Abnormal Response to the Anorexic Effect of GHS-R Inhibitors and Exenatide in Male Snord116 Deletion Mouse Model for Prader-Willi Syndrome

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Prader-Willi syndrome (PWS) is a genetic disease characterized by persistent hunger and hyperphagia. The lack of the Snord116 small nucleolar RNA cluster has been identified as the major contributor to PWS symptoms. The Snord116 deletion (Snord116del) mouse model manifested a subset of PWS symptoms including hyperphagia and hyperghrelinemia. In this study, male Snord116del mice were characterized and tested for their acute and chronic responses to anorexic substances related to the ghrelin pathway. In comparison with their wild-type littermates, the food intake rate of Snord116del mice was 14% higher when fed ad libitum, and 32% to 49% higher within 12 hours after fasting. Fasted Snord116del mice were less sensitive to the acute anorexic effect of competitive antagonist [D-Lys3]-GHRP6, YIL-781, and reverse agonist [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P (SPA) of ghrelin receptor GHS-R. All 3 GHS-R inhibitors failed to inhibit chronic food intake of either Snord116del or wild-type mice due to rapid adaptation. Although fasted Snord116del mice had normal sensitivity to the acute anorexic effect of glucagon-like peptide 1 receptor agonist exenatide, those fed ad libitum required a higher dose and more frequent delivery to achieve ~15% suppression of long-term food intake in comparison with wild-type mice. Ghrelin, however, is unlikely to be essential for the anorexic effect of exenatide in fed mice, as shown by the fact that exenatide did not reduce ghrelin levels in fed mice and food intake of ghrelin−/− mice fed ad libitum could be suppressed by exenatide. In conclusion, this study suggests that GHS-R may not be an effective therapeutic target, and in contrast, exenatide may produce anorexic effect in PWS individuals. (Endocrinology 155: 2355–2362, 2014)
anxiety and deficiency in motor learning ability (10). Although not obese (10, 11), Snord116 deletion (Snord116del) mice are hyperghrelinemic and moderately hyperphagic (10), and may serve as a PWS model for testing drug candidates.

Ghrelin is an orexigenic peptide hormone (12), and the intriguing discovery that circulating ghrelin levels were elevated in PWS (13–15) had led to the proposal that hyperghrelinemia might be the cause for hyperphagia. Effective suppression of ghrelin levels in PWS had been achieved by somatostatin or its analog octreotide, but hyperphagia was not alleviated, since somatostatin suppressed anorexic hormones as well (16–18). For the past decade, specific antagonists of ghrelin receptor GHS-R were proposed as promising candidates for treating hyperphagia in PWS (19, 20), yet they have not been tested.

Exenatide is an agonist for glucagon-like peptide 1 receptor (GLP-1R). It was shown to reduce ghrelin levels in fasted rats, and therefore was suggested as a candidate drug for hyperphagia in PWS (21). Recently, the beneficial effects of GLP-1R agonist exenatide and liraglutide on appetite and weight control were reported in three isolated PWS cases (22–24). However, two of the studies were complicated by the cotreatment with metformin (22, 23), and appetite and weight control were reported in three isolated PWS cases (22–24). However, two of the studies were complicated by the cotreatment with metformin (22, 23), which by itself suppresses appetite (25). Hence the effect of GLP-1R agonist alone on PWS requires further evaluation.

In this study, four drug candidates were tested for their anorexic effect on Snord116del mice. The acute and long-term experiments showed that all of them had reduced anorexic efficacy in Snord116del mice, highlighting their abnormal feeding regulation.

**Materials and Methods**

**Animals**

All animal experiments were approved by the Xiamen University Ethical Committee on Animal Research. Mice were housed in temperature- and moisture-controlled facility with free access to water and standard chow unless indicated otherwise. Snord116del mice (JAX: B6(Cg)-Snord116tm1.1Uta/J) were obtained from Uta Francke lab (10). Male Snord116del mice and their wide-type littermates of 6–12 months on congenic C57/BL6 obtained from Uta Francke lab (10). Male mice were housed in temperature- and moisture-controlled facility with free access to water and standard chow unless indicated otherwise. Snord116del mice (JAX: B6(Cg)-Snord116tm1.1Uta/J) were obtained from Uta Francke lab (10). Male Snord116del mice and their wide-type littermates of 6–12 months on congenic C57/BL6 background, and ghrelin-/—mice of 3–6 months on conegenic C57/BL6 background (26, 27) were used.

**Test substances**

Peptides were synthesized by Bambio Inc. The purity was 99.43% for [D-Lys3]-GHRP6, 99.10% for [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P (SPA), and 95.0% for exenatide acetate. They were dissolved in PBS, frozen, and thawed once before use. YIL-781 (Adoqol Bioscience) was dissolved in PEG400/10 mM methanesulfonic acid (80:20, v/v).

**Measurement of food intake**

Mice were individually caged and handled daily for 5 days for acclimation. The peptides were injected ip. After 24 hr fasting from food, 5 μl/g solution were injected. Food were provided and weighed at indicated time. For long-term studies, mice were acclimated by PBS injection at the onset of dark phase for 5 days, followed by daily injection of test solution, and recovered with daily PBS injection.

YIL-781 was delivered by oral gavage 5 hours before provision of food in fasted mice. For long-term study, mice were acclimated to oral gavage at onset of dark phase for 5 days. To control for potential adverse effect of oral gavage on ingestion, mice receiving solvent were included.

**Blood glucose and ghrelin measurements**

Tail tips were incised, and after discarding two drops of blood, the third drop was used for glucose measurement with OneTouch Ultra glucometer (Life First). Serum from tail blood was measured with Rat/Mouse total Ghrelin ELISA kit (Millipore) according to manufacturer’s instruction.

**Data analysis**

Food intake rate was calculated as [weight of food intake/(body weight)0.6667] (28). All data were analyzed by Graphpad Prism for 1-way or 2-way ANOVA, and presented as (mean±SEM).

**Results**

**Feeding abnormalities of Snord116del mice**

Snord116del mice were moderately hyperphagic when housed in Columbus CLAMS metabolic cages, which were small (<1/4 of footprint of regular cage) without bedding (10). In the current study, food intake was measured in regular cages (Figure 1A). For the first 2 days upon single housing, Snord116del mice ate more than wild-type littermates, although both ate less in comparison to later days, possibly due to that the stress in novel environment suppressed appetite (25). Hence the effect of GLP-1R agonist alone on PWS requires further evaluation.

In this study, four drug candidates were tested for their anorexic effect on Snord116del mice. The acute and long-term experiments showed that all of them had reduced anorexic efficacy in Snord116del mice, highlighting their abnormal feeding regulation.
Hence, Snord116del mice are abnormal in feeding and weight maintenance.

Effect of [d-Lys³]-GHRP6

It was reported that 6 μmol/kg [D-Lys³]-GHRP6, a competitive antagonist for GHS-R, decreased food intake and body weight in ob/ob obese mice over 6 days (19). Three to 12 μmol/kg [D-Lys³]-GHRP6 (Figure 2A) significantly reduced food intake of fasted wild-type mice (F = 6.217, df = 3, P < .0001 for dose), but not Snord116del mice (F = 2.394, P > .05) by 2-way ANOVA of time and dose.

Daily injection of 12 μmol/kg [D-Lys³]-GHRP6 in mice fed ad libitum, which mimics PWS individuals living without food restriction, did not suppress intake of Snord116del or wild-type mice (Supplemental Figure 2A). On day 1, food intake of Snord116del mice was inhibited only at the first hour (P < .01) but recovered by the seventh hour, and the suppressive effect of the initial hour disappeared by day 2 (Figure 2B). A higher dose of 24 μmol/kg [D-Lys³]-GHRP6 led to severe toxicity as indicated by that 3 out of 20 mice died, and those survived showed reduced motility right after injection. Hence, [D-Lys³]-GHRP6 is not an effective treatment for suppressing chronic food intake of Snord116del mice.

Effect of SPA

Next, we tested whether reverse agonist for GHS-R, SPA, would be more effective, since SPA’s binding to GHS-R is less affected by ghrelin concentration (29) and it has a longer half-life in circulation (30). Based on the previous study which used up to 6.6 μmol/kg SPA (30), we tested a range of 3–9 μmol/kg. SPA significantly reduced intake of fasted wild-type (F = 8.295, df = 3, P < .0001) and Snord116del mice (F = 16.04, P < .0001) (Figure 2C). At 3 μmol/kg, SPA significantly reduced intake of wild-type mice (P < .001), but marginally for Snord116del mice (P = .04).

Daily injection of 4.5 μmol/kg SPA in mice fed ad libitum did not suppress food intake in wild-type or Snord116del mice (Supplemental Figure 2B). No significant inhibition of 7hr cumulative intake was observed on any day for Snord116del mice, whereas wild-type mice had decreased 7hr intake (P < .05) only on day 1 and 2 (Figure 2D). When SPA was increased to 9 μmol/kg, 4 out of 20 mice died, and the rest showed little movement after injection, indicative of severe toxicity. Hence, SPA is not an effective anorexic treatment for these mice, either.

Effect of YIL-781

Both [D-Lys³]-GHRP6 and SPA have nonspecific effects on other receptors (31, 32). To further evaluate the anorexic effect of GHS-R inhibition, YIL-781, a quinazoline derivative with improved specificity (33), was tested. Food intake of fasted wild-type mice was moderately suppressed by 134 μmol/kg YIL-781 between 6–12 hr (P < .05) (Figure 2E and Supplemental Figure 2C), consistent with the ~14 hr half-life of YIL-781 in circulation (33). No significant suppression of intake were observed for fasted Snord116del mice.

YIL-781 was found to reduce body weight of diet-induced-obese mice at 67 μmol/kg (33). When wild-type and Snord116del mice fed ad libitum received daily delivery of 134 μmol/kg YIL-781, reduced intake was observed only on day 1, but not thereafter in comparison to those receiving solvent (Figure 2F). Since the amount used was ~50- and ~10-fold more than the dose required to reach Ki in circulation and brain, respectively (33), we conclude...
that YIL-781 is ineffective in suppress long-term intake in these mice.

**Effect of exenatide**

When 3–12 μg/kg (0.7–2.9 nmol/kg) exenatide were injected in fasted mice, suppressed food intake were observed (Figure 3C) for both wild-type and Snord116del mice (F = 17.11 and 11.68, respectively, df = 3, P < .0001). Remarkably, Snord116del mice were as sensitive as wild-type mice to the acute effect of exenatide (F 0.5, df = 3 for genotype and dose interaction). When mice fed ad libitum were injected daily with 12 μg/kg (2.9 nmol/kg) exenatide (Supplemental Figure 3A), overall intake of wild-type mice decreased to 85.6 ± 3.1% (P < .0005) of their regular levels, and that of Snord116del mice reduced to 92.0 ± 1.9% (P < .01) (Figure 3B). Wild-type mice had reduced intake for both 0–6 hr and 7–12 hr intervals after exenatide injection; whereas Snord116del mice only had reduced intake for 0–6 hr (Supplemental Figure 3C, right). Hourly analysis revealed that intake of wide-type mice was inhibited continuously for 7 hr, but that of Snord116del recovered on the seventh hour (Supplemental Figure 3C, left, P < .0005 for genotype).

Once daily injection of 24 μg/kg (5.7 nmol/kg) exenatide (Supplemental Figure 3B) had similar anorexic effect as 12 μg/kg exenatide on both genotypes, 86.9 ±
1.6% (P < .0001) and 92.5 ± 3.6% (P < .05) for wild-type and Snord116del mice, respectively (Figure 3C). Although feeding suppression lasted for 12 hr in Snord116del mice (Supplemental Figure 3D, left), consumption in the following light phase increased (right). Even once daily injection of 48 μg/kg (11.5 nmol/kg) exenatide did not prevent light phase compensatory feeding in Snord116del mice (Supplemental Figure 3E).

To suppress compensatory feeding in the light phase, 24 μg/kg exenatide was injected at the onset of dark and light phases for 17 days (Figure 3E and Supplemental Figure 3F). Notably, intakes of wild-type and Snord116del mice were reduced to 84.6 ± 1.4% and 83.5 ± 1.3% (P < .0001), respectively (Figure 3D). Reduced intake, however, did not result in weight loss (Figure 3F and Supplemental Figure 3G). After an initial decline, the weight of wild-type mice returned to normal, with the average of last 3 days of treatment at 99.7 ± 1.1% of their original weight; whereas that of Snord116del mice increased to 104.3 ± 1.6% (P < .05) (Figure 3F), consistent with their better weight maintenance upon feeding restriction (Figure 1C).

Role of ghrelin in exenatide’s anorexic effect on fed mice

Next, we addressed whether exenatide suppressed food intake in fed mice by decreasing ghrelin levels. Exenatide significantly reduced ghrelin levels in fasted (Figure 4A) but not fed mice (Figure 4B), although their blood glucose levels were significantly decreased (Supplemental Figure 4, A and B). Moreover, when ghrelin−/− mice were treated with 12 μg/kg exenatide for 11 days (Figure 4C, left), their overall intake were reduced to 79.3 ± 3.8% (P < .005) (Figure 4C). Hence, exenatide could suppress food intake independent of ghrelin in mice fed ad libitum.
Discussion

In this study, four structurally and functionally distinct anorectic substances were tested for the first time in a mouse model for Prader-Willi syndrome. Although neither hypotonic or obese, Snord116del mice showed an intriguing subset of PWS-like phenotypes, such as growth retardation, hyperghrelinemia, and feeding abnormality (10), which is further evident by their reduced sensitivity to all 4 tested anorectic substances (Figures 2 and 3). It has been noted that mutations in human and mouse orthologues may not always manifest the same phenotypes (34, 35). The discrepancy may lie in the basic difference in metabolism of the two species. For instance, the daily intake of dry food in human is ~1.3% of the body weight (2000–2500 kcal/d), whereas a mouse consumes 13% of their body weight (~4 g rodent chow). Hence, the regulation of the ingestive and digestive functions may differ. Nevertheless, the study of endophenotypes in mouse models is invaluable for elucidating the molecular mechanism and developing therapeutic treatments for human disorders (36, 37). In this regard, Snord116del mice may serve as a useful model in preliminary testing of drug candidates.

Three different GHS-R inhibitors, namely [D-Lys³]-GHRP6, SPA, and YIL-781, suppressed acute food intake in fasted wild-type mice but showed reduced efficacy in Snord116del mice (Figure 2 A, C, and E). In long term studies, however, they were ineffective for both genotypes due to desensitization in 1–2 days (Figure 2 and Supplemental Figure 2), suggesting that fast adaptability might be an intrinsic feature for ghrelin pathway in these mice. Although decreased intake and body weight were observed for [D-Lys³]-GHRP6 treatment in obese ob/ob mice (19), or YIL-781 in diet-induced-obese mice (33), we found no evidence that GHS-R might a therapeutic target for PWS.

Encouragingly, food intake of Snord116del was reduced by ~8% upon once daily injection of 12 or 24 μg/kg exenatide (Figure 3, B and C and Supplemental Figure 3, A and B), and by ~15% upon twice daily injection of 24 μg/kg exenatide (Figure 3 D and E). Ghrelin levels in these mice, however, was not significantly changed by exenatide (Figure 4B). Moreover, ghrelin⁻/⁻ mice were also sensitive to the anorectic effect of exenatide (Figure 4C). Furthermore, a single injection of exenatide after a meal was found to suppress appetite without affecting ghrelin levels in PWS and control obese subjects (38). These observations indicate that ghrelin is not essential for the anorectic effect of exenatide on nonfasted individuals, although it decreased ghrelin levels to reduce intake in fasted individuals (21).

Exenatide has been shown to reduced body weight of obese rodents (39–43). In human, recent studies demonstrated that GLP-1R agonists led to weight loss in overweight and obese patients (44). Although exenatide did not reduce body weight of Snord116del mice (Figure 3F), it may do so in obese PWS individuals with the additional benefit on glycemic control, as supported by the positive outcome of the three case reports (22–24). For lean PWS subjects, who are under strict feeding constrains, exenatide may relieve persistent hunger and therefore improve their psychological well-being. The current study suggests that PWS individuals may require a higher dose of exenatide with more frequent delivery for effective suppression of appetite in comparison to those without the genetic condition. In clinical practice, the once-weekly (44) or the oral delivery type of GLP-1R agonists (45) might be considered instead of frequent injections. The safety for such treatment, however, must be carefully evaluated.

Why were Snord116del mice less sensitive to the anorectic effect of all four substances? In the case of GHS-R competitive inhibitor [D-Lys³]-GHRP6 or YIL-781 (Figure 2, A and E), a higher dose might be required to compete with elevated ghrelin in Snord116del mice. For reverse antagonist SPA, however, its effect should not be influenced much by ghrelin levels (29). The reduced sensitivity of Snord116del mice to SPA (Figure 2C) suggests that mechanism other than hyperghrelinemia might contribute to their excessive orexigenic drive.

Could defect in GLP-1 pathway cause hyperphagia in Snord116del mice? Fasted Snord116del mice had a normal response to exenatide (Figure 3A), and those fed ad libitum showed a normal feeding suppression for the initial hours after exenatide injection (Supplemental Figure 3, C–E). Moreover, GLP-1 levels in PWS were comparable...
to those in nonsyndromic obese subjects (46). These observations suggest that GLP-1/GLP-1R system is likely to be intact in Snord116del mice. Notably, the reduced efficacy of exenatide on fed Snord116del mice is due to faster recovery from feeding suppression (Supplemental Figure 3C), indicating that excessive orexigenic drive prevails once exenatide subsides. The molecular and cellular nature of such drive, however, remains elusive. Snord116 RNA is expressed in brain (47), with a higher expression in specific hypothalamic nuclei (48). Microarray profiling showed that hypothalamic gene expression of young Snord116del mice was apparently normal (49). In future, thorough evaluation of feeding regulators in specific brain regions may shed light on the cellular defects in PWS.

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