

# In Vivo and in Vitro Structure-Activity Relationships and Structural Conformation of Kisspeptin-10-Related Peptides

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## ABSTRACT

Kisspeptins, the natural ligands of the G protein-coupled receptor KISS1R, comprise a family of related peptides derived from the proteolytic processing of a common precursor encoded by the *KISS1* gene. Among those, Kisspeptin-10 (Kp-10) contains the basic residues to retain full functional activity and exhibits higher receptor affinity and biopotency than longer forms of the peptide. Although kisspeptins were first characterized by their ability to inhibit tumor metastasis, recent studies have revealed that the KISS1/KISS1R system plays an essential role in the neuroendocrine control of the reproductive axis. In this context, development and functional analysis of Kp-10 analogs may help in the search for new agonists and antagonists as valuable tools to manipulate the KISS1/KISS1R system and hence fertility. We report herein functional and structural analyses of a series of Ala-substituted rat kp-10 analogs, involving  $[Ca^{2+}]_i$  responses in rat kiss1r-transfected Chinese hamster ovary cells, dynamic luteinizing hormone (LH) responses in vivo, and NMR structural studies. In vitro assays revealed that Ala substitutions in positions 6 or 10 of kp-10 resulted in a significant increase in  $EC_{50}$  values ( $>6.46 \times 10^{-6}$  M versus  $1.54$  to  $2.6 \times 10^{-8}$  M for rat and human Kp-10, respec-

tively) and a substantial decrease in the proportion of responsive cells coupled to a marked increase in the time required to reach maximal response. In vivo assays showed that Ala<sup>6</sup> substitution diminished and Ala<sup>10</sup> substitution eliminated LH secretory responses, whereas coadministration of each analog failed to affect the LH-releasing ability of kp-10. Molecular modeling under NMR restraints revealed that kp-10 exhibits a helical structure between the Asn<sup>4</sup> and Tyr<sup>10</sup> residues, with mixed  $\alpha$ - and  $3_{10}$ -helix characteristics. Ala<sup>6</sup> substitution induced limited destabilization of the helix around the position of the substitution. Ala<sup>10</sup> substitution was found to totally disrupt the helical structure in the C-terminal region of the molecule. Taken together, our results indicate that positions 6 and 10 are critical for kp-10 action at kiss1r and suggest that modifications in these positions could lead to the generation of new kisspeptin agonists and/or antagonists with altered functional and perhaps binding properties. Furthermore, they emphasize the importance of using combined, multidisciplinary approaches, including in vivo studies, to reliably evaluate structure function properties of novel kisspeptin analogs.

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The ligand-receptor tandem comprising kisspeptins and kiss1r has been the focus of intense research over the last decade owing to its peculiar dual pathophysiological relevance. The *KISS1* gene was initially discovered in 1996 as a tumor suppressor gene and was subsequently found to inhibit metastasis in melanoma and breast cancer cells (Lee et al., 1996; Lee and Welch, 1997). Five years later, three different groups identified kiss1r as the receptor for the family of peptides derived by proteolytic processing of a precursor

**ABBREVIATIONS:** Kp-10, Kisspeptin-10; CHO, Chinese hamster ovary; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; PCR, polymerase chain reaction; LH, luteinizing hormone; ORX, orchidectomized; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; NOE, nuclear Overhauser enhancement; AUC, area under the curve; ANOVA, analysis of variance; Fmoc, 9-fluorenylmethoxycarbonyl; rmsd, root mean square deviation.

protein encoded by *kiss1*, the kisspeptins, and characterized the basic pharmacological, signaling, and functional properties of the kisspeptin/kiss1r system (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). These studies also showed that KISS1 is widely produced throughout the central nervous system and other organs, such as liver, lung, gonads, and prostate (Muir et al., 2001; Ohtaki et al., 2001). A series of subsequent reports added convincing evidence indicating that this system was able to counteract metastasis and cell migration/proliferation in different cancer cell models and established the presence and potential pathological relevance of kisspeptins and kiss1r in different types of cancers (Nash and Welch, 2006; Mead et al., 2007).

In a completely different scenario, two independent groups identified in late 2003 mutations in *KISS1R* as the underlying cause of hypogonadotropic hypogonadism in humans (de Roux et al., 2003; Seminara et al., 2003). This finding, coupled to the ensuing demonstration that lack of *kiss1r* (Funes et al., 2003; Seminara et al., 2003) and, more recently, *kiss1* (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007) genes caused an identical reproductive disruption phenotype in mouse, paved the way for the unexpected establishment of kisspeptin/kiss1r as a novel, critical component for the control of the reproductive axis (Popa et al., 2008; Roa et al., 2008). In fact, there is now growing evidence that hypothalamic kiss1 and kiss1r act as key integrative players in the hierarchical control of reproduction, mainly by conveying to the neurons releasing gonadotropin-releasing hormone the central and peripheral signals that regulate (at least) puberty onset, ovulation, and sensing of metabolic status (Popa et al., 2008; Roa et al., 2008).

Despite the obvious interest raised by the potential pathophysiological implications of kisspeptins and kiss1r, relatively few studies have been addressed at elucidating in detail the structural and molecular features of this tandem, their pharmacology, and related signal transduction pathways (Castaño et al., 2009). In particular, only a limited number of reports have analyzed the structure-activity relationships of kisspeptins and kiss1r (Niida et al., 2006; Tomita et al., 2006, 2007a,b, 2008; Orsini et al., 2007), and none has integrated the chemical structure of this peptide family, its functional features in cell models *in vitro*, and its effects *in vivo* in a physiologically relevant target.

Proteolytic processing of the precursor encoded by the *kiss1* gene predominantly generates Kisspeptin-54, also known as metastatin, as well as lower amounts of shorter peptide fragments, including Kisspeptin-14, Kisspeptin-13, and Kisspeptin-10 (Nash and Welch, 2006; Mead et al., 2007; Popa et al., 2008; Roa et al., 2008). Earlier reports showed that kisspeptins share the 10-amino acid C-terminal region (H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH<sub>2</sub>), which contains the canonical Arg-Phe-NH<sub>2</sub> motif of the Arg-Phe-amide peptide family. This structure is highly conserved across species, because in rat, mouse, platypus, sheep, and cow, Kp-10 differs only by one amino acid from the sequence of human and nonhuman primates (a C-terminal Tyr instead of Phe) (Roa and Tena-Sempere, 2007). Functional studies indicated that all kisspeptins are highly potent in terms of receptor activation, with shorter forms being even more active than Kisspeptin-54 (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Using different cell lines expressing kiss1r, it has been established that coupling of kisspeptins to this

receptor induces, as its major signaling cascade, activation of phospholipase C and polyphosphoinositide hydrolysis, followed by accumulation of inositol-(1,4,5)-triphosphate, Ca<sup>2+</sup> mobilization, as well as phosphorylation of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinases (Castaño et al., 2009). In support of this, we have shown that, in rat hypothalamic explants, kp-10 elicits gonadotropin-releasing hormone secretion through the required activation of phospholipase C, mobilization of intracellular Ca<sup>2+</sup>, and recruitment of extracellular signal-regulated kinase 1/2 and p38 kinases (Castellano et al., 2006).

Studies from two different groups have analyzed the structure-activity relationships of Kp-10 (Niida et al., 2006; Tomita et al., 2006, 2007a,b, 2008; Orsini et al., 2007). These analyses indicate that the nature and the orientation of the side chains of the five C-terminal residues are important for receptor binding and activation, whereas the N-terminal amino acids are more tolerant to substitution by L-alanine or enantiomer residues (Niida et al., 2006; Orsini et al., 2007). In addition, in pursuing the development and analysis of new analogs for kisspeptin, one group has generated short-chain analogs, notably pentapeptides (Tomita et al., 2006, 2007a,b, 2008). To date, compound 4-fluorobenzoyl Phe-Gly-Leu-Arg-Trp-NH<sub>2</sub> is the shortest kiss1r ligand that exhibits the same efficacy and potency as Kp-10 (Tomita et al., 2007b, 2008). In SDS micelles, Kp-13 encompasses an  $\alpha$ -helix from residues Asn<sup>7</sup> to Phe<sup>13</sup> (Asn<sup>4</sup> and Phe<sup>10</sup> in Kp-10, respectively) suggesting that the disruption of this  $\alpha$ -helix could be responsible for the lower activity of inappropriately substituted compounds (Orsini et al., 2007).

Although these studies have provided valuable information regarding the individual contribution of each residue to kiss1r activation, there is still a lack of coordinate information on the potential activity of these modified kisspeptin compounds in a physiologically relevant target. Accordingly, in the present work, we have applied an interdisciplinary approach to attain an integrated analysis of the structure-activity relationships of a series of rat kp-10 analogs obtained by chemical substitution of each residue by alanine. To this end, we have evaluated the potential functional activity of Ala-substituted kp-10 analogs using an *in vitro* assay model of kiss1r-transfected Chinese hamster ovary (CHO)-K1 cells, assessed the *in vivo* action of kp-10 and two analogs (selected from *in vitro* assays) on a well characterized, physiologically relevant target for kisspeptins [i.e., stimulation of luteinizing hormone (LH) release in rats], and finally studied the three-dimensional structure by molecular modeling under NMR restraints of these three selected compounds.

## Materials and Methods

### Chemicals and Reagents for Peptide Synthesis

L-Amino acid residues and the resins *O*-benzotriazol-1-yl-*N,N,N'*-tetramethyluronium hexafluorophosphate and 1-hydroxybenzotriazole were purchased from APPLERA France (Courtaboeuf, France). Acetonitrile and *N*-methylpyrrolidin-2-one were obtained from Carlo Erba (Val-de-Reuil, France). Diisopropylethylamine, piperidine, trifluoroacetic acid (TFA), and other reagents were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Phospholipids were acquired as follows: dodecylphosphocholine-d<sub>38</sub> (99.1%) was from C/D/N Isotopes (Pointe-Claire, QC, Canada); D<sub>2</sub>O and sodium 2,2-dimethyl-2-silapentane-5-sulfonate were from Eurisotop (Gif-sur-Yvette, France).

## Peptide Synthesis

Rat metastatin (rat kisspeptin-52; TSPCPPVENPTGHRPPCA-TRSLRIPAPRGSLVQ REKDMSAYNWNFSGLRY-NH<sub>2</sub>), rat kp-10 (YNWNSFGLRY-NH<sub>2</sub>), human Kp-10 (YNWNSFGLRF-NH<sub>2</sub>) and L-alanine analogs of rat kp-10 (Table 1) were synthesized (0.25-mmol scale for metastatin; 0.1-mmol scale for all other peptides) on a Fmoc-PAL-polyethylene glycol-polystyrene resin (Applied Biosystems, Foster City, CA) for metastatin and on a Rink amide 4-methylbenzhydrylamine resin for the other peptides using an Applied Biosystems model 433A automatic peptide synthesizer and the standard procedures, as described previously (Leprince et al., 1998). All Fmoc-amino acids (1 mmol, 4 Eq, or 10 Eq) were coupled by in situ activation with *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate/1-hydroxy-benzotriazole (1.25/1.25 mmol, 5 Eq, or 12.5 Eq) and diisopropylethylamine (2.5 mmol, 10 Eq, or 25 Eq) in *N*-methylpyrrolidin-2-one. Reactive side chains were protected as follows: Arg, pentamethyldihydrobenzofuran sulfonylamide; Asn and Gln, trityl amide; Ser, Thr, and Tyr, *tert*-butyl ether; Asp and Glu, *O*-*tert*-butyl ester; Cys, acetamidomethyl thioether; His, trityl amine; and Lys and Trp, *tert*-butyloxycarbonyl carbamate. After completion of the chain assembly, cyclization of rat metastatin was performed by Ti(OCOCF<sub>3</sub>)<sub>3</sub> oxidation as described previously (Chatenet et al., 2006). Peptides were deprotected and cleaved from the resin by TFA as described previously (Leprince et al., 1998; Chatenet et al., 2006). Crude peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Vydac 218TP1022 C<sub>18</sub> column (2.2 × 25 cm; Grace Davison Discovery Sciences Alltech, Tempe, Arizona, France) using a linear gradient (10–50% over 50 min) of acetonitrile/TFA [99.9:0.1 (v/v)] at a flow rate of 10 ml/min. Peptides were analyzed by RP-HPLC on a Vydac 218TP54 C<sub>18</sub> column (0.46 × 25 cm; Grace Davison Discovery Sciences Alltech) using a linear gradient (10–60% over 25 min) of acetonitrile/TFA at a flow rate of 1 ml/min. All peptides were characterized by MALDI-TOF mass spectrometry on a Voyager DE-PRO (APPLERA France) in the reflector mode with  $\alpha$ -cyano-4-hydroxycinnamic acid as a matrix.

## Cloning of the Rat *kiss1r* and Transfection and Selection of Monoclonal Cell Lines

Genomic DNA was extracted from rat pituitaries by using the Tripure Isolation Reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. The full-length rat *kiss1r* was amplified by two PCR rounds, using a high-fidelity Taq polymerase (Ecozyme; Ecogen, Barcelona, Spain). For the first PCR round, specific primers were designed at the 5' and 3' regions of the coding sequence, respectively (sense, 5'-ATGGATAT-

GGCG-3'; antisense, 5'-AGATACTGGTCTGG-3'), according to the known sequence of rat *kiss1r* (GenBank accession no. AF115516). The PCR product was subjected to a second amplification using the same sense and antisense primers but including HindIII (upper, HindIII; 5'-TACAAGCTTGCCATGGATATGGCG-3') and EcoRI (lower, EcoRI; 5'-TCAGGGAA TTCAGATACTGGTCTGG-3') restriction sites. After the second PCR amplification, the rat *kiss1r* full-length cDNA was subcloned into the corresponding restriction sites of the pCDNA3.1 vector (Invitrogen, Barcelona, Spain). Identity of the cloned sequence was confirmed by sequencing at the Central Facilities of the University of Córdoba.

CHO-K1 cells were cultured to semiconfluence in 12-well plates using Ham's F12 medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution, and transfected with different concentrations of the recombinant plasmid, ranging from 3 to 5  $\mu$ g, using Lipofectamine 2000 (Invitrogen, Barcelona, Spain). Twenty-four hours after transfection, media were replaced by fresh F12 medium containing 1 mg/ml G418 (Geneticin; Life Technologies, Inc.). One week later, surviving cells were detached, diluted to 70 cells/ml and plated on 96-well plates at 0.7 cells/well. Monoclonal cell lines expressing rat *kiss1r* were followed daily by contrast phase microscopy. Seven independent monoclonal stable cell lines were obtained after a 3-week period of selection. By using quantitative RT-PCR, two cell lines were selected based on their expression levels being closest to physiological levels among all cell lines. Of these, one cell line was definitely selected based on its best performance in showing clear [Ca<sup>2+</sup>]<sub>i</sub> responses to treatment with kp-10.

## Measurement of [Ca<sup>2+</sup>]<sub>i</sub> Using FlexStation Technology

To compare the *kiss1r* agonistic activities of kisspeptin-52, kisspeptin-10, and Ala-substituted decapeptide analogs, the level of [Ca<sup>2+</sup>]<sub>i</sub> after stimulation of CHO-K1 cells stably expressing *kiss1r* by these peptides was monitored by spectrofluorometry. The agonistic activity of each compound was assessed by calculating two distinct values: the percentage activity (i.e., relative maximum agonistic activity induced by each compound compared with the maximum signal induced by the addition of kp-10) and the EC<sub>50</sub> value (i.e., the concentration required for 50% of the full agonistic activity induced by 10<sup>-12</sup> to 10<sup>-5</sup> M doses of each compound).

Functional assays were performed by first seeding 96-well black/clear microplates (Costar; Corning Life Sciences, Acton, MA) with 4 × 10<sup>4</sup> cells/well 24 h before assay. After overnight incubation at 37 °C, cells were loaded for 1 h at 37 °C with 4 mM Fluo-4 AM (Invitrogen) in standard Hank's balanced salt solution buffer (Invitrogen) containing 5 mM HEPES (Sigma) and 2.5 mM probenecid (Sigma). Cells were then washed three times with standard Hank's balanced salt solution and changes in [Ca<sup>2+</sup>]<sub>i</sub> induced by compound addition were measured using the FlexStation II system (Molecular Devices, Sunnyvale, CA) during the next 135 s. Responses were measured as peak fluorescent intensity minus basal fluorescent intensity at each concentration of agonist. Sigmoidal concentration-response curves were fitted with variable Hill slopes, and EC<sub>50</sub> values were generated using the Prism software (version 4.0; GraphPad Software, San Diego, CA).

## Measurement of [Ca<sup>2+</sup>]<sub>i</sub> in Single Cells

CHO-K1 cells stably transfected with rat *kiss1r* were incubated for 30 min at 37 °C with 2.5 mM Fura-2 AM, a Ca<sup>2+</sup> indicator dye (Invitrogen) in phenol red-free Dulbecco's modified Eagle's medium containing 20 mM Na<sub>2</sub>HCO<sub>3</sub>, pH 7.4. Coverslips were washed with phenol red-free Dulbecco's modified Eagle's medium and mounted on the stage of a microscope (Eclipse TE2000-E; Nikon, Tokyo, Japan) with attached back-thinned charge-coupled device cooled digital camera (ORCA II BT; Hamamatsu Photonics, Hamamatsu, Japan). Cells were examined under a 40× oil immersion objective during exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 505 nm was measured every 5 s. Changes in

TABLE 1

Analytical data for compounds 1–13

Calculated indicates the theoretical monoisotopic MH<sup>+</sup> value. Observed values were assessed by MALDI-TOF in the reflector mode unless otherwise noted.

Peptide	HPLC		Mass Spectrometry	
	Retention Time	Purity	Calculated	Observed
	min	%	m/z	
Rat metastatin	20.35	99.95	5833.91	5833.12 <sup>a</sup>
rKp-10	19.35	99.07	1318.62	1318.54
hKp-10	20.64	99.83	1302.63	1302.80
[Ala <sup>1</sup> ]kp-10	18.37	99.99	1226.60	1226.52
[Ala <sup>2</sup> ]kp-10	19.43	99.66	1275.62	1275.65
[Ala <sup>3</sup> ]kp-10	16.40	99.96	1203.58	1203.63
[Ala <sup>4</sup> ]kp-10	19.84	99.89	1275.62	1275.54
[Ala <sup>5</sup> ]kp-10	19.62	99.80	1302.63	1302.54
[Ala <sup>6</sup> ]kp-10	16.62	99.70	1242.59	1242.68
[Ala <sup>7</sup> ]kp-10	19.33	99.99	1332.64	1332.79
[Ala <sup>8</sup> ]kp-10	17.92	99.78	1276.58	1276.38
[Ala <sup>9</sup> ]kp-10	19.97	99.62	1233.56	1233.25
[Ala <sup>10</sup> ]kp-10	17.77	99.85	1226.60	1226.43

<sup>a</sup> Assessed by MALDI-TOF in the linear mode.



$[Ca^{2+}]_i$  after administration of the compounds were recorded as background-subtracted ratios of the corresponding excitation wavelengths ( $F_{340}/F_{380}$ ) using the MetaFluor software (Molecular Devices, Sunnyvale, CA).

### In Vivo Studies

Adult Wistar male rats bred in the Experimental Animal Department of the University of Córdoba were used. The animals were maintained under constant conditions of light (14 h of light, starting at 7:00 AM) and temperature (22°C), with free access to pelleted food and tap water. Procedures of intracerebroventricular administration of the testing compounds (kp-10 and/or Ala-substituted analogs) under conscious conditions were as described previously (Navarro et al., 2004; Castellano et al., 2006), after implantation (>24–48 h before pharmacological testing) of intracerebroventricular cannulae. To allow delivery of compounds into the lateral cerebral ventricle, the cannulae were lowered to a depth of 4 mm beneath the surface of the skull; the insert point was 1 mm posterior and 1.2 mm lateral to bregma. Correct positioning of the cannulae was visually inspected in the course of the experiments and confirmed post mortem. Experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals.

To characterize the functionality of the selected kp-10 analogs in vivo, three different experiments were conducted.

**Experiment 1.** The effects on LH secretion of a single bolus of kp-10 or the Ala-substituted analogs were tested in intact male rats. To this end, groups of male rats ( $n = 10$ –12) received 10- $\mu$ l i.c.v. injections of kp-10, [Ala<sup>6</sup>]kp-10, or [Ala<sup>10</sup>]kp-10 (dose, 1 nmol), and blood samples were taken by jugular venipuncture at 0 (basal), 15, 60, and 120 min after central administration. Animals intracerebroventricularly injected with saline served as controls.

**Experiment 2.** The effects on LH secretion of the coadministration of each analog with kp-10 were explored in intact males. Thus, groups of male rats ( $n = 10$ –12) received 10- $\mu$ l i.c.v. injections of 100 pmol of kp-10 alone or kp-10 together with 1-nmol doses of [Ala<sup>6</sup>]kp-10 or [Ala<sup>10</sup>]kp-10; rats intracerebroventricularly injected with saline were used as controls. Blood samples for hormonal determinations were taken by jugular venipuncture at 0 (basal), 15, 60, and 120 min after central administration.

**Experiment 3.** The effects of kp-10 analogs on prestimulated LH secretion were explored in orchidectomized (ORX) rats. Groups of male rats ( $n = 10$ –12) were subjected to bilateral ORX via the scrotal route as described previously (Navarro et al., 2004). One week after surgery, the animals received three consecutive (at 60-min intervals) 1-nmol [Ala<sup>6</sup>]kp-10 or [Ala<sup>10</sup>]kp-10 i.c.v. injections; rats receiving saline served as controls. Blood samples for hormonal determinations were taken by jugular venipuncture at 15 min after each intracerebroventricular injection of the analogs.

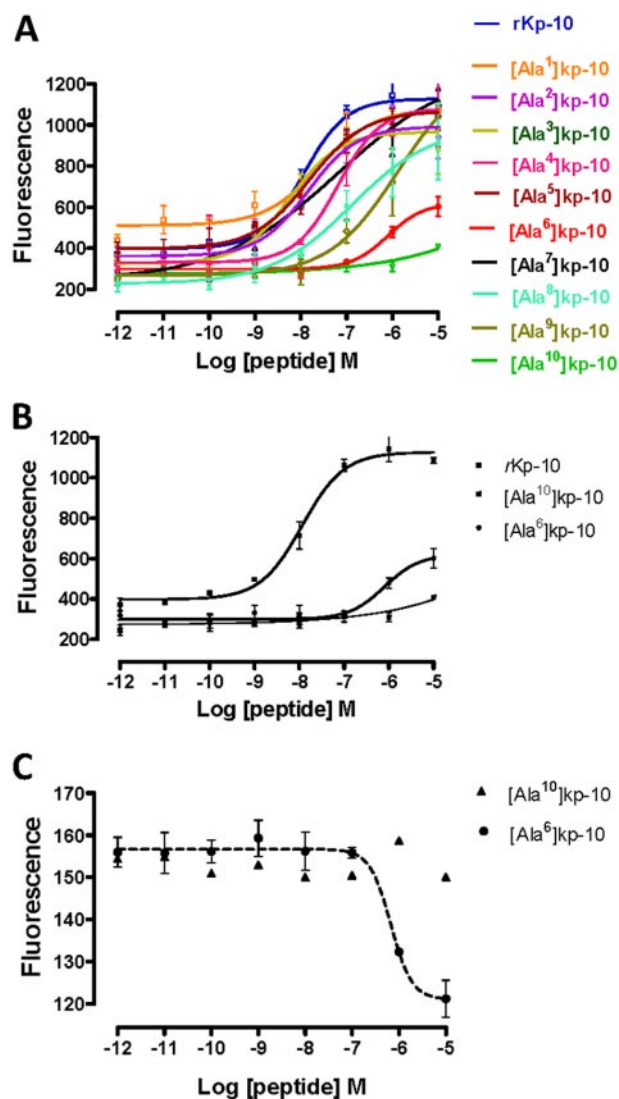
### Hormone Measurements

Serum LH levels were measured in duplicate using a double-antibody radioimmunoassay method and reagents 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-10 was labeled with <sup>125</sup>I using Iodo-Gen precoated tubes, following the instructions of the manufacturer (Pierce, Rockford, IL). Hormone concentrations were expressed using reference preparation LH-RP-3 as standard. The intra-assay coefficient of variation was <10%, and the sensitivity of the assay was 3.75 pg/tube. All samples were measured in the same assay. Accuracy of hormone determinations was confirmed by assessment of rat serum samples of known hormone concentrations used as external controls.

### NMR Spectroscopy

Each peptide was dissolved at a concentration of 1.0 mM in 10% D<sub>2</sub>O/90% H<sub>2</sub>O in the presence of 150 mM dodecylphosphocholine-d<sub>38</sub> for a micelle concentration of approximately 3 mM. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was added as an internal <sup>1</sup>H chemical shift reference.

All NMR experiments were performed on an NMR spectrometer (Avance DMX 600; Bruker, Wissembourg, France) equipped with a triple resonance cryoprobe including shielded z-gradients. For all samples, one-dimensional spectra were recorded at 5 K intervals from 293 to 313 K. The following conventional two-dimensional experiments were carried out: two-dimensional correlation spectroscopy, TOCSY, and NOESY at 295 K and 308 K. NOESY spectra were collected at mixing times of 50, 80, 100, 150, 200, and 250 ms. TOCSY experiments were performed with a 80-ms MLEV-17 spin-



**Fig. 1.** In vitro evaluation of the functional properties of rat kp-10 and Ala-substituted analogs of kp-10. Effects of increasing concentrations of the different kisspeptins were assessed by using FlexStation technology to measure the levels of free cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) on cultured CHO-K1 cells stably transfected with kiss1r. A, average dose-response curves for  $[Ca^{2+}]_i$  observed for [Ala<sup>1</sup>]-, [Ala<sup>2</sup>]-, [Ala<sup>3</sup>]-, [Ala<sup>4</sup>]-, [Ala<sup>5</sup>]-, [Ala<sup>6</sup>]-, [Ala<sup>7</sup>]-, [Ala<sup>8</sup>]-, and [Ala<sup>9</sup>]kp-10. B, average dose-response curves for  $[Ca^{2+}]_i$  for [Ala<sup>6</sup>] and [Ala<sup>10</sup>]kp-10. C, competition assay of [Ala<sup>6</sup>]kp-10 and [Ala<sup>10</sup>]kp-10. Increasing doses of Ala-substituted analog were applied in presence of a fixed concentration of rat kp-10 and changes in  $[Ca^{2+}]_i$  levels were measured. Values represent the mean  $\pm$  S.E.M. from at least three independent experiments.

lock mixing pulse. During data acquisition, the water signal was suppressed by using the pulse-field gradient based water gate technique (Piotto et al., 1992) except for the correlation spectroscopy experiment, where a low power presaturation was applied during the relaxation delay. TOCSY and NOESY experiments were performed in the phase-sensitive mode, using proportional phase incrementation method for quadrature detection (states-time-proportional phase incrementation) (Marion et al., 1989). Spectra were collected with 512 and 2048 complex data points in  $t_1$  and  $t_2$  dimensions. Data were processed on Silicon Graphics Indigo 2 XL (SGI, Mountain View, CA) or Windows (Microsoft, Redmond, WA) workstations, using XWINNMR or TOPSPIN softwares (Bruker). The  $f_1$  dimension was zero-filled to 1024 real data points. Time domain data were multiplied by shifted sine bell or gaussian functions in both dimensions before Fourier transformation. Proton chemical shifts were reported relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate taken as an internal reference. The sequential assignment was performed at 308 K using the standard sequential assignment strategy (Wüthrich, 1986).  $^3J_{\text{HN-H}\alpha}$  coupling constants were measured on one-dimensional spectra.

Experimental NMR Restraints

Distance restraints for structure calculations were derived from NOE cross-peaks in the NOESY spectrum recorded at 308 K with a mixing time of 150 ms. A NOESY spectrum recorded at 295 K allowed the solving of some ambiguities that were due to overlapping of the resonance signals. NOE cross-peaks were integrated into distances by volume integration using the Felix software (Accelrys, Orsay, France). The NOE volumes were calibrated from well resolved geminal  $\text{H}^\beta$  cross-peaks. The consistency of this calibration was verified on other geminal proton distances. A range of  $\pm 25\%$  of the calculated distance was used to define the upper and lower bounds of the restraints between  $\text{H}^N$ ,  $\text{H}^\alpha$ , and  $\text{H}^\beta$  atoms. For other restraints, a range of  $\pm 12.5\%$  of the squared calculated distance was used to take into account spin diffusion. Structure calculations were conducted with 65, 72, and 93 unambiguous and 19, 15, and 12 ambiguous distance restraints for kp-10, [Ala<sup>6</sup>]kp-10, and [Ala<sup>10</sup>]kp-10, respectively.  $\phi$  dihedral angles were obtained from  $^3J_{\text{HN-H}\alpha}$  coupling constants using the Karplus relation with the Pardi coefficients. For coupling constants with multiple solutions, the value was selected according to its consistency with secondary information provided by the NOE data.

Three-Dimensional Structure Calculation

Three-dimensional structures were calculated in vacuo on a Silicon Graphics Indigo 2 XL station with the CNX program (Accelrys, San Diego, CA), using a random simulated annealing calculation. The target function was similar to that used by Nilges et al. (1988)

TABLE 2  
Functional parameters of the response of kiss1r-CHO-K1 cells to treatment with different kisspeptins as measured using FlexStation technology  
The maximum efficacy is expressed as a percentage of the amplitude induced by  $10^{-6}$  M rkp-10.

Peptide	Agonist			Antagonist		
	<i>n</i>	Maximum Efficacy	EC <sub>50</sub>	<i>n</i>	Maximum Efficacy	IC <sub>50</sub>
		%			%	
rkp-10	11	100	$1.5 \pm 0.53 \times 10^{-8}$			
hKp-10	4	$100.3 \pm 7.3$	$2.6 \pm 0.88 \times 10^{-8}$			
[Ala <sup>1</sup> ]kp-10	6	$87.2 \pm 2.3$	$1.7 \pm 0.48 \times 10^{-8}$			
[Ala <sup>2</sup> ]kp-10	6	$91.2 \pm 5.4$	$1.4 \pm 0.28 \times 10^{-8}$			
[Ala <sup>3</sup> ]kp-10	5	$92.2 \pm 5.5$	$1.0 \pm 0.52 \times 10^{-8}$			
[Ala <sup>4</sup> ]kp-10	5	$89.6 \pm 5.6$	$7.3 \pm 0.17 \times 10^{-8}$			
[Ala <sup>5</sup> ]kp-10	4	$79.0 \pm 2.8$	$1.6 \pm 0.16 \times 10^{-8}$			
[Ala <sup>6</sup> ]kp-10	7	$49.9 \pm 2.8$	$6.5 \pm 3.0 \times 10^{-6}$	10		$9.3 \pm 1.0 \times 10^{-7}$
[Ala <sup>7</sup> ]kp-10	4	$88.9 \pm 3.7$	$3.4 \pm 1.4 \times 10^{-8}$			
[Ala <sup>8</sup> ]kp-10	5	$84.1 \pm 5.5$	$1.0 \pm 0.63 \times 10^{-7}$			
[Ala <sup>9</sup> ]kp-10	6	$74.2 \pm 4.2$	$1.7 \pm 0.49 \times 10^{-7}$			
[Ala <sup>10</sup> ]kp-10	4	$24.1 \pm 5.6$		10	Inactive	

and a force field adapted for NMR structure determination (parallhdg.pro and topallhdg.pro in X-PLOR) was used. A first set of structures was generated, based on the amino acid sequence and on a starting set of distance restraints, using an ab initio-simulated annealing protocol. During the whole process, the distance restraint force was kept to 50 kcal · Å<sup>-2</sup> and the NOE intensities were averaged with the “sum” option. Starting from an extended conformation, a first phase of 40-ps dynamics (time step = 2 fs) at 1000 K was followed by a 20-ps slow cooling step to 100 K (time step = 2 fs). A low weight of the van der Waals repulsive term was used at high temperature to allow a large conformation sampling. The variation of the slope of the asymptote in the NOE potential function allowed a progressive fit of the distance restraints. The structures leading to no systematic distance violation larger than 0.20 Å were submitted for further refinement. A 40-ps simulated annealing procedure from 1000 K to 100 K (time step = 2 fs) with smoothing of the van der Waals repulsions and square-shaped NOE potential, was applied. A final minimization was carried out using the force field derived from CHARMM22 (files parallh22.pro and topallh22.pro) (Brooks et al., 1983) to yield a final set of optimized structures. Analysis of the structures made it possible to resolve ambiguities in the assignment of NOE cross-peaks, which arose from chemical shift degeneracy. This procedure was iterated several times by calculating, at each step, a new set of structures with an improved list of restraints. Nevertheless, some ambiguous assignments still remained and were introduced with an appropriate treatment in CNX (Nilges, 1995). A final set of structures was then generated. The final structures were analyzed by CNX and displayed by using SYBYL (Tripos, Saint Louis, MO) and MOLSCRIPT (Kraulis, 1991).

Statistical Analysis

Hormonal determinations were conducted in duplicate; data are presented as mean ± S.E.M. of at least 10 independent determinations per group. When appropriate, integrated LH secretory responses after i.c.v. administration of the testing compounds were calculated as the area under the curve (AUC), following the trapezoidal rule. Functional experiments were performed at least in triplicate, and data, expressed as mean ± S.E.M, were analyzed with Prism software (GraphPad Software). The EC<sub>50</sub> from the concentration-response curve values were determined using a sigmoidal dose-response fit with variable slope. Statistical comparisons of hormonal values, as well as potencies of kp-10 and kp-10 analogs, were analyzed by ANOVA followed by a Dunnett’s multiple comparison test or a Student-Newman-Keuls multiple range test. Differences were considered significant at  $P < 0.05$

## Results

Earlier studies have shown that the C-terminal decapeptide of human Kisspeptin (45–54; Kp-10) exhibits higher receptor affinity and evokes more potent functional responses than its extended counterparts (Ohtaki et al., 2001). To further explore the structural requirements of this core sequence, we have carried out Ala scan of rat kp-10 to identify important residues for kiss1r-agonistic activity. To this aim, we synthesized rat metastin, rat and human Kp-10, and 10 rat kp-10 Ala-analogs (Table 1) by standard Fmoc-based solid phase peptide synthesis, and we tested their functional activity in vitro on *rkiss1r*-transfected CHO-K1 cells and, for selected compounds, their ability to elicit LH responses in vivo.

**Peptide Analysis.** RP-HPLC analysis of rat metastin, rat and human Kp-10, and Ala-substituted rat kp-10 revealed that the purity of all peptides was higher than 99% (Table 1). For all peptides, the molecular weight observed by MS analysis was consistent with the theoretical value (Table 1).

**In Vitro Functional Assay for Kisspeptins and Synthetic Substituted Analogs.** Activities of the peptides were determined based on the kinetics of  $[Ca^{2+}]_i$  in CHO-K1 cells stably transfected with *rkiss1r*, by using a multimode Flex-Station system. As shown in Fig. 1A and Table 2, kp-10 analogs generated by Ala point substitutions of amino acid residues 1 to 5 and 7 to 9, displayed  $EC_{50}$  values ranging from  $1.04 \times 10^{-8}$  M to  $1.27 \times 10^{-7}$  M, which closely resemble those found for rat and human Kp-10 ( $EC_{50} = 1.54$ – $2.6 \times 10^{-8}$  M), suggesting that amino acids located at those positions are not critical for kp-10 functional activity, and possibly, for its binding to kiss1r. In clear contrast,  $[Ala^6]kp-10$  and  $[Ala^{10}]kp-10$  showed an increased  $EC_{50}$  value ( $>6.46$   $\mu$ M), well above that found for kp-10 and the other analogs studied (Fig. 1B and Table 2).

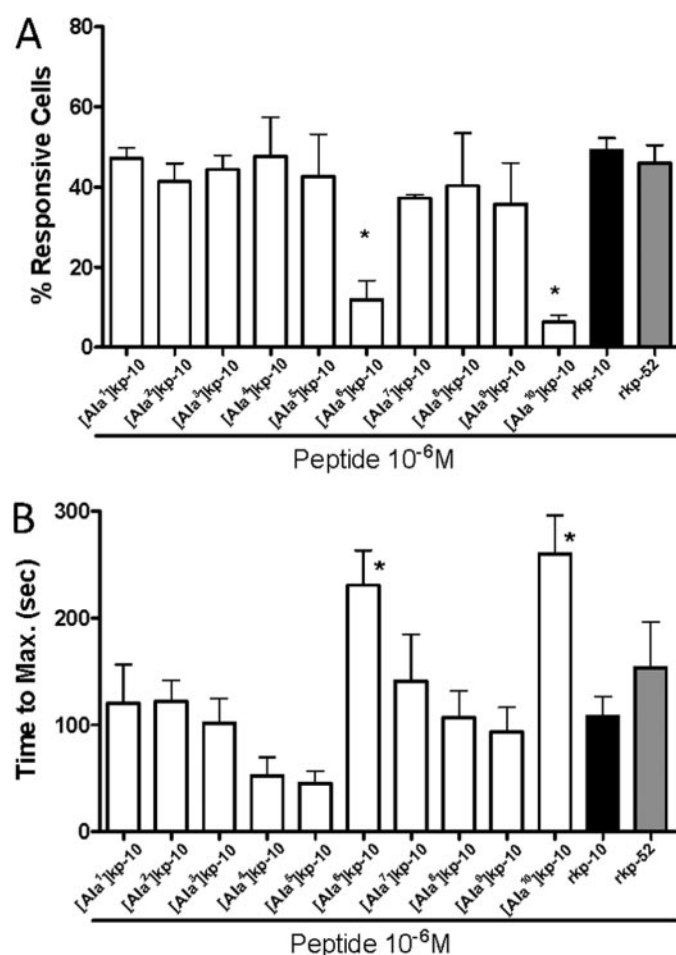
Based on the above data, we performed specific assays to test the potential antagonistic activity of the  $[Ala^6]kp-10$  and  $[Ala^{10}]kp-10$  analogs. The data showed that only the  $[Ala^6]kp-10$  analog had an apparent antagonistic property, with an  $IC_{50}$  value of  $9.31 \times 10^{-7}$  M (Fig. 1C and Table 2), which would reflect a potential competitive action. However, a Schild analysis of the data obtained in these in vitro assays evidenced that  $[Ala^6]kp-10$  did not behave as a typical competitive reversible antagonist (data not shown).

To further understand the properties of the Ala-substituted kisspeptin analogs, we examined their actions on the dynamics of  $[Ca^{2+}]_i$  in individual CHO-K1 cells stably expressing *rkiss1r*. In line with the studies on cell populations, administration of kisspeptin analogs substituted in position 1 to 5 and 7 to 9 did not result in appreciable changes in response as compared with those caused by rat kp-10 and rat metastin (Fig. 2A). Conversely, treatments with  $[Ala^6]kp-10$  and  $[Ala^{10}]kp-10$  showed a significant decrease in the proportion of responsive cells which was accompanied by a marked increase in the time required by responsive cells to reach maximal amplitude of response (Fig. 2B). Taken together, the results obtained in vitro indicate that position 6 and 10 are critical for kiss1r activation and thus possibly for kiss1r binding.

**Effects of kp-10 and Ala-Substituted Analogs on LH Secretion in Vivo.** In vivo studies monitored the ability of  $[Ala^6]kp-10$  and  $[Ala^{10}]kp-10$  to mimic or antagonize the ef-

fects of rat kp-10 in terms of dynamic LH secretion. First, analysis of LH responses to administration of a single bolus of kp-10 or the substituted analogs revealed that  $Ala^6$  substitution resulted in a significant reduction in the LH-releasing effects of the compound, yet  $[Ala^6]kp-10$  retained its capacity to stimulate LH secretion. Thus, although kp-10 evoked an 8-fold increase in serum LH levels that persisted up to 120-min after intracerebroventricular administration, an equimolar dose of  $[Ala^6]kp-10$  induced a 4-fold increase of circulating LH only at 15 min after injection. In good agreement, integrated (120-min) LH responses to  $[Ala^6]kp-10$  were  $\sim 20\%$  of those elicited by kp-10. In contrast,  $Ala^{10}$  substitution fully prevented the ability of the compound to induce LH secretion after intracerebroventricular administration, with individual and integrated LH responses that were strictly similar to those observed in vehicle-injected controls (Fig. 3).

The effects of the combined administration of kp-10 and each of the above analogs were also tested in vivo. To underscore potential antagonistic effects of the Ala-substituted peptides, and considering the enormous potency of kp-10 in

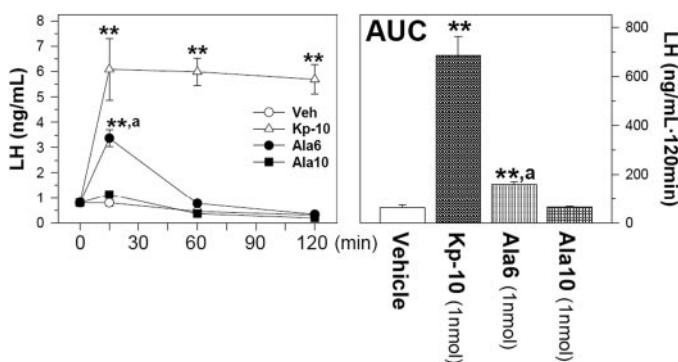


**Fig. 2.** In vitro analysis of the functional features of rat metastin (rat kp-52), rat kp-10, and kp-10 analogs on single cells. Microfluorimetry was applied to determine the effects of treatment with metastin, kp-10 and Ala-substituted analogs of kp-10 on the levels of  $[Ca^{2+}]_i$  in single CHO-K1 cells stably transfected with *kiss1r*. A, percentage of cells that was responsive to a challenge with  $10^{-6}$  M of the corresponding kisspeptin. B, time required by responsive cells to reach maximal level of  $[Ca^{2+}]_i$  after application of the corresponding kisspeptin ( $10^{-6}$  M). Values represent the mean  $\pm$  S.E.M. of a minimum of  $n = 3$  independent experiments, and within each experiment a minimum of 80 individual cells were measured.

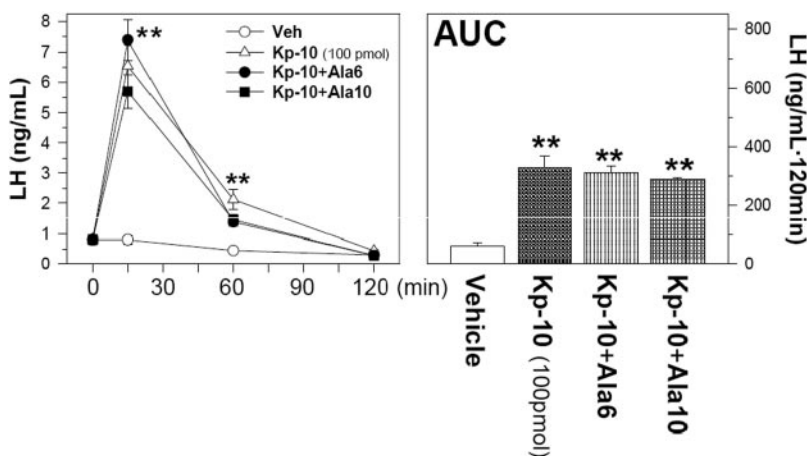


inducing LH release after intracerebroventricular administration (Navarro et al., 2005), 1-nmol doses of the analogs were centrally coadministered with submaximal 100-pmol doses of kp-10. As shown in Fig. 4, injection of 100 pmol kp-10 i.c.v. elicited robust LH secretory responses that peaked at 15-min and progressively decreased thereafter, in keeping with previous reports (Navarro et al., 2005). Similar secretory profiles were observed after intracerebroventricular coinjection of kp-10 and [Ala<sup>6</sup>]kp-10 or [Ala<sup>10</sup>]kp-10, with individual time-point responses that were analogous to those elicited by the administration of kp-10 alone. In good agreement, integrated (120-min) LH responses after intracerebroventricular injection of kp-10 were similar regardless of the coadministration of [Ala<sup>6</sup>]kp-10 or [Ala<sup>10</sup>]kp-10 (Fig. 4).

Finally, the effects of selected Ala-substituted kp-10 analogs on stimulated LH secretion were also assayed in ORX rats. It is noteworthy that ORX has been proven to induce a state of increased expression of *kiss1* in the hypothalamus that seems mechanistically relevant for the elevation of circulating LH levels (Smith et al., 2005). Thus, it was predicted that central blockade of kisspeptins should bring about a detectable reduction of circulating LH in ORX animals. Pro-



**Fig. 3.** In vivo analysis of the effects of Ala<sup>6</sup> and Ala<sup>10</sup> kp-10 analogs on LH secretion in adult male rats. The experimental animals were injected i.c.v. with a single dose of 1 nmol of [Ala<sup>6</sup>]kp-10, [Ala<sup>10</sup>]kp-10, or kp-10, and blood samples for LH determination were obtained before (0 min) and at 15, 60, and 120 min after compound administration. Rats injected intracerebroventricularly with vehicle served as controls. In addition to the time course profiles (left), integrated secretory responses to central administration of kp-10 analogs (calculated as AUC over the 120-min study period) are also shown on the right. Hormonal values are the mean  $\pm$  S.E.M. of at least 10 independent determinations per group. \*\*,  $p < 0.01$  versus corresponding vehicle-injected group; a,  $p < 0.01$  versus corresponding kp-10 injected group (ANOVA followed by Student-Newman-Keuls multiple range test).



**Fig. 4.** In vivo analysis of the effects of coadministration of kp-10 and Ala<sup>6</sup> or Ala<sup>10</sup> analogs on LH secretion in adult male rats. The experimental animals were injected intracerebroventricularly with a 100-pmol dose of kp-10, alone or in combination with a 1-nmol bolus of [Ala<sup>6</sup>]kp-10 or [Ala<sup>10</sup>]kp-10 or kp-10. Blood samples for LH determination were obtained before (0-min) and at 15, 60, and 120 min after compound administration. Rats intracerebroventricularly injected with vehicle served as controls. In addition to the time course profiles (left), integrated secretory responses to central administration of kp-10 analogs (calculated as AUC over the 120-min study period) are also shown on the right. Hormonal values are the mean  $\pm$  S.E.M. of at least 10 independent determinations per group. \*\*,  $p < 0.01$  versus corresponding vehicle-injected group (ANOVA followed by Student-Newman-Keuls multiple range test).

tolocs of repeated injection of 1-nmol i.c.v. boluses of [Ala<sup>6</sup>]kp-10 or [Ala<sup>10</sup>]kp-10, with a total of three injections at 60-min intervals, were implemented, and serum LH levels were monitored 15 min after each injection. As expected, 1-week ORX resulted in the elevation of basal LH levels over control values in intact male rats ( $10.5 \pm 0.9$  ng/ml in ORX versus  $0.8 \pm 0.16$  ng/ml). Repeated administration of 1 nmol [Ala<sup>6</sup>]kp-10 i.c.v. evoked further increases in circulating LH levels at 15-min after each injection of the compound. In contrast, central injection of [Ala<sup>10</sup>]kp-10 failed to modify the pre-elevated LH levels, at any of the time-points studied (Fig. 5).

**NMR Solution Structure of Rat kp-10, [Ala<sup>6</sup>]kp-10, and [Ala<sup>10</sup>]kp-10.** The three-dimensional structures of kp-10, [Ala<sup>6</sup>]kp-10, and [Ala<sup>10</sup>]kp-10 were calculated by a simulated annealing protocol using NMR restraints collected on the 150-ms NOESY spectra recorded at 308 K.

For kp-10, among the 100 generated structures, 88 structures were consistent with the experimental data and the standard covalent geometry. The structures presented no distance violation larger than 0.2 Å and no ideal geometry violations. Analysis of the  $\phi/\psi$  angles (data not shown) revealed a good convergence for residues Asn<sup>4</sup> to Tyr<sup>10</sup>. These residues were found in the  $\phi/\psi$  space energetically allowed and were located in the right-handed helix region of the Ramachandran plot (data not shown). In this region, all kp-10 structures exhibited a similar helical backbone folding, as shown by the superimposition of the 88 structures (Fig. 6A,  $\langle \text{rmsd} \rangle = 0.36$  Å). The  $\phi/\psi$  values obtained for Asn<sup>4</sup>-Tyr<sup>10</sup> residues were intermediate between the characteristic values of the  $\alpha$ - and  $3_{10}$ -helix types. Consistent with these observations, both  $\text{HN}_{i+4} \rightarrow \text{CO}_i$  and  $\text{HN}_{i+3} \rightarrow \text{CO}_i$  hydrogen bonds, typical of  $\alpha$ - and  $3_{10}$ -helix, respectively, were observed in most of the structures.

For [Ala<sup>6</sup>]kp-10 and [Ala<sup>10</sup>]kp-10, 77 and 81 structures, respectively, were retained among the 100 generated structures. As for kp-10, these structures were consistent with the experimental data and the standard covalent geometry, with no distance violation larger than 0.2 Å and no ideal geometry violations. They exhibited a well defined helicoidal folding for residues Trp<sup>3</sup>-Tyr<sup>10</sup> in [Ala<sup>6</sup>]kp-10 structures (Fig. 6B,  $\langle \text{rmsd} \rangle = 0.41$  Å) and for residues Asn<sup>4</sup>-Leu<sup>8</sup> in [Ala<sup>10</sup>]kp-10 structures (Fig. 6C,  $\langle \text{rmsd} \rangle = 0.38$  Å).

For [Ala<sup>6</sup>]kp-10, analysis of the  $\phi/\psi$  angles and hydrogen bonds revealed  $\alpha$ -helical characteristics for residues Trp<sup>3</sup>,

Asn<sup>4</sup>, Leu<sup>8</sup>, and Arg<sup>9</sup>, and 3<sub>10</sub>-helical characteristics for residues Ser<sup>5</sup>, Arg<sup>6</sup>, and Tyr<sup>10</sup>. Although Gly<sup>7</sup> was found in the right-handed helix region of the Ramachandran plot, it could not be classified in any canonical helicoidal type. For [Ala<sup>10</sup>]kp-10, as for kp-10,  $\phi/\psi$  angles and hydrogen bonds of residues Asn<sup>4</sup>-Leu<sup>8</sup> presented mixed characteristics of  $\alpha$ - and 3<sub>10</sub> helices. The two last residues, Arg<sup>9</sup> and Ala<sup>10</sup>, did not exhibit any helicoidal folding.

## Discussion

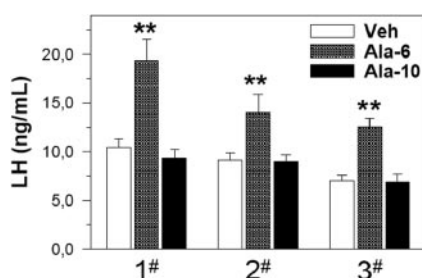
Structure-activity relationship studies constitute a valuable approach to obtain critical information in the development of novel analogs of biologically active peptides with potential clinical applications. The strength of these types of studies is greatly enhanced by applying a combined, interdisciplinary approach in which structural analysis and in vitro activity assays of the compounds under investigation are complemented with in vivo functional testing of selected compounds. In the present work, an Ala scan of rat kp-10 was performed to determine the essential structural requirements for kiss1r agonistic activity.

Our two-pronged in vitro approach to assess the functional features of the Ala-substituted analogs revealed that the Phe<sup>6</sup> and Tyr<sup>10</sup> residues of kp-10 are critical for its kiss1r-agonistic activity. Indeed, when examined both in whole cell populations by using a FlexStation system and at the single

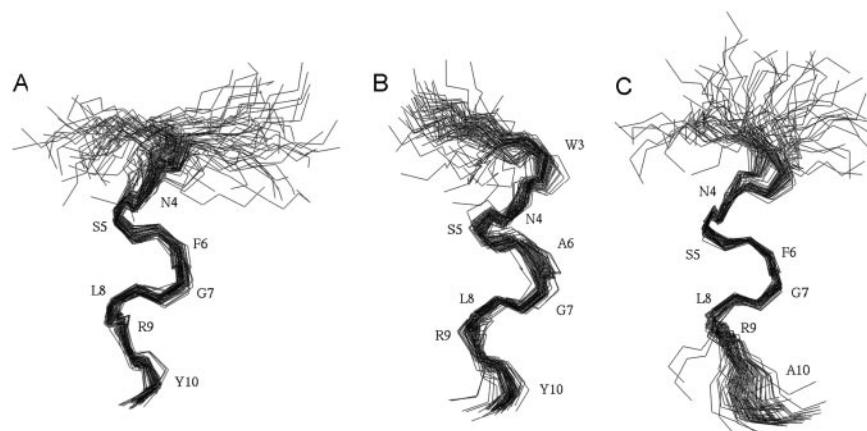
cell level, amino acids 6 and 10 appeared as key functional residues for kp-10. The essential role of the C-terminal residue was expected, because previous studies have shown the indispensable role of this amino acid to maintain an appropriate function not only of kisspeptin (Ohtaki et al., 2001; Niida et al., 2006; Orsini et al., 2007) but also of other members of the RF-amide family of peptides, which includes prolactin-releasing peptide and neuropeptide FF, where the C-terminal Arg-Phe is also critical for their biological activity (Mazarguil et al., 2001; Boyle et al., 2005). However, the native C-terminal phenylalanine residue can be replaced by different aromatic moieties, such as tyrosine, tryptophan, substituted phenylalanine, or naphthylalanine, without significant loss of potency or efficacy of Arg-Phe-amide peptides (Mazarguil et al., 2001; Boyle et al., 2005; Tomita et al., 2006). Conversely, replacement of the C-terminal phenylalanine by saturated side-chain residues such as cyclohexylalanine suppresses the biological activity of kp-10 (Orsini et al., 2007). In the case of neuropeptide FF, the distance of the aromatic ring from the peptide backbone seems to be crucial for access to the binding pocket because homophenylalanine and phenylglycine moieties markedly reduce the affinity of the peptide for its cognate receptor (Mazarguil et al., 2001).

The present results extend and complement previous studies that have analyzed the pharmacological and functional features of Ala-substituted analogs for human Kp-10 and Kp13 (Niida et al., 2006; Orsini et al., 2007) by applying an approach similar to that used herein. In vitro testing of human Kp-10 showed that residues Phe<sup>6</sup>, Leu<sup>8</sup>, Arg<sup>9</sup>, and Phe<sup>10</sup> are crucial to the activity of the peptide (Niida et al., 2006; Orsini et al., 2007). Consistent with these data, we found that Phe<sup>6</sup> and Tyr<sup>10</sup> in rat kp-10 are also critical for *rkiss1r* activation. Conversely, in our study, Leu<sup>8</sup> and Arg<sup>9</sup> do not seem to be playing such a key role. Based on the above, [Ala<sup>6</sup>]kp-10 and [Ala<sup>10</sup>]kp-10 analogs were selected for integral analysis, including assessment of their effects in terms of LH secretion in vivo and comparison of their three-dimensional structure under NMR restraints.

In vivo tests on the LH-releasing capacities of [Ala<sup>6</sup>]kp-10 and [Ala<sup>10</sup>]kp-10 compounds confirmed our initial observations in vitro on the essential roles of Phe<sup>6</sup> and Tyr<sup>10</sup> for full agonistic activity of kisspeptins at the *kiss1r* level. However, such in vivo analyses also unveiled interesting differences in the functional relevance of these two residues. Thus, Phe<sup>6</sup> in rat kp-10 seems critical for maximal activation of *kiss1r* in vivo but perhaps dispensable for receptor binding, given that



**Fig. 5.** In vivo analysis of the effects of Ala<sup>6</sup> and Ala<sup>10</sup> kp-10 analogs on LH hypersecretion in ORX rats. One week after ORX, the animals received three consecutive intracerebroventricular injections (at 60-min intervals) of 1 nmol of [Ala<sup>6</sup>]kp-10 or 1 nmol of [Ala<sup>10</sup>]kp-10, and blood samples for LH determinations were taken by jugular venipuncture at 15-min after each intracerebroventricular injection of the analogs. ORX rats intracerebroventricularly injected with vehicle served as controls. Hormonal values are the mean  $\pm$  S.E.M. of at least 10 independent determinations per group. \*\*,  $p < 0.01$  versus corresponding vehicle-injected group (ANOVA followed by Student-Newman-Keuls multiple range test).



**Fig. 6.** Backbone (N, C $\alpha$ , C' atoms) superimposition of the lowest conformers of kp-10 between residues Asn<sup>4</sup>-Tyr<sup>10</sup> (A), [Ala<sup>6</sup>]kp-10 between residues Trp<sup>3</sup>-Tyr<sup>10</sup> (B), and [Ala<sup>10</sup>]kp-10 between residues Asn<sup>4</sup>-Leu<sup>8</sup> (C).



[Ala<sup>6</sup>]kp-10 was still able to evoke significant LH secretory responses (albeit of lower magnitude than those of kp-10). This suggests its function as partial agonist. In keeping with this view, even in a 10-fold excess, [Ala<sup>6</sup>]kp-10 was unable to inhibit the stimulatory effect of exogenous kp-10 or to suppress the stimulated LH levels in a model of sustained elevation of the prevailing kisspeptin tone, such as the ORX rat (Navarro et al., 2004). Thus, in contrast to that suggested by the results obtained in vitro on rkiss1r-CHO cells, we did not find comparable evidence to support that [Ala<sup>6</sup>]kp-10 might operate as antagonist of kisspeptin actions in vivo.

Our in vivo work also confirmed that Ala<sup>10</sup>-substituted kp-10 is completely devoid of detectable activity in vivo: 1) it did not induce LH release per se, 2) it did not prevent the effects of kp-10 on LH secretion, and 3) it did not affect pre-elevated LH levels in ORX rats after its repeated administration. Given the conspicuous lack of agonistic or antagonistic activity of [Ala<sup>10</sup>]kp-10, it is proposed that such C-terminal position could be essential for kisspeptin binding to kiss1r, a possibility that needs to be experimentally confirmed. From a more general standpoint, our results emphasize the importance of an integral characterization of kisspeptin derivatives, involving in vivo testing. In this context, it is noticeable that previously developed pentapeptides, with apparent full agonistic activity at kiss1r in cellular models, have not been evaluated in terms of gonadotropin secretion in vivo. Studies on pentapeptides derived from C-terminal kisspeptin have been mainly focused on the discovery and/or design of analogs with superagonistic properties, which could be used to profit the antimetastatic abilities of kisspeptin (metastatin). However, in the field of reproductive endocrinology, the functional potency and efficacy of kp-10 is already remarkable in in vivo studies, and this makes difficult the discovery and/or design of superagonistic kisspeptin analogs. On the other hand, in this particular field, it would be highly valuable to obtain compounds with antagonistic capacity for the kisspeptin/kiss1r system, because this pair seems to play a key, hierarchically relevant role in the control of the reproductive axis.

The conformations of kp-10, [Ala<sup>6</sup>]kp-10, and [Ala<sup>10</sup>]kp-10 were investigated by <sup>1</sup>H-NMR spectroscopy and restrained molecular dynamics in a diphenylcarbodiimide-water mixture, a membrane-mimetic medium. The structure of rat kp-10 encompasses a helix spanning from Asn<sup>4</sup> to Tyr<sup>10</sup>, with mixed  $\alpha$  and  $3_{10}$  characteristics, preceded by a disordered N-terminal region. The solution structure of human Kp-13 has been determined in a SDS-water mixture, another membrane mimetic medium (Orsini et al., 2007). Human Kp-13 differs from rat kp-10 by an N-terminal extension (Leu-Pro-Asn) and the substitution of the C-terminal residue (Phe-NH<sub>2</sub> instead of Tyr-NH<sub>2</sub>). It is noteworthy that the helical structure of human Kp-13 runs from Asn<sup>7</sup> to Phe<sup>13</sup> (i.e., at the same relative position as in rat kp-10, but the type of helix in human Kp-13 had not been determined) (Orsini et al., 2007). The observation that human Kp-13 and rat kp-10 share a common helical domain, together with the fact that both peptides behave as full kiss1r agonists (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001) strongly suggests that the helical structure plays a relevant role in kiss1r binding and/or activation.

Substitution of native Phe<sup>6</sup> by an Ala moiety slightly modified the helical structure of the peptide. Specifically,

the helix was extended by one residue in its N-terminal region (Trp<sup>3</sup>-Tyr<sup>10</sup>), and the Gly<sup>7</sup> moiety did not show canonical helical characteristics. These findings suggest that Ala<sup>6</sup> substitution did not dramatically impair the helical conformation of the peptide but only weakly modified the local arrangement around the alanine residue. This mild perturbation is compatible with the partial agonistic profile of [Ala<sup>6</sup>]kp-10.

Substitution of native Tyr<sup>10</sup> residue by an Ala moiety provoked a marked destabilization of the helical structure in the C-terminal part of the peptide. Although a helix with characteristics similar to those of kp-10 was observed between the Asn<sup>4</sup> and Leu<sup>8</sup> residues, no structuration was detected for the last two C-terminal residues, Arg<sup>9</sup> and Ala<sup>10</sup>. Because, [Ala<sup>10</sup>]kp-10 was totally devoid of agonistic and antagonistic properties, it seems that the shortening of the C-terminal helix may be responsible, at least in part, for the lack of activity of the peptide, as already suggested (Orsini et al., 2007).

In summary, our data demonstrate that the replacement of the Tyr<sup>10</sup>-NH<sub>2</sub> residue of kp-10 by an L-Ala-NH<sub>2</sub> moiety abolishes the agonistic activity of this peptide at kiss1r both in vitro and in vivo, whereas the replacement of the Phe<sup>6</sup> residue by L-Ala yields a weak antagonist in vitro that shows severely reduced agonistic activity in vivo. The altered activities of the [Ala<sup>10</sup>]kp-10 and [Ala<sup>6</sup>]kp-10 analogs are attributable to complete destabilization of the C-terminal region of the helix and to a moderate distortion of the helical structure around position 6, respectively. These data, coupled to the type of experimental strategy used herein to examine structure-activity relationships, pave the way for the future design and characterization of new kiss1r agonists and/or antagonists with potential experimental and clinical applications.

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