*Neuroscience* 173 (2011) 37–56

# CHARACTERIZATION OF *Kiss1* NEURONS USING TRANSGENIC MOUSE MODELS

**R. M. CRAVO,[a] L. O. MARGATHO,[a,b] S. OSBORNE-LAWRENCE,[a] J. DONATO JR,[a] S. ATKIN,[c] A. L. BOOKOUT,[a,c] S. ROVINSKY,[a] R. FRAZÃO,[a] C. E. LEE,[a] L. GAUTRON,[a] J. M. ZIGMAN[a1] AND C. F. ELIAS[a1]***

[a]*Department of Internal Medicine, Division of Hypothalamic Research, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard Dallas, TX, USA*

[b]*Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP 14049-900, Brazil*

[c]*Department of Pharmacology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard Dallas, TX, USA*

**Abstract—Humans and mice with loss-of-function mutations of the genes encoding kisspeptins (*Kiss1*) or kisspeptin receptor (*Kiss1r*) are infertile due to hypogonadotropic hypogonadism. Within the hypothalamus, *Kiss1* mRNA is expressed in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (Arc). In order to better study the different populations of kisspeptin cells we generated *Kiss1*-Cre transgenic mice. We obtained one line with Cre activity specifically within *Kiss1* neurons (line J2–4), as assessed by generating mice with Cre-dependent expression of green fluorescent protein or β-galactosidase. Also, we demonstrated *Kiss1* expression in the cerebral cortex and confirmed previous data showing *Kiss1* mRNA in the medial nucleus of amygdala and anterodorsal preoptic nucleus. *Kiss1* neurons were more concentrated towards the caudal levels of the Arc and higher leptin-responsivity was observed in the most caudal population of Arc *Kiss1* neurons. No evidence for direct action of leptin in AVPV *Kiss1* neurons was observed. Melanocortin fibers innervated subsets of *Kiss1* neurons of the preoptic area and Arc, and both populations expressed melanocortin receptors type 4 (MC4R). Specifically in the preoptic area, 18–28% of *Kiss1* neurons expressed MC4R. In the Arc, 90% of *Kiss1* neurons were glutamatergic, 50% of which also were GABAergic. In the AVPV, 20% of *Kiss1* neurons were glutamatergic whereas 75% were GABAergic. The differences observed between the *Kiss1* neurons in the preoptic area and the Arc likely represent neuronal evidence for their differential roles in metabolism and reproduction. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: leptin, melanocortin, Cre-recombinase, reporter mouse, GAD-67, vGluT2.**

Humans and mice with loss-of-function mutations of the gene encoding kisspeptins (*Kiss1*) or kisspeptin receptor (*Kiss1r*) are infertile due to hypogonadotropic hypogonadism. They show abnormal sexual maturation and decreased circulating levels of sex steroids and gonadotropins (de Roux et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007). In many species, administration of kisspeptins induce a rapid increase in luteinizing hormone (LH) secretion—an effect mediated via direct action on gonadotropin releasing hormone (GnRH) neurons (Gottsch et al., 2004; Navarro et al., 2004a; Dhillo et al., 2005; Plant et al., 2006).

*Kiss1* is expressed in different organs and tissues including pancreas, gonads, placenta and brain (Ohtaki et al., 2001; Colledge, 2008). Within the central nervous system, *Kiss1* is expressed in neurons of several hypothalamic nuclei, including the anteroventral periventricular nucleus (AVPV), the anterior periventricular nucleus (PeN) and the arcuate nucleus (Arc) (Gottsch et al., 2004). In these sites, virtually all *Kiss1* neurons colocalize sex steroids receptors, particularly estrogen receptor α (ERα) and androgen receptors and are differentially modulated by changing levels of gonadal steroids. For example, high estrogen levels stimulate *Kiss1* gene expression in the AVPV and decrease Kiss1 expression in the Arc (Smith et al., 2005a, 2007; Gottsch et al., 2009). In cycling females, increases in estrogen levels induce a GnRH surge, which is observed as an increase in the frequency of pulses and sustained high levels of GnRH secretion; this response in turn triggers an LH surge and ovulation (Levine et al., 1982; Levine and Ramirez, 1982; Moenter et al., 1992; Caraty et al., 1995; Herbison, 2008). Thus, *Kiss1* in the AVPV is thought to mediate the estrogen positive feedback action on LH secretion. In contrast, *Kiss1* expression in the Arc is high in conditions of low estrogen levels, suggesting that these *Kiss1* Arc neurons relay the estrogen negative feedback action on LH secretion (Smith et al., 2005a; Dungan et al., 2006; Popa et al., 2008; Gottsch et al., 2009). As defined in estrogen receptors knockout mice, the estrogen positive and negative feedback action on GnRH secretion are mediated by ERα (Couse et al., 2003; Wintermantel et al., 2006). Therefore binding of estrogen to ERα is thought to drive the presumably distinct kisspeptin action on LH secretion (Smith et al., 2005a; Dungan et al., 2006; Popa et al., 2008; Gottsch et al., 2009). However, the mechanism by which kisspeptin released from different populations of neurons mediate both the negative and positive feedback action of estrogen on LH secretion has not been determined.

[1] JMZ and CFE contributed equally to this work.

*Corresponding author. Tel: +1-214-648-0248; fax: +1-214-648-5612.
E-mail address: carol.elias@utsouthwestern.edu (C. F. Elias).

*Abbreviations:* Arc, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BACs, bacterial artificial chromosomes; ERα, estrogen receptor α; FACS, fluorescence activated cell sorting; GFP, green fluorescent protein; GnRH, gonadotropin releasing hormone; HBSS, Hank's balanced salt solution; LepR, leptin receptor; MC4R, melanocortin receptors type 4; PeN, periventricular nucleus; qPCR, quantitative RT-PCR; βGal, β-galactosidase.


doi:10.1016/j.neuroscience.2010.11.022

A role for kisspeptin in pubertal development has also been described. Hypothalamic levels of *Kiss1r* and *Kiss1* increase during sexual maturation, and administration of kisspeptin to juvenile rodents precipitates puberty (Navarro et al., 2004a,b; Han et al., 2005). In addition, electrophysiological responses of GnRH neurons to kisspeptin increase across puberty (Han et al., 2005). However, the signals that impinge on *Kiss1* neurons to drive their development during puberty initiation are not known. Several studies have suggested that one possible candidate is the adipocyte-derived hormone leptin. Leptin receptors are expressed in a subpopulation of Kiss1 neurons and leptin signaling-deficient mice and humans are hypogonadotropic hypogonadal, remaining in a prepubertal state (Coleman, 1978; Zhang et al., 1994; Tartaglia et al., 1995; Farooqi et al., 1999; Smith et al., 2006). However, leptin's effects on the various *Kiss1* neuronal populations have been inconsistently reported. In one study, obese leptin-deficient (*ob*/*ob*) male mice showed decreased expression of *Kiss1* in the Arc, which was partially restored by leptin treatment (Smith et al., 2006). In another study, total hypothalamic expression of *Kiss1* (which includes the AVPV, the PeN and the Arc) was not changed in *ob*/*ob* mice following leptin administration, except when matched with food restricted *ob*/*ob* control mice (Luque et al., 2007). In a third study, following fasting (a state of low leptin levels), female rats showed decreases in *Kiss1* expression in the AVPV but not in the Arc (Kalamatianos et al., 2008).

Thus, in order to better study and determine the characteristics of specific populations of *Kiss1* neurons, we have generated new transgenic mouse lines in which Cre recombinase is expressed within *Kiss1* cells. We created reporter mice in which Cre-mediated expression of green fluorescent protein (GFP) or $\beta$-galactosidase ($\beta$Gal) marks the location of *Kiss1* neurons. Since the neurotransmitters GABA and glutamate have been identified as fundamental players in GnRH neuron activity and in steroidal feedback control of GnRH secretion (Clarkson and Herbison, 2006; Moenter et al., 2009), we postulated that differences in GABA and glutamate synthesis and neurotransmission reflect the potential roles played by the distinct populations of *Kiss1* neurons in GnRH secretion and puberty initiation. In addition, we aimed at defining the subpopulations of *Kiss1* neurons directly responsive to leptin and those comprising likely downstream targets of leptin-responsive neurons.

## EXPERIMENTAL PROCEDURES

### Subjects

Adult male and female Kiss1-Cre, LepR-IRES-Cre/LacZ, MC4R-GFP and C57BL/6 mice were housed in the University of Texas Southwestern Medical Center Animal Resource Center, in a light- (12 h on/12 h off) and temperature- (21–23 °C) controlled environment. They were fed standard chow diet (Harlan Teklad Global Diet, Harlan Laboratories Inc., Indianapolis, IN, USA), unless otherwise mentioned and had free access to water. All experiments were carried out in accordance with the guidelines established by the National Institute of Health Guide for the Care and Use of Laboratory Animals, as well as with those established by the University of Texas Institutional Animal Care and Use Committee.

### Generation of Kiss1-Cre BAC transgenic mice

We generated several lines of transgenic mice that express Cre recombinase eutopically within kisspeptin-expressing cells, and which we called J2–3, J2–4 and J2–6. These animals were made through the use of various ET-cloning "recombineering" technologies (Lee et al., 2001; Muyrers et al., 2001). We constructed two different Kiss1-Cre transgene-containing bacterial artificial chromosomes (BACs). The original *Kiss1* BACs were purchased from BACPAC Resources Center at Children's Hospital Oakland Research Institute (Oakland, CA, USA). Both spanned the entire coding region of *Kiss1* gene. The first BAC (RP24-186J14) contained approximately 88.03 kb sequence upstream of the *Kiss1* start codon and approximately 86.26 kb sequence downstream of the *Kiss1* stop codon. The second BAC (RP24-299B2) contained approximately 109.49 kb sequence upstream of the *Kiss1*start codon and approximately 69.01 kb sequence downstream of the *Kiss1*stop codon. These BACs were transformed into EL250 cells by electroporation. EL250 cells were provided by N. Copeland; they contain heat-inducible recE and recT recombinases for homologous recombination and arabinose-inducible Flp-recombinase for site-specific recombination at *frt* sites (Lee et al., 2001). Next, a DNA fragment containing the coding sequence of Cre recombinase followed by an SV40 polyadenylation (polyA) signal and a kanamycin resistance gene flanked by *frt* sites (FKF) was inserted into the *Kiss1* BACs, at the translational start site of *Kiss1*, by ET-cloning. The construction of the Cre-polyA-FKF cassette was described previously (Dhillon et al., 2006). This insertion also resulted in the replacement of the entire coding region of *Kiss1* and an additional 48-bp downstream of the *Kiss1* stop codon. Finally, the kanamycin resistance gene was removed by arabinose induction of Flp-recombinase, and the Cre recombinase coding region was sequenced to ensure that no mutations had been introduced. The Cre-modified *Kiss1* BACs were submitted to the UTSW Medical Center Transgenic Core Facility for microinjection into pronuclei of fertilized one-cell stage embryos of C57BL/6 mice. Oligonucleotide primers used to confirm the genotype of mice harboring the Kiss1-Cre transgenes were as follows: M358: 5′-GCTCTGGTGAAGTACGAACTCTGA-3′ and M247: 5′-TGCGAACCTCATCACTCGTTGCAT-3′. The mice used in this study were on a pure C57BL/6 genetic background (Fig. 1).

### Validation of Kiss1-Cre mouse models

In order to validate our lines, we crossed the Kiss1-Cre mice with reporter mice that express either GFP [B6.Cg-Tg(ACTB-Bgeo/GFP)21Lbe/J; Jackson Labs, Bar Harbor, ME, USA] or $\beta$Gal [B6.129S4-Gt(ROSA)26–Sortm1Sor/J; Jackson Labs] in a Cre-dependent manner (Srinivas et al., 2001; Scott et al., 2009). The lines demonstrating Cre activity (as assessed by Cre-mediated expression of GFP and/or $\beta$Gal) in the hypothalamic arcuate nucleus (Arc) and anteroventral periventricular nucleus (AVPV, line J2–4) were used in additional histochemical experiments for assessment of colocalization of Cre activity and *Kiss1* mRNA expression. Adult (60-day old) males and females from each line ($n$=8 females and $n$=4 males per line) were perfused in the afternoon (2:00 to 4:00 PM). Females were further divided into two groups: those ovariectomized 15 days prior perfusion ($n$=4) and those normally cycling ($n$=4). Brains were dissected, cryoprotected overnight and cut in the frontal plane into 25-$\mu$m sections on a freezing microtome. Five series were collected into antifreeze solution and stored at −20 °C.

### Quantitative RT-PCR (qPCR)

In order to further assess the expression of *Kiss1* mRNA, we collected tissue from specific brain sites from wild-type C57BL/6 mice ($n$=3 males and $n$=3 females). These sites included olfactory bulb, cerebral cortex, amygdala, hypothalamus, cerebellum