Peripheral kisspeptin reverses short photoperiod-induced gonadal regression in Syrian hamsters by promoting GNRH release

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Abstract

In seasonal breeders, reproduction is synchronised by day length via the pineal hormone melatonin. In short winter days (short day, SD), the Syrian hamster displays a complete gonadal atrophy together with a marked reduction in expression of kisspeptins (Kp), a family of potent hypothalamic stimulators of GNRH neurons. Both central and peripheral acute injections of Kp have been reported to activate the gonadotrophic axis in mammals. The aim of this study was to determine if and how peripheral administration of Kp54 could restore gonadal function in photo-inhibited hamsters. Testicular activity of hamsters kept in SD was reactivated by two daily i.p. injections of Kp54 but not by chronic subcutaneous delivery of the same peptide via mini-pumps. Acute i.p. injection of Kp54-induced FOS (c-Fos) expression in a large number of GNRH neurons and pituitary gonadotrophs together with a strong increase in circulating testosterone. The activation of pituitary cells by Kp was inhibited by preadministration of the GNRH receptor antagonist acyline. Altogether, our results demonstrate that peripheral Kp54 activates the gonadotrophic axis by stimulating GNRH release and indicate that an appropriate protocol of long-term systemic Kp administration can recrudescence a photo-inhibited reproductive axis.

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Introduction

Important seasonal variations in environmental parameters such as photoperiod, temperature or hygrometry occur throughout the year. Most seasonal breeders rely on annual changes in photoperiod to restrict their fertility to a particular season, ensuring the birth of the offspring occurs at the most favourable time of year. Thus, transfer of male Syrian hamsters from long days (LD) to short days (SD) inhibits their reproductive activity within 8–10 weeks with a tenfold reduction in testes weight and a dramatic decline in gonadotropins and sex steroid plasma levels (Goldman 2001). Inversely, when animals are transferred back to LD condition reproductive activity is restored within 6 weeks (Goldman 2001). SD-adapted Syrian hamsters constitute an adult model of reversible hypogonadism. Annual variations of the photoperiod are translated into a release of the pineal hormone melatonin, the nocturnal production of which relies on night length. It has been documented that the annual variations of melatonin secretion synchronise reproductive activity to season (Reiter 1980). However, the sites and mechanisms of action of melatonin on the hypothalamo-pituitary–gonadotrophic (HPG) axis remain

undetermined, and it is unclear whether melatonin acts at a site upstream or downstream of the GNRH neuron. We reported that melatonin inhibits the expression of several hypothalamic genes in the Syrian hamster (Revel *et al.* 2006a, 2006b, 2008), especially *Kiss1*, which is considered to relay the photoperiodic information to the reproductive axis (Simonneaux *et al.* 2009).

Disruption of either the *Kiss1* gene or the KISS1 receptor gene (*Kiss1r*; formerly referred to as *Gpr54*) generates a hypogonadic phenotype in mice (Funes *et al.* 2003, Seminara *et al.* 2003, d'Anglemont de Tassigny *et al.* 2007, Lapatto *et al.* 2007) and humans (de Roux *et al.* 2003), demonstrating the key role of the *Kiss1*/*Kiss1r* system in the regulation of the HPG axis. In rodents, the *Kiss1* gene is mainly expressed in two hypothalamic nuclei: the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei (Gottsch *et al.* 2004, Smith *et al.* 2005a, 2005b). It is translated into a 145 amino acid protein processed to generate one or several biologically active peptides named kisspeptins (Kp). Both human and rat Kp10 and Kp54 (Kp52 in the rat) share a 10 amino acid common C-terminus and bind to the KISS1 receptor with similar pharmacological

characteristics (Kotani *et al.* 2001). Kp are very potent activators of the HPG axis since central administration of doses as low as 1 fmol triggers the release of LH (Gottsch *et al.* 2004).

In the Syrian hamster, we have shown that *Kiss1* mRNA levels in AVPV and ARC are significantly reduced under SD conditions and this effect is mediated by melatonin (Ansel *et al.* 2010). Furthermore, chronic administration of Kp to SD hamsters via a central route reverses the inhibitory effect of melatonin by reactivating the HPG axis, pointing to Kp as a pivotal gatekeeper of seasonal reproduction (Revel *et al.* 2006a, Simonneaux *et al.* 2009). Since peripheral administration of Kp has been reported to activate the HPG axis as does central infusion (Thompson *et al.* 2004, Navarro *et al.* 2005, Mikkelsen *et al.* 2009), we investigated whether long-term i.p. administration of Kp can restore gonadal activity in SD photo-inhibited male hamsters and further analysed the sites of action of peripheral Kp.

Results

Experiment 1: effect of long-term peripheral administration of Kp54 on testicular activity of photo-inhibited male hamsters

To examine whether peripheral administration of human Kp54 could restore reproductive activity of photo-inhibited hamsters, two types of long-term administration were tested. Chronic delivery (4 weeks) of Kp54 via s.c. mini-pumps failed to increase testes weight and plasma testosterone of male hamsters kept in SD. Twice daily Kp54 injections (twice 10 nmol/hamster) for 5 weeks significantly increased testes weight more than twofold ($P=0.001$; Fig. 1B) and tended to increase basal levels of testosterone ($P=0.07$; Fig. 1B) compared with vehicle-injected hamsters.

Experiment 2: effect of an acute peripheral administration of Kp54 on the HPG axis in the hamster

To reveal the sites of action of peripheral Kp, we studied the expression of the immediate early gene FOS in the forebrain and the pituitary after a single Kp54 i.p. injection. Kp54 strongly increased FOS immunoreactivity in hamsters' anterior pituitary compared with vehicle-treated hamsters ($P=0.005$; Fig. 2A and B). Dual FOS and LHB/FSHB labelling of pituitaries from animals injected with Kp54 revealed that over 90% of FOS-positive cells were gonadotrophs (Fig. 2C). The activation of gonadotrophs was accompanied by a large release of testosterone ($P<0.001$; Fig. 2D). Strikingly, the effect of acute peripheral injection of GNRH on pituitary FOS expression and testosterone production was qualitatively and quantitatively similar to those of Kp54 (Fig. 2B–D). This finding suggests that peripheral Kp may act via a release of GNRH. To test this hypothesis, the

Figure 1 Effect of continuous or repeated long-term peripheral administration of Kp54 on testes weight and testosterone release of short day (SD)-adapted Syrian hamsters. (A) Mean value of paired testes weight (left panel) and concentration of free plasma testosterone (right panel) were measured in SD Syrian hamsters after 4 weeks of chronic s.c. administration of vehicle (Ringer) or Kp54 (10 nmol/hamster per day) via osmotic mini-pumps. Values are mean $\pm$ s.e.m. ($n=5-6$). (B) Mean value of paired testes weight (left panel) and concentration of plasma free testosterone (right panel) were measured in SD Syrian hamsters after 5 weeks of daily i.p. injections of vehicle (Ringer) or Kp54 (twice 10 nmol/hamster). Values are mean $\pm$ s.e.m. ($n=5-7$), *** $P<0.001$ compared with vehicle-injected hamsters (Student's *t*-test).

effect of Kp54 and GNRH was tested in the presence of a GNRH receptor antagonist, acyline.

Pretreatment with acyline not only completely blocked GNRH stimulatory effect, but also the effect of Kp54 ($P=0.017$ and $P=0.028$, respectively; Fig. 3).

To determine the site of action of peripheral Kp54, we analysed the effect of peripherally administered Kp54 on FOS expression in GNRH neurons and downstream testosterone levels. Kp54 i.p. injection significantly induced FOS expression in about 60% of the GNRH neurons ($P=0.0045$; Fig. 4A and B) and testosterone release ($P<0.001$; Fig. 4C) compared with vehicle-treated counterparts.

Experiment 3: effect of photoperiod on FOS expression in the hamster anterior pituitary

To analyse the effect of photoperiod on GNRH- or Kp54-induced FOS expression in the pituitary, an i.p. injection of vehicle (Ringer; Braun Medical, Boulogne, France), GNRH or Kp54 was given to either LD- or SD-adapted hamsters. Both GNRH- and Kp-induced FOS expression in LD ($P<0.05$ and $P<0.001$, respectively; Fig. 5). While photoperiod did not affect

Figure 2 Effect of acute peripheral injection of Kp54 or GNRH on pituitary FOS expression and plasma testosterone level in the Syrian hamster. (A) Mean value of FOS immunoreactivity (arbitrary units, a.u.) in the pituitary of long day-adapted Syrian hamsters was analysed 2 h after an i.p. injection of vehicle (Ringer) or Kp54 (15 nmol/hamster). The scale bar on pictures represents 50 μ m. Values are mean $\pm$ S.E.M. ($n=5$), ** $P<0.01$ compared with vehicle-injected hamsters (Student's t -test). (B) Phenotype of FOS-positive cells after GNRH or Kp54 i.p. injection in the pituitary of Syrian hamsters. Representative images of dual immunohistochemistry for FOS (blue, open arrows) and LHB/FSHB (brown, black arrows) in the anterior pituitary of long day-adapted Syrian hamsters 2 h after i.p. injection of GNRH (30 nmol/hamster) or Kp54 (15 nmol/hamster). (C) Proportion of FOS-positive cells co-localising LHB/FSHB in the anterior pituitary of Syrian hamsters 2 h after an i.p. injection of GNRH (30 nmol/hamster) or Kp54 (15 nmol/hamster). Values are mean $\pm$ S.E.M. ($n=3$). (D) Concentration of free plasma testosterone (pg/ml) 2 h after vehicle (Ringer), GNRH (30 nmol/hamster) or Kp54 (15 nmol/hamster) i.p. injection. Values are mean $\pm$ S.E.M. ($n=5-6$), *** $P<0.001$ compared with vehicle-injected hamsters (ANOVA followed by Tukey's *post hoc* analysis).

GNRH-induced FOS expression, Kp54 induced a significantly larger expression of FOS in SD hamsters compared with LD hamsters ($P<0.01$; Fig. 5).

Discussion

Our study reports that long-term administration of Kp54 via a peripheral route reactivates the HPG axis of photo-inhibited SD hamsters, providing the protocol is appropriately defined. Our study also indicates that peripheral Kp54 is acting by promoting GNRH release and downstream activation of pituitary gonadotrophs.

We observed that continuous s.c. administration of Kp54 to SD male hamsters using mini-pumps failed to reactivate the HPG axis as attested by low testes weight and low plasma testosterone levels. We previously reported that chronic central administration of Kp10 to SD male hamsters during 4 weeks is sufficient to restore LD-like testes weight and testosterone levels (Revel *et al.* 2006a). While centrally administered Kp could reach non-GNRH neurons targets, this might not be the case for peripherally given peptide. One can hypothesise that the activation of non-GNRH targets is required for the proper stimulation of the reproductive axis in a constant delivery protocol. The absence of effect of continuous s.c. administration of Kp54 is consistent with the finding that in SD Siberian hamsters, continuous peripheral administration of Kp10 failed to increase testes weight (Greives *et al.* 2008). Since the stability of Kp54 in the Alzet osmotic mini-pumps for several weeks was previously established (Revel *et al.* 2006a, Thompson *et al.* 2006), it appears unlikely that the lack of Kp54 effect in this protocol is only due to the degradation of the peptide in the pumps. Hence, one would speculate that the dose used was not appropriate; however, a prior study reported that continuous administration of a similar dose of Kp54 induced testicular degeneration in rats (Thompson *et al.* 2006). Therefore, we propose that the failure of continuous administration of Kp54 to activate testicular activity results from a downregulation/desensitisation of the KISS1 receptor. Indeed, it has been reported that a preliminary continuous i.v. administration of Kp10 in male rhesus monkeys prevented the LH rise induced by a further bolus of Kp10 (Seminara *et al.* 2006, Ramaswamy *et al.* 2007). Moreover, continuous Kp exposure of mice mediobasal hypothalamus explants induced a sustained GNRH release for 4 h followed by a decrease, suggesting a desensitisation of the KISS1 receptor in the median eminence area (d'Anglemont de Tassigny *et al.* 2008). Alternatively, one might speculate that constant exposure to Kp induces a constant release of GNRH, which is known to desensitise pituitary GNRH receptors (Belchetz *et al.* 1978) and this could also explain the absence of effect of chronic Kp54 administration on gonadal activity in photo-inhibited hamsters. By contrast, we found that two daily injections of Kp54 for 5 weeks to SD-acclimated, sexually inactive, Syrian hamsters significantly increased testes weight, indicating a reactivation of the HPG axis despite the inhibitory photoperiod. The stimulatory effect of repeated versus continuous peripheral administration of Kp54 may indicate an absence of desensitisation/downregulation of the KISS1 receptor or GNRH receptor using this protocol. This assumption is supported by the previous findings that intermittent infusions of Kp10 to juvenile agonadal male monkeys induce sustained GNRH-dependant LH pulsatile release (Plant *et al.* 2006) and that repeated i.v. administration of low doses of Kp10 to adult male rats increases LH levels

Figure 3 Effect of a GNRH antagonist on Kp-induced FOS expression in the anterior pituitary of the Syrian hamster. Mean value of FOS immunoreactivity (a.u.) in the pituitary of long day-adapted Syrian hamsters pretreated or not with acyline, a GNRH receptor antagonist (50 µg/hamster), then injected intraperitoneally with GNRH (30 nmol/hamster) or Kp54 (15 nmol/hamster) 2 h before killing. Values are mean $\pm$ S.E.M. ($n=3-6$), ** $P<0.01$ compared with vehicle-injected hamsters (ANOVA followed by Tukey's *post hoc* analysis); # $P<0.05$, ### $P<0.001$ compared with acylin-pretreated hamsters (ANOVA followed by Tukey's *post hoc* analysis).

without evidence of desensitisation (Tovar *et al.* 2006). However, further experiments are required to clearly establish the existence of this phenomenon and its mechanisms in the Syrian hamster.

Another study reported that one daily i.p. injection of Kp10 failed to reactivate the HPG axis in photo-inhibited Siberian hamsters (Greives *et al.* 2008). Discrepancies between this study and ours can be explained by the form of the peptide used, the frequency of peripheral administration and the animal model chosen. First, longer forms of the human peptide tend to act in a prolonged manner compared with shorter forms (Mikkelsen *et al.* 2009). Secondly, one daily injection might not be enough to induce a sustained activation of the HPG axis. Indeed, so far only a frequency of two daily peripheral bolus of Kp10 over 60 h caused ovulation in seasonally acyclic ewes (Caraty *et al.* 2007), underlining the importance of the frequency of administration. Thirdly, Syrian and Siberian hamsters display different photoperiodic responses (Bartness *et al.* 1993) and *Kiss1* mRNA regulation by photoperiod is opposite in the arcuate nucleus of the two species (Greives *et al.* 2007, Simonneaux *et al.* 2009, Ansel *et al.* 2010). These observations suggest there exist important differences regarding the effect and physiological roles of Kp in mediating photoperiodic control of the reproductive axis between Siberian and Syrian hamsters.

To elucidate the target site(s) of peripheral Kp54, we examined FOS expression as a marker of cellular activation in the brain and pituitary. Peripheral Kp54 strongly induced labelling experiment showed that Kp-FOS expression in the anterior pituitary, an effect followed by the downstream increase in levels of circulating testosterone reflecting increased LH secretion. The dual-induced FOS expression in the anterior pituitary is restricted to gonadotrophs. A direct effect of Kp on the pituitary is still a matter of debate. In cultured pituitary cells from peripubertal rats (Gutierrez-Pascual *et al.* 2007) or glands from pubertal rats (Navarro *et al.* 2005) application of 10 nM Kp10 increased LH release but did not affect FSH levels.

However, on cultured anterior pituitaries (Thompson *et al.* 2004) or dissociated cells of anterior pituitaries (Matsui *et al.* 2004) from adult rats exogenous Kp10 failed to increase LH or FSH even at doses as high as 1 µM. In the adult Syrian hamster, the effect of Kp54 on FOS immunoreactivity in the anterior pituitary was completely blocked by acyline, a GNRH antagonist. Thus, peripheral Kp54 activates the pituitary in a GNRH-dependant manner. Our results are in line with prior studies in sheep showing that peripheral Kp-induced GNRH release in cerebrospinal fluid (Caraty *et al.* 2007) but failed to induce LH release in hypothalamo-pituitary disconnected animals (Smith *et al.* 2008), and that in rodents, acyline prevented Kp-induced LH release (Gottsch *et al.* 2004, Irwig *et al.* 2004, Mikkelsen *et al.* 2009). These observations indicate that peripheral Kp54 activates the pituitary gonadotrophs via the release of GNRH and exclude a direct effect of the peptide on the pituitary. However, the precise site of action of

Figure 4 Effect of peripheral Kp54 injection on FOS expression in GNRH neurons and subsequent testosterone in the same animals. (A) The images show FOS immunoreactivity in GNRH neurons of Syrian hamsters kept in long days and injected peripherally with vehicle (Ringer, left panel) or Kp54 (15 nmol/hamster, right panel) 2 h before killing. An open arrow indicates a FOS-negative GNRH neuron while a black arrow points to cells expressing both GNRH and FOS. The scale bar represents 20 µm. (B) Percentage of GNRH neurons expressing FOS in the anterior hypothalamus of Syrian hamsters 2 h after an i.p. injection of vehicle or Kp54. Values are mean $\pm$ S.E.M. ($n=6$), ** $P<0.001$ compared with vehicle-treated hamsters (Student's *t*-test). (C) Concentration of free plasma testosterone (pg/ml) 2 h after vehicle, or Kp54 i.p. injection. Values are mean $\pm$ S.E.M. ($n=6$), *** $P<0.001$ compared with vehicle-injected hamsters (Student's *t*-test).

Figure 5 Effect of photoperiod on GNRH or Kp54-induced FOS expression in the Syrian hamster pituitary. Mean value of FOS immunoreactivity (arbitrary unit, a.u.) in the anterior pituitary of Syrian hamsters kept in long days (LD) or short days (SD) injected intraperitoneally with vehicle (Ringer), GNRH (30 nmol/hamster) or Kp54 (15 nmol/hamster) 2 h before killing. Values are mean $\pm$ S.E.M. ($n=5-6$), * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ when compared with Kp54-injected LD hamsters (two-way ANOVA followed by Bonferroni's *post hoc* analysis).

peripheral Kp on GNRH neurons remains unknown. We found that peripheral Kp54 increased FOS in GNRH neurons. In a prior study in mice, we showed that peripheral mouse Kp10 failed to induce FOS expression in GNRH cell bodies (Mikkelsen *et al.* 2009). The reason for this difference could be a matter of peptide sequence. Our new findings confirm a number of other studies showing that peripheral Kp54 is acting upstream of the pituitary, on GNRH neurons. Peripheral Kp could act on GNRH nerve terminals of the median eminence to induce GNRH release as suggested by the presence of a few Kp immunoreactive fibres in the external layer of the median eminence of the Syrian hamster (L Ansel, D Streicher, J Bartzen, JD Mikkelsen & V Simonneaux 2009, unpublished observations) and the female rat (Desroziers *et al.* 2010). Furthermore, the ability of Kp to induce GNRH release from mice mediobasal hypothalamus explants despite tetrodotoxin pretreatment (d'Anglemont de Tassigny *et al.* 2008) strengthens this hypothesis. However, the possibility that peripheral Kp crosses the blood-brain barrier requires experiments to be substantiated.

Because Syrian hamsters display marked photoperiodic variations in both *Kiss1* and Kp expression with lower levels in SD (Revel *et al.* 2006a, Simonneaux *et al.* 2009, Ansel *et al.* 2010), we investigated whether gonadotrophs responsiveness to Kp or GNRH depends on photoperiod. An acute injection of Kp54 increased FOS immunoreactivity in the pituitary of SD than of LD-adapted animals. The increased FOS immunoreactivity in SD animals is not due to a photoperiodic change in the anterior pituitary's responsiveness to GNRH since there was no effect of photoperiod on GNRH-induced FOS expression. Insufficient dose of GNRH cannot account for the lack of photoperiodic difference in GNRH effect on FOS expression in our

study since a dose as low as 0.1 nmol of GNRH given subcutaneously induces a massive LH release in Syrian hamsters (Pickard & Silverman 1979). Moreover, previous studies reported no photoperiodic difference of pituitary response to exogenous GNRH (Pickard & Silverman 1979). Therefore, Kp54 sensitivity to photoperiod probably occurs at the level of the GNRH neurons. One might speculate that low levels of Kp in SD hamsters (Revel *et al.* 2006a) could upregulate/resensitise KISS1 receptor at the GNRH neurons. Indeed, in fasting prepubertal rats, a condition where *Kiss1* mRNA is decreased and *Kiss1r* mRNA is increased (Castellano *et al.* 2005), Kp10 is more potent to induce LH release (Castellano *et al.* 2005), indicating a higher sensitivity of the HPG axis in undernutrition. However, a feedback effect of testosterone, a direct effect of photoperiod/melatonin on GNRH neurons sensitivity to Kp or an increase in the releasable pool of GNRH cannot be excluded.

In conclusion, our data demonstrate that exogenous peripheral Kp54 reactivates a photo-inhibited HPG axis by stimulating GNRH release and this sensitivity to Kp54 is photoperiod-dependant. Taken together, our data confirm the key role played by Kp in the seasonal control of reproduction in the Syrian hamster and demonstrate that peripheral Kp54 is also effective under certain conditions. In light of the existing literature, our results also suggest that long and short forms of Kp are not interchangeable and that Siberian and Syrian hamsters display considerable differences in the effects and physiological roles of Kp on the reproductive axis.

Materials and Methods

Animals

Male Syrian hamsters (*Mesocricetus auratus*) weighing 100–180 g were raised in our animal facilities (INCI, Strasbourg, France) with food and water *ad libitum*, under controlled temperature ($22 \pm 1^\circ\text{C}$) and long photoperiod (LD; 14 h light (200 lux):10 h darkness (2 lux, dim red light) cycle with lights on at 0600 h). When required, hamsters were exposed to short photoperiod (SD; 10 h light:14 h darkness cycle with lights on at 1000 h) for 10 weeks. All experiments were conducted in accordance with the French National Law (licence n° 67-32) implementing the European Communities Council Directives of 24 November 1986 (86/609/EEC).

Peptide and reagents

Human Kp54 was selected to investigate the effect of peripheral Kp on the gonadotropic axis because it exerts a long-lasting effect on the HPG axis compared with Kp10 (Mikkelsen *et al.* 2009). It was synthesised by GenScript (Piscataway, NJ, USA). GNRH was purchased from Sigma and acyline, a selective GNRH antagonist (Herbst 2003), was kindly provided by Drs John Amory and William J Bremner from the University of Washington, Seattle, USA.

Experimental designs

Experimental design 1: effect of long-term peripheral administration of Kp54 on testicular activity of photo-inhibited male hamsters

To examine whether peripheral administration of Kp54 could restore reproductive activity of photo-inhibited hamsters, two types of long-term administration were tested. Hamsters ($n=6-7/\text{group}$) were transferred to SD for 10 weeks, after which testicular regression was verified by scrotal palpation. In the first protocol, s.c. chronic administration was tested with osmotic mini-pumps (Alzet, Charles River, Arbresle, France; model 2004, 4-week treatment) filled with either vehicle (Ringer) or Kp54 (140 μM , i.e. 10 nmol/hamster per day) delivered at a rate of 2.5 $\mu\text{l/h}$ over a period of 28 days. The dose of Kp54 was chosen according to previous studies in rats (Thompson *et al.* 2006). The hamsters were anaesthetised with 3% isoflurane and a small incision of the skin was made in the interscapular region. A haemostat was used to create a pocket into which the pump was inserted with the flow moderator facing away from the incision. Xylocain was administered in the wound before the skin was sutured with wound clips. In the second protocol, hamsters were intraperitoneally injected (10 nmol/hamster) twice daily (1 h after lights on and when lights off) with either vehicle (Ringer) or Kp54 (100 μM). The hamsters were kept in SD for the 4 or 5 weeks (first and second protocol, respectively) of Ringer or Kp54 administration. In twice daily intraperitoneally injected hamsters, 4 weeks of treatment increased testes size as examined by scrotal palpation. Injections were performed for one more week to maximise Kp54 effect. Hamsters' body weight was measured at the beginning and at the end of the period of Kp54 chronic administration. At the end of the experiments, the hamsters were killed by decapitation after deep isoflurane anaesthesia (AErrane; Baxter, Maurepas, France). Trunk blood was collected in heparinised tubes. The plasma was separated and stored at -20°C until testosterone assay. The testes were immediately dissected and weighed.

Experimental design 2: effect of an acute peripheral administration of Kp54 on the HPG axis in the hamster

To identify Kp sites of action, we examined the effect of peripheral administration of Kp54 on FOS expression in GNRH neurons and the pituitary. LD-acclimated hamsters ($n=5/\text{group}$) were intraperitoneally (100 $\mu\text{l}/\text{hamster}$) injected with a vehicle solution (Ringer) or 150 μM Kp54 solution (15 nmol/hamster). Two hours after injection, hamsters were deeply anaesthetised with isoflurane vapours and perfused transcardially with 4% formaldehyde in 0.1 M phosphate buffer pH 7.4. The rostral area of the forebrain known to contain GNRH neurons and the entire pituitary tissue (both anterior and posterior lobes) were sectioned and the expression of FOS was analysed by semi-quantitative immunohistochemistry.

Dual labellings were performed to phenotype FOS responsive cells in the pituitary and forebrain. The pituitaries of three GNRH-injected and three Kp54-injected hamsters were processed for dual FOS and LHB/FSHB immunohistochemistry. Three sections per pituitary were processed and for each section, three different areas were analysed.

To determine whether the effect of Kp54 on the pituitary is direct or mediated via GNRH, five groups of LD-acclimated hamsters ($n=4-6/\text{group}$) were intraperitoneally injected (100 $\mu\text{l}/\text{hamster}$) first with either vehicle (Ringer) or 50 μg of the GNRH receptor antagonist acyline, then, 1 h later, with vehicle or 300 μM GNRH or 150 μM Kp54. Two hours after the last injection, animals were killed as described previously and pituitary tissues were examined for FOS expression. Moreover, to find out if pituitary cell activation is correlated to downstream activation of the HPG axis, blood was taken by cardiac puncture. The plasma was extracted, stored at -20°C and processed for testosterone assay. Testosterone was preferred to LH to assess the downstream activation of the HPG axis because peripheral Kp effect on LH release is no longer detectable 60 min after the injection (Navarro *et al.* 2005) whereas Kp54 exerts a long-lasting effect on testosterone secretion in the rat (Mikkelsen *et al.* 2009).

Experimental design 3: effect of photoperiod on FOS expression in the hamster anterior pituitary

Hamsters were kept in LD or SD for 10 weeks (three groups per photoperiod, $n=6/\text{group}$). For each photoperiod, groups of hamsters received a single i.p. injection of either vehicle (Ringer), 300 μM GNRH or 150 μM Kp54. Two hours after the injection, the hamsters were deeply anaesthetised with pentobarbital (5 ml/kg) and perfused transcardially with 4% formaldehyde in 0.1 M PBS pH 7.4. The fixed testes were dissected and weighed to examine for photoperiodic regression. The pituitaries were removed and processed for FOS immunohistochemistry.

Immunohistochemistry

After perfusion of 4% formaldehyde, brain with the pituitary attached was immediately removed from the skull, postfixed for 24 h at 4°C in the same fixative, rinsed overnight in PBS, dehydrated and embedded in polyethylene glycol as described by Klosen *et al.* (1993). Microtome sections (12 μm thick) were cut and processed (one every 120 μm) for immunohistochemistry. For FOS detection, non-specific binding sites were saturated for 1 h with 3% dry skimmed milk. Primary anti-FOS rabbit polyclonal antibody (1:2000, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was applied overnight. This antibody is unlikely to detect Fos-related antigens because it was raised against the N-terminus of the protein, region showing little similarity with Fos-related antigens. The sections were then incubated for 1 h with a secondary biotinylated donkey anti-rabbit antibody (1:2000, Jackson Immunoresearch Laboratories, West Grove, PA, USA) followed by a 1 h incubation with streptavidin-peroxidase (1:2000, Roche). Peroxidase activity was detected using 3,3'-diaminobenzidine (Sigma) as a chromogen. The slides were then dehydrated and mounted with Eukitt.

For the quantification of FOS immunohistochemistry, photomicrographs of pituitaries were taken with a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France) equipped with an Olympus DP50 digital camera (Olympus France, Rungis, France). All parameters of the microscope and

the camera software (Viewfinder Lite, Olympus) were standardised. Photomicrographs were taken with a 5× objective and quantified with ImageJ software (Rasband, W.S., US National Institutes of Health, Bethesda MD, USA). The quantification method used was adapted with minor modifications from Salingre *et al.* (2009). Briefly, pictures were thresholded according to the intensity of the background and the same threshold was applied for each individual experiment. A particle analysis was performed on the pituitary and particles whose diameter was inferior to 30 pixels were excluded. The sum of the integrated density of each immunoreactive particle (i.e. FOS-positive cell) was calculated and divided by the total area quantified to obtain a mean integrated density per surface unit taking into account both the number of immunoreactive particles and the intensity of the staining.

FOS-LHB/FSHB dual immunohistochemistry in pituitary tissue

For dual FOS and LHB/FSHB immunohistochemistry, endogenous alkaline phosphatase was inhibited with 15% acetic acid for 5 min. The sections were first processed for FOS immunohistochemistry as described previously except for the enzyme label (streptavidin–alkaline phosphatase, 1:2000, Roche). The enzymatic activity was detected for 40 min using Naphthol AS MX phosphate as substrate and Fast Blue BB as a chromogen. The FOS and the biotinylated donkey anti-rabbit antibodies were then eluted with 100 mM glycine and 0.3% Triton X-100 for twice 15 min. Gonadotrophs were labelled with a mix of anti-LHB and anti-FSHB antibodies (LHB: 1:60 000 rabbit polyclonal antibody; FSHB: 1:40 000 rabbit polyclonal antibody, both from National Hormone and Peptide Program). LHB and FSHB presence was detected with peroxidase and 3,3'-diaminobenzidine as described earlier.

For FOS and LHB/FSHB dual staining, the total number of blue positive nuclei and brown LHB/FSHB-FOS-positive cells was counted manually by an observer unaware of the treatment of individual animals. The proportion of gonadotrophs expressing FOS and the proportion of FOS-positive cells expressing LHB/FSHB were calculated for each animal.

FOS-GNRH dual immunohistochemistry

After fixation, brain sections were processed as described previously with minor modifications (Mikkelsen *et al.* 2009). Briefly, sections were incubated in a rabbit serum against FOS (1:12 000 (Mikkelsen *et al.* 1998)), immunoreacted with biotinylated donkey anti-rabbit serum (1:1000, cat#711-066-152, Jackson Labs) and reaction product visualised using a nickel-enhanced diaminobenzidine method resulting in a black staining of the FOS-labelled nuclei. After several PBS washes, the FOS-stained sections were incubated with a rabbit antiserum against GNRH (1:4000, cat#H-040-02; Phoenix Pharmaceuticals, Burlingame, CA, USA) stained with the same avidin–biotin method described earlier, but the reaction product was detected using 3,3'-diaminobenzidine as a chromogen. Finally, the sections were mounted on gelatinised glass slides, dried and coverslipped in Depex.

For quantification, all GNRH-immunoreactive neurons were counted by an observer unaware of the treatment

group. Double-labelled neurons characterised by a black-stained nucleus within a brown bipolar neuron were counted, and for each animal the percentage of observed GNRH-immunoreactive neurons that co-expressed nuclear FOS staining were calculated.

Testosterone assay

Free testosterone levels were measured using a direct RIA kit (DPC coat-a-count RIA method; Siemens Medical Solutions, Mölndal, Sweden) as described previously (Mikkelsen *et al.* 2009). The kit analytical sensitivity is of 15 pg/ml. The coefficients of variation are of 18.3, 8.5 and 8.0% for mean testosterone values of 1.2, 8.9 and 20 pg/ml respectively.

Statistical analysis

Student's *t*-test or one-way ANOVA was carried out followed by Tukey's multicomparisons. For experiment 3, a two-way ANOVA was performed followed by Bonferroni's multicomparisons. Two-tailed *P* values are expressed as **P*<0.05, ***P*<0.01 and ****P*<0.001.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research project reported.

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