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PhD thesis

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Characterisation of the kisspeptin system:
The role of sex, obesity, and endocrine disruptors

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PhD Thesis

Characterisation of the kisspeptin system: The role of sex, obesity, and endocrine disruptors

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Title: Characterisation of the kisspeptin system: The role of sex, obesity, and endocrine disruptors

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Contents

Preface

This thesis is the result of a three year PhD programme at The Faculty of Health and Medical Sciences, University of Copenhagen. The work was primarily conducted at Neurobiology Research Unit, Copenhagen University Hospital, Rigshospitalet.

The thesis is based on the following original manuscripts, which in the text are referred to by their Roman numerals:

I. **Overgaard A**, Tena-Sempere M, Franceschini I, Desroziers, E, Simonneaux V, Mikkelsen JD (2013) Comparative analysis of kisspeptin-immunoreactivity reveals genuine differences in the hypothalamic Kiss1 systems between rats and mice. Peptides 45:85-90

II. **Overgaard A**, Ruiz-Pino F, Castellano J, Tena-Sempere M, Mikkelsen JD. Disparate changes in kisspeptin and neurokinin B expression in the arcuate nucleus following sex steroid manipulation reveal differential regulation of the two KNDy peptides in rats. *Manuscript*

III. **Overgaard A**, Holst K, Mandrup KR, Boberg J, Christiansen S, Jacobsen PR, Hass U, Mikkelsen JD (2013) The effect of perinatal exposure to ethinyl oestradiol or a mixture of endocrine disrupting pesticides on kisspeptin neurons in the rat hypothalamus. Neurotoxicology 37:154-62

IV. Castellano JM, **Bentsen AH**, Sánchez-Garrido MA, Ruiz-Pino F, Romero M, Garcia-Galiano D, Aguilar E, Pinilla L, Diéguez C, Mikkelsen JD, Tena-Sempere M (2011) Early metabolic programming of puberty onset: impact of changes in postnatal feeding and rearing conditions on the timing of puberty and development of the hypothalamic kisspeptin system. Endocrinology 152(9):3396-408

V. **Overgaard A**, Axel AMD, Lie MEK, Hansen H, Mikkelsen JD. High plasma triglyceride levels strongly correlate with low kisspeptin in the arcuate nucleus of male rats. *Manuscript*

The following original and review papers are related to the work described in the thesis, but not included in the thesis, and are referred to as regular references:

Bentsen AH, Ansel L, Simonneaux V, Tena-Sempere M, Juul A, Mikkelsen JD (2010) Maturation of kisspeptinergic neurons coincide with puberty onset in male rats. Peptides 31: 275-83

Castellano JM, **Bentsen AH**, Mikkelsen JD, Tena-Sempere M (2010) Kisspeptins: Bridging energy homeostasis and reproduction. Brain Research 1364:129-38 (review)

Ruiz-Pino F., Navarro VM, **Bentsen AH**, Garcia-Galiano D, Sanchez-Garrido MA, Ciofi P, Steiner RA, Mikkelsen JD, Pinilla L, Tena-Sempere M (2012) Neurokinin B and the control of the gonadotropic axis in the rat: developmental changes, sexual dimorphism, and regulation by gonadal steroids. Neuroendocrinology 153(19):4818-29

Desroziers E, Droguerre M, **Bentsen AH**, Robert V, Mikkelsen JD, Caraty A, Tillet Y, Duittoz A, Franceschini I (2012) Embryonic development of kisspeptin neurones in rat. Journal of Neuroendocrinology 24:1284-95

Lie MEK, **Overgaard A**, Mikkelsen JD (2013) Effects of a postnatal high-fat diet exposure on puberty onset, the estrous cycle, and kisspeptin in female rats. Reproductive Biology 13:298-308

I changed my name from Agnete Høyer Bentsen to Agnete Overgaard in July, 2012.

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Agnete Overgaard
Copenhagen, September 2013

Summary

The recently discovered hypothalamic neuropeptide kisspeptin potently stimulates gonadotropin-releasing hormone secretion and thereby the hypothalamic-pituitary-gonadal (HPG) axis. Neurones expressing kisspeptin are sensitive to key regulators of the HPG axis, such as sex steroids and metabolic status, and accordingly play an important role in control of the HPG axis. Kisspeptin neurones are therefore considered to be important gatekeepers for puberty onset and for the positive and negative steroid control of reproductive function.

The aims of the thesis were twofold: 1) To characterise the kisspeptin system with respect to sex and species differences and possible co-localisation of kisspeptin and neurokinin B, which is another peptide important for HPG axis regulation. 2) To investigate whether kisspeptin neurones are sensitive to known environmental risk factors for advanced puberty and decreased reproductive function, such as obesity and exposure to endocrine disruptors.

We found differences in kisspeptin immunoreactivity between the closely related species of mice and rats in both the anteroventral nucleus (AVPV) and the arcuate nucleus (ARC). The mouse AVPV contained kisspeptin-immunoreactive neurones, whereas this was only the case in the rat after inhibition of axonal transport. In the ARC, the rat had clearly discernable neurones, whereas the mouse had dense fibres. These findings are in contrast to findings at the mRNA level, and suggest posttranscriptional species differences. Further, we found more kisspeptin- and neurokinin B-immunoreactive neurones in the ARC of female compared to male rats. Topographical mapping revealed that the largest sex difference in immunoreactive neurones was in the caudal ARC both for kisspeptin and neurokinin B. Both female kisspeptin and neurokinin B expression were reduced to male levels after neonatal estrogenisation. On the contrary, adult gonadectomy and sex steroid replacement had opposite effects on the number of kisspeptin- and neurokinin B-immunoreactive neurones. The number of kisspeptin-immunoreactive neurones decreased and neurokinin B-immunoreactive neurones increased upon sex steroid replacement in both males and females, which suggests differential regulation of the two peptides.

In a rat model of postnatal over- and underfeeding, obtained by raising the pups in small and large litters, respectively, we showed that pubertal timing was sensitive to both early-life subnutrition and overfeeding. Specifically, we found that the overfed group had increased *Kiss1* mRNA and advanced puberty onset, whereas the underfed group had decreased *Kiss1* mRNA and kisspeptin levels and delayed puberty. This suggests that changes in kisspeptin expression

may be involved in the altered pubertal timing. In adult male rats subjected to high-fat diet for three months after weaning, we found no change in the number of kisspeptin-immunoreactive neurones. However, a strong negative correlation between kisspeptin expression and plasma triglyceride levels was observed, suggesting that it is the high plasma triglycerides and not the diet, leptin, or bodyweight, which affect kisspeptin expression.

Finally, we assessed the effect of perinatal exposure to ethinyl oestradiol or to endocrine disrupting pesticides on adult *Kiss1* mRNA expression. Although our aforementioned study using a neonatal estrogenisation protocol clearly showed effects on kisspeptin immunoreactivity, we found no effects on adult *Kiss1* mRNA expression after chronic perinatal exposure to lower doses of endocrine disruptors.

Collectively, the results of this work emphasises the dynamic regulation of kisspeptin neurones in different models, indicating their important role in puberty onset and reproduction.

Summary in Danish

Det hypothalamiske neuropeptid kisspeptin stimulerer frigivelsen af gonadotropinfrigørende hormon, og stimulerer dermed hypothalamus-hypofyse-gonade (HPG)-aksen. Både kisspeptin neuroner og HPG-aksen reguleres af bl.a. kønshormoner og den metaboliske tilstand. Kisspeptin neuroner betragtes derfor som vigtige regulatorer af HPG-aksen og er dermed vigtige for pubertetsudvikling samt for kontrollen af den reproduktive funktion, som styres af positiv og negativ feedback fra kønshormoner.

Formålet med denne afhandling er: 1) At karakterisere køns- og artsforskelle i kisspeptinsystemet samt mulig co-lokalisering af kisspeptin og neurokinin B, som er et andet neuropeptid, der er vigtig for regulering af HPG-aksen 2) At undersøge om kisspeptin neuroner er følsomme overfor fedme og eksponering til hormonforstyrrende stoffer, som er kendte risikofaktorer for nedsat reproduktiv funktion samt for tidlig pubertet.

Vi fandt artsforskelle i kisspeptin-immunreaktivitet mellem rotte og mus i både den anteroventrale periventrikulære kerne (AVPV) og i arkuatus (ARC). Mens musens AVPV indeholdt kisspeptin-immunreaktive celler, var dette kun tilfældet i rotten, når axon transporten var blokeret. I ARC havde rotten klart definerede celler, mens musens ARC var karakteriseret ved et tæt fibernetværk. Disse observationer er i kontrast til mRNA fund og tyder på posttranskriptionelle artsforskelle. Desuden fandt vi flere kisspeptin- og neurokinin B-immunreaktive celler i ARC i hunrotter i forhold til i hanner. Detaljeret kortlægning viste, at den største forskel i immunreaktive neuroner fandtes i den kaudale ARC for både kisspeptin og neurokinin B. Neonatal østrogenisering i hunner reducerede niveauet af både kisspeptin og neurokinin B til niveau med hanner. Derimod viste det sig, at gonadektomi samt kønshormon erstatning i voksne rotter havde modsatte effekter på antallet af kisspeptin og neurokinin B neuroner i begge køn. Kønshormon erstatning reducerede antallet af kisspeptin neuroner, og øgede antallet af neurokinin B neuroner, hvilket tyder på at de to peptider er reguleret forskelligt.

I en rottemodel hvor ændret kuldstørrelse resulterede i postnatal overfodring og underernæring, viste vi, at tidspunktet for indtrædelse af pubertet var sensitiv overfor både overfodring og underernæring tidligt i livet. Den overfodrede gruppe gik tidligere i pubertet og havde øget *Kiss1* mRNA, mens den underernærede gruppe havde sænket *Kiss1* mRNA, kisspeptin immunoreaktive neuroner og forsinket pubertet, hvilket tyder på, at ændringer i kisspeptin ekspressionen er involveret i det forskudte tidspunkt for pubertet. Hos voksne

hanrotter udsat for fedtrig kost i tre måneder efter fravænning fandt vi ingen ændring i antallet af kisspeptin-immunreaktive celler. Til gengæld fandt vi en stærk negativ korrelation mellem kisspeptin ekspression og plasmakoncentrationen af triglycerid. Det antyder, at det var den høje koncentration af plasma triglycerider og ikke kosten, leptin eller kropsvægt, som påvirkede kisspeptin neuronerne.

Endelig vurderede vi effekten af perinatal eksponering til ethinylestradiol og hormonforstyrrende pesticider på *Kiss1* mRNA ekspressionen i voksne rotter. Mens vores ovennævnte neonatale østrogeniseringsstudie tydeligt viste effekter på kisspeptin-immunreaktivitet, fandt vi ingen effekt på *Kiss1* mRNA ekspression efter kronisk perinatal eksponering til lavere doser af hormonforstyrrende stoffer.

Samlet set understreger resultaterne af dette arbejde den dynamiske regulering af kisspeptin neuroner i forskellige modeller, hvilket antyder deres integrerede rolle i reproduktion.

Abbreviations

#067	antiserum against N-terminal rat kisspeptin-52
ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus
BSA	bovine serum albumin
CA1	cornu ammonis 1
cDNA	complementary deoxyribonucleic acid
C_T	threshold cycles
DG	dentate gyrus
DAB	**3,3´-**diaminobenzidine
Dyn	dynorphin
EE_2	ethinyl oestradiol
ER	estrogen receptor
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotropin-releasing hormone
HFD	high-fat diet
HPG	hypothalamic-pituitary-gonadal
IHC	immunohistochemistry
ir	immunoreactive
IS-39	antiserum against neurokinin B
JLV-1	antiserum against murine kisspeptin-52
kg	kilogram
Kiss1	gene encoding kisspeptin
Kiss1R	the kisspeptin receptor
KNDy	kisspeptin - neurokinin B - dynorphin
KOR	the dynorphin receptor
KP	kisspeptin
LH	luteinising hormone
LL	large litters, underfeeding
ME	median eminence
mg	milligram
mM	millimolar
mm	millimetre
mRNA	messenger ribonucleic acid
n	number of separate samples
NK3R	the neurokinin B receptor
NKB	neurokinin B
NN	normal litters, control
OD	optical density
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PD	pup day
PFC	prefrontal cortex
PND	postnatal day
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SEM	standard error of mean
SL	small litters, overfeeding
Tac2	gene encoding neurokinin B
µg	microgram
µM	micromolar
VO	vaginal opening

Introduction

What triggers puberty is a question which the journal Science has listed in 2005 as one of the top hundred questions for researchers to answer in the coming quarter of a century (Science, 2005). This question is still unanswered here in 2013, although we have come a bit closer with the discovery of the central role of kisspeptin in the initiation of puberty and in the central regulation of reproduction in general. Further, kisspeptin is sensitive to environmental signals and is a likely messenger of environmental cues to the reproductive system. Understanding of the function of kisspeptin will thus potentially help us understand the fundamental mechanisms behind pubertal maturation and the regulation of reproduction in response to environmental signals. Answering such fundamental questions may have several implications for human health, including optimal treatment of precocious puberty, prostate cancer, and endometriosis, and for assisted fertilisation as well as for development of new contraceptives.

The role of kisspeptin in the regulation of reproduction has been a focus of research in the laboratory of Jens D. Mikkelsen for almost a decade, beginning in 2006 with the finding that kisspeptin mediates the photoperiodic control of reproduction in the seasonal reproductive hamsters (Revel et al., 2006). One focus of research has been the anatomical mapping of kisspeptin in rodents, using our own well-characterised antiserum (Bentsen et al., 2010; Desroziers et al., 2010; Mikkelsen and Simonneaux, 2009). Particularly, we have characterised the pubertal development of kisspeptin in the rat hypothalamus (Bentsen et al., 2010; Desroziers et al., 2012b; Mikkelsen and Simonneaux, 2009). We have also worked with the *in vivo* effects and mechanisms of action of kisspeptins administered peripherally (Mikkelsen et al., 2009) as well as the effects on the kisspeptin system of different factors known for their inhibitory impact on reproduction (Ansel et al., 2011; Castellano et al., 2010b). To elaborate on these findings, the main focus of this thesis was to further characterise the kisspeptin system (Papers I-II) and to determine the effect of external factors associated with reduced fertility on the kisspeptin system (Papers III-V). To give the reader an opportunity to comprehend and interpret the work presented in these papers, this chapter contains a short introduction to the field, including a description of the anatomy, function and development of the kisspeptin system, in addition to a background for the hypothesis that the kisspeptin system is a potential target for environmental signals, such as endocrine disruptors and obesity, known to affect reproduction.

The kisspeptin system

Kiss1, the gene encoding kisspeptins, was discovered in 1996 (Lee et al., 1996). It was initially known for its anti-metastatic properties (Lee et al., 1996), but since mutations in the gene encoding the kisspeptin receptor *Kiss1r* were found to cause hypogonadotropic hypogonadism (de Roux et al., 2003; Seminara et al., 2003), the main focus of kisspeptin research has revolved around its role in reproduction. In humans, the gene product of *Kiss1* is translated into a pre-pro-peptide which is cleaved into a 54 amino acids long peptide, and amidated at the carboxy-terminal; this peptide is called kisspeptin-54 (Muir et al., 2001; Ohtaki et al., 2001). In rodents, the same cleavage sites give rise to a 52 amino acids long amidated peptide with some sequence differences compared to human kisspeptin-54 (Terao et al., 2004). Shorter kisspeptins have also been found, all sharing the amidated carboxy-terminal (Ohtaki et al., 2001), making kisspeptins members of the RF-amide family (Dockray, 2004). Despite these differences, all amidated kisspeptins containing at least the 10 amino acids of the carboxy-terminal (in this thesis collectively referred to as kisspeptin) have been shown to effectively activate Kiss1R (Kotani et al., 2001; Mikkelsen et al., 2009). Kiss1R activation gives rise to gonadotropin-releasing hormone (GnRH)-dependent release of luteinising hormone (LH) and sex steroids (Gottsch et al., 2004; Mikkelsen et al., 2009), and kisspeptin is as such a potent activator of the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis comprises hypothalamic GnRH neurones, the gonadotropins LH and follicle-stimulating hormone (FSH) at the pituitary level and the sex steroids testosterone and oestradiol from the gonads. The central control of sex steroids are maintained by the pulsatile release of GnRH to the pituitary portal system, which mediates pulsatile release of LH and FSH from the anterior pituitary, which then stimulate sex steroid release from the gonads. Sex steroids in turn have negative feedback on GnRH secretion, except during the preovulatory GnRH surge, where a positive feedback loop, essential for proper ovulation, is created. Although GnRH is regulated by sex steroids, GnRH neurones do not express sex steroid receptors and sex steroids thus regulate GnRH release indirectly. Kisspeptin neurones on the other hand, express sex steroid receptors (Lehman et al., 2010b), and several lines of evidence suggest that kisspeptin regulates GnRH release, as described in the following section and depicted in Fig. 1.

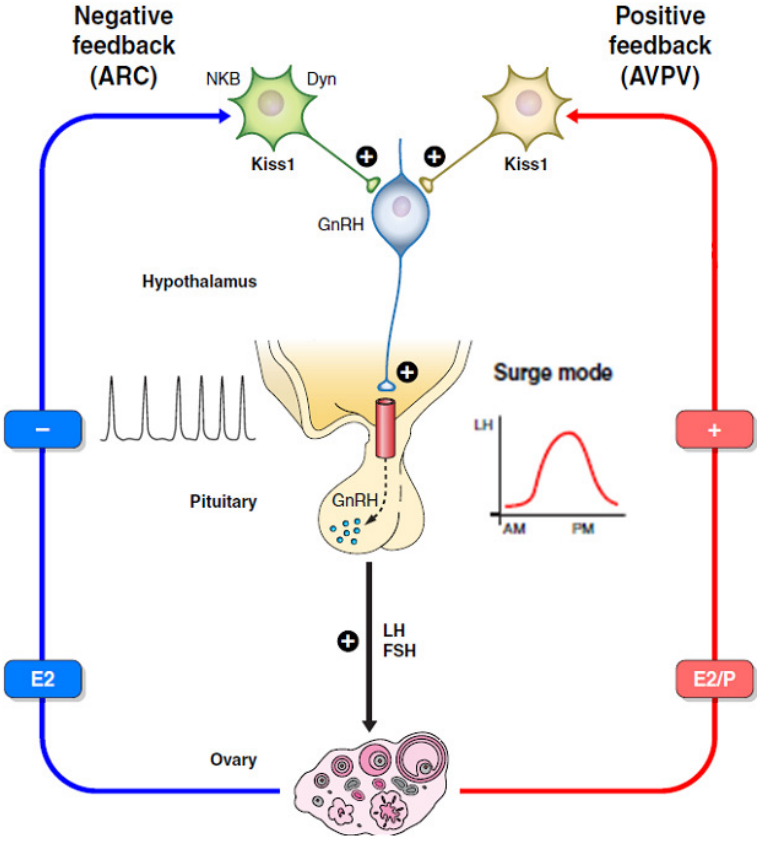

Fig. 1 The HPG axis and the role of kisspeptin in GnRH secretion in rodents. A schematic depiction of the hypothalamic-pituitary-gonadal axis, and the proposed regulation of GnRH secretion by the two kisspeptin neurone populations in the female rodent. Sex steroids (E2) mediate negative feedback to kisspeptin neurones in the ARC, whereas the preovulatory increase in E2 stimulates *Kiss1* mRNA expression in the AVPV. The kisspeptin neurones in the ARC are therefore proposed to be involved mainly in the pulsatile release of GnRH in basal conditions, and the kisspeptin neurones in the AVPV are suggested to be mainly involved in the preovulatory LH surge. The projections from the kisspeptin neurones to the GnRH neurones are uncertain, but these fibres likely terminate at the GnRH fibres.
ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; NKB, neurokinin B; Dyn, Dynorphin, GnRH, gonadotropin-releasing hormone; E2, oestradiol; P, progesterone; LH, luteinising hormone; FSH, follicle stimulating hormone.
Modified from (Pinilla et al., 2012).

Kisspeptin was found to be important for GnRH secretion when it was discovered that humans with an inactivating mutation in Kiss1R suffered from hypogonadotropic hypogonadism, and that Kiss1R knockout mice displayed the same infertile phenotype (de Roux et al., 2003; Seminara et al., 2003). These findings prompted scientists to investigate the nature of the hypothalamic kisspeptin system, and major progression in the understanding of hypothalamic regulation of GnRH release has been made. Findings supporting the central role of kisspeptin in regulation of GnRH secretion include the discovery that GnRH neurone soma (Clarkson and Herbison, 2006) as well as GnRH fibres at the level of the median eminence (Ramaswamy et al., 2008) are innervated by kisspeptin-immunoreactive (-ir) fibres and GnRH neurones express *Kiss1r* mRNA (Han et al., 2005; Irwig et al., 2004), suggesting that kisspeptin regulates GnRH neurones directly. This is further supported by the finding that kisspeptin depolarises isolated GnRH neurones (Han et al., 2005), and the finding of synchrony between kisspeptin pulses and GnRH pulses in monkeys (Keen et al., 2008). Further, kisspeptin neurones are activated during the preovulatory LH surge, and infusion of anti-kisspeptin antibody into the preoptic area prevents the preovulatory LH surge in rats (Kinoshita et al., 2005). Finally, it is well-documented that kisspeptin administration elicits release of LH to the circulation, which is blocked by GnRH antagonist pre-treatment (Gottsch et al., 2004; Irwig et al., 2004; Mikkelsen et al., 2009).

Concomitantly, these findings strongly suggest a direct role for kisspeptin in the regulation of GnRH neurones.

In the adult rodent, kisspeptin is expressed in two hypothalamic areas; one is the rostral periventricular area of the third ventricle including the anteroventral periventricular nucleus (collectively termed AVPV in this thesis) and the other is the arcuate nucleus (ARC) (Clarkson et al., 2009b; Mikkelsen and Simonneaux, 2009; Smith et al., 2005a). However, species differences exist and the kisspeptin neurone population in the AVPV is only found in rodents, whereas e.g. in humans the magnocellular paraventricular nucleus (Hrabovszky et al., 2010) and in sheep the preoptic area (Smith, 2009) is a possible equivalent to the rodent AVPV. Even in rodents, consensus in distribution is obtained mainly at the mRNA level, where rats and mice have similar *Kiss1* mRNA distribution (Kauffman et al., 2007; Smith et al., 2005b; Smith et al., 2005a), whereas at the peptide level differences exist, which will be addressed in Paper I.

In rodents, sex steroids differentially regulate the two populations of kisspeptin neurones, such that sex steroids increase *Kiss1* mRNA expression in the AVPV and decrease *Kiss1* mRNA expression in the ARC (Kauffman et al., 2007; Quennell et al., 2010; Smith et al., 2005b; Smith et al., 2005a). Both the positive and negative feedback mechanisms are regulated by oestrogen receptor (ER) α expressed in kisspeptin neurones (Adachi et al., 2007; Clarkson et al., 2008; Mayer et al., 2010; Smith et al., 2005a).

Development of the kisspeptin system

Kisspeptin neurones are born in the late embryonic neurogenesis period in rats, starting at embryonic day 12.5 and proceeding to at least embryonic day 17.5 (Desroziers et al., 2012a). These newborn neurones produce kisspeptin soon after maturation, with detection of *Kiss1* mRNA and kisspeptin immunoreactivity from embryonic day 14.5 in the ARC (Desroziers et al., 2012a). There is a prenatal increase in kisspeptin expression in both males and females reaching approximately half of adult kisspeptin levels, which is followed by a marked decrease just prior to birth (Desroziers et al., 2012a).

Sex differences in *Kiss1* and kisspeptin expression are thus not evident prenatally, however, within the first week of life a sex difference become evident in the ARC of rats, with the female displaying higher *Kiss1* mRNA and kisspeptin levels compared to males (Desroziers et al., 2012b; Takumi et al., 2010). From the third postnatal week, differences between the sexes are present in the AVPV, with female rats displaying more *Kiss1* mRNA expressing cells

(Takumi et al., 2010) and female mice having more kisspeptin-ir cells compared to males (Clarkson and Herbison, 2006). Kisspeptin-immunoreactivity in the AVPV of rats is less investigated due to absence of kisspeptin-ir cell bodies in this nucleus as discussed in Paper I, but sex differences in density of kisspeptin-ir fibres across the postnatal period has been described in the rat AVPV (Desroziers et al., 2012b). Concomitantly, these findings suggest that the kisspeptin system sexually differentiate in the perinatal period.

Pubertal maturation is characterised by increased frequency of GnRH pulses which mediate a rise in sex steroid levels, mediating maturation to achieve reproductive capacity. It is well recognised that in humans, pubertal maturation is accompanied by growth spurt, development of secondary sex characteristics, and psychological development. In rodents, vaginal opening and balano-preputial separation, in females and males respectively, is regarded as reliable external markers of pubertal maturation. Although the hormone-dependent pubertal development is well characterised, the upstream regulation of increased GnRH secretion at the onset of puberty is not understood. The central role for kisspeptin signalling in the increased GnRH secretion and thereby in pubertal development, as first observed in humans (de Roux et al., 2003; Seminara et al., 2003), has been confirmed in mice without proper kisspeptin function (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007; Mayer et al., 2010; Seminara et al., 2003). In the same line, kisspeptin antagonist administration delays puberty onset (Pineda et al., 2010). Conversely, activating mutations in Kiss1R has been reported to cause precocious puberty (Bianco and Kaiser, 2013; Teles et al., 2008) and kisspeptin administration to prepubertal female rats and monkeys resulted in advanced puberty onset (Navarro et al., 2004b; Shahab et al., 2005). In further support of the central role of kisspeptin in pubertal maturation, the second peak of *Kiss1* and kisspeptin expression occurs around pubertal maturation (Bentsen et al., 2010; Clarkson and Herbison, 2006; Desroziers et al., 2012b; Han et al., 2005; Navarro et al., 2004a; Takase et al., 2009; Takumi et al., 2010). This peripubertal peak is accompanied by an increase in kisspeptin-ir fibres that appose GnRH neurones during puberty in mice (Clarkson and Herbison, 2006). Further, an increase in kisspeptin secretion is observed in pubertal monkeys (Keen et al., 2008) and the sensitivity of GnRH neurones to kisspeptin increases in the same period (Han et al., 2005). Finally, the involvement of kisspeptin in puberty progression is dependent upon ERα, as shown by ablation of ERα specifically in kisspeptin neurones, which results in both advanced puberty onset mediated by decreased inhibition of GnRH by kisspeptin neurones in the ARC, and failure to acquire normal ovulatory cyclicity, mediated by decreased

stimulation of GnRH by kisspeptin neurones in the AVPV (Mayer et al., 2010). Concomitantly, these findings underline the essential role for kisspeptin in pubertal maturation.

The physiology of kisspeptin neurones

The AVPV is well known to be involved in the preovulatory surge in rodents, and because sex steroids stimulate kisspeptin expression in this region, these kisspeptin neurones are believed to be involved in the preovulatory rise in LH (Adachi et al., 2007; Clarkson et al., 2008). Further, *Kiss1* mRNA expressing cells in the AVPV are activated during surge conditions (Adachi et al., 2007; Smith et al., 2006b). With this in mind, it is not surprising that *Kiss1*/kisspeptin expressing cells are more abundant in the rodent female compared to the male AVPV (Clarkson and Herbison, 2006; Kauffman et al., 2007). However, as mentioned, species differences exist and the AVPV is therefore not the site for the preovulatory surge in all species.

Kisspeptin neurones in the ARC are suggested to mediate the negative feedback of sex steroids to GnRH neurones, and are thus considered important for the pulsatile release of GnRH characteristic of basal conditions. The fibre projections from kisspeptin fibres in the ARC is not fully understood; in the female mouse kisspeptin fibres from the ARC project to the medial preoptic area, where GnRH neurone soma are localized (Yeo and Herbison, 2011), whereas this is not the case in the female rat (True et al., 2011). However, the majority of kisspeptin neurones in the ARC co-express the neuropeptides neurokinin B (NKB, encoded by *Tac2*) and dynorphin, and these neurones, called KNDy neurones, are proposed to generate the GnRH pulses by the release of pulsatile kisspeptin (Goodman et al., 2007; Lehman et al., 2010a). The KNDy neurones form an extensive interconnected network (Rance et al., 2010) which create a synchronised pulsatile release of kisspeptin at the level of the median eminence, where to both KNDy neurones and GnRH neurones project (True et al., 2011). KNDy neurones also express the NKB receptor NK3R (Amstalden et al., 2010) and the working model for KNDy neurone function is based on the findings that NKB stimulate *Kiss1* expressing neurones (Navarro et al., 2011), and NK3R stimulation thereby results in kisspeptin-dependent LH release (Garcia-Galiano et al., 2012). Dynorphin inhibits kisspeptin release, supposedly via an indirect mechanism (Goodman et al., 2012), thereby terminating the kisspeptin pulse, and NKB and dynorphin thereby create kisspeptin pulses *via* a dynamic activation-inactivation interplay. The KNDy neurones also express ERα and oestradiol inhibits both *Kiss1* and *Tac2* mRNA expression

(Navarro et al., 2011). The KNDy neurone model, as first proposed by Navarro and colleagues (Navarro et al., 2009a; Wakabayashi et al., 2010), is depicted in Fig. 2.

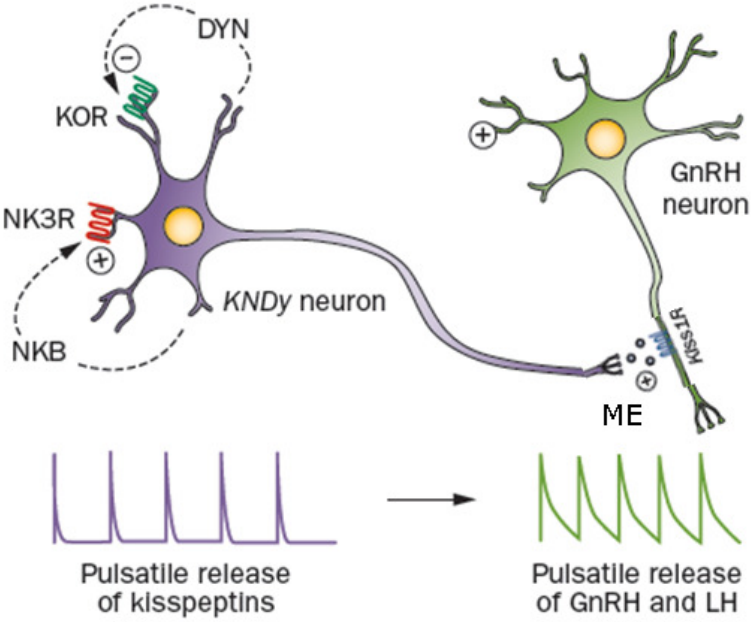

Fig. 2. The KNDy neurone model.
Kisspeptin neurones in the ARC co-express neurokinin B (NKB) and dynorphin (Dyn), and are therefore called KNDy neurones. NKB has auto-stimulatory effects on KNDy neurones *via* the NKB receptor NK3R, whereas dynorphin has inhibitory effect *via* the dynorphin receptor KOR, and these peptides are therefore suggested to be important for the pulsatile kisspeptin release stimulating the pulsatile release of GnRH release *via* the kisspeptin receptor Kiss1R on GnRH fibres at the level of the median eminence (ME).
Modified from (Navarro and Tena-Sempere, 2012).

Kisspeptin and NKB do not possess as pronounced sex differences in the ARC compared to kisspeptin expression in the AVPV, but although some studies find no sex difference at *Kiss1* or *Tac2* mRNA levels in the ARC (Kauffman et al., 2007; Kauffman et al., 2009), other studies find that females have more *Kiss1* mRNA positive, kisspeptin-ir and NKB-ir neurones than males (Cheng et al., 2010; Desroziers et al., 2012b; Goubillon et al., 2000; Hrabovszky et al., 2010; Takumi et al., 2010). Sex differences in the ARC are relevant in the perspective that several studies suggest a role for KNDy neurones in the regulation of the preovulatory surge, as supported by the finding that KNDy neurones are activated both during the GnRH surge and during the basal pulsatile release of GnRH in the ewe (Merkley et al., 2012), and the finding that the positive feedback of oestradiol on LH release was blocked in monkeys with destroyed ARC (Plant et al., 1978). In further support, the number and the activation of kisspeptin-ir cells are greater in proestrous rats compared to diestrous rats (Kinoshita et al., 2005) and *Kiss1* expression in the ARC of both rats and ewes varies across the estrous cycle (Estrada et al., 2006; Merkley et al., 2012; Navarro et al., 2004a; Plant et al., 1978; Smith et al., 2006b; Smith et al., 2009). In Paper II we propose that kisspeptin and NKB have differential posttranscriptional regulation, and we report sex differences in the numbers of kisspeptin- and NKB-ir neurones and in the regulation of release of these peptides, which may be involved in specific functions, such as

generation of the preovulatory surge. Further, we emphasise the possibility that the peptides involved in KNDy neurone function, may be involved in unrelated functions as well.

Kisspeptin as a putative target for endocrine disruption

The organisation of sexual dimorphic hypothalamic nuclei takes place in the perinatal period, where a peak in sex steroids is present only in males, as reviewed by Kauffman (Kauffman, 2010). This organisation occurs at the same time as the aforementioned timing of sexual differentiation of the kisspeptin system. It has been shown that neonatal estrogenisation results in masculinisation of the female hypothalamic *Kiss1* expression (Bateman and Patisaul, 2008; Dickerson et al., 2011; Kauffman et al., 2007; Losa-Ward et al., 2012; Navarro et al., 2009b), which suggests that the sexual differentiation of the kisspeptin system, like the sexual differentiation of several other brain structures (Parent et al., 2011), is dependent on the perinatal difference in sex steroids. The increase in *Kiss1* mRNA expression during pubertal development is also dependent on sex steroids (Clarkson et al., 2009a; Clarkson et al., 2012), and puberty could therefore be a second susceptible period for the kisspeptin system. Kisspeptin neurones express several steroid receptors, including ERα, ERβ, androgen receptor, and progesterone receptor (Adachi et al., 2007; Cheng et al., 2010; Lehman et al., 2010b; Smith et al., 2005b; Smith et al., 2005a). Influences on the kisspeptin system could thus stem from activation of all these receptors, and not only from the well-recognised regulation of kisspeptin by ERα (Smith et al., 2005a). In sum, the development of the kisspeptin system is regulated by sex steroids and is as such a potential target for endocrine disruption.

Endocrine disruptors (EDs) are substances that disrupt the normal function of endogenous hormones. The term EDs covers a large spectrum of substances with various mechanisms of action, e.g. by interfering with steroidgenesis and aromatase activity, through agonist or antagonist activity on steroid receptors or by changing the level of steroid receptors (Frye et al., 2012; Roa et al., 2008). Thousands of chemicals in our everyday life are suspected to possess endocrine disrupting activity. EDs include plastic products, flame retardants, pesticides, and pharmaceuticals, and EDs are often spread to the environment such that it is present in drinking water and in the human food chain (Frye et al., 2012). The developing foetus is especially vulnerable to endocrine disruption, and many EDs readily cross both into the foetal blood circulation and into breast milk (Frye et al., 2012).

It is well documented that injection of potent sex steroid agonists in early postnatal life, particularly for ERα, have long-term organisational effects on kisspeptin expression (Bateman and Patisaul, 2008; Dickerson et al., 2011; Kauffman et al., 2007; Losa-Ward et al., 2012; Navarro et al., 2009b). Studies modelling human exposure of compounds present in the environment, however, have not fully elucidated whether the human kisspeptin system is a potential target for endocrine disruption. Injection of the estrogenic EDs genistein and bisphenol-A in early postnatal life affects kisspeptin expression only at high doses (Bateman and Patisaul, 2008; Losa-Ward et al., 2012; Patisaul et al., 2009), and mixture effects of environmental endocrine disruptors on *Kiss1* mRNA expression have only been investigated in the foetal and not in the adult hypothalamus (Bellingham et al., 2009). In Paper III, *Kiss1* mRNA expression in the adult rat was evaluated after chronic perinatal exposure to ethinyl oestradiol (EE_2) or to a mixture of endocrine disrupting pesticides.

Kisspeptin and metabolic stress

Reproduction is very energy-demanding, especially for the female mammal. During evolution this has led to sophisticated signals which regulate the HPG axis to influence fertility and the timing of puberty, thereby ensuring that reproduction occurs at optimal energy conditions (Evans and Anderson, 2012; Tena-Sempere, 2007). These signals of metabolic status, ultimately regulating GnRH, include ghrelin, leptin, insulin, galanin and NPY; however, GnRH neurones lack receptors for most of these metabolic signals, as for instance leptin (Quennell et al., 2009), and now kisspeptin is suggested as one of the central intermediates and gatekeepers of some of these signals (Backholer et al., 2010; Castellano et al., 2010a; Castellano and Tena-Sempere, 2013; Evans and Anderson, 2012; Maeda et al., 2010; Michalakis et al., 2013; Navarro and Tena-Sempere, 2012). Energy insufficiency is well-known to cause delayed puberty and decreased fertility, and a connection to the kisspeptin system is supported by our finding that the kisspeptin system is particularly vulnerable to fasting in the peripubertal period, where the number of kisspeptin-ir cells in the ARC is significantly decreased upon fasting (Castellano et al., 2010a). In that line, prepubertal subnutrition inhibits pubertal maturation in female rats, and kisspeptin administration is able to partially rescue this, without changing bodyweight (Castellano et al., 2005), and kisspeptin thus circumvent the repressive effect of negative energy balance on pubertal maturation. In addition, other forms of metabolic stress, such as acute

inflammation and diabetes, also decrease *Kiss1* mRNA expression (Castellano et al., 2006; Castellano et al., 2009; Castellano et al., 2010b).

Energy intake and expenditure is regulated at the hypothalamic level, and leptin, an adipose-derived hormone which is secreted in proportion to fat mass, plays a key role in that regard (Schwartz et al., 2000). Kisspeptin neurones in the ARC express leptin receptors (Smith et al., 2006a) and leptin deficient mice have reduced *Kiss1* mRNA expression (Cravo et al., 2013; Quennell et al., 2011; Smith et al., 2006a). It has therefore been proposed that leptin regulates GnRH secretion in a kisspeptin-dependent manner. On the other hand, leptin receptor deletion specifically in kisspeptin neurones do not compromise puberty or fertility (Donato et al., 2011), leptin treatment to leptin deficient mice do not rescue *Kiss1* mRNA expression (Smith et al., 2006a), and re-introcution of the leptin receptor specifically in kisspeptin neurones in leptin receptor deficient mice do not rescue the compromised puberty and fertility characteristic of absent leptin receptor signalling (Cravo et al., 2013). The latest data therefore suggest that leptin signalling in kisspeptin neurones are neither required nor sufficient for fertility in mice.

In addition to the role of leptin as a signal for energy sufficiency, leptin also has neurotrophic effects on the organisation of hypothalamic nuclei in the perinatal period where a leptin surge is essential for proper development of neurone projections from the ARC (Bouret, 2013). This neurotrophic function of leptin may explain why perinatal metabolic insults can dispose to adult disturbances in appetite control and bodyweight homeostasis, with a concomitant increased risk for obesity. It remains to be investigated whether perinatal alterations in leptin affect the organisation of the kisspeptin system.

Kisspeptin and obesity

The kisspeptin system is particularly sensitive to some types of metabolic stress, e.g. acute inflammation and diabetes, which down-regulate *Kiss1*/kisspeptin expression in adult rats and subsequently down-regulate the HPG axis (Castellano et al., 2006; Castellano et al., 2009; Castellano et al., 2010b), whereas the kisspeptin system is less sensitive to other types of metabolic stress such as high-fat diet (HFD) exposure. Some studies find that HFD exposure increase pubertal *Kiss1* mRNA (Brown et al., 2008; Li et al., 2012), whereas other studies report no effect of HFD exposure on adult *Kiss1* expression (Lie et al., 2013; Luque et al., 2007; Quennell et al., 2011). The increased peripubertal *Kiss1* expression could reflect advanced pubertal maturation after HFD exposure, as advanced puberty onset is observed in several

studies of prepubertal HFD/obesity in both humans and rodents (Aksglaede et al., 2009; Boukouvalas et al., 2008; Sloboda et al., 2009). One study, however, find decreased *Kiss1* mRNA expression after HFD exposure in a mouse strain with increased sensitivity to obesity-induced infertility (Quennell et al., 2011). The reason why the kisspeptin system is less sensitive to HFD exposure compared to other metabolic challenges may be that HFD exposure *per se* is not a challenge. Rather, in evolutionary terms, energy excess is a favourable condition. On the other hand, the growing obesity epidemic has clarified that obesity has co-morbidities, such as the metabolic syndrome and type II diabetes (Sorensen et al., 2010) and energy excess is thus not always beneficial for the fitness of the individual. Further, advanced puberty onset has been related to decreased fertility later in life, and the peripubertal increase in kisspeptin expression may not be beneficial for kisspeptin signalling later in life (Brewer and Balen, 2010; Ibanez et al., 1999). Adding to the complexity, it is well recognised that the risk of co-morbidities of obesity varies significantly, such that some severely obese individuals are perfectly healthy and *vice versa* (Capeau et al., 2005; Karelis et al., 2004). These opposing effects of excess energy availability and the variety in susceptibility to co-morbidities are challenging when designing animal models of the co-morbidities of human obesity. For instance, it may be that only a subset of the obese animals is unable to cope with the increased load of fatty acids.

In relation to the HPG axis, it is well known that morbid obesity is related to hypogonadotropic hypogonadism and reduced fertility, but the mechanism behind is not fully understood (Loret de Mola, 2009; Pasquali et al., 2007). The mouse strain DBA/2J, which is prone to obesity-induced infertility, provides a good animal model to study this phenomenon, and DBA/2J is the only model of adult obesity showing reduced kisspeptin-ir cells (Quennell et al., 2011). The central mechanisms behind the decreased kisspeptin expression in this model of obesity remain to be determined. Leptin is considered an important central signal as previously mentioned, but although leptin is a signal of sufficient energy stores, and stimulate *Kiss1* mRNA expression in diabetic rats (Castellano et al., 2006) and underfed ewes (Backholer et al., 2010), it may not be involved in the signal of the detrimental effects of excessive energy stores. Rather, it has been proposed that circulating triglycerides reflect the detrimental effects of obesity since circulating triglycerides increase when storage capacity in the adipose tissue is reached, and triglycerides are then stored in non-adipose tissue, which has a very low storage capacity (Sorensen et al., 2010). Triglycerides are therefore degraded *via* non-oxidative pathways, resulting in endoplasmic reticulum stress and ultimately apoptosis; this phenomenon is called

lipotoxicity (Sorensen et al., 2010). Lipotoxicity can explain the concomitant deterioration of various tissues affected in the co-morbidities of obesity, and hypertriglycemia may thus be a good marker for the co-morbidities of obesity. Of note, lipotoxicity has also been observed in the hypothalamus (De Souza et al., 2005; Milanski et al., 2009; Moraes et al., 2009). One study shows that intracerebroventricular injection of saturated fatty acids produce endoplasmic reticulum stress in the hypothalamus (Milanski et al., 2009). In further support of hypothalamic lipotoxicity, a study in rats showed hypothalamic increase in several inflammatory markers upon HFD exposure (De Souza et al., 2005). Further, inhibition of the pro-inflammatory JNK pathway restored hypothalamic insulin signalling, underlining the functional importance of hypothalamic inflammation (De Souza et al., 2005). In the same line, another study found that HFD, in addition to causing inflammation, also induced increased apoptosis in the ARC (Moraes et al., 2009). In Paper V we suggest that lipotoxicity may cause a decrease in kisspeptin-ir neurones in the ARC, thereby mediating the detrimental effect of obesity on the HPG axis.

Aim

The overall aim of the thesis is to characterise the kisspeptin system and investigate the implications of high-fat diet and endocrine disrupting chemicals, which are environmental risk factors for altered puberty and fertility, on kisspeptin expression and puberty onset.

Specifically, the aims are to:

- Characterise species and sex differences in kisspeptin expression between rats and mice.
- Compare in anatomical detail the male and female kisspeptin and NKB expression, including the sensitivity to changes in sex steroid levels.
- Study changes in the kisspeptin system and in the timing of puberty onset after perinatal exposure to endocrine disruptors.
- Study the effect of perinatal over- and underfeeding on the kisspeptin system and on puberty onset.
- Correlate metabolic markers of obesity with kisspeptin expression after high-fat diet exposure to identify potential regulators of kisspeptin.

Experimental Methods

This section describes some general aspects of the experimental methods used. For details regarding the specific experiments, please consult the Materials & Methods sections of the respective manuscripts.

Immunohistochemistry

Single labelling immunohistochemistry (IHC) is a method for detection of an antigen in tissue sections with the use of labelled antibodies. IHC allows detection with subcellular resolution, but if the signal is not amplified, the method is not very sensitive. To increase sensitivity, we visualised the antigen by letting an unlabelled primary antibody bind the antigen, and subsequently a secondary antibody coupled to either a fluorescent molecule or to biotin was applied. This step allows amplification of the final signal, because several secondary antibodies can bind the primary antibody. For the biotinylated secondary antibody, the reaction was further amplified by the use of avidin, which binds several biotin molecules with high affinity. Avidin-biotin-peroxidase complexes coupled to the secondary antibody are thereby created. In the presence of hydrogen peroxide, peroxidase converts the chromagen 3,3´-diaminobenzidine (DAB) into a brownish precipitate which can be identified using light microscopy. The use of avidin-biotin-complexes to amplify the immunohistochemical signal was first described by Hsu et al. (Hsu et al., 1981), and provides a sensitive method for the detection of antigens in paraformaldehyde-fixated tissue.

It is possible to label multiple antigens in the same specimen by visualising each primary antibody with substrates of different colour. By choosing primary antibodies raised in different species, cross reaction between primary and secondary antibodies is minimised. If the antigens of interest are both located in the same cellular compartment (e.g. in the cytoplasm), fluorescent detection of the antigens are needed to visualise the overlay of staining.

It is important to verify the specificity of the primary antibody, as cross reactions with similar antigens are a potential problem in IHC. One possibility is to verify lack of staining when preincubating with excess free antigen, which will verify that the antiserum indeed binds the antigen of interest. However, it does not preclude concomitant binding to other antigens containing similar immunoreactive epitopes, and therefore adsorption with similar antigens is also recommended (Saper and Sawchenko, 2003). Further, a dot blot with related antigens can be

performed to directly visualise any cross-reactivity. The disadvantage of these methods is that only antigens with known similarities to the antigen of interest are tested. The optimal test is therefore to stain tissue from knockout mice which lack the specific antigen (Saper and Sawchenko, 2003). Absence of staining confirms specificity in the mouse, though there is still a risk that other species express cross-reacting antigens. Another good validation test is to show overlapping expression patterns of mRNA and peptide by performing *in situ* hybridisation and compare with the IHC.

In the present thesis, a purified polyclonal antiserum raised in a rabbit against rat kisspeptin-52, termed JLV-1, is used for quantification of kisspeptin-ir neurones and fibres. Because kisspeptin is a member of the RF-amide family (Dockray, 2004), it is important to verify that no cross-reactivity with other RF-amides occurs. Adsorption tests with several RF-amides have been performed, which show no decrease in JLV-1 staining (Desroziers et al., 2010). Adsorption with kisspeptin-52 abolished all staining of cells and varicose fibres; however, a faint cross-reactivity with ependymocytes was detected (Desroziers et al., 2010). Further, on a western blot of extract from the ARC, JLV-1 has been shown to stain a single band with the size corresponding to kisspeptin-52 (Desroziers et al., 2010), and a dot blot shows no cross-reactivity with the RF-amides RFRP-1 and RFRP-3 (Fig. 3).

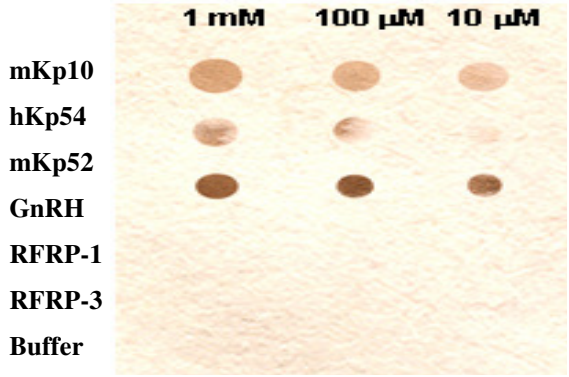

Fig. 3. Dot blot of RF-amides using the kisspeptin antiserum JLV-1.
Peptides were dissolved in water and applied to a filter membrane at the denoted concentrations and fixated in paraformaldehyde. The immuno-histochemical protocol was followed, and revealed binding to mKP10, hKp54 and mKp52, and no cross-reaction with GnRH, or the related RF-amides RFRP-1 or RFRP-3.
mKp10, mouse kisspeptin-10; hKp54, human kisspeptin-54; mKp52, mouse kisspeptin-52; GnRH gonadotropin-releasing hormone; RFRP, RF-amide related peptide. mM, millimolar; µM, micromolar.

In Paper II we viualised NKB with the polyclonal antiserum IS-39 (a generous gift from Dr P. Ciofi, INSERM U862, Neurocentre Magendie, Bordeaux, France). IS-39 is raised in a rabbit against the precursor of NKB to avoid cross-reaction with the related peptides in the tachykinin family, neurokinin A and substance P (Ciofi et al., 2006). Preadsorption with related epitopes revealed no cross-reactivity (Ciofi et al., 2006).

In order to dual-label for NKB and kisspeptin in Paper II, a recently developed kisspeptin antiserum raised in sheep (Franceschini et al., 2013) was used to avoid dual labelling with two

antisera raised in rabbits. This antiserum is called #067 (a generous gift from Dr. Alain Caraty, PRC-INRA, 37380 Nouzilly, France), and was raised against amino acids 5 to 18 of rat kisspeptin-52. The antiserum was subsequently purified to isolate specific kisspeptin-52 antibodies, and to test specificity, preadsorption with kisspeptin-52 was performed (Franceschini et al., 2013). This antiserum is not optimal for labelling of kisspeptin in intact animals (Franceschini et al., 2013), and in Paper II #067 is therefore only used in gonadectomised rats, and the dual labelling was not used for quantification. Dual labelled cells were visualised by taking photomicrographs under identical microscope settings at 20x magnification using a Zeiss Imager Z.1 microscope with the software AxioVision 4.7.3 (Carl Zeiss Imaging Solutions, Germany).

Quantification of immunoreactivity

As the brains were cut into free-floating sections, the identities of the animals were blinded and the identities were revealed after all quantification had ended. All rat brains were cut in series of four in 40 µm sections (thereby separating adjacent sections in a series by 160 µm), and the entire AVPV and ARC were cut. For quantification of immunoreactive neurones, all discernable cells were counted in the entire nucleus; for the ARC meaning counting the sections in the area covering minimum 2.12 to 4.88 mm posterior to bregma. In Paper II, topographical mapping was performed by paying special attention to anatomical cues, such as the shape of the optical nerves, the third ventricle, and the hippocampus, ensuring proper assignment of each section relative to bregma.

Due to a dense kisspeptin-ir fibre network in the mouse ARC (Paper I) discernable cells could not be reliably detected in the ARC of mice. Therefore immunoreactivity was quantified by measuring optical density (OD). OD was measured by outlining the ARC on a photomicrograph of a section using ImageJ (National Institute of Health, USA) and OD was subtracted the background OD, measured outside the ARC. This method was also used for quantification of immunoreactive fibres in the rat AVPV in Paper IV and for quantification of fibres in the rostral ARC of rats in Paper II. For quantification of fibres in the rat ARC, a rostral section with very few immunoreactive neruones was chosen to ensure that OD reflected fibre immunoreactivity and not immunoreactive cells. OD was measured in the triangular shape of the ARC, and the background was measured close to the ventromedial hypothalamus. For the AVPV, OD was measured between the anterior commissure and the optic chiasm and 100 µm

into the parenchyma. In all cases, pictures of a single section (using anatomical cues to ensure identical rostro-caudal level) were taken under identical microscope and camera settings at 10x magnification using a Zeiss Axioskop 2plus microscope (Carl Zeiss Imaging Solutions, Germany) and a PixeLINK PL-A622C camera (PixeLINK, US). In addition, in Papers I and IV, fibre density in the rat AVPV was measured by counting fibres crossing arbitrary lines. Specifically, an area including the AVPV, defined as the area between the anterior commissure and the optic chiasm and 100 or 200 μm into the parenchyma, was covered with randomly positioned horizontal lines with a spacing of 43.44 μm, using the quantification software Axiovision (Zeiss MicroImaging, NY, USA) and the number of immunoreactive fibres crossing was counted. This arbitrary number thus reflects the level of innervation, with special emphasis on vertical fibres, and reflecting more the number of fibres rather than the intensity of the fibres. The vast majority of kisspeptin-ir fibres in the AVPV area are oriented vertically, and the number of fibres, rather than the overall density of immunoreactivity adds additional information to the level of kisspeptin fibres.

In situ hybridisation

In situ hybridisation is a method for localisation of DNA or RNA in tissue sections, and is commonly used for localisation of mRNA expression. An oligonucleotide probe complementary to the gene transcript is labelled with radioactive, colorimetric, or fluorescent methods, to enable visualisation. We used oligonucleotides labelled with [α^{35}S] using the enzyme terminal transferase, a method developed in the 1980s (Lewis et al., 1985). After purification of the labelled probe, hybridisation of the probe to the tissue section, and thorough wash to remove excess probe, we exposed the sections to either a photographic film or immersed the sections in liquid emulsion. The radioactive probe darkens the film, which is a measure of mRNA levels. For quantification of the photographic film, OD in the area of interest was measured using the image analysis software QuantityOne (Bio-Rad, USA), and background OD measured next to each section was subtracted. This was the method used for quantification of *synaptophysin* mRNA presented in Fig. 8 and for quantification of *Kiss1* mRNA after pesticide exposure in Paper III. For quantification of emulsified sections, mRNA-positive cells were counted manually, defined as all thionin-labelled cells containing a cluster of silver grains, and an average of three sections is presented. This method was used for quantification of *Kiss1* mRNA

in Paper I and in Fig. 4 in the Results and Discussion chapter. For both methods, the identities of the animals were blinded.

To obtain a good signal it is important to ensure efficient labelling of the oligonucleotide to avoid binding of unlabelled probe to the mRNA of interest. Further, optimised pretreatment of the tissue to allow proper penetration of the probe, efficient hybridisation (optimising buffer and temperature) as well as efficient washing to remove any unbound probe after the hybridisation will improve the signal to noise ratio. A single test to confirm specificity of the probe is not possible, but showing identical labelling with probes directed against different parts of the transcript is a good test, as is confirmation of overlapping signal with IHC using a validated antiserum (Lewis et al., 1985). Detection of a band of expected size in a Northern blot analysis, lack of signal when hybridising with the sense probe, and lack of signal when pre-incubating with unlabelled probe are other useful validation methods (Lewis et al., 1985). The *Kiss1* probe used in the thesis is previously described (Bentsen et al., 2010). The probe sequence is [CCT GCC TCC TGC CGT AGC GCA GGC CAA AGG AGT TCC AGT TGT AGG] and for validation, two *Kiss1* probes were shown to produce identical signals occuring only in the AVPV and ARC. *Synaptophysin* mRNA data is presented in the Results and Discussion chapter when discussing Paper III, and this probe has also been previously described (Larsen et al., 2008). The sequence of the probe complimentary to *synaptophysin* mRNA is [5´- TGT TGG CAC ACT CCA CGC TCA GCC GAA GCT CCC CGG TGT AGC TGC - 3´], and the same *in situ* hybridiation protocol as described for detection of *Kiss1* mRNA in Paper III was used with the exception of exposure time, which was 2 days for *synaptophysin* mRNA.

RT-qPCR

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a widely used method for analysing gene expression in tissue and cell cultures (VanGuilder et al., 2008). First, RNA is extracted from the tissue. In the present work, the TRIzol method was used, but a column-based extraction can also be used. The mRNA is converted to cDNA in a reverse transcriptase-dependent step, and the cDNA is then quantified using PCR (Nolan et al., 2006). Amplified cDNA is measured by the use of SYBR green, which provide a fluorescent signal corresponding to the amplified cDNA. Data obtained from RT-qPCR reflects the number of cycles before entering the exponential phase, and this number is denoted threshold cycles (C_T). C_T is therefore inversely related to the amount of amplified cDNA. The fold change in

expression was calculated using the comparative C_T method (Schmittgen and Livak, 2008), and the expression was normalised to a housekeeping gene. For this study, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was stable across sex and treatment and was used as a reference gene.

RT-qPCR was used in Paper III to detect the expression of *Kiss1* mRNA in the AVPV and ARC of rats. Tissue was isolated on a cryostat: sections were cut until the AVPV approached (0.20 mm posterior to bregma). Then a square was cut around the AVPV area (borders were set 1 mm lateral to the ventricle and just dorsal to the anterior commissure) and sections were collected in eppendorf tubes until the end of the AVPV was reached (0.70 mm posterior to bregma). The ARC was cut in the same way, cutting from 2.30 mm to 4.70 mm posterior to bregma with the borders of the isolated area being 2 mm lateral from the ventricle and with the upper border just dorsal to reuniens thalamic nucleus. This method was chosen to ensure precise isolation of the tissue, such that the two nuclei were properly separated and to ensure equal dilution of the *Kiss1* mRNA.

For the detection of *Kiss1* mRNA (GenBank ID: NM_181692) the following primers, with a product size of 139 base pairs, were used: forward primer 5'-AGC TGC TGC TTC TCC TCT GT-3'; reverse primer 5'-GCA TAC CGC GGG CCC TTT T-3', and for the reference gene GAPDH (GenBank ID: NM_017008) the following primers, with a product size of 93 base pairs, were used: forward primer 50-CAT CAA GAA GGT GGT GAA GCA-30; reverse primer 50-CTG TTG AAG TCA CAG GAG ACA-30. As a general note, it is important to avoid genomic DNA contamination of the PCR reaction. We avoided this by treating the samples with DNAse. Constructing primers to span introns is an additional precaution, ensuring amplification exclusively of transcribed DNA. Further, it was verified on a DNA gel that a product with the correct size was produced and that no primer dimerisation occurred.

Evaluation of puberty onset

To evaluate the onset of puberty vaginal opening and balano-preputial separation was monitored in female and male rats, respectively. These are external markers of the action of sex steroids and are reliable external signs of puberty onset, marking the proper activation of all levels of the HPG axis (Chehab et al., 1997; Navarro et al., 2004b). As these external events occur after full activation of the HPG axis, they are rather late markers of puberty; however, in rodent experiments they are the first reliable sign of puberty, and this method has become

commonly accepted. In this thesis, puberty onset was monitored by collaborators in the groups of Prof. M. Tena-Sempere and Prof. U. Hass. The time of puberty onset is reported as the postnatal day (PND), with the day of birth designated PND 1, except in Paper III where the time of puberty onset is reported as pup day (PD), where the expected day of birth calculated from the day of conception, is designated PD 1. The reason for this approach is that some EDs prolong gestation.

Statistical analysis

This section covers only the statistics used for the results presented in the Results and Discussion chapter. For statistics used in Papers I-V please consult the respective papers.

Data presented in histograms are presented as the mean ± standard error of mean (SEM). Puberty onset is depicted in a survival plot, and the graphical representation of the association between puberty onset and *Kiss1* mRNA expression in the ARC is a simple XY plot. Student's t-test was applied to investigate differences in mRNA levels between two groups, and for analysis of differences in numbers of immunoreactive neurones after neonatal estrogenisation a two-way ANOVA followed by Tukey's posthoc test was used. $p < 0.05$ was considered statistical significant and GraphPad Prism version 6.00 (GraphPad Software, CA, USA) was used for statistical analyses and for creation of graphs.

Results and discussion

Genuine species differences in the adult kisspeptin system

In situ hybridisation reveal similar *Kiss1* mRNA profiles between rats and mice (Kauffman et al., 2007; Smith et al., 2005b; Smith et al., 2005a), which has delayed the recognition of different expression profiles at the protein level between these closely related species. In Paper I we compared the immunohistochemical profile of kisspeptin in the AVPV and ARC in rats and mice, and we showed that this profile indeed differed between these two species. We found that female rats had higher numbers of *Kiss1* mRNA positive neurones in the AVPV. This observation, however, was not reflected in the detection of kisspeptin, as we found that the AVPV was virtually devoid of kisspeptin immunoreactivity in both sexes in the rat. After inhibition of axonal transport in the rat we found distinct kisspeptin-ir cell bodies in the AVPV with more kisspeptin-ir neurones in the female compared to the male, which was in accordance with our *Kiss1* mRNA findings. Although others have found that *Kiss1* mRNA and kisspeptin levels in the AVPV are comparable in mice (Clarkson et al., 2009b; Han et al., 2005), we thus found that this was only true in rats after inhibition of axonal transport. Collectively, these data suggest that kisspeptin accumulate in the soma in the mouse AVPV, whereas in the rat kisspeptin is transported out of the soma immediately after synthesis, suggesting species differences in the translation and/or axonal transport of kisspeptin.

In the rat ARC some have found no sex difference in *Kiss1* mRNA expression (Kauffman et al., 2007; Kauffman et al., 2009) whereas others find more *Kiss1* mRNA in the female compared to the male ARC (Takumi et al., 2010). We found a tendency to more *Kiss1* mRNA positive neurones in the female rat ARC compared to the male (Fig. 4). At the protein level we found that females express more kisspeptin compared to males in both mice and rats (Paper I).

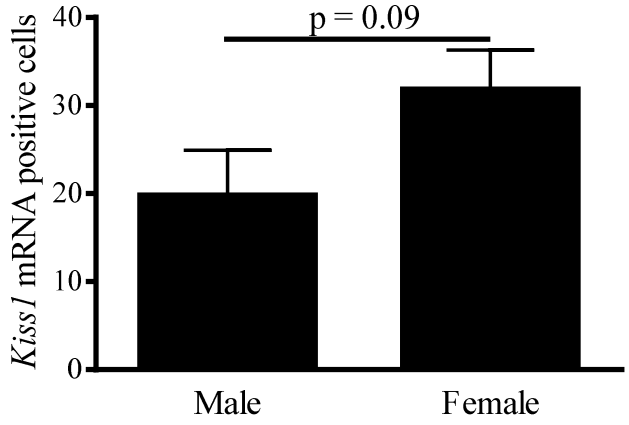

Fig. 4. *Kiss1* mRNA positive cells in the ARC of male and female rats.
The number of *Kiss1* mRNA positive cells in the ARC of intact male and females rats, as quantified by *in situ* hybridisation, is not significantly different (Student's *t*-test, n = 9 per group, average of 3 sections).

However, we found species differences also in this nucleus, as the rat ARC was characterised by clearly discernable cells and few fibres, whereas the mouse ARC contained a dense fibre network with poorly visible cells.

Our finding of higher kisspeptin levels in the female ARC compared to the male could suggest that differential translational regulation occurs, such that sex differences are evident only at the peptide level. Such a translational regulation has been observed across the estrous cycle, where the level of kisspeptin-ir neurones changes and *Kiss1* mRNA does not (Kinoshita et al., 2005). Translational regulation of kisspeptin has also been observed in the lactational state (True et al., 2011), and is further discussed in the next section.

In summary, Paper I revealed differences in kisspeptin-immunoreactivity between rats and mice both in the AVPV and ARC. In the AVPV, the *Kiss1* mRNA level reflected kisspeptin level in the mouse, whereas in rats this was only the case after inhibition of axonal transport, indicating species differences in the translation and/or axonal transport of kisspeptin. In the ARC, we found sex differences in peptide levels in both species, and we found differences in peptide distribution in fibres *versus* cell bodies, also in this nucleus. Comparison of studies in different species as well as studies at the mRNA level contra the protein level should therefore be compared with caution. These findings led us to further explore the regulation and distribution of kisspeptin in the ARC (Paper II), where kisspeptin neurones have recently been found to co-express NKB and dynorphin (Lehman et al., 2010a).

Regulation of the KNDy neuronal population by sex steroids

In Paper II we investigated the topographical distribution of kisspeptin- and NKB-ir neurones in the ARC of male and female rats and we explored the organisation and regulation of these neurones by sex steroids to localise sensitive subpopulations of neurones and to characterise the posttranscriptional dynamics of these two peptides.

We found a marked sex difference in the number of both kisspeptin- and NKB-ir neurones, with females having higher numbers of neurones compared to males, especially in the caudal ARC. Although the largest difference in cell numbers was found in the caudal ARC, females also expressed significantly more kisspeptin in the rostral ARC, whereas no sex difference was found for NKB in the rostral ARC. Abundant kisspeptin expression in the caudal relative to the rostral ARC of females has also been shown in sheep (Cheng et al., 2010; Estrada

et al., 2006; Merkley et al., 2012), monkeys (Alcin et al., 2013) and in a female rat (Desroziers et al., 2012a). Kisspeptin and NKB dual labelling in the caudal ARC of gonadectomised rats revealed extensive co-localisation of kisspeptin and NKB in the female, whereas in the male a subpopulation of neurones expressing only NKB and not kisspeptin was evident, which is in line with findings in humans (Hrabovszky et al., 2011; Hrabovszky et al., 2012). As we find that kisspeptin increase upon gonadectomy, whereas NKB is unchanged (Paper II), the population of neurones expressing only NKB may be larger in sham conditions and may also be present in the female. Investigation of this issue requires other antisera or further protocol optimisation, as the used kisspeptin antiserum is not suitable for fluorescent detection of kisspeptin immunoreactivity in sham animals (Franceschini et al., 2013). The function of these NKB-only neurones remains speculative at present, but they could play a role in the regulation of KNDy neurones, optimising synchronicity between neurones. However, based on the extensive NKB projections from the ARC (Krajewski et al., 2010), another possibility could be that these neurones participate in functions unrelated to KNDy neuron function, e.g. thermoregulation which has recently been proposed (Rance et al., 2013).

In addition to the differential sensitivity of kisspeptin and NKB to gonadectomy, the number of kisspeptin- and NKB-ir cells changed in opposite directions in response to sex steroid replacement; kisspeptin-ir cell numbers decreased and NKB-ir cell numbers increased. This immunhistochemical evaluation of sex steroid-dependent changes in NKB is in contrast with findings at the mRNA level, where several studies have shown inhibitory effects of sex steroids (Abel et al., 1999; Danzer et al., 1999; Dellovade and Merchenthaler, 2004; Kauffman et al., 2009). The difference in levels of *Tac2* mRNA and NKB suggests that NKB is not regulated at the translational level. Further, NKB accumulate in the soma, and NKB may thus be regulated by sex steroids both at the level of transcription and the level of axonal transportation.

In the female rat we found that sex steroids increase both NKB- and kisspeptin-ir fibres in the ARC whereas no change upon sex steroid manipulation was observed in males. This change in fibre density suggests that vesicles containing KNDy peptides are regulated at the level of release in females, which could be important in relation to regulation of the estrous cycle where rapid changes in release occur. Along with our findings of more kisspeptin- and NKB-ir neurones in the female compared to the male, these findings support the recently proposed role of KNDy neurones in the control of the preovulatory surge (Merkley et al., 2012; Smith et al., 2009). Topographical mapping of kisspeptin- and NKB-ir neurones after adult sex steroid

manipulation revealed regulation of expression across the entire rostro-caudal extension of the ARC, and no particularly sensitive subpopulation was thus identified.

Neonatal estrogenisation resulted in a pronounced decrease of both kisspeptin- and NKB-ir neurones in the female ARC, whereas only the male NKB expression was affected (Paper II). The decrease in kisspeptin-ir cells upon neonatal estrogenisation is in line with previous findings (Losa-Ward et al., 2012; Patisaul et al., 2009). Topographical mapping revealed that the largest decrease in immunoreactive neurones occurred in the caudal ARC, but analysing the rostral and caudal ARC separately revealed comparable sensitivity to neonatal estrogenisation in both areas (Fig. 5).

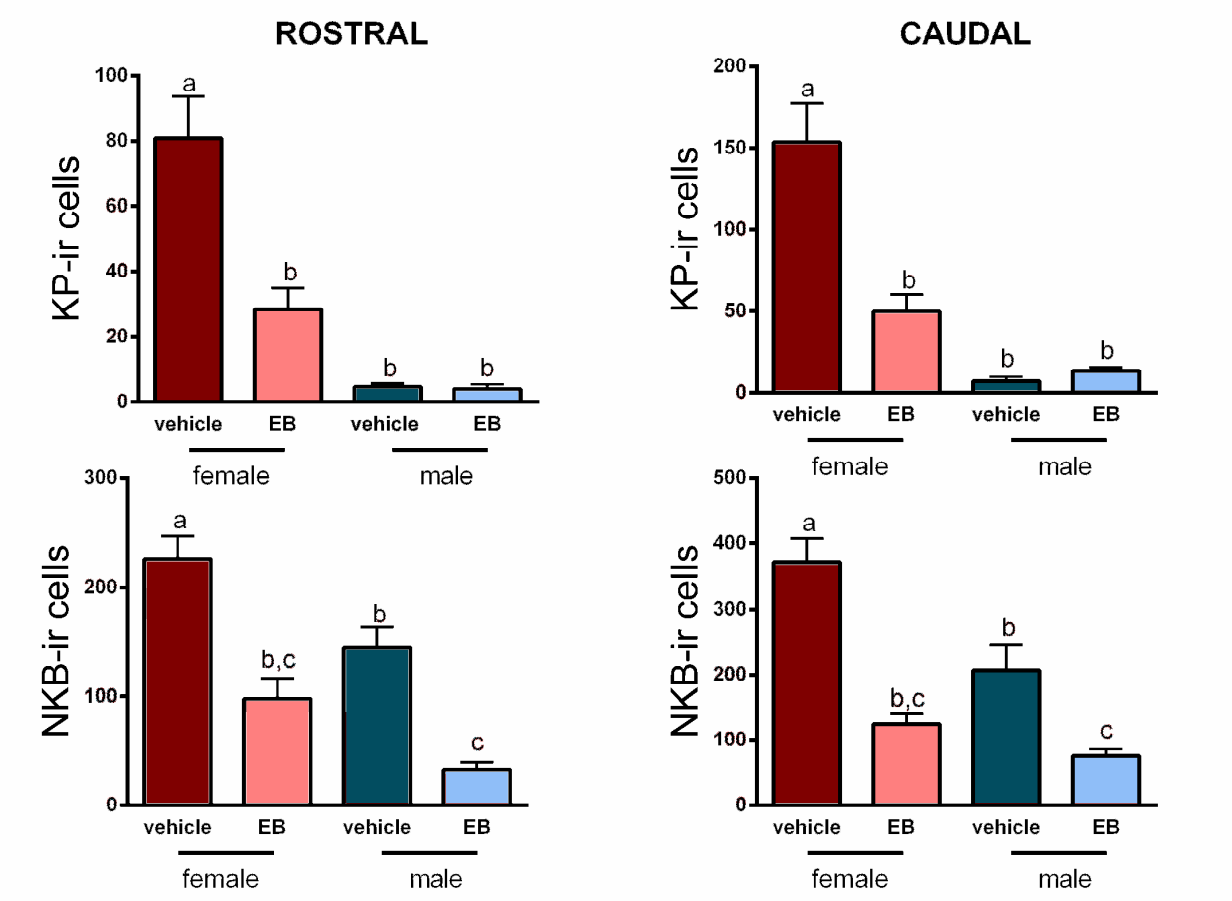

Fig. 5. Rostral and caudal numbers of kisspeptin- and NKB-ir neurones after neonatal estrogenisation. The total number of kisspeptin-ir neurones (top) and NKB-ir neurones (bottom) in the rostral (left panel) and the caudal ARC (right panel). The rostral ARC was defined as the sections 2.12 – 3.40 mm posterior to bregma, and the caudal ARC was defined as the sections 3.56 – 4.68 mm posterior to bregma. Separate analysis of the rostral and caudal ARC revealed comparable sensitivity to neonatal estrogenisation in the two areas. Different letters designate significant difference between groups ($p < 0.05$, two-way ANOVA followed by Tukey's posthoc test, n = 5-7 per group). EB, oestradiol benzoate; ir, immunoreactive; KP, kisspeptin; NKB, neurokinin B.

The decrease in female kisspeptin and NKB expression upon neonatal estrogenisation suggests that expression is sexually differentiated during early postnatal development, a period where sexual differentiation of the hypothalamus takes place and where low sex steroid levels are important for the development of the female brain (Kauffman, 2010). The decrease only in male NKB and not in kisspeptin expression after neonatal estrogenisation underlines the different sensitivity to sex steroids of these two peptides. Moreover, decrease of female kisspeptin and NKB expression in adulthood after neonatal estrogenisation is in line with the finding of anovulation in rats subjected to a similar protocol (Bateman and Patisaul, 2008).

A general concern to consider is whether studies exploring the KNDy neuron model tend to anticipate that all KNDy peptides are equally expressed and regulated, and therefore infer that investigation of a single KNDy peptide covers the general regulation of KNDy neurones. Our study showing NKB-only neurones in addition to differential regulation of expression and different sensitivity to sex steroids of kisspeptin and NKB emphasise the complexity of KNDy neuron regulation. This is supported by the finding that the effects of NK3R stimulation is dependent on the sex steroid milieu (Ruiz-Pino et al., 2012) and by the report of differential regulation of *Kiss1* and *dynorphin* gene expression by different ERα signalling pathways (Gottsch et al., 2009). Accordingly, interpretation of studies investigating only one KNDy peptide/mRNA should be interpreted with caution, both to avoid overemphasising the role of KNDy neurones and to avoid underestimating the role of the KNDy peptides in functions unrelated to KNDy neurone function.

In summary, we found more kisspeptin- and NKB-ir neurones in the female comared to the male whith extensive overlap in the female, and we found NKB-only neurones in the gonadectomised male. Further, we found sex differences in the regulation of release of kisspeptin and NKB. Finally, we found evidence for differential regulation of kisspeptin and NKB by sex steroids, with regulation of kisspeptin at the transcriptional level, and NKB regulation at the level of transport which may expand our view on the KNDy neuron model.

The effect of perinatal exposure to endocrine disruptors on adult *Kiss1* expression

In Paper III we tested the hypothesis that early life exposure to EE_2 or to pesticides with documented endocrine disrupting effects present in our environment possesses a relevant risk of interfering with the development of the kisspeptin system. The hypothesis was tested by measuring *Kiss1* mRNA in adult rats subjected to perinatal exposure. An overview of previously

reported endocrine disrupting effects of the tested compounds is given in Table 1 in Paper III, along with doses and dosing periods.

Perinatal EE_2 exposure did not affect *Kiss1* mRNA expression, assessed by RT-qPCR, in the AVPV or ARC of adult male or female rats. However, when the two sexes were analysed together, the effect of EE_2 in the ARC was significantly different in females compared to males ($p < 0.05$, simultaneous simple interaction model). The effect was characterised by increased *Kiss1* mRNA at the lowest EE_2 dose and decreased *Kiss1* mRNA at the highest EE_2 dose in the females. The mechanism behind the inverted U-shape can be explained by stimulation at low doses and inhibition at high doses, termed hormesis (Cook and Calabrese, 2006; Welshons et al., 2003). For instance, hormone levels (or levels of EDs) in the high physiological range will occupy the majority of receptors, whereas higher levels will cause receptor desensitisation and down regulation (Welshons et al., 2003). Although our observed effect should be interpreted with caution, inverted U-shape dose-response curves are seen for related environmental pollutants and endpoints. For instance, similar dose-response curves have been found after estrogenic exposure for dopamine uptake in hypothalamic dopaminergic neurones (Christian and Gillies, 1999), and for running wheel activity and sleep-wake cycle related gene expression in the preoptic area of adult female mice (Ribeiro et al., 2009). It is thus possible that studies finding a decrease in kisspeptin expression after high postnatal doses of estrogens (subcutaneous injection of 25 µg /kg oestradiol benzoate or 10 µg/kg EE_2 or 50 mg/kg bisphenol A, PND 1-4) (Losa-Ward et al., 2012; Patisaul et al., 2009) would find increased kisspeptin expression if testing lower doses in the same experimental setup. The maximal effect on mRNA expression and running wheel activity in the aforementioned study was seen after subcutanous implantation of a capsule containing 1.25 µg oestradiol benzoate (Ribeiro et al., 2009), and this dose is thus low compared to the studies finding decreased kisspeptin expression after postnatal estrogenisation (Losa-Ward et al., 2012; Patisaul et al., 2009); however, the studies are not directly comparable, as the developmental effects contra the immediate effects of oestradiol are investigated, and these effects may be obtained at different doses.

In line, we showed that puberty onset was delayed in both sexes after exposure to the lowest EE_2 dose; however, the overall dose effect was only significant in males. To further characterise the progression through puberty, accumulative percentage of animals entering puberty at two peripubertal time points is presented in Paper III. Further, in Fig. 6 the accumulated percentage of animals entering puberty is presented, which shows a trend for an inverted U-shaped effect of EE_2 on puberty onset in the female groups, with advanced puberty onset in the group exposed to 50 µg/kg/day, and delayed puberty onset in the group exposed to 5 µg/kg/day. In the males, no effect of the 50 µg/kg/day dose was observed, possibly due to the different mechanisms by which *Kiss1* mRNA and puberty onset is regulated in males and females (Kauffman et al., 2009).

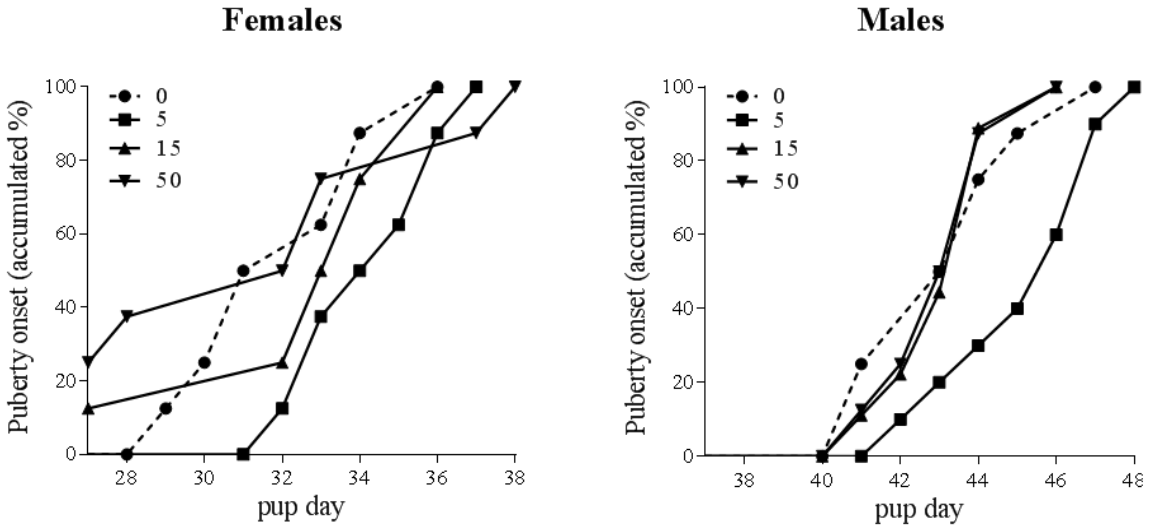

Fig. 6. Accumulated percentage of puberty onset in female and male rats exposed to perinatal ethinyl oestradiol.
Accumulated percentage of puberty onset in female (left) and male (right) rats exposed via maternal oral gavage to ethinyl oestradiol in the periods gestational day 7-21 and pup day 1 (approximately the day of birth) – pup day 22 (weaning). The control group (●) is marked with dotted lines, and the groups exposed to 5 (■), 15 (▲), or 50(▼) µg/kg/day ethinyl oestradiol are marked with solid lines. Puberty was determined by the external marker of vaginal opening in the females and balano-preputial separation in the males. n = 8-10 per group.

Because we found similar inverted U-shaped effects of EE_2 on puberty onset and *Kiss1* mRNA expression in the female, we investigated whether the timing of puberty onset and *Kiss1* mRNA expression in adulthood were associated. A graphical representation (not providing the basis for the statistical analysis) of the association is presented in Fig. 7. We found that puberty onset and adult *Kiss1* mRNA expression were not statistically associated ($p = 0.12$), and we thus did not find that the timing of puberty could predict adult *Kiss1* mRNA levels. Although

advanced puberty has been linked to decreased fertility in humans (Ibanez et al., 1999), in this model where only modest changes in the timing of puberty were found, timing of puberty was not associated to adult *Kiss1* mRNA levels. However, since kisspeptin is important for puberty onset, it is possible that although changes did not persist into adulthood, peripubertal *Kiss1* mRNA levels in these rats could indeed be changed. Overall, our findings in Paper II and III are in line with previous studies which show that the female kisspeptin system is more sensitive to disruption by estrogenic compounds compared to males (Dickerson et al., 2011; Patisaul et al., 2009). Further, we found that the kisspeptin system was not as sensitive to our protocol of perinatal oral exposure of the mother, compared to early postnatal injection of estrogenic compounds as we reported in Paper II and as previously published (Dickerson et al., 2011; Patisaul et al., 2009). In Paper III, the purpose was to model human exposure rather than investigating the effect of high acute doses. The reason for the discrepancies between the findings after high acute exposure and the model of human exposure investigated in Paper III, which is characterised by indirect chronic exposure through the placenta and breast milk at doses which are low compared to other studies (Dickerson et al., 2011; Patisaul et al., 2009), could be that the foetus is protected from exogenous estrogens by α-fetoprotein, a foetal plasma protein that has high affinity for estrogens (Bakker and Baum, 2008), only at low and not at high doses.

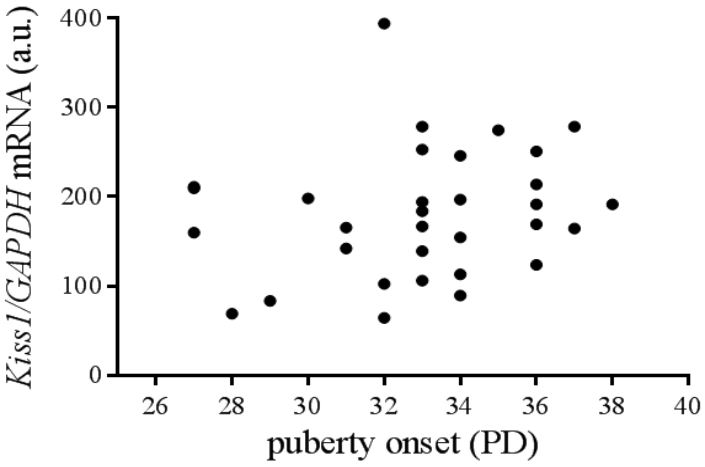

Fig. 7. Association between age at puberty onset and *Kiss1* mRNA expression in the ARC of female rats.
Graphical representation of the association between puberty onset, determined as the day of vaginal opening, and the level of *Kiss1* mRNA expression in the ARC of the young adult female rats from all perinatal EE_2 groups (n=32). *Kiss1* mRNA was determined by RT-qPCR and normalised to the housekeeping gene GAPDH. There is no association between age at puberty onset and adult *Kiss1* mRNA levels (p = 0.12; random intercept model, see Paper III for details). PD, pup day; a.u., arbitrary units.

Perinatal exposure to a mixture of five different pesticides with multiple endocrine disrupting effects did not have any persistent effects on *Kiss1* mRNA expression in the AVPV or ARC of adult female rats, assessed by *in situ* hybridisation (Paper III). Because many endpoints were assessed in this comprehensive study, we know that the pesticide mixture possesses additive effects on other endpoints such as gestational length, pup survival and nipple retention

(Hass et al., 2012), and we therefore consider the kisspeptin system to be less prone to endocrine disruption, compared to these endpoints.

Because perinatal exposure to EDs has been associated with adverse neurodevelopment in humans (de Cock et al., 2012), we wanted to also assess the general vulnerability of the brain to the pesticide mixture. We therefore measured *synaptophysin* mRNA in the cortex and prefrontal cortex, and in the hippocampal areas dentate gyrus (DG) and cornu ammonis 1 (CA1) (see Fig.8 for the outline of the areas). We chose these areas because perinatal exposure to endocrine disruptors have been found to alter these areas (Parent et al., 2011). Synaptophysin is an integral protein of synaptic vesicles which is important for synaptic function (Alder et al., 1992; Evans and Cousin, 2005), and decreased *synaptophysin* mRNA levels thus suggest degeneration of synapses or decreased neuronal sprouting during development. In this study, we found no difference between control group and the highest pesticide mixture group (Fig. 8). This is in line with behavioural tests from the same study showing no effect on motor activity or spatial learning after exposure to the pesticide mixture (Jacobsen et al., 2012). Overall, we found that kisspeptin expression and neuronal plasticity were less vulnerable to endocrine disruption compared to peripheral target tissues.

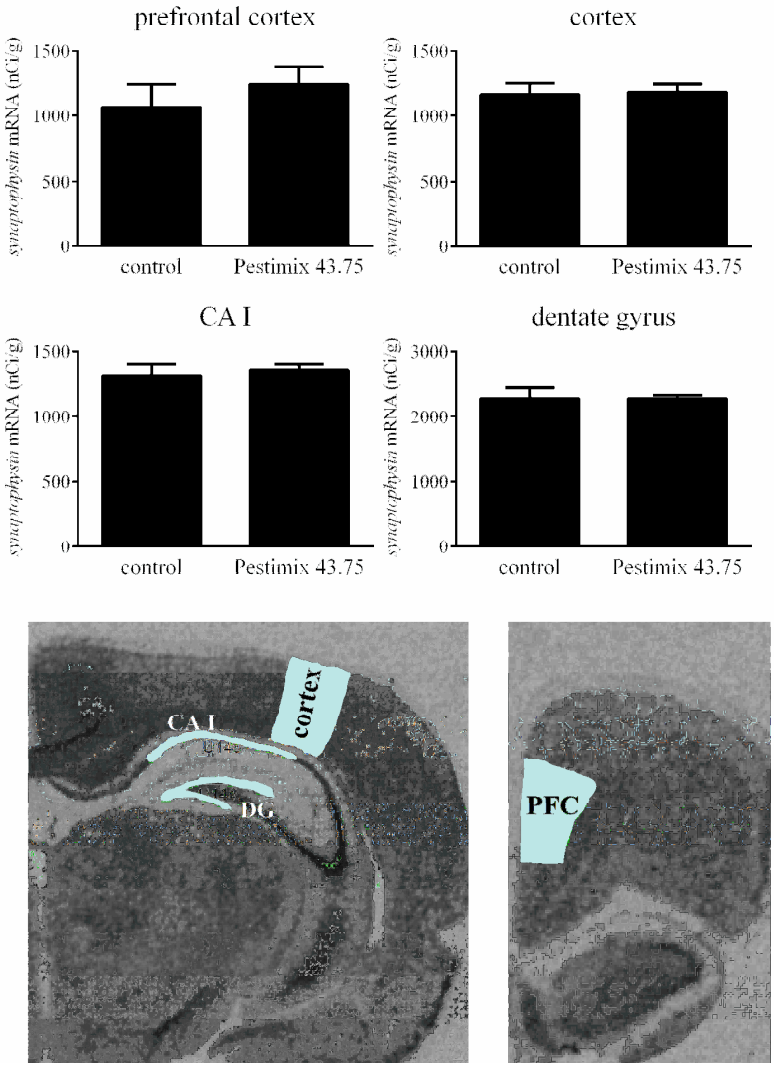

Fig. 8. *Synaptophysin* mRNA in cortex and hippocampus after perinatal pesticide exposure.
Synaptophysin mRNA, assessed by *in situ* hybridisation, was measured in hippocampal (CA1 and DG) and in cortical areas (PFC and cortex; areas are outlined in bottom panel on representative sections), in young adult female rats exposed to vehicle or the highest pesticide mixture (43.75 mg/kg/day) in perinatal life. We found no effect of pesticide exposure on *synaptophysin* mRNA (top panels). controls, n = 6; Pestimix 43.75, n = 7.
CA1, cornu ammonis 1; DG, dentate gyrus; PFC, prefrontal cortex.

When the pesticides were tested alone, two pesticides tended to affect *Kiss1* mRNA expression; tebuconazole tended to increase *Kiss1* mRNA levels in the AVPV ($p = 0.05$) and mancozeb tended to increase *Kiss1* mRNA levels in the ARC ($p = 0.08$) at the highest dose tested (Paper III). Mancozeb has endocrine disrupting effects on the thyroid gland (Hurley, 1998), whereas the sex steroid levels are unaffected by this pesticide (Hass et al., 2012). In addition, the manganese component of mancozeb has been shown to possess neurotoxic effects, especially on dopaminergic neurones, and mancozeb is associated with neurodegenerative diseases (Harrison Brody et al., 2013; Kamel et al., 2007). Although it cannot be ruled out that changes in thyroid hormones affect *Kiss1* mRNA expression, a more likely explanation is that degeneration of dopaminergic neurones caused decreased inhibition of *Kiss1* neurones in the ARC, since dopamine has been shown to suppress the activity of kisspeptin neurones in the ARC (Goodman et al., 2012).

The mechanism of action for tebuconazole in the AVPV *Kiss1* neurones is more difficult to explain, because tebuconazole possesses the same endocrine disrupting effects on the sex steroids as several of the other pesticides tested (see Table 1 in Paper III). However, it may be that tebuconazole crosses the blood-brain barrier more readily than the other pesticides, and that this accounts for its tendencies to increase *Kiss1* mRNA in the AVPV. If this is the case, the ER antagonistic effects of tebuconazole would cause a hyperfeminisation of the AVPV in a period of female development where endogenous sex steroid levels are low (Semaan and Kauffman, 2010).

In conclusion, our findings on *Kiss1* mRNA expression and puberty onset after perinatal exposure to EDs suggest that the kisspeptin system is rather insensitive to endocrine disruption compared to the well-documented effects on peripheral targets in the same and similar experiments (Hass et al., 2012; Jacobsen et al., 2010; Jacobsen et al., 2012; Mandrup et al., 2012; Taxvig et al., 2007; Vinggaard et al., 2005). On the other hand, mancozeb tended to change *Kiss1* expression, presumably through neurotoxic rather than endocrine disrupting pathways, and attention should be paid to disruption of the kisspeptin system through multiple mechanisms.

Early metabolic programming of pubertal timing and the kisspeptin system

Metabolic challenges during the early postnatal period where hypothalamic circuits are forming (Bouret, 2010) are another risk factor for impaired pubertal development (Ahmed et al.,

2009; Boukouvalas et al., 2008; Sloboda et al., 2009). In Paper IV we tested whether early postnatal over- or underfeeding affected the kisspeptin system by raising female rat pups in litters of different sizes: small litters (SL; 4 pups per dam, overfeeding), normal litters (NL; 12 pups per dam) and large litters (LL; 20 pups per litter, underfeeding). We found that bodyweights diverged from PND 7 and the difference in bodyweights persisted after weaning, where all animals had equal access to food, which is in line with similar studies (Caron et al., 2012; Smith and Spencer, 2012). The SL group entered puberty earlier whereas the LL group entered puberty later compared to the NL group, as assessed by the age for completion of vaginal opening (VO) for 50% of the animals (VO50). Although the SL and NL groups differed in bodyweight and puberty onset, these two groups reached puberty at the same bodyweight. This indicates that under conditions of normal to increased energy availability a threshold bodyweight is controlling the timing of puberty. Intriguingly, the LL group reached VO50 at a much lower bodyweight, suggesting compensatory mechanisms allowing pubertal progression under suboptimal nutritional conditions. This relationship between puberty onset at a particular bodyweight has been reported in other studies (Caron et al., 2012; Smith and Spencer, 2012).

Kiss1 mRNA and kisspeptin immunoreactivity were assessed at peripuberty (PND 36). We found that hypothalamic *Kiss1* mRNA, comprising both the AVPV and the ARC, was increased in SL and decreased in LL relative to NL. The picture was not as clear for kisspeptin immunoreactivity; while the number of kisspeptin-ir neurones was decreased in the LL group, we found a 20% non-significant increase in the SL group relative to NL animals. We found no change in fibre density in the periventricular area (including the AVPV), using two different methods; however, a trend towards more fibres in the SL group was present. These findings suggest an increased turnover of kisspeptin in the overfed group, which is supported by increased serum LH levels in this group. On the contrary, the LL group displayed a 70% reduction in the number of kisspeptin-ir neurones, which along with the observed increase in gonadotropin sensitivity towards exogenous kisspeptin, suggests a decrease in endogenous kisspeptin tone.

Because we assessed *Kiss1* and kisspeptin expression at PND 36 where there was a large difference in puberty onset between the groups, the difference in *Kiss1* and kisspeptin expression may be due to the increased expression reported to occur around puberty in both the AVPV and ARC (Bentsen et al., 2010; Clarkson and Herbison, 2006; Desroziers et al., 2012b; Takase et al., 2009; Takumi et al., 2010). This is supported by the finding that when measuring *Kiss1* mRNA expression at the day of vaginal opening, no difference is found between rats raised in small and

normal litters even though the rats from small litters enter puberty earlier than controls (Smith and Spencer, 2012). We therefore propose that the advanced puberty in the SL group is accompanied by an advanced peak in *Kiss1* expression. The model of introducing postnatal overnutrition by raising pups in small litters may be advantageous to exposing the dams to HFD during lactation, which has showed varying effects on the timing of puberty (Lie et al., 2013; Sloboda et al., 2009). It is possible that the effect on milk composition may be less prominent, compared to the increased access to food experienced by pups raised in small litters. Although we have observed unchanged puberty onset and *Kiss1* mRNA expression after postweaning HFD (Lie et al., 2013), another study found advanced puberty onset along with an advanced peak in *Kiss1* mRNA expression (Li et al., 2012), which supports that in the presence of advanced puberty, the peripubertal peak in *Kiss1* expression will also advance.

Moreover, all groups in Paper IV were raised under identical conditions after weaning with free access to food, and the early postnatal conditions must therefore cause persistent changes which resulted in the change in the timing of puberty and in *Kiss1*/kisspeptin expression. Whether these persistent changes is a result of changes in the perinatal leptin levels, which is involved in the perinatal shaping of hypothalamic circuits (Bouret, 2010), remains to be investigated. Nevertheless, the persistent changes reflected in the observed peripubertal effects, may also affect the function of the kisspeptin system in the adult. A study of different litter sizes in the mouse found only a minor effect of postnatal overnutrition on puberty onset and kisspeptin expression, whereas postnatal undernutrition had a pronounced effect on both puberty onset and kisspeptin expression (Caron et al., 2012). Interestingly they found long-term effects on fertility in both postnatally overfed and underfed mice, which may be caused by early postnatal organisation of kisspeptin fibres, since both groups had decreased kisspeptin fibres projecting from the ARC to the area of Kiss1R expressing GnRH neurones (Caron et al., 2012). Similarly, in our postweaning HFD study (Lie et al., 2013), where we found no peripubertal effects on *Kiss1*/kisspeptin expression, we do find disturbed estrous cycle in a third of the rats after only 40 days of HFD exposure. In addition, other studies have found that a prolonged HFD exposure causes more severe cycle disturbances (Akamine et al., 2010) and disturbances in *Kiss1* and kisspeptin expression (Quennell et al., 2011). This suggests that in addition to changing the timing of the peripubertal *Kiss1* mRNA peak, postnatal changes in nutrition also cause impaired development of kisspeptin projections and persistent reduction in expression, which ultimately can lead to impaired reproductive capacity. Human studies suggesting that early puberty onset is

linked to decreased fertility (Brewer and Balen, 2010; Ibanez et al., 1999) makes the translational aspects of such studies even more interesting.

Kisspeptin is inversely correlated with plasma triglyceride levels

As discussed above, early postnatal overfeeding advanced puberty onset and increased peripubertal kisspeptin tone (Paper IV). Since both advanced puberty onset (Brewer and Balen, 2010; Ibanez et al., 1999) and obesity (Loret de Mola, 2009; Pasquali et al., 2007) are related to decreased fertility, and since kisspeptin is a putative link between metabolic status and regulation of the HPG axis (Castellano et al., 2010a), it is suspected that the adult kisspeptin system is adversely affected in models of adult obesity. In women with polycystic ovary syndrome plasma kisspeptin levels are found to be lower in overweight/obese patients compared to normal weight patients (Panidis et al., 2006), whereas polycystic ovary syndrome patients with normal bodyweight actually have increased serum kisspeptin levels compared to normal weight controls (Chen et al., 2010). However, these two human studies are conflicting on the relation between kisspeptin levels and LH and testosterone levels, and should be interpreted with caution. In animal experiments, previous studies have showed no change in *Kiss1*/kisspeptin expression in adult obese animals (Li et al., 2012; Lie et al., 2013; Luque et al., 2007; Quennell et al., 2011), except in one mouse model susceptible to obesity-induced infertility (Quennell et al., 2011). Hence, obesity or diet *per se* does not appear to affect kisspeptin expression.

In Paper V we investigated which components of obesity that affect the number of kisspeptin-ir neurones in a model of diet-induced obesity. Specifically, male Sprague-Dawley rats were fed a high-fat diet (HFD; 45% or 60% energy from fat) upon weaning, and after three months the number of kisspeptin-ir neurones in the ARC, the bodyweight and the plasma levels of leptin, insulin, triglycerides and testosterone were evaluated. The metabolic parameters were then correlated to kisspeptin. We found that HFD increased bodyweight and plasma leptin concentrations, but neither leptin nor bodyweight were correlated to the number of kisspeptin-ir neurones. Further, although there was a 25-50% increase in numbers of kisspeptin-ir neurones in the HFD groups, this difference did not reach statistical significance, and the diet *per se* did thus not change the number of kisspeptin-ir neurones in the ARC of rats.

Intriguingly, we found that the number of kisspeptin-ir neurones was inversely correlated with plasma triglyceride levels (Paper V). This strong correlation was found despite that plasma

triglyceride concentrations were not increased in the HFD groups, and a correlation between the number of kisspeptin-ir neurones and plasma triglyceride concentration was present even within the control group (R^2 = 0.99, p = 0.004). Collectively, these findings suggest that it is hypertriglycemia, and not increased bodyweight or HFD exposure, which is detrimental to kisspeptin expression in the ARC. In line with the liptoxicity hypothesis, it seems that it is not the degree of obesity but rather the ability to clear circulating triglycerides which is important for consequences on kisspeptin expression. In humans, this ability varies considerably, and some severely obese individuals therefore show no metabolic dysfunctions and *vice versa* (Capeau et al., 2005; Karelis et al., 2004). Accordingly, we found no correlation between bodyweight and plasma concentration of triglycerides. Our data support that hypertriglycemia cause lipotoxic inflammation in the hypothalamus, thereby decreasing the number of kisspeptin-ir neurones. Although other studies have found inflammation and apoptosis in the hypothalamus upon HFD (De Souza et al., 2005; Mayer and Belsham, 2010; Milanski et al., 2009; Moraes et al., 2009), further studies localising inflammatory markers in kisspeptin-ir neurones are needed to confirm our hypothesis.

In summary, we found that energy excess is supportive of kisspeptin expression in the ARC as both peripubertal female rats raised in small litters (Paper IV) and adult male diet-induced obese male rats (Paper V) tended to have increased numbers of kisspeptin-ir neurones. Because reproduction is energy demanding, reproduction is favoured in times of energy excess. In that sense, increased kisspeptin expression in models of energy abundance could be hypothesised. On the other hand, our finding of a negative correlation between plasma triglyceride levels and the number of kisspeptin-ir neurones in the ARC could be part of the link between obesity and poor fertility, which has been observed in humans (Loret de Mola, 2009; Pasquali et al., 2007). Moreover, advanced puberty has been linked to reduced fertility in humans (Ibanez et al., 1999) and our finding of advanced puberty and increased peripubertal *Kiss1* mRNA expression may thus not be beneficial for reproductive outcomes in adulthood. Hopefully, future studies will determine the mechanisms by which energy excess affects both the developmental organisation of the kisspeptin system and the direct effect on kisspeptin neurones in the adult.

Conclusion and Perspectives

This thesis provides novel findings which will improve the understanding of the kisspeptin system. Specifically, differential anatomy and posttranscriptional regulation of the kisspeptin system in mice and rats was revealed in Paper I and the quantification of kisspeptin- and NKB-ir neurones after neonatal and adult sex steroid manipulation revealed that these two KNDy peptides have differential sensitivity towards sex steroids and are differentially regulated by sex steroids (Paper II). Further, we found a population of neurones expressing NKB and not kisspeptin in the gonadectomised male rat and we found that the kisspeptin and NKB-ir fibres are regulated differently between sexes. Future development of the immunohistochemical protocol to achieve optimised dual labelling of kisspeptin- and NKB-ir neurones in intact rats will determine the degree of overlap in expression during endogenous sex steroid levels, and clarify whether neurones expressing only NKB are also present in the female ARC.

In Paper III we found that in a model of human perinatal exposure to EE_2 or to different pesticides with documented endocrine disrupting effects, *Kiss1* mRNA expression was not affected, although tendencies towards inverted U-shaped effects was observed for *Kiss1* mRNA and puberty onset in females exposed to EE_2. Although we showed in Paper II that the kisspeptin system was indeed sensitive to neonatal estrogenisation, we thus found that *Kiss1* mRNA expression was relatively robust to perinatal sex steroid exposure, compared to peripheral endpoints found to be affected in the same animals.

In Paper IV we found that pubertal timing was sensitive to both postnatal over- and underfeeding, and this change in pubertal timing was reflected in *Kiss1* mRNA and kisspeptin expression. Although postnatal overfeeding led to advanced puberty and increased *Kiss1* mRNA expression, the longterm effects of postnatal overfeeding are likely not beneficial for the function of the kisspeptin system. Studies to further elucidate the relationship between the kisspeptin system and the metabolic disturbances related to energy excess are a future goal. Specifically, the long-term effects of early life overnutrition on estrous cyclicity and kisspeptin expression would elucidate whether postnatal overfeeding is associated with a risk for life-long decreased reproductive function. Our model of rats raised in small litters has proven particularly effective in inducing early life overnutrition compared to studies using HFD exposure, and manipulation of litter size is therefore preferred. Further, measurement of serum kisspeptin levels in peripubertal boys and girls which have been evaluated for adiposity and exposure to endocrine

disruptors would enhance the translational perspectives of the findings in Paper III and IV. In collaboration with Department of Growth and Reproduction, Copenhagen University Hospital Rigshospitalet, Denmark, we have such serum samples available from The Copenhagen Puberty Study, and assay development to reliably detect serum kisspeptin at levels present in children is a future goal.

In Paper V we found that the level of circulating triglycerides was negatively correlated with the number of kisspeptin-ir neurones in the ARC of male rats. On the contrary, kisspeptin-ir cell numbers were not correlated with leptin, insulin or bodyweight, neither did high-fat diet change the number of kisspeptin-ir neurones in the ARC of male rats. Concomitantly, these data suggest a negative effect of circulating triglycerides, which is in line with the lipotoxicity hypothesis. To further investigate the importance of lipotoxicity in mediating the detrimental effects of obesity on adult fertility, localisation of inflammation in the ARC, and preferably in kisspeptin neurones would be a desirable future experiment to perform. Also, an adult HFD study in female rats, with the concomitant evaluation of kisspeptin, estrous cycle regularity, triglyceride levels and inflammation in the ARC, and ideally a similar study in the mice strain prone to obesity-induced infertility, would help shedding light on possible mechanisms by which kisspeptin could be involved in the link between obesity and poor fertility.

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Appendices

Paper I-V

I. **Overgaard A**, Tena-Sempere M, Franceschini I, Desroziers, E, Simonneaux V, Mikkelsen JD (2013) Comparative analysis of kisspeptin-immunoreactivity reveals genuine differences in the hypothalamic Kiss1 systems between rats and mice. Peptides 45:85-90

II. **Overgaard A**, Ruiz-Pino F, Castellano J, Tena-Sempere M, Mikkelsen JD. Disparate changes in kisspeptin and neurokinin B expression in the arcuate nucleus following sex steroid manipulation reveal differential regulation of the two KNDy peptides in rats. *Manuscript*

III. **Overgaard A**, Holst K, Mandrup KR, Boberg J, Christiansen S, Jacobsen PR, Hass U, Mikkelsen JD (2013) The effect of perinatal exposure to ethinyl oestradiol or a mixture of endocrine disrupting pesticides on kisspeptin neurons in the rat hypothalamus. Neurotoxicology 37:154-62

IV. Castellano JM, **Bentsen AH**, Sánchez-Garrido MA, Ruiz-Pino F, Romero M, Garcia-Galiano D, Aguilar E, Pinilla L, Diéguez C, Mikkelsen JD, Tena-Sempere M (2011) Early metabolic programming of puberty onset: impact of changes in postnatal feeding and rearing conditions on the timing of puberty and development of the hypothalamic kisspeptin system. Endocrinology 152(9):3396-408

V. **Overgaard A**, Axel AMD, Lie MEK, Hansen H, Mikkelsen JD. High plasma triglyceride levels strongly correlate with low kisspeptin in the arcuate nucleus of male rats. *Manuscript*

Paper I

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Comparative analysis of kisspeptin-immunoreactivity reveals genuine differences in the hypothalamic Kiss1 systems between rats and mice

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ABSTRACT

Kiss1 mRNA and its corresponding peptide products, kisspeptins, are expressed in two restricted brain areas of rodents, the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC). The concentration of mature kisspeptins may not directly correlate with *Kiss1* mRNA levels, because mRNA translation and/or posttranslational modification, degradation, transportation and release of kisspeptins could be regulated independently of gene expression, and there may thus be differences in kisspeptin expression even in species with similar *Kiss1* mRNA profiles. We measured and compared kisspeptin-immunoreactivity in both nuclei and both sexes of rats and mice and quantified kisspeptin-immunoreactive nerve fibers. We also determined *Kiss1* mRNA levels and measured kisspeptin-immunoreactivity in colchicine pretreated rats. Overall, we find higher levels of kisspeptin-immunoreactivity in the mouse compared to the rat, independently of brain region and gender. In the female mouse AVPV high numbers of kisspeptin-immunoreactive neurons were present, while in the rat, the female AVPV displays a similar number of kisspeptin-immunoreactive neurons compared to the level of *Kiss1* mRNA expressing cells, only after axonal transport inhibition. Interestingly, the density of kisspeptin innervation in the anterior periventricular area was higher in female compared to male in both species. Species differences in the ARC were evident, with the mouse ARC containing dense fibers, while the rat ARC contains clearly discernable cells. In addition, we show a marked sex difference in the ARC, with higher kisspeptin levels in females. These findings show that the translation of *Kiss1* mRNA and/or the degradation/transportation/release of kisspeptins are different in mice and rats.

1. Introduction

Over relatively few years major progression in the understanding of the kisspeptinergic system's role in the regulation of reproduction has been made [29,32,34]. Both central and peripheral administration of kisspeptins has been shown to elicit LH release in several mammalian species including mouse, rat, hamster, sheep, monkey, and man [3,5,15,16,18,21,27,39]. Species differences exist, but in rodents, kisspeptin expressing neurons have been demonstrated in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) [26,28]. These neurons receive both neural and hormonal inputs and *via* neuronal projections, these neurons stimulate gonadotropin-releasing hormone (GnRH) release to the portal circulation [29,34]. Kisspeptin neurons at the two anatomical locations are regulated by circulating sex steroids, such that sex steroids increase *Kiss1* mRNA expression in the AVPV neurons, and reduce it in the ARC in both mice and rats [2,22,35,36]. In female rodents, AVPV neurons are involved in the positive feed-forward control of GnRH and mediate the circadian regulated LH surge *via* a direct innervation from the suprachiasmatic nucleus [40,42]. Given the different roles of the hypothalamo-pituitary-gonadal axis in males and females, the regulation of the *Kiss1* gene, translation of its mRNA, posttranslational modification of kisspeptins, peptide package in granula and release may be different between the sexes. In the rat a higher level of *Kiss1* mRNA expression in the female AVPV compared with males has been reported, while sex differences in *Kiss1* mRNA expression in the rat ARC remains more controversial [22,38].

Further, Kiss1 gene expression increases during puberty [5,14,20,38]. However, the mechanisms by which kisspeptin

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increase during pubertal development are not fully understood, and species differences may be present. In mice, *Kiss1* mRNA in the ARC is differentially regulated by sex steroids in prepubertal males and females, such that sex steroids inhibit prepubertal Kiss1 expression in females, while male *Kiss1* expression is mainly inhibited by sex steroid-independent mechanisms prepubertally, and this is reflected in the levels of LH [23]. Such a prepubertal sex difference in *Kiss1* regulation may not be present in rats, since both sexes show comparable increases in LH upon prepubertal gonadectomy [17,31].

Today kisspeptin research is carried out in a wide range of species. However, initially most anatomical studies were carried out in rats and mice [8,16,18,22,35,36], and many of these papers fundamental for our perception of the kisspeptin system are being translated to other species. We have been unable to replicate immunohistochemical staining patterns in rat AVPV previously published in mice [8,10] and we have validated our antiserum and compared it to another widely used antiserum [13]. Hence, our objective of this study was to perform a comparative analysis of kisspeptin immunoreactivity in mice and rats under controlled conditions. To elucidate the mechanisms resulting in the observed different immunohistochemical staining patterns, we also carried out *in situ* hybridization in the rat to compare kisspeptin gene expression levels. To assess the level of kisspeptin being transported to the axon terminals, we also measured the level of nerve fiber structures in the anterior periventricular area of both species. In addition, we were interested in investigating the level of kisspeptin neurons in the AVPV of both sexes in the rat after inhibition of axonal transport. In this comprehensive study we thus provide evidence of prominent species differences in kisspeptin immunoreactivity in the closely related species of mice and rats.

2. Methods

Adult male ($n = 7$; 330 g) and diestrous female ($n = 5$; 250 g) Wistar rats bred and raised in the animal facility of the University of Córdoba were selected for immunohistochemistry studies. Diestrous, characterized by non-fluctuating and low sex steroid levels, were chosen to assure homogenous and minimal sex steroid influence on kisspeptin expression. The rats were kept under constant conditions of light (14 h of light, from 07.00 h) and temperature (22 °C), with free access to standard laboratory chow and tap water until perfusion during the early light-phase at three month of age, and brains were shipped to Denmark for immunohistochemical processing.

Adult male and diestrous female NMRI mice ($n = 4$; males 32 g, females 27 g; age 2 months) for immunohistochemistry and adult Wistar rats ($n = 9$; males 250 g, females 190 g; age 2 months) for *in situ* hybridization were obtained from Taconic Inc., Denmark, and housed for at least one week in the animal facility (12 h light/dark cycles) with free access to normal chow and water, in order to acclimatize. Mice were perfused and rats were decapitated during the early light-phase.

In addition, brains from adult male ($n = 5$; 300 g; age 3 months) and female rats ($n = 5$; 280 g; age 3 months) injected with 50 μg colchicine (Sigma–Aldrich, USA) in the lateral ventricle under anesthesia (10 mg ketamine and 3 mg xylazine i.p. per rat) 24 h before perfusion (ChemPartners Inc., Shanghai, China) was collected as previously reported [1]. All procedures were approved by the corresponding local Ethics Committees for animal experimentation.

2.1. Immunohistochemistry

All animals were deeply anesthetized and transcardially perfused with 0.9% saline for 5 min, followed by 4% paraformaldehyde–phosphate buffer for 10 min (0.1 M; pH 7.4). The brains were rapidly removed from the skull and immersed overnight in the same fixative and transferred to 0.05 M phosphate-buffered saline (PBS) at 4 °C until use. Importantly, all perfused brains were subjected to the same immunohistochemical procedure in the same laboratory. Two days prior to being sectioned on a sliding microtome in 40 μm free-floating sections, the brains were dehydrated in a 30% sucrose–PBS solution in order to avoid tissue damage during freezing. Rat brains were cut in coronal series through the AVPV and the ARC with sections in each series being 160 μm apart, while mouse AVPV sections in each series were 80 μm apart and ARC sections were 160 μm apart.

The individual sections were washed for 3 × 10 min in PBS, followed by 10 min wash in 1% H_2O_2 in PBS and incubated for 20 min in PBS containing 0.3% Triton X-100, 5% swine serum, and 1% bovine serum albumin (BSA). After this treatment, the sections were directly transferred to one of the primary antisera in dilutions as described below diluted in 0.3% Triton-X 100 and 1% BSA and gently shaken overnight at 4 °C. The sections were washed in PBS containing 0.1% Triton X-100 (T-PBS) and incubated for 1 h in biotinylated donkey anti-rabbit (Jackson Labs, 711-066-152) diluted 1:1000 in T-PBS with 0.3% BSA. After washing in T-PBS, the sections were incubated 1 h in 0.4% avidin–biotin–peroxidase complex (Vector Elite Kit™, Vector Labs, USA) diluted in T-PBS. Finally, sections were developed in 0.05% diaminobenzidine (Sigma–Aldrich, USA) with 0.05% H_2O_2 in 0.05 M (in 5 °C) Tris–HCl buffer (pH 7.6) for 10 min and then washed 4 × 5 min in PBS. The sections were mounted on glass slides using a 0.5% gelatine solution with 0.05% chromalun and the dry slides were cover slipped in Pertex® (HistoLab, Sweden).

Two polyclonal antisera raised against either the full length rodent kisspeptin (kisspeptin-52) or the C-terminal part (kisspeptin-10) were used in these investigations. The purified antiserum (JLV-1) against kisspeptin-52 was used in a dilution of 1:200 and the crude antiserum (code #564) raised against kisspeptin-10 was diluted 1:1000. Both antisera have previously been characterized [11,13,14,24,28]. We have recently reported differences in the distribution of fibers using the two antisera, likely due to co-labeling of other RF peptides [13], and accordingly, the quantification of fiber densities was only carried out in sections immunoreacted with JLV-1.

2.2. In situ hybridization

Male and female adult rats were decapitated and the brains were carefully removed from the skull and immediately frozen in powdered dry ice and stored at −80 °C. Brains were cut in 8 parallel series of 12 μm sections through the AVPV and ARC.

One AVPV and one ARC series were fixed in 4% paraformaldehyde in 0.2 M PBS, acetylated, delipidated and dehydrated as described previously [25]. An oligonucleotide complementary to the bases 325–370 of the rat *Kiss1* gene [CCT GCC TCC TGC CGT AGC GCA GGC CAA AGG AGT TCC AGT TGT AGG] was used for detection of *Kiss1* mRNA. As described previously, this probe produced labeling only in the AVPV and the ARC and the complementary sense probe did not label the rat brain [5]. The oligonucleotide was labeled at the 3′-end with α-[^{35}S]ATP (>3000 Ci/mmol, GE Healthcare, UK) using terminal deoxynucleotidyl transferase (Roche Diagnostics GmbH, Germany). The specific activity of the hybridization buffer was 1×10^6 cpm per 100 μl. The sections were hybridized, washed and emulsified as described previously [25]. After 7 days, the sections were developed and sections were counterstained in 0.1% thionin (pH 4.0), dehydrated in ethanol and xylen and mounted in Pertex® (HistoLab, Sweden). All sections for analysis were processed simultaneously.

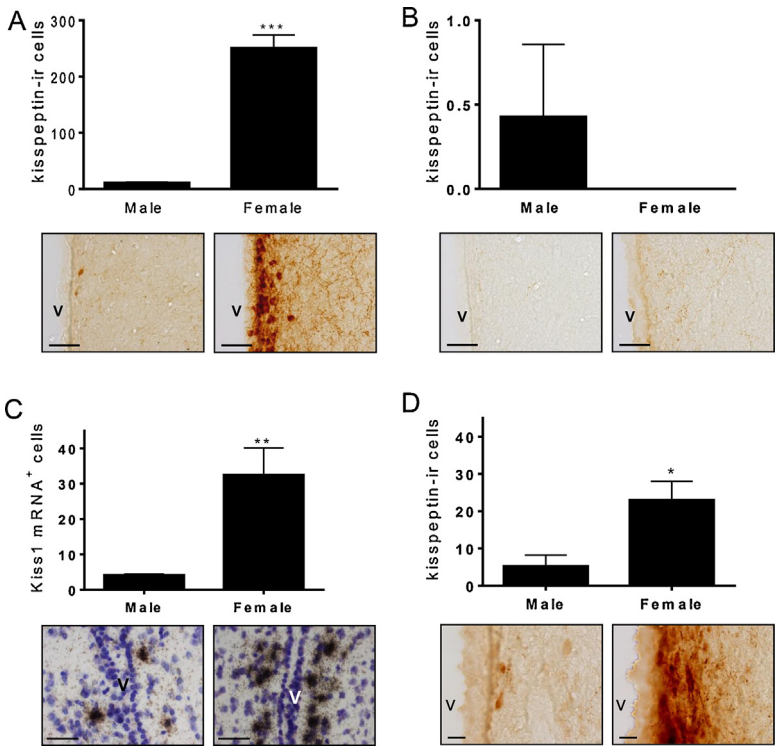

Fig. 1. Kisspeptin expression in the AVPV of mice and in rats. Mean number of kisspeptin-immunoreactive (-ir) cells in the AVPV of naïve mice (A) and rats (B). The number of *Kiss1* mRNA positive cells in the AVPV of naïve rats (C) and the number of kisspeptin-ir cells after axonal transport inhibition (D) is depicted below. Below the graphs are representative photos. Bar = 50 μm in figures A–C, bar = 20 μm in figure D. Kisspeptin antiserum JLV-1 (1:200). V, 3rd ventricle. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, Student's *t*-test.

2.3. Quantitative analysis of positive cells and fibers

Analysis of kisspeptin-immunoreactive cell bodies in the AVPV of mice and rats and in the ARC of rats were conducted by counting all immunoreactive cells within the full extension of the respective areas in one series of sections under bright field illumination in a Zeiss Imager.Z1 microscope. The number of kisspeptin-immunoreactive neurons in the rat AVPV and ARC was quantified in series of sections reacted for either of the two antisera applied.

For optical density quantification of immunoreactivity in the mouse ARC, images of the posterior ARC (−2.2 mm relative to bregma) containing the largest contingent of kisspeptin-immunoreactivity in the nucleus were captured for each animal. Pictures for analysis in ImageJ (NIH, USA) were taken under identical microscope and camera settings using an AxioCam ICc1camera at 10× magnification on a Zeiss Imager.Z1, and optical density was measured in the ARC.

Kisspeptin-immunoreactive fibers were stereotactically quantified as the number of immunoreactive fibers crossing horizontal lines with a spacing of 43.44 μm in the anterior periventricular area (covering 100 μm from the ventricle, including the AVPV), using the microscope software AxioVision (Zeiss MicroImaging), as previously described [6].

Kiss1 mRNA-positive cells were quantified manually under bright field illumination. Three representative sections of the AVPV and the ARC from each animal were examined. All thionin-stained cells containing a cluster of silver grains were included, and the mean was calculated. The identity of all sections was blinded to the evaluator during processing and quantification, and all sections were evaluated by the same person.

The StatPrism software was used for the statistical analysis, and data was subjected to Student's *t*-test. $P \leq 0.05$ was considered significant. All data are represented as mean ± SEM.

3. Results

Adjacent sections were immunostained with the two different antisera directed against either the full length rat kisspeptin-52 or the C-terminal decapeptide. We find that the two antisera stain the same number of cells in the AVPV and ARC of male and female rats (data not shown), and therefore the differences presented here appear to be genuine and not due to divergences in antisera affinities. However, we have previously reported that the number of fibers in the AVPV region was higher using the antiserum #564 against the C-terminal decapeptide likely reflecting cross-reactivity with RFRP [13], and quantifications presented here is performed with the antiserum against rat kisspeptin-52.

3.1. Distribution of kisspeptin expressing neurons in the AVPV in mice and rats

In the mouse, while virtually no positive neurons were found in the male AVPV (11 ± 1.5 cells/animal), numerous kisspeptin-positive cell bodies (251 ± 23.1 cells/animal) were located near the ependyma of the AVPV and extending posterior into the periventricular area of the female mouse hypothalamus (Fig. 1A). Quantification of positive cell bodies along the entire extension of the AVPV revealed significantly higher numbers in the female mouse than in males ($P < 0.001$; Fig. 1A). In contrast to the mouse, much fewer cells were observed in the rat AVPV of both sexes, actually only in one rat visible immunoreactive soma could be identified, and no difference in the number of immunoreactive cells between the two sexes was detected ($P = 0.42$; Fig. 1B). By contrast, we find a strong sex difference at the mRNA level ($P = 0.002$), with numerous *Kiss1* mRNA-positive cells in the female rat (32.5 ± 7.6 cells/animal) compared to the male (4.1 ± 0.3 cells/animal), as detected by *in situ* hybridization (Fig. 1C). Because the lack of immunoreactive cell bodies in

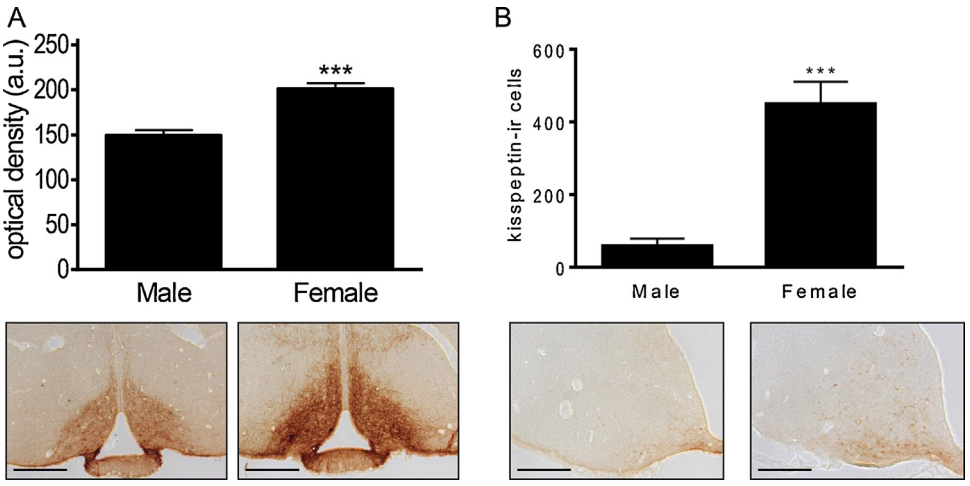

Fig. 2. Kisspeptin-immunoreactivity in the ARC of mice and rats. Optical density measures of kisspeptin-immunoreactivity in the ARC of male and female mice (A) and mean number of kisspeptin-immunoreactive cells in the ARC of male and female rats (B). Below the graphs are representative images. V, 3rd ventricle. Bar = 250 μm. ***$P < 0.001$, Student's *t*-test.

the rat could be either a result of rapid transport of kisspeptin out of the soma or limited translation of mRNA to mature propeptide, rats of both sexes were treated with the axonal transport inhibitor colchicine. This treatment resulted in clearly visible kisspeptin-immunoreactive cells in the AVPV of both male (5.2 ± 3.0 cells/animal) and female (23.0 ± 5.0 cells/animal) rats (Fig. 1D), with significantly more cells in the female ($P = 0.016$).

3.2. Quantitative analysis of kisspeptin-immunoreactivity in the ARC of both sexes in rats and mice

The ARC of rats is characterized by defined cell bodies surrounded by scattered positive fibers (Fig. 2B), whereas the ARC of mice contains very dense fiber plexuses making it difficult to discern individual cells among the fibers (Fig. 2A). Optical density was therefore chosen for quantification of immunoreactivity in the mouse ARC, revealing a higher density of kisspeptin-immunoreactivity in the diestrous female compared to males ($P < 0.001$; Fig. 2A). The number of cell bodies in the ARC of the diestrous female rat (450.4 ± 60.4 cells/animal) was also found to be significantly higher than for the male (59.4 ± 19.5 cells/animal) ($P < 0.001$; Fig. 2B). In addition, the number of *Kiss1* mRNA containing cells tended to be greater in females (31.8 ± 4.4 cells/animal) compared to males (19.8 ± 5.1 cells/animal), although this was not statistically significant ($P = 0.09$; data not shown).

3.3. Comparative analysis of fiber densities in the anterior periventricular area

Kisspeptin-immunoreactive fibers in the anterior periventricular area were numerous, and axons spread to neighboring structures such as the organum vasculosum lamina terminalis, the periventricular hypothalamic area and laterally into the basal forebrain. By counting the number of immunoreactive fibers in the region covering the AVPV and the surrounding anterior periventricular area, we found that the number of fibers in the female of both species was significantly higher than in the male (mice $P < 0.0001$ and rats $P < 0.05$; Fig. 3).

4. Discussion

The overall aim of the present study was to compare the number and distribution of kisspeptin expressing neurons and fibers between rats and mice and males and females. We found that the female rat AVPV neurons are virtually devoid of kisspeptin-immunoreactivity, despite high numbers of *Kiss1* mRNA containing cells. This is in contrast with our and other findings in mice showing a large contingent of immunoreactivity in this region [8,10,40]. The differences in kisspeptin-immunoreactivity between species were quite remarkable and genuine and could not be explained by the different antisera used. These findings suggest pronounced species

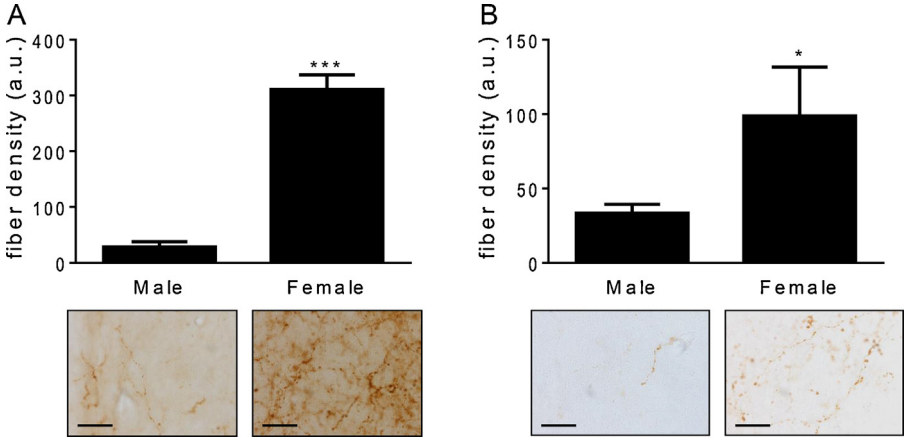

Fig. 3. Kisspeptin-immunoreactive fibers in the anterior periventricular area of mice (A) and rats (B). Below the graphs are representative photos. Fibers are quantified in a 100 μm band from the ventricle, counting fiber crossings of horizontal lines with 43, 44 μm spacing. Bar = 20 μm. ***$P < 0.001$, *$P < 0.05$, Student's *t*-test.

and sex differences in the synthesis and/or axonal transportation of kisspeptin, and our study does not merely suggest species differences in the pattern of kisspeptin-immunoreactivity between rats and mice, but rather documents, on the basis of our detailed analysis of mRNA and peptide expression data, that there are genuine differences in the translation and processing of kisspeptins in the AVPV population of *Kiss1* neurons in these two species, despite the assumption that this population behaves similarly in mice and rats, thus providing a call of caution for this general assumption. Further, it is noteworthy that between these two closely related species, in which the population of kisspeptin neurons in the AVPV is crucial for the surge generation, there are apparently important differences, not at the mRNA, but rather at the peptide processing level, which might be of functional interest.

The distribution of kisspeptin cells in the AVPV of mice has been described before [8,10,35], but a direct comparison of the level of kisspeptin between mice and rats using the same antisera and immunohistochemical technology has not been performed. Most striking, the level of kisspeptin-immunoreactivity in the AVPV is much lower in the rat as compared to the mouse. While previous studies show correlation between the levels of *Kiss1* mRNA and kisspeptin-immunoreactivity in the AVPV of the female mouse [10,18], such a correlation was found in the rat only after colchicine treatment. Notably, *Kiss1* expression fluctuates across the estrous cycle [1,37], but as both mice and rats were in the diestrous phase, the species differences reported here cannot be explained by cycle fluctuations. The reasons for such a divergence in the kisspeptin profiles in these two closely related species remain obscure, and raise questions as to whether there are different mechanisms in the kisspeptin synthesis, processing or transportation between mammals. For example, in female goat and horse, kisspeptin-immunoreactive cells were not observed in the preoptic area despite the presence of high number of cells in the arcuate in the same brains [12,30,41], resembling the distribution in rats reported here. While it has previously been assumed that the kisspeptin distribution in mice and rats are identical, it might thus be that the rat has more similarities with other mammals.

Even our data suggest differences between closely related species with regard to the posttranslational processing of kisspeptins, the mechanisms behind these species-specific differences are not clear. One possible explanation is that in the female rat AVPV a large amount of kisspeptin is trafficked from the soma to the pre-synaptic terminal immediately after translation of the mRNA, which accounts for the lack of kisspeptin-immunoreactivity in cell bodies, unlike in the female mouse where the cells accumulate substantial amounts of peptide in the cytoplasm of the soma. The strongly labeled cells in the AVPV of rats treated with colchicine support this explanation. In addition, the absolute number of kisspeptin neurons in the rat AVPV as revealed after colchicine treatment, was lower compared to the naïve female mouse, suggesting that in addition to the differences in cellular trafficking, kisspeptin expression in the AVPV is more pronounced in the mouse compared to the rat.

In addition to the species differences, our data confirm previous reports of a clear sexual dimorphism in *Kiss1* expression in the AVPV of all mammalian species examined to date, with females displaying significantly higher levels than males [2,7,8,22,38]. The number of fibers in the region covering both the AVPV and the surrounding medial preoptic area was also higher in diestrous female compared to males in both species.

With regard to the ARC, species differences were evident as the ARC of mice is densely packed with fibers and no clear cell bodies can be detected, in contrast to the rat ARC which contains clearly discernable cells and fewer fibers, and the distribution and numbers of kisspeptin-immunoreactive cell bodies of the two species were thus not directly comparable. The inability to detect kisspeptin-immunoreactive cells in the ARC of mice has been reported by others [4,33]. This difference in kisspeptin distribution in the ARC suggests that cellular trafficking of kisspeptin may differ between species, also in this nucleus. Nevertheless, a higher level of kisspeptin-immunoreactivity was detected in females of both species using semi-quantitative methods.

Detection of sex differences at the mRNA level in the rat differs between studies, one study showing no sex difference [22] and another study finding more *Kiss1* mRNA in female compared to male ARC [38]. We have recently shown that female rats have a greater number of kisspeptin-immunoreactive neurons and a higher kisspeptin fiber density in the ARC compared to their male counterparts [14]. Also, higher numbers of kisspeptin-immunoreactive cells in females compared to males have been reported in ARC of sheep and human [7,19]. Here a similar sex difference in the protein level in the mouse ARC, as measured by optical density, was found. As the AVPV and the ARC are differently regulated by sex steroids, the mechanism behind a higher peptide level in the female in this nucleus is likely regulated by other factors than steroids. One option is that some kisspeptinergic fibers in the ARC originate from the AVPV. A study in aromatase knockout mice supports this idea, showing absence of kisspeptin neurons in the AVPV along with a reduction of fibers in the ARC, whereas kisspeptin neurons in the ARC were unaffected [9].

In summary, this study shows the existence of a prominent species difference in the kisspeptin system involving cell bodies in the AVPV. Based on detailed comparative immunohistochemical analyses using two different antisera, we conclude that this difference is genuine and likely reflects important differences in the dynamics of peptide processing in these two species in a neuronal population which is essential for the preovulatory surge. In addition, we find evidence for a sex difference in the ARC of both rats and mice, with females having higher peptide levels than males. In addition, species differences in kisspeptin distribution in the ARC are evident, with rats having clearly discernable cell bodies and the mouse ARC containing dense fibers and no clear cell bodies. These differences between rats and mice in kisspeptin distribution and processing calls for caution when translating kisspeptin results to results obtained in even closely related species.

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Paper II

Disparate changes in kisspeptin and neurokinin B expression in the arcuate nucleus following sex steroid manipulation reveal differential regulation of the two KNDy peptides in rats

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Abstract

It has recently been discovered that kisspeptin, neurokinin B (NKB), and dynorphin (Dyn) are co-expressed in a population of neurons in the arcuate nucleus (ARC) termed KNDy neurons, and these neurons are important for the generation of GnRH pulses. However, the neuroanatomical distribution of these peptides and the regulation of expression by sex steroids are still not well understood. In this study, a detailed topographical mapping of NKB and kisspeptin immunoreactivity in the ARC was carried out in adult rats, including comparison between sexes with and without sex steroid replacement. Interestingly, we found that kisspeptin- and NKB-immunoreactive neurons were most prominent in the caudal ARC of the female and neurons expressing NKB, but not kisspeptin, were most prominent in the male ARC. In addition, we found that NKB and kisspeptin were differentially regulated by adult sex steroid manipulation. Whereas the number of kisspeptin-immunoreactive cells was increased by gonadectomy and reduced by sex steroid replacement, the number of NKB-ir cells did not change after gonadectomy and increased upon sex steroid replacement. Only in the female did the density of immunoreactive fibers in the ARC increase upon sex steroid replacement for both kisspeptin and NKB, suggesting inhibition of release in this setting.

In addition, we found that the level of both kisspeptin- and NKB-immunoreactive neurons was sensitive to neonatal imprinting by gonadal steroids. In the adult female both peptides decreased to male levels after neonatal estrogenisation, whereas in the male only NKB was down-regulated in under these conditions.

In conclusion, our data reveal sex differences in the expression of both kisspeptin and NKB which are most prominent in the caudal ARC. Further, we showed that kisspeptin and NKB were differentially regulated by sex steroid manipulation in the adulthood and that both peptides were sensitive to the effects of neonatal estrogenisation. Concomitantly, these findings expand our understanding of the posttranscriptional dynamics of KNDy neurons in the ARC.

Introduction

Recently a new model of the GnRH pulse generator has been suggested, in which neurons in the arcuate nucleus (ARC) co-expressing kisspeptin (encoded by *Kiss1)*, dynorphin (Dyn), neurokinin B (NKB, encoded by *Tac2*), the NKB receptor NK3R and estrogen receptor (ER)α facilitate the pulsatile release of GnRH (Cheng et al., 2010; Navarro et al., 2009a). These neurons have been termed KNDy neurons (Lehman et al., 2010). The pulses are created via NKB-NK3R auto-stimulation and the pulse is terminated by the inhibitory actions of dynorphin, thereby creating a dynamic activation-inactivation interplay (Amstalden et al., 2010; Burke et al., 2006; Wakabayashi et al., 2010). KNDy neurons thereby release kisspeptin pulses, which communicate via the kisspeptin receptor (Kiss1R) on GnRH neurons (Keen et al., 2008; Messager et al., 2005).

The KNDy neuron concept implies that KNDy neurons are a homogenous population of neurons, but actually the expression of the three KNDy peptides only overlaps partially in females. In the intact ewe for instance, 80% of kisspeptin-immunoreactive (-ir) cells co-express NKB and 73% of NKB-ir neurons co-express kisspeptin (Foradori et al., 2006; Goodman et al., 2007). In mice, approximately 90% of *Kiss1* mRNA expressing cells also express *Tac2* and *Dyn* mRNA, whereas the percentage of *Tac2* mRNA expressing cells co-expressing *Kiss1* mRNA was not assessed (Navarro et al., 2009a). In males, the overlap in expression is even less prominent than in the female, with more than double NKB immunoreactivity compared to kisspeptin in the human (Hrabovszky et al., 2011; Hrabovszky et al., 2012; Molnar et al., 2012) and more than double *Tac2* mRNA expressing cells compared to *Kiss1* mRNA expressing cells in mice (Navarro et al., 2011b), suggesting a population of NKB cells not expressing kisspeptin.

The regulation of KNDy neurons by sex steroids has been investigated mainly at the mRNA level, and it is well documented that expression levels in the ARC increase upon gonadectomy and decrease upon steroid replacement for both *Kiss1* mRNA (Kauffman et al., 2009; Quennell et al., 2010; Rometo et al., 2007; Smith et al., 2007; Smith et al., 2006; Smith et al., 2005a; Smith et al., 2005b) and *Tac2* mRNA (Abel et al., 1999; Danzer et al., 1999; Dellovade and Merchenthaler, 2004; Kauffman et al., 2009; Pillon et al., 2003; Sandoval-Guzman et al., 2004). However, it is noteworthy that *Kiss1* mRNA is more sensitive to sex steroids compared to *Tac2* mRNA, as *Kiss1* mRNA is reduced at low levels of estradiol replacement, whereas *Tac2* mRNA is only reduced at a higher estradiol replacement level (Gill et al., 2012). To further add to the complexity of sex steroid regulation of gene expression in KNDy neurons, it has been shown that *Kiss1* and *Dyn* mRNA

expression in the ARC is regulated by different ERα signalling pathways, allowing differential ERα-dependent regulation of KNDy peptides within the same neuron (Gottsch et al., 2009).

Sex differences in the expression levels of KNDy peptides have also been investigated, and immunocytochemical studies agree that kisspeptin and NKB are more abundant in the female than in the male ARC in several species (Cheng et al., 2010; Desroziers et al., 2012b; Goubillon et al., 2000; Hrabovszky et al., 2011; Overgaard et al., 2013). However, most studies find no sex difference in the mRNA expression of *Kiss1* and *Tac2* mRNA (Kauffman et al., 2007; Kauffman et al., 2009; Overgaard et al., 2013), with the exception of one study that show more *Kiss1* mRNA expressing neurons in the female at certain stages of postnatal developmental (Takumi et al., 2010). Overall, these findings suggest posttranscriptional regulatory mechanisms so that sex differences become evident at the peptide level, and importantly kisspeptin neurons in the ARC have been found to be involved in the preovulatory LH surge (Estrada et al., 2006; Merkley et al., 2012; Smith et al., 2009b). This raises some interesting aspects as to whether the two peptides are regulated by sex steroids in the same manner, and whether this sex-specific regulation is established during the perinatal sexual differentiation of the brain. Actually, sex differences in kisspeptin immunoreactivity in the ARC of rats are evident within the first week of postnatal life (Desroziers et al., 2012b). This is a developmental period where sex steroid levels are low in females compared to males, and low sex steroid levels are important for development of the female brain (Kauffman, 2010), making the early postnatal period a sensitive window for sexual differentiation of the kisspeptin system. In the same line, previous studies have shown that early postnatal exposure to estrogenic compounds results in decreased *Kiss1* mRNA and decreased overall kisspeptin density in the ARC (Kauffman et al., 2007; Losa-Ward et al., 2012; Navarro et al., 2009b).

In addition, NKB has been shown to be either inhibitory or stimulatory on LH release, dependent on the sex steroid milieu (Billings et al., 2010; Kinsey-Jones et al., 2012; Navarro et al., 2009a; Navarro et al., 2011b; Navarro et al., 2012; Ruiz-Pino et al., 2012; Sakamoto et al., 2012; Sandoval-Guzman et al., 2004), whereas kisspeptin always stimulate LH release.

Based on the above mentioned evidence for perinatal organisation of sex differences in KNDy neuron expression, the posttranscriptional manifestation of sex differences, the regulation of mRNA by adult sex steroid manipulation and the sex steroid-dependent function of NKB, it is evident that the KNDy neurons are controlled by complex regulatory mechanisms, involving possibly differential translation, transport and release. In the light of this complex regulation, we investigated in neuroanatomical detail the organisation and regulation of kisspeptin and NKB by sex steroid

manipulation to localise sensitive subpopulations of neurons and to characterise the posttranscriptional dynamics of these two peptides.

Materials and Methods

Animals and drugs

Male and female Wistar rats were bred and raised in the animal facility of the University of Córdoba, where they were kept under constant conditions of light:dark cycles (14 hours of light from 7am) and temperature (22°C), with free access to standard laboratory chow and tap water. The experimental procedures were approved by the University of Córdoba Ethical Committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals.

Gonadectomy and hormone replacement was performed under ketamine/xylaxine anesthesia; female rats underwent bilateral ovariectomy via abdominal incisions, and male rats were castrated via a single abdominal incision. Immediately after gonadectomy, capsules filled with 17β-estradiol (abbreviated estradiol or E) or testosterone (T) were implanted subcutaneously (sc) via a small incision at the base of the neck. Silastic tubing (Dow Corning Corp., MI; 1.98 mm inner diameter, 3.18 mm outer diameter) was used for capsule preparation. For estradiol replacement, crystalline estradiol (E8875, Sigma Chemical, MO) was dissolved in olive oil (100 μg/ml) and filled in capsules of 20 mm effective length. For testosterone replacement, crystalline testosterone (86500, Sigma Chemical, MO) was filled in capsules of 10 mm effective length. Capsules were filled as described previously (Navarro et al., 2011a; Navarro et al., 2011b). Based on previous studies, doses were chosen to obtain replacement in the low physiological range (Navarro et al., 2011a; Navarro et al., 2011b). Sham-operated animals served as controls, and olive oil-filled and empty capsules were used in females and males respectively, not receiving hormone replacement.

For the neonatal estrogenisation protocol, female rats were injected sc with a single dose of 100 μg β-Estradiol 3-benzoate (EB, E8515 purchased from Sigma Chemical, MO) in 100 μl olive oil and male rats were injected sc with a single dose of 500 μg EB in 100 μl olive oil at the day of birth. This dosing regime ensures depot release over several days, covering most of the postnatal window for sex differentiation of the rat brain (Navarro et al., 2009b). Animals injected sc with 100 μl of olive oil served as controls.

Experiment 1 – Adult sex steroid manipulation

In Experiment 1, the regulatory effects of gonadal steroids on kisspeptin and NKB immunoreactivity were evaluated in male and female rats subjected to sex steroid manipulation in adulthood. Further, topographical mapping across the ARC was performed as described below, and kisspeptin and NKB co-localisation was assessed by dual labelling. Rats (age 5-6 months; females ~230 g and males ~280 g) were randomly assigned into three groups per sex (females, n= 6-8 per group; males 8-10 per group): sham female, ovariectomised (OVX), ovariectomised and estradiol replaced (OVX+E), sham male, orchidectomised (ORX), and orchidectomised and testosterone replaced (ORX+T). The estrous cycle of the female sham group was followed by vaginal cytology to confirm regular estrous cyclicity. Three weeks after surgery the animals were perfused and the brains were processed as described below. Sham females were perfused in diestrous 1.

Experiment 2 – Neonatal sex steroid manipulation

In Experiment 2, male and female rats were subjected to neonatal estrogenisation to assess whether the development of the kisspeptin and NKB systems were affected in the adult rat. Topographical mapping of kisspeptin-ir and NKB-ir neurons was performed to identify the localisation of neurons sensitive to neonatal estrogenisation. At the day of birth female (EB, n = 7; vehicle, n = 6) and male (EB, n = 5; vehicle, n = 6) rats were injected as described above. In adulthood, the animals were perfused as described below. The total number of NKB-ir cells in the ARC of animals from this experiment has been previously published (Ruiz-Pino et al., 2012), and this study was extended to examine the detailed topography of immunoreactive NKB and kisspeptin neurons.

Tissue preparation

Animals were deeply anaesthetised with pentobarbital (50 mg/kg) and perfused transcardially with 0.9% saline for 5 min, followed by 4% paraformaldehyde-phosphate buffer for 10 min (0.1M; pH 7.4). The brains were rapidly and carefully isolated from the skull and postfixated in the same fixative overnight and thereafter kept in 0.05 M phosphate-buffered saline (PBS) at 4°C until immunocytochemical processing.

Immunocytochemistry

The fixed brains were dehydrated in 30% sucrose-PBS solution before sectioning into four parallel series of 40 μm free-floating coronal sections through the entire rostro-caudal extension of the ARC; the sections of each series were thus separated by 160 μm. For the mapping of immunoreactive neurons, one series of sections was used to evaluate kisspeptin immunoreactivity and the adjacent series was evaluated for NKB immunoreactivity using 3,3′-diaminobenzidine (DAB) as chromagen, as previously described (Bentsen et al., 2010; Ruiz-Pino et al., 2012). For evaluation of kisspeptin immunoreactivity a purified polyclonal primary antiserum (JLV-1, diluted 1:200) was used. This antiserum is raised against rat kisspeptin-52 in rabbit and has been shown not to cross-react with a number of RF-peptides (Desroziers et al., 2010; Overgaard et al., 2013). For detection of NKB immunoreactivity, a purified polyclonal antiserum raised against NKB in rabbit (IS-39, diluted 1:5000), which has also been previously characterised, was used (Ciofi et al., 2007; Ciofi et al., 2006). For each experiment, all sections reacted for the same antigen was reacted simultaneously. For the DAB staining procedure a series of sections was washed in PBS, endogenous peroxidases were blocked in 1% H_2O_2 in PBS for 10 min, followed by blockade of non-specific binding in PBS containing 0.3% Triton X-100, 5% swine serum, and 1% bovine serum albumin (BSA) for 20 min. The sections were then incubated in primary antiserum diluted in PBS containing 0,3% Triton-X 100 and 1% BSA and gently shaken overnight at 4°C. After washing in PBS containing 0.1% Triton X-100 (T-PBS), sections were incubated 1 h in biotinylated donkey anti-rabbit (1:1000, Jackson Labs, USA) in PBS containing 0.3% Triton X-100 and 1% BSA. After another T-PBS wash, sections were incubated in 0.4% avidin-biotin-peroxidase complex (Vector Elite Kit™, Vector Labs, USA) diluted in T-PBS and thereafter developed in 0.05% DAB (Sigma-Aldrich, USA) with 0.05% H_2O_2 in Tris-HCl buffer (0.05M, pH 7.6, 5°C) for 10 min. The sections were mounted in a rostral to caudal order and coverslipped in Pertex® (HistoLab, Sweden).

For each section, the number of NKB or kisspeptin-ir neurons in both hemispheres was quantified under bright field illumination (Zeiss Axioskop 2plus) with special attention to anatomical cues, in order to map the correct rostro-caudal position of each section. The identities of the rats were blinded through the immunocytochemical procedure and during quantification.

For quantification of immunoreactive fiber density, a photomicrograph of the section 2.44 mm posterior to bregma was used. This section contains only few immunoreactive neurons, and optical density (OD) therefore reflects the amount of immunoreactive fibers in the rostral ARC, rather than cell bodies. The section was selected based on anatomical cues, with the shape of the ARC and the

median eminence and the retraction of the optical nerves as the major anchor points. Photomicrographs were taken at 10x magnification under identical light and microscope settings using the Zeiss microscope Axioskop 2plus (Carl Zeiss Imaging Solutions, Germany) and a PixeLINK PL-A622C camera (PixeLINK, US), and the photomicrographs were analysed using the software ImageJ (National Institute of Health, USA), measuring OD unilaterally in a triangle covering the ARC. OD outside of the ARC, close to the ventromedial hypothalamic nucleus, which contained no immunoreactive fibers, served as background measure and was subtracted OD of the ARC. The identities of all photomicrographs were blinded during analysis, and all photomicrographs were analysed simultaneously.

Fluorescent double labelling

To evaluate the degree of co-expression of kisspeptin and NKB the section 3.88 mm posterior to bregma, which displays the largest sex difference in expression, was stained for both kisspeptin and NKB using double immunofluorescence. The kisspeptin antiserum #067 against the N-terminal part of rat kisspeptin-52 is raised in sheep and is therefore appropriate for double labelling with the NKB antibody IS-39, which is raised in rabbit. #067 has been characterised previously and specificity has been validated in knockout tissue (Franceschini et al., 2013). Further, it was reported that this antibody is suitable for staining of kisspeptin in the ARC of rats, only if the rats are gonadectomised (Franceschini et al., 2013), and we therefore double labelled the OVX and ORX groups. For the double labelling procedure, the sections were washed thoroughly in PBS, and non-specific binding was then blocked in blocking buffer (5% normal swine serum and 0.3% Triton X-100 in PBS) for 30 min. The sections were thereafter transferred directly to the primary antiserum #067 diluted 1:1000 in blocking buffer. After incubation overnight at 4 ºC, sections were washed in PBS + 0.3% Triton X-100 and then incubated 3 h in fluorochrome-coupled secondary antibody (Alexa Fluor® 488 anti-sheep IgG, Invitrogen, US) diluted 1:333 in blocking buffer. From this point, the wells were covered at all times to protect the fluorochrome. The sections were washed thoroughly in PBS + 0.3% Triton X-100, and the protocol was repeated using the polyclonal NKB antiserum IS-39 (diluted 1:4000) and the secondary antibody Alexa Fluor® 568 anti-rabbit IgG, (Invitrogen, US). After washing in PBS, the sections were mounted and coverslipped with Fluoromount-G (SouthernBiotech, US). Representative photomicrographs were taken at 20x magnification using a Zeiss Imager Z.1 microscope with the software AxioVision 4.7.3 (Carl Zeiss

Imaging Solutions, Germany), and an AxioCam MRm camera (Carl Zeiss Imaging Solutions, Germany).

Statistical analysis

Differences between the three groups in Experiment 1 were analysed using a one-way ANOVA followed by Tukey's multiple comparisons test. Due to the caudal peak in NKB- and kisspeptin-ir cells in the females, the ARC was divided in a rostral part (3.56 mm posterior to bregma and rostral hereof) and a caudal part (caudal to 3.56 mm posterior to bregma) which were also analysed separately using a one-way ANOVA followed by Tukey's multiple comparisons test to reveal whether sex differences and sex steroid sensitivity differed in the rostral and caudal populations. Differences between the sham male and sham female group were analysed using a Student's t-test. In Experiment 2, a two-way ANOVA followed by Tukey's multiple comparisons test was used to analyse differences in the number of immunoreactive neurons in the ARC, as well as separate analysis for the rostral and the caudal ARC (defined as for Experiment 1). For all analyses $p < 0.05$ was considered statistical significant. Data are presented as mean ± standard error of mean (SEM). Mapping of cell numbers in the ARC are presented both as the total cell count for the entire ARC, and as a rostral-caudal mapping of the total cell count of each section.

Results

Sex differences in kisspeptin- and NKB-ir neurons in the ARC

We investigated the potential sex differences in the neuroanatomical distribution of kisspeptin- and NKB-ir cells throughout the ARC of male and diestrous female sham rats. Overall, the female ARC contained both more kisspeptin- (fig. 1A, $p < 0.0001$) and NKB-ir cells (fig. 1B, $p < 0.05$) compared to males. In females, there was a steady rostro-caudal increase in the number of kisspeptin- and NKB-ir cells in the rostral ARC, and from 3.56 mm posterior to bregma, a marked increase in cell numbers occured for both peptides, peaking 3.88 mm posterior to bregma (fig. 1A+B). In males, the number of kisspeptin-ir cells was very low throughout the extent of the ARC, and no peak was observed in sham males (fig. 1A). Accordingly, females had more kisspeptin-ir cells in both the rostral ($p < 0.0001$) and the caudal ARC ($p < 0.0001$). For NKB, the number of immunoreactive cells was similar to female levels in the rostral ARC ($p < 0.44$). In the caudal ARC of the male there was a peak in NKB-ir cells, which was in contrast to male kisspeptin expression.

However, the caudal peak was less prominent than in the female, as was the total number of NKB-ir cells (fig. 1B, $p < 0.01$). Kisspeptin and NKB double labelling of the caudal ARC in OVX and ORX rats supported the finding that kisspeptin- and NKB-ir neurons are more abundant in the female compared to the male and suggested that kisspeptin and NKB are co-expressed to a large extent in OVX females, whereas in the ORX male, cells expressing NKB, but not kisspeptin, were evident (Fig. 2). Concomitantly, these expression patterns suggest that there are more neurons expressing both NKB and kisspeptin in the caudal ARC of females, and suggest the existence of a subpopulation of cells in the male ARC expressing only NKB and not kisspeptin; we define these as NKB-only neurons.

Kisspeptin- and NKB-ir neurons are differentially regulated by adult sex steroid manipulation

To investigate the sex steroid feedback on kisspeptin- and NKB-ir neurons, we subjected one group of male and female rats to gonadectomy, and another group to gonadectomy with physiological level of sex steroid replacement for a total of three weeks. The number of kisspeptin-ir cells in the ARC increased after gonadectomy in both females (fig. 3A, $p < 0.05$) and males (fig. 3C, $p < 0.001$) and sex steroid replacement reduced the number of kisspeptin-ir cells to sham level in the female. In the male, the kisspeptin level in the ORX+T group was reduced compared to ORX (fig. 3C, $p < 0.001$), but had not reached sham male levels ($p < 0.05$).

Examination of the number of kisspeptin-ir neurons along the rostro-caudal axis (females, fig. 3B; males, fig. 3D) revealed that gonadectomy increased the number of kisspeptin-ir cells across the entire ARC, and steroid replacement lowered the number of kisspeptin-ir cells across the entire ARC in both males and females.

Regulation of NKB-ir cells by adult sex steroid manipulation was different from the regulation of kisspeptin. Firstly, the number of NKB-ir cells was not significantly affected by gonadectomy in either the female (fig. 3E) or the male (fig. 3G). Secondly, sex steroid replacement resulted in an increase in the number of NKB-ir cells relative to the gonadectomised group in both sexes (females, $p < 0.01$; males, $p < 0.001$), and the OVX+E group expressed more NKB-ir cells relative to sham (fig. 3G, $p < 0.05$). The topographical expression profiles (females, fig. 3F; males, fig. 3H) showed that, as for kisspeptin, the expression was equally affected by changes in sex steroid levels across the extension of the ARC. The equal sensitivity of the rostral and caudal ARC to sex steroid manipulation was confirmed when analyzing the rostral and caudal ARC separately.

Sex steroids increase kisspeptin- and NKB-ir fiber density in the female ARC

To assess the potential association between the number of immunoreactive cells and the density of fibers in the ARC, OD was measured in a rostral ARC section (2.44 mm posterior to bregma) as a marker of fiber density. This section contains immunoreactive fibers but only few immunoreactive cells, and OD is therefore an indirect measure of fiber density. A significant sex difference in fiber density was observed in sham animals for kisspeptin ($p < 0.0001$), but not for NKB ($p < 0.075$). However, both kisspeptin and NKB were sensitive to adult sex steroid manipulation in the female, where the OVX+E group had higher fiber density compared to sham or OVX, respectively (fig. 4A,C, $p < 0.05$). In contrast, male fiber density was not affected by sex steroid manipulation for either kisspeptin or NKB (fig. 4B,D).

Neonatal estrogenisation decreases female kisspeptin and NKB to male levels

To determine whether kisspeptin and NKB were sensitive to the developmental effects of neonatal estrogenisation, kisspeptin- and NKB-ir cells in the ARC were quantified in adult male and female rats subjected to a neonatal estrogenisation protocol. In females, both the number of kisspeptin- and NKB-ir cells were reduced to lower levels after neonatal estrogen exposure (fig. 5A,D; $p < 0.0001$) not differing from the level in males. In males, the number of NKB-ir cells was also reduced by neonatal estrogenisation (fig. 5D; $p < 0.01$). However, the number of kisspeptin neurons was not altered (fig. 5A). The topographical expression profiles of both peptides (kisspeptin, fig. 5B; NKB, fig. 5D) suggested that the caudal population of kisspeptin- and NKB-ir cells was most sensitive to the effects of neonatal estrogenisation. However, analyzing the rostral and caudal ARC separately showed comparable sensitivity to neonatal estrogenisation of the rostral and caudal ARC.

Discussion

Sex differences in kisspeptin and NKB immunoreactivity

Lately, the validity of the KNDy neuron model has been debated, and it has become more evident that sex and species differences may be pronounced (Cheng et al., 2010; Hrabovszky et al., 2011; Hrabovszky et al., 2012; Overgaard et al., 2013). Our topographical mapping of kisspeptin- and NKB-ir neurons in the ARC of intact male and female rats showed that whereas there was a sex difference only in the number of kisspeptin-ir cells and not of NKB-ir cells in the rostral ARC, there was a pronounced sex difference in the caudal ARC, with the female expressing both more

kisspeptin and NKB. Based on the finding of extensive overlap in kisspeptin and NKB expression in the female, as revealed by double labeling, these neurons are suggested to be KNDy neurons. Such a caudal population of kisspeptin/NKB neurons has also been described in ewes (Cheng et al., 2010; Estrada et al., 2006; Merkley et al., 2012), OVX monkeys (Alcin et al., 2013), and in a female rat (Desroziers et al., 2012a). Our finding of more NKB- and kisspeptin-ir cells in intact adult females compared to males is supported by previous findings in different mammalian species (Cheng et al., 2010; Desroziers et al., 2012b; Goubillon et al., 2000; Hrabovszky et al., 2011; Overgaard et al., 2013). Further, our finding of sex differences in kisspeptin fiber density and not in NKB fiber density in the ARC is in line with reports in humans (Hrabovszky et al., 2011). Concomitant with the relative abundance of NKB-ir cells in the female relative to the male, this could suggest that a fraction of female NKB-ir neurons project to areas outside the ARC.

Changes as a result of adult sex steroid manipulation

Our finding of increased numbers of NKB-ir neurons in OVX+E relative to OVX is not in line with studies at the mRNA level, where hormone replacement results in a decrease in *Tac2* mRNA expression (Danzer et al., 1999; Dellovade and Merchenthaler, 2004; Navarro et al., 2011b; Pillon et al., 2003). A potential explanation for this discrepancy could be that sex steroids not only inhibit *Tac2* mRNA transcription but also suppress NKB axonal transport without affecting the translational activity of the mRNAs, resulting in increased intracellular NKB levels in the ARC.

On the contrary to NKB, both *Kiss1* mRNA (Merkley et al., 2012; Rometo et al., 2007; Smith et al., 2007; Smith et al., 2005b) and kisspeptin levels decrease upon sex steroid replacement, and kisspeptin expression is therefore regulated also at the translational level, with decreased mRNA resulting in decreased numbers of kisspeptin-ir cells. To our knowledge, we are the first to quantify kisspeptin- and NKB-ir fibers in the ARC upon sex steroid replacement, and the increased fiber density specifically in the female suggests that inhibited release of kisspeptin and NKB is occurring in the OVX+E state. Although the mechanisms causing inhibition of peptide release are not understood, inhibition of NKB and kisspeptin release at the level of the median eminence has also been observed in lactating rats (True et al., 2011). The additional regulation of kisspeptin at the translational level may serve as an extra precaution to ensure inhibition of kisspeptin signalling. The inhibition of NKB and kisspeptin release from fibers in the OVX+E group is not found in the ORX+T group, suggesting a female-specific regulation of release. This regulation may be important for rapid changes in release, and could serve a function in regulation of the estrous cycle. In

addition, it remains a possibility that NKB release from NKB-ir fibers projecting to areas outside the ARC, as described e.g. for the involvement of NKB in regulation of body temperature (Mittelman-Smith et al., 2012b), is regulated differently.

In line with findings in humans, where postmenopausal decrease in the negative sex steroid feedback has a greater effect on kisspeptin compared to NKB levels (Hrabovszky et al., 2011; Molnar et al., 2012), we found that kisspeptin was more sensitive than NKB to gonadectomy. Differential regulation of *Tac2* and *Kiss1* mRNA has also been reported in mice, where peripubertal lack of sex steroids markedly increase *Kiss1* expression without affecting *Tac2* mRNA levels, and in adult sex steroid replaced OVX mice, *Kiss1* expression is affected at lower levels of replacement compared to *Tac2* expression (Gill et al., 2012). Species differences in the sensitivity of NKB to sex steroid feedback likely exist, because whereas we find no difference in NKB-ir cells upon gonadectomy, in humans there is an increase in NKB-ir cells upon decreased negative sex steroid feedback (Kallo et al., 2012) and in sheep, both pre- and postpubertal gonadectomy increase the number of NKB-ir cells in the ARC (Nestor et al., 2012).

The NKB-only population

We found more NKB-ir cells compared to kisspeptin-ir cells in the male rat, which is in line with findings in male mice (Navarro et al., 2011b) and humans (Hrabovszky et al., 2011; Hrabovszky et al., 2012). In the same line, we found NKB-ir cells not co-expressing kisspeptin in the male rat, concomitantly supporting the existence of a NKB-only population in the male ARC. It is possible that the major function of the NKB-only neurons is to integrate in the KNDy neuron population to ensure optimal coordination of kisspeptin pulses but the projection of NKB-ir fibers to several areas also allows speculation in multiple functions for NKB unrelated to KNDy neuron function, e.g. in homeostasis, behaviour and neuroendocrine functions (Krajewski et al., 2010). Co-localisation was investigated in gonadectomised rats to obtain optimal immunoreactive signal. Even in this state, which has increased number of kisspeptin-ir cells and unchanged NKB cells relative to sham, we detect neurons expressing NKB and not kisspeptin in the male. Dual-labelling in sham conditions would possibly reveal NKB-only neurons, also in the female, but this remains to be investigated.

In addition to a NKB-only population, we also found a small fraction of cells expressing only kisspeptin. In the present study in rats as well as in a study in mice (Kallo et al., 2012), the kisspeptin-only neurons are few, but kisspeptin neurons project to several limbic structures (Yeo

and Herbison, 2011), and a specific function for this population other than participation in the KNDy neuron function can not be ruled out. Actually, in agonadal male monkeys, the kisspeptin-only population is rather significant (only 50% of kisspeptin-ir cells co-express NKB) and a NKB-only population does not exist (Ramaswamy et al., 2010), whereas in the goat there is an almost complete overlap of kisspeptin and NKB-ir cells (Wakabayashi et al., 2010) emphasizing the species differences of this system.

We acknowledge the KNDy neuron concept and its important implications in the generation of GnRH pulses, but we find the recent tribute to the KNDy neuron concept as an isolated cell type in which the implicated neuropeptides serve only a function as part of the KNDy neuron circuitry, troublesome; for instance some studies conclude that certain projections or regulation of certain neurons are KNDy neurons, when in fact only one KNDy peptide/mRNA/receptor is investigated (Krajewski et al., 2010; Merkley et al., 2012; Mittelman-Smith et al., 2012a; Rance et al., 2013). This may lead to overestimation of the function of KNDy neurons and also overlook other functions of these neuropeptides. For instance, it is possible that it is a NKB-only population and not the KNDy neuron population participating in the hormonal influence on body temperature during menopause (Rance et al., 2013).

Changes as a result of neonatal estrogenisation

To investigate whether the sex differences in kisspeptin and NKB expression in sham male and females stem from early sex steroid-dependent neurodevelopment, we assessed whether early postnatal estrogenisation resulted in long-term changes in expression of the two peptides. We found that in the female rat, neonatal estrogenisation reduced kisspeptin and NKB expression to the level of vehicle-treated males, whereas in males a reduction was found only for NKB-ir cells. The topographical expression profiles showed a large decrease in caudal cell numbers upon neonatal estrogenisation in the females, which suggested that the caudal population of kisspeptin and NKB cells was particularly sensitive to neonatal estrogenisation; however, analyzing the rostral and the caudal ARC separately revealed similar sensitivity in both areas. It is likely that the higher levels of KNDy neurons in females are involved in the female-specific regulation of the HPG axis, such as the preovulatory LH surge, and the decrease in immunoreactive neurons after neonatal estrogenisation is in line with findings of anovulation after neonatal etrogenisation (Bateman and Patisaul, 2008).

Adult ewes subjected to testosterone in the first half of pregnancy have reduced NKB expression in the middle and caudal ARC (Cheng et al., 2010; Goubillon et al., 2000) whereas kisspeptin expression is unchanged (Cheng 2010), suggesting that the sensitive window for kisspeptin is later in development, whereas NKB may be sensitive to long-term changes in a longer period of perinatal development. Together with our results of neonatal estrogenisation only affecting the male NKB system and not the male kisspeptin system, and the report of different sensitivity to sex steroids in the peripubertal period (Gill et al., 2012), these findings suggest that NKB and kisspeptin have differential sensitivity to regulation by sex steroids across several stages in life.

In conclusion, we report here that kisspeptin- and NKB-ir neurons are differentially regulated by sex steroid manipulation, with regulation of kisspeptin at the translational level, and NKB regulation at the level of transportation, resulting in increased NKB-ir cells and decreased kisspeptin-ir cells in the ARC of sex steroid replaced rats. Further, we found a population of NKB-only neurons in the male and a population of neurons co-expressing kisspeptin and NKB which was more pronounced in females compared to males, and we found that the expression of kisspeptin and NKB was sensitive to neonatal estrogenisation. Collectively, these findings reveal differential regulation of kisspeptin and NKB, suggest a role for NKB in addition to KNDy neuron function, and underlines that the peptides in the KNDy neuron are regulated in a complex manner, which may not fit perfectly in our current view on the KNDy neuron model.

Acknowledgements

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Figures and figure legends

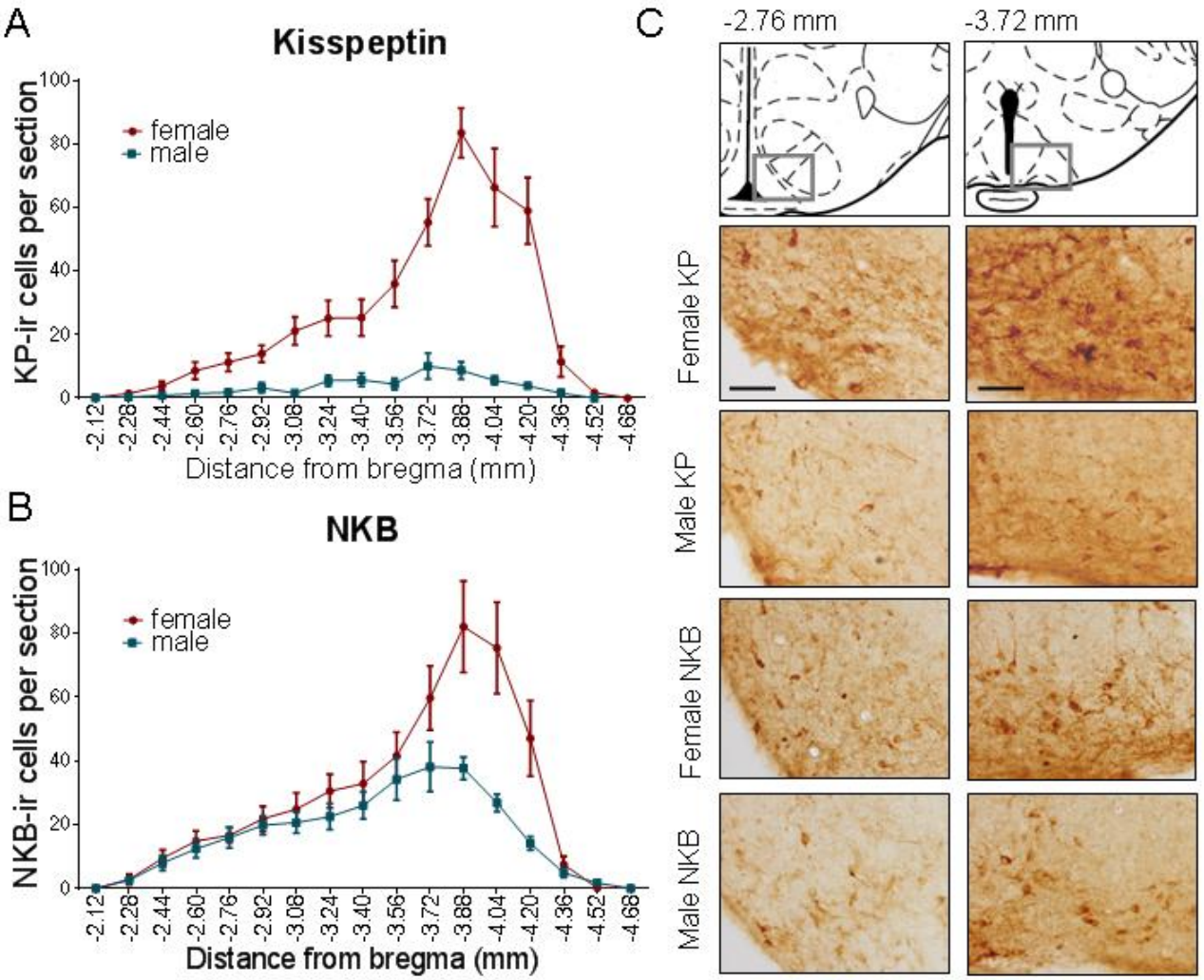

Fig. 1. Topographical mapping of sex differences in kisspeptin- and NKB-immunoreactive cells in the ARC. Topographical mapping of kisspeptin- (A) and NKB-immunoreactive cells (B) across the extension of the ARC revealed sex differences in expression of both peptides, especially in the caudal ARC. C) Representative photomicrographs of a rostral (2.76 mm posterior to bregma) and a caudal (3.72 mm posterior to bregma) section in males and females for kisspeptin and NKB. Bar = 50 μm. KP, kisspeptin; NKB, neurokinin B.

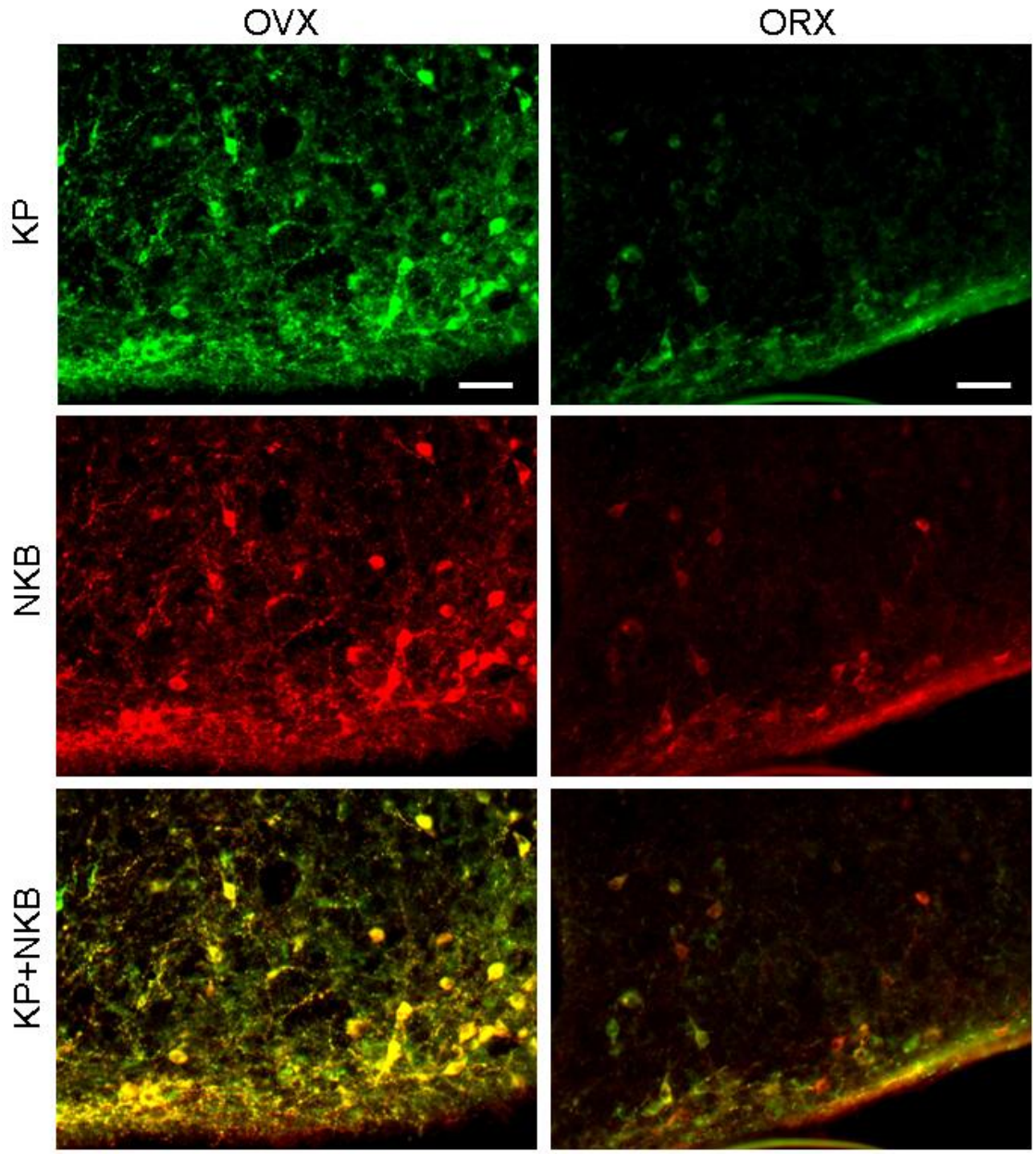

Fig. 2. Co-localisation of kisspeptin- and NKB-immunoreactive neurons in the ARC of gonadectomized female and male rats. Representative photomicrographs of the section 3.88 mm posterior to bregma from OVX and ORX animals dual labelled for kisspeptin (top) and NKB (middle) were merged (bottom). Neurons co-localising kisspeptin and NKB were more numerous in the female compared to the male. In the male, kisspeptin and NKB co-localisation was also evident, and in addition cells expressing NKB and not kisspeptin were detected. Bar = 50 μm. KP, kisspeptin; NKB, neurokinin B; ORX, orchidectomised; OVX, ovariectomised.

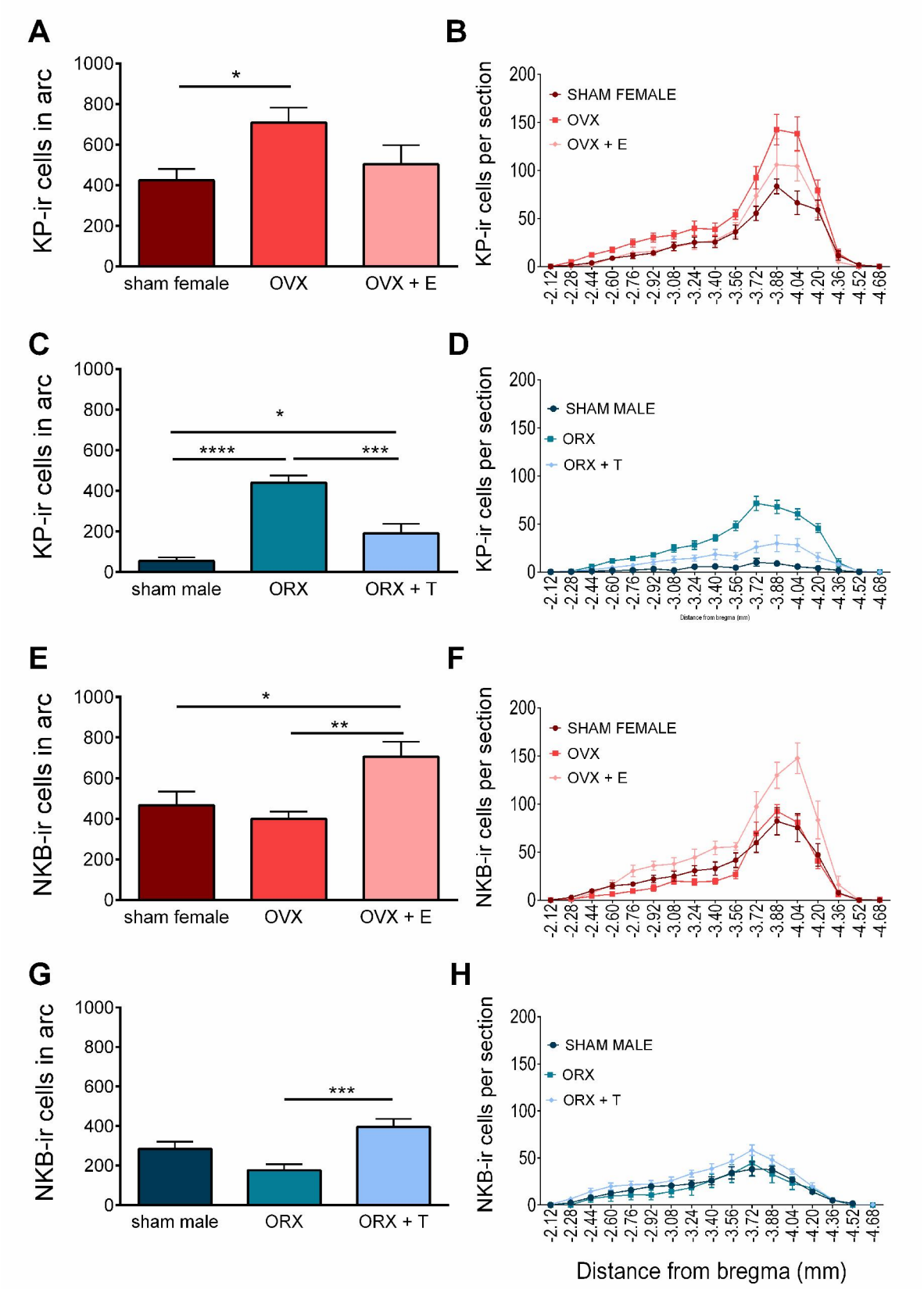

Fig. 3. **Kisspeptin- and NKB-immunoreactive neurons in the ARC after adult sex steroid manipulation.** Total number of immunoreactive cells in the ARC (left panel) as well as the

topographical mapping across the extension of the ARC (right panel) are presented for kisspeptin in females (A,B) and males (C,D) and for NKB in females (E,F) and males (G,H). Gonadectomy increased kisspeptin-ir cells and sex steroid replacement lowered kisspeptin expression across the extention of the ARC in both males and females. In contrast, NKB expression was unchanged upon gonadectomy and increased in both sexes after sex steroid replacement across the entire ARC. One-way ANOVA followed by Tukey's posthoc test (A,C,E,G), *P<0.05, **P<0.01, ***P<0.001. E, estradiol; ir, immunoreactive; KP, kisspeptin; NKB, neurokinin B; ORX, orchidectomy; OVX, ovariectomy; T, testosterone.

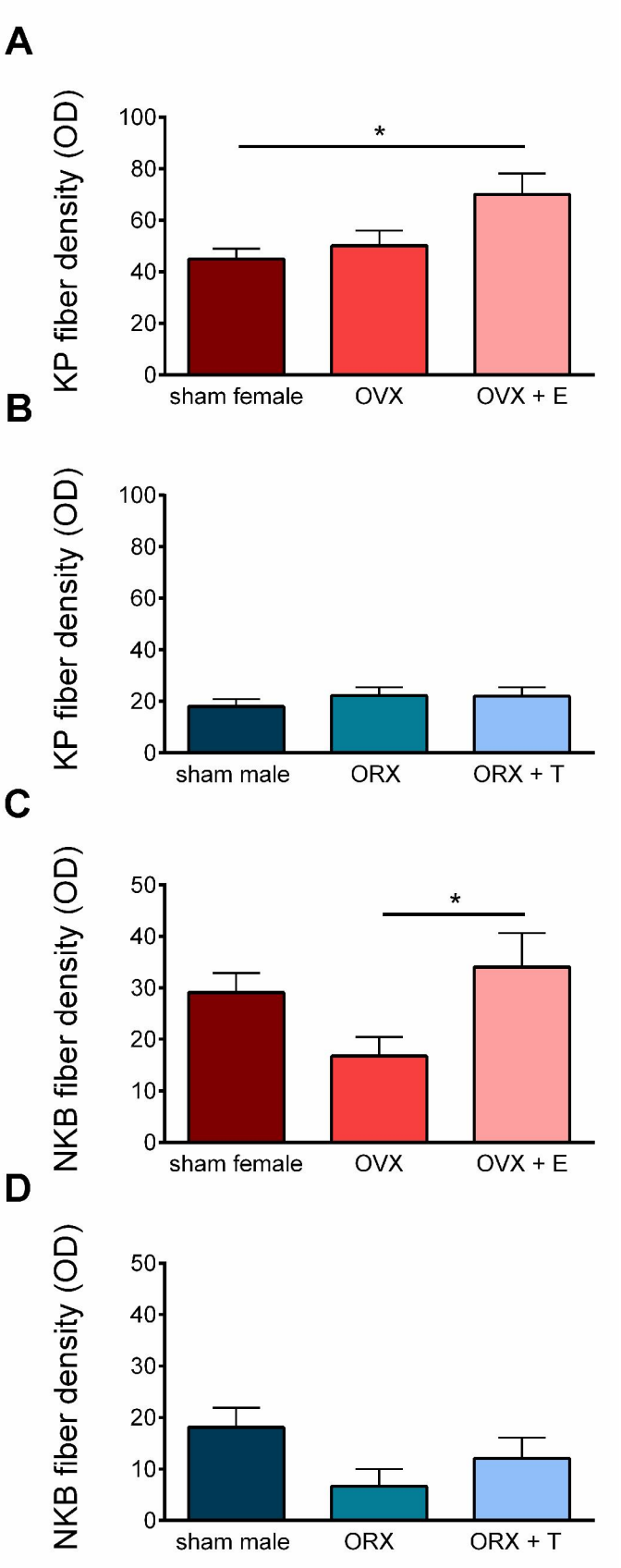

Fig. 4. Kisspeptin- and NKB-immunoreactive fiber density in the ARC after adult sex steroid manipulation. Kisspeptin and NKB fiber density in the rostral ARC, reflected by the optical density (OD) measure, in male and female rats subjected to adult gonadectomy and sex steroid replacement. In females, kisspeptin fiber density was increased in the OVX+E group relative to sham (A) whereas the NKB fiber density was increased in the OVX+E group relative to OVX (C). In males, no changes in kisspeptin fiber density (B) or NKB fiber density (D) were observed. One-way ANOVA followed by Tukey's posthoc test, *P<0.05. E, estradiol; KP, kisspeptin; NKB, neurokinin B; OD, optical density; ORX, orchidectomy; OVX, ovariectomy; T, testosterone.

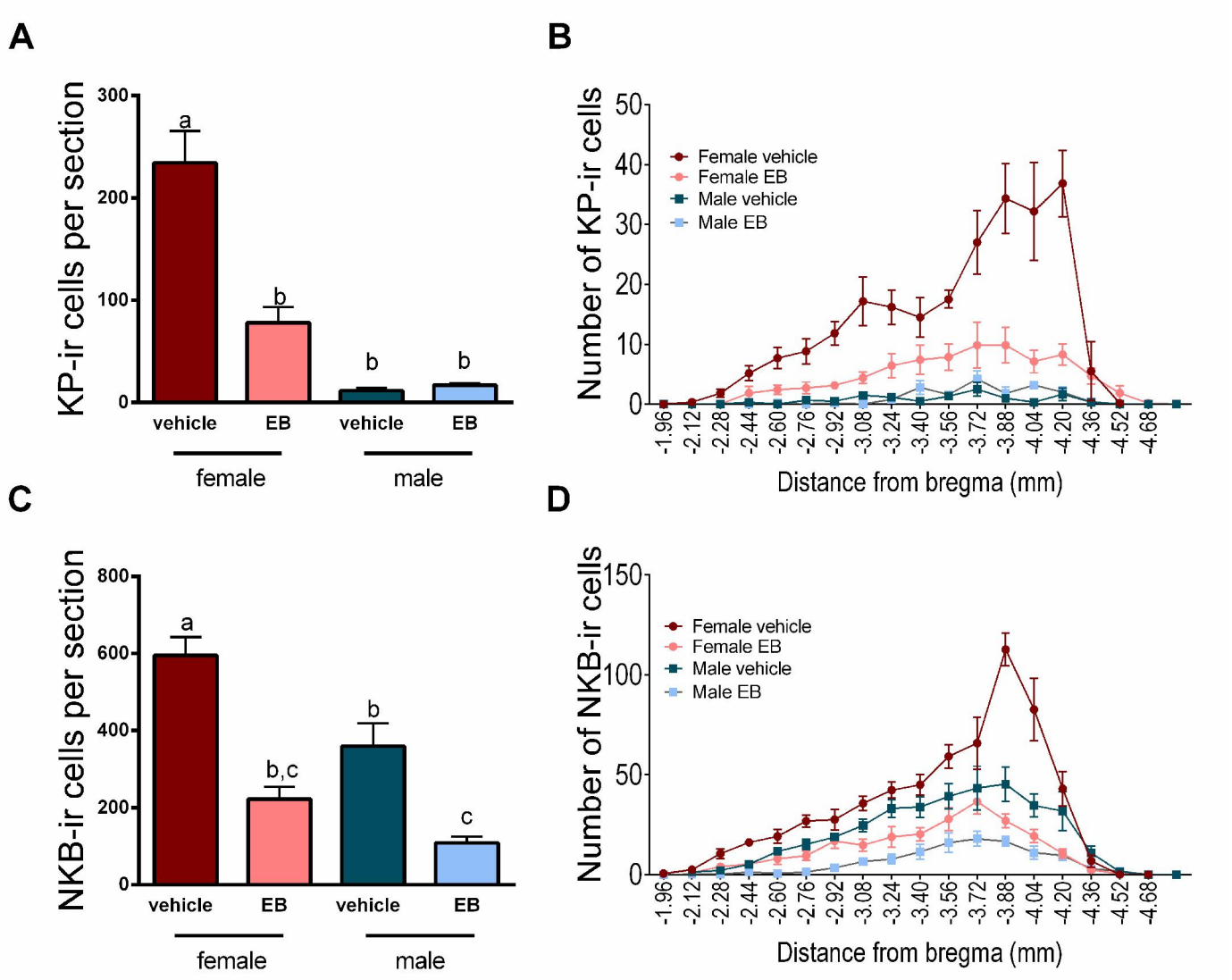

Fig. 5. Kisspeptin- and NKB-immunoreactive neurons in the ARC after neonatal sex steroid manipulation. Total number of immunoreactive cells in the ARC is presented for kisspeptin (A) and NKB (C), which shows that neonatal estrogenisation lowered adult expression of both kisspeptin and NKB in the female, whereas in the male only NKB expression was affected. Topographical mapping of kisspeptin- (B) and NKB-ir cells (D) across the extension of the ARC revealed the largest difference in immunoreactive cells in the caudal ARC. However, the sensitivity was similar after analyzing the rostral and caudal ARC separately. Two-way ANOVA followed by Tukey's posthoc test, "a" is different from "b" and "c", $P<0.0001$, and "c" is different from "b", $P<0.01$ (A, D). EB, estrogen benzoate; ir, immunoreactive; KP, kisspeptin.

Paper III

NeuroToxicology 37 (2013) 154–162

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NeuroToxicology

The effect of perinatal exposure to ethinyl oestradiol or a mixture of endocrine disrupting pesticides on kisspeptin neurons in the rat hypothalamus

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Estrogen

ABSTRACT

Early life exposure to endocrine disruptors is considered to disturb normal development of hormone sensitive parameters and contribute to advanced puberty and reduced fecundity in humans. Kisspeptin is a positive regulator of the hypothalamic–pituitary–gonadal axis, and plays a key role in the initiation of puberty. In the adult, *Kiss1* gene expression occurs in two hypothalamic nuclei, namely the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC), which are differentially regulated by peripheral sex steroid hormones. In this study we determined the effects on puberty onset and Kiss1 mRNA levels in each of the two nuclei after long-term perinatal exposure of rats to ethinyl oestradiol (EE_2) or to five different pesticides, individually and in a mixture. Rat dams were per orally administered with three doses of EE_2 (5, 15 or 50 μg/kg/day) or with the pesticides epoxiconazole, mancozeb, prochloraz, tebuconazole, and procymidone, alone or in a mixture of the five pesticides at three different doses. Kiss1 mRNA expression was determined in the AVPV and in the ARC of the adult male and female pups in the EE_2 experiment, and in the adult female pups in the pesticide experiment.

We find that perinatal EE_2 exposure did not affect Kiss1 mRNA expression in this study designed to model human exposure to estrogenic compounds, and we find only minor effects on puberty onset. Further, the Kiss1 system does not exhibit persistent changes and puberty onset is not affected after perinatal exposure to a pesticide mixture in this experimental setting. However, we find that the pesticide mancozeb tends to increase Kiss1 expression in the ARC, presumably through neurotoxic mechanisms rather than *via* classical endocrine disruption, calling for increased awareness that Kiss1 expression can be affected by environmental pollutants through multiple mechanisms.

1. Introduction

Perinatal exposure to substances that interfere with endogenous hormones, thereby disrupting their normal function, is suspected to contribute to advanced pubertal maturation and reduced fecundity in women (Buck Louis et al., 2008; Crain et al., 2008; Euling et al., 2008; Mouritsen et al., 2010; Wohlfahrt-Veje et al., 2012). Such substances, termed endocrine disruptors (EDs), have various mechanisms of action, such as changing the rate of steroidogenesis and aromatase activity, agonizing/antagonizing steroid receptors, and changing steroid receptor level or function (Frye et al., 2012; Roa et al., 2008). Girls of mothers exposed to pesticides in early pregnancy experience advanced breast development (Wohlfahrt-Veje et al., 2012), and there is an increased prevalence of cryptorchidism in boys of mothers exposed to pesticides (Weidner et al., 1998). Many EDs cross the placenta and are found in breast milk (Frye et al., 2012). Fetuses and children are especially vulnerable to EDs, since steroid hormone levels during this period are critical for proper development of both the reproductive organs and the nervous system, including the hypothalamus (Frye et al., 2012; Patisaul and Adewale, 2009a).

Kisspeptin, encoded by *Kiss1*, is a hypothalamic peptide expressed in two hypothalamic nuclei; the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC), and kisspeptin potently elicits GnRH release, thereby stimulating the hypothalamic–pituitary–gonadal axis (Pinilla et al., 2012). Kisspeptin is essential for puberty onset, as described by the hypogonadotropic hypogonadistic phenotype of humans with loss-of-function mutations in the kisspeptin receptor Kiss1R (de Roux et al., 2003; Seminara et al., 2003). Kiss1 neurons express

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estrogen receptor (ER)-α, progesterone receptor and androgen receptor (AR) in a high degree, while fewer Kiss1 neurons express ER-β (Lehman et al., 2010). Kiss1 neurons are thus considered to be important targets for steroid feedback in the adult, and *Kiss1* expression is differentially regulated by steroid hormones in the two areas, with sex steroids stimulating kisspeptin expression in the AVPV and inhibiting expression in the ARC (Clarkson et al., 2012; Smith et al., 2005a, b). During pubertal development, sex steroids are essential for the increase in kisspeptin (Clarkson et al., 2009, 2012; Smith et al., 2005a, b), and ablation of ER-α in kisspeptin neurons advances puberty, decreases kisspeptin expression, and disrupts estrous cyclicity in mice (Mayer et al., 2010). Furthermore, the Kiss1 system is highly sexual dimorphic and sexual dimorphic hypothalamic regions are shaped during developmental windows where differences in sex steroid levels are high, particularly the perinatal period (Bakker and Brock, 2010; Clarkson et al., 2009; Kauffman et al., 2007). Collectively, kisspeptin is a putative target for perinatal endocrine disruption.

Several studies have shown that perinatal or prepubertal exposure to estrogenic EDs can advance puberty onset in female rats (Bateman and Patisaul, 2008; Dickerson et al., 2011; Marty et al., 1999; Rasier et al., 2007), whereas other EDs have the capability to delay puberty onset (Davis et al., 2011; Laws et al., 2000; Marty et al., 1999; Stanko et al., 2010). The delay in puberty onset was induced by atrazine, which disturbs GnRH release (Cooper et al., 2007; Davis et al., 2011; Laws et al., 2000; Stanko et al., 2010), and ketoconazole, which decreases oestradiol levels through aromatase inhibition (Marty et al., 1999). Reduced kisspeptin expression after perinatal exposure to potent ER agonists has also been reported (Bateman and Patisaul, 2008; Bellingham et al., 2009; Dickerson et al., 2011; Losa-Ward et al., 2012; Navarro et al., 2009; Patisaul et al., 2009b), whereas the environmental EDs bisphenol-A and genistein only affect kisspeptin expression at high doses (four neonatal sc. injections of 50 mg/kg and 10 mg/kg, respectively) and not at low doses (four sc. neonatal injections of 50 μg/kg BPA) (Bateman and Patisaul, 2008; Losa-Ward et al., 2012; Patisaul et al., 2009b). Also, a reduced kisspeptin level has been reported in fetuses of ewes exposed to a mixture of environmental EDs at an undefined exposure level, but timing of puberty was not assessed (Bellingham et al., 2009).

These studies have a number of limitations in comparison to human exposure, as high doses of a single compound are injected in early postnatal life in most studies. By contrast, humans are exposed to several EDs at low levels, primarily *via* skin and digestion, and pesticide residues in food are an important exposure route. Pesticides have therefore been tested for their potential endocrine disrupting effects, and European recommendations to limit human exposure of single compounds have been made (Regulation(EC)No. 396/2005). However, it is increasingly recognized that the effects of EDs can be additive, such that exposure to

Table 1
Compounds, dosing, effects and mechanisms of action.

Study 1 compound	Doses (μg/kg/day)	Dosing period	Effects and mechanisms of action	References
Ethinylestradiol	5; 15; 50	GD 7–21 + PD 1–22	Endogenous estrogen receptor agonist	Ryan et al. (2010)
			Accelerated mammary gland development	Mandrup et al. (2012)
			Increased anogenital distance and advanced puberty in female offspring	Ryan et al. (2010)
Study 2 compounds	**Doses (mg/kg/day)**	**Dosing period**	**Effects and mechanisms of action**	**References**
Epoxiconazole	3.75; 15	GD 7–21 + PD 1–16	AR antagonism *in vitro*	Hass et al. (2012) and Kjaerstad et al. (2010a)
			Reduction of estradiol and testosterone and increase in progesterone synthesis *in vitro*	Hass et al. (2012) and Kjaerstad et al. (2010a)
			Increased anogenital distance in female rats	Taxvig et al. (2007)
			Anti-estrogenic and aromatase inhibiting	Kjaerstad et al. (2010b)
Mancozeb	6.25; 25	GD 7–21 + PD 1–16	Manganese-dependent mitochondrial dysfunction in neurons	Harrison Brody et al. (2013)
			Thyroid hormone disruption	Axelstad et al. (2011) and Hurley (1998)
			Increased risk of Parkinson's disease in exposed humans	Kamel et al. (2007)
Prochloraz	8.75; 35	GD 7–21 + PD 1–16	AR antagonism *in vitro*	Hass et al. (2012) and Kjaerstad et al. (2010a)
			Central and peripheral AR antagomism *in vivo*	Vinggaard et al. (2002)
			Reduction of estradiol and testosterone and increase in progesterone synthesis *in vitro*	Hass et al. (2012) and Kjaerstad et al. (2010a)
			Anti-estrogenic and aromatase inhibiting	Kjaerstad et al. (2010b)
Tebuconazole	12.5; 50	GD 7–21 + PD 1–16	AR antagonism *in vitro*	Hass et al. (2012), Orton et al. (2011) and Kjaerstad et al. (2010a)
			Reduction of estradiol and testosterone and increase in progesterone synthesis *in vitro*	Hass et al. (2012) and Kjaerstad et al. (2010a)
			Increased anogenital distance in female rats	Taxvig et al. (2007)
			Anti-estrogenic and aromatase inhibiting	Kjaerstad et al. (2010a)
Procymidone	12.5; 50	GD 7–21 + PD 1–16	AR antagonism *in vitro*	Hass et al. (2012) and Ostby et al. (1999)
			Decreased anogenital distance in male rats	Ostby et al. (1999)
Pesticide mixture	14.58; 29.17; 43.75	GD 7–21 + PD 1–16	Additive effects on gestational length, pup survival and nipple retention	Hass et al. (2012)
			Additive effects on sperm count, decreased weight of reproductive organs and altered spatial learning in males	Jacobsen et al. (2012)

In vivo mechanisms of actions is reported predominantly for females.
GD, gestational day; PD, pup day; AR, androgen receptor.

many EDs in low doses may affect human health, even if the tolerable limit of each ED has not been reached (Bellingham et al., 2009; Birkhøj et al., 2004; Christiansen et al., 2009; Rajapakse et al., 2002; Rider et al., 2008).

In that perspective, the pesticides tested in this study have been chosen based on information on pesticide use in Denmark and in the EU, and based on their multiple endocrine disrupting properties (Table 1). The aim of this study is thus to model human exposure to relevant pesticides, rather than testing additivity of compounds with a single mode of action, *e.g.* purely estrogenic compounds.

The azole fungicides epoxiconazole, tebuconazole, and prochloraz have previously shown multiple endocrine disrupting activities *in vitro*, including aromatase inhibition and sex steroid receptor antagonism (Hass et al., 2012; Kjaerstad et al., 2010a,b; Orton et al., 2011; Vinggaard et al., 2002). Endocrine disrupting effects of these conazoles or mixture of these have also been reported *in vivo*, with increased gestational length and external signs of female virilisation and incomplete masculinization of the male rat offspring (Hass et al., 2012; Jacobsen et al., 2012; Taxvig et al., 2007; Vinggaard et al., 2005).

Mancozeb disturbs dopaminergic and serotonergic neurotransmission as well as thyroid function, and is associated with neurogenerative diseases (Harrison Brody et al., 2013; Hurley, 1998; Kamel et al., 2007). In pregnant rats, oral exposure to mancozeb changes thyroid hormone levels in dams during gestation, whereas no changes in behavior or thyroid hormone levels were found in the pups (Axelstad et al., 2011). *In vitro* studies show that mancozeb does not have AR antagonistic activity neither does it affect estradiol or testosterone synthesis (Hass et al., 2012); instead, the effect of mancozeb can be partly accounted for by its manganese component, which cause mitochondrial dysfunction and oxidative stress (Benedetto et al., 2010; Harrison Brody et al., 2013).

Procymidone is found, both *in vivo* and *in vitro*, to be an AR antagonist, which is capable of causing incomplete masculinization of male pups after perinatal oral gavage of the dams (Hass et al., 2012; Ostby et al., 1999).

The endocrine disrupting effects of these pesticides are thus previously described, and their combined effects *in vivo* have previously been shown to include *e.g.* affected gestational length and pup survival, as well as impaired sexual differentiation at doses where the single pesticides does not affect these parameters (Hass et al., 2012; Jacobsen et al., 2010, 2012). We thus test the hypothesis that the effects on the Kiss1 mRNA expression will be additive.

In this study we determine the effects on puberty onset and Kiss1 mRNA levels in the two hypothalamic nuclei after long-term perinatal exposure of male and female rats to ethinyl oestradiol (EE_2), as a model for exposure to estrogenic EDs. In addition, the effect on puberty onset and Kiss1 mRNA levels in AVPV and ARC is determined in female rats after perinatal exposure to five different pesticides, individually and in a mixture, to explore possible additive effects of the pesticides.

2. Materials and methods

Two experiments were conducted; Study 1 investigated perinatal exposure to ethinyl oestradiol (EE_2) while Study 2 investigated perinatal exposure to five pesticides individually and in a mixture.

2.1. Chemicals, animals and dosing

Ethinyl oestradiol (EE_2; CAS no. 57-63-6) was obtained from Steraloids Inc. (USA) and procymidone (CAS no. 32809-16-8), epoxiconazole (CAS no. 106325-08-8), tebuconazole (CAS no. 107534-96-3), mancozeb (CAS no. 8018-01-7) and prochloraz (CAS no. 67747-09-5) were obtained from VWR-Bie & Berntsen (Denmark). Corn oil from Sigma–Aldrich (Denmark) served as vehicle.

All dams were housed in pairs until gestational day (GD) 18, and had access to a complete rodent diet for growing animals (Soy- and alfalfa free ALTROMIN 1314, Altromin GmbH, Germany) and acidified tap water during the entire experiments, under conditions of 12 h light–dark cycle (light on at 9 p.m.) and constant temperature (21 ± 2 °C) and humidity (50% ± 5%). Time-mated adult Wistar rats (HanTac:WH, Taconic, Denmark) arrived at GD 3 and were sorted in groups with equal body weight distributions. The dams were dosed daily by oral gavage (2 ml/kg), from GD 7 to GD 21, and again from the day after delivery to pup day (PD) 22 in Study 1 and to PD 16 in Study 2. The expected day of delivery, GD 23, was designated PD 1. For an overview of dosing period and dosages, please consult Table 1. All animals used for Kiss1 determination were euthanized between 9 a.m. and 2 p.m., and animals from all groups were euthanized in a random manner.

2.2. Studies and dose levels

In Study 1, 40 pregnant dams were distributed in four groups (n = 10); control (0 μg/kg/day), E-5 (5 μg/kg/day), E-15 (15 μg/kg/day) and E-50 (50 μg/kg/day). For Kiss1 mRNA determination, groups of 8–10 adult male and female pups (PD 50, males ∼210 g, females ∼140 g; from 5 to 9 different litters, as shown in Table 2),

Table 2
Body weight and puberty onset for Study 1.

Estradiol exposure	Control	E-5	E-15	E-50
Male (n=)[a]	8 (8)	10 (6)	10 (9)	8 (6)
BW juvenile (PD22) (g)	63.8 ± 6.9	61.3 ± 3.8	63.3 ± 7.6	57.0 ± 5.3
BW adult (g)	214 ± 18	206 ± 15	212 ± 20	197 ± 8
Accumulative PO PD41 (%)	25	0	10	12.5
Accumulative PO PD45 (%)	87.5	40	90	87.5
PO – 95% confidence interval in relation to controls (days)		0.19–3.69	−1.67–1.54	−2.02–1.44
Female (n=)[a]	8 (8)	8 (5)	8 (8)	8 (8)
BW juvenile (PD22) (g)	62.4 ± 3.0	56.4 ± 7.7	58.9 ± 6.1	56.1 ± 6.5
BW adult (g)	143 ± 8	138 ± 16	140 ± 8	136 ± 8
Accumulative PO PD27 (%)	0	0	12.5	25
Accumulative PO PD30 (%)	25	0	12.5	37.5
PO – 95% confidence interval in relation to controls (days)		0.23–4.85	−1.73–3.74	−4.47–3.63

BW data represent group means ± SEM.
[a] Number in parentheses is the litter n-value.
Adult body weight is at the time of sacrifice.
BW is analyzed in a random intercepts model, adjusting for littermates.
PO is analyzed with a Tobit regression with adjustment for littermates and juvenile body weight.
BW, body weight; PD, pup day; PO, puberty onset.

were randomly selected, and females were not selected in a specific cycle stage. The dissected brains were analyzed for gene expression using RT-qPCR as described in Section 2.3.1. Juvenile (PD 22) and adult body weights and puberty onset data for these animals are presented in Table 2. Data on estrous cyclicity and puberty onset data including all weaned pups are presented in Mandrup et al. (under review).

In Study 2, 198 pregnant dams were distributed in ten groups (n = 10–12). The groups with individual pesticide exposure were as follows: epoxiconazole 3.75 mg/kg/day (Epo-3.75) or 15 mg/kg/day (Epo-15), mancozeb 6.25 mg/kg/day (Man-6.25) or 25 mg/kg/day (Man-25), prochloraz 8.75 mg/kg/day (Prochlo-8.75) or 35 mg/kg/day (Prochlo-35), tebuconazole 12.5 mg/kg/day (Tebu-12.5) or 50 mg/kg/day (Tebu-50) and procymidone 12.5 mg/kg/day (Procymi-12.5) or 50 mg/kg/day (Procymi-50). In addition, four groups (n = 22) were dosed with vehicle or a mixture of the five pesticides in different concentrations; 14.58 mg/kg/day (Pestimix-14.58), 29.17 mg/kg/day (Pestimix-29.17) and 43.75 mg/kg/day (Pestimix-43.75). The pesticides were mixed in the following ratio: epoxiconazole:mancozeb:prochloraz:tebuconazole:procymidone – 15:50:35:50:50, according to the NOAEL for effects on gestation length of the individual pesticides. For Kiss1 mRNA determination, groups of 2–9 adult (6 month old, ~250 g) female pups (from 1 to 8 different litters, as shown in Table 3) were euthanized in proestrous and analyzed for Kiss1 mRNA levels using *in situ* hybridization, as described in Section 2.3.2. Pups from each litter were used for multiple analyses, and thus euthanized at different time points, and in addition the number of dams with successful pregnancies and the size of litters varied between groups (Hass et al., 2012). Based on the number of successful pregnancies and the number of live pups in each group, a dissection plan for the different analyses was made. In addition, littermates were included when possible to increase group size, for which correction is made in the statistical analysis. *In situ* hybridization was chosen for this experiment to gain optimal resolution and permit detection of subtle changes. Data for puberty onset and juvenile (PD 28) and adult body weight for these animals are presented in Table 3.

In Study 1, we investigated the effect of EE_2 exposure during gestation and lactation on Kiss1 mRNA expression and puberty onset in male and female offspring. Doses of EE_2 covering the low to medium exposure levels were chosen based on earlier reports (Mandrup et al., 2012).

In Study 2, we used the same model of perinatal exposure, to investigate the effect of a mixture of five different pesticides (procymidone, epoxiconazole, tebuconazole, mancozeb and prochloraz) in three different doses, on Kiss1 mRNA and puberty onset in female offspring. In addition, the effect of each of the pesticides was tested at two different doses. Based on initial range-finding studies (Jacobsen et al., 2010; Taxvig et al., 2007; Vinggaard et al., 2005), the no observed adverse effect level (NOAEL) for gestational length and pup survival was defined for the individual pesticides. This dose was chosen as the low dose in the single pesticide exposure groups. The high dose was set to be four times higher than the NOAEL. The high pesticide mixture (Pestimix-43.75) contains the single pesticides in the same doses as the low exposure group for the single pesticides. The two other mixture doses were 33% and 66% of the highest dose. For an overview of the dosing scheme please consult Table 1 and for further details on dosing, please consult Hass et al. (2012).

Effects on mammary gland development in animals from Study 1 (Mandrup et al., 2012), and effects on the reproductive tract, postnatal development, behavior and maternal endpoints in animals from Study 2, as well as *in vitro* studies using the same pesticide mixture, have recently been previously published (Hass et al., 2012; Jacobsen et al., 2012).

The animal studies were performed under conditions approved by the Danish Animal Experiments Inspectorate and by the in-house Animal Welfare Committee.

2.3. Determination of Kiss1 mRNA

Rats were decapitated in CO_2/O_2 anesthesia, and brains were removed instantly. The brains were frozen in powdered dry ice and kept at −80 °C until processed.

2.3.1. RT-qPCR

In Study 1, the AVPV and ARC were isolated by isolating the respective areas on a cryostat at −20 °C. Specifically, for the AVPV a square were cut 1 mm lateral to the ventricle and just dorsal to the anterior commissure, and sections were collected from 0.20 mm to 0.70 mm posterior to bregma, thus also containing the preoptic periventricular nucleus. For the ARC a square were cut 2 mm lateral to the ventricle and just dorsal to reuniens thalamic nucleus and sections were collected from 2.30 mm to 4.70 mm posterior to bregma. All animals were processed simultaneously and the identities of all animals were blinded to allow determination of both treatment and sex effects. The tissue was extracted for RNA with Trizol Reagent (Sigma–Aldrich, USA) and dissolved in RNase-free water and quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA). Reverse transcription of the RNA to single stranded cDNA using the ImPromII™ reverse

Table 3
Body weight and puberty onset for Study 2.

Single pesticide exposure	Control	Epo 3.75	Epo 15	Man 6.25	Man 25	Prochlo 8.75	Prochlo 35	Tebu 12.5	Tebu 50	Procymi 12.5	Procymi 50
Female (n=)[a]	9 (8)	6 (5)	2 (1)	5 (4)	4 (3)	8 (7)	3 (3)	6 (6)	4 (4)	4 (3)	2 (1)
BW juvenile (PD28) (g)	62.4 ± 3.7	73.1 ± 9.0	71.5 ± 0.8	63.1 ± 2.3	64.7 ± 2.2	64.3 ± 8.9	57.9 ± 8.2	64.0 ± 3.2	66.4 ± 1.0	61.0 ± 2.0	67.0 ± 1.0
BW adult (g)	250 ± 12	266 ± 17	267 ± 4	239 ± 10	265 ± 5	253 ± 28	229 ± 6	247 ± 18	251 ± 10	242 ± 8	245 ± 9
Puberty onset (PD)	33.8 ± 1.9	34.3 ± 1.8	31.5 ± 0.7	36.4 ± 2.1	33.0 ± 1.6	33.9 ± 1.9	35.8 ± 3.0	35.8 ± 1.5	35.8 ± 1.7	33.3 ± 2.4	33.5 ± 2.1

Pesticide cocktail exposure	Control	Pestimix 14.58	Pestimix 29.17	Pestimix 43.75
Female (n=)[a]	9 (8)	9 (9)	7 (5)	7 (7)
BW juvenile (PD28) (g)	62.4 ± 3.7	60.4 ± 4.9	58.3 ± 5.6	65.8 ± 7.7
BW adult (g)	250 ± 12	254 ± 19	247 ± 20	251 ± 27
Puberty onset (PD)	33.8 ± 1.9	34.6 ± 2.1	35.0 ± 2.3	34.3 ± 2.8

Data represent means ± SEM.
[a] Number in parentheses is the litter n-value.
Adult body weight is at the time of sacrifice.
Data is analyzed in a random intercepts model, adjusting for littermates. Puberty onset is also adjusted for juvenile body weight.
BW, body weight; PD, pup day.

transcription kit (Promega, USA) was performed according to the directions provided by the manufacturer. RT-qPCR was conducted using 10 μl of 2× Brilliant II SYBR® Green mastermix (Agilent Technologies, USA), 5 μl sample cDNA and 15 pmol of the reverse and forward primer (DNA Technology, Denmark), adding distilled water to a total volume of 20 μl. A LightCycler® 480 Real-Time PCR System (Roche, USA) was used for qPCR, with a program of 10 min preincubation at 94 °C, followed by 40 cycles of 30 s at 94 °C, 45 s of 60 °C and 90 s of 72 °C. The *Kiss1* primer has the following characteristics: forward primer 5′-AGC TGC TGC TTC TCC TCT GT-3; reverse primer 5′-GCA TAC CGC GGG CCC TTT T-3′, GenBank ID: NM_181692, and a product size of 139 bp. The size of the product was validated on a DNA gel and it was verified that there was no primer dimerisation. The comparative C_T method was applied for quantification of mRNA expression as described in Schmittgen and Livak (2008). Kiss1 mRNA was normalized to the reference gene GAPDH (GenBank ID: NM_017008; forward primer 5′-CAT CAA GAA GGT GGT GAA GCA-3′; reverse primer 5′-CTG TTG AAG TCA CAG GAG ACA-3′, product size 93 bp) for each sample, and depicted as fold change compared to male vehicle.

2.3.2. In situ *hybridization*

In study 2, the frozen brains were cut in a cryostat in 12 μm sections in parallel series of three through the AVPV and series of six through the ARC. Accordingly, for the AVPV the two sections analyzed were separated by 36 μm and for the ARC the analyzed sections were 72 μm apart. One series of AVPV sections and one series of ARC sections were used for *in situ* hybridization, as previously described (Bentsen et al., 2010; Larsen et al., 1993). The oligonucleotide probe used, was complimentary to the bases 325–370 of the rat *Kiss1* gene (DNA Technology, Denmark), and the probe specificity to Kiss1 mRNA has been characterized previously (Bentsen et al., 2010). The hybridized sections and a ^{14}C standard were exposed to Kodak BiomaxMR film (GE Healthcare, country). The film was analyzed for mean optical density in the areas of interest, bilaterally in two sections per area per animal, and background measures were obtained from every slide, using the image analysis software Quantity One® (Bio-Rad, CA, USA). Specifically, the regions were hand traced, and the areas of interest were defined according to anatomical cues; for the AVPV the shape of the ventricle, the optic chiasm and the anterior commissure were used and for the ARC, the section in which the 3rd ventricle is split in two (approximately 3.80 mm posterior to bregma) was selected in addition to the section 72 μm anterior to this section. This is the area in which we detect the highest level of kisspeptin expression (unpublished observation). The identities of the animals were blinded during the quantification. The presented value for each animal is the average activity (nCi/g) of the measured values subtracted individual background measurements.

2.4. Assessment of puberty onset

Onset of puberty was determined by physical examination of the animals for balano-preputial separation (males) or vaginal opening (females). The males were examined from PD 34 and the females were examined from PD 27 in Study 1 and from PD 30 in Study 2, and continued every day until all animals had entered puberty.

2.5. Data analysis

In both studies, the effect of dose on Kiss1 mRNA expression was analyzed in a linear model with a random intercept to adjust for litter effects. For analysis of dose effect on puberty onset in Study 1 a Tobit regression including dose (categorical) and juvenile bodyweight as covariates was used, in order to take the left censoring of these data into account. Robust standard errors was calculated using a cluster-specific sandwich estimator (generalized estimating equation approach), and the statistical significance of dose was tested using a Wald test. In addition, accumulative percentage of animals entering puberty at two peripubertal time points is shown in Table 2, to illustrate the progression of puberty in Study 1, whereas in Study 2, no left censoring occurred, and puberty onset is therefore depicted as the mean ± SEM. In Study 2, a linear model with random intercepts to adjust for both litter effects and juvenile bodyweight was used.

Association between puberty onset and Kiss1 mRNA levels in the ARC of females in Study 1 was investigated using a random intercept model, categorizing puberty onset into three groups: early (until PD30), normal and late (after PD35) puberty onset, and adjusting for administered dose.

The effect of dose on juvenile and adult bodyweight was analyzed in a linear model with a random intercept to adjust for litter effects. Bodyweights are depicted as mean ± SEM.

Males and females were analyzed separately, and the different pesticides were analyzed separately. R (version 2.15, R Foundation for Statistical Computing, Austria) was used for the statistical analysis, and $p < 0.05$ was considered statistically significant. *p*-values are based on a likelihood ratio test for the overall dose effect.

3. Results

3.1. Effects on puberty onset

Puberty onset was delayed in both sexes in the animals evaluated for Kiss1 mRNA, only after exposure to the lowest EE_2 dose of 5 μg/kg/day ($p = 0.03$ for both genders), however, the overall dose effect of EE_2 was only statistically significant in the males (male $p = 0.02$, female $p = 0.07$). In Table 2, the 95% confidence intervals of changes in puberty onset in relation to controls are listed for both males and females. In addition, the accumulative percentage of animals entering puberty at two peripubertal time points is listed to illustrate pubertal progression. This accumulative overview shows that none of the females in the control group and the lowest exposure group had entered puberty at PD 27, whereas 25% of the highest exposure group had entered puberty by this time.

In Study 2, no effect of single pesticides or pesticide mixtures on puberty onset was found (Table 3). Juvenile and adult bodyweight was unaffected by perinatal EE_2 exposure (Table 2) and perinatal pesticide exposure, except for adult bodyweight in the Man-25 group, which was increased ($p < 0.05$; Table 3).

3.2. Perinatal EE_2 exposure and Kiss1 expression

No effect of perinatal EE_2 on Kiss1 expression was found either in the AVPV (males $p = 0.29$, females $p = 0.76$; Fig. 1 left) or in the ARC of males ($p = 0.61$; Fig. 1 right). However, in the female ARC perinatal EE_2 tended to affect Kiss1 expression ($p = 0.07$), with the lowest EE_2 dose tending to increase Kiss1 mRNA levels and the highest dose tending to reduce Kiss1 mRNA levels in the female ARC (Fig. 1 bottom right).

3.3. Association between Kiss1 mRNA expression and puberty onset in females after perinatal EE_2 exposure

As described in Sections 3.1 and 3.2, we find similar patterns for puberty onset and Kiss1 mRNA expression in the ARC of females after EE_2 exposure, with delayed puberty and increased Kiss1 mRNA expression in the lowest exposure group and tendency toward advanced puberty and decreased Kiss1 mRNA expression

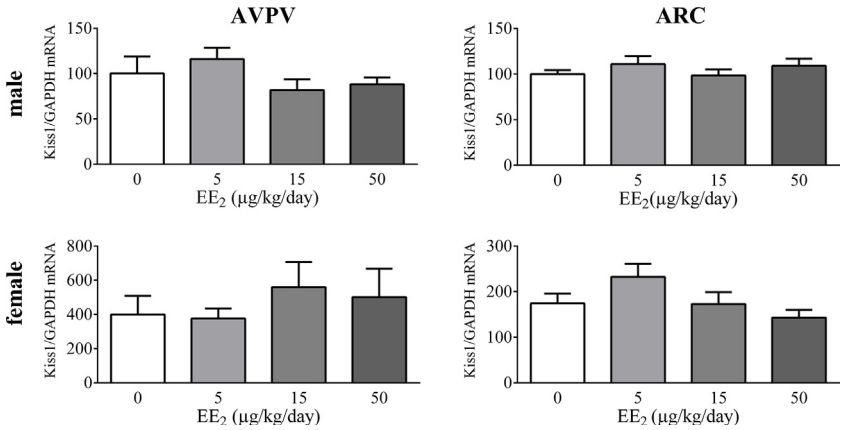

Fig. 1. Kiss1 mRNA expression after perinatal ethinyl oestradiol (EE_2) exposure. The graphs depict Kiss1 mRNA levels in the AVPV (left) and ARC (right) in adult male (upper panel) and female (lower panel) pups of dams exposed to oral EE_2 in the perinatal period (GD 7–GD21 and PD1–PD 22). Kiss1 mRNA expression in the AVPV and the ARC was not affected by perinatal EE_2 exposure. Kiss1 mRNA was determined by RT-qPCR, normalized to GAPDH mRNA levels and depicted as fold change in relation to control males.

in the highest exposure group. However, no significant association between puberty onset and Kiss1 mRNA expression in the ARC of females after perinatal EE_2 exposure was found ($p = 0.12$).

3.4. Perinatal pesticide exposure and Kiss1 expression

Exposure to individual pesticides had no effect on Kiss1 mRNA expression in the AVPV or ARC (Fig. 2). In addition, no dose effect was observed in the pesticide mixture groups, neither in the AVPV ($p = 0.38$; Fig. 3 left) nor in the ARC ($p = 0.51$; Fig. 3 right). However, tebuconazole tended to increase Kiss1 mRNA expression in the AVPV ($p = 0.05$; Fig. 2 left panel) and mancozeb tended to increase Kiss1 mRNA expression in the ARC ($p = 0.08$; Fig. 2 right panel).

4. Discussion

4.1. Perinatal EE_2 exposure and Kiss1 expression

Study 1 is designed to model human perinatal exposure to low levels of estrogenic compounds, and we explored the effects of estrogenic exposure chronically during gestation and lactation. No significant change in Kiss1 expression was found, suggesting that exposure to estrogenic compounds at low levels has no impact on sexual differentiation of the Kiss1 system, but rather affect mainly peripheral sites (Mandrup et al., 2012). In contrast, injection of higher doses produce lower or unchanged Kiss1 gene expression dependent on the nature of the estrogenic substance and the dose (Bateman and Patisaul, 2008; Bellingham et al., 2009; Dickerson et al., 2011; Losa-Ward et al., 2012; Navarro et al., 2009; Patisaul et al., 2009b). Notably, the development of the sexual dimorphic Kiss1 expression in the AVPV is strongly affected by manipulations designed to masculinize brain development (Kauffman et al., 2007), whereas ARC is less sensitive to the same treatment (Clarkson et al., 2009; Kauffman et al., 2007). We have been interested to model human exposure using low but prolonged exposure to EE_2, but found no significant effects. The lower chronic exposure used in our study may not be sufficient to induce changes in Kiss1 gene expression, possibly because the fetal α-fetoprotein system is able to maintain low levels of free circulating estradiol with this type of exposure, thus reducing effects on the Kiss1 system. Even the effects were not significant, the tendencies were quite interesting because the lowest EE_2 dose tended to produce an increase and the highest dose a reduction in Kiss1 mRNA levels; hence, we found a tendency to a dose-response curve with an inverted U-shape in the female ARC. While such a tendency should be interpreted with caution, similar dose–response curves of estrogenic exposure have been found for sleep-wake cycle related gene expression in the preoptic area (Ribeiro et al., 2009) and for dopamine uptake in hypothalamic dopaminergic neurons (Christian and Gillies, 1999).

In males, the Kiss1 mRNA levels were not affected by EE_2, which is in agreement with a number of studies (Dickerson et al., 2011; Patisaul et al., 2009b), and collectively the data support that the female Kiss1 system is more sensitive than the male to endocrine disruption by estrogenic compounds.

4.2. Puberty onset is affected after perinatal EE_2 exposure

Puberty onset data reported here is for the subset of animals analyzed for Kiss1 expression, and the present findings should be interpreted with caution, as a delay in puberty onset is not found in the complete Study 1 dataset (Mandrup et al., under review) or in similar studies (Axelstad et al., 2011; Kauffman et al., 2009). It is interesting that similar U-shaped dose response relationships are seen for Kiss1 mRNA expression in the ARC and for the time of puberty onset in the female. This curve shape can be explained by different effects at different targets for low and high doses of EE_2; this is used in the clinic, where low doses of EE_2 is acting peripherally to promote childhood growth without affecting the pubertal development, while higher doses promote puberty onset *via* a central effect (Janner et al., 2012; Ross et al., 2011). The tendency of advanced puberty in the female rats treated with the highest EE_2 dose is of interest in relation to the fact that puberty onset has declined in recent years in girls; where EDs are suspected to be involved (Mouritsen et al., 2010; Sorensen et al., 2012).

Tendencies toward changes in Kiss1 expression were observed in the female, but not in males. By contrast, puberty onset was affected in both sexes. This indicates that the persistent changes in Kiss1 expression after EE_2 exposure during prepubertal development is sex specific. Accordingly, only the female kisspeptin system is restrained mainly by gonadal steroids during pubertal development (Kauffman et al., 2009).

4.3. Pesticide exposure and Kiss1 mRNA expression

In Study 2 we explored the effects of relevant EDs present in the environment on Kiss1 mRNA expression and puberty onset. Hence, five pesticides with multiple modes of action were investigated for their individual and additive effects in female rats (see Table 1 for an overview). We have recently shown that the same pesticide mixture increased gestational length and nipple retention, at doses where the pesticides had no effect alone (Hass et al., 2012;

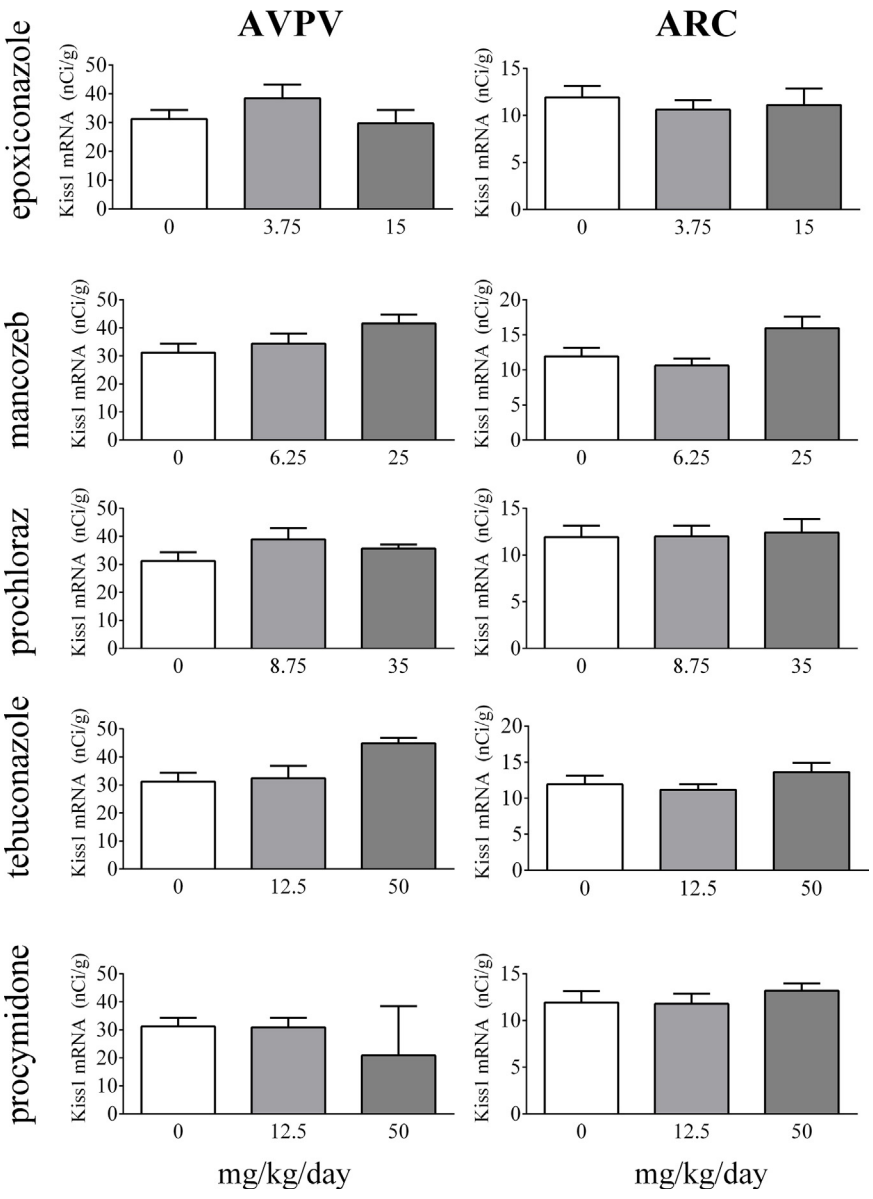

Fig. 2. Kiss1 mRNA expression after perinatal exposure to single pesticides. The graphs depict Kiss1 mRNA levels in the AVPV (left) and ARC (right) in adult female pups of dams orally exposed to the pesticides epoxiconazole, mancozeb, prochloraz, tebuconazole or procymidone in two doses during the perinatal period (GD 7–GD21 and PD 1–PD 16). Exposure to single pesticides did not change Kiss1 mRNA expression in the AVPV or ARC. Kiss1 mRNA was determined by *in situ* hybridization, quantified using a ^{14}C standard and depicted as nCi/g.

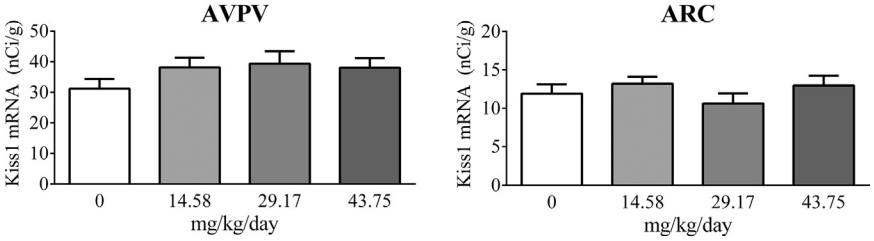

Fig. 3. Kiss1 mRNA expression after perinatal exposure to a mixture of five pesticides. The graphs depict Kiss1 mRNA levels in the AVPV (left) and ARC (right) in adult female pups of dams orally exposed to a mixture of the pesticides epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone at three doses during the perinatal period (GD 7–GD 21 and PD1–PD 16). Exposure to the pesticide mixture did not affect Kiss1 mRNA levels in the AVPV or ARC of the adult female pups. Kiss1 mRNA was determined by *in situ* hybridization, quantified using a ^{14}C standard and depicted as nCi/g.

Jacobsen et al., 2010, 2012; Taxvig et al., 2007; Vinggaard et al., 2005). In general, none of the pesticides had effect on Kiss1 gene expression in female rats. However, while no effect was found when the pesticides were mixed, tebuconazole and mancozeb tended to have minor effects on hypothalamic Kiss1 mRNA expression when tested alone.

Each pesticide displays a very heterogeneous target profile (see Table 1), and tebuconazole can thus affect Kiss1 expression

through multiple mechanisms. While prochloraz is a more potent estrogen antagonist and aromatase inhibitor compared to tebuconazole, tebuconazole is a more potent AR antagonist (Kjaerstad et al., 2010a), and the effect of tebuconazole on Kiss1 expression in the AVPV could thus stem from its AR antagonism. However, while Kiss1 neurons express AR (Clarkson et al., 2012; Lehman et al., 2010), non-aromatizable testosterone does not lower Kiss1 expression in the AVPV (Smith et al., 2005b). Kiss1 neurons are thus suspected to be regulated by sex steroids mainly through ER-α in the AVPV, and the AR antagonistic properties of tebuconazole is thus not likely to be its primary mechanism of action on Kiss1 expression. The pharmacokinetics of the tested pesticides, including the capacity to cross the blood–brain barrier, is not fully defined. In that perspective, it is difficult to compare central and peripheral effects of the different pesticides. Given tebuconazole is a potent ER antagonist in the neonatal brain, feminization of the Kiss1 neurons in AVPV would be promoted, as the sexual differentiation of the female brain is dependent on low sex steroid levels (Semaan and Kauffman, 2010). However, as estradiol levels in the female brain are low during development; the window for further reduction is limited.

Prenatal mancozeb exposure is known to affect cognition in children and is associated with neurodegenerative diseases due to its disrupting effects both on the thyroid gland and on dopamine neuron survival (Harrison Brody et al., 2013; Kamel et al., 2007). Kisspeptin neurons in the ARC receive inhibitory input from dopaminergic neurons (Goodman et al., 2012), thus reduction of inhibitory inputs to the kiss1 neurons would lead to an increase in Kiss1 mRNA expression in the ARC. Notably these effects are not mediated *via* steroid receptors, but rather through toxic mechanisms which cause neurodegeneration.

Kiss1 mRNA expression was unaffected by the pesticide mixture even at the highest doses. This exposure is probably the most relevant for humane exposure, because several pesticides are present in the environment and their effects must be considered as additive. Since the tested pesticides have various modes of action, and because the dose of the individual pesticides in the highest pesticide mixture is equal to the lowest dose tested individually, an effect is only expected in the pesticide mixture groups, if the pesticides have additive effects on Kiss1 mRNA expression. This additive effect was demonstrated for gestational length and nipple retention in the same study (Hass et al., 2012), but was not found to influence puberty onset or Kiss1 mRNA expression. In accordance, the adult pups from the same pesticide mixture experiment did not change motor activity levels or mating behavior, and only males in the highest pesticide mixture group displayed decreased performance in a spatial learning test (Jacobsen et al., 2012). Collectively, we find that the rodent brain is less sensitive to endocrine disruption by this pesticide mixture, compared to other endpoints.

It has been reported that fetuses of ewes maintained on pastures fertilized with sewage sludge, which is a model for human exposure to a mixture of relevant EDs, have reduced hypothalamic Kiss1 mRNA expression in the neonatal brain (Bellingham et al., 2009), indicating that Kiss1 mRNA expression, in contrast to our findings in rats, was sensitive to endocrine disruption after oral exposure to a mixture of environmental pollutants of the mother. However, the effects of the individual EDs in the mixture was not tested to explore the role of possible dose addition, and the concentration of the EDs that the ewes were exposed to was unknown (Bellingham et al., 2009). It is therefore unclear whether the discrepancies in the effects of ED mixtures are due to species, dose or compound differences.

4.4. Conclusions

In summary, we find that perinatal EE_2 exposure did not affect Kiss1 mRNA expression in this study designed to model human exposure to estrogenic compounds. We find minor effects on puberty onset. Further, the Kiss1 system did not exhibit persistent changes and puberty onset was not affected after perinatal exposure to the pesticides. Hence, we find that hypothalamic Kiss1 mRNA expression is less sensitive to EE_2 and to the selected pesticides compared to peripheral target tissues. Additive effects of the pesticides on either Kiss1 mRNA expression or puberty onset were not found. However, we find that mancozeb tends to increase Kiss1 expression in the ARC, presumably through neurotoxic mechanisms rather than *via* classical endocrine disruption, calling for increased awareness that Kiss1 expression can be affected by environmental pollutants through multiple mechanisms.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Paper IV

Early Metabolic Programming of Puberty Onset: Impact of Changes in Postnatal Feeding and Rearing Conditions on the Timing of Puberty and Development of the Hypothalamic Kisspeptin System

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Kiss1 neurons have recently emerged as a putative conduit for the metabolic gating of reproduction, with leptin being a regulator of hypothalamic *Kiss1* expression. Early perturbations of the nutritional status are known to predispose to different metabolic disorders later in life and to alter the timing of puberty; however, the potential underlying mechanisms remain poorly defined. Here we report how changes in the pattern of postnatal feeding affect the onset of puberty and evaluate key hormonal and neuropeptide [*Kiss1*/kisspeptin (Kp)] alterations linked to these early nutritional manipulations. Female rats were raised in litters of different sizes: small (four pups per dam: overfeeding), normal (12 pups per dam), and large litters (20 pups per litter: underfeeding). Postnatal overfeeding resulted in persistently increased body weight and earlier age of vaginal opening, as an external sign of puberty, together with higher levels of leptin and hypothalamic *Kiss1* mRNA. Conversely, postnatal underfeeding caused a persistent reduction in body weight, lower ovarian and uterus weights, and delayed vaginal opening, changes that were paralleled by a decrease in leptin and *Kiss1* mRNA levels. Kisspeptin-52 immunoreactivity (Kp-IR) in the hypothalamus displayed similar patterns, with lower numbers of Kp-IR neurons in the arcuate nucleus of postnatally underfed animals, and a trend for increased Kp-positive fibers in the periventricular area of early overfed rats. Yet, gonadotropin responses to Kp at puberty were similar in all groups, except for enhanced responsiveness to low doses of Kp-10 in postnatally underfed rats. In conclusion, our data document that the timing of puberty is sensitive to both overfeeding and subnutrition during early (postnatal) periods and suggest that alterations in hypothalamic expression of *Kiss1*/kisspeptin may underlie at least part of such programming phenomenon. **(*Endocrinology* 152: 3396–3408, 2011)**

In mammals, many neuroendocrine networks responsible for the homeostatic control of essential body functions become organized during the intrauterine and perinatal stages of development (1). This organizational process is essential for the generation of proper adaptative responses throughout the lifespan (2–4). Because the developing organism uses early external cues to anticipate future environmental settings (5, 6), exposure to extreme

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Abbreviations: ARC, Arcuate nucleus; AVPV, anteroventral periventricular nucleus; BW, body weight; IHC, immunohistochemistry; icv, intracerebroventricular; IR, immunoreactive/immunoreactivy; LL, large litter; NL, normal litter; semi-Q, semiquantitative; SL, small litter; VO, vaginal opening; VO50, 50% VO.

conditions during this period results in perturbed maturation of key neuroendocrine networks and, hence, may compromise the capacity to generate homeostatic responses later in life (7–9). As a paradigmatic example, data from different species, including humans, have documented that severe alterations of the nutritional status or hormonal milieu during the gestational and early postnatal life predispose to later development of metabolic diseases, such as type 2 diabetes and obesity (2, 3, 10–14). Accordingly, early nutritional malprogramming of different metabolic and hypothalamic pathways is considered as a potential contributing factor for the rising incidence of obesity and its related sequelae (12, 15).

Although the impact of metabolic programming on other physiological functions tightly coupled to energy homeostasis is yet to be fully characterized, compelling evidence has mounted recently, suggesting that puberty onset and reproductive capacity are also sensitive to nutritional influences during early stages of development (16–23). In this context, human studies evidenced that puberty onset is accelerated in girls with low birth weight that subsequently develop obesity in childhood (24, 25). In rats, different manipulations of gestational and postnatal feeding have been shown to variably impact puberty onset, although the nature of such changes depends on the type and timing of the nutritional challenge. Thus, in females rats, prenatal overfeeding has been reported to cause early puberty onset (19), whereas delayed puberty has been observed in rats submitted to intrauterine growth retardation or early postnatal underfeeding (16, 20–22). However, caloric restriction during pregnancy and lactation has also been reported to advance puberty in the female offspring (19). Most of the above studies were descriptive, and little is known regarding the neuroendocrine mechanisms involved (21). However, the pathophysiological relevance of the above observations is stressed by the plethora of adult reproductive defects described in animal and human models of early nutritional challenge (23, 24, 26, 27), a phenomenon the translational interest of which is reinforced by the rising incidence of disorders of body weight, especially obesity, during gestation and childhood.

Puberty is triggered by the heightening of the secretory activity of GnRH neurons, which results in the full activation of the gonadotropic axis and attainment of reproductive capacity (28, 29). The mechanisms of puberty onset have been the subject of active investigation, especially after recent reports of possible changes in the timing of puberty in humans (30, 31), and the potentially associated health problems (32–36). A major breakthrough in our understanding of the neuroendocrine basis of puberty came from the identification of Kp, products of the *Kiss1* gene, and their receptor, GPR54 (also termed Kiss1R), as major regulators of GnRH secretion and, hence, reproductive maturation and function (37, 38). Indeed, compelling evidence indicates that the hypothalamic Kiss1 system, which in rodents comprises two major neuronal populations, located in the arcuate (ARC) and anteroventral periventricular (AVPV) nuclei (37, 38), undergoes a complex pattern of activation during puberty that appears essential for its proper timing (39, 40). This maturational process involves not only an increase in the hypothalamic Kp tone (41, 42) but also an elevation of the number of Kp neurons and their projections to GnRH neurons (43, 44). These plastic changes are sensitive to the organizing and activational effects of sex steroids during postnatal and prepubertal development (40, 44). Whether pubertal maturation of the hypothalamic Kiss1 system is influenced by additional modifiers has not been explored in detail to date.

Hypothalamic Kiss1 neurons are highly sensitive to body energy status and metabolic cues, as evidenced by suppressed *Kiss1*/kisspeptin expression in conditions of negative energy balance, which are also linked to inhibition of the reproductive axis (45, 46). Likewise, deregulated hypothalamic *Kiss1*/kisspeptin levels have been reported in mouse models of obesity (47). The metabolic signals that regulate the Kiss1 system are likely multiple, but the adipokine, leptin, has been proposed as a key modulator, and Kiss1 neurons have been suggested to participate in mediating leptin regulation of GnRH secretion, therefore defining a pathway for the permissive actions of leptin on puberty onset (45, 46). It must be stressed, however, that the experimental data supporting a role of leptin in the control of hypothalamic Kiss1 system come from studies in adult models of metabolic stress and may not be fully representative of physiological conditions at puberty. In addition, it is noted that recent functional genomic studies in mice lacking leptin receptors in Kiss1 neurons suggest that the effects of leptin might be indirect and/or dispensable, at least in conditions of congenital absence of leptin signaling in this neuronal population (48).

In the present study, we evaluated the impact of nutritional challenges during the critical postnatal period on the onset of puberty, and the putative neurohormonal mechanisms involved. To this end, a rat model of manipulation of litter size, to induce conditions of underfeeding (large litters) or overnutrition (small litters) during lactation, was used (49). This model has been proposed to mimic nutritional challenges during the last trimester of human gestation (50). As neuroendocrine parameters, leptin levels and hypothalamic expression of *Kiss1* mRNA, as well as Kp immunoreactivity (IR) and gonadotropin responses to exogenous Kp-10, were selected for analysis (37, 45). Of note, whereas the effects of acute metabolic

stress on Kiss1 expression and gonadotropin responsiveness to Kp had been previously explored in pubertal and adult animals (45, 46), the impact of early (postnatal) metabolic challenges on the subsequent expression and function of the hypothalamic Kiss1 system later during puberty remains largely unexplored to date.

Materials and Methods

Animals and drugs

Wistar female rats bred in the vivarium of the University of Córdoba were used. Pregnant dams were obtained by mating with adult Wistar male rats and were kept under constant conditions of light (14 h of light, from 0700 h) and temperature (22 C), with free access to standard laboratory chow and tap water. On d 1 postpartum, female pups were selected, cross-fostered, and grouped into three different litter sizes: small litters (SL; four pups per litter), normal litters (NL; 12 pups per litter), and large litters (LL; 20 pups per litter), as described elsewhere (16, 18, 49, 51). This procedure has been previously used to induce over- or underfeeding during early postnatal maturation (*i.e.* lactation). The animals were weaned on d 21 postpartum, when the pups of each litter size were housed in groups of four rats per cage, with free access to pelleted food and tap water. The experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation and conducted in accordance with the European Union guidelines for use of experimental animals. Mouse Kiss1 (110–119)-NH_2, also termed Kp-10, was obtained from Phoenix Pharmaceuticals (Belmont, CA). For immunohistochemical (IHC) analyses, a mouse Kp-52 antiserum (code JLV-1), which recognizes the longer form of rodent Kp (Kp-52) without any detectable cross-reactivity with Arg-Phe-related peptide, was used in accordance with previous literature (52–54).

Experimental design

In experiment 1, the effects of changes in postnatal feeding on the timing of puberty were evaluated in female rats derived from different litter sizes (SL, NL, and LL). Body weight (BW) gain was evaluated in the different groups (n = 36/group) at weekly intervals during the first month of postnatal life. Thereafter, BW and vaginal opening (VO; as external sign of puberty) were monitored daily between d 30 and d 37 postpartum. Detailed inspection was conducted in each animal to determine the age of complete canalization of the vagina (55). In addition, in a subset of animals from each group, ovarian and uterus weights were recorded, and blood samples were collected for hormonal determinations, upon decapitation of the animals at d 31 or d 36 postpartum (n = 12/age-point).

In experiment 2, analyses of hypothalamic expression of *Kiss1* mRNA and Kp-IR were conducted in 36-d-old female rats, raised in SL, NL, and LL. For RNA analyses, hypothalamic samples (n = 6/group) were excised immediately after decapitation of the animals, by dissection with a horizontal cut of approximately 2 mm depth, using the following limits: 1 mm anteriorly from the optic chiasm, the posterior border of the mammillary bodies, and the hypothalamic fissures. Brain samples for IHC analyses (n = 5/group) were obtained after *in vivo* perfusion and fixation of the animals (see below).

In experiment 3, gonadotropin responsiveness to Kp was studied in our models of early nutritional manipulation by assessing the effects of acute central injection of Kp-10 on LH and FSH secretion in 31-d-old females. The procedure of intracerebroventricular (icv) injection of Kp-10 was as previously published (55); icv cannulae were implanted to a depth of 3 mm beneath the surface of the skull, with an insert point 1 mm posterior and 1.2 mm lateral to bregma (55). Two doses of Kp-10 were tested: 1 nmol (maximal stimulation) and 10 pmol (submaximal stimulation), to estimate potential changes in the sensitivity to Kp. Blood samples were obtained 15 min after Kp-10 injection. Animals injected with vehicle served as controls.

Hormone measurements

LH and FSH levels were measured using RIA kits supplied by the National Institutes of Health (National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 and FSH-I-9 were labeled with ^{125}I using Iodo-gen tubes (Pierce Chemical Co., Rockford, IL). Hormone concentrations were expressed using reference preparations LH-RP-3 and FSH-RP-2 as standards. Intra- and interassay coefficients of variation (CV) were less than 8 and less than 10%, respectively. Leptin levels were determined using a commercial RIA kit from MP Biomedicals (Costa Mesa, CA). The sensitivity of the assay was 0.05 ng/tube, and the intraassay CV was less than 5%.

RNA analysis by semi-Q (semiquantitative) RT-PCR

Relative *Kiss1* mRNA levels were assayed in hypothalamic preparations by RT-PCR, optimized for semi-Q detection, using previously defined primer pairs and conditions (41, 56). As internal control, amplification of a 240-bp fragment of S11 ribosomal protein mRNA was carried out in parallel in each sample. In keeping with previous optimization tests, 32 and 24 PCR cycles were chosen for semi-Q analysis of the specific target (*Kiss1*) and *RP-S11* internal control, respectively (41, 56). Specificity of PCR products was confirmed by direct sequencing. Quantification of intensity of RT-PCR signals was carried out by densitometric scanning, and values of the specific target were normalized to those of internal control to express arbitrary units of relative expression. Liquid controls and reactions without reverse transcription resulted in negative amplification.

Immunohistochemistry

IHC assays were conducted in hypothalamic sections of brain samples from 36-d-old female rats. The animals were perfused through the ascending aorta, under thiobarbital anesthesia, with 250 ml fixative solution (4% paraformaldehyde) (57, 58). Brains were collected, immersed in fixative overnight, and dehydrated in 30% sucrose for 2–4 d. Coronal sections (40 μm) were cut in parallel series of four on a freezing microtome, and one series of sections were processed for Kp-IR. The sections were incubated in 1% H_2O_2-PBS to block endogenous peroxidase activity and in 0.01 M PBS with 0.3% Triton X-100, 5% swine serum, and 1% BSA to block nonspecific binding sites. The sections were then incubated at 4 C for 24 h with a purified rabbit antiserum against mouse Kp-52 (JLV-1), diluted 1:200. Kp-IR was detected by the avidin-biotin method using diaminobenzidine as chromagen. The sections were incubated for 60 min in biotinylated secondary anti-rabbit IgG (The Jackson Laboratory, Bar Harbor, ME) diluted 1:1000, washed, and transferred to the avidin-biotin complex (Vector Laboratories, Burlingame, CA) diluted 1:250. Thereafter, the

sections were incubated in 0.1% diaminobenzidine (Sigma Chemical Co., St. Louis, MO). The sections were mounted on gelatinized glass slides, dried, and coverslipped in Pertex.

In keeping with our previous protocols (54), IHC analyses involved counting of the number of immunoreactive (IR) neuronal cell bodies in the ARC and AVPV. Nevertheless, the numbers of identifiable Kp-positive cell bodies in the AVPV were very low in all groups; thus, quantitative analyses were only conducted in the ARC. In addition, Kp fibers were stereotaxically quantified in the anterior periventricular area. To this end, horizontal lines with a spacing of 43 μm were placed randomly across a region covering the area between the anterior commissure and the optic chiasm, with a maximum limit of 200 μm into the parenchyma from the ventricle, including the AVPV. The number of fiber crossings of these arbitrary lines was counted, using the microscope software AxioVision (Zeiss MicroImaging, Thornwood, NY). As complement of this stereotactic approach, Kp-IR fibers were also quantified by measuring OD in the same anterior periventricular region (maximum limit of 200 μm into the parenchyma from the ventricle), using the software Image-J (National Institutes of Health, Bethesda, MD). Background was measured in a region with no immunoreactivity (IR) outside the region of interest, and this value was subtracted from the specific OD of the anterior periventricular region and expressed as arbitrary units. Because of high density, fiber numbers could not be reliably estimated in ARC.

Presentation of data and statistics

Hormonal determinations were conducted in duplicate, with a minimum of 10 samples per group. Semi-Q RT-PCR analyses were carried out in duplicate from at least five independent RNA samples per group. IHC analyses were carried out in five brain samples of each group. The numbers of Kp-positive (IR) cells and fibers were counted using a light microscope (Nikon Biophot-20 × magnification; Nikon, Inc., Melville, NY) by an observer blind to treatment regimens. Kp-IR cells and fibers, located within the ARC and periventricular regions, were counted in one of the series of brain sections. Data are presented as the means ± SEM. Results were analyzed for statistical differences, using unpaired Student's *t* test or ANOVA followed by Student-Newman-Keuls multiple-range tests (SigmaStat 8.0; Jandel, San Rafael, CA). $P \leq 0.05$ was considered significant.

Results

Impact of changes in postnatal feeding on the timing of puberty and related parameters

The effects of changes in early postnatal feeding on BW gain and puberty onset were monitored in female rats bred in litters of different size (SL, NL, and LL), in order to induce conditions of early overfeeding or undernutrition (49). BW curves progressively diverged between the three groups, starting from d 7 (Fig. 1A), so that at weaning, SL rats were 33% heavier than NL controls, whereas LL rats were 38% leaner that NL animals. Such differences in BW persisted after weaning, even though the animals were allowed to eat *ad libitum*. Indeed, BW curves from the three groups displayed parallel slopes, with SL females being

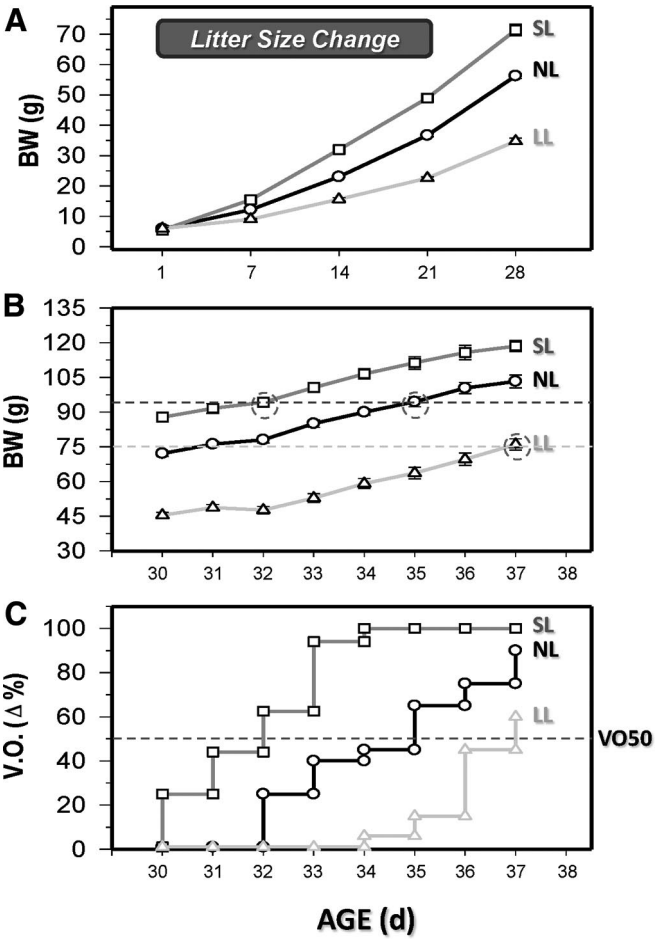

FIG. 1. Impact of changes in early postnatal feeding on BW gain and puberty onset, assessed by VO. Female rats were raised in litters of different size: four pups per dam (SL), 12 pups per dam (NL), and 20 pups per dam (LL), as a mean to induce overfeeding or undernutrition during early postnatal stages of development. The period of litter size manipulation (between d 1 and d 21 postpartum) is denoted by the *gray box* in panel A. BW was monitored in the groups on a weekly basis up to d 30 postpartum (panel A). Thereafter, BW was daily monitored up to d 37 postpartum (panel B). In addition, between d 30 and d 37 the animals were inspected daily for canalization of vagina; the day of complete canalization being considered as VO. For presentation of data, cumulative percentage data of VO are presented for the three groups in panel C. For each group, the age when 50% of the animals displayed VO was considered as VO50, indicated in panel C as a *gray dotted line*. In addition, the values of BW when each group achieved VO50 are *circled* and represented by *dotted lines* in panel B.

20% heavier than control NL rats throughout pubertal maturation and LL animals showing approximately 30% lower BW than controls during this period (Fig. 1B). These changes in BW were paralleled by similar trends in terms of VO (Fig. 1C). Thus, SL females displayed earlier VO, with all animals showing complete canalization of vagina by d 34 postpartum and a mean age of VO of approximately 32 d. In contrast, by d 37, 10% and 40% of females from NL and LL had not yet displayed VO, which prevented calculation of mean ages of VO. Alternatively, the mean age for completion of 50% VO (VO50) was estimated, with values of 32 d, 35 d, and 37 d for SL, NL, and

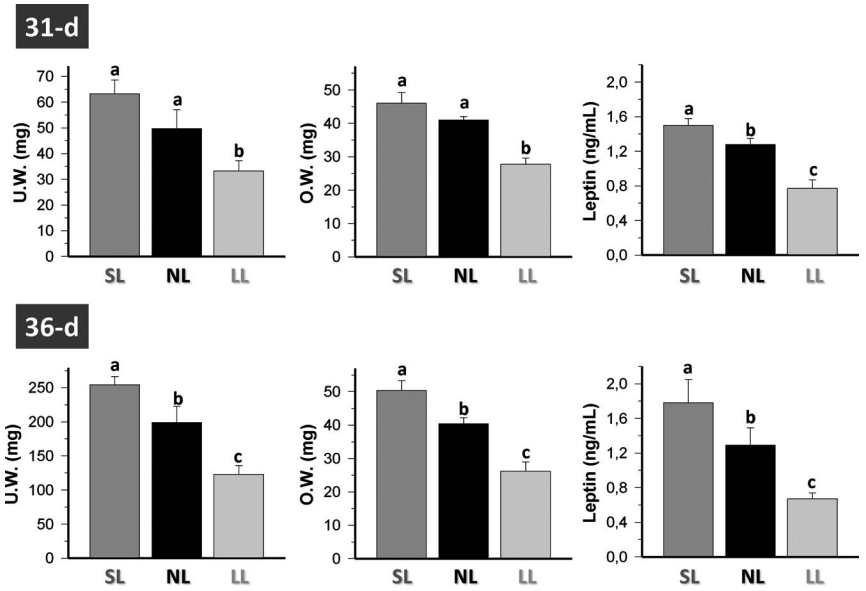

FIG. 2. Impact of changes in early postnatal feeding on key phenotypic and neurohormonal parameters of puberty. Uterus and ovarian weights (U.W. and O.W., respectively), as well as serum leptin levels (ng/ml), were recorded in female rats from three groups: SL (overnutrition), NL (normal nutrition), and LL (subnutrition). Determinations were conducted at two age points: d 31 and d 36 postpartum (*upper* and *lower panels*, respectively). Values are given as the mean ± SEM of at least 10 independent determinations per group. Groups with *different superscript letters* are statistically different. ($P < 0.05$; ANOVA followed by Student-Newman-Keuls multiple range test).

LL, respectively (*dotted line* in Fig. 1C). BW at VO50 was similar between SL and NL groups (~95 g BW); in contrast, VO50 was achieved at a much lower BW in LL females (~75 g BW) (see *dotted lines* in Fig. 1B).

As additional indices of pubertal maturation and activation of the reproductive axis, uterus and ovarian weights were also monitored in the different groups, at d 31 and d 36 postpartum (*i.e.* during the pubertal transition). Postnatally overfed rats had higher ovarian and uterus weights than control NL animals on d 36 postpartum (Fig. 2); similar trends were detected on d 31, but these differences did not reach statistical significance. In clear contrast, underfed females had severely reduced uterus and ovarian weights at the two age points, which were 37% and 50% lower than in control (NL) and overfed (SL) animals, respectively. Serum leptin levels on d 31 and d 36 were higher in SL animals than in NL rats. In contrast, leptin concentrations were significantly lower in underfed LL rats at both age points. Indeed, serum leptin levels in LL females on d 36 (close to VO50) were lower than in SL and NL rats on d 31, *i.e.* before VO50 was achieved (0.67 ± 0.07 ng/ml in LL rats on d 36 *vs.* 1.28 ± 0.07 ng/ml in NL and 1.51 ± 0.07 ng/ml in SL on d 31; $P < 0.01$). In addition, basal serum LH levels in SL females on d 31 were higher than in NL females (3.55 ± 0.5 *vs.* 2.3 ± 0.29 ng/ml; $P < 0.01$), whereas LH concentrations in LL females tended to be lower (1.78 ± 0.25 ng/ml), but this trend was not statistically significant.

Kiss1 expression and Kp-IR in pubertal female rats after early nutritional challenges

Based on the above data, changes in the hypothalamic expression of *Kiss1* mRNA and the patterns of Kp-IR were evaluated in our models. Pubertal levels of *Kiss1* mRNA in whole hypothalamic fragments were significantly higher in SL females than in NL animals (Fig. 3). Conversely, *Kiss1* mRNA expression in LL females was significantly decreased *vs.* corresponding control values on d 36, with its relative levels being approximately half of those detected in SL animals at the same age.

RNA expression analyses were complemented by IHC studies. As shown in Fig. 4, SL and NL rats displayed similar number of Kp-IR neurons in the ARC at puberty; the slightly higher mean number of Kp cells in the ARC of SL rats (20% over NL values) was not statistically significant. In contrast, the number of Kp-positive cells in the ARC of LL females was significantly decreased, with approximately 70–75% reduction in the mean cell number *vs.* NL and SL rats. In addition, Kp-positive fibers in the AVPV region were evaluated by a combination of stereotactic and densitometric approaches; the former provides an estimate of fiber numbers whereas the latter gives an integral account of fiber peptide content (including fiber and terminal densities). The maximum area of parenchyma for analysis was set at 200 μm from the third ventricle. Nevertheless, to provide a more thorough estimation of regional changes in fibers and density, additional quantitative analyses were selectively conducted at the limit of the first 100 μm from the ventricle. As a whole, these data indicate that postnatally overfed animals tend to have higher numbers and density of Kp fibers in the AVPV area, although substantial variations were detected across this region (as indicated by comparison of results at 100- and 200-μm width areas) and the high in-group variability prevented these differences from reaching statistical significance. In any event, our analyses indicated that pubertal SL females had a mean 2-fold elevation in the number of Kp fibers in the 200-μm periventricular region compared with control NL rats ($P = 0.058$; Student's t test), whereas no difference was detected between NL and LL groups (Fig. 5). Like-

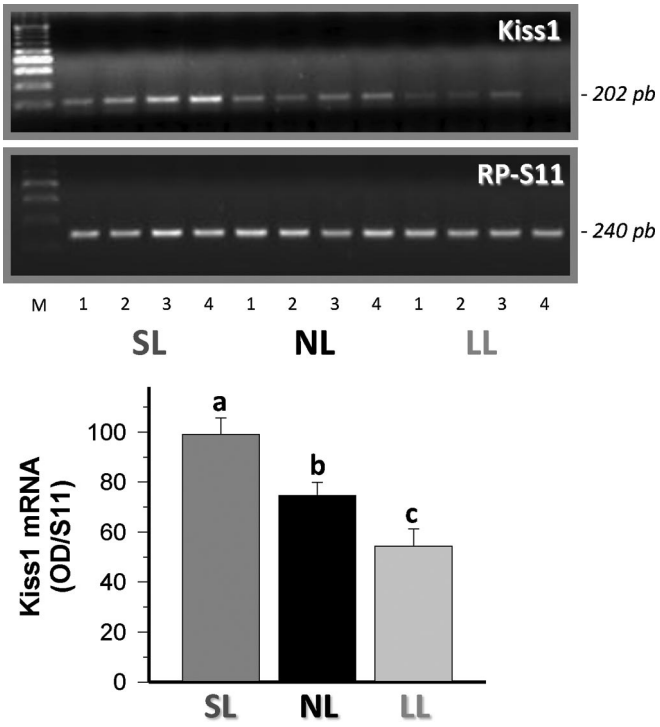

FIG. 3. Profiles of hypothalamic expression of *Kiss1* mRNA in the three experimental groups of female rats: SL (overnutrition), NL (normal nutrition), and LL (subnutrition). Hypothalamic samples were obtained at d 36 postpartum, as described in *Materials and Methods*. In the *upper panel*, a representative RT-PCR assay is shown of expression levels of *Kiss1* mRNAs in hypothalamic samples from the three experimental groups under analysis. Four independent samples per group are presented. Parallel amplification of S-11 ribosomal protein mRNA served as an internal control. In the *lower panel*, semi-Q relative levels of *Kiss1* mRNA are presented as the mean ± SEM of at least five independent determinations. Groups with *different superscript letters* are statistically different. ($P < 0.05$ by ANOVA followed by Student-Newman-Keuls multiple range test).

wise, the mean Kp fiber density within the first 100-μm width periventricular area was 2-fold higher in SL than in NL and LL rats, although this difference was not statistically significant. Admittedly, no detectable change in mean fiber density was observed among the groups when the total 200-μm width periventricular region was considered for quantitative analysis (Fig. 5).

Gonadotropin responses to Kp-10 in pubertal female rats after early nutritional challenges

Acute gonadotropic responses to central administration of Kp-10 were also explored in peripubertal (31-d-old) female rats raised in SL, NL, and LL, as indirect index of changes in endogenous Kp tone and proxy measurement of altered sensitivity to Kp (59, 60). icv injection of 1 nmol Kp-10 evoked very robust LH and FSH responses in control (NL) animals at 15 min after injection (maximal stimulation). In contrast, the submaximal dose of 0.01 nmol induced a 3-fold lower LH secretory response, with no effect on FSH secretion (Fig. 6). Similar patterns were detected in SL rats, with potent LH and FSH responses to 1 nmol Kp-10 and modest LH secretion after icv injection of 0.01 nmol Kp-10; the absolute magnitude of these responses was similar to that of NL rats. In contrast, pubertal LL females displayed enhanced gonadotropic responses to Kp-10 *in vivo*, as evidenced by 1) higher net LH responses to 1 nmol Kp-10 ($P < 0.05$ *vs.* SL rats); 2) enhanced LH responses to 0.01 nmol Kp-10 ($P < 0.05$ *vs.* NL rats); and 3) significant FSH secretory responses to 0.01 Kp-10, which were not detected for this dose in SL or NL females (Fig. 6).

Discussion

We provide herein an integral characterization of the impact on female puberty of different conditions of nutritional distress during the early postnatal period. Of note, rat models of nutritional challenge during early postnatal life have been proposed to mimic conditions of metabolic perturbation during late gestation in humans (50). Indeed, a substantial component of neuroendocrine maturation, which in primates occurs in late intrauterine life, takes place during the early postnatal period in rodents (50, 61). Accordingly, rat models of nutritional manipulation during these postnatal stages are likely more appropriate to analyze conditions of neurohormonal malprogramming of eventual translational interest (50). To gain a mechanistic insight into the pubertal phenotypes observed, we focused our studies on the hypothalamic Kiss1 system, as essential element for the timing of puberty, sensitive to the activational regulatory effects of metabolic signals (37, 45, 46).

Although the impact of early nutritional or metabolic cues on the later development and functioning of the hypothalamic Kiss1 system has received limited attention to date, a very recent report suggested that intrauterine undernutrition impairs the rise of *Kiss1* mRNA levels at the hypothalamus during the pubertal transition (21). However, that study was focused on the effect of underfeeding during the gestation, a model that probably mimics metabolic insults during human midgestation (16), *i.e.* before completion of the organization of multiple neuroendocrine systems. Our present results complement and extend that previous study and unveil also interesting differences between these two models of perinatal subnutrition, which are discussed in detail below.

Postnatal subnutrition and puberty onset

Most of the rodent studies addressing the impact of early nutritional programming of puberty have focused on the consequences of undernutrition during pregnancy or

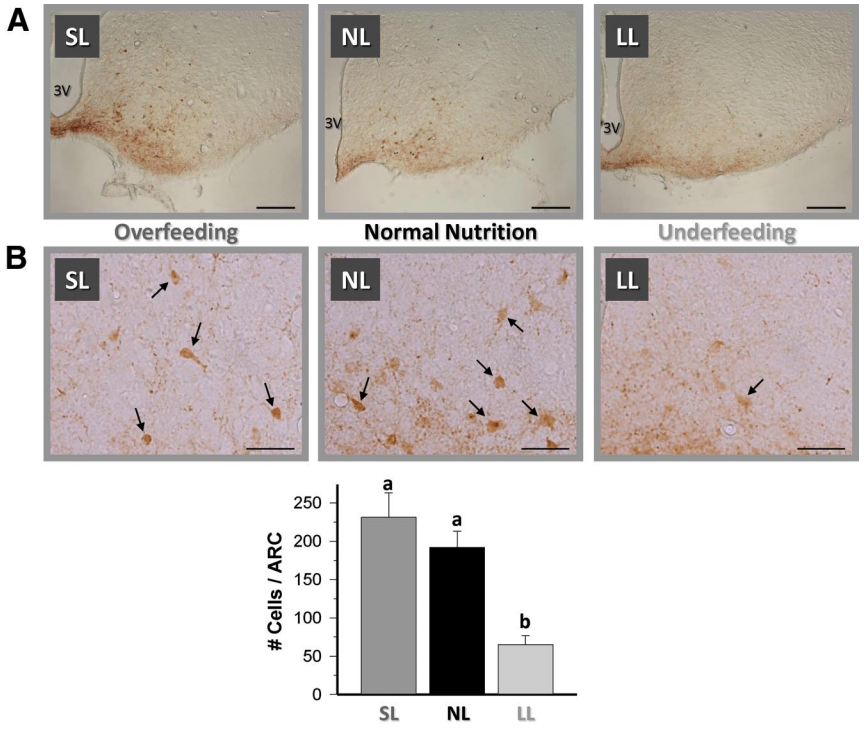

FIG. 4. IHC analysis of the number of Kp neurons (detected as cell bodies with discernible Kp-IR) in the ARC in the three experimental groups under analysis: SL (overnutrition), NL (normal nutrition), and LL (subnutrition). In the *upper panels*, representative photomicrographs of Kp-IR cell bodies in the ARC from SL, NL, and LL female rats at puberty are shown, at two different magnifications: Panels A (lower magnification) and B (higher magnification). In the latter, Kp-IR cell bodies are indicated with *arrows*. In addition, in the *lower panel*, quantification of the number of Kp-positive cells in the ARC is shown for each experimental group. Cell numbers are represented as the mean ± SEM. Groups with *different superscript letters* are statistically different ($P < 0.05$ by ANOVA followed by Student-Newman-Keuls multiple range test). *Scale bars*, 100 μm (*panels A*, lower magnification) or 50 μm (*panels B*, higher magnification). 3V, Third ventricle.

lactation (16, 18, 20–22). However, these studies remain scarce and have yielded contradictory findings. In most cases, gestational subnutrition or postnatal underfeeding have been reported to induce a variable delay in puberty, as monitored by the age of VO and/or first estrous cycle, as well as other indices of ovarian maturation (16, 20–22). However, overtly delayed VO has not been detected in all studies involving postnatal malnutrition due to large litter size (16, 18). Our results in LL rats are in keeping with the predominant literature and document an unambiguous delay of the age of VO, together with decreased uterus and ovarian weights at puberty, in females subjected to postnatal subnutrition. Interestingly, a recent study reported a slight advancement in the age of VO in the offspring of mothers submitted to 50% calorie restriction during lactation; yet, in that study, indirect evidence for some degree of postpubertal ovarian failure was also observed (19). It is possible that the impact of nutritional manipulations on the developmental programming of puberty might depend not only on the magnitude and timing of energy restriction, but also on the type/features of such challenge, because food deprivation of the lactating mother or the pups may associate with different covariables (50) with different impact on the onset of puberty.

The mechanism behind the above alterations is likely to involve disturbed development and/or expression of the hypothalamic Kiss1 system. Thus, relative *Kiss1* mRNA levels at the hypothalamus were notably decreased in peripubertal female rats submitted to postnatal undernutrition. Although our data did not allow us to discriminate the hypothalamic site (ARC *vs.* AVPV) where mRNA levels are actually decreased, they provide indirect evidence for the disruption by postnatal malnutrition of the increase in *Kiss1* expression reported to occur during normal pubertal maturation in the female rat. This phenomenon was analogous to that described recently in a model of intrauterine undernutrition (21), suggesting that both gestational and early postnatal conditions of energy deficit have a durable inhibitory impact on the expression levels of *Kiss1* mRNA in the hypothalamus.

As complement to RNA expression data, our anatomical IHC analyses demonstrated a marked suppression of the number of Kp-IR neurons in the ARC of LL rats at puberty, without changes in the number of Kp fibers at the periventricular area. The putative function of ARC Kiss1 neurons in the control of male and female puberty remains somewhat contentious. Nonetheless, *in situ* hybridization and quantitative RT-PCR analyses of specific hypothalamic regions have demonstrated a rise of *Kiss1* mRNA levels in the ARC during the pubertal transition in the rat (62, 63). Moreover, Kp-IR has been shown to increase in the ARC of male and female rats during puberty (53, 62). Importantly, *Kiss1* mRNA expression at the ARC is also sensitive to metabolic cues, as documented by its partial suppression in conditions of transient subnutrition at puberty (64). Thus, the decrease in Kp-positive neurons in the ARC of LL females may contribute to the delay in puberty onset in this model.

Previous studies demonstrated a marked increase in the number of Kp-IR neurons in the AVPV of female mice during postnatal maturation, especially between d 15 and d 30 postpartum (43, 44). Our IHC analyses, using the validated JLV-1 antiserum against rodent Kp-52 (52–54), intended to

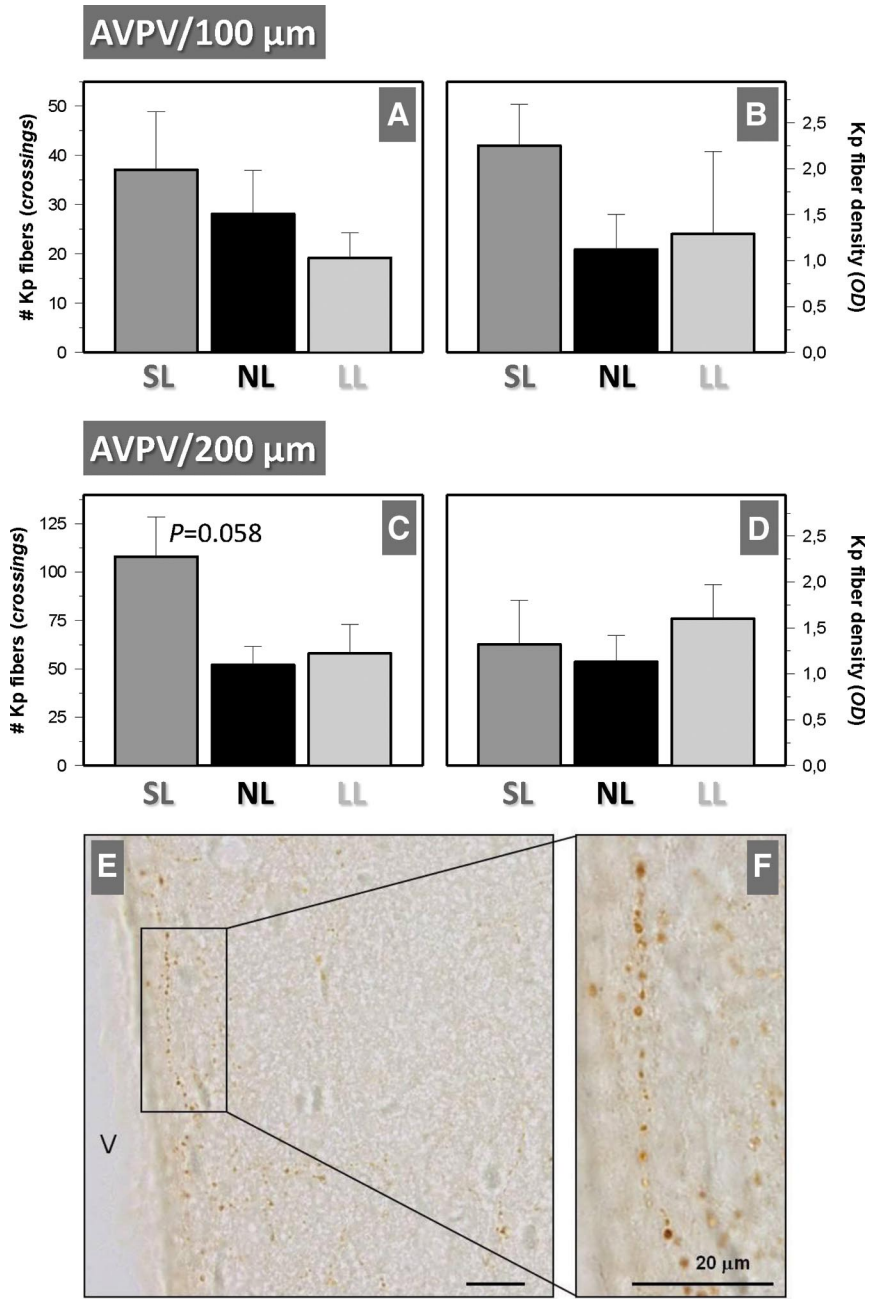

FIG. 5. In the *upper panels*, quantitative data from immunohistochemical analyses of the number and density of Kp-positive fibers in the hypothalamic periventricular region. The area of interest encompassed a section of up to 200 μm apart from the third ventricle. However, to provide a more thorough analysis of potential regional variations, additional quantitative analyses were specifically conducted in a subsection of the above area, spanning only the first 100 μm from the third ventricle. Two complementary quantitative analyses were conducted: 1) by counting the number of IR-fiber crossings (panels A and C); and 2) by measuring OD of the Kp-IR fibers (panels B and D), in the above hypothalamic areas of the three experimental groups: SL (overnutrition), NL (normal nutrition), and LL (subnutrition). For further details, see *Materials and Methods*. In addition to quantitative data, in the *lower panels*, representative photomicrographs of Kp-IR fibers and terminals in the same coronal section, containing the anterior periventricular region at the level of the preoptic nucleus and the AVPV, are shown from a NL female rat. Two different magnifications are presented in panels E and F. Most Kp-positive fibers possessed many boutons, were predominantly located near the ependyma of the third ventricle (3V), and displayed a ventrodorsal orientation. In panel C, $P = 0.058$ *vs.* NL group, (Student's *t* test). *Scale bar*, 20 μm.

evaluate IR cells in the ARC and AVPV regions (37, 38). However, only the ARC data were quantitatively analyzed, because very few Kp-IR cell bodies were detected in the AVPV. Considering the proven expression of *Kiss1* mRNA at the AVPV in rats (65, 66), the lack of detection of Kp-IR neurons at this site is intriguing. Nonetheless, we consider that this is not due to technical limitations of our approach, because we obtained similar results with another (universally used) antibody targeting rodent Kp-10 (Caraty's antiserum, ref. AC 566; data not shown). Moreover, a recent study in adult female rats confirmed that, in contrast to mice, Kp-IR cell bodies are not easily visualized in the rat AVPV regardless of the use of JLV-1 or AC-566 antiserum (67), unless colchicine is used, an approach that is likely to have an impact on quantitative IHC results. Indeed, the differential labeling of Kiss1 neurons between the ARC and AVPV in the rat might reflect physiological differences in the processing and/or secretory dynamics of mature Kp that warrant specific investigation.

The potential changes in responsiveness and sensitivity to Kp were also analyzed in LL rats. Acute fasting at puberty has been previously shown to augment gonadotropin responses to Kp-10 (59), a phenomenon that was associated to the lowering of Kp tone caused by energy deficit, which could induce a compensatory state of enhanced responsiveness. Albeit modest, a similar trend for higher gonadotropin responsiveness to Kp-10 was detected in pubertal LL female rats. In the above context, and considering our current expression/IHC data, it is tempting to propose that this moderate elevation of Kp responsiveness may reflect a proportional decrease of its basal hypothalamic tone in pubertal female rats after postnatal underfeeding.

Postnatal overfeeding and puberty onset

There was so far limited information regarding the impact of overfeeding

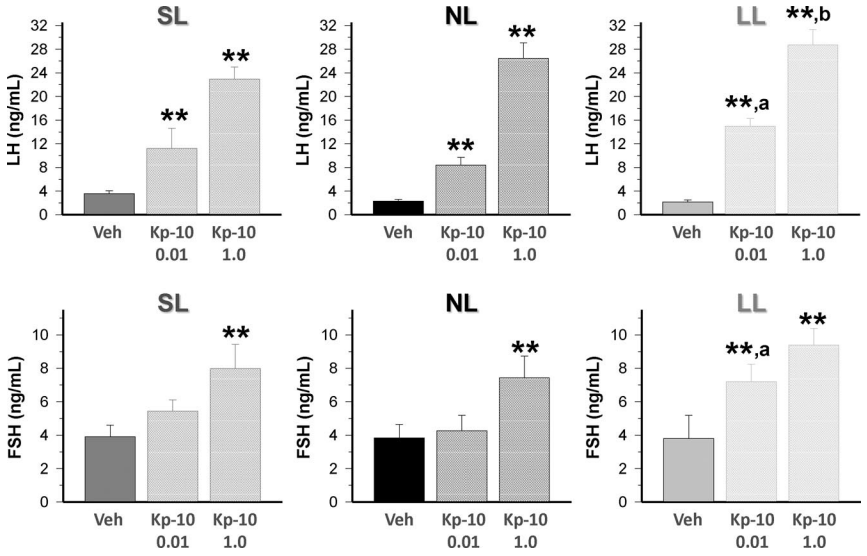

FIG. 6. Gonadotropin responses (LH, *upper panel*; FSH, *lower panel*) after central (icv) injection of a single bolus of Kp-10. Tests were conducted in 31-d-old female rats from one of the experimental groups: SL (overnutrition), NL (normal nutrition), and LL (subnutrition). Two doses of Kp-10 (0.01 and 1 nmol) were tested in icv tests. Hormonal data were assayed 15 min after icv injection of Kp-10; LH and FSH values are mean ± SEM of at least 10 independent determinations per group. **, $P < 0.01$ *vs.* corresponding control values (rats injected with vehicle); a, $P < 0.05$ *vs.* responses to a similar dose of Kp-10 in NL rats; b, $P < 0.05$ *vs.* responses to a similar dose of Kp-10 in SL rats (ANOVA followed by Student-Newman-Keuls multiple range test). Veh, Vehicle.

during early critical periods on the developmental programming of puberty. This, however, has become an issue of translational interest, given the rising incidence of obesity during human gestation and childhood. Of note, whereas our model of overfeeding during lactation had been extensively used to assess the effects of early overweight on the developmental programming of BW homeostasis and metabolism, to our knowledge this is the first report to characterize changes in the timing of puberty and the Kiss1/kisspeptin system in such conditions of early overfeeding. In keeping with a previous study involving maternal feeding on a high-fat diet during pregnancy and/or lactation (19), our model of postnatal overnutrition induced a persistent increase in BW gain and advanced the timing of puberty. These data demonstrate that persistent energy excess during early development might contribute to precocious pubertal activation, a phenomenon that, in face of the escalating incidence of child obesity, may contribute to the trends of earlier puberty onset reported recently, especially in girls (30, 31).

Our data in SL rats suggest that postnatal overfeeding is associated with elevated Kp tone in the hypothalamus at puberty, as evidenced by higher expression levels of *Kiss1* mRNA and the trend for a higher density of Kp-positive fibers in the anterior periventricular area of the hypothalamus. In addition, a nonsignificant 20% increase in the number of Kp-IR neurons in the ARC was detected in the overfed group. Considering that our IHC analysis of Kp-positive cell bodies does not allow quantification of discrete/continuous changes in Kp expression, but rather variations in the number of Kp-positive cells, it remains possible that the increase of Kp expression might have not been of sufficient magnitude as to result in a significant rise of IR cell bodies in the ARC. In addition, we detected some degree on interindividual variation (eventually linked to subtle changes in BW within the same group), which might have masked clearer changes between SL and NL groups. In the same vein, although a tendency for increased numbers of Kp fibers and density was observed in SL animals at this periventricular area, these changes displayed some regional and interindividual variability, which prevented them from reaching statistical significance. In any event, the functional relevance of such trends of increased Kp-IR is yet to be defined, in particular as to whether this leads to enhanced release of mature Kp, because changes in peptide content or even Kiss1 mRNA levels may not necessarily translate into equivalent fluctuations in neuropeptide release. Similarly, the origin, projections, and eventual neuronal targets of Kp fibers remain unknown and merits specific investigation. In this sense, solid evidence for the origin of the Kp fibers in the periventricular region is still missing, although it is most likely that they stem from the neurons in the vicinity. Notwithstanding these uncertainties, our expression data are compatible with an increase in the turnover of Kp synthesis and axonal transport in postnatally overfed animals. Such putative elevation of Kp tone is further supported by the observed increase of basal LH levels in SL females at puberty. However, such elevation does not appear to be associated with any detectable desensitization to further stimulation with Kp, in keeping with our previous data that pubertal female rats are less prone to desensitization than adult rats (68).

Mechanistic implications

A key issue emerging from our data are the underlying mechanisms for the observed changes in the timing of puberty. Previous studies on the metabolic control of puberty have mainly focused on the impact of acute nutritional stress, mainly subnutrition, or actual BW; analyses that have supported the contention that threshold energy (fat) stores are permissive for puberty (46). Nonetheless, limited attention has been paid to the eventual neurohor-

monal mechanisms for early developmental programming of puberty. Noteworthy, leptin has been shown to be a relevant neurotrophic factor during early postnatal life in rodents, promoting the development of hypothalamic neural pathways essential for BW homeostasis (69, 70). Such actions appear to be conducted by the postnatal surge of leptin, the timing of which is affected by perinatal malnutrition (71–73). Our current data make it tempting to propose that postnatal development of the Kiss1 system is also affected by metabolic cues, eventually including leptin, a phenomenon that may impact on the timing of puberty and its modulation by later challenges. Importantly, the different models of early nutritional challenge used to evaluate puberty induce also persistent modifications in BW gain after weaning, with the potential confounding influence of changes in fat stores and leptin levels at the time of puberty. However, detailed analysis of our results unveils potential interactions between developmental (early) influences and later changes in the modulation of puberty onset by BW and (putatively) leptin levels. With regard to the latter, however, it is stressed that a recent report failed to demonstrate a substantial role of Kiss1 signaling in mediating leptin effects on puberty in mice (48), although the use of models of congenital lack of leptin receptors in Kiss1 neurons does not allow us to exclude the possibility of indirect actions of leptin on Kp networks and/or the occurrence of compensatory phenomena during development.

In SL rats, early onset of puberty takes place at a BW and, presumably, leptin levels equivalent to those of control NL animals. This is illustrated by analogous values of BW at VO50 between these two groups. This would suggest that within the nutritional range from normal nutrition to overfeeding the timing of puberty may be the consequence of achieving threshold levels of BW and fat stores, in keeping with the original Frisch hypothesis (74); early developmental influences would operate mainly as durable modifiers of postnatal BW gain. In this sense, maternal obesity has been described to amplify the neonatal surge of leptin in the offspring, with deferred influences in terms of leptin resistance and perturbation of key hypothalamic networks, such as AgRP, which may lead to hyperphagia and obesity (72). Whether these early alterations of the postnatal surge of leptin also affect (directly or indirectly) the developmental maturation of the hypothalamic Kiss1 system merits further investigation.

In clear contrast, BW values at VO50 in animals subjected to postnatal undernutrition were substantially lower (>20%) than those in the control NL group. Similarly, leptin levels in LL rats were persistently decreased throughout the pubertal transition, with values in 36-d-old LL females being approximately half of those in control NL rats on d 31. In the same vein, pubertal maturation of the hypothalamic Kiss1 system, as estimated by *Kiss1* mRNA levels and number of ARC Kp-IR cells, was severely impaired by postnatal subnutrition. Yet, approximately 60% of LL females displayed VO on d 37 postpartum. The above data would suggest that early malnutrition resets the permissive threshold levels of BW (and eventually leptin), allowing pubertal maturation even in the face of suboptimal metabolic conditions. Indeed, puberty took place in LL rats earlier than it would be predicted on the basis of their BW/leptin levels. This adaptative response is reminiscent of other catch-up manifestations of developmental malprogramming associated with early subnutrition (19). It is tempting to propose, although yet to be proven, that the mechanism for pubertal changes in the expression of Kiss1 system in postnatally underfed rats may involve perturbations in the shape and trophic actions of the postnatal surge of leptin.

As a final note, early nutritional manipulations in rodents have been shown to disturb the development of several hypothalamic pathways involved in energy homeostasis that might have an impact of the Kiss1 system as well. For instance, perinatal under- and overfeeding has been shown to persistently alter hypothalamic neuropeptide Y expression (49), neuropeptide Y being a putative regulator of *Kiss1* expression (75). Similarly, altered metabolic responses to melanin-concentrating hormone and melanocortins (α-MSH) have been described in postnatally overfed rats; melanin-concentrating hormone and α-MSH have been shown to affect Kp responsiveness and *Kiss1* mRNA expression in mice and sheep, respectively (76, 77). In addition, peripheral hormones other than leptin, such as ghrelin, with potential effects on *Kiss1* expression and puberty (78, 79), might also be altered in our models of early nutritional challenge and, thus, could contribute to the observed effects in terms of pubertal timing reported herein. Finally, it is noted that manipulations of litter size may have an impact on additional hormonal signals (*e.g.* sex steroids) and neuroendocrine systems (*e.g.* the adrenal/stress axis), that might have contributed to part of the changes in the maturation of the hypothalamic Kiss1 system and the timing of puberty reported herein (80, 81).

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Paper V

High plasma triglyceride levels strongly correlate with low kisspeptin in the arcuate nucleus of male rats

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Short title: High plasma triglyceride levels correlate with low kisspeptin

Keywords: kisspeptin, high fat diet, triglycerides, obesity, lipotoxicity

Abstract

It is well known that reproductive capacity is lower in obese individuals, but what mediators and signals are involved is unclear. Kisspeptin is a potent stimulator of GnRH release, and it has been suggested that kisspeptin neurones located in the arcuate nucleus (ARC) transmit metabolic signals to the GnRH neurones. In this study we measured bodyweight and plasma concentrations of leptin, insulin, testosterone and triglycerides after high fat diet (HFD) exposure, and correlated these parameters with the number of kisspeptin-immunoreactive (-ir) neurones in the ARC of the male rat. In this model, a HFD (45% or 60% energy from fat, respectively) or a control diet (10% energy from fat) was provided after weaning for three months. We found a significant increase in bodyweight and plasma leptin concentration, but no change in the number of kisspeptin-ir cells with increased fat in the diet. The number of kisspeptin-ir cells was not correlated with body weight, testosterone, leptin or insulin. However, we found that the number of kisspeptin-ir cells was strongly and negatively correlated with the level of plasma triglycerides (R^2=0.49, P=0.004). Our results are in line with the hypothesis of lipotoxicity, stating that circulating triglycerides cause endoplasmic reticulum stress in non-adipose tissue leading to the concomitant deterioration of the different tissues affected in co-morbidities of obesity. Our results suggest that it is the inability to clear circulating triglycerides and not the diet or body weight causing a decrease in kisspeptin-ir neurones.

Introduction

Obesity has become a matter of great public concern due to the increased risk of co-morbidity such as metabolic dysfunctions, hypertension, and reduced fertility (Khaodhiar et al., 1999; Loret de Mola, 2009; Pasquali et al., 2007). Kisspeptin, encoded by the *Kiss1* gene, is a potent stimulator of GnRH release and kisspeptin is eliciting the pre-ovulatory luteinising hormone surge, and kisspeptin is thus considered essential for a proper reproductive function (Pinilla et al., 2012). Kisspeptin expression in the arcuate nucleus (ARC) is sensitive to metabolic status and these kisspeptin neurones have been considered to play an important role as mediators of peripheral signals to the GnRH neurones, thereby linking obesity with reduced fertility (Castellano et al., 2010; George et al., 2010). Several studies have investigated the effect of high fat diet (HFD) exposure on kisspeptin expression in rodents, but reduced *Kiss1* mRNA and kisspeptin expression have only been shown in a mouse strain susceptible to HFD-induced fertility (Quennell et al., 2011), whereas wild-type mice and rats have unchanged or increased *Kiss1* mRNA expression after HFD exposure (Brown et al., 2008; Li et al., 2012; Lie et al., 2013; Luque et al., 2007; Quennell et al., 2011).

It has long been known that leptin-deficient mice are infertile, and that their fertility is rescued by leptin treatment (Mounzih et al., 1997); however, the site of action for leptin in relation to fertility is still not clear. Kisspeptin neurones are proposed to express the leptin receptor (Backholer et al., 2010; Cravo et al., 2011; Quennell et al., 2011; Smith et al., 2006), and leptin has thus been suggested to be the major messenger of information on sufficient energy stores directly to kisspeptin neurones. However, recent studies fail to detect leptin receptor-dependent signalling in kisspeptin neurones (Louis et al., 2011; True et al., 2011), and although mice lacking leptin signalling have reduced *Kiss1* mRNA expression (Quennell et al., 2011; Smith et al., 2006), this phenotype is only partly rescued by leptin treatment (Smith et al., 2006). Further, transgene mice lacking the functional leptin receptor only in kisspeptin neurones do not have compromised puberty or fertility (Donato et al., 2011). Hence, the effect of leptin on fertility is likely to be, at least partly, independent of kisspeptin signalling.

It is well known that co-morbidities are linked to obesity; however significant variation exists between individuals, with some severely obese showing no signs of metabolic dysfunctions and *vice versa* (Capeau et al., 2005; Karelis et al., 2004). A hypothesis explaining this variation is the hypothesis of lipotoxicity, which states that it is not the volume of adipose tissue, but rather the capability of the body to clear lipids from the circulation, which determines the adverse effects of

adiposity (Sorensen et al., 2010). When the fat storage capacity in the adipose tissue is reached, fat is directed to non-adipose tissue, where storage capacity is very limited. Lipids are therefore degraded through non-oxidative pathways resulting in toxic reactive lipids and ultimately apoptosis of cells in non-adipose tissue (Sorensen et al., 2010). Hence, the lipotoxicity hypothesis explains the concomitant deterioration of pancreas, liver, kidney, skeletal muscle, blood vessels, and heart observed in patients with the metabolic syndrome.

Lipotoxicity has also been reported in the hypothalamus (De Souza et al., 2005; Milanski et al., 2009; Moraes et al., 2009). Specifically, a long-term HFD exposure has been shown to increase inflammatory markers such as TNFα, pJNK , IL-1β and IL-6 in the hypothalamus of male rats (De Souza et al., 2005; Milanski et al., 2009; Moraes et al., 2009), and an increase in neuronal apoptosis and reduction of synaptic inputs specifically in the ARC after HFD exposure has also been reported (Moraes et al., 2009). In the same line, pJNK mediated endoplasmic reticulum (ER) stress after exposure to a non-esterified fatty acid in a hypothalamic neuronal cell line has been reported (Mayer and Belsham, 2010).

Moreover, obesity models in rodents have varying efficacy on co-morbidity parameters of obesity and even on bodyweight, depending on species, genetic background, and diet (Nilsson et al., 2012; Rosini et al., 2012). Further, even within a single rat strain, such as Sprague-Dawley, there is variation in the sensitivity towards becoming obese on a specific high caloric diet (Levin and Keesey, 1998). This variation in the susceptibility to experiencing adverse effects of HFD, suggests that it is not the diet *per se*, which induces the co-morbidity of obesity. In the same line, previous studies do not find an adverse effect of HFD on kisspeptin expression (Brown et al., 2008; Li et al., 2012; Lie et al., 2013; Luque et al., 2007; Quennell et al., 2011). We therefore hypothesise that a metabolic marker, rather than HFD *per se*, is important for kisspeptin expression.

Materials and Methods

Male Sprague-Dawley rats were obtained from Taconic Inc., Denmark, and kept under constant conditions of light (12 hours of light from 2pm), temperature (18-22°C), and relative air humidity (55±10%). Upon arrival the rats (n=6 per group; age 24 days) were randomly assigned into three groups with free access to tap water and either standard chow (Altromin 1324 with 10% kcal from fat and energy density of 2.85 kcal/g, Altromin GmbH, Germany) or a HFD with either 45% (#D12451; energy density 4.73 kcal/g, Research Diets, USA) or 60% kcal from fat (#D12492;

energy density 5.24 kcal/g, Research Diets, USA). After 12 weeks on their respective diets, tail vein blood was collected in heparinised tubes and animals were anaesthetised with Mebumal and perfused transcardially with 0.9% saline for 5 min, followed by 4% paraformaldehyde-phosphate buffer for 10 min (0.1M; pH 7.4). The brains were rapidly isolated and postfixated in the same fixative overnight and thereafter kept in 0.05 M phosphate-buffered saline (PBS) at 4°C.

The experiment was approved (J.no. 2005/561-1055) and conducted in accordance with the guidelines of the Animal Experimentation Inspectorate, Ministry of Justice, Denmark.

Immunocytochemistry

The fixed brains were dehydrated in 30% sucrose-PBS solution for two days, and where hereafter cut in 4 series of 40 µm free-floating coronal sections through the ARC. The sections were evaluated for kisspeptin-immunoreactivity, as previously described (Bentsen et al., 2010). In brief, one series of sections were washed in PBS, incubated in 1% H_2O_2 in PBS for 10 min to block endogen peroxidases followed by 20 minutes in PBS containing 0.3% Triton X-100, 5% swine serum, and 1% bovine serum albumin (BSA), to block non-specific binding. The sections were then incubated in the primary antiserum JLV-1 diluted 1:200 in 0,3% Triton-X 100 and 1% BSA and gently shaken overnight at 4°C. This primary antiserum raised against N-terminal kisspeptin-52 has been shown not to cross-react with related RF-peptides (Desroziers et al., 2010; Overgaard et al., 2013). After washing in PBS containing 0.1% Triton X-100 (T-PBS), the sections was incubated for 1 hour in biotinylated donkey anti-rabbit (Jackson Labs, 711-066-152) diluted 1:1000 in T-PBS with 0.3% BSA. After another T-PBS wash, the sections were incubated in 0.4% avidin-biotin-peroxidase complex (Vector Elite Kit™, Vector Labs, USA) diluted in T-PBS and developed in 0.05% diaminobenzidine (Sigma-Aldrich, USA) with 0.05% H_2O_2 in Tris-HCl buffer (0.05M, pH 7.6, 5°C) for 10 minutes. The sections were mounted and cover slipped in Pertex® (HistoLab, Sweden).

The total number of kisspeptin-immunoreactive (-ir) cells in the entire ARC in one series of sections was quantified for each rat under bright field illumination (Zeiss Imager Z.1 microscope) by the same person, and the identity of the rats were blinded through the immunocytochemical procedure and during quantification.

Plasma analysis

Plasma insulin was measured using a standard sandwich ELISA assay (10-1250-01, Mercodia AB, Sweden), total triglycerides were measured using a biochemical assay (TR0100, Sigma-Aldrich, Denmark), and free testosterone was measured using a coat-a-count RIA kit based on ^{125}I labelled testosterone (TKTF1; Siemens Medical Solutions, USA), following the instructions of the manufacturer of the respective assays. Plasma leptin was determined with a Rat Leptin Elisa kit (90040, Crystal Chem Inc, USA) using a standard sandwich technique. The instructions of the manufacturer were followed, except from the background absorbance measurement, which was measured at 655nm absorbance.

Limited plasma volume was available, and therefore not all animals were included in all plasma analyses. See Table 1 for the number of animals in the different groups analysed for each analyte.

Statistical analysis

Differences between the three diet groups were analysed using a one-way ANOVA followed by Tukey's multiple comparisons test, except for analysis of body weight, where the groups showed significant different standard deviations ($p < 0.05$, Brown-Forsythe test). Therefore a Kruskal-Wallis test was applied to analyse differences in body weight between the three diet groups. Data are presented as mean ± standard error of mean (SEM) in Fig. 1A and Table 1. Pearson's correlations were used in all correlations, and for all correlations the three diet groups were pooled. The P-value and R^2 of the correlations are presented in Fig. 1B and Table 2. For all analyses $p < 0.05$ was considered statistical significant.

Results

High fat diet and metabolic markers

Male rats were put on HFD after weaning, and after three months on the 45% HFD or the 60% HFD body weights were significantly increased ($p < 0.001$, Table 1). In addition, exposure to 60% HFD caused a significant increase in plasma leptin levels ($p < 0.05$, Table 1). It is noteworthy that both HFD groups had significantly increased variability in body weight compared to the control group ($p < 0.05$), indicating different susceptibility to body weight gain on the given diets.

The other metabolic markers in plasma were not different between the diet groups, as neither insulin, triglyceride nor testosterone plasma concentrations were affected by the HFD *per se* (Table 1). However, both plasma concentrations of insulin ($p < 0.05$) and leptin ($p < 0.001$) were

positively correlated with body weight (Table 2). On the contrary, plasma concentrations of triglyceride ($p = 0.91$) and testosterone ($p = 0.74$) were not correlated with body weight (Table 2).

Triglyceride levels correlate to kisspeptin irrespective of diet

To relate the metabolic markers to kisspeptin expression, immunocytochemistry was performed in a series of sections covering the entire ARC. The number of kisspeptin-ir cells in the ARC was not different between the three diet groups ($p = 0.22$; Fig. 1A), and neither body weight nor plasma concentrations of leptin, insulin, or testosterone correlated with the number of kisspeptin-ir cells in the ARC (Table 2).

We found a significant negative correlation between plasma concentration of total triglycerides and the number of kisspeptin-ir cells in the ARC ($p < 0.004$, $R^2 = 0.49$; Fig. 1B and Table 2). When the diet groups were analysed separately, significant correlation was only found in the control group (controls, $R^2 = 0.99$, $p = 0.004$; 45% HFD, $R^2 = 0.58$, $p = 0.078$; 60% HFD, $R^2 = 0.66$, $p = 0.095$).

Discussion

Previous studies investigating the effect of HFD on *Kiss1* mRNA expression have reported only plasma leptin and not triglyceride levels, and no correlations have been made (Li et al., 2012; Quennell et al., 2011). Our study is thus the first to show that kisspeptin expression is correlated with plasma triglyceride levels and not with leptin levels or body weight. Intriguingly, this correlation is evident also within the control group, emphasizing the relative importance of plasma triglyceride levels compared to other obesity parameters. This finding is in line with the lipotoxicity hypothesis, stating that triglycerides stored in adipose tissue are physiologically inert, whereas circulating triglycerides can be detrimental (Sorensen et al., 2010). Moreover, HFD has been shown to cause cellular stress in the hypothalamus leading to increased hypothalamic apoptosis and central leptin and insulin resistance (De Souza et al., 2005; Moraes et al., 2009). Further, it has been demonstrated *in vitro* that fatty acid exposure causes ER stress in a hypothalamic neuronal cell model, supporting the importance of triglyceridemia in the pathophysiology of obesity, also at targets within the hypothalamus (Mayer and Belsham, 2010). Although acute intracerebroventricular injection of fatty acids inhibits food intake and glucose production (Obici et al., 2002), further studies with prolonged fatty acid injection, and subsequent localisation of inflammatory markers in kisspeptin neurones, will be needed to determine whether

it is indeed the fatty acids that reduce kisspeptin expression directly in the hypothalamus and whether this is accompanied by inflammation and apoptosis. We therefore conclude that elevated plasma triglyceride levels itself, or another mechanism induced by this biomarker, cause a down-regulation of kisspeptin expression in the ARC.

We found that the number of kisspeptin-ir cells after HFD exposure was unchanged compared to control diet, which is in line with previous reports, showing no change or increased levels of *Kiss1* mRNA or kisspeptin expression in obese rodents (Brown et al., 2008; Castellano et al., 2011; Li et al., 2012; Lie et al., 2013; Luque et al., 2007; Quennell et al., 2011). Decreased testosterone levels are reported in obese men and mice (Corona et al., 2013; Luque et al., 2007). Because kisspeptin expression in the ARC is inhibited by sex steroids (Smith et al., 2005), the tendency to increased kisspeptin in the HFD groups could be explained by decreased testosterone levels.

There is some controversy whether leptin is an important regulator of *Kiss1* mRNA expression (Castellano et al., 2006; Donato et al., 2011; Luque et al., 2007; Quennell et al., 2011; Smith et al., 2006). However, we propose that leptin could be important as a permissive signal during energy deficit, whereas leptin may not be the key signal for the detrimental metabolic effects of obesity to kisspeptin neurones. Likewise, we found no correlation between plasma insulin levels and the number of kisspeptin-ir cells. Inactivation of the insulin receptor specifically in the brain gives rise to an infertile and metabolic disturbed mouse phenotype, suggesting an important central role of insulin for fertility (Bruning et al., 2000). However, the central actions of insulin is not likely to be mediated by kisspeptin, since reduced *Kiss1* mRNA expression in diabetic mice is not rescued by insulin administration (Castellano et al., 2006), and *in vitro* studies confirm the lack of effect of insulin on *Kiss1* mRNA expression (Luque et al., 2007).

In conclusion, we find a strong negative correlation between plasma triglyceride concentrations and kisspeptin neuronal numbers in the rat ARC regardless of the percentage of fat in the diet. In line with the lipotoxicity hypothesis, our results suggest that it is the level of hypertriglyceridemia *per se* that is a detrimental factor for kisspeptin expression in the ARC.

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Figures and figure legends

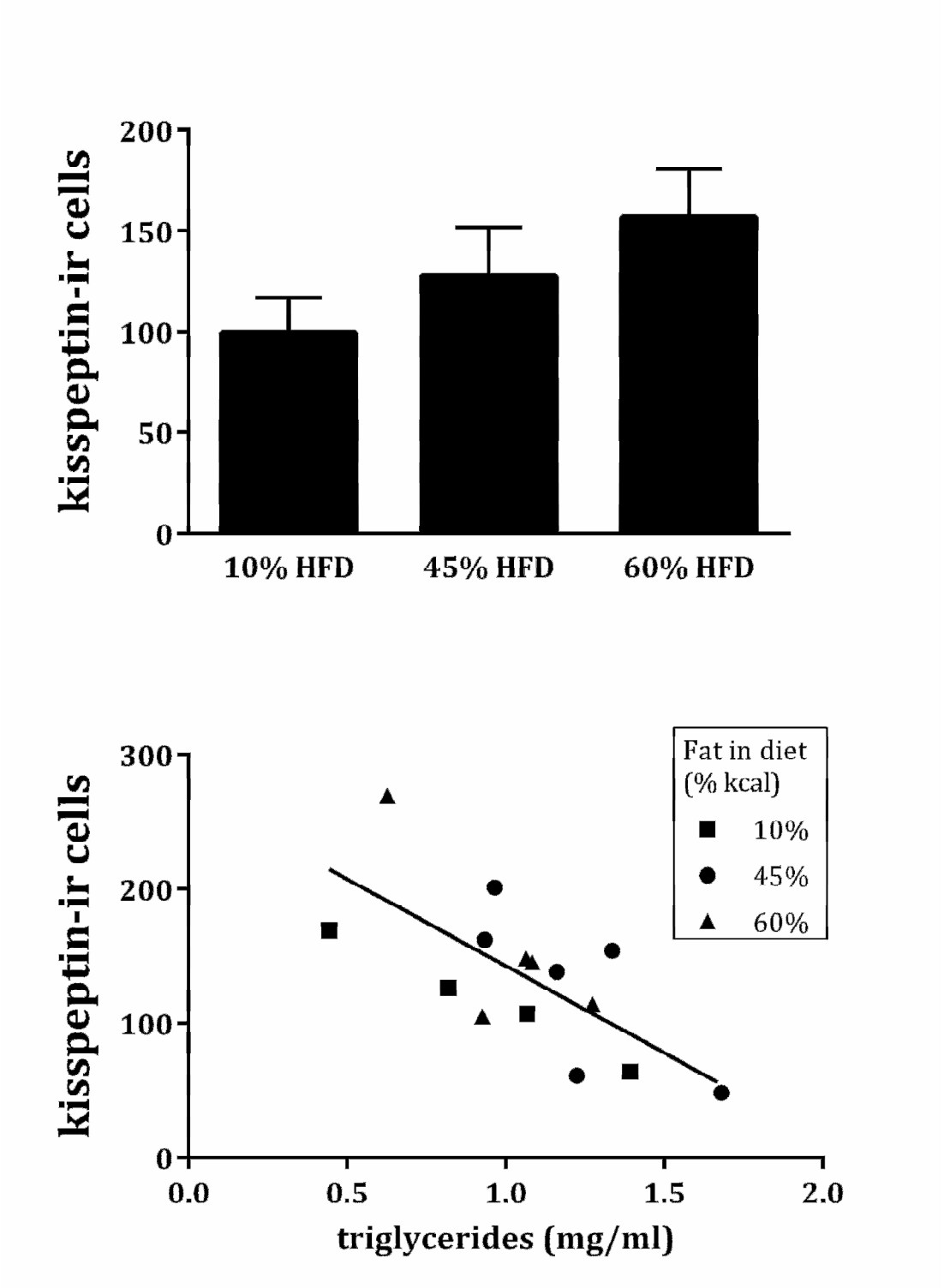

Figure 1. The number of kisspeptin-immunoreactive (-ir) cells in the arcuate nucleus (ARC) in relation to diet and total plasma triglycerides.
A) The number of kisspeptin-ir cells in the ARC of male rats on a diet with 45% and 60% energy from fat is not different from controls ($p = 0.22$, one-way ANOVA). B) Plasma triglyceride concentrations are negatively correlated with kisspeptin-ir cells in the ARC ($p = 0.004$, $R^2 = 0.49$). HFD, high fat diet.

Table 1
Body weight and plasma concentrations of metabolic markers

Fat in diet (% kcal)	10%	45%	60%
Body weight (g)	405.8 ± 3.0 (6)	**505.2 ± 25.4**[a] (6)	**547.7 ± 15.7**[a] (6)
Leptin (ng/ml)	4.34 ± 1.37 (2)	10.90 ± 1.79 (4)	**19.43 ± 3.93*** (4)
Insulin (ng/ml)	0.45 ± 0.03 (2)	1.05 ± 0.26 (6)	1.81 ± 0.69 (4)
Triglycerides (mg/ml)	0.93 ± 0.20 (4)	1.22 ± 0.11 (6)	0.99 ± 0.11 (5)
Testosterone (pg/ml)	5.30 ± 2.40 (2)	1.86 ± 0.19 (2)	7.39 ±5.06 (3)

Data represent group means ± SEM.
Numbers in parentheses are the n-values.
[a] High fat diet significantly affects bodyweight ($p < 0.001$, Kruskal-Wallis test)

Table 2
Correlation of kisspeptin, body weight and metabolic plasma markers

Correlation with body weight	P-value	R^2	n
Leptin (ng/ml)	**0.001**	**0.85**	10
Insulin (ng/ml)	**0.017**	**0.53**	12
Triglycerides (mg/ml)	0.91	0.001	15
Testosterone (pg/ml)	0.74	0.025	7
Correlation with kisspeptin	**P-value**	**R^2**	**n**
Body weight (g)	0.14	0.14	17
Leptin (ng/ml)	0.26	0.17	10
Insulin (ng/ml)	0.11	0.26	12
Triglycerides (mg/ml)	**0.004**	**0.49**	15
Testosterone (pg/ml)	0.15	0.37	7

Correlations based on data from all three diet groups.
Kisspeptin is the number of immunoreactive cells in the ARC.
Leptin, insulin, triglycerides and testosterone are plasma concentrations.