2. Feeding and locomotion in free-feeding *Magel2 KO* mice. (A) Food intake and (B) locomotor activity in male *Magel2 KO* mice and their wild-type (WT) littermates. Activity was measured in the animal's home cage. Results are expressed as mean ± SEM. ***, $P < 0.001$ by unpaired Student's *t*-test (n = 5–7 per group).

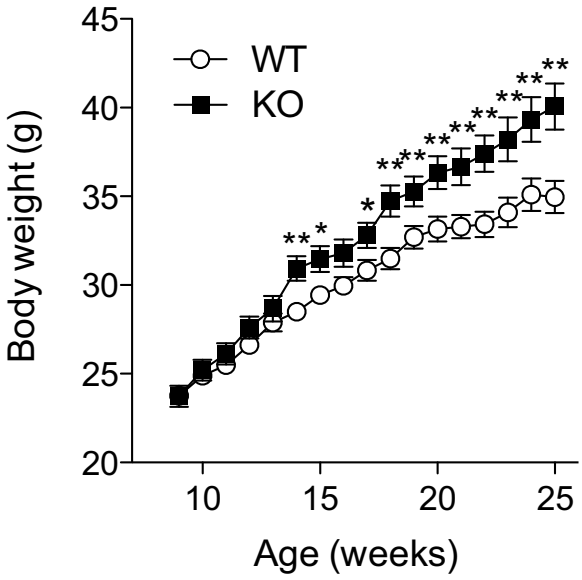

Fig. 3. *Magel2 KO* mice develop age-dependent weight increase. Body-weight gain in male *Magel2 KO* (filled squares) and wild-type (WT, circles) mice maintained on a standard diet. Results are expressed as mean ± SEM. *, $P < 0.05$ and **, $P < 0.01$ by unpaired Student's *t*-test (n = 15 per group).

bottom cages to avoid coprophagia and were acclimated to this housing condition for at least 3 days. Food deprivation was conducted for 24 h (from 9 a.m. to 9 a.m.). The animals were provided access to food and feeding behavior was recorded for 1 h. The following feeding parameters were analyzed: total food intake (g/kg)—the amount of food consumed during 24 h (free feeding) or first 1 h (food deprivation-re-feeding); feeding latency (min)—the time interval from the onset to the first eating episode; meal size (g/kg)—the amount of food consumed during the meal; eating rate [(g/kg)/min]; and meal frequency (meals/h). For biochemical experiments, animals were sacrificed after 30 min of re-feeding. Animals were anesthetized using isoflurane and the jejunum was collected, opened and rinsed with ice-cold phosphate-buffered saline. The mucosal layer was scraped onto a glass plate using glass slides, and immediately frozen in liquid N_2. All tissue samples were stored at −80 °C until time of processing.

2.3. Lipid extractions

Tissue (20–50 mg) from jejunum mucosa was homogenized in methanol (1 ml) containing the following internal standards: [$^2H_4$]OEA (100 pmol), [$^2H_4$]palmitoylethanolamide (PEA) (100 pmol) and [$^2H_4$]anandamide (10 pmol). Lipids were extracted using chloroform (2 ml) and water (1 ml) [27]. The organic phases were dried under N_2, reconstituted in chloroform (2 ml) and applied to open-bed silica gel columns to fractionate lipid groups based on polarity, as described previously [27]. Eluted fractions containing fatty acid ethanolamides (FAE) (chloroform/methanol, 9:1, v/v) were dried under N_2 and the residue was reconstituted in a solvent mixture of chloroform and methanol (1:3, v/v) for liquid chromatography-mass spectrometry (LC–MS) analyses.

2.4. FAE analyses

FAEs levels were measured using an LC system consisting of an Agilent 1100 system and 1946D mass spectrometer detector equipped with electrospray ionization interface (Agilent Technologies, Santa Clara, CA, USA). FAEs were separated on a ZORBAX Eclipse XDB-C18 column (2.1 × 100 mm, 1.8 μm, Agilent Technologies) using an acetonitrile gradient. Solvent A consisted of water containing 0.1% formic acid, and Solvent B consisted of acetonitrile containing 0.1% formic acid. The gradient profile of the solvents was as follows: 0–15 min, 65% B; 15–16 min, 65–100% B linear gradient; 16–26 min, 100% B; 26–28 min, 100–65% B linear gradient; 28–30 min, 65% B. The flow rate was 0.3 ml/min, and the column temperature was maintained at 15 °C. Electrospray ionization interface was in the positive ionization mode, capillary voltage was set at 3 kV, and the fragmentor voltage was set at 70 V. N_2 was used as a drying gas at a flow rate of 12 l/min and a temperature of 350 °C. The nebulizer pressure was set at 40 psi. Selected ion monitoring (SIM) mode was used to monitor protonated molecular ions $[M+H]^+$ of FAEs (endogenous and [$^2H_4$] standard). Absolute amounts of FAEs were quantified using a calibration curve.

2.5. OEA analyses in human plasma

This study was approved by the University of California,Irvine (UCI) Institutional Review Board (IRB) (IRB 2007- 5605) and signed IRB consents were obtained from the subjects >18 years or their parents if <the age of 18 years or not able to provide consent because of cognitive impairment. Children over the age of 7 years also provided written assent in addition to parental consent. Venous blood samples from 18 patients with PWS were collected by nurses in the ICTS (Institute of Clinical and Translational Science) at UCI from a periperal vein, spun down immediately and frozen until testing was performed. Patients had molecular confirmation of PWS (5 with maternal uniparental disomy, 13 with 15q11-13 deletions) and ranged in age from 9 to 21 y (mean 15.2 y). There were 14 males and 4 females. Anonymized samples were also obtained from 15 age and sex matched obese individuals. Plasma samples (1 ml) were spiked with 25 pmol of [$^2H_4$]-OEA and subjected to acetone precipitation of proteins. The supernatants were collected and their volumes were reduced under a stream of N_2. Lipids were extracted with chloroform/methanol (2:1, vol/vol), and chloroform phases were fractionated by open-bed silica column chromatography. Lipid fractions were reconstituted in methanol/chloroform (80 μl total) and analyzed by LC–MS as described above.

2.6. Statistical analyses

Results are expressed as mean ± SEM. Data were analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were considered significant if $P < 0.05$. Statistical significance was evaluated by unpaired Student's *t*-test, one-way analysis of variance (ANOVA) or two-way ANOVA, as appropriate. *Post hoc* analyses were conducted using

Table 1
Fatty acid ethanolamides in *Magel2 KO* mice. Levels of palmitoylethanolamide (PEA) and anandamide (AEA) in jejunum and plasma from male wild-type (WT) and *Magel2 KO* mice under food-deprived (FD) or re-fed (RF) conditions (n = 5–7 per group).

Genotype/condition	WT		*Magel2 KO*	
	FD	RF	FD	RF
Jejunum (pmol/g)				
PEA	2351 ± 564	1672 ± 356	2309 ± 413	1554 ± 195
AEA	30.6 ± 11.6	25.5 ± 4.1	46.5 ± 14.4	50.3 ± 11.2
Plasma (pmol/ml)				
PEA	637 ± 105	614 ± 64	768 ± 110	572 ± 55
AEA	2.6 ± 0.6	2.2 ± 0.1	3.3 ± 0.4	2.6 ± 0.2

Newman–Keuls multiple comparison test or Dunnett's test, to compare means when significant differences were found.

3. Results

Previous work has shown that mice carrying a deletion of the *Magel2* gene, which is frequently mutated in persons with PWS, display a deficit in the ability to regulate food intake [1–3]. Consistent with those data, we found that *Magel2 KO* mice that had been deprived of food for 24 h consumed significantly more food than did their wild-type littermates (wild-type, 33.88 ± 3.20; *Magel2 KO*, 55.63 ± 9.94 g/kg/h, P < 0.05; two-tailed Student's *t* test) (Fig. 1A). To determine the behavioral basis of this effect, we investigated the effect of *Magel2* gene deletion on meal patterns in fasted/refed and free-feeding mice. Following an established procedure [17,28], we assessed two categories of feeding parameters: 'first meal parameters' and 'average meal parameters'. The first meal parameters include latency of feeding onset (the time interval from trial inception to the first eating episode), first meal size (amount of food consumed during the first meal), first postmeal interval (the time interval between end of the first meal and beginning of the second meal) and first satiety ratio (the ratio between first postmeal interval and first meal size). Average meal parameters are the average of each meal parameter over all meals during the trial period, calculated for each animal. The analyses revealed that the increase in food intake observed in *Magel2 KO* mice is associated with significant increases in meal size and meal duration, without changes in average post-meal interval (Fig. 1B–D). No significant difference was noted between *Magel2 KO* and wild-type mice in other meal parameters (Fig. 1E–H), although a trend toward decreased latency, which did not reach statistical significance, was observed (Fig. 1E). Free-feeding male *Magel2 KO* mice maintained on a standard diet showed no overt change in food intake (Fig. 2A), but displayed reduced locomotion compared to wild-type controls (Fig. 2B). Together, the meal pattern alterations described above are suggestive of an impaired ability of *Magel2 KO* mice to generate feedback signals in response to feeding after a 24-h fast.

Until 14 weeks of age, the body weight of male *Magel2 KO* mice was identical to that of their wild-type littermates (at week 9: wild-type, 23.73 ± 0.41; *Magel2 KO*, 23.73 ± 0.60 g, n = 15 per genotype). Starting from week 14, however, the body-weight trajectory of *Magel2 KO* mice became significantly different from that of controls (wild-type, 28.47 ± 0.43; *Magel2 KO*, 30.93 ± 0.69 g at 14 week-old) (Fig. 3). At 25 weeks, *Magel2 KO* mice weighed about 15% more than their control littermates. Two-way ANOVA for body weight from 9 to 25 weeks of age gave the following results: F(genotype) = 85.92, Df = 1, $p < 0.0001$; F (age) = 65.74. Df = 16, $p < 0.0001$; F (interaction) = 1.993, Df = 16, $p < 0.012$, indicating that *Magel2 KO* mice develop an age-dependent overweight.

We hypothesized that deletion of the *Magel2* gene might alter food-induced OEA production in the jejunum [8,9]. Consistent with this prediction, we found that re-feeding evoked significantly higher OEA accumulation in the jejunum of *Magel2 KO* mice, relative to wild-type controls (Fig. 4A). Moreover, plasma OEA levels were significantly elevated in fasted *Magel2 KO* mice, compared to wild-type mice (Fig. 4B). No significant changes were found in other FAE species, including PEA (another PPAR-α agonist that is

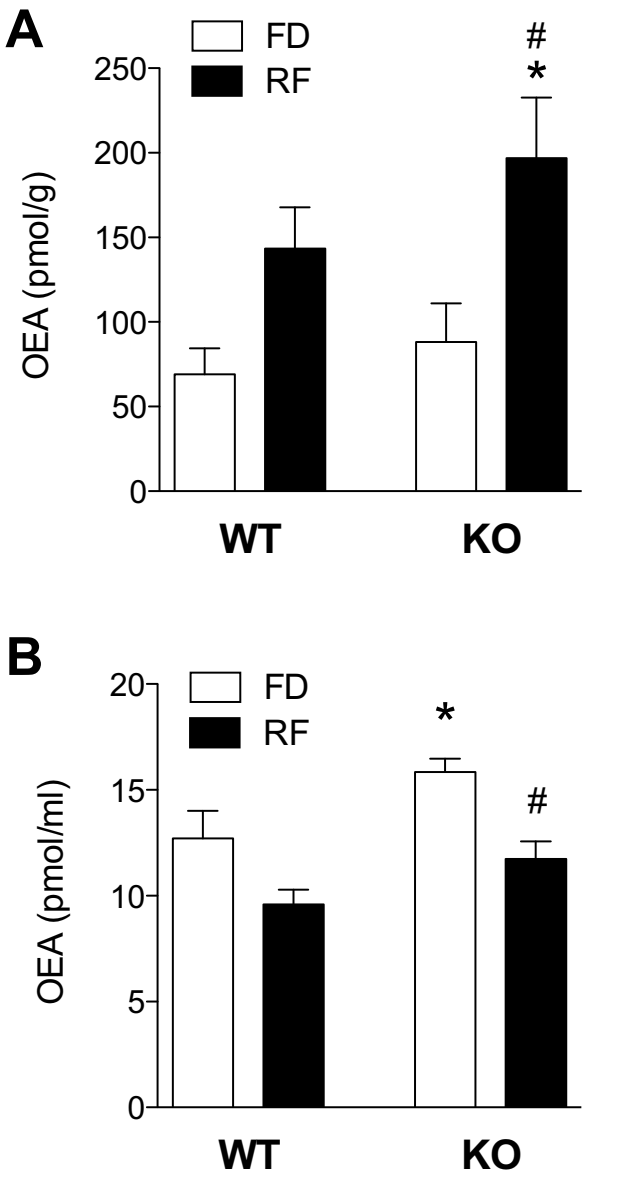

Fig. 4. Elevated OEA levels in *Magel2 KO* mice. OEA levels in (A) jejunum and (B) plasma (cardiac blood) of male wild-type (WT) and *Magel2 KO* mice under food-deprived (FD) or re-fed (RF) condition. Results are expressed as mean ± SEM. *, $P < 0.05$ compared to WT-FD; and #, $P < 0.05$ compared to KO-FD, by unpaired Student's *t*-test (n = 5–7 per group).

not primarily involved in feeding regulation) and anandamide (an endocannabinoid agonist), in the jejunum or plasma of *Magel2 KO* mice under food-deprived or re-fed conditions (Table 1).

Previous studies have shown that systemic administration of OEA inhibits food intake in free-feeding and food-deprived mice and rats [4,17]. We tested whether exogenous OEA is able to suppress feeding in *Magel2 KO* mice. The results show that intraperitoneal administration of OEA (10 mg-kg^{-1}) produced a significant reduction of food intake in *Magel2 KO* mice, compared to vehicle (Fig. 5A). This effect was attributable to a decrease in meal size (Fig. 5B) and an accompanying increase in satiety ratio (post-meal interval/meal size)(Fig. 5C). In the wild-type littermates used as control for *Magel2 KO* mutants, the same dose of OEA did not produce any significant effect on food intake (Fig. 5A–C).

Finally, we asked whether circulating OEA levels might be altered in persons with PWS. We used LC–MS to measure the concentration of OEA in plasma of 18 children with PWS and 15 age-matched control children. The results show that PWS is accompanied by a significant elevation in plasma OEA levels (Fig. 6).

4. Discussion

In the present study, we report that mice carrying a deletion of the *Magel2* gene, which is relatively common in persons with PWS, eat more than do their wild-type littermates, and become overweight with age. Importantly, our meal pattern analyses show that this enhanced feeding response results from increased meal size and meal duration, which is suggestive of an impaired ability to generate feedback signals in response to food ingestion. Furthermore, we found that systemic administration of exogenous OEA reduces food intake and heightens satiety in *Magel2 KO* mice. Together, the results suggest that *Magel2* gene deletion is associated with dysfunctional OEA-mediated satiety signaling, and that supplementation with exogenous OEA normalizes feeding in this mouse model of PWS.

Current theories emphasize three non-exclusive mechanisms as potential causes of hyperphagia in PWS: (i) deficits in the ability to generate satiety signals in response to feeding; (ii) dysfunctions of hypothalamic centers that control energy homeostasis; and/or (iii) abnormally high activation of reward pathways in the brain by food-related stimuli [1,29]. PWS is caused by the absence of paternally expressed, maternally silenced genes at 15q11–q13. Inactivating mutations in one Prader-Willi syndrome candidate gene, *MAGEL2*, cause a Prader-Willi-like syndrome called Schaaf-Yang syndrome, highlighting the importance of loss of *MAGEL2* in Prader-Willi syndrome phenotypes [30–33]. The *MAGEL2* gene is frequently deleted or mutated in persons with PWS, and consistently, *Magel2 KO* mice also show increased food intake [1–3]. The protein encoded for by the *Magel2* gene is part of a ubiquitin ligase complex involved in the control of cell vesicle trafficking [34], but the molecular mechanism through which this protein might regulate energy balance is unknown. The present study demonstrates that removal of the *Magel2* gene enhances food intake by increasing meal size and meal duration, without changing post-meal interval or feeding frequency. This pattern of effects suggests that the predominant feeding abnormality in *Magel2 KO* mice involves mechanisms underlying the control of satiation (the phasic termination of feeding resulting from the act of food ingestion) rather than satiety (the tonic state of inhibition over eating). This result is consistent with the high levels of expression of *Magel2* in the hypothalamus [35,36], a brain region that is crucially involved in the control of satiation and satiety [6].

A substantial body of evidence indicates that OEA inhibits food intake in mice and rats [9,17]. Indeed, OEA-mediated signaling has been documented throughout vertebrate phylogeny [16–20,23,24]

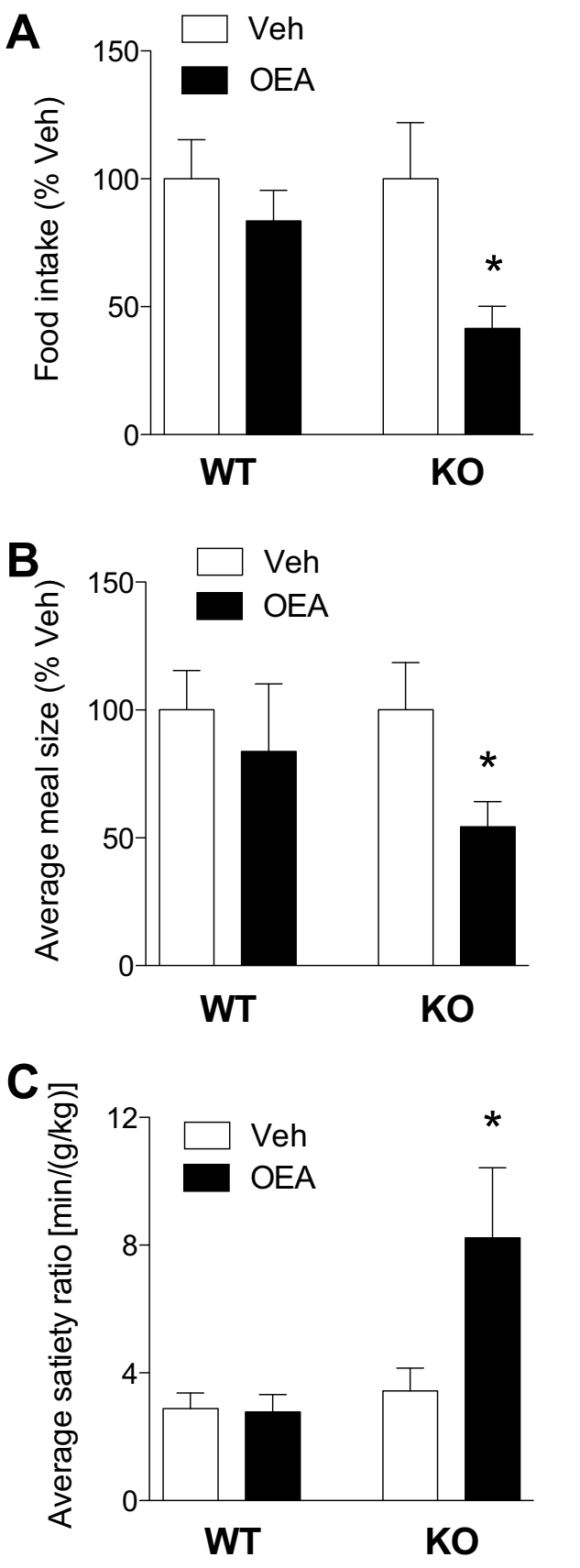

Fig. 5. OEA normalizes food intake in *Magel2 KO* mice. Effects of vehicle (open bars) or OEA (10 mg/kg, intraperitoneal; closed bars) on (A) total food intake, (B) meal size and (C) satiety ratio in male *Magel2 KO* mice and their wild-type (WT) littermates. Results are expressed as mean ± SEM. *, $P < 0.05$ compared to KO-Veh, by unpaired Student's *t*-test (n = 14–15 per group).

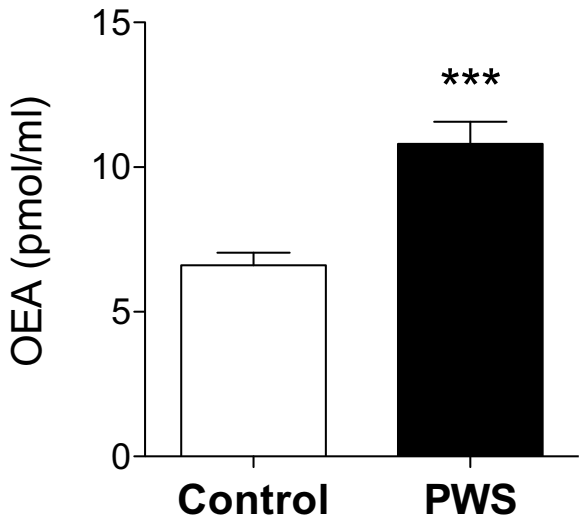

Fig. 6. Plasma OEA levels in children with PWS and normal children. Results are expressed as mean ± SEM. ***, $P < 0.001$ by unpaired Student's *t*-test (n = 15 for controls, and 18 for PWS).

and is likely to exist also in humans [37,38]. Importantly, recent evidence from genetic and human laboratory studies indicates that deficits in the activity of this signaling system may be implicated in human obesity. Studies in a Norwegian population-based cohort have associated a single-nucleotide polymorphism in the gene encoding for *NAPE-PLD*, the enzyme responsible for the production of OEA, with severe obesity [39]. Furthermore, functional imaging experiments have suggested a role for OEA in the hedonic regulation of food craving and obesity in humans [40]. The present results show that deletion of the PWS-related gene, *Magel2*, is accompanied by a paradoxical enhancement of food-induced OEA production in the jejunum, as well as by increased OEA levels in plasma. Significantly elevated OEA levels were also found in plasma obtained from a sample of 18 children with PWS, and compared to 15 age-matched controls. We interpret this enhancement in OEA signaling as an attempt to compensate for the loss of satiating factors, the expression of which is impaired following *Magel2* deletion. The identity of these hypothetical factors is unknown, however. It is notable that a recent study found an abnormal increase in 2-arachidonoylglycerol (2-AG) system, which may contribute to hyperphagia and obesity in *Magel2*-null mice [41]. Interestingly, the changes in OEA production found in *Magel2*-null mice are markedly different from those seen in mice that are made obese either by exposure to a high-fat diet or by genetic deletion of leptin (*ob/ob* mice). In diet-induced obese mice, the enzymatic machinery involved in OEA formation is dramatically suppressed, whereas in *ob/ob* obese mice it remains unchanged [42,43].

Our results also indicate that administration of exogenous OEA lowers food intake in *Magel2 KO* mice, suggesting that the main effector systems engaged by this lipid mediator are not affected by *Magel2* deletion, and that OEA may help attenuate the life-threatening hyperphagia experienced by persons with PWS. It is worth noting that OEA is a naturally occurring compound and is present at relatively high concentrations in several foods as well as in the human body [44,45]. Thus, if shown to be safe and effective, OEA may qualify to be used as dietary supplement or medical food, rather than a conventional medication, which might expedite its regulatory approval.

5. Conclusions

Small-intestinal OEA signaling is abnormally elevated in *Magel2 KO* mice, possibly resulting from deficits in other signaling mechanisms underlying the control of satiation in this mouse model of PWS. Administration of OEA as a drug normalizes feeding behavior in *Magel2 KO* mice, pointing to a possible therapeutic use of this naturally occurring lipid in the management of PWS.

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