Characterization of the reproductive effects of the anorexigenic VGF-derived peptide TLQP-21: in vivo and in vitro studies in male rats

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TLQP-21 is a 68-kDa protein encoded by the homonymous gene, which is expressed abundantly at the hypothalamus and has been involved in the control of metabolism and body weight homeostasis. Different active peptide fragments are generated from VGF, including TLQP-21. Circumstantial evidence has suggested that VGF might also participate in the control of reproduction. Yet its mechanisms of action and the eventual role of specific VGF-derived peptides on the hypothalamic-pituitary-gonadal (HPG) axis remain unknown. Herein we report a series of studies on the reproductive effects of TLQP-21 as evaluated in male rats by a combination of in vivo and in vitro analyses. Central administration of TLQP-21 induced acute gonadotropin responses in pubertal and adult male rats, likely via stimulation of GnRH secretion, as documented by static incubations of hypothalamic tissue. In addition, in pubertal (but not adult) males, TLQP-21 stimulated LH secretion directly at the pituitary level. Repeated central administration of TLQP-21 to pubertal males subjected to chronic undernutrition was able to ameliorate the hypergonadotropic state induced by food deprivation. In contrast, chronic administration of TLQP-21 to fed males at puberty resulted in partial desensitization and puberty delay. Finally, in adult (but not pubertal) males, TLQP-21 enhanced hCG-stimulated testosterone secretion by testicular tissue in vitro. In summary, our data are the first to document a complex and multifaceted mode of action of TLQP-21 at different levels of the HPG axis with predominant stimulatory effects, thus providing a tenable basis for the (direct) reproductive role of this VGF-derived peptide.

VGF was identified initially as a nerve growth factor-inducible transcript in PC12 cells (18). This was later shown to be distributed widely in hypothalamus, pituitary, and several peripheral tissues (19, 33, 35). The entire protein encoded by the VGF gene comprises 615 amino acids in the human and 617 amino acids in the rat and mouse. The VGF protein contains several consensus cleavage sites for protein convertases that predict the multiple proteolytic cleavage of the precursor. Indeed, different peptide fragments have been proposed to derive from VGF, including NAPP-129, neuroendocrine regulatory peptide (NERP)-I, NERP-2, TLQP-62, TLQP-21, AQEE-30, and LQEQ-19 (19, 33, 41).

Initial efforts in VGF research were focused in the characterization of its potential involvement in energy homeostasis as well as in its putative role in central cognitive functions and depression (1, 16, 20, 38). To dissect out these eventual physiological roles, Vgf-null mice were generated. Phenotypic analyses of these lines revealed that Vgfknockout (KO) mice are small, hypermetabolic, and hyperactive, thus suggesting a role of VGF peptides in metabolic control (11). Indeed, the catabolic state induced by the congenital lack of VGF prevented overweight even in the presence of potent obesogenic factors, such as high-fat diet, and resulted in decreased body fat content and leptin expression (12). Nonetheless, these analyses also unveiled a significant complexity of the function of VGF system, since the hypermetabolic phenotype associated with null mutations of Vgf gene was similar to that evoked by the pharmacological administration of some of its peptide fragments, such as TLQP-21 (see below).

Circumstantial evidence has suggested that VGF peptides might also be involved in the control of reproduction, since Vgf-KO mice displayed delayed puberty, reduced fertility, and decreased ovarian and uterus weights, with the absence of mature follicles or corpora lutea, as indices of failure of ovulation (11). A central defect of the hypothalamic-pituitary-gonadal (HPG) axis in conditions of VGF deficiency was suggested by the observation that mRNA and protein contents of LH and FSH β-subunits at pituitary levels were reduced in Vgf-null mice (11). Of important note, however, is that such a plethora of reproductive defects was considered to be secondary to the primary metabolic alterations observed in this model and explained through reduced serum leptin levels (11). Whether additional mechanisms, linked to the absence of VGF itself, contribute to the reproductive phenotype of Vgf-null animals remains unknown.

Among the different VGF-derived peptides, TLQP-21 has drawn considerable attention as a potential regulator of energy homeostasis in certain species and physiological settings. Thus, Bartolomucci and colleagues (1, 2) described that intracerebro-

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ventricular (icv) injection of 15 μg of TLQP-21 for 14 days increased energy expenditure and prevented the early phase of diet-induced obesity. These results suggested a catabolic role of this peptide fragment opposite to the predominant anabolic function of VGF suggested by KO studies (11). Indeed, as observed for vgf−/− mice, TLQP-21-treated mice displayed hyperthermia, increased lipolysis, and enhanced energy expenditure and tissue sensitivity to insulin (2, 11, 42). These observations have been later confirmed by studies showing that chronic treatment with TLQP-21 caused a sustained reduction in food intake and body weight and decreased abdominal fat depots in Syrian hamsters (17).

Despite the suggested neuroendocrine roles of VGF peptides, there is a substantial paucity of data on their potential roles in the control of hypothalamic-pituitary secretion. Indeed, to our knowledge, studies on the neurosecretory activity of this family have been limited so far to the demonstration of the ability of NERPs to suppress hypertonic saline- or angiotensin II-induced vasopressin release from hypothalamus and the pituitary (40). In addition, specific analyses have suggested that neither VGF nor TLQP-21 has an impact on the growth hormone-IGF-I axis in pigs or mice (3, 32). Considering the previous proposal for a reproductive role of VGF-derived peptides, and given the specific biological features of TLQP-21 (as one of the major products of VGF with distinct metabolic effects), in this work we intended to evaluate the potential reproductive effects of this peptide as assessed by a combination of in vivo and in vitro approaches in male rats, with the ultimate aim to provide new mechanistic insights for the effects of VGF deficiency on the HPG axis.

MATERIALS AND METHODS

Animals and drugs. Wistar male rats, bred in the vivarium of the University of Córdoba, were used. Animals were kept under controlled conditions of light (10 h of darkness, lights on at 0500) and temperature (22°C). On day 1 of life, each dam was left with 10 pups. Animals were weaned at 21 days of age in groups of four rats per cage until initiation of the experimental procedures. Experimental procedures were approved by the Córdoba University Ethics Committee and in accordance with the European Union normative for care and use of experimental animals. Experiments were carried out between 1000 and 1200. Special care was taken to avoid any stressing influences upon the animals (all animals were handled daily before the experiment and euthanized by the same person, and different drugs were used at random). TLQP-21 was purchased from Tocris (Bristol, UK), gonadotropin-releasing hormone (GnRH) was purchased from Sigma (Barcelona, Spain), and human chorionic gonadotropin (hCG; Profasi) was obtained from Serono (Madrid, Spain). For in vivo experiments, TLQP-21 was dissolved in physiological saline immediately before use. For in vitro experiments, TLQP-21, GnRH, and hCG were dissolved in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose and without L-glutamine and phenol red (BioWhittaker, Verviers, Belgium) immediately before use. Experimental design. Assessment of the role of TLQP-21 in the control of reproductive function in male rats was carried out using different experimental approaches. In a first group of experiments, the potential functional role of TLQP-21 signaling in the control of gonadotropin secretion was explored in prepubertal rats. To this end, in experiment 1, 22-day-old male rats were implanted under light ether anesthesia with icv cannulae into the lateral cerebral ventricle. The cannulae were lowered to a depth of 3 mm beneath the surface of the skull; the insert point was 1 mm posterior and 1.2 mm lateral to bregma (23, 27). Three days later, 25-day-old rats were icv injected with TLQP-21 (1 or 5 nmol/rat) or vehicle. All animals were humanely euthanized by decapitation 15 or 30 min after injection, and trunk blood was collected for LH and FSH determinations. Given that data obtained showed that icv administration of TLQP-21 induced a significant increase in gonadotropin secretion, in experiment 2 we analyzed, through a static incubation system, the possible direct effect of TLQP-21 upon hypothalamic GnRH secretion. Twenty-five-day-old male rats were humanely euthanized by decapitation, and the hypothalamus (excised by a horizontal cut of ~2 mm depth with the following limits: 1 mm anteriorly from the optic chiasm, the posterior border of the mammillary bodies, and the hypothalamic fissures) was rapidly dissected out. Tissue samples were subsequently incubated in 250 μl DMEM in a Dubnoff shaker incubator under an atmosphere of 95% O2 and 5% CO2 at 37.5°C. After a 30-min preincubation, the media were removed and hypothalami challenged for 45 min with TLQP-21 (10−8 or 10−6 M) or medium alone. At the end of the incubation period, medium samples were boiled for 30 min to inactivate endogenous protease activity and stored at −80°C until they were used for Ghrelin measurement. In addition, to detect a potential primary action of TLQP-21 at the pituitary level in the regulation of gonadotropin secretion, in experiment 3, 25-day-old male rats were decapitated, and the pituitaries (n = 9–10 pituitaries/ group) were immediately dissected, and the posterior lobe was discarded and placed in glass scintillation vials (1 pituitary/vial) in a Dubnoff shaker at 37°C in an atmosphere of 95% O2 and 5% CO2. Each vial contained 1 ml of DMEM. After preincubation for 60 min, the medium was replaced by fresh medium alone or medium containing TLQP-21 (10−8 or 10−6 M). Samples of medium were obtained at 60 and 120 min of the incubation period.

In a second set of experiments, the effects of chronic intermittent administration of TLQP-21 on puberty onset were evaluated in male rats. In experiment 4, male rats fed ad libitum (n = 10/group) received twice daily (at 0900 and 1900) icv injections of TLQP-21 (1 nmol·rat−1·12 h−1) or vehicle between postnatal days 38 and 45. This period was selected on the basis of previous references on the normal timing of puberty in the male rat and local data on the occurrence of balanopreputial separation (BPS) in our animal stock (9, 21). Selection of treatment regimen was set on an empirical basis, considering previous studies from our group on the effects of other neuropeptides on the timing of puberty (21, 24) and our preliminary observations on the time course of gonadotropic responses to this dose of TLQP-21 and its lack of effects on body weight. The procedure for implantation of icv cannulae was similar to that described in experiment 1. Because puberty is particularly sensitive to changes in energy stores, in all experimental animals body weight and food intake were daily monitored. The rats were euthanized by decapitation on day 45, 15 min after the last injection, when trunk blood was collected for determination of circulating LH, FSH, and testosterone concentrations, and hypothalami were obtained to analyze the expression levels of key genes in pubertal development, namely Kiss1, G protein-coupled receptor 54 (GPR54), and GnRH. In addition, in these animals, testis and prostate weights were recorded, and testes from both experimental groups were collected for histological examination.

Since it is well known that the onset of reproductive function needs sufficient fuel stores, and given the putative metabolic roles of TLQP-21, we also analyzed the effects of chronic treatment with TLQP-21 on puberty onset in food-restricted male rats. Thus, in experiment 5, groups of male rats (n = 11) were submitted to a protocol of 30% restriction in daily food intake from 23-day-old rats. Food-restricted animals received twice daily injections (at 0900 and 1900) of TLQP-21 (1 nmol·rat−1·12 h−1; icv) or vehicle between postnatal days 40 and 47. In all experimental animals, body weight and BPS were monitored daily. The procedure for implantation of icv cannulae was similar to that described in experiment 1. The rats were euthanized by decapitation on day 47, 15 min after the last injection, when trunk blood was collected for determination of circulating LH, FSH, and testosterone concentrations, and hypothalami were obtained.
to analyze the expression levels of Kiss1, GPR54, and GnRH mRNAs. In addition, in these animals, testis and prostate weights were recorded, and testes from both experimental groups were collected for histological examination.

In a third set of experiments, we assessed whether, as initially observed for pubertal animals, TLQP-21 may also have a discernible role in the control of gonadotropin and GnRH secretion in adult male rats. For this purpose, in experiment 6, 90-day-old male rats were icv injected with TLQP-21 (1 or 5 nmol/rat) or vehicle, and blood samples were collected by jugular venipuncture after light ether anesthesia 15 and 60 min after the injections. The procedure for implantation of icv cannulae was similar to that described for prepubertal animals, except that cannulae were lowered to a depth of 4 mm beneath the skull (23). In addition, in experiment 7, hypothalami (n = 10 hypothalamus/group) obtained from adult male rats were incubated with TLQP-21 (10^-6 M) or DMEM, as described in experiment 2. Finally, in experiment 8, hemipituitaries obtained from adult male rats were incubated in the presence of DMEM alone, TLQP-21 (10^-6 M), GnRH (10^-6 M), or TLQP-21 (10^-6 M) plus GnRH (10^-8 M), following the same experimental procedure described in experiment 3. Finally, the potential functional role of TLQP-21 signaling in the direct control of testicular function was also explored. To this end, in experiment 9, we assessed the effect of TLQP-21 upon basal and stimulated testosterone secretion in vitro, using static incubations of 25-day-old and adult rat testicular tissue. The general procedure for static incubations of testicular tissue has been described in detail elsewhere (36, 37). In this setting, testis samples were incubated in fresh medium or medium containing TLQP-21 (10^-6 M) alone (basal conditions) or hCG (10 UI/ml; stimulated conditions). Testosterone was measured at 180 min of the incubation period.

**Hormone assays.** Concentrations of LH and FSH were measured in serum and incubation medium using a double-antibody method and radioimmunoassay kits kindly supplied by the National Institutes of Health (Dr. A. F. Parlow; National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Torrance, CA). Rat LH-I-9 and FSH-I-9 were labeled with 125I using the Iodo-Gen method, following the instructions of the manufacturer (Pierce, Rockford, IL), and hormone concentrations were expressed using LH-RP-3 and FSH-RP-2 as standards. All samples were measured in duplicate, and all samples from each experiment were measured in the same assay. Intra-assay coefficients of variation were <8%, and the sensitivities of the assays were 20 and 7.5 pg/50 μl for LH and FSH, respectively. Accuracy of hormone determinations was confirmed by assessment of rat serum samples of known hormone concentrations used as external controls. GnRH concentrations in the incubation medium were measured in 100-μl aliquots using a commercial RIA kit from Peninsula Laboratories (Hercules, CA) and primer pairs and conditions indicated in Table 1. General procedures for real-time RT-PCR were as described previously (7, 23). The synthesized cDNAs were further amplified (one-tenth) in duplicate by PCR, using SYBR green I as fluorescent dye and 1× iQ Supermix containing 60 mm KCl, 20 mm Tris-HCl, 0.2 mM dNTP, 3 mm MgCl2, and 2.5 U Taq DNA polymerase (Bio-Rad Laboratories) in a final volume of 25 μl. The PCR cycling conditions were as follows: initial denaturation and enzyme activation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 66°C (Kiss1 and GPR54), 64°C (GnRH), or 58°C (S11) for 15 s, and extension at 72°C for 1 min. Calculation of relative expression levels of the target mRNAs was conducted based on the cycle threshold (CT) method (14). The CT for each sample was calculated using the iCycler iQ Real-Time PCR detection system software with an automatic fluorescence threshold setting. Accordingly, fold expression of target mRNAs over reference values was calculated by the equation 2^-ΔΔCT, where ΔΔCT is determined by subtracting the corresponding S11 CT value (internal control) from the specific CT of the target (Kiss1, GPR54, or GnRH) and ΔΔCT obtained by subtracting the ΔCT of each experimental sample from that of the reference sample. No significant differences in CT values were observed for S11 between the treatment groups. In all assays, liquid controls and reactions without RT resulted in negative amplification.

**Histological analysis.** Testes were dissected, fixed in Bouin for 48 h, and routinely processed for paraffin embedding. Six-micrometer-thick sections were cut and stained with hematoxylin and eosin, following previously established procedures (28).

**Presentation of data and statistics.** Real-time RT-PCR analyses were performed in duplicate from at least four independent RNA samples of each experimental group. Weight and hormonal and quantitative RNA data are presented as means ± SE. Results were analyzed for statistically significant differences using Student’s t-test or ANOVA followed by the Student-Newman-Keuls multiple range test (SigmaStat 2.0; Jandel, San Rafael, CA). Statistical comparison of data for percentages of BPS was conducted by x^2 tests and survival plot type analyses. P ≤ 0.05 was considered significant.

Table 1. Primer pairs used for real-time RT-PCR in hypothalamic samples

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligo Primers (5’-3’)</th>
<th>Annealing Temperature, °C</th>
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<tbody>
<tr>
<td>Kiss1(NM_1816921.1)</td>
<td>GCT GCT GCT TCT CCT CTG TG</td>
<td>66</td>
</tr>
<tr>
<td>Sense</td>
<td>GCA TAC CGC GGG CCC TTT T</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>GGC ACA GAT GTC ACT TTC CTT C</td>
<td></td>
</tr>
<tr>
<td>GPR54(NM_023992.1)</td>
<td>GCC GAA CAC AGT CAC GAA GTA CCA</td>
<td>66</td>
</tr>
<tr>
<td>Sense</td>
<td>GCG CTG TTC TCT TTC TGC CAG TGA CTC TG</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>GGG GTT CTG CCA TTT CAT CCT C</td>
<td></td>
</tr>
<tr>
<td>GnRH(NM_012767.2)</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>GAT TCA GAC GGA GGG TGC TTA C</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>TGC AAT TGC TAT TGC GTC AC</td>
<td>58</td>
</tr>
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GPR54, G protein-coupled receptor 54; GnRH, gonadotropin-releasing hormone.

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RESULTS

Effects of TLQP-21 on LH, FSH, and GnRH secretion in prepubertal male rats. Intracerebral injection of TLQP-21 (1 or 5 nmol/rat) evoked robust LH and FSH secretory responses in 25-day-old male rats, as detected 15 min after injection (Fig. 1). In contrast, at 30 min none of the doses of TLQP-21 were able to modify LH secretion, and only the higher dose of the peptide significantly increased FSH secretion (Fig. 1). In prepubertal animals, TLQP-21 (10^{-8} or 10^{-6} M) elicited a significant rise in GnRH secretion by hypothalamic explants ex vivo (Table 2). In addition, in vitro experiments at this age (25-day-old males) revealed that TLQP-21 can stimulate gonadotropin secretion directly at the pituitary level. Thus, at 10^{-6} M, TLQP-21 induced a consistent elevation of LH secretion at 60 and 120 min of the incubation period as well as FSH release at 60 min (Fig. 2). However, at lower (10^{-8} M) doses TLQP-21 could only increase LH secretion at 120 min, whereas it failed to modify FSH secretion at any of the time points studied.

Effects of chronic intermittent administration of TLQP-21 in pubertal male rats. Central (icv) administration of TLQP-21 (1 nmol·rat^{−1}·12 h^{−1}) between postnatal days 38 and 45 in male rats fed ad libitum failed to induce changes in body weight gain (Fig. 3A) or daily food intake (data not shown). However, this treatment induced a delay in the timing of puberty, as evidenced by the later occurrence of BPS as a consensus external sign of male puberty (Fig. 3B). At the end of experiment, the expression levels of Kiss1, GPR54, or GnRH mRNAs were not significantly changed in TLQP-21-treated animals (Fig. 3C). In line with the observed delay in the

Table 2. GnRH released (pg/hypothalamus) in vitro by hypothalamus from 25- and 90-day-old male rats incubated in the presence of TLQP-21 (10^{-8} or 10^{-6} M) or medium (DMEM) alone

<table>
<thead>
<tr>
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<th>Rats</th>
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<tr>
<td></td>
<td>25 days old</td>
<td>90 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>0.31 ± 0.08</td>
<td>11.23 ± 2.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLQP-21 (10^{-8} M)</td>
<td>0.74 ± 0.15*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLQP-21 (10^{-6} M)</td>
<td>0.93 ± 0.22**</td>
<td>18.54 ± 2.24*</td>
<td></td>
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</tr>
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</table>

Values are expressed as means ± SE (n = 9–10 animals/group). **P ≤ 0.01 and *P ≤ 0.05 vs. control (DMEM) group (ANOVA followed by Student-Newman-Keuls multiple range test).

![Fig. 1. Serum LH (top) and FSH (bottom) concentrations (ng/ml) in 25-day-old male rats 15 and 30 min after intracerebroventricular administration of TLQP-21 (1 or 5 nmol/rat) or vehicle (Veh). Data are expressed as means ± SE (n = 9–10 animals/group). **P ≤ 0.01 and *P ≤ 0.05 vs. corresponding Veh-injected group (1-way repeated-measures ANOVA followed by Student-Newman-Keuls multiple range test).](image1)

![Fig. 2. LH (top) and FSH (bottom) concentrations (ng/ml) in the medium 60 and 120 min after incubation of pituitaries from 25-day-old male rats. Incubations were carried out in the presence of TLQP-21 (10^{-8} and 10^{-6} M) or DMEM alone. Values are expressed as means ± SE (n = 8–10 pituitaries/group). **P ≤ 0.01 and *P ≤ 0.05 vs. DMEM at the corresponding time point; *P ≤ 0.01 vs. corresponding values at 60 min (1-way repeated-measures ANOVA followed by Student-Newman-Keuls multiple range test).](image2)
age of BPS, a trend for reduction in serum LH and T levels at terminal blood determinations (which did not reach statistical significance because of the high variability among samples) was detected in TLQP-treated animals, whereas FSH levels remained unchanged (Fig. 3D). In addition, a significant decrease in testicular and prostate weights was observed in animals chronically treated with TLQP-21 (Fig. 3E). However, histological analyses of testicular sections failed to reveal major structural changes in this group, which despite the
signs of delayed puberty displayed normal spermatogenesis in the seminiferous tubules and fully differentiated Leydig cells in interstitial areas (Fig. 3F).

A similar protocol of repeated injections of TLQP-21 was applied to pubertal male rats subjected to a protocol of 30% restriction in daily food intake from 23 days old onward. These animals showed the expected decrease in body weight (30 to 25%) vs. pair-aged males fed ad libitum (see Fig. 4A vs. Fig. 3A). This protocol of food restriction resulted in delayed timing of puberty, as monitored by BPS (Fig. 4B vs. Fig. 3B) and LH.

Fig. 4. Effects of intracerebroventricular administration of TLQP-21 (1 nmol·rat⁻¹·12 h⁻¹) or Veh between 40 and 47 postnatal days in male rats submitted to a protocol of 30% restriction in daily food intake from 23-day-old rats. A: body weight (g) between 40 and 47 postnatal days. B: dates of BPS expressed as percentage over total number of animals (per group). C: hypothalamic expression of Kiss1, GPR54, and GnRH mRNA (2⁻ΔΔCT) levels obtained by real-time RT-PCR 15 min after the last injection. D: serum FSH, LH, and testosterone concentrations (ng/ml) 15 min after the last injection. E: testis and prostate weights (mg/100 g body Wt) 15 min after the last injection. F: representative hematoxylin and eosin-stained testicular sections showing ST and differentiated Leydig cells (arrows in the insets). Data are expressed as means ± SE (n = 11 animals/group). ***P ≤ 0.01 and *P ≤ 0.05 vs. corresponding Veh-injected group (1-way repeated-measures ANOVA followed by Student-Newman-Keuls multiple range test).
secretion (Fig. 4D vs. Fig. 3D). Central administration of TLQP-21 (1 nmol·rat\(^{-1}\)·12 h\(^{-1}\)) to this group between postnatal days 40 and 47 did not significantly modify body weight gain along treatment (Fig. 4A), nor did it induce BPS (Fig. 4B vs. Fig. 3B). However, at the end of the experiment, food-restricted animals injected with TLQP-21 showed a significant increase in Kiss1 mRNA levels at the hypothalamus without detectable changes in the mRNA levels of GPR54 or GnRH (Fig. 4C). In addition, undernourished males injected with TLQP-21 displayed significantly elevated LH and T levels in terminal blood determinations, whereas FSH levels remained unchanged (Fig. 4D). Yet despite the observed elevations of LH and T levels in this group, testicular and prostate weights were not affected (Fig. 4E), nor was there a significant alteration in different histological indices of spermatogenesis and Leydig cell maturation, both of which were grossly normal in the treated animals (Fig. 4F).

**Effects of TLQP-21 on LH, FSH, and GnRH secretion in adult male rats.** In adult male rats, central administration of TLQP-21 (1 or 5 nmol/rat) was followed by a significant augmentation of LH levels 15 min after injection, which remained elevated at 60 min (Fig. 5, top). In contrast, none of the doses of TLQP-21 used were able to modify serum FSH levels at the time points studied (Fig. 5, bottom). At this age, TLQP-21 (10\(^{-6}\) M) elicited a significant rise in GnRH secretion by hypothalamic explants (Table 2).

In pituitaries obtained from adult male rats, TLQP-21 (10\(^{-6}\) M) did not alter either basal or GnRH-stimulated gonadotropin secretion at 60 min of the incubation period but significantly decreased basal LH and FSH secretion at 120 min (Fig. 6).

**Direct effects of TLQP-21 on T secretion in prepubertal and adult age.** Basal T secretion by testes from 25-day-old animals, stimulated or not with TLQP-21 (10\(^{-6}\) M) in vitro, were below the limit of detection of our assay and could not be determined reliably. However, hCG-stimulated T secretion by prepubertal testes was measurable, and our data showed evidence that TLQP-21 did not alter T responses to hCG at this age (Fig. 7, top). In contrast, in the adult age, T secretion to the incubation medium was readily detectable in both basal and hCG-stimu-

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**Fig. 5.** Serum LH (top) and FSH (bottom) concentrations (ng/ml) in adult male rats 15 and 60 min after intracerebroventricular administration of TLQP-21 (1 or 5 nmol/rat) or vehicle. Values are expressed as means ± SE (n = 10 animals/group). **P ≤ 0.01 vs. corresponding Veh-injected groups (1-way repeated-measures ANOVA followed by Student-Newman-Keuls multiple range test).

**Fig. 6.** LH (top) and FSH (bottom) concentrations (ng/ml) in the medium 60 and 120 min after incubation of pituitaries from adult male rats. Incubations were carried out in the presence of DMEM alone, TLQP-21 (10\(^{-6}\) M), GnRH (10\(^{-8}\) M), or TLQP-21 (10\(^{-6}\) M) plus GnRH (10\(^{-8}\) M). Values are expressed as means ± SE (n = 10–12 pituitaries/group). **P ≤ 0.01 and *P ≤ 0.05 vs. DMEM at the corresponding time point; aP ≤ 0.01 vs. corresponding values at 60 min (1-way repeated-measures ANOVA followed by Student-Newman-Keuls multiple range test).
The reproductive phenotype of Vgf-KO mice, with delayed puberty, reduced fertility, decreased ovarian and uterus weights, and reduced mRNA levels and protein contents of LH and PSH β-subunits at the pituitary, strongly suggested that the products of VGF are directly or indirectly involved in the control of the reproductive axis (11). However, given the prominent metabolic alterations in these null mice, with a hypercatabolic state, decreased body weight, and reduced leptin levels, it was assumed that such reproductive impairment stemmed mainly from the congenital state of leptin deficiency linked to VGF inactivation, since leptin is a well-known stimulator of the reproductive axis (6, 22). Yet, those original studies did not rule out the potential contribution of direct reproductive effects of the products of VGF (11). Our current data, testing the effects of TLQP-21 at the hypothalamus, pituitary, and testis of prepubertal and adult rats, provide conclusive demonstration for discernible (mainly stimulatory) actions of this particular VGF-derived peptide at different levels of the HPG axis, thus supporting the concept that the reproductive phenotype of VGF-null mice may be caused, at least partially, by the lack of the actions of TLQP-21.

As major evidence for its stimulatory effects on the gonadotropin axis, central injection of TLQP-21 consistently increased LH and, to a lesser extent, FSH secretion in both prepubertal and adult male rats. The consistency of this observation is further supported by the fact that stimulatory effects of TLQP-21 have also been detected by our group in 25-day-old (prepubertal) female rats, in which administration of 1 nmol of the peptide induced a significant increase in serum LH concentrations at 15 min of administration (Pinilla L, Tena-Sempere M, and Aguilar E, unpublished observations). Furthermore, the gonadotropin-releasing effect of TLQP-21 was detected at relatively low doses of the peptide (5 μg/animal), whereas previous studies addressing its metabolic effects generally required the use of higher doses (in the range of 15 μg·mice⁻¹·day⁻¹) to unveil such metabolic actions (2, 4).

From a mechanistic standpoint, the above stimulatory effects are likely due to the ability of TLQP-21 to elicit GnRH release at the hypothalamus, as demonstrated ex vivo at both ages. The fact that gonadotropin responses to TLQP-21 were acutely detected after icv injection of the peptide in vivo and the concomitant stimulation of GnRH release from hypothalamic fragments by TLQP-21 in an ex vivo setting casts doubts on the possibility of an indirect metabolic mechanism (e.g., mediated by changes in leptin secretion) for the effects of TLQP-21 on gonadotropin secretion and strongly suggests a genuine primary excitatory action on the GnRH system. Moreover, previous data had demonstrated that chronic administration of TLQP-21 did not cause significant changes in circulating leptin levels in control animals, which were even decreased in animals on high-fat diets treated with TLQP-21 (2). These observations add further arguments against a major leptin-mediated effect of TLQP-21 on GnRH secretion. Whether TLQP-21 actions are conducted directly on GnRH neurons or rather take place via afferent pathways involved in the stimulatory control of GnRH release is yet to be determined. However, it is worth noting that, in conditions of undernutrition, TLQP-21 was able to significantly increase the hypothalamic expression of Kiss1, namely one of the most potent activators of GnRH secretion with key roles in the control of puberty, gonadotropin secretion, and the metabolic regulation of fertility (7, 23, 24, 30).

Therefore, it is possible that, at least in some states, the positive effects of TLQP-21 on GnRH/gonadotropin secretion may partially derive from its primary actions at sites upstream of GnRH neurons, such as Kiss1 neurons. Detailed analyses of the effects of TLQP-21 at different levels of the reproductive axis in prepubertal and adult males showed evidence of interesting age-dependent differences, which is suggestive of maturational changes in the roles and putative sites of action of this VGF-derived peptide. Thus, in prepubertal rats, TLQP-21 not only activated GnRH/gonadotropin-releasing activity in vitro in the presence of DMEM alone or DMEM containing TLQP-21 (10⁻⁶ M) plus hCG (10 UI/ml). Values are expressed as means ± SE (n = 10–12/group). *P ≤ 0.05 vs. DMEM at the corresponding time point; **P ≤ 0.01 vs. corresponding basal values (1-way repeated-measures ANOVA followed by Student-Newman-Keuls multiple range test). ND, not detected (all animals of both groups showed testosterone levels below the limit of detection by radioimmunoassay).
tropin secretion acting at central levels but was also able to increase LH and FSH release directly at the pituitary, whereas it failed to alter T secretion by testicular tissue in vitro. Of note, our analyses showed evidence of some disparities in the time course of the stimulatory gonadotropin responses to TLQP-21 between in vivo and in vitro studies, which may be suggestive of differences in the physiological mechanisms behind the central and pituitary effects of the peptide but might also be caused (at least partially) by divergences in the experimental settings. In clear contrast, in adult males, TLQP-21 did not stimulate but rather inhibited LH and FSH secretion directly at the pituitary level, whereas it significantly increased hCG-stimulated T secretion acting on the testis. Taken together, these observations would suggest a consistent stimulatory action of TLQP-21 on the gonadotropic axis at puberty, with convergent central effects at the hypothalamus and the pituitary, whereas in adulthood the actions of TLQP-21 may be more diverse (stimulatory and inhibitory) and could take place at the three levels of the HPG axis. The physiological relevance of these actions warrants specific investigation. In this context, the biological testing of pharmacological antagonists of TLQP-21, when they become available, may prove instrumental to ascertain the actual roles and major sites of action of this VGF-derived peptide. Similarly, although pharmacological evidence strongly suggests the presence of a receptor(s) for TLQP-21 in different tissues, including the brain, pituitary, and testis (our present data), it is striking that molecular cloning of such a receptor(s) has remained elusive and is eagerly awaited as a mean to further characterize its pattern of tissue/cellular distribution as well as its signaling pathways and major biological targets.

Further analysis of the putative roles of TLQP-21 in the control of puberty involved testing of the effects of chronic administration of the peptide to male rats during the pubertal transition in two different metabolic states: control animals fed ad libitum and male rats subjected to a protocol of 30% reduction in their daily food intake. At first glance, results from these experiments may suggest a marginal role of TLQP-21 in the control of the progression of puberty since 1) repeated administration of this peptide to control males failed to advance the onset of puberty and 2) chronic administration of TLQP-21 did not rescue BPS (as an external sign of puberty) in males with puberty arrest due to persistent subnutrition. Nonetheless, detailed analysis of our results in these models unveils interesting features of the biological effects of TLQP-21, which may be relevant in terms of control of puberty onset.

First, repeated administration of the effective doses of TLQP-21 to control males affected the onset of puberty, but (quite counterintuitively) it caused an apparent delay, as reflected by a reduced percentage of BPS as well as decreased prostate and testis weights, and trended toward lowering of LH and T levels. Considering the acute stimulatory actions of TLQP-21 on gonadotropin secretion also reported here, these observations are suggestive of a state of desensitization, which has been documented previously for GnRH analogs (8, 15, 31). Likewise, repeated administration of kisspeptin has been shown to cause testicular degeneration in adult rats (39). Thus, our present observations show evidence that, as is the case for other key regulators, the gonadotrophic axis is sensitive to excessive stimulation by TLQP-21 during male puberty. On the other hand, our unpublished data suggest that even repeated administration of kisspetin-10 (despite it being one of the most potent elicitors of gonadotropin secretion) failed to significantly advance male puberty, in contrast with observations of earlier puberty onset induced by kisspeptin injections in the female rat (24), thus suggesting that puberty in the male might be resistant to precocious activation, in keeping with clinical findings showing that virtually all forms of idiopathic precocious puberty are observed in girls (26).

Second, although repeated TLQP-21 administration to pubertal male rats subjected to chronic subnutrition failed to rescue BPS (as consensus index of male puberty), it did induce a number of responses (increased serum levels of LH and T, enhanced expression of Kiss1 mRNA at the hypothalamus) that jointly suggest a conserved stimulatory effect of TLQP-21 in conditions of negative energy balance. Assuming that the blockade of puberty progression in undernourished males is due to a decreased activity of the GnRH-gonadotropin axis (5, 10), it is plausible that, despite its stimulatory effects, the magnitude (in terms of amplitude and duration) of LH and T responses to TLQP-21 was insufficient to overtly rescue puberty onset (or at least BPS) in our experiment. Yet, as demonstrated previously for kisspeptins (7), the HPG axis of male rats in conditions of negative energy balance remains responsive to the stimulatory actions of TLQP-21, and therefore, a suppression of its endogenous levels might contribute to the defects of the male HPG axis observed in those conditions. The lack of specific assays to detect the actual changes in TLQP-21 levels at the hypothalamus in situations of food deprivation prevented us from addressing this issue. Despite this potential limitation, our analyses of the effects of acute and repeated administration of TLQP-21 conclusively document that the male HPG axis is sensitive to the actions of this VGF-derived peptide at puberty whose stimulatory effects may be dependent on changes in its endogenous tone or the metabolic state.

Since previous studies revealed that treatment with TLQP-21 induced a decrease in body weight in Siberian hamsters (17), we expected a similar effect in our experimental models. However, administration of TLQP-21 for 1 wk was not followed by changes in body weight in prepubertal animals, regardless of their feeding status (ad libitum or under 30% restriction). Since the effects of TLQP-21 on body weight in Siberian hamsters took place rather rapidly (as detectable in the first 24 h, even after 5-μg administration) (17), the lack of effect of the peptide on this parameter in our experiments cannot be explained by the duration of treatment or by the dose used. More likely is that the effects of the peptide may depend on the species, since mice treated chronically with TLQP-21 failed to display significant alteration in body weight (2). In addition, we have also detected that signals with proven impact on energy homeostasis in adulthood do not cause overt changes in body weight when administered during puberty (9, 21).

Finally, it is stressed that although the effects of TLQP-21 are in line with the predominant role of VGF in the control of the HPG axis, as evidenced by studies on Vgf-KO mice, our present findings do not exclude the possibility of additional (even opposite) actions of other VGF-derived peptides on the reproductive system. Similarly, despite the direct stimulatory actions of TLQP-21 documented here, it remains possible that part of the reproductive defects observed in Vgf-null mice are caused by their primary metabolic alterations.
In conclusion, our data are the first to provide direct, conclusive evidence for the effects (mostly stimulatory) of the VGF-derived peptide TLQP-21 on the function of the HPG axis in male rats. Our studies document a multifaceted mode of action of this signal, with potential effects at the hypothalamic, pituitary, and testis, both during puberty and in adulthood. Although further analyses on the dynamic changes of the endogenous levels of TLQP-21 in those tissues are warranted (when methodologically feasible), our present results expand our perception of the biological profile of TLQP-21 as pleiotropic molecule with multiple putative effects on metabolism, neuronal survival, and inflammatory pain (1, 2, 4, 16, 17, 29, 34).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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