Charakterisierung und funktionelle Analyse des humanen KiSS1-Promotors

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I. Abkürzungsverzeichnis

ARC	Nucleus arcuatus
AVPV	Nucleus periventricularis anteroventralis
AS	Aminosäure
α-MSH	α-Melanozyten stimulierendes Hormon
bp	Basenpaar
cDNA	complementary deoxyribonucleic acid
CUX1	Cut homeobox 1
DAG	Diacylglycerin
EAP1	Enhanced At Puberty 1
ER-α	Estrogenrezeptor α
ERE	Estrogen-Response-Element
FSH	Follikel-stimulierendes Hormon
GABA	γ-Aminobuttersäure
GnRH	Gonadotropin Releasing Hormon
GPR	G-Protein gekoppelter Rezeptor
HHG-Achse	Hypothalamus-Hypophysen-Gonaden-Achse
IP_3	Inositol-1,4,5-triphosphat
LH	Luteinisierendes Hormon
MBH	medio-basaler Hypothalamus
mRNA	messenger ribonucleic acid
OCT2	Octamer-binding transcription factor 2
PIP_2	Phosphatidylinositolbisphosphat
POA	Area praeoptica
POMC	Proopiomelanocortin
RACE-PCR	rapid amplification of cDNA-ends with polymerase chain reaction
TSG	Tumor Suppressor Gen
TSS	Transkriptionsstartstelle
TTF1	Thyroid Transcription Factor 1
YY1	Yin Yang 1
ZNS	Zentrales Nervensystem

1. Einleitung

Die Pubertät stellt eine Entwicklungsphase des Lebens dar, die mit einer Reihe anatomischer, physiologischer und emotionaler Veränderungen einhergeht, welche zur sexuellen Reifung und somit zur Fortpflanzungsfähigkeit führen.
In den letzten Jahren konnten enorme Fortschritte im Hinblick auf die Erforschung des Beginns der Pubertät und dessen auslösende Faktoren erzielt werden. Es zeigt sich, dass die Regulation der Pubertät sowohl auf neuronaler, neuroendokriner und genetischer Ebene stattfindet. Gene, die an der Kontrolle der zeitlichen Koordination der Pubertät beteiligt sind, sind in einem hierarchischen Netzwerk miteinander verknüpft und ihre Aktivität wird maßgeblich durch epigenetische Mechanismen beeinflusst *(Lomniczi und Ojeda, 2016a)*. Der aktuelle Wissensstand soll im Folgenden, mit besonderem Augenmerk auf das KiSS1/KiSS1R-System, kurz zusammengefasst werden.

1.1. Funktion der Hypothalamus-Hypophysen-Gonadenachse

Von zentraler Bedeutung für den Beginn der Pubertät und die Regulation der Fortpflanzung ist das Gonadotropin Releasing Hormon (GnRH), ein Dekapeptid *(Matsuo et al., 1971)*, welches von spezialisierten Neuronen im Hypothalamus synthetisiert wird. Die ca. 1000-2000 GnRH-Neurone entstammen der olfaktorischen Plakode und wandern während der 6.-16. Woche der Embryonalentwicklung ins Gehirn ein *(Schwanzel-Fukuda und Pfaff, 1989, Wray et al., 1989)*. Ihre Zellkörper liegen weit verteilt im medio-basalen Hypothalamus (MBH), die neurosekretorischen Axone projizieren in die Eminentia mediana, wo GnRH in den portalen Blutkreislauf der Hypophyse freigesetzt wird. Dort induziert GnRH die Freisetzung des Follikel - stimulierenden Hormons (FSH) und des luteinisierenden Hormons (LH) aus der Adenohypophyse. Die Gonadotropine fördern die Produktion und Sekretion von Sexualhormonen und damit die Ausprägung sekundärer Geschlechtsmerkmale, stimulieren die Gametogenese und regulieren die normale Reproduktionsfunktion im späteren Leben. Durch Feedback-Mechanismen auf allen Ebenen der Hypothalamus-Hypophysen-Gonadenachse (HHG-Achse) entstehen regulatorische Kreisläufe.
Die HHG-Achse ist bei Geburt und in den ersten Lebensmonaten bereits voll funktionsfähig, stellt jedoch während der Kindheit ihre Aktivität bis zur Pubertät ein *(Boyar et al., 1972)*. Es ist mittlerweile akzeptierte Lehrmeinung, dass für den Beginn der Pubertät eine synchrone, pulsatile GnRH-Ausschüttung obligat ist. Diese Sekretionssteigerung der GnRH-Neurone geschieht nicht durch eine Erhöhung ihrer intrinsischen Aktivität, sondern durch Änderungen transsynaptischer *(Kordon et al., 1994; Ojeda und Terasawa, 2002)* und glialer *(Ojeda et al., 2003)* Einflüsse.
Man geht davon aus, dass es einerseits zur Abnahme transsynaptischer, vorwiegend GABA- und opioiderger Inhibition kommt *(Terasawa, 1999; Plant, 1994, Ojeda und Skinner, 2006)* und andererseits der Einfluss aktivierender Neurotransmitter wie Glutamat *(Brann und Mahesh, 1997; Gay und Plant, 1987)* und Norepinephrin *(Terasawa et al., 1988; Gearing und Terasawa, 1991)* zunimmt.

In den letzten Jahren wurden weitere Komponenten dieses regulatorischen Systems entdeckt. So entdeckten Tsutsui und Kollegen im Jahr 2000 in Wachteln ein hypothalamisches Neuropeptid, das die Sekretion von GnRH aktiv hemmt, das Gonadotropin-Inhibitor Hormon (GnIH) *(Tsutsui et al., 2000).* In nachfolgenden Studien konnte gezeigt werden, dass GnIH bei Wirbeltieren hochkonserviert ist und eine bedeutende Rolle bei der Regulation der Fortpflanzung spielt *(Tsutsui et al., 2015)*. Bei Säugetieren entsprechen diesem die Peptide RFRP1 und RFRP3, welche zur Superfamilie der RFamide-related Peptides (RFRP) gehören. Sie entfalten ihre inhibierende Wirkung direkt über den auf den GnRH-Neuronen exprimierten GPR147-Rezeptor *(Hinuma et al., 2000; Ducret et al., 2009).*

Eine weitere exzitatorische Komponente kam mit der Entdeckung des Kisspeptins und des dazugehörigen Rezeptors KiSS1R hinzu**,** welcher ebenfalls direkt auf den GnRH-Neuronen exprimiert wird *(Messager et al., 2005; Seminara et al., 2003; Ojeda und Terasawa, 2002).* Auf die Funktion von Kisspeptin und die Neuroanatomie der zugehörigen Neurone wird später genauer eingegangen.

Neben diesen transsynaptischen Mechanismen spielen auch gliale Einflüsse eine entscheidende Rolle für das Erreichen der Pubertät und die Erhaltung der Fortpflanzungsfähigkeit. Der vorwiegend aktivierende Einfluss der Astroglia wird durch Wachstumsfaktoren und diffusionsfähige, neuroaktive Substanzen vermittelt und führt sowohl zu einer direkten als auch indirekten Stimulation der GnRH-Sekretion *(Ojeda et al., 2010).*

In den letzten 20 Jahren wurden die genetischen Hintergründe, die der neuroendokrinen Regulation der GnRH-Sekretion zugrunde liegen, umfangreich beforscht. Störungen im Pubertätsverlauf konnten auf diverse aktivierende und inaktivierende Mutationen in Genen, die an der Kontrolle der Pubertät beteiligt sind, zurückgeführt werden. Hierzu zählen u.a. die Gene, die für den GnRH-Rezeptor, Kisspeptin, den Kisspeptin Rezeptor, Neurokinin B und dessen Rezeptor codieren *(rez. in Lomniczi und Ojeda, 2016a)*. Durch die bekannten Mutationen lässt sich jedoch nur ein kleiner Teil der Pubertätsstörungen erklären. Dies deutet darauf hin, dass weit mehr Gene an der Regulation der Pubertät beteiligt sind.

In einem systembiologischen Ansatz wurde ein komplexes, hierarchisch aufgebautes Gennetzwerk beschrieben. Durch ihre hohe Interkonnektivität beeinflussen sich diese Gene in ihrer Funktion gegenseitig *(Lominczi et al., 2013a)* (Abb.1, S.52). Trotz unterschiedlicher zellulärer Funktionen, ist den übergeordneten Genen dieses Netzwerks gemeinsam, dass sie ursprünglich den Tumorsuppressoren zugeordnet wurden. Als zentrale Knotenpunkte stellen sie wichtige Regulatoren dar. *GnRH* und *KiSS1* gehören als untergeordnete Gene ebenfalls zu diesem Netzwerk *(Roth et al., 2007; Ojeda et al., 2010; Lomniczi et al., 2013a).*

Im Verlauf der Pubertät ändert sich zum einen die Aktivität dieser verschiedenen Aktivatoren und Repressoren *(Lomniczi und Ojeda, 2016a),* zum anderen ist bekannt, dass Pubertät und Fortpflanzungsfähigkeit sehr stark von internen und externen Milieuänderungen wie Ernährungsstatus, Umweltfaktoren, zirkadianem Rhythmus und Hormonstatus abhängig sind *(Kurian und Terasawa, 2013)*. Diese Tatsachen legen den Einfluss epigenetischer Kontroll-mechanismen nahe.

Obwohl die Erforschung epigenetischer Einflüsse auf die pubertätsregulierenden Gene noch in Anfängen steckt, konnten einige Studien deren wichtige Rolle in Bezug auf den Prozess der

Pubertät nachweisen *(Tomikawa et al., 2012; Lomniczi et al., 2013b; rez. in Kurian und Terasawa, 2013; rez. in Semaan und Kauffman, 2013; rez. in Uenoyama et al., 2016)*.
So zeigten Kurian und Kollegen an kultivierten GnRH-Neuronen von Rhesusaffen, dass es durch Abnahme der DNA-Methylierung an CpG-Inseln in der 5`flankierenden Region des *GnRH*-Gens zu verstärkter Transkription von *GnRH* kommt. Dieser epigenetische Prozess konnte sowohl perinatal als auch zu Beginn der Pubertät nachgewiesen werden *(Kurian und Terasawa, 2013)*, beides Zeitpunkte mit erhöhter Aktivität der HHG-Achse.
Neben DNA-Methylierung konnte auch der Einfluss anderer epigenetischer Mechanismen, wie nicht-codierende RNAs und posttranslationale Modifikation der Histone, auf das pubertäts-regulierende Gennetzwerk nachgewiesen werden *(rez. in Lomiczi und Ojeda, 2016a,b)*.
Die Vielzahl der an der Regulation der HHG-Achse beteiligten Elemente und Gene bietet, durch redundante und kompensatorische Pfade des Systems, die Möglichkeit zur funktionellen Integrität im Falle des Funktionsverlustes einer Komponente. Zusätzliche epigenetische Einflüsse erlauben eine flexible Anpassung an sich ändernde Ausgangsbedingungen *(Lomniczi et al., 2013a)*.
Im Folgenden soll das KiSS1/KiSS1R-System und seine entscheidende Schlüsselrolle in diesen komplexen Regelkreisen näher beleuchtet werden.

1.2 Das KiSS1/KiSS1R-System

1.2.1 Aufbau, Entdeckung und Funktion der Gene

Das KiSS1-Gen liegt auf dem langen Arm des Chromosoms 1 (Chr1q32.1) *(West et al., 1998)* und besteht aus drei Exonen, wobei der Translationsstart im zweiten Exon liegt *(www.ncbi.nlm.nih.gov/gene; www.ensembl.org Gene-ID: 3814; Luan et al., 2007).* Es kodiert für Prepro-Kisspeptin, einen aus 145 Aminosäuren (AS) bestehenden Precursor. Durch proteolytische Spaltung entsteht das Kisspeptin-54 als Hauptpeptid mit einer Länge von 54 AS, welches anfänglich unter dem Namen Metastin bekannt wurde *(Kotani et al., 2001; Ohtaki et al., 2001; Muir et al., 2001).* Es wurden weitere Derivate entdeckt und in Abhängigkeit von ihrer Länge Kisspeptin-14, Kisspeptin-13 und Kisspeptin-10 benannt *(Kotani et al., 2001)*. Aufgrund ihrer strukturellen und funktionellen Ähnlichkeit werden sie in der Literatur meist unter dem Begriff Kisspeptin zusammengefasst. Allen gemeinsam ist ein C-terminales Arginin-Phenylalanin-NH_2-Motiv, typisch für die Familie der RF-amid-Peptide *(Tena-Sempere, 2006; Yun et al., 2014).*
Initial wurde *KiSS1* mRNA in wenig metastasierenden Tumorzellen gefunden und aufgrund dieser antimetastatischen Eigenschaft den Tumorsuppressor-Genen zugordnet. Der Name KiSS enthält sowohl den Hinweis auf diese Suppressor-Sequenz (SS) als auch auf seinen Entdeckungsort in Hershey, Pennsylvania, berühmt für seine Hershey`s Chocolate Kisses *(Lee et al., 1996).*
Die Tatsache, dass Kisspeptin in der Plazenta, dem ZNS, einschließlich des Hypothalamus, und in geringerer Menge auch in den Gonaden exprimiert wird *(Ohtaki et al., 2001; Muir et al., 2001; Smith et al., 2005b; Shahab et al., 2005)***,** gibt einen Hinweis darauf, dass es auch in normalen Geweben eine physiologische Funktion erfüllt.
Kisspeptin ist der natürliche Ligand des G-Protein gekoppelten Rezeptors (GPR) KiSS1R *(Kotani et al., 2001)***,** welcher 1999 von Lee und Kollegen als „orphan receptor“ im Gehirn von Ratten

entdeckt wurde. Das Gen für KiSS1R liegt auf dem kurzen Arm des Chromosoms 19, Chr 19p13.3, und besitzt 5 Exone *(Lee et al., 1999).* In älterer Literatur wird der KiSS1R auch als GPR54 bezeichnet. Die Expression von KiSS1R scheint weit verteilt, mit maximalen Expressionsraten in Plazenta, Gonaden und dem ZNS, einschließlich des Hypothalamus. Es konnte gezeigt werden, dass GnRH-Neurone KiSS1R exprimieren *(Messager et al., 2005; Irwig et al., 2005).*

Nach Bindung von Kisspeptin an seinen Rezeptor kommt es über die Aktivierung der Phospholipase C zur Spaltung von Phosphatidylinsositolbisphosphat ($PIP_{2)}$ zu Inositol-1,4,5-triphosphat(IP_3) und Diacylglycerin (DAG). IP_3 sorgt für die Freisetzung von Calcium aus dem endoplasmatischen Retikulum und damit zur Erhöhung der intrazellulären Ca^{2+}-Konzentration. Damit verbunden ist eine Änderung der Permeabilität von Ionenkanälen und die daraus entstehende Depolarisation *(Kotani et al., 2001; Stafford et al., 2002).* Zusätzlich aktiviert DAG die Proteinkinase C, was wiederum zur Phosphorylierung verschiedener mitogenaktivierter Proteinkinasen (MAPKs) führt. Letzteres scheint stark zellkontextabhängig zu sein, erklärt aber zumindest teilweise den antimetastatischen Effekt des KiSS1/KiSS1R-Systems *(Pinilla et al., 2012).*

1.2.2 Bedeutung des KiSS1/KiSS1R-Systems für die GnRH-Funktion

Der Zusammenhang des KiSS1/KiSS1R-Signalkomplexes mit der Regulation der Fortpflanzung und des Pubertätsbeginns wurde im Jahr 2003 von zwei unabhängigen Forschergruppen entdeckt: Mutationen in *KiSS1R* führen beim Menschen zu hypogonadotropen Hypogonadismus *(de Roux et al., 2003; Seminara et al., 2003),* einem Krankheitsbild, dass durch mangelnde hypothalamische GnRH-Ausschüttung und nachfolgend mangelnder hypophysärer Gonadotropinsekretion zu ausbleibender bzw. verzögerter Geschlechtsreife und verringerter Fortpflanzungsfähigkeit führt. In den folgenden Jahren wurden weitere Missense-Mutationen entdeckt, die zu unterschiedlich starker Rezeptorinaktivierung führten *(Semple et al., 2005; Lanfranco et al., 2005).*

Kiss1r knock-out Mäuse zeigten die gleichen Charakteristika wie betroffene Patienten, was auf die hochkonservierte Rolle dieses Signalkomplexes in der Reproduktionskontrolle von Säugetieren hindeutet *(Funes et al., 2003; Seminara et al., 2003).* Es konnte gezeigt werden, dass das Antwortverhalten der Hypophyse auf exogenes GnRH erhalten blieb und es, sowohl bei Mäusen als auch beim Menschen, zu einer adäquaten Ausschüttung von LH und FSH kam *(Seminara et al., 2003).*

Auf der anderen Seite führt eine aktivierende Mutation von *KiSS1R* zu zentraler Pubertas praecox *(Teles et al., 2008).*

Kisspeptin nutzt ausschließlich den KiSS1R als Zielrezeptor. Dies zeigt die Tatsache, dass *Kiss1* knock-out Mäuse ein positiv auf Kisspeptin-Administration ansprachen, während dies bei *Kiss1r* null Mäusen nicht der Fall war *(Lapatto et al., 2007).*

Kisspeptin ist ein sehr starker Stimulator für die Gonadotropin-Sekretion. Dies konnte in einer Vielzahl von Studien an verschiedenen Säugetierarten, einschließlich dem Menschen, gezeigt werden *(Shahab et al., 2005; Han et al., 2005; Gottsch et al., 2004; Matsui et al., 2004; Thompson et al., 2004; Castellano et al., 2005; Dhillo et al., 2005).*

So führte die zentrale und periphere Gabe von Kisspeptin bei Versuchstieren sowohl vor als auch nach Beginn der Pubertät zu einer Aktivierung der HHG-Achse *(Shahab et al., 2005; Han et al., 2005; Gottsch et al., 2004; Matsui et al., 2004; Thompson et al., 2004).* Durch zentrale Verabreichung von Kisspeptin konnte bei untergewichtigen Tieren die Pubertätsentwicklung wiederhergestellt *(Navarro et al., 2004a)* und bei weiblichen Ratten ein vorzeitiger Pubertätsbeginn ausgelöst werden *(Castellano et al., 2005).* Der stimulatorische Effekt des Kisspeptins wird durch eine direkte Aktivierung des, auf den GnRH-Neuronen exprimierten, KiSS1R hervorgerufen**.** Dies belegen Experimente, in denen die Sekretionssteigerung von LH und FSH durch Kisspeptin bei gleichzeitiger Gabe eines GnRH-Antagonisten ausblieb *(Gottsch et al., 2004; Matsui et al., 2004).* Die Aktivierung der GnRH-Neurone durch KiSS1 geschieht im Wesentlichen auf zwei Wegen: erstens durch eine Erhöhung der *KiSS1*-Expressionsmenge und zweitens durch eine zunehmende Sensitivität der GnRH-Neurone auf Kisspeptin *(Han et al., 2005).*

Trotz seiner zentralen Rolle konnte gezeigt werden, dass scheinbar auch für das KiSS1/KiSS1R-Systems, wenn auch in begrenztem Maße, Kompensationsmechanismen bestehen. So erreichten weibliche Mäuse, bei denen die KiSS1-Neuronen in einer frühen Entwicklungsphase abladiert wurden, eine normale Pubertät und waren fertil. Bei späterer, akuter Ablation war dies nicht der Fall *(Mayer und Boehm, 2011)*.

Am Rande sei erwähnt, dass der Effekt von Kisspeptin auf die Gonaden und dessen physiologische Bedeutung noch als unzureichend erforscht und insgesamt eher gering zusammengefasst werden kann *(Pinilla et al., 2012)*. Auch die Funktion während der Schwangerschaft ist noch unklar, obwohl beide Gene in Plazentagewebe in hohen Mengen exprimiert werden *(Seminara et al., 2008).*

Zusammenfassend lässt sich sagen, dass die Identifizierung der Rolle des KiSS1/KiSS1R-Systems, das Verständnis um die Regulationsmechanismen der Pubertät und der Fortpflanzungsfähigkeit revolutioniert hat. Es hat eine wichtige, regulierende Aufgabe und trägt entscheidend zur Integration zentraler und peripherer Einflüsse bei. Dies soll im nächsten Abschnitt näher beleuchtet werden.

1.2.3 Neuroendokrine Funktion von Kisspeptin und der KiSS1-Neuronen

Um die neuroendokrine Funktion des KiSS1/KiSS1R-Systems besser verstehen zu können, lohnt sich ein Blick auf die neuroanatomische Verteilung der KiSS1-Neurone und den sich daraus ergebenden Besonderheiten (Abb.2, S. 53).

Bereits 2005 konnten mittels in-situ-Hybridisierung in Nagern zwei Hauptpopulationen von KiSS1-Neuronen im Hypothalamus identifiziert werden *(Smith et al., 2005a,b)*, zum einen im Nucleus arcuatus (ARC) und zum anderen im Bereich um den Nucleus periventricularis anteroventralis (AVPV), welcher sich als Kontinuum bis in den rostralen Bereich des 3. Ventrikels fortzusetzen scheint *(Clarkson et al., 2006; Herbison et al., 2008).*

Dass diese KiSS1-Neuronenpopulationen unterschiedliche funktionelle Bedeutung haben, wird durch folgende Zusammenhänge deutlich: Es konnte gezeigt werden, dass die synchronisierte Aktivierung der ARC-KiSS1-Neurone hauptverantwortlich für die pulsatile LH-Freisetzung ist,

wobei LH als Surrogatparameter für GnRH zu sehen ist *(Han et al., 2015; Beale et al., 2014).* Des Weiteren exprimieren ARC-KiSS1-Neurone neben Kisspeptin die Neurotransmitter Dynorphin (Dyn) und Neurokinin B (NKB) und deren dazugehörige Rezeptoren, was zu dem Vorschlag führte, diese Neuronengruppe KNDy zu nennen *(Lehman et al., 2010).* Das freigesetzte NKB bindet an andere ARC-KiSS1-Neurone und stimuliert dort die zusätzliche Freisetzung von Kisspeptin. Durch den inhibitorischen Effekt des periodisch und phasenverschoben sezernierten Dynorphins, entsteht ein oszillatorisches Kisspeptin-Sekretionsmuster, welches aktuell als Teil des „Pulsgenerators" betrachtet wird (*Wakabayashi et al, 2010; Navarro et al., 2011; Lomniczi und Ojeda, 2016b).*

Die AVPV-KiSS1-Neurone sind nicht an der pulsatilen GnRH-Freisetzung beteiligt und nehmen erst später Einfluss auf die pubertäre Entwicklung *(Clarkson et al., 2010)*. Ihre Hauptaufgabe liegt in der Estrogen-abhängigen Verstärkung der GnRH-Aktivität und damit der Generierung des zyklischen, präovulatorischen Anstiegs der Gonadotropine bei erwachsenen, weiblichen Individuen *(Clarkson et al., 2009a; Pinilla et al., 2012).*

Ihre Funktion spiegelt sich auch in einem geschlechtsspezifischen Dimorphismus wieder, was bedeutet, dass weibliche Tiere eine deutlich höhere Anzahl dieser Neuronen haben als männliche (*Clarkson et al., 2009b; Kauffman et al., 2007).* Ob ein solcher Dimorphismus auch für die ARC-Population existiert, wird aktuell kontrovers diskutiert *(rez. in Herbison, 2015).*

Verantwortlich für diesen und andere geschlechtsspezifische Differenzierungsvorgänge im Gehirn ist ein perinataler Testosteronanstieg bei männlichen Individuen *(Morris et al., 2004; Kauffman et al., 2007; Homma et al., 2009)*. Es konnte gezeigt werden, dass dieser Vorgang ebenfalls Kisspeptin abhängig ist. So führte eine gezielte Deletion des KiSS1R an GnRH-Neuronen von männlichen Mäusen zu einem signifikant geringeren perinatalen Anstieg an Testosteron im Vergleich zum Wildtyp mit nachfolgender fehlerhafter sexueller Differenzierung des Gehirns *(Clarkson et al., 2014).*

Des Weiteren sind KiSS1-Neurone an der Feedback-Regulation von Sexualhormonen beteiligt. Dabei zeigen beide Neuronengruppen ein gegensätzliches Antwortverhalten *(Smith et al., 2005a,b).* KiSS1-Neuronen im AVPV reagieren auf Estrogen mit zunehmender *Kiss1* Expression. Bei Nagern zeigte sich, dass in der Embryonalphase und zur Geburt in dieser Subgruppe keine *Kiss1* Expression stattfindet, diese aber während der Pubertät stetig zunimmt *(Semaan und Kauffman, 2013).* Der stimulatorische Einfluss des Estrogens auf die AVPV-Kiss1-Neuronen ermöglicht ihnen die Generierung des präovulatorischen LH-Anstiegs *(Semaan und Kauffman, 2013).*

Im Gegensatz dazu ist die Expression von *Kiss1* in den ARC-KiSS1-Neuronen bereits in der Embryonalentwicklung und zur Geburt nachweisbar *(Desroziers et al., 2012)*. Unter dem Einfluss von Estrogen kommt es im ARC zu sinkenden Expressionsmengen von *Kiss1 (Smith et al., 2005a,b).* Auf diese Weise sind die ARC-KiSS1-Neurone an der Vermittlung des negativen Feedbacks der Sexualhormone auf die GnRH-Sekretion beteiligt *(Semaan und Kauffman, 2013).* Nachgewiesen wurde dies an gonadektomierten Versuchstieren *(Navarro et al., 2004b).* So konnte durch beidseitige Gonadektomie bei männlichen wie weiblichen Ratten eine konstante Erhöhung von *Kiss1* mRNA provoziert werden, die einen parallelen Verlauf zur LH-Konzentration zeigte. Durch Gabe von Sexualhormonen konnte dieser Effekt wieder aufgehoben werden. Wie oben erwähnt, zeigt die Neuronenpopulation des AVPV ein gegensätzliches Verhalten. Hier führt die

Gonadektomie bei Mäusen zu einer Zunahme der *Kiss1* mRNA Expression, welche sich nach Gabe von Sexualhormonen wieder reduziert *(Smith et al., 2005a,b).*
Die Estrogenwirkung wird in beiden Neuronengruppen durch den von ihnen exprimierten Estrogenrezeptor alpha (ERα) vermittelt *(Smith et al., 2005b)*. Die bidirektionale Wirkung des Estrogen-ERα-Komplexes scheint durch einen Estrogen-Response-Element-(ERE)-abhängigen Signalweg im AVPV und einen ERE-unabhängigen im ARC zustanden zu kommen *(Uenoyama et al., 2016; Gottsch et al., 2009; Huijbregts und de Roux, 2010).*
Auch an der Integration von Informationen über metabolische Lage und Ernährungsstatus, welche einen entscheidenden Einfluss auf den Beginn der Pubertät und die Fortpflanzungsfähigkeit haben, ist Kisspeptin beteiligt. Bekannt ist, dass GnRH-Neurone keine *(Cunnimgham et al., 1999)* und KiSS1-Neurone nur in geringem Maße Leptin-Rezeptoren exprimieren *(Smith et al., 2006; Donato et al., 2011)*.
Es konnte gezeigt werden, dass der permissive Effekt von Leptin über einen Leptin → α-Melanozyten stimulierendes Hormon (α-MSH) → Kisspeptin → GnRH-Signalweg vermittelt wird *(Manfredi-Lozano et al., 2016).* Dabei führte die zentrale Stimulation von im ARC gelegenen Proopiomelanocortin (POMC)-Neuronen bei Nagern zu einer Aktivierung der HHG-Achse. Die direkte Beteiligung der KiSS1-Neuronen an diesem Signalweg wurde durch zentrale Blockade der α-MSH-Repzetoren MC3/4R nachgewiesen. Dies führte in den ARC-KiSS1-Neuronen zu einer Abnahme der Kiss1-Expression und zu einem verzögerten Eintritt der Pubertät. Die *Kiss1*-Expressionsmenge im AVPV blieb währenddessen unverändert *(Manfredi-Lozano et al., 2016).*
All diese Ergebnisse veranschaulichen, dass das KiSS1/KiSS1R-System eine entscheidende Rolle sowohl bei der zeitlichen Koordinierung des Pubertätsbeginns als auch bei der Erhaltung der Fortpflanzungsfähigkeit hat. Im nächsten Abschnitt soll auf Mechanismen der *KiSS1*-Expressionskontrolle eingegangen werden.

1.3 Mechanismen der *KiSS1*-Expressionskontrolle

1.3.1 Epigenetische Regulation des *KiSS1*-Gens

Auch wenn es nicht unmittelbarer Gegenstand dieser Arbeit ist, soll die epigenetische Kontrolle des *KiSS-1* hier kurz Erwähnung finden, wobei lediglich auf die beiden besser erforschten und synergistisch arbeitenden Mechanismen der DNA-Methylierung und der posttranslationalen Modifikation der Histone eingegangen werden soll. Auch hierbei müssen ARC- und AVPV-KiSS1-Neurone getrennt betrachtet werden.
Lomniczi und Kollegen untersuchten den Einfluss epigenetischer Mechanismen an weiblichen Ratten während des Übergangs von juveniler zu pubertärer Phase *(Lomniczi et al., 2013b)*. Dabei konnten sie nachweisen, dass die DNA-Methylierung der Promotorregionen von *Eed* und *Cbx7* während dieser Phase zunimmt. *Eed und Cbx7* sind zwei Gene aus der Polycomb-Silencing-Gruppe (PcG) und werden in den ARC-KiSS1-Neuronen exprimiert. Die aufgrund der DNA-Methylierung verringerte Expressionsmenge von *Cbx7* und *Eed* führt wiederum zu steigender *Kiss1*-Expression. Dieser Mechanismus der Reduktion des repressiven Einflusses des PcG auf die

Kiss1-Expression konnte nur für den MBH (ARC), nicht jedoch für den POA (AVPV) nachgewiesen werden *(Lomniczi et al., 2013b)*.
Direkte DNA-Methylierung des *Kiss1*-Promotors scheint, zumindest bei Ratten, in beiden Neuronengruppen keine signifikante Rolle während der pubertären Entwicklung zu spielen *(Tomikawa et al. 2012; Lomniczi et al., 2013b).* Interessanterweise ist der repressive Einfluss von DNA-Methylierung auf den humanen *KiSS1*-Promotor im Rahmen der Tumorbiologieforschung nachgewiesen *(Cebrian et al.,2011; Moya et al., 2013)*. Die im humanen Promotor methylierten CpG-Inseln sind im *Kiss1*-Gen von Nagern nicht vorhanden *(Seeman und Kauffman, 2013)*, sodass eine Beteiligung der DNA-Methylierung an der Kontrolle der Pubertät beim Menschen nicht ausgeschlossen werden kann.
Des Weiteren ändert sich der Chromatinstatus des *Kiss1*-Promotors während der pubertären Entwicklung *(Lomniczi et al., 2013b).* So nehmen aktivierende Modifikationen wie die Acetylierung und Trimethylierung des Histon3 (H3K9,14ac und H3K4me3) zu. Interessanterweise kommt es erst im späteren Verlauf der Pubertät, genauer während des präovulatorischen LH-Anstiegs, zur Abnahme reprimierender Histonenmodifikationen wie H3K27me3. Die Existenz bivalenter Histonenmodifikationen in enger räumlicher Nähe am *Kiss1*-Promotor unterstützt die These, dass die Promotoraktivität je nach eingehenden Informationen beeinflusst werden kann *(Lomniczi et al., 2013b)*.
Auch Estrogen spielt im Zusammenhang mit epigenetischer Regulation eine wichtige Rolle. Tomikawa und Kollegen zeigten, dass die präovulatorische Zunahme an Estrogen zu einer vermehrten H3-Actetlyierung am *Kiss1*-Promotor der AVPV-Neuronen und konsekutiv zu steigenden Mengen an Kisspeptin führte *(Tomikawa et al., 2012)*. Im Gegensatz dazu führte Estrogen zu einer Deacetylierung von H3 am *Kiss1*-Promotor in den ARC-Neuronen. Diese bidirektionale Wirkung lässt sich auf unterschiedliche Chromatin-Konformation des *Kiss1*-Gens zurückführen. Durch Estrogen kommt es im ARC und im AVPV zu unterschiedlichen Formen der Chromatin-Schleifenbildung *(Tomikawa et al., 2012; Goto et al., 2015)*, die wiederum die Bildung aktivierender oder reprimierender Komplexe begünstigten. So ist auch die oben bereits erwähnte unterschiedliche ERE-Abhängigkeit des *Kiss1*-Promotors im ARC und im AVPV teilweise zu erklären *(Uenoyama et al., 2016).*
Zusammenfassend lässt sich sagen, dass mit Pubertätsbeginn der repressive Einfluss der PcG-Gene abnimmt und sich der Chromatinstatus von Inhibierung zu Aktivierung verschiebt *(Lomniczi et al., 2016b)*.

1.3.2 Regulation durch verschiedene Transkriptionsfaktoren

Neben der epigenetischen Kontrolle hat auch die Regulation durch übergeordnete, transkriptionelle Faktoren einen wichtigen Einfluss auf die *KiSS1*-Expression.
Ojeda und Kollegen schlugen 2006 in einem systembiologischen Ansatz vor, dass es sich bei *KiSS1* und *KiSS1R* um untergeordnete Gene handelt, die in einem hierarchisch aufgebauten Netzwerk durch übergeordnete, zentrale Gene auf transkriptioneller Ebene reguliert werden *(Ojeda et al., 2006).*

Tatsächlich konnte durch high-throughput DNA Arrays bei Ratten und Rhesusaffen der Beweis für die Existenz eines solchen Netzwerkes, welches die Pubertät auf einer übergeordneten Ebene zu regulieren scheint, geführt werden *(Roth et al., 2007).* Es wurde gezeigt, dass zum Zeitpunkt der Pubertät die Expression einiger Gene im Hypothalamus deutlich zunimmt, wobei eine bestimmte Gruppe überpräsentiert war. Diese erfüllen zwar unterschiedliche zelluläre Funktionen, gemeinsam ist ihnen jedoch, dass sie initial den Tumorsuppressor-Genen (TSG) zugeordnet wurden. Computeranalysen ergaben, dass die Kontrolle dieser Gene in einem Netzwerk hierarchisch arrangiert ist und durch einige zentrale, scheinbar übergeordnete Gene reguliert wird *(Roth et al., 2007). KiSS1* gehört, als untergeordnetes Gen ebenfalls zu diesem Netzwerk *(Ojeda et al., 2010)* (Abb.1, S.52).
In anderen Studien konnte gezeigt werden, dass *TTF1(Mastronardi et al., 2006), EAP1 (Heger et al., 2007)* und *OCT2 (Ojeda et al., 1999)* als nicht-TSG-Gene an der transkriptionellen Kontrolle der Pubertät beteiligt sind.
Weitere Computeranalysen konnten nicht nur starke Interaktionen dieser nicht-TSG mit dem oben erwähnten Netzwerk zeigen, sondern erbrachten Hinweise, dass zumindest *TTF1* und *OCT2* diesem sogar übergeordnet sind *(Roth et al., 2007).*
Aus diesen Überlegungen ergeben sich Kandidatengene für die transkriptionelle Kontrolle von *KiSS1*. Auf der einen Seite sind dies *TTF1* und *EAP1*, als Vertreter der nicht-TSG und auf der anderen Seite zwei Gene des TSG-Netzwerkes, *YY1* und *CUTL1* (in neuerer Literatur *CUX1*).
TTF1 (Transcription Factor 1) gehört zur Familie der NKX Homeoboxgene *(Civitareale et al., 1989; Guazzi et al., 1990)* und steuert unter anderem die Entwicklung des Dienzephalons *(Kimura et al., 1996).* Postnatal bleibt die *TTF1* Expression besonders in den Hirnarealen, die die Pubertät und die Fortpflanzungsfähigkeit kontrollieren, erhalten *(Lee et al., 2001)*. Durch in situ Hybridisierung konnte dies auch für KiSS1-Neurone nachgewiesen werden *(Mastronardi et al., 2006).*
TTF1 bindet und transaktiviert den *GnRH*-Promotor, aber unterdrückt die Transkription des Preproenkephalin-Gens *(Lee et al., 2001),* welches eine inhibierende Wirkung auf die GnRH-Neurone ausübt *(Ojeda und Skinner, 2006).*
In späteren Studien konnte gezeigt werden, dass es durch konditionelle Deletion des *Ttf1*-Gens in hypothalamischen Neuronen bei Mäusen zu einem verzögerten Pubertätsbeginn und einer verminderten Reproduktionsfähigkeit kommt *(Mastronardi et al., 2006).*
EAP1 (Enhanced At Puberty 1) ist ein intronloses Gen, welches auf dem Chromosom 14q24.3 liegt und ursprünglich als *C14ORF4* bezeichnet wurde *(Rampazzo et al., 2000).* Heger und Kollegen konnten 2007 die Expression dieses Gens mittels DNA-Mikroarrayuntersuchungen im Gehirn sich entwickelnder weiblicher Rhesusaffen nachweisen und zeigen, dass diese zu Beginn der Pubertät deutlich anstieg, was zur Umbenennung des Gens führte *(Heger et al., 2007)***.** *EAP1* kodiert für ein nukleäres Protein mit einer dualen, promotorabhängigen, transkriptionellen Aktivität. So führt EAP1 zur Aktivierung des *GnRH*-Promotors und, wie auch TTF1, zur Hemmung des Preproenkephalin-Promotors *(Heger et al., 2007).* Versuche mit small-interference RNA demonstrierten, dass eine erniedrigte Expression von *Eap1* zu einem verzögerten Pubertätsbeginn, einer gestörten Zyklusfunktion und erniedrigten Gonadotropin- und Estrogenspiegeln bei weiblichen Ratten führte *(Heger et al., 2007).*

CUX1 (Cut homeobox 1, auch bekannt als CCAAT displacement protein CDP) ist ein konserviertes Homeoboxgen und kodiert für einen Transkriptionsfaktor *(Xu und Li, 2016).* Dieser kann, abhängig vom zellulären Kontext, zur Aktivierung oder zur Repression der Transkription führen *(Dufort und Nepveu, 1994; Superti-Furga et al., 1989; Valarche et al., 1993; Harada et al., 2008; Kim et al., 1997*). Auch die, durch proteolytische Spaltung entstehenden, Isoformen p200 und p110 scheinen diesen dualen Charakter zu teilen *(Sansregret und Nepveu, 2008; Goulet et al., 2002).* Ein Verlust der *Cux1*-Genfunktion führte bei männlichen Mäusen zu einer schweren Einschränkung der Fortpflanzungsfähigkeit *(Luong et al., 2002)***.**
YY1 (Yin Yang 1) ist ein ubiquitär vorkommender Transkriptionsfaktor *(Gordon et al., 2006),* der zum Polycomb-Silencing-Komplex gehört *(Schwartz und Pirrotta, 2007).* Er hat die Fähigkeit, Transkription zu initiieren, zu aktiveren oder zu unterdrücken, immer abhängig vom Kontext, in dem er bindet. Dies kann über direkte oder indirekte Aktivierung oder Repression mit oder ohne Bindung von Co-Faktoren geschehen. YY1 spielt eine fundamentale Rolle bei normalen biologischen Prozessen wie Embryogenese, Replikation, Zelldifferenzierung und –proliferation *(Gordon et al., 2006).*

1.4 Zielsetzung

Die zentrale Bedeutung des *KiSS1* für die Kontrolle der Pubertät und den Erhalt der Fortpflanzungsfähigkeit ist hinreichend belegt. Auch konnten in den letzten Jahren viele Erkenntnisse sowohl bezüglich der Physiologie der neuroendokrinen Funktionen von Kisspeptin als auch der Neuroanatomie der KiSS1-Neurone gewonnen werden. Über die transkriptionelle Kontrolle des *KiSS1*-Gens im Hypothalamus und deren Zusammenhänge mit dem Zeitpunkt des Pubertätsbeginns ist wenig bekannt.
Ziel dieser Dissertation war es, die Promotorregion des *KiSS1*-Gens durch funktionelle Analysen genauer zu untersuchen. Die dabei gestellten Zielsetzungen lassen sich wie folgt zusammenfassen:

Arbeit 1:

1. Identifizierung der Transkriptionsstartstelle (TSS) durch experimentelle Untersuchung der 5`flankierenden Region als Voraussetzung für funktionelle Analysen
2. Identifizierung, Klonierung und funktionelle Analyse der putativen Promotorregion in neuronalen und nicht-neuronalen Zelllinien
3. Überprüfung des Einflusses der übergeordneten Transkriptionsfaktoren TTF1, EAP1, CUX1 und YY1 auf die Promotoraktivität durch funktionelle Analysen

Arbeit 2:

4. Nachweis der Bedeutung der TTF1-Bindungsstelle für die *KiSS1*-Promotoraktiviät durch gerichtete Promotormutagenese mit anschließender funktioneller Analyse

2. Originalarbeiten

2.1 Arbeit 1: Transcriptional regulation of the human *KiSS1* gene

Im Rahmen dieser Arbeit erfolgte die Klonierung der putativen Promotorregion und experimentelle Identifizierung der Transkriptionsstartstelle des *KiSS1*-Genes. Die funktionelle Analyse des Promotors erfolgte in nicht-neuronalen und neuronalen Zelllinien mittels Dual-Luciferase-Reporter-Assays. Es wurde sowohl der Kernpromotor identifiziert als auch dessen basale Aktivität bestimmt. Anschließend wurde das Antwortverhalten bei Anwesenheit der oben genannten Transkriptionsfaktoren untersucht.
Mittels Chromatin-Immunopräzipitationsassay (ChIP) wurde die Rekrutierung der Transkriptionsfaktoren an den *KiSS1*-Promotor im Zellmodell nachgewiesen.

2.2 Arbeit 2: Deletion of the *Ttf1* Gene in Differentiated Neurons Disrupts Female Reproduction without Impairing Basal Ganglia Function

Hauptanliegen dieser Arbeit war es, die Beteiligung von TTF1 an der Kontrolle des Pubertätsbeginns und der Fortpflanzungsfähigkeit zu untersuchen. Es wurde für die Deletion des *Ttf1*-Gens ein konditionelles knock-out Mausmodell gewählt, da konventionelle knock-out Mäuse tot geboren werden *(Kimura et al., 1996).* Zusammenfassend lässt sich sagen, dass diese Tiere eine normale Entwicklung und eine normale Morphologie und Funktion der Basalganglien hatten, jedoch eine verspätete Pubertät und eine reduzierte Fortpflanzungsfähigkeit zeigten.
Im Rahmen dieser Arbeit wurde auch die Wirkung von TTF1 auf den *KiSS1*-Promotor getestet. Durch gerichtete Mutagenese einer bzw. beider vermuteten TTF1-Bindungsstellen im klonierten Promotorkonstrukt und anschließender funktioneller Analyse mittels Dual-Luciferase-Reporter-Assays konnte die funktionelle Bedeutung der TTF1-Bindung an den Promotor untermauert werden.

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Transcriptional regulation of the human *KiSS1* gene

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ABSTRACT

Kisspeptin, the product of the *KiSS1* gene, has emerged as a key component of the mechanism by which the hypothalamus controls puberty and reproductive development. It does so by stimulating the secretion of gonadotropin releasing hormone (GnRH). Little is known about the transcriptional control of the *KiSS1* gene. Here we show that a set of proteins postulated to be upstream components of a hypothalamic network involved in controlling female puberty regulates *KiSS1* transcriptional activity. Using RACE-PCR we determined that transcription of *KiSS1* mRNA is initiated at a single transcription start site (TSS) located 153–156 bp upstream of the ATG translation initiation codon. Promoter assays performed using 293 MSR cells showed that the *KiSS1* promoter is activated by TTF1 and CUX1-p200, and repressed by EAP1, YY1, and CUX1-p110. EAP1 and CUX-110 were also repressive in GT1-7 cells. All four TFs are recruited *in vivo* to the *KiSS1* promoter and are expressed in kisspeptin neurons. These results suggest that expression of the *KiSS1* gene is regulated by trans-activators and repressors involved in the system-wide control of mammalian puberty.

1. Introduction

It is well established that an increase in pulsatile gonadotropin-releasing hormone (GnRH) release from GnRH neurons is required for the initiation of puberty. The pubertal increase in GnRH secretion is determined by changes in transsynaptic (Kordon et al., 1994; Ojeda and Terasawa, 2002) and glial (Ojeda et al., 2003; Ojeda and Terasawa, 2002) inputs to the GnRH neuronal network. The transsynaptic control of GnRH neurons has two components: an excitatory arm that is activated at puberty and an inhibitory arm that loses strength at the time when the excitatory inputs to GnRH neurons are increasing (Ojeda and Terasawa, 2002; Plant and Witchel, 2006; Terasawa and Fernandez, 2001).

Although glutamatergic neurons have been known for many years to be major contributors to the excitatory transsynaptic system controlling GnRH secretion (Brann, 1995; Ojeda and Skinner, 2006; Plant and Witchel, 2006), compelling evidence now exists demonstrating the existence of another, more critical set of neurons providing excitatory inputs to the GnRH neuronal network. These neurons use a peptide known as kisspeptin for neurotransmission, and the G protein-coupled receptor GPR54 for signaling. While inactivating mutations of the GPR54 gene result in hypogonadotrophic hypogonadism (HH) in humans (De Roux et al., 2003; Seminara et al., 2003; Tenenbaum-Rakover et al., 2007; Semple et al., 2005), activating mutations and certain types of polymorphisms of this gene have been reported to be associated with precocious puberty (Luan et al., 2007; Teles et al., 2008). Both *Kiss1* and *Gpr54* null mice fail to undergo sexual development; they are infertile and have reduced gonadotropin levels (Seminara et al., 2003; Lapatto et al., 2007). These and other observations demonstrate that an intact KiSS1/GPR54 signaling system is mandatory for normal reproductive maturation and fertility in mammals [reviewed in D'Anglemont de Tassigny et al. (2007) and Oakley et al. (2009)].

Kisspeptin is a product of the *KiSS1* gene which encodes a 145 amino acid precursor. Post-translational modifications of this peptide result in a C-terminally amidated 54-amino acid peptide and several shorter fragments (e.g. kisspeptin-10, kisspeptin-13, kisspeptin-14) (Ohtaki et al., 2001). The first biological actions assigned to the KiSS1 system were its capacity to inhibit cell migration and tumor metastasis (Ohtaki et al., 2001; Lee et al., 1996). For this reason the 54-amino acid peptide is also known as metastin.

Although kisspeptin plays a pivotal role of in the hypothalamic control of reproductive function, the transcription factors controlling *KiSS1* expression have not been identified. Using a high-throughput approach (DNA arrays) we used rats and rhesus

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monkeys to provide evidence for the existence of a gene network that, according to our results, contributes to the hypothalamic control of puberty (Roth et al., 2007). Genes composing this network have diverse cellular functions, but they share the common feature of having been earlier identified as involved in tumor suppression/tumor formation (Roth et al., 2007). Computational analysis revealed that the *KiSS1* gene is a subordinate member of this tumor suppressor gene (TSG) network. As such, *KiSS1* was predicted to be under the transcriptional control imposed by central TSG hubs and to be regulated by non-TSG genes. These non-TSG genes, which are also connected to the TSG network, are involved in the transcriptional regulation of the pubertal process.

In the present study, we verified this computational prediction by determining if the human *KiSS1* gene is transcriptionally regulated by two postulated central hubs of the TSG network, CCAAT displacement protein (CDP, also known as CUTL1 and CUX1) and Ying Yang 1 (YY1) (Roth et al., 2007), in addition to two non-TSGs previously shown to be transcriptional regulators of puberty, enhanced at puberty 1 (EAP1) (Heger et al., 2007) and thyroid transcription factor 1 (TTF1) (Mastronardi et al., 2006). *CUTL1* (henceforth called *CUX1*) is an evolutionary conserved homeobox gene encoding a protein that functions as a transcriptional repressor in several systems (Nepveu, 2001), but that can be proteolytically processed into isoforms with trans-activational capabilities (Sansregret and Nepveu, 2008). Due to this, CUX1 can either repress (Dufort and Nepveu, 1994; Superti-Furga et al., 1989; Valarche et al., 1993) or activate gene transcription (Harada et al., 2008; Kim et al., 1997). *Cux1*-null mutant male mice are subfertile (Luong et al., 2002). In our studies we utilized two isoforms of CUX1, p200, and p110, which have been previously shown to have divergent transcriptional activity in breast cancer cells (Goulet et al., 2002). YY1 is a member of the Polycomb (PcG) group silencing complex and as such, it plays an important role in gene silencing, because it recruits PcG repressive proteins in addition to histone 3 trimethylated at lysine 27 (H3K27me3) to gene promoters to silence transcription (Wilkinson et al., 2006; Woo et al., 2010). EAP1 is a newly identified transcription factor that displays mostly repressive activity, and that is required for both the timing of puberty and normal female reproductive cyclicity (Heger et al., 2007; Roth et al., 2007). Finally, TTF1 (NKX2.1) is a member of the NKX family of homeobox genes (Pera and Kessel, 1998; Price et al., 1992) required for embryonic development of the diencephalon and pituitary gland (Kimura et al., 1996). Studies involving peripubertal female rats demonstrated that *Ttf1* expression persists in hypothalamic regions involved in the control of reproductive development (Lee et al., 2001). Within these regions, kisspeptin neurons were later identified as cells that express *Ttf1* (Mastronardi et al., 2004). Conditional deletion of the *Ttf1* gene from neurons leads to a delayed onset of puberty and reduced fertility in female mice (Mastronardi et al., 2004).

Here we report that TTF1, YY1, EAP1, and CUX1 interact with the *KiSS1* promoter *in vivo*, and that Eap1, Yy1, and Cux1 are expressed in kisspeptin neurons of the rodent hypothalamus. We also show that the *KiSS1* gene is trans-activated by TTF1 and repressed by YY1, EAP1, and CUX1-p110. CUX-p200 exhibits a dual transcriptional activity depending on the cell context. These results suggest that *KiSS1* expression depends on a balance between trans-activators and repressors involved in the system-wide hypothalamic control of puberty.

2. Materials and methods

2.1. Identification of the human KiSS1 transcription start site

To map the transcription start site (TSS) of *KiSS1*, total RNA was isolated from human placental tissue (kindly provided by R. Faber, Leipzig, Germany) using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The integrity of the RNA was verified by agarose gel electrophoresis. Five-prime rapid amplification of cDNA ends (RACE) was performed using the GeneRacer kit (Invitrogen, Karlsruhe, Germany). Briefly, 1 μg of total human placental RNA was treated with calf intestinal phosphatase (provided with the kit) to dephosphorylate truncated mRNAs and non-mRNA species. Subsequent treatment with tobacco acid phosphatase removed the 5′ cap structure from intact, full-length mRNAs. The GenRacer RNA oligodeoxynucleotide was ligated to pre-treated human placental RNA using the T4 RNA ligase provided with the kit. Linked RNA was reversed transcribed (1 h at 37 °C followed by 5 min at 93 °C) using an oligo-DT primer (Omniscript Kit, Qiagen). PCR amplification utilizing HotStarTaq DNA Polymerase (Qiagen) and the primers 1F/R (Table 1) were used for hot start touchdown PCR employing the following conditions: 15 min at 95 °C, 30 s at 94 °C, and 4 min at 72 °C for 5 cycles, 30 s at 94 °C followed by 4 min at 70 °C for 5 cycles, and 30 s at 94 °C followed by 4 min at 68 °C for 20 cycles. Final extension was 10 min at 72 °C. The amplicon was used as a template for nested PCR (primers 2F/R, Table 1). The PCR product was visualized on a 1% agarose gel and cloned into pCRII® TOPO®-Vector (TOPO TA Cloning Kit, Invitrogen). DH5-α competent cells (Invitrogen) and XL1-Blue super competent cells (Stratagene, Heidelberg, Germany) were used for transformation. Seventeen clones were analyzed by sequencing using the primer provided with the kit. To confirm the results, the experiment was repeated using random hexamer primers for reverse transcription, pGEM®-T Easy Vector (Promega, Mannheim, Germany) for PCR product cloning, and XL1-Blue cells for transformation. The use of human tissue has been approved by the Ethical Committee of the University of Leipzig, Germany.

2.2. Gene reporter constructs

To generate reporter constructs containing different lengths of the *KiSS1* 5′-flanking region, DNA fragments were amplified from human genomic DNA (Promega) using the primers 3F/R and 4F/R (Table 1). PCR products of a length of 556 and 1339 bp were cloned into the pCRII® TOPO®-Vector (Invitrogen). To generate a 1975 bp long DNA fragment, a 951 bp PCR-product, amplified by primers 5F/R (Table 1), was subcloned into the pCRII® TOPO®-Vector (Invitrogen). From there a 636 bp fragment was excised with HpaI and KpnI and cloned into the HpaI and KpnI sites of pCRII® TOPO®-*KiSS1*p1339 utilizing the Rapid Ligation Kit (Fermentas, St. Leon-Rot, Germany). The sequence and orientation of each construct (pCRII® TOPO®-*KiSS1*p1975, pCRII® TOPO®-*KiSS1*p1339, and pCRII® TOPO®-*KiSS1*p556) were verified by automatic sequencing, and the fragments were subcloned into the KpnI and XhoI sites of the luciferase pGL4.10 basic vector (Promega).

2.3. Expression vectors

Human EAP1: To determine if EAP1 regulates *KiSS1* gene expression, we used an expression vector containing the coding region of *EAP1* mRNA (Heger et al., 2007). A mutated EAP1, lacking the RING finger domain (Heger et al., 2007; Waterman et al., 1999) was used to determine the structural requirements for EAP1 action on *KiSS1* transcription.

Human YY1: To express *YY1*, a construct containing the *YY1* coding region tagged with a hemagglutinin epitope (HA) was excised with XbaI and ApaI from pCMV-HAYY1 (kindly provided by Y. Shi, Harvard Medical School, Boston, USA), and cloned into pcDNA3.1$^+$/zeo. The sequence of the construct was verified by DNA sequencing.

Human CUX1: We used two different *CUX1* constructs, both cloned into the vector pXJ42 (both kind gifts from A. Nepveu, Goodman Cancer Center and Departments of Oncology,

Table 1
Primer sequences.

Name	Sequence	Position according to Accession No. NT_004487
Primer1F	5′-CGA CTG GAG CAC GAG GAC ACT GA-3′	Provided by GenRacer Kit
Primer1R	5′-CTC TCG GTG CAC GGC AGG CTC T-3′	bp 54,650,216–54,650,237
Primer2F	5′-GGA CAC TGA CAT GGA CTG AAG GAG TA-3′	Provided by GenRacer Kit
Primer2R	5′-GAG GCC CAG TTC TAG CTG CT-3′	bp 54,650,254–54,650,276
Primer3F	5′-AGA ATC TCT GCC ACC ACC AC-3′	bp 54,656,479–54,656,503
Primer3R	5′-GCT GGG CTC CCG GTC TCA AG-3′	bp 54,655,947–54,655,966
Primer4F	5′-GGA CAG GCC AAC GTA CAC ATC ATC-3′	bp 54,657,263–54,657,286
Primer4R	5′-GCT GGG CTC CCG GTC TCA AG-3′	bp 54,655,947–54,655,966
Primer5F	5′-CTT GAA CTT GGA TCA TTG GTT GAG-3′	bp 54,657,899–54,657,922
Primer5R	5′-AGG GCA GAG ACT GTT TCT TCT ATC-3′	bp 54,656,972–54,656,995
		Position according to TSS = +1 Accession No. NM_002256
Primer6F	5′-CCC CCG CAC CTT CTC CAT TTG A-3′	bp −2456 to −2434
Primer6R	5′-CCG CAC TTA GCC AGA TCC CCA GAA-3′	bp −2288 to −2264
Primer7F	5′-TGT CCC TGT CCT CAA AGT GCT GTA-3′	bp −839 to −816
Primer7R	5′-CTT CCT TCC TGC TTC CCT TCT TTC-3′	bp −562 to −538
Primer8F	5′-CGG CCC CGG GTG TCG TT-3′	bp 11,149–11,166
Primer8R	5′-CTG GGC AGT GTG GGG TTA TTT TCT-3′	bp 11,225–11,249

Primer 1F/1R and 2F/2R were used for RACE-PCR, Primer 3F/3R, 4F/4R, and 5 F/5R for promoter cloning, and Primer 6F/6R to 8F/8R for PCR detection of chromatin immunoprecipitated DNA.

Biochemistry and Medicine, McGill University, Montreal, Canada). The pXJ42/MCH construct contains the full-length *CUX1* (termed p200); pXJ42/MCH/878-1505 contains *CUX1* isoform p110. Both isoforms differ in length and transcriptional activity. P200 has been reported to be inhibitory, while p110 exhibit either repressive or trans-activating activity, depending on the promoter context (Sansregret and Nepveu, 2008).

Rat TTF1: rTTF1 was expressed from a pcDNA3.1$^+$/zeo-rTTF1 construct containing the rTTF1 coding region (Lee et al., 2001).

Human SP1: hSP1 (2891 bp) was purchased from Deutsches Ressourcenzentrum für Genomforschung GmbH (RZPD, Berlin, Germany), cleaved out from the pOTB7-hSP1 vector by EcoRI and XhoI, and cloned into pcDNA3.1/zeo.

2.4. Cell culture

GripTite™ 293 MSR cells (Invitrogen) were cultured in a humidified atmosphere containing 5% CO_2 and 37 °C. They were maintained in Dulbecco's modified Eagle's medium containing high glucose (4.5 g/L; PAA Laboratories, Pasching, Germany), supplemented with 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany), 0.1 mM non-essential amino acids (PAA Laboratories), and 600 μg/ml G-418 sulfate (PAA Laboratories).

GT1-7 cells (kind gift of Pam Mellon, Department of Reproductive Medicine and Center for Reproductive Science and Medicine, University of California, San Diego, La Jolla, CA, USA), were cultured in Dulbecco's modified Eagle's medium containing high glucose (4.5 g/L; PAA Laboratories), supplemented with 10% FBS (Biochrom), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

HeLa cells (LGC Standards GmbH, Wesel, Germany) were grown in a humidified atmosphere containing 5% CO_2 and 37 °C. The culture medium was Dulbecco's modified Eagle's medium containing high glucose (4.5 g/L; PAA Laboratories), supplemented with 10% fetal bovine serum, 2 mM glutamine (PAA Laboratories), 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.5. Transient transfection and dual-luciferase promoter assays

The luciferase reporter constructs used in these assays contained fragments of the *KiSS1* gene 5′-flanking region of different lengths: −1948 to +27 (1975 bp); −1312 to +27 (1339 bp); or −529 to +27 (556 bp). The transregulatory effects of TTF1, EAP1, YY1, and CUX1 on the *KiSS1* promoter activity were examined in the human embryonic kidney cell line GripTite™ 293 MSR, and GT1-7 cells (mouse immortalized gonadotropin-releasing hormone neurons). The cells were seeded in antibiotic-free medium at a density of 2×10^5 cells per well in 24-well dishes (Greiner bio-one, Frieckenhausen, Germany) 18 h before transfection. Each of the reporter plasmids was transiently transfected (at 500 ng/well) for 4 h using Nanofectin (PAA Laboratories) for GripTite™ 293 MSR, or TurboFect™ *in vitro* Transfection Reagent (Fermentas) for GT1-7 cells, in conjunction with either the empty expression vector (pcDNA3.1zeo, Invitrogen at 500 ng/well) or vectors expressing EAP1, TTF1, YY1, or CUX1. Each well received a total of 0.5 ml of culture medium. Transfection efficiency was normalized by co-transfecting the *Renilla* plasmid pGL4.70 [hRluc] (Promega) at 20 ng/well.

Cells were harvested 48 h after transfection and lyzed in lysis buffer (Promega). Luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) in a luminometer according to the manufacturer's protocol. Relative firefly luciferase activities were calculated following correction for transfection efficiency using the *Renilla* luciferase activities as the normalizing unit. To ensure that the transcriptional effects observed were exerted on the *KiSS1* promoter and not on the pGL4.1 basic vector, basal pGL4.1 luciferase activity was measured after transfecting the cells with each of the four TFs examined. Experiments were performed three times using three or four wells per group each time.

2.6. Chromatin immunoprecipitation (ChIP) assay

To determine the association of EAP1 and TTF1 to the *KiSS1* promoter, we transfected HeLa cells with either an expression vector carrying the *EAP1* coding region tagged with an influenza hemagglutinin (HA) epitope (kindly provided by H. Samuels, New York University School of Medicine, New York) or the rat TTF1 construct described in Section 2.3. Subsequent immunoprecipitation was performed using antibodies that recognize either the HA tag of the EAP1 construct or rat TTF1 itself (see below). In the case of YY1 and CUX1, we used antibodies against the endogenous YY1 and CUX1 proteins (see below). For transfection, HeLa cells

(25×10^6) were seeded in 15 cm diameter plates, and 24 h later 45 μg of each expression vectors were transiently transfected for 5 h using Lipofectamine LTX (Invitrogen). Forty-eight hours after transfection, the cells were harvested for chromatin immunoprecipitation. They were washed once in ice-cold PBS containing a protease inhibitor cocktail (1 mM phenylmethylsulfonylfluoride, 7 μg/ml aprotinin, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin), a phosphatase inhibitor cocktail (1 mM β-glycerophosphate, 1 mM sodium pyrophosphate, and 1 mM sodium fluoride), an HDAC inhibitor (20 mM sodium butyrate), and a tyrosine phosphatase inhibitor (1 mM). Cross-linking was performed by incubating the cell suspension in 1% formaldehyde for 10 min at room temperature. After two additional washing steps in PBS the cells were lysed with 200 μl SDS buffer (1% SDS, 50 mM Tris–HCl, and 10 mM EDTA) containing protease, phosphatase, and HDAC inhibitors and sonicated to yield chromatin fragments of 500–1000 bp using a Kontes Micro Ultrasonic Cell Disruptor (Vineland, NJ, USA) (power 4.5 and tune 1.5). Size fragmentation was confirmed by agarose gel electrophoresis. The sonicated chromatin was clarified by centrifugation at 14,000 rpm for 10 min at 4 °C, brought up to 1 ml in chip dilution buffer (CDB) (16.7 mM Tris–HCl, pH 8.1, 150 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, and 0.01% SDS) containing protease, phosphatase, and HDAC inhibitors and stored at −80 °C for subsequent immunoprecipitation. For this step, chromatin was pre-cleared with Protein A/G beads (Dynabeads, Invitrogen, Carlsbad, CA) for 1 h at 4 °C. One aliquot (200 μl of 1000 μl) of the cleared chromatin was stored at −80 °C as input DNA. Aliquots (200 μl) of the remaining chromatin were then incubated with 5 μg of a mouse monoclonal antibody against HA (Covance, Berkeley, CA) to detect EAP1, rabbit polyclonal antibodies against TTF1 (kindly provided by C. Mendelson, Southwestern Medical Center, Dallas, TX, USA), a mouse monoclonal antibody (sc-7341 X, Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect YY1, or rabbit polyclonal antibodies (sc-13024, rabbit polyclonal, Santa Cruz Biotechnology) to detect CUX1, each reaction in final volume of 1 ml CDB. Two additional aliquots of chromatin were incubated with either normal mouse IgG (sc-2025, Santa Cruz Biotechnology) or rabbit IgG (sc-2027, Santa Cruz Biotechnology) and used as negative controls. Antibody-chromatin complex mixtures were incubated at 4 °C overnight with gentle agitation. Immunocomplexes were then collected by adding 25 μl of protein A or G beads solution (Dynabeads) and incubated for 2 h at 4 °C with gentle agitation. Immunocomplexes were washed sequentially with 0.5 ml low salt wash buffer (20 mM Tris–HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS), high salt wash buffer (20 mM Tris–HCl, pH 8.1, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS), LiCl buffer (10 mM Tris–HCl, pH 8.1, 250 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 1 mM EDTA), and with TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Immunocomplexes were eluted with 300 μl of 0.1 M $NaHCO_3$ and 1% SDS at room temperature for 30 min. Cross-linking was reversed by adding 20 μl of 5 M NaCl and incubating at 65 °C overnight. Proteins were then digested by adding 6 μl of proteinase K (20 mg/ml), 6 μl of 0.5 M EDTA, and 6 μl of Tris–HCl, pH 6.5, and incubating the mixture for 2 h at 55 °C. DNA was recovered by using the QIAquick PCR purification Kit (Qiagen, Germantown, MD) and stored at −80 °C until subsequent PCR analysis. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.7. PCR detection of chromatin immunoprecipitated DNA

To detect CUX1, EAP1, YY1, and TTF1 binding to the promoter region of the *KiSS1* gene we PCR-amplified, using Hot Star Taq polymerase (Qiagen), a DNA fragment derived from the *KiSS1* proximal promoter (NM_002256). As controls for the ChIP assays, we amplified an upstream fragment located more than 2000 bp from the gene's TSS, and a second segment located in intron 2. The regions amplified were (1) a 192 bp segment (amplicon 1) located at position −2456 to −2264 from the *KiSS1* TSS, (2) a 301 bp segment (amplicon 2) located at −839 to −538, and (3) a 100 bp fragment (amplicon 3) located in intron 2 (nucleotides 11,149–11,249 in the *KiSS1* genomic sequence). Amplicon 1 was generated using primers 6F/R, amplicon 2 with primers 7F/R, and amplicon 3 with primers 8F/R (Table 1). All primers were purchased from Eurofins MWG Operon, Huntsville, AL. The thermocycling conditions used were 95 °C for 5 min, followed by 31 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min. PCR products were resolved in a 1.5% agarose gel, stained with ethidium bromide.

2.8. Immunohistofluorescent detection of EAP1, YY1, and CUX1 in kisspeptin neurons

To determine if kisspeptin neurons in the rat hypothalamus contain Yy1, Eap1, and/or Cux1, we detected kisspeptin neurons with a sheep antibody against kisspeptin (diluted 1:50,000) (Ramaswamy et al., 2008). Eap1 was detected using rabbit polyclonal antibodies (diluted 1:3000) (Heger et al., 2007); Cux1 and Yy1 were detected using mouse monoclonal antibodies (Abcam, Cambridge, MA, USA; 1:1000, and Santa Cruz; 1:3000, respectively). Frozen sections (30 μm) obtained from brains perfusion-fixed with 4% paraformaldehyde–PBS, pH 7.4, were mounted on Superfrost glass slides, dried for 2 h under an air stream and subjected to an antigen retrieval protocol (Shi et al., 1991a). Thereafter, the sections were incubated for 48 h at 4 °C with the primary antibodies indicated above. At the end of this period, kisspeptin immunostaining was visualized using Alexa 488-donkey antisheep IgG (1:500; Invitrogen), Eap1 staining with Alexa 594-donkey anti-rabbit IgG (1:500), and Cux1 staining with Alexa 568 donkey anti-mouse IgG (1:500). Yy1 staining was developed using biotinylated donkey antimouse IgGs (1:250; Invitrogen) followed by incubation with Streptavidin-Alexa 568 (1:500; Invitrogen). Before this incubation, the sections were subjected to the biotyramine enhancement method, as previously described (Jung et al., 1997). Fluorescent images were acquired as reported (Heger et al., 2007). The specificity of the TTF1, EAP1, and kisspeptin antibodies used has been reported in earlier publications from either our laboratory (Mastronardi et al., 2006; Heger et al., 2007) or others investigators (Ramaswamy et al., 2008). The specificity of the YY1 and CUX1 antibodies is described by the respective manufacturers (Santa Cruz and AbCAM, respectively). We also verified that the pattern of expression of these two proteins in the hypothalamus corresponds to that of their respective mRNA detected by *in situ* hybridization (Allan Mouse Brain Atlas; http://mouse.brain-map.org). In the case of CUX1, we stained brain sections using rabbit polyclonal antibodies (Ellis et al., 2001), kindly provided by M. Busslinger (Research Institute of Molecular Pathology, Vienna, Austria) and found a pattern of expression identical to that seen with the commercial monoclonal antibody (not shown). In addition, for every reaction were included sections incubated without the first antibodies. Animal usage was duly approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center, USA.

2.9. PCR detection of transcription factors in different cell lines

We used reverse transcription (RT)-PCR to assess the presence of *KiSS1*, *EAP1*, *CUX1*, *YY1*, and *TTF1* mRNAs in the three cell lines used for study (GripTite™ 293 MSR, GT1-7, and HeLa cells). Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and RNA integrity was assessed by gel electrophoresis. The RT-PCR was performed using 1 μg RNA and the Omniscript RT Kit (Qiagen) according to the manufacturer's protocol. The PCRs were performed using

Table 2
Primer sequences for *KiSS1* and other transcription factors in cell lines.

Name	Sequence	Primer for PCR amplification in different cell lines
KiSS1		
Primer9F	5′-ACC TGC CGA ACT ACA ACT GG-3′	Human bp 477–697 (NM_002256.3)
Primer9R	5′-CTT TTA TTG CCT CGG GTT GG-3′	
KiSS1		
Primer 10F	5′-GAG CCG CTG GCA AAA GTG-3′	Mouse bp 124–187 (NM_178260.3)
Primer 10R	5′-GCA TAC CGC GAT TCC TTT TC-3′	
EAP1		Human bp 937–1063 (NM_024496)
Primer11F	5′-CTT GCT ACC TGT GCG AC-3′	Mouse bp 952–1077 (AF525300)
Primer11R	5′-GTG CTG TCT CGA TCA CG-3′	Rat bp 366–491 (AY879229)
CUX1		Human bp 1574–2705 (NM_181552)
Primer12F	5′-CAG CAC AAA CTC CAT ATC-3′	Mouse bp 1454–2576 (NM_009986)
Primer12R	5′-GTA TGG GGA CTC AGC G-3′	Rat bp 1825–2947 (XM_347163)
YY1		Human bp 826–940 (NM_003403.3)
Primer13F	5′-GAC GAC TCG GAC GGG-3′	Mouse bp 454–568 (NM_009537.3)
Primer13R	5′-CCA CGG TGA CCA GCG-3′	Rat bp 343–457 (NM_173290.1)
TTF1		Human bp 203–278 (NM_001079668)
Primer14F	5′-AGC ACA CGA CTC CGT TCT CA-3′	Mouse bp 630–705 (NM_009385.3)
Primer14R	5′-CCC TCC ATG CCC ACT TTC TT-3′	Rat bp 17–92 (NM_013093.1)

500 ng RT product, 1 μM primer (Table 2; Primer9F/9R to Primer14F/14R), 2.5 mM dNTPs, 2.5 Dream Taq Buffer which includes 20 mM $MgCl_2$ and 2.5 U Dream Taq Polymerase (Fermentas). The PCR program employed an initial activation step of 3 min at 95 °C, and 35 cycles of denaturing at 94 °C for 45 s, annealing at 58 °C (KiSS1), 56 °C (EAP1), 48 °C (CUX1), 62 °C (YY1), and 60 °C (TTF1), for 45 s and 1 min extension at 72 °C, followed by a final extension of 7 min at 72 °C.

2.10. Statistics

Differences between two groups were analyzed using one-way ANOVA and Student's *t*-test for individual means. Data are expressed as means ± SEM. The level of significance was $p < 0.05$.

3. Results

3.1. Transcription of the human KiSS1 gene is initiated at a single TSS

To identify the TSS of *KiSS1* we analyzed 17 clones derived from RACE-PCR of human placental cDNA. Nine out of 17 clones exhibited a transcriptional start at 155 bp upstream of the ATG translational start site. All 17 clones mapped to a narrow region of 4 bp, indicating that the *KiSS1* gene utilizes a single TSS mapping to a region located between 153 and 156 bp upstream of the ATG (Fig. 1A). This finding is consistent with the computer-predicted location of *KiSS*1 TSS (NM_002256).

3.2. The 5′-flanking region of KiSS1 contains putative binding sites for transcription factors implicated in the hypothalamic control of female puberty

In silico analysis of the region upstream the *KiSS1* TSS, using TESS [http://www.cbil.upenn.edu/cgi-bin/tess/tess; Schug (2008)], Vector NTI 9.0.0 (Invitrogen; September 02, 2003; InforMax, Frederick, USA) and information from literature search (PubMed; http://preview.ncbi.nlm.nih.gov/pubmed/), revealed the presence of several putative TTF1 recognition sites located within 1200 bp upstream of the TSS (Bruno et al., 1995; Guazzi et al., 1990; Son et al., 2003) (Fig. 1B). Several CUX1 binding sites (Harada et al., 1995) were also detected, in addition to recognition sites for YY1 (Fig. 1B), a member of the PcG silencing complex (Park and Atchison, 1991; Shi et al., 1991b) that has both repressive and activating functions (Shrivastava and Calame, 1994; Thomas and Seto, 1999; Wilkinson et al., 2006), and recruits other PcG proteins and histone 3 (H3) trimethylated a lysine 27 to gene promoters to silence transcription (Wilkinson et al., 2006; Woo et al., 2010). *TTF1*, *CUX1*, and *YY1* have been implicated as upstream transcriptional regulators of the pubertal process (Mastronardi et al., 2006; Roth et al., 2007). We did not search for EAP1 binding sites, because it is not yet known if EAP1 regulates gene transcription by binding directly to DNA. Table 3 provides an overview of the expression of *KiSS1*, *EAP1*, *TTF1*, *CUX1*, and *YY1* mRNAs in the three cell lines used for experiments.

3.3. The human KiSS1 promoter is active in both neuronal and non-neuronal cell lines

We used luciferase assays to determine the transcriptional activity of the *KiSS1* 5′-flanking region using three segments of different lengths (1975, 1339, and 556 bp) (Fig. 1B). These assays showed that the shorter 556 bp fragment was similarly active in non-neuronal GripTite™ 293 MSR cells and neuronal GT1-7 cells, but the longer fragments (1339 and 1975 bp) tended to be more active in the non-neuronal cells (Fig. 2). Because of this, all subsequent promoter assays were conducted in GripTite™ 293 MSR cells. We used GT1-7 cells to determine if transcriptional effects on the *KiSS1* promoter observed in the non-neuronal cells can be also observed in neuronal cells.

3.4. CUX1, YY1, EAP1, and TTF1 are recruited to the KISS promoter in vivo

CUX1, an evolutionary conserved homeobox transcription factor, has been postulated to be an upstream regulator of female puberty and to target the *KiSS1* gene for transcriptional control (Roth et al., 2007). To determine if CUX1 can be recruited to the *KiSS1* promoter *in vivo*, we performed ChIP assays using HeLa cells, specific antibodies against CUX1, and the primers listed in Table 1 for PCR amplification. The results show that endogenous CUX1 binds to a region located ~2000 bp upstream of the TSS (Fig. 3; Amplicon 1) and to the *KISS1* proximal promoter (Amplicon 2), but not to a region located in intron 2 (Amplicon 3). While the PCR product showing CUX1 binding in the *KiSS1* proximal promoter contains several putative CUX1 binding sites (Fig. 1B), the more distal (−2456 to −2264 bp) product likely derives from

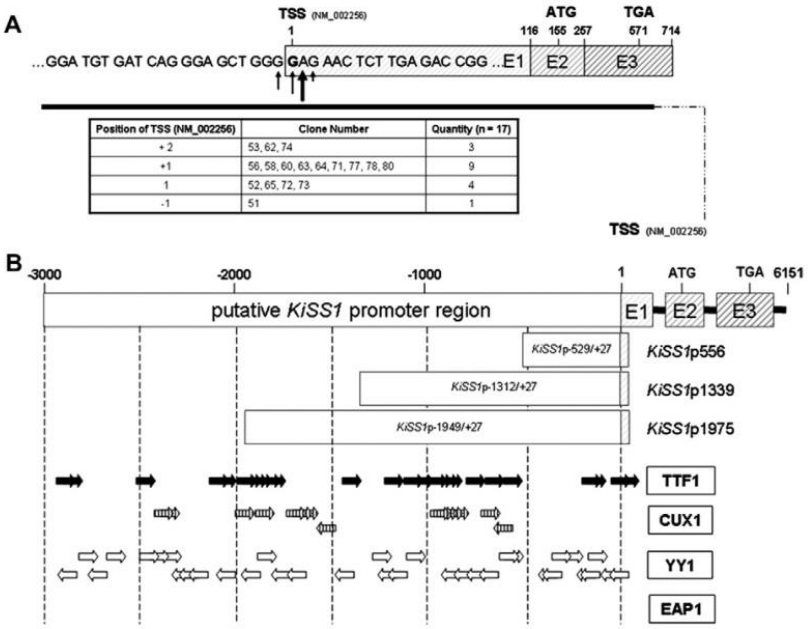

Fig. 1. Identification of the *KiSS1* transcription start site (TSS), and *in silico* prediction of transcription factor recognition sites in the *KiSS1* 5′-flanking region. (A), The *KiSS1* gene is transcribed from a single TSS. The translational start site ("ATG") is located within exon 2 (at nt +155 from the TSS) and the translational stop codon is located in exon 3 (at nt +571 from the TSS). Vertical arrows and the table below the sequence illustrate the TSSs found by RACE-PCR. The bold arrow denotes the nucleotide most frequently used as a TSS. (B) Three constructs containing *KiSS1* 5′-flanking region segments of different lengths (*KiSS1*p556, *KiSS1*p1339, and *KiSS1*p1975) were generated to assess *KiSS*1 transcriptional activity. Putative binding sites for three transcription factors (TTF1, CUX1, and YY1) implicated in the control of puberty were identified *in silico* (TESS, Vector NTI) and are shown by horizontal wide arrows. The direction of each arrow indicates the presence of each binding site on either the sense or antisense DNA strand. EAP1 binding sites, if present, are currently unknown.

Table 3
Expression of *KiSS1* and transcription factor mRNAs in different cell lines.

	KiSS1	EAP1	TTF1	CUX1	YY1
GripTite™ 293 MSR	–	+	–	+	+
GT1-7	+	+	–	–	+
HeLa	+	+	+	+	+

+ = expressed; – = not expressed.
The non-neuronal cell line GripTite™ 293 MSR expresses *EAP1*, *CUX1*, and *YY1*, whereas neuronal GT1-7 cells express *KiSS1*, *EAP1*, and *YY1*. Only HeLa cells express all four transcription factors examined in this study, in addition to *KiSS1*.

DNA fragments that include putative CUX1 binding sites predicted to be present directly adjacent to the amplified region (Fig. 1B).

YY1 is a transcriptional regulator postulated to be a central node in a gene network involved in the hypothalamic control of female puberty (Roth et al., 2007). As such, YY1 was also predicted by *in silico* analysis to control a host of subordinate genes, including *KiSS1*. CHIP assays, using HeLa cells, specific antibodies against YY1, and the same primers used to demonstrate CUX1 association to the *KiSS1* promoter (Table 1) showed that endogenous YY1 is most efficiently recruited to the *KiSS1* proximal promoter (Fig. 3; Amplicon 2), and to a much lesser extent to the upstream −2456 to −2264 bp region (Amplicon 1). In contrast, no YY1 binding to intron 2 (Amplicon 3) was detected.

EAP1 is a transcription factor with trans-activating and repressive activities, previously implicated in the transcriptional control of female puberty (Heger et al., 2007). In the rat, *Eap1* is highly expressed in the arcuate nucleus and preoptic area, the two regions where kisspeptin neurons are located (Heger et al., 2007). ChIP assays using HeLa cells transfected with an HA-tagged construct expressing the *EAP1* coding region demonstrated that EAP1 is

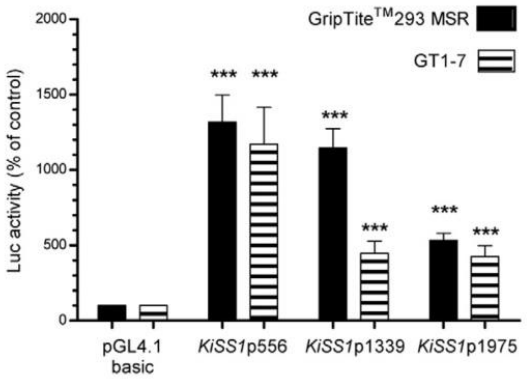

Fig. 2. Assessment of transcriptional activity of three *KiSS1* promoter constructs using Griptite™ 293 MSR, and GT1-7 cells, and a luciferase reporter system. Luciferase activity is shown as percent of the activity displayed by pGL4.10 basic, the luciferase reporter plasmid in which the three *KiSS1* constructs were cloned. Bars are means and vertical lines represent SEM. Griptite™ 293 MSR cells $n = 3$, GT1-7 cells $n = 3$; $^{***}P < 0.001$ compared to pGL4.1 basic vector.

exclusively recruited to the *KiSS1* proximal promoter (Fig. 3; Amplicon 2)

TTF1 (Nkx2.1, T/ebp) is a homeodomain gene required for basal forebrain morphogenesis (Kimura et al., 1996), but that remains expressed in the hypothalamus after birth (Lee et al., 2001). In the postnatal hypothalamus, TTF1 transactivates genes involved in the stimulatory control of puberty, and represses genes inhibitory to the pubertal process (Mastronardi et al., 2006). ChIP assays using HeLa cells transfected with a construct expressing the rat *Ttf1* coding region, and the primers described above for PCR

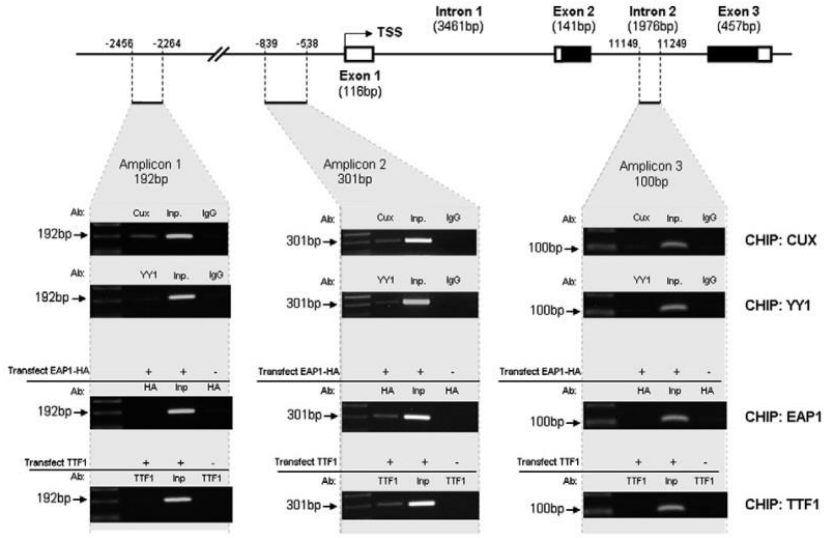

Fig. 3. CUX1, YY1, EAP1, and TTF1 are recruited to the *KiSS1* promoter in HeLa cells, as assessed by ChIP assays. Endogenous CUX1 and YY1, as well as transfected HA-tagged EAP1 and untagged TTF1, are recruited to the proximal promoter region of the *KiSS1* gene (Amplicon 2). An upstream region (Amplicon 1) showed Cux1 association, some YY1 binding, and neither EAP1 nor TTF1 association. A downstream region localized in intron 2 (Amplicon 3) showed no binding of any of the four proteins examined. When endogenous proteins (CUX1 and YY1) were immunoprecipitated, we used monoclonal antibodies against each of these proteins and mouse IgGs as a negative control for the immunoprecipitation. When the immunoprecipitating proteins derived from transfected expression vectors (EAP1-HA and TTF1), immunoprecipitation was performed using a mouse monoclonal antibody against HA (for EAP) or rabbit polyclonal antibodies against TTF1. Chromatin prepared from cells transfected with the empty expression vector was used as the negative control. Inp: input DNA, chromatin precleared with protein A beads before immunoprecipitation. Transfect = transfected.

amplification, demonstrated that, as is the case of EAP1, TTF1 is only recruited to the *KiSS1* proximal promoter (Fig. 3; Amplicon 2).

3.5. EAP1, TTF1, CUX1, and TTF1 have different effects on KiSS1 transcription

Luciferase assays, using GripTite™ 293 MSR and neuronal GT1-7 cells, demonstrated that the shorter CUX1 isoform (p110) potently inhibited transcription of all three *KiSS1* promoter fragments in both non-neuronal and neuronal cells. In contrast, the p200 isoform was strongly trans-activational in GripTite™ 293 MSR cells (Fig. 4A). Surprisingly, p200 was repressive in GT1-7 cells. This activity, however, was limited to the shorter (556 bp) *KiSS1* promoter segment (Fig. 4B). These results suggest that CUX1 can either activate or inhibit *KiSS1* expression, depending on the predominant isoform present and the cell context in which the protein–DNA interaction takes place.

Similar assays performed in GripTite™ 293 MSR, indicate that YY1 significantly inhibited the transcriptional activity of the *KiSS1* promoter constructs (Fig. 5A). Because YY1 interacts with Sp1 to regulate gene transcription (Lee et al., 1993), and the *KiSS1* proximal promoter has a number of SP1 binding sites (Mitchell et al., 2006, 2007), we tested this potential interaction on *KiSS1* promoter activity, using GripTite™ 293 MSR cells. The results showed that Sp1 does not affect the repressive effect of YY1 on *KiSS1* promoter activity (data not shown). Thus YY1 represses *KiSS1* transcription without recruiting Sp1 as a co-repressor. We did not observed an effect of YY1 on *KiSS1* gene expression in GT1-7 cells (Fig. 5B).

Co-transfection of GripTite™ 293 MSR with pcDNA3.1^{+}/zeo-*EAP1* and each of the *KiSS1* promoter constructs showed that

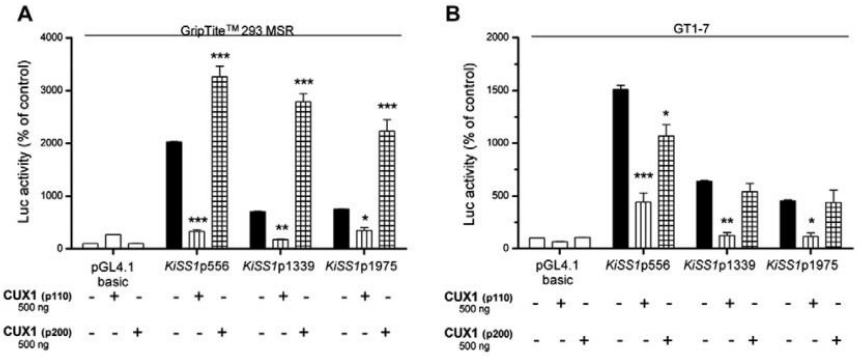

Fig. 4. CUX1 regulates *KiSS1* gene expression dually. (A) The p110 isoform of CUX1 inhibits, whereas the full-length CUX1 isoform (p200) trans-activates *KiSS1* transcription in GripTite™ 293 MSR cells, $n = 3$. (B) Both isoforms repress *KiSS1* transcription in GT1-7 cells, $n = 3$. Bars are mean and vertical lines are SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to *KiSS1* promoter alone.

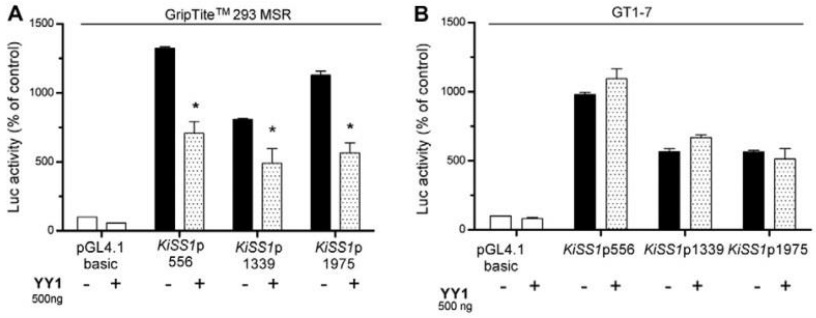

Fig. 5. YY1 inhibits *KiSS1* transcriptional activity. (A) YY1 inhibits *KiSS1* transcription in GripTite™ 293 MSR cells, $n = 3$, (B) YY1 does not modify *KiSS1* transcription in GT1-7 cells. Bars are mean and vertical lines are SEM, $n = 3$; $^{*}P < 0.05$ compared to *KiSS1* promoter alone.

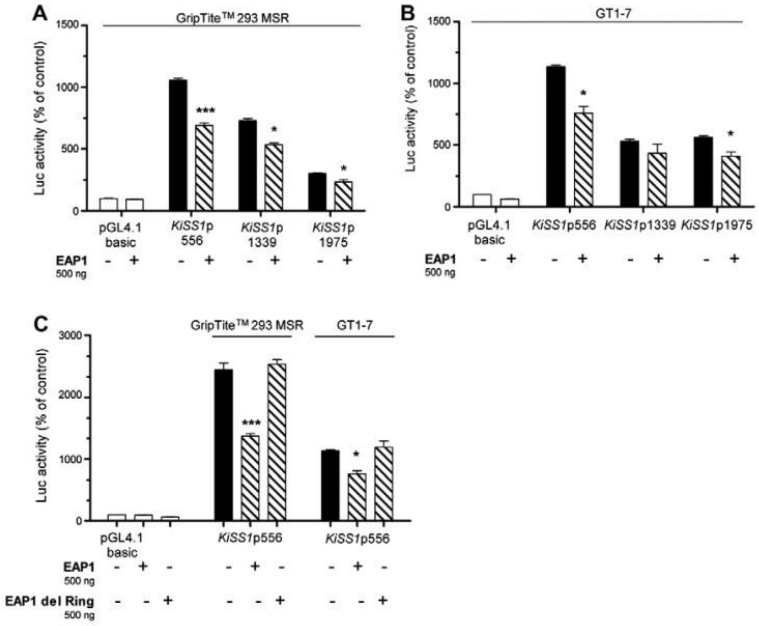

Fig. 6. EAP1 represses *KiSS1* transcriptional activity in non-neuronal and neuronal cell lines. (A) EAP1 represses transcription of all three *KiSS1* promoter constructs in non-neuronal (GripTite™ 293 MSR) cells, $n = 3$, (B) EAP1 represses the activity of two of the three constructs in the neuronal (GT1-7) cell line, $n = 3$. (C) The repressive effect of EAP1 is abolished in both cell lines by deletion of the EAP1 RING finger domain. Bars are mean and vertical lines are SEM, $n = 3$; $^{*}P < 0.05$, and $^{***}P < 0.001$ compared to *KiSS1* promoter alone.

EAP1 exerts a modest, but significant, inhibitory effect on *KiSS1* transcriptional activity of all *KiSS1* promoter fragments (Fig. 6A). A similar, though less consistent effect was observed in GT1-7 cells (Fig. 6B). EAP1 lacking the RING finger domain, tested with the 556 bp *KiSS1* promoter construct, failed to affect transcriptional activity of this DNA fragment in both non-neuronal cells (GripTite™ 293 MSR) and GT1-7 neurons (Fig. 6C). These results indicate that EAP1 modestly represses *KiSS1* transcriptional activity in two different cell lines and that the RING finger domain of EAP1 is required for this effect.

Luciferase reporter assays, using GripTite™ 293 MSR cells, showed that TTF1 consistently trans-activates the short *KiSS1* promoter construct (Fig. 7A). However, this effect was not observed in GT 1-7 cells (Fig. 7B). In a previous study we showed that deletion of the TTF1 recognition site most proximal to the TSS (−109 to −100) or the simultaneous deletion of this site plus another site located at −1019 to −1010 in the *KiSS1* promoter abolished the trans-activational effect of TTF1 on *KiSS1* promoter activity (Mastronardi et al., 2006). Altogether, these results indicate that TTF1 is a trans-activator of *KiSS1* transcription.

3.6. CUX1, YY1, EAP1, and TTF1 are expressed in kisspeptin neurons

We previously demonstrated that the nuclei of kisspeptin neurons contain Ttf1 immunoreactive material (Mastronardi et al., 2006). To determine if they are also Yy1, Eap1, and/or Cux1 immunopositive, we performed double immunofluorescence studies using hypothalami from late juvenile female rats. The results indicated that each of these proteins is present in kisspeptin neurons of the medial basal hypothalamus (Fig. 8), and that as expected, they have a nuclear localization. However, not all kisspeptin neurons contain all three transcription factors, as shown by the absence

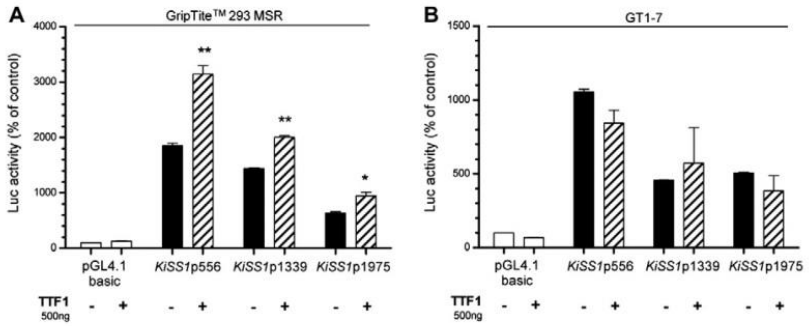

Fig. 7. TTF1 enhances *KiSS1* transcription in a non-neuronal cell line, but not in GT1-7 cells. (A) TTF1 trans-activates transcription of each *KiSS1* construct tested in GripTite™ 293 MSR cells, $n = 3$, (B) TTF1 does not modify *KiSS1* promoter transcription in GT1-7 cells. Bars are mean and vertical lines are SEM, $n = 3$; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared to *KiSS1* promoter alone.

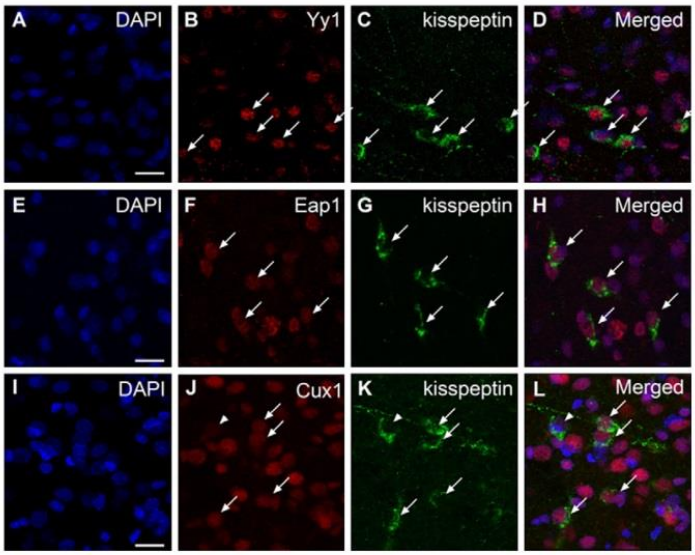

Fig. 8. Kisspeptin neurons of the prepubertal female rat hypothalamus contain Eap1, Yy1, and Cux1 as detected by double immunohistofluorescence. (A–D) Yy1. (E–H) Eap1. (I–L) Cux1. Arrows point to examples of colocalization. Arrowhead in (J–L) points to a kisspeptin positive neuron lacking detectable Cux1 immunoreactivity. Notice the nuclear localization of Eap1, Yy1, and Cux1. Bar = 10 μm.

of Cux1 in some kisspeptin neurons (Fig. 8; example denoted by arrowhead).

4. Discussion

Following the pioneer observations of two different groups (De Roux et al., 2003; Seminara et al., 2003) showing that mutations in the *GPR54* gene result in hypogonadotropic hypogonadism, a substantial body of evidence has accumulated demonstrating that the kisspeptin–GPR54 receptor system is a key regulator of puberty and adult reproductive function [reviewed in Oakley et al. (2009)]. This progress notwithstanding, the factors controlling *KiSS1* expression at the transcriptional level have been only partially identified (Mitchell et al., 2006, 2007). In the present study, we have experimentally tested computational biology predictions implicating four TFs, previously postulated to be involved in the hypothalamic control of female puberty, as regulators of *KiSS1* transcriptional activity (Roth et al., 2007). Not surprisingly, the picture that has emerged from this study is one of complexity, but also one which is consistent with the basic concept that ultimately *KiSS1* transcription depends on a delicate balance between activators and repressors.

We found that the *KiSS1* TSS is located in a region between −153 and −156 bp upstream of the ATG which is consistent with *in silico* predictions (NM_002256). The absence of other transcripts suggests that at least in placental tissue the *KiSS1* gene utilizes a single TSS to generate a primary mRNA transcript. Analysis of the genomic region 5′ to this site revealed the presence of specific promoter elements such as a TATA-box, GC-rich regions, and binding sites for various members of the basic transcriptional machinery, like SP1 and AP1. To the best of our knowledge only one TSS (identical to the one we identified here) has been described for the human *KiSS1* gene. An alternative upstream promoter expressed in placenta that includes sequences from the *Golt1a* has been reported to exist in the mouse (NM AY707856, AY707857, AY707859), but its biological significance has never been assessed experimentally. When examining *in silico* potential transcripts derived from this hypothetical TSS, we have not been able to obtain a translated kisspeptin sequence, suggesting that utilization of this

upstream TSS may not generate a translatable transcript. Nevertheless, the possibility of an alternative TSS usage in nervous tissue is an issue that requires future consideration.

Promoter activity was highest when shorter 5′-flanking region fragments were used, suggesting that enhancer elements are preferentially located near the TSS, whereas repressor elements are located further upstream. Interestingly, the *KiSS1* promoter activity of longer DNA fragments was dependent on the cell system used. It tended to be higher in the non-neuronal cell line GripTite™ 293 MSR than in neuronal GT1-7 cells. A potential explanation for the difference in basal transcriptional activity noted between these two cell lines is that they do not have the same complement of repressors/activators or the same abundance/availability of these proteins. Alternatively, the differences may have been simply due to different transfection efficiencies.

Because the transcriptional control of female puberty appears to be exerted by both TSGs and genes without tumor suppressor activity, we selected two members of each class for our study. EAP1 and TTF1 represent the latter category; both have been suggested to be upstream transcriptional regulators of mammalian puberty, based on physiological, cellular and genetic evidence (Heger et al., 2007; Mastronardi et al., 2006). Of the two, EAP1 has dual transcriptional activity. It trans-activates the GnRH promoter, but inhibits preproenkephalin gene transcription (Heger et al., 2007). Because RNAi-mediated knock-down of *Eap1* in the preoptic region of prepubertal female rats results in delayed pubertal onset and disruption of estrous cyclicity (Heger et al., 2007), we expected that EAP1 would increase *KiSS1* gene expression; instead, a repressive effect was observed. We do not understand well the physiological significance of such an inhibitory action, but speculate that EAP1 may behave differently in the presence of partners specifically expressed in kisspeptin neurons. It was recently shown that EAP1 physically associates with two other proteins to form a repressive complex that inhibits the expression of a downstream transcriptional repressor (Yeung et al., in press). These findings suggest that in some cells EAP1 functions as a repressor of repressors. Within the hypothalamus, a function of EAP1 may be to repress genes involved in the inhibitory control of puberty/reproductive cyclicity, so that loss of EAP1 expression would result in pubertal delay and loss of reproductive cyclicity. This is, indeed, the case because knock-down of EAP1 expression in either the preoptic area of rats (Heger et al., 2007) or the arcuate nucleus of non-human primates (Dissen et al., 2011) interrupts reproductive cyclicity. These findings and our own observation that EAP1 trans-activates, instead of repressing, the GnRH promoter in GT1-7 cells (Heger et al., 2007), suggest that the absence/presence of appropriate partners or downstream targets in a given cell context may determine whether EAP1 will act as a transactivator or repressor. More work is obviously needed to clarify this issue.

In contrast to EAP1, our results show that TTF1 enhances *KiSS1* expression. A previous study showed that this trans-activation requires a TTF1 binding site located near the TSS. TTF1 is expressed in kisspeptin neurons of the arcuate nucleus (Mastronardi et al., 2006). An earlier report showed that TTF1 expression increases in the hypothalamus during prepubertal development (Lee et al., 2001), preceding the increase in *KiSS1* expression (Clarkson and Herbison, 2006; Navarro et al., 2004; Takase et al., 2009). The possibility that this two events are causally related is supported by the present results showing that TTF1 is not only recruited to the *KiSS*1 promoter *in vivo*, but it also strongly trans-activates *KiSS1* transcription *in vitro*. Unexpectedly, this trans-activational effect was observed in GripTite^{MSR}293 cells, but not in GT1-7 cells, suggesting that the ability of TTF1 to trans-activate the *KiSS1* promoter is cell-dependent. Although GT1-7 cells are neuronal, they may not have the same complement of co-activators present in GripTite^{MSR}293 cells (and perhaps also in kisspeptin neurons themselves).

The two TSGs we examined also displayed contrasting activities. The activity of CUX1 was found to be isoform-dependent. Previously we showed that CUX1 mRNA abundance increases in the monkey hypothalamus at the time of puberty, and postulated that CUX1 acts as a central node of a TSG network that operates within the hypothalamus to control the onset of puberty (Roth et al., 2007; Ojeda et al., 2006). In general, CUX1 functions as a transcriptional repressor (Dufort and Nepveu, 1994; Superti-Furga et al., 1989; Valarche et al., 1993; Coqueret et al., 1998). However, depending on the cellular context, CUX1 can also act as a transcriptional activator (Harada et al., 2008; Kim et al., 1997). In our experiments we used two different isoforms of CUX1 previously shown to have divergent transcriptional activities (Goulet et al., 2002; Sansregret and Nepveu, 2008). Our results confirm this isoform-specific activity. While p200, the full- length form of CUX1, behaved as a trans-activator in non-neuronal cells and displayed limited repressive activity in GT1-7 cells, the proteolytically derived form p110 was consistently repressive. Whether p200 behaves as a repressor or activator in kisspeptin neurons themselves remains an open question. Our immunohistochemical studies show that the CUX1 protein is present in kisspeptin neurons. However, the lack of isoform-specific antibodies does not allow us to distinguish between the predominant form or to screen for a shift in relative abundance at the time of puberty. It may be speculated that if expression of the p200 and p110 forms decreases at puberty in kisspeptin neurons, a potentially repressive effect of CUX1 on *KiSS1* transcription would be lifted. This shift would be expected to contribute to the pubertal increase in *KiSS1* mRNA abundance (Clarkson and Herbison, 2006; Navarro et al., 2004; Takase et al., 2009).

Like CUX1 and EAP1, YY1 can display both repressive and trans-activating activities (Shi et al., 1997; He and Casaccia-Bonnefil, 2008; He et al., 2007). Our results show that YY1 is repressive in GripTite^{MSR}293 cells, but had no effect in GT1-7 cells. As in the case of TTF1, this difference may be due to the absence of appropriate partners in GT1-7 cells. It is not known if the repressive effect of YY1 we observed in GripTite™ 293 MSR cells occurs in kisspeptin neurons themselves. These cells may contain a different complement of trans-activators/repressors that may either reinforce the repressive effect of YY1 or switch it to activation, as shown in other systems (Gordon et al., 2006). Further studies are required to resolve this issue.

The repressive effect of YY1 on *KiSS1* transcription is consistent with its well-established gene silencing activity (He et al., 2007; Wilkinson et al., 2006; Woo et al., 2010), and its contribution to the recruitment of PcG repressive proteins to gene promoters (Schwartz and Pirrotta, 2007; Woo et al., 2010). Because of its known interaction with Sp1 (Lee et al., 1993), YY1 action was analyzed in combination with Sp1 and observed that, at least in GripTite™ 293 MSR cells, Sp1 does not alter the repressive activity of YY1. The increase in *Kiss1* mRNA abundance observed in the rodent hypothalamus at puberty (Clarkson and Herbison, 2006; Navarro et al., 2004; Takase et al., 2009) and the repression of *KiSS1* transcription by YY1 we observed in GripTite^{MSR}293 cells, suggest that this repression is regulated developmentally. Should kisspeptin neurons expressed the appropriate partners for YY1 to exert its well-established repressive effects, one could envision a strong repression taking place during early juvenile days when *KiSS1* expression is low, and loss of this inhibition near the time of puberty when *KiSS1* expression increases.

Taken together, the present study provides experimental evidence supporting the *in silico* prediction that upstream regulators of the pubertal process control *KiSS1* transcription by directly modifying *KiSS1* promoter activity. The results also suggest that *in vivo* expression of the *KiSS1* gene depends on a balance between repressors and inhibitors able to modify *KiSS1* promoter activity. Although the above discussion focuses on the significance of these

interactions for female puberty, it seems reasonable to infer that the *KiSS1* promoter is similarly regulated in males.

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Development/Plasticity/Repair

Deletion of the *Ttf1* Gene in Differentiated Neurons Disrupts Female Reproduction without Impairing Basal Ganglia Function

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Thyroid transcription factor 1 (TTF1) [also known as Nkx2.1 (related to the NK-2 class of homeobox genes) and T/ebp (thyroid-specific enhancer-binding protein)], a homeodomain gene required for basal forebrain morphogenesis, remains expressed in the hypothalamus after birth, suggesting a role in neuroendocrine function. Here, we show an involvement of TTF1 in the control of mammalian puberty and adult reproductive function. Gene expression profiling of the nonhuman primate hypothalamus revealed that TTF1 expression increases at puberty. Mice in which the *Ttf1* gene was ablated from differentiated neurons grew normally and had normal basal ganglia/hypothalamic morphology but exhibited delayed puberty, reduced reproductive capacity, and a short reproductive span. These defects were associated with reduced hypothalamic expression of genes required for sexual development and deregulation of a gene involved in restraining puberty. No extrapyramidal impairments associated with basal ganglia dysfunction were apparent. Thus, although TTF1 appears to fulfill only a morphogenic function in the ventral telencephalon, once this function is satisfied in the hypothalamus, TTF1 remains active as part of the transcriptional machinery controlling female sexual development.

Key words: **TTF1; homeobox genes; conditional gene deletion; hypothalamus; basal ganglia; female puberty**

Introduction

Morphogenesis of the basal forebrain is controlled by several homeobox genes showing spatially and temporally overlapping patterns of expression (Alvarez-Bolado et al., 1995; Shimamura et al., 1995). The ventral telencephalon, a forebrain component, gives origin to the basal ganglia and the preoptic area (POA) (Shimamura et al., 1995); the basal ganglia are, in turn, composed of two subdivisions: the striatum, located dorsally, and the pallidum, located ventrally (Gerfen, 1992). The diencephalon, the other forebrain component, also has two subdivisions, the thalamus and the hypothalamus. Development of both the pallidum and the hypothalamus requires the temporally restricted expression of thyroid transcription factor 1 (TTF1) [also known as Nkx2.1 (related to the NK-2 class of homeobox genes) and T/ebp (thyroid-specific enhancer-binding protein)] (Kimura et al., 1996; Sussel et al., 1999), a member of the Nkx family of homeobox genes (Price et al., 1992; Pera and Kessel, 1998). In the absence of TTF1, the pallidum fails to form (Sussel et al., 1999), and development of the hypothalamus is severely impaired (Kimura et al., 1996; Takuma et al., 1998; Marin et al., 2002).

TTF1 expression persists in some postmitotic basal forebrain neurons, suggesting a role for TTF1 in maintaining neuronal differentiated functions (Sussel et al., 1999; Marin et al., 2000, 2002). Such a role might extend to postnatal life, because TTF1 remains expressed after birth in selected striatal/pallidum interneurons (Marin et al., 2000), as well as in defined glial and neuronal subsets of the hypothalamus and the POA (Lee et al., 2001). Specifically, TTF1 is detected in POA neurons that control repro-

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ductive function via release of the peptide luteinizing hormone (LH)-releasing hormone (LHRH) (Lee et al., 2001), in preproenkephalinergic neurons of the lateral ventromedial nucleus (Lee et al., 2001; Davis et al., 2004), which restrain the initiation of puberty by transsynaptically inhibiting LHRH neurons (Ojeda and Skinner, 2006), in unidentified neurons of the arcuate nucleus, and in ependymoglial cells of the third ventricle and median eminence (ME). The ME is the final common pathway for LHRH neuronal axons converging to release LHRH into the portal system that connects the hypothalamus to the pituitary gland (Silverman et al., 1994). In female rats, TTF1 mRNA abundance increases in the hypothalamus preceding the natural onset of puberty and after puberty-inducing hypothalamic lesions, suggesting an involvement of TTF1 in the control of female puberty (Lee et al., 2001).

Mutations in the TTF1 gene result in neurological symptoms that include hypotonia, dyskinesia, and choreoathetosis (Krude et al., 2002; Pohlenz et al., 2002), a condition consisting of rapid involuntary and slow writhing movements. These symptoms, which are caused by impairments in basal ganglia function, are also observed in mice carrying only one *Ttf1* allele disrupted by gene targeting (Pohlenz et al., 2002). In the absence of both alleles, the pallidum is absent, cholinergic neurons of the basal forebrain fail to develop, and migration of GABAergic neurons from the pallidum to the striatum and cortex is impaired (Sussel et al., 1999; Marin et al., 2002). These abnormalities suggest that loss of TTF1 may result in cognitive deficiencies (because of the absence of cholinergic neurons) (Ginsberg et al., 2006) and behavioral abnormalities (attributable in part to the loss of cortical GABAergic neurons) (Siniscalchi et al., 2003). It is also possible that the functional integrity of these neuronal populations requires continuous TTF1 support. Unfortunately, neither possibility can be addressed using conventional *Ttf1* knock-out (KO) mice, because they are born dead (Kimura et al., 1996).

In the present study, we use Cre-loxP technology to demonstrate an involvement of TTF1 in the hypothalamic control of puberty and show that in contrast to the need for TTF1 to sustain this process, TTF1 deficiency instituted after establishment of telencephalic neuronal fates does not diminish differentiated basal ganglia-dependent neurological functions.

Materials and Methods

Animals

Nonhuman primates. The rhesus monkeys (*Macaca mulatta*) used in this study were classified into different stages of pubertal development according to the criteria reported by Watanabe and Terasawa (1989). Plasma LH levels obtained from a different set of age-matched, contemporary animals ($n = 10$ per group) increased from 2.59 ± 0.97 in juvenile animals to 6.48 ± 1.64 ng/ml at midpuberty, reflecting pubertal maturation of the hypothalamic–pituitary axis. Brain tissue was obtained through the Tissue Distribution Program of the Oregon National Primate Research Center. Gene expression profiling and real-time PCR studies were conducted using mediobasal hypothalamus (MBH) and cerebral cortex (CTX) samples from juvenile (1–2 years of age) and midpubertal (3–4 years of age) animals.

Mice. The mice were housed under a controlled photoperiod (12 h of light and 12 h of darkness; lights on at 07:00 A.M.) and temperature (23–25°C) and were given *ad libitum* access to rodent chow and tap water. They were used in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The experimental protocols were approved by the Animal Care and Use Committee of the Oregon National Primate Research Center (ONPRC). Mice used for behavioral experiments were transferred from the ONPRC to the Oregon Health and Sciences University (OHSU) main campus and kept under the same environmental conditions described above. All behavioral procedures used were approved by the OHSU Animal Care and Use Committee.

Mice expressing Cre under the control of the Synapsin I promoter were those previously described by Zhu et al. (2001). Because the Synapsin I promoter becomes active only in terminally differentiated neurons (Hoesche et al., 1993), Cre-mediated recombination deletes the *Ttf1* gene only from postmitotic neurons. Mice carrying floxed *Ttf1* alleles, produced by one of our laboratories, were described recently (Kusakabe et al., 2006). Crossing these two mutant lines generated hemizygote SynCre mice (SynCre+/−) carrying one floxed allele (*Ttf1 flox/+*). Breeding these animals to *Ttf1 flox/flox* mice resulted in a progeny consisting of wild-type (WT) animals (SynCre−/−, *Ttf1 flox/flox*, and SynCre−/− *Ttf1 flox/+*), heterozygous (HT) animals [SynCre+/− *Ttf1 flox/+* (*Ttf1*^{SynCre} *flox/+*), and SynCre+/− *Ttf1 flox/flox* mice (*Ttf1*^{SynCre} KO)]. A potential problem of using SynCre mice is that the transgene is frequently expressed in the testis and is transmitted to the progeny (Rempe et al., 2006). Although this might represent a problem in some cases, TTF1 is not expressed in the gonads. Its main sites of expression are in the forebrain, circumventricular organs, lungs, and pituitary gland. In our experience, *Ttf1*^{SynCre} KO mice grow normally, indicating that neither the pituitary–thyroid axis nor the respiratory system is affected. We also observed that male *Ttf1*^{SynCre} KO mice are reproductively indistinguishable from wild-type animals.

Genotyping

The primers used to detect the WT and floxed alleles (sense 5Neo2, 5′-TGCCGTGTAAACACGAGGAC-3′; and antisense 3Neo2, 5′-GACTCTCAAGCAAGTCCATCC-3′) were those described recently (Kusakabe et al., 2006). The PCR conditions used consisted of an initial activation step of 5 min at 95°C and 36 cycles as follows: 30 s of denaturing at 94°C, 30 s of annealing at 60°C, a 1 min extension at 72°C, and a final extension of 10 min at 72°C. Because tail DNA from *Ttf1*^{SynCre} flox/+ mice did not always yield a clear 540 bp PCR product that identifies the WT allele, the genotype of these animals was confirmed in a second PCR using primers that amplify a 271 bp fragment from a DNA segment only present in the wild-type allele. The sense primer 5′-CAGCTGGAGGGCGGTGGATTGG-3′ and the antisense primer 5′-TGAACGCCGGTCTCGCTAACTGGA-3′ were located within a *Xho–XhoI* genomic fragment located downstream from *Ttf1* exon 2. This segment was replaced by a loxP-flanked pGK-neo cassette in *Ttf1* floxed mice (Kusakabe et al., 2006). The PCR conditions were similar to those described above, except that the annealing temperature was 60°C. The SynCre transgene was identified with a set of primers (sense, 5′-CTTG-GTCGCGTCCGTGCA-3′; and antisense, 5′-CAGGCAAATTTTGG-TGTA-3′) that amplifies a PCR product of 376 bp. The touch-down PCR program used consisted of an initial activation of 5 min at 95°C, followed by two cycles of denaturing at 95°C for 30 s, annealing at 65°C for 1 min, and extension at 72°C for 2 min. Then, rounds of two cycles were performed, decreasing the annealing temperature by 2°C in each round, until 55°C was reached. The program was completed with 20 more cycles at an annealing temperature of 50°C.

Degree of Cre-mediated recombination

The procedure used to assess degree of Cre-mediated recombination (DCMR) was that reported by Leneuve et al. (2001). DCMR was assessed in genomic DNA extracted from several brain regions, including the MBH, basal ganglia, hippocampus, cerebral cortex, and cerebellum, between postnatal day 6 and 40 in both WT and *Ttf1*^{SynCre} KO mice. In addition to the 5Neo2 and 3Neo2 primers described above, we used a second sense primer (5′TTF1 Exon2Del; 5′-ATGCTCAAGACTTCA-GGGAGCTAAG-3′) corresponding to a sequence upstream of the first loxP site in intron 1 (see Fig. 2*a*). In the absence of Cre-mediated recombination, the primer pair 5Neo2–3Neo2 amplifies a 220 bp fragment; after recombination, the pair 5′TTF1 Exon2Del–3Neo2 amplifies a 269 bp fragment. The PCR conditions were similar to those described above for genotyping with the primer pair 5Neo2/3Neo2, except that in this instance, we used 30 cycles. To estimate the degree of recombination, the intensity of the 269 bp and 220 bp bands was determined using the Quantity One software, their respective background was subtracted, and

the prevalence of the deleted allele was calculated by using the following equation: degree of recombination = *VRA*/(*VRA* + *VFA*), where *VRA* is the value of the recombined allele, and *VFA* is the value of unrecombined floxed allele.

Tissue collection and RNA isolation

The MBH from rhesus monkeys was dissected with a cut along the posterior border of the optic chiasm, a caudal cut immediately in front of the mammillary bodies, and two lateral cuts along the hypothalamic sulci. The prefrontal cortex was dissected as a shallow slice along the superior frontal gyrus. The thickness of both tissue fragments was ~4 mm. The collected tissue samples were placed in vials containing RNAlater (Ambion, Austin, TX) and left overnight at 4°C. The following day, tissues were frozen on dry ice and stored at −85°C until RNA extraction. Dissection of mouse brain regions was performed as described previously (Rogers et al., 1991; Ma et al., 1992). The monkey and mouse tissues were homogenized in 1 ml (<100 mg of tissue per milliliter) of TriReagent solution (Molecular Research Center, Cincinnati, OH), and the aqueous and organic phases were separated by the addition of 0.1 vol of bromochloropropane (Sigma Chemicals, St. Louis, MO) followed by centrifugation at 4°C. RNA was precipitated from the aqueous phase with 1 vol of isopropanol followed by centrifugation at 13,000 rpm for 15 min at 4°C. The pellets were resuspended in DEPC-treated H_2O, and the suspension was incubated with DNA-free DNase I (2 U per reaction; Ambion) for 30 min at 37°C. RNA concentrations were determined spectrophotometrically, and RNA integrity was verified on denaturing agarose gels.

DNA microarrays

Hybridization of monkey hypothalamic total RNA to DNA microarrays containing human cDNAs was performed by the OHSU Gene Microarray Shared Resource. The arrays were prepared from the IMAGE Consortium human cDNA library [Research Genetics, now Invitrogen (Carlsbad, CA)]. The cDNA sequences were amplified by PCR with DyNAzyme EXT (New England Biolabs, Ipswich, MA). Amplified DNA was separated from unincorporated primers and nucleotides using MultiScreen PCR filtration plates (Millipore, Billerica, MA) on a BioRobot 8000 (Qiagen, Valencia, CA). All amplifications were examined for product by agarose gel electrophoresis. PCR products were dried in a CentriVap vacuum evaporator (LabConCo, Kansas City, MO) and then resuspended in 30 μl of 50% DMSO/Tris-EDTA and stored at −80°C. Printing plates were made by transferring 3 μl aliquots of each PCR product to a 384-well microtiter plate (Whatman, Florham Park, NJ). Arrays were printed with a PyxSys 5500XL microarray printer [Cartesian Technologies, now Genomic Solutions (Ann Arbor, MI)] using sixteen CMP3 printing pins (TeleChem, Sunnyvale, CA) and UltraGAPS array slides (Corning, Corning, NY). Control plant PCR products (Stratagene, La Jolla, CA) were printed but not used for these experiments. After printing, slides were baked for 3.5 h at 80°C and then stored in a desiccator cabinet in the dark until use. Total RNA was labeled using the Tyramide Signal Amplification protocol (TSA; PerkinElmer, Boston, MA) with substantial modifications to increase throughput and reproducibility. Briefly, total RNA template was used to make cDNA using either fluorescein–dCTP or biotin–dCTP. Paired samples were hybridized to arrays in TSA hybridization buffer under M-Series LifterSlips (Erie Scientific, Portsmouth, NH) and incubated overnight at 60°C in a water bath using deep-well hybridization chambers (TeleChem). After hybridization, slides were developed as described in the TSA protocol manual, but with modifications for higher throughput and reproducibility. The specific protocol is available for download from www.ohsu.edu/gmsr/smc. Results from this protocol have been validated in other studies (Reddy et al., 2004). Arrays were scanned on a ScanArray 4000 (PerkinElmer). Cyanine 5 (Cy5) and Cy3 results were saved as separate 16-bit tagged image file format images. Paired images were analyzed using ImaGene (BioDiscovery, El Segundo, CA). Low-quality spots were flagged manually for exclusion within ImaGene. Spot values were calculated with 5% trimming of highest and lowest pixel values. Pairwise comparisons of the data were examined in GeneSight (BioDiscovery) using the following protocol: (1) exclude flagged spots; (2) transform data to $\log_2$(intensity); (3) five-bin piecewise linear regression of the entire dataset, (4) calculation of log differences for Cy5 versus Cy3, and (5) combination of the replicates. For increased robustness of the analysis, two arrays were printed per slide so that each target RNA pair is interrogated twice. In addition, each RNA pair was hybridized to two slides, providing four measurements per gene analyzed. Because we compared five individuals at midpuberty to juvenile controls, each gene was interrogated a total of 20 times to take into account both assay and biological variability. A basic confidence analysis of the data identified those genes that were significantly changed with 95% confidence. Pairwise comparison did not include scaling across pairs.

Real-time PCR

Monkey TTF1 mRNA and the mRNAs encoding mouse TTF1, preproenkephalin, KiSS1, and LHRH were quantified by real-time PCR, as described previously (Romero et al., 2002; Shahab et al., 2005). After reverse transcription of 200 ng of total RNA, aliquots of each reaction (10 ng of cDNA per microliter) were diluted (1:10 to 1:50) before using 2 μl for PCR amplification. Each sample was run in triplicate along with a relative standard curve. Relative standard curves were generated by diluting one sample 1:10 to 1:10,000; they served to estimate the relative content of 18S ribosomal RNA in each sample. The primers used to detect 18S ribosomal RNA were purchased as a kit (TaqMan Ribosomal RNA Control Reagents Kit; Applied Biosystems, Foster City, CA). All gene-specific primers (Invitrogen) and fluorescent probes (Applied Biosystems) were selected with the assistance of the program Primer Express (Applied Biosystems). The monkey TTF1 primers (XM_001089890) were as follows: forward, 5′-AGCACACGACTCCGTTCTCA-3′; and reverse, 5′-CCCTCCATGCCCACTTTCTT-3′. The internal fluorescent probe was 5′-TGACATCTTGAGTCCCCTGGAGGAAAGC-3′. The mouse TTF1 primers (mmU19755) were as follows: forward, 5′-GCTCGAGCGACGTTTCAAG-3′; and reverse, 5′-GCGTGGGTGTCAGGTGAATC-3′. The internal fluorescent probe sequence was 5′-CGCCGGAGCGCGAGCATC-3′. The LHRH primers (NM_008145) were as follows: forward, 5′-ACTGATGGCCGGCATTCTAC-3′; and reverse, 5′-CCAGGGCGCAACCCATAGG-3′. The internal fluorescent probe was 5′-ACTGTGTGTTTGGAAGGCTGCTCCAGC-3′. The preproenkephalin primers (BC049766) were as follows: forward, 5′-GCCCAGGCGACATCAATTT-3′; and reverse, 5′-ATCCTTGCAGGTCTCCCAGAT-3′. The internal probe was 5′-GAAGGCAGCTGTCCTTCACATTCCAGTG-3′. The KiSS1 primers (NM_178260) used were as follows: forward, 5′-GAGCCGCTGGCAAAAGTG-3′; and reverse, 5′-GCATACCGCGATTCCTTTTC-3′. The internal fluorescence probe was 5′-ATCCACAGGCCAGCAGTCCGGA-3′. All internal fluorescent probes were covalently linked to the fluorescent dye 6-carboxyfluorescein at the 5′ end and to the quencher dye 6-carboxytetramethylrhodamine at the 3′ end.

Real-time PCRs were performed in a total volume of 10 μl, each reaction containing 2 μl of the diluted reverse-transcribed sample or 2 μl of standard, 5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 250 nM each gene-specific and ribosomal fluorescent probe, 300 nM each gene-specific primer, and 10 nM each ribosomal primer. The real-time PCR program used consisted of an initial annealing period of 2 min at 50°C, followed by 10 min of denaturing at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

In situ *hybridization*

The hybridization procedure was performed as recommended by Simmons et al. (1989) and described by us (Junier et al., 1991; Ma et al., 1994), using a ^{35}S-UTP-mTTF1-labeled cRNA probe transcribed from an mTTF1 cDNA (described below). Control sections were incubated with a sense probe transcribed from the same plasmid but linearized on the 3′ end to transcribe the coding strand of the cDNA template. After an overnight hybridization at 55°C, the slides were washed and processed for cRNA detection (Junier et al., 1991; Ma et al., 1994). After dehydration, the slides were dipped in NTB-2 emulsion and were exposed to the emulsion for 3 weeks at 4°C. At this time, the slides were developed, counterstained with 0.1% thionin, quickly dehydrated in ascending concentrations of alcohol, and coverslipped for microscopic examination.

Immunohistochemistry

To identify the cells and cellular sites containing TTF1 protein in the mouse hypothalamus, we used rabbit polyclonal antibodies previously characterized by Li et al. (1998). Frozen sections (30 μm) derived from the brain of adult WT and *Ttf1*^{SynCre} KO mice (perfusion fixed with 4% paraformaldehyde-borate, pH 9.5) were incubated overnight at 4°C with TTF1 antibodies diluted 1:2000. The next day, the sections were incubated with a biotinylated donkey anti-rabbit γ globulin (1:250; Vector Laboratories, Burlingame, CA) for 1 h at room temperature, followed by incubation in avidin–biotin complex (Vector Laboratories) for another 1 h. The immunohistochemical reaction was then developed to a brown color with 3,3′-DAB-HCl and 0.005% H_2O_2.

*Combined immunohistochemistry/*in situ *hybridization*

To determine whether TTF1 is expressed in KiSS1 neurons of the mouse hypothalamus, we used a combined immunohistochemistry/*in situ* hybridization procedure (Jung et al., 1997; Rage et al., 1997). After completing the TTF1 immunohistochemical procedure, the sections were mounted on glass slides and dried overnight under vacuum before hybridization with the ^{35}S-UTP-labeled rKiSS1 (rat KiSS1) cRNA probe described below. All reagents used for the immunohistochemical procedure were prepared in DEPC-treated water.

Gross morphology

To determine whether SynCre-mediated deletion of neuronal TTF1 affects the normal morphology of the forebrain, brains from WT and TTF1 KO mice ($n = 3$ each) were perfusion-fixed in 4% paraformaldehyde-borate buffer, pH 9.5, sectioned at 30 μm, and stained with 0.1% thionin before microscopic examination.

DNA cloning

To PCR clone a cDNA from the mouse TTF1 mRNA (GenBank accession number mmU19755), we used a sense primer (5′-CGCCGGGTGCTCTTCTCC-3′) corresponding to nucleotides 490–507 and an antisense primer (5′-GCCACCGCTGCCCACTGAG-3′) complementary to nucleotides 915–933. PCR conditions consisted of an initial activation step of 15 min at 95°C, followed by 36 cycles of denaturing at 94°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 1 min, and a final extension of 10 min at 72°C. The resulting single PCR product of 444 bp was cloned into the plasmid pGEM-T (Promega, Madison, WI) and sequenced from both ends to verify its identity. This cDNA was used as a template to generate either sense or antisense RNA for *in situ* hybridization assays. To generate a KiSS1 cDNA, we used a sense primer (5′-ATGATCTCGCTGGCTTCTTG-3′) that corresponds to nucleotides 1–20 in rat KiSS1 mRNA (GenBank accession number AY196983) and an antisense primer (5′-CCCGCGCCACCTGCCTCCTG-3′) complementary to nucleotides 359–379. The PCR conditions used were identical to those used for TTF1, except that the annealing step was at 61°C.

Gene reporter constructs

A luciferase (luc) gene reporter construct containing 5 kb of the rat TTF1 gene 5′ flanking region (Oguchi and Kimura, 1998) was used to determine the effect of estradiol on TTF1 gene transcription.

To prepare a reporter vector containing a segment of the human KiSS1 (hKiSS1) gene 5′ flanking region, we PCR amplified a 1298 bp DNA fragment from human genomic DNA (Promega). The 5′ sense primer used (5′-GGACAGGCCAACGTACACATCATC-3′) corresponds to nucleotides 54657264–54657286 in the human chromosome 1 genomic contig NT_004487.18 gi:88943682. The antisense primer (5′-GCTGGGCTCCCGGTCTCAAG-3′) is complementary to nucleotides 54655947–54655966. The PCR was performed using Platinum PFX DNA Polymerase (Invitrogen) following the manufacturer's recommendations. The PCR program used consisted of an initial denaturing step of 2 min of 94°C and 34 cycles as follows: 15 s at 94°C, 30 s at 66°C, 2 min at 68°C, and a final extension for 10 min at 68°C.

The single PCR product obtained was purified (PCR Purification Kit; Qiagen), an A-extension was performed (BD Advantage cDNA Polymerase Mix; Clontech, Mountain View, CA), and the cDNA was cloned into the pCR–TOPOII vector (TOPO TA Cloning Kit; Invitrogen). Before excising the putative promoter region by *Sac*I–*Xho*I digestion and subcloning it into the *Bam*HI–*Xho*I sites of the reporter vector pGL2-Basic-luciferase (Promega), the sequence of the hKiSS1 promoter was verified by sequencing.

Promoter assays

Assessment of the transregulatory activity of TTF1 on the hKiss1 promoter was performed as described previously (Ojeda et al., 1999; Lee et al., 2001), using 293T cells. We first verified the transcriptional activity of the promoter alone. Twenty-four hours after seeding 300,000 cells per well in six-well plates, different concentrations of hKiSS1p–pGL2 (100–500 ng per well) were transiently transfected for 5 h using Lipofectamine (Invitrogen) in conjunction with 20 ng/ml reporter plasmid constitutively expressing β-galactosidase (β-gal) (CMV-Sport β-gal; Invitrogen) to normalize for transfection efficiency. The cells were collected 48 h after transfection and assayed for luciferase and β-gal activity, as reported previously (Ojeda et al., 1999). To examine the ability of TTF1 to transregulate the hKiSS1 promoter, 293T cells were cotransfected with 100 ng/ml wild-type hKiSS1p–pGL2 or hKiSS1p mutants lacking one or both putative TTF1 recognition motifs, plus 250 ng/ml an expression vector (pcDNA3.1Zeo; Invitrogen) containing the coding region of rTTF1 (Lee et al., 2001). As before, the cells were collected 48 h later.

To assess the ability of estradiol to transregulate the TTF1 promoter, we used C6 glioma cells stably transfected with plasmids expressing estrogen receptor α (ERα) or ERβ (Mhyre et al., 2006). The cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 μg/ml penicillin, and 100 μg/ml streptomycin. They were reseeded 24 h before the assay in 12-well plates (at 120,000 cells per well) in 10% charcoal-stripped FBS (Hyclone) DMEM without phenol red or antibiotics and were supplemented with glutamine (20 mM). Twenty-four hours later, the various reporter gene constructs were premixed at a ratio of 1 μg of DNA to 2 μl of Lipofectamine 2000 (Invitrogen) for 30 min before adding the mixture to the cells. Each well was transiently transfected with a control plasmid (pGL2-Basic; Promega), 500 ng of TTF-1 expression vector, or 750 ng of a tkERE–luc plasmid (Mhyre et al., 2006). This plasmid contains estrogen-responsive elements fused to the thymidine kinase promoter and serves as a positive control for the assay. Variability in transfection efficiency was determined by cotransfecting the cells with 250 ng of CMV-Sport-β-gal plasmid (Invitrogen). The total amount of DNA transfected was kept constant at 1 μg per well by adding the appropriate amount of pGL2–Basic to each well. After 6 h of incubation, the cells were washed with PBS, and fresh medium was added. The day after transfection, cell medium was replaced by medium containing the vehicle (ethanol) or 17β-estradiol (Sigma) at 1 or 10 nM. The cells were harvested 48 h after transfection and assayed for luciferase and β-galactosidase, as previously reported (Ojeda et al., 1999; Lee et al., 2001).

Site-directed mutagenesis of TTF1 binding sites

In silico analysis of the predicted 5′ flanking region of the *hKiSS1* gene using the Transcription Element Search System tool (http://www.cbil.upenn.edu/cgi-bin/tess/tess) yielded two putative TTF1 binding sites. To test the functional importance of these sites, we prepared two mutants by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit; Stratagene). One of them lacked the TTF1 binding motif proximal to the putative transcription start site (nucleotides 54656068–54656076; NT_004487.18); the other lacked both this site and the more distal motif (nucleotides 54656968–54656976; NT_004487.18). The hKiSS1 promoter–TOPOII construct described above was used as the template for the proximal mutant. The forward mutagenesis primer used (5′-CTCCCAAGGTGCCATGCTGAGGGTCTGAGGAGGAGG-3′) corresponds to nucleotides 54656053–54656091 of the *Homo sapiens* chromosome 1 genomic contig (NT_004487.18). The reverse mutagenesis primer had a sequence complementary to the forward primer. The mutation was verified by sequencing. To prepare a mutant lacking both TTF1 sites, the hKiSS1p–TOPOII construct carrying the proximal deletion was used as the template. The forward mutagenesis primer used (5′-AAGAAACAGTCTCTGCCAGCCCCTTGTCCAAGGG-3′) corresponds to nucleotides 54656951–54656990 of the *Homo sapiens* chromosome 1 genomic contig (NT_004487.18). The sequence of the antisense

mutagenesis primer was complementary to the forward primer. The deletion of the second TTF-1 binding site was also verified by sequencing.

Both KiSS1 promoter mutants were cloned into the *Kpn*I and *Xho*I sites of the pGL2–Basic–luciferase vector (Promega).

Evaluation of puberty and reproductive performance

Litters consisting of WT, HT, and *Ttf1*^{SynCre} KO mice were weaned on day 21, the males were discarded, and the females were placed in groups of four per cage. Starting on day 25, the animals were inspected daily for perforation of the vaginal membrane, an event known as vaginal opening. Thereafter, vaginal lavages were performed daily to detect the appearance of cornified cells, which identify the estrous phase of the rodent estrous cycle. Both vaginal opening and cornification of the vaginal epithelium result from the rise in estrogen secretion that accompanies the onset of puberty in rodents (Ojeda and Skinner, 2006). Ovulation normally occurs on the day of estrus, but in mice it is not certain (Stiff et al., 1974; Nelson et al., 1990) unless vaginal cornification is followed by the appearance of a predominance of leukocytes in the vaginal lavage (Prevot et al., 2003a). This abundance of leukocytes occurs in the diestrous phase of the estrous cycle and indicates that a functional corpus luteum was formed after ovulation. Therefore, we recorded the age at both vaginal opening and at first estrus, considering that a true first estrus (and thus the age at first ovulation) occurs only when the cornified cells in the vaginal lavages were replaced by at least 2 d of lavages containing mostly leukocytes (Prevot et al., 2003a). The time of first ovulation was functionally confirmed by mating 30-d-old WT, HT, and *Ttf1*^{SynCre} KO females (i.e., at the age of vaginal opening) with WT males and determining the length of the mating–delivery interval (MDI) for each group.

To determine whether neuronal deletion of *Ttf1* gene affects adult reproductive capacity, young adult (60 d of age) wild-type and mutant females were exposed to a fertile WT male (one male per female), and the MDI, litter size, and weight were recorded. The same parameters were recorded from subsequent litters born during a 1 year period. The percentage of reproducing dams per experimental group was calculated defining a reproductively active female as an animal showing an MDI no longer than the MDI mean of the first litter of the WT group + 2 SD of the mean. Body weights were recorded from postnatal day 6, every 6 d until day 30, and then monthly until the animals were 8 months of age.

Behavioral testing

Fourteen-month-old WT and Ttf1^{SynCre} KO male mice ($n = 5$ mice per genotype) were tested in the open field and elevated zero maze (week 1), for novel location and novel object recognition (week 2), in the water maze (week 3), and for rotarod performance and passive avoidance (week 4). The person testing the mice was blinded to the genotype of the mice.

Open field. To evaluate exploratory behavior, mice were placed in a 40.64 × 40.64 cm brightly lit (luminescence, 200 lux) open arena equipped with infrared photocells interfaced with a computer (Hamilton-Kinder, Poway, CA) for a single 10 min session.

Elevated zero maze. To assess measures of anxiety, the elevated zero maze was used. The custom-built elevated zero maze (Hamilton-Kinder) consisted of two enclosed areas and two open areas. Mice were placed in the closed part of the maze and allowed *ad libitum* access for 10 min (luminescence, 200 lux). They could spend their time either in a closed safe area or in an open area. A video tracking system (set at six samples per second; Noldus Information Technology, Sterling, VA) was used to calculate the time spent in the open and closed areas.

Novel location and novel object recognition. For 3 consecutive days, mice were individually habituated (for 5 min) to the open field described above (luminescence, 200 lux). On the fourth day, the mice were first given three 10 min trials with three plastic objects (monkey, horse, and man) placed in different corners of the enclosure and occupying an area of 6 cm^2. Objects were chosen based on preliminary data showing that they were of equal interest to naive C57BL/6J wild-type mice (data not shown). There was a 3 min intertrial interval during which mice were placed back in their home cage, and the maze and the objects were cleaned with 5% acetic acid to remove odors. All familiar objects were exchanged with replicas in subsequent trials. For the fourth 10 min trial, the monkey was moved from one corner of the enclosure to another to evaluate novel location recognition. For the fifth 10 min trial, the horse was replaced by a cow to assess novel object recognition. For each trial, the total time the mouse spent exploring each object within 2 cm was recorded by an observer blinded to the genotype and treatment of the mice. The time spent exploring all objects over the first three trials assessed familiarization with the objects. The difference between the percentages of time spent exploring the object in the familiar (trial 3) and novel (trial 4) locations was calculated to assess novel location recognition. The percentage of time spent exploring the novel object during trial 5 was calculated to assess novel object recognition.

Water maze. The water maze was used to assess spatial learning and memory. A circular pool (diameter, 140 cm) was filled with opaque water (24°C), and mice were trained to locate a submerged platform to escape from the water (luminescence, 40 lux). First, they were trained to locate a clearly marked platform (days 1 and 2). Subsequently, they were trained to locate a platform hidden beneath the surface of water made opaque using white chalk (days 3–5). During the training with the hidden platform, the mice had to navigate to it using the available spatial cues. There were two daily sessions 3.5 h apart, each consisting of three 60 s trials (with 10–15 min intertrial intervals). Mice that failed to find the platform within 60 s were led to the platform by the experimenter and allowed to stay on the platform for 3 s. During the visible platform training, the platform was moved to a different quadrant of the pool for each session. For the hidden platform training, the platform location was kept constant for each group of mice. The mice were assigned to four groups using a randomized block design to avoid any potential quadrant bias. Mice were placed into the water facing the wall at the side of the pool in nine different locations around the pool circumference, and the starting location was changed for each trial. The swimming patterns of the mice were recorded with the Noldus Ethovision video tracking system, set at six samples per second. Time to locate the platform (latency), distance moved, cumulative distance to the platform, and swim speeds were analyzed. Probe trials (no platform present) were used to assess spatial memory retention. Probe trials were conducted 1 h after the last hidden trial on each day of hidden training (total of three probe trials).

Rotarod. To assess sensorimotor ability, mice were tested using a rotarod apparatus (Hamilton-Kinder). Mice were placed on an elevated rod (7.0 mm in diameter) initially rotating at 4 rpm. Every 15 s, the rod was accelerated by 15 rpm. Fall latency was recorded by timers, which stopped when the mouse broke the photobeams at the bottom of the chamber. Mice received three trials per day for 3 subsequent days.

Passive avoidance. To assess emotional learning and memory, the passive avoidance test was used. Each mouse was placed in a brightly lighted compartment of a chamber also containing a dark compartment (Hamilton-Kinder). After 5 s of acclimation, the bright house light turned on, and a connecting gate to the dark compartment opened. The mouse, preferring the darkened left side, steps quickly through the gate to enter the dark compartment. After doing so, the mouse received a brief and slight foot shock (0.3 mA for 3 s). Each mouse was trained until it met a learning criterion of three consecutive 120 s trials without entering the dark compartment or up to ten trials, whichever came first. After 24 h, the mouse was again placed in the lighted compartment, and the time to reenter the dark compartment was measured up to 300 s.

Statistical analysis

Differences between two groups were analyzed using the Student's *t* test. When comparing several groups, the differences were analyzed by one-way ANOVA followed by the Student–Newman–Keuls multiple-comparison test for unequal replications. To analyze the behavioral data, we used ANOVA, followed by Tukey–Kramer *post hoc* test or by *t* test as indicated. Data are expressed as mean ± SEM. The null hypothesis was rejected at the 0.05 level for all analyses.

Results

TTF1 gene expression increases in the nonhuman primate hypothalamus at the time of puberty

To identify genes that may participate in the control of primate puberty, we used total RNA and human cDNA arrays containing

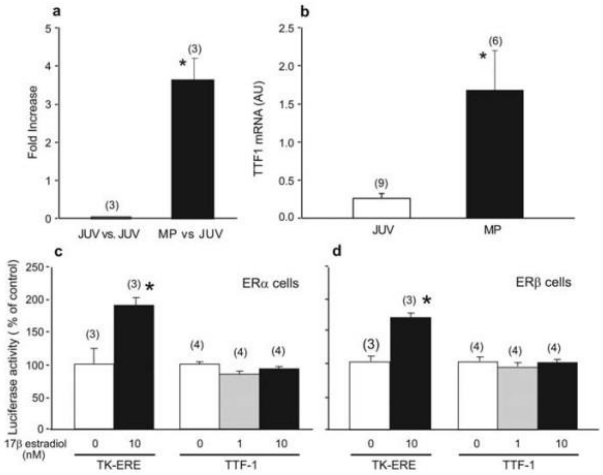

Figure 1. TTF1 mRNA abundance increases in the female monkey hypothalamus at the time of puberty. ***a***, Puberty-related increase in hypothalamic TTF1 mRNA content detected using human cDNA microarrays. Changes in mRNA content are expressed as fold increase over values present in prepubertal juvenile (JUV) animals. MP, Midpuberty. ***b***, Verification of the array results by real-time PCR. $*p < 0.05$ versus JUV controls. AU, Arbitrary units. ***c***, Lack of effect of 17β-estradiol-dependent stimulation of ERαs on TTF1 promoter activity measured in transcription activity assays using a luciferase reporter gene driven by the rat TTF1 gene 5′ flanking region. ***d***, Lack of effect of ERβ stimulation by estradiol on TTF1 promoter activity. C6 cells stably expressing either ERα or ERβ were exposed to estradiol (1 or 10 nM), and luciferase activity was measured after 48 h of exposure to the steroid. Tk-ERE, Reporter plasmid in which luciferase expression is driven by estrogen-responsive elements fused to the thymidine kinase promoter. $*p < 0.05$ versus control wells not exposed to estradiol. In this and the following figures, bars represent group means, error bars represent SEM, and numbers in parentheses indicate the number of animals or independent observations per group.

8500 probes to interrogate the MBH of female rhesus monkeys at the juvenile and midpubertal stages of development. As in humans, the midpubertal stage of rhesus monkeys precedes the first ovulation by several months (Watanabe and Terasawa, 1989). The CTX was used as a control tissue. The arrays revealed that TTF1 was one of the genes whose expression increased in the MBH during primate puberty (Fig. 1*a*). This increase was verified using a different set of arrays (Institute for Systems Biology, Seattle, WA) containing 40,000 probes (data not shown) and by real-time PCR (Fig. 1*b*). TTF1 mRNA was not detected in the CTX (data not shown).

Estradiol does not transregulate TTF1 gene transcription

Because the puberty-related increases in hypothalamic TTF1 mRNA abundance that we previously detected in female rats (Lee et al., 2001) and have now detected in female monkeys could be attributable to the pubertal rise in estrogen levels, we first analyzed *in silico* 10 kb of the mouse, rat, and human TTF1 5′ flanking region (http://sdmc.lit.org.sg/ERE-V2/index; home page, http://research.i2r.a-star.edu.sg/promoter/) and found this region to be devoid of detectable putative estrogen-responsive sites. We then performed functional promoter assays using a cell line that stably expresses either ERα or ERβ receptors (Mhyre et al., 2006) and a reporter plasmid in which luciferase expression is driven by 5 kb of the rTTF1 gene 5′ flanking region (Oguchi and Kimura, 1998). Estradiol did not modify TTF1 promoter activity in either ERα- (Fig. 1*c*) or ERβ-expressing cells (Fig. 1*d*) when used at a physiological dose (1 nM) or at supraphysiological concentrations (10 nM). In contrast, the tkERE–luc control plasmid responded to estrogen-dependent ERα or ERβ receptor stimulation with distinct increases in luciferase activity. These results indicate that the TTF1 gene is not an estrogen target and suggest that the pubertal increase in hypothalamic TTF1 mRNA is an ovary-independent phenomenon.

Loss of neuronal TTF1 perturbs expression of genes involved in the control of puberty

TTF1 remains expressed after birth not only in hypothalamic neurons but also in ependymoglial cells lining the third ventricle (Lee et al., 2001) and circumventricular organs (Son et al., 2002), precluding the use of conventional gene targeting approaches to identify neuronal versus glial sites of TTF1 action. To circumvent this limitation, we bred mice carrying *Ttf1* floxed alleles at the *Ttf1* locus (Kusakabe et al., 2006) to transgenic mice in which Cre recombinase is expressed under the control of the synapsin I gene promoter (henceforth referred to as SynCre mice). The SynCre transgene is specifically expressed in postmitotic, terminally differentiated neurons (Zhu et al., 2001). Because synapsin I expression is low during embryonic and perinatal development, reaching a peak by the third postnatal week of life (Hoesche et al., 1993) and because the SynCre transgene is not expressed in glial cells (Zhu et al., 2001), substantial Cre-mediated recombination is expected to occur postnatally and in a neuron-specific manner (Zhu et al., 2001). Figure 2 shows the PCR strategy we used to genotype the mice and to assess the DCMR of the targeted *Ttf1* floxed locus in brain tissue. The primers 5Neo2 and 3Neo2, used for genotyping (Kusakabe et al., 2006), amplify PCR products of 540 bp and 220 bp from the WT or *Ttf1* floxed allele, respectively (Fig. 2*a*). To assess the DCMR, we used a multiplex PCR approach (Leneuve et al., 2001) using an additional forward primer (5′TTF1 Exon2Del). The primer pair 5′TTF Exon2Del–5Neo2 amplifies a PCR product of 269 bp if the *Ttf1* floxed allele is excised (Fig. 2*b*). We refer to these animals as $Ttf1^{SynCre}$ KO. We found a DCMR of 50–55% by postnatal day 6 in both brain regions, with peak levels being attained soon afterward (by day 12) in basal ganglia and much later (days 30–40) in the MBH (Fig. 2*b*). The lack of complete recombination in whole-tissue samples likely reflects the presence of an intact *Ttf1* gene in glial cells. In agreement with a previous report using the same SynCre transgene (Zhu et al., 2001), little recombination was observed in the cerebellum (data not shown). In contrast to conventional *Ttf1*-null mice (Kimura et al., 1996; Sussel et al., 1999), basal forebrain gross morphology was normal in $Ttf1^{SynCre}$ KO mice, a feature illustrated by the similarity that exists between the MBH of WT and $Ttf1^{SynCre}$ KO animals (Fig. 3).

Cre-mediated deletion of the *Ttf1* floxed alleles resulted in selective loss of TTF1 mRNA in the MBH with no changes in the ME, a region of the hypothalamus that lacks neurons and only contains astroglial and ependymoglial cells. We first quantified these changes by real-time PCR in 60-d-old female mice (Fig. 4*a*,*b*) and then verified their cellular distribution by both *in situ* hybridization (Fig. 4*c–f*) and immunohistochemistry (Fig. 4*g*,*h*). As expected, loss of TTF1 mRNA was evident in both the ventromedial and arcuate nucleus, two neuronal sites of postnatal TTF1 expression in the hypothalamus (Lee et al., 2001; Davis et al., 2004). In contrast, TTF1 mRNA and protein levels appeared to be unchanged in the ME and ependymoglial cells of the ME (Fig. 4*c–h*). Scattered cells of the pallidum exhibited low levels of immunoreactive TTF1 protein in WT mice (Fig. 4*g*, inset), and, as observed in the hypothalamus, the staining was very much reduced in $Ttf1^{SynCre}$ KO animals (Fig. 4*h*, inset)

In the hypothalamus, TTF1 displays dual transcriptional activity in regulating the transcription of genes involved in the con-

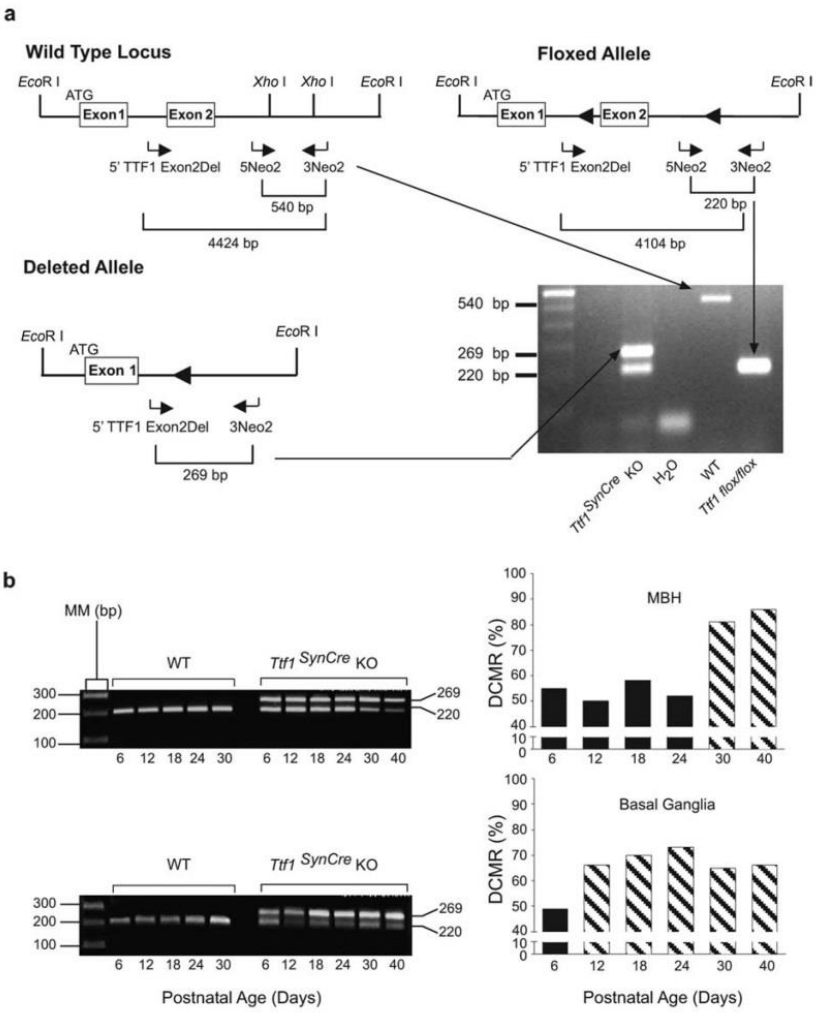

Figure 2. PCR strategies used to genotype and calculate the DCMR in brain tissue. ***a***, Three primers (horizontal arrows) were designed to distinguish among three possible TTF1 alleles. The wild-type allele is identified by a 540 bp PCR product amplified by primers 5Neo2 and 3Neo2. The intact floxed allele is detected by a 220 bp PCR product amplified by the same pair of primers. To estimate DCMR in different brain regions, the 5Neo2 and 3Neo2 primers were used in conjunction with a second forward primer (5′TTF1 Exon2Del). When paired with 3Neo2, this primer amplifies a 269 bp band that identifies a deleted *Ttf1* allele. Although three exons have been described in the mouse genome (http://www.ensembl.org/Mus_musculus/transview?db=core&transcript=ENSMUST00000001536), we represent in the diagram only two exons, because the exact location of an exon upstream from the exon containing the main ATG (as shown in the figure) is uncertain (Lonigro et al., 1996; Oguchi and Kimura, 1998). It is also unclear whether just one or more upstream exons might be present. ***b***, DCMR in MBH and basal ganglia. The PCR products shown on the left correspond to the floxed allele (220 bp band) and the recombined allele (269 bp band). The bar graphs on the right represent the DCMR calculated to occur in *Ttf1*^{SynCre} KO mice at the indicated postnatal ages. Each bar is the mean of 2–3 animals. AU, Arbitrary units; MM, molecular marker.

trol of puberty (Lee et al., 2001). It transactivates the LHRH gene, which plays an essential role in controlling sexual development and reproductive function, but it represses the preproenkephalin gene, which restrains the pubertal process (Ojeda and Skinner, 2006). Consistent with these findings, preproenkephalin mRNA content, measured in early adulthood (60 d of age), was increased in the MBH of *Ttf1*^{SynCre} KO animals, suggesting derepression of transcriptional activity in the absence of TTF1 (Fig. 5*a*). Although LHRH mRNA content in the POA was not changed at this age (Fig. 5*b*), the age-related rise in LHRH mRNA abundance observed in 18-month-old WT mice did not occur in *Ttf1*^{SynCre} KOs (Fig. 5*c*).

Kisspeptin (also known as metastin) is a product of the *KiSS1* gene (Kotani et al., 2001; Ohtaki et al., 2001) that serves as a ligand for the receptor G-protein-coupled receptor 54 (GPR54) (Kotani et al., 2001; Muir et al., 2001). Because mutations of the *GPR54* gene result in hypothalamic hypogonadism in humans (de Roux et al., 2003; Seminara et al., 2003) and impaired sexual development in mice (Funes et al., 2003; Seminara et al., 2003), it is now clear that activation of GPR54 receptors is a critical transsynaptic input to LHRH neurons required for the initiation of puberty. A major population of KiSS1-expressing neurons is located in the periventricular and arcuate nucleus of the MBH (Gottsch et al., 2004; Shahab et al., 2005; Smith et al., 2005). We found that KiSS1 mRNA levels are significantly reduced in the MBH of 60-d-old *Ttf1*^{SynCre} KO mice compared with WT controls (Fig. 5*d*), suggesting that *KiSS1* is a TTF1 target gene. To assess this possibility, we first used immunohistochemistry combined with *in situ* hybridization to determine whether KiSS1 neurons of the arcuate nucleus express TTF1. As shown in Figure 5*e*, many KiSS1 mRNA-expressing neurons (black grains) also contain the TTF1 protein (brown stained nuclei). Next, we cloned 1.3 kb of the 5′ flanking region of the human *KiSS1* gene into a luciferase expression vector. Using this construct in functional promoter assays, we found that TTF1 transactivates the KiSS1 promoter in a dose-related manner (Fig. 5*f*). A search for transcriptional regulatory motifs revealed the existence of two putative TTF1 binding sites in the KiSS1 promoter (nucleotides −1010 to −1019 and −100 to −109 relative to the putative transcription start site). Both sites conform to the consensus TTF1 binding site sequence GCNCTNNAG (Bohinksi et al., 1994). Deletion of either the proximal site or both sites by site-directed mutagenesis abolished the transactivating effect of TTF1 (Fig. 5*g*), demonstrating that TTF1 enhances KiSS1 gene expression via specific recognition motifs present in the KiSS1 promoter.

TTF1 is required for the timing of female puberty and maintenance of adult reproductive function

The age at vaginal opening, an indication of increased estrogenic stimulation of the vaginal epithelium, was similar in WT mice, HT animals expressing SynCre but carrying only one floxed *Ttf1* allele, and *Ttf1*^{SynCre} KOs (Fig. 6*a*). However, the age at puberty, defined by the age at which the first ovulation takes place, was delayed in *Ttf1*^{SynCre} KOs (Fig. 6*b*). The length of the first estrous cycle in these animals was almost three times longer than in WT controls, with some lengthening also observed in HT mice (Fig. 6*c*). After this initial disruption of estrous cyclicity, subsequent

cycles assessed during a 30 d period had a slightly longer duration (5–6 d) in *Ttf1*^{SynCre} KO mice than in WT or HT animals (4–5 d). The delay in puberty was not secondary to differences in body weight gain, because the three experimental groups showed a similar gain in body weight over a 240 d period (Fig. 6*d*). Representative estrous cycle profiles illustrating the delayed initiation of cyclicity and the slight lengthening of subsequent estrous cycles in *Ttf1*^{SynCre} KO mice are shown in Figure 6*e*. The delay in first ovulation was functionally assessed by mating 30-d-old females (i.e., at the age of vaginal opening) of the three different genotypes with WT males and determining the length of the mating–delivery interval for each group. In agreement with the 10 d delay of the first ovulation assessed by the age at first estrus (Fig. 6*b*), *Ttf1*^{SynCre} KO mice delivered their pups almost 2 weeks later than WT and HT animals (Fig. 7). This delay does not appear to be related to a defect in mating behavior, because the age at first ovulation calculated from the age at delivery (using 22 d as the length of gestation) is similar (47 vs 42 d) to the age at first ovulation determined by vaginal lavages in mice not exposed to males (compare Figs. 6*b*, 7). Interestingly, conventional Ttf1+/− mice did not show a delay in the age at first ovulation (data not shown), indicating that the presence of one functional allele is sufficient to maintain normal reproductive development.

In addition to delaying the time of puberty, neuronal deletion of *Ttf1* compromised adult reproductive capacity (Fig. 8). The reproductive life span of *Ttf1*^{SynCre} KO females was shorter than that of WT and HT mice, with 50% of TTF1-deficient mice failing to become pregnant by 6–9 months of age, a time at which all WT animals and 90% of HT mice were reproductively competent (Fig. 8*a*). There was also a reduction in the number of litters produced by the *Ttf1*^{SynCre} KO mice. We determined the total number of litters per dam every 90 d for a period of 1 year and found that by 3 months of age, the number of litters per dam had decreased to almost 50% in the *Ttf1*^{SynCre} KO group compared with WT and HT controls (Fig. 8*b*). We also observed that the total number of pups per dam produced in a 1 year period was significantly lower in the TTF1-deficient group than in WT and HT groups (Fig. 8*c*). Despite these differences, the pups born to TTF1-deficient mice appeared to be normal and showed body weights similar to WT and HT animals (Fig. 8*d*).

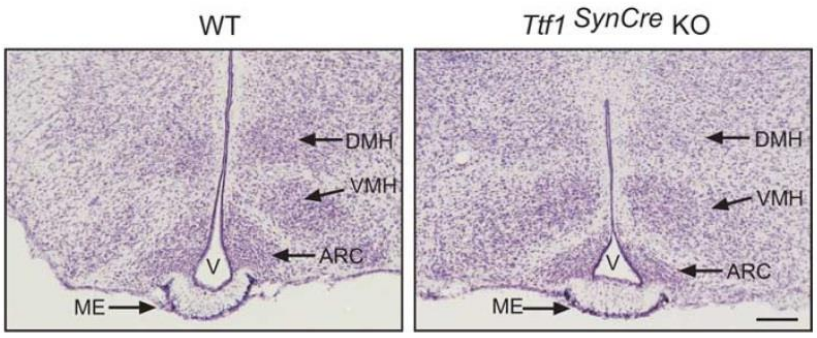

Figure 3. Gross morphology of the MBH is similar in WT and *Ttf1*^{SynCre} KO mice. The images depict the morphological aspect of 30 μm tissue sections stained with 0.1% thionin. DMH, Dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; ARC, arcuate nucleus; V, third ventricle. Scale bar, 400 μm.

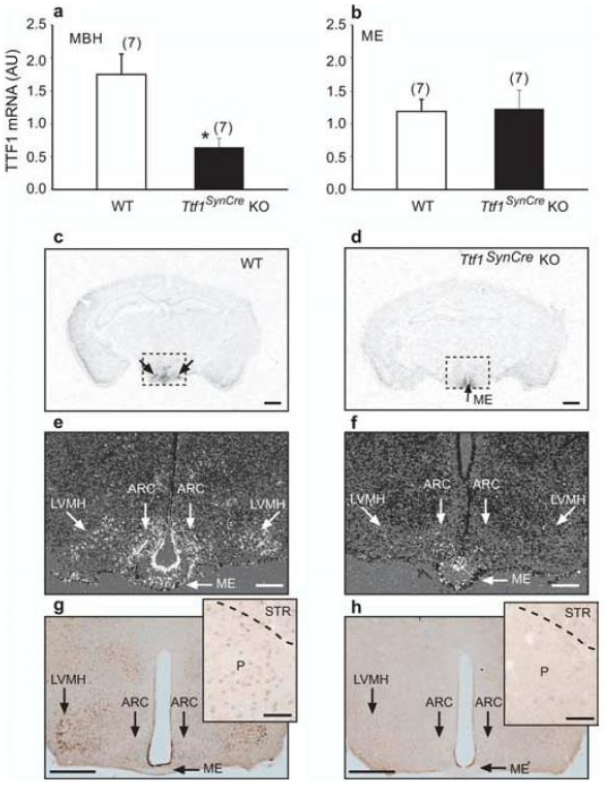

Figure 4. SynCre-mediated deletion of the *Ttf1* gene reduces TTF1 expression in hypothalamic neurons but not ependymoglial cells, as assessed by real-time PCR ($n = 7$ per group), *in situ* hybridization ($n = 3$ per group), and immunohistochemistry ($n = 3$ per group) analysis of the brains from 60-d-old female mice. ***a***, ***b***, TTF1 mRNA content decreases in the MBH (***a***) but not in the ME (***b***) of *Ttf1*^{SynCre} KO mice compared with WT animals, as assessed by real-time PCR. ***c***, ***d***, Coronal sections of the brain at the level of the MBH illustrating the presence of TTF1 mRNA in the MBH of WT mice (***c***; double arrows) and the loss of expression in the MBH but not the ME (***d***; single arrow) in *Ttf1*^{SynCre} KO mice, as determined by *in situ* hybridization using a ^{35}S-UTP-labeled TTF1 cRNA. ***e***, ***f***, Dark-field, higher-magnification *in situ* hybridization images showing the presence of TTF1 mRNA in specific MBH subregions including the arcuate (ARC), lateroventral medial nuclei (LVMH), and the ME of WT mice (***e***) and the selective loss of TTF1 mRNA in the same nuclei of *Ttf1*^{SynCre} KO mice, without a change of expression in the ME (***f***). ***g***, Detection of TTF1 protein in the LVMH, ARC, and ME of WT mice. Inset, TTF1 protein is also detected in cells of the pallidum (P), although at lower levels. ***h***, Selective loss of the protein in the LVMH and ARC, with persistent expression in the ME of *Ttf1*^{SynCre} KO mice, as determined by immunohistochemistry. Inset, Loss of TTF1 in the pallidum of *Ttf1*^{SynCre} KO mice. STR, Striatum. Scale bars: ***c***, ***d***, 400 μm; ***e***, ***f***, 200 μm; ***g***, ***h***, 400 μm; insets, 50 μm. *$p < 0.05$ versus WT.

Neuronal deletion of the Ttf1 gene does not diminish basal ganglia-dependent functions

To determine whether basal ganglia functions are compromised in TTF1-deficient mice, we used two complementary tests: the open field test to assess general locomotor activity and exploratory behavior in a novel environment (Wilson et al., 1976) and the rotarod test to evaluate sensorimotor function (Jones and Roberts, 1968). In both conventional *Ttf1*+/− mice (Pohlenz et al., 2002) and patients carrying TTF1 mutations (Krude et al., 2002; Pohlenz et al., 2002), sensorimotor function is impaired. In contrast to these observations, *Ttf1*^{SynCre} KO mice showed higher, instead of lower, locomotor activity than age-matched WT mice in the open field test (Fig. 9*a*). Similarly, on the rotarod, KO mice were able to stay longer on the rod than WT mice (Fig. 9*b*). These data show that basal ganglia function, instead of being dimin-

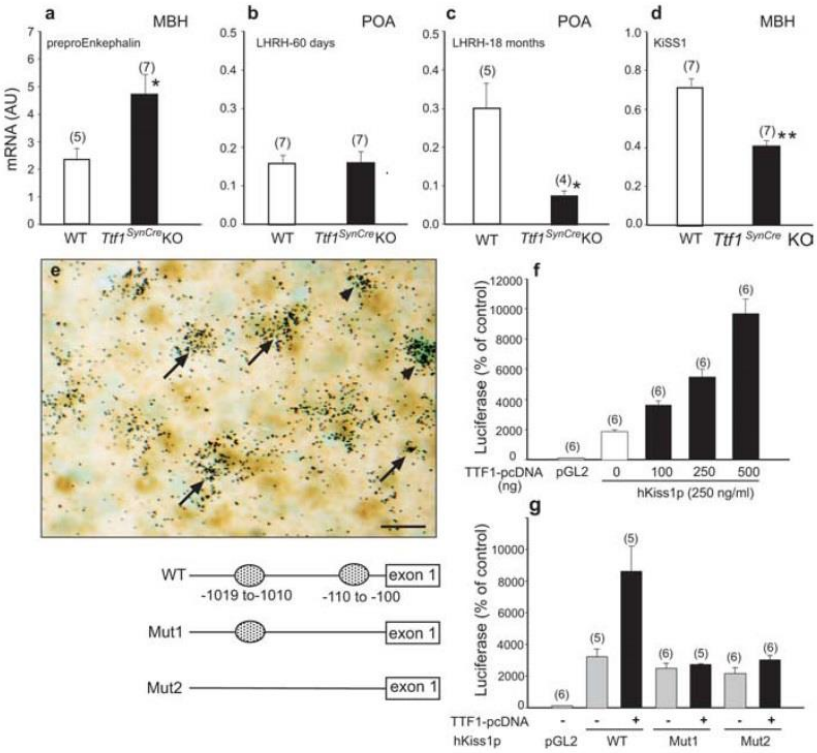

Figure 5. Hypothalamic expression of TTF1 target genes is altered in 60-d-old *Ttf1*^{SynCre} KO mice. ***a***, Preproenkephalin mRNA levels are increased in the MBH. Normally, TTF1 represses preproenkephalin gene transcription. ***b***, LHRH mRNA abundance in the POA is similar to WT mice at 60 d. ***c***, However, the age-related increase in LHRH mRNA levels seen in WT mice is abolished in *Ttf1*^{SynCre} KO animals. Normally, TTF1 transactivates the LHRH promoter. ***d***, KiSS1 mRNA abundance in the MBH is decreased. ***e***, KiSS1 neurons of the arcuate nucleus identified by *in situ* hybridization (black grains) also express TTF1 protein, identified by immunostaining (brown color). Examples of colocalization are denoted by arrows. Some KiSS1 mRNA-containing cells are TTF1 negative (arrowheads). Scale bar, 20 μm. ***f***, TTF1 transactivates the KiSS1 promoter, as assessed by functional promoter assays using a luciferase reporter gene. ***g***, Deletion of either a single proximal putative TTF1 recognition motif [located at −110 to −100 relative to the presumed transcription start site; mutation 1 (Mut1)] or both this motif and an additional site (located at −1019 to −1010; Mut2) in the 5′ flanking region of the hKiSS1 gene obliterates the transactivating effect of TTF1 on the KiSS1 promoter. $^{*}p < 0.05$; $^{***}p < 0.001$.

ished, is enhanced in *Ttf1*^{SynCre} KO mice. Constitutive deletion of the *Ttf1* gene results in substantial loss of GABAergic neurons in the cerebral cortex, because these neurons fail to migrate from the pallidum into the cortex in the absence of TTF1 (Sussel et al., 1999). Should this migratory pathway be affected in *Ttf1*^{SynCre} KO mice, these mice might exhibit changes in anxious behavior (Siniscalchi et al., 2003). To measure anxiety, we used the elevated zero maze test (Shepherd et al., 1994). Surprisingly again, *Ttf1*^{SynCre} KO mice showed higher, instead of lower, measures of anxiety than WT mice in the elevated zero maze (Fig. 9*c*). Because most, if not all, tyrosine receptor kinase A (trkA)-positive neurons of the basal forebrain are cholinergic (Sobreviela et al., 1994), and trkA-containing neurons fail to develop in the forebrain of constitutive *Ttf1* KO mice (Sussel et al., 1999), it is likely that adult *Ttf1*^{SynCre} KO mice would suffer from learning and memory deficits, should basal forebrain cholinergic neurons be affected by the loss of TTF1. To evaluate this possibility, we tested *Ttf1*^{SynCre} KO mice for cognitive function. When object recognition was assessed, WT mice predictably spent more time exploring a familiar object in a novel than a familiar location (Fig. 9*d*). The response of TTF1-deficient mice was more variable and not statistically different from the response to an old location, indicating reduced novel location recognition (Fig. 9*d*). However, in a subsequent trial, both WT and KO mice spent more time exploring a novel than a familiar object, indicating intact novel object recognition in both genotypes (Fig. 9*e*). When spatial learning and memory was assessed in the water maze, there was no genotype difference in acquisition or spatial memory retention (data not shown). Last, emotional learning and memory was assessed in the passive avoidance test. There was no genotype difference in trials to criterion, a measure of passive avoidance learning (WT, 7 ± 1.4; KO, 3.8 ± 1.1) or memory retention 24 h later (WT, 250.6 ± 39.0; KO, 300 ± 0; test cutoff set at 300 s; 0 indicates that none of the KO mice reentered within 300 s).

Discussion

Our results unveil the existence of a remarkable dichotomy in the manner by which a homeobox gene controls the functional integrity of two basal forebrain regions. Although morphogenesis of both the pallidum and the hypothalamus require the presence of TTF1, only the hypothalamus necessitates the continuous expression of TTF1 to carry out specific differentiated functions. Our study indicates that one such function is the control of the pubertal process and adult reproductive activity. It also suggests that TTF1 exerts this control by regulating the transcriptional activity of genes that, expressed in defined hypothalamic neuronal subsets, are indispensable for the central regulation of reproduction.

Consistent with the fact that SynCre-mediated recombination only occurs in terminally differentiated neurons (Zhu et al., 2001), we observed no overt abnormalities in the gross morphological makeup of either telencephalic or diencephalic derivatives (pallidum and hypothalamus, respectively) in *Ttf1*^{SynCre} KO mice. It has been known for some time that several cellular subsets of the hypothalamus, including LHRH and preproenkephalin neurons, express TTF1 after birth (Lee et al., 2001). We previously showed that TTF1 controls in an opposite manner the transcription of genes expressed in these cellular subsets. Thus, TTF1 transactivates the LHRH gene, but it represses preproenkephalin gene transcription (Lee et al., 2001). In addition, TTF1 transactivates the erbB2 gene (Lee et al., 2001), which is abundantly expressed in ependymoglial cells (Prevot et al., 2003b). LHRH is essential for reproductive function, and erbB2 is a crucial component of the erbB-dependent signaling complex used by glial cells to facilitate sexual development (Ojeda et al., 2003). In contrast, preproenkephalinergic neurons contribute to restraining the pubertal process (for review, see Ojeda and Skinner, 2006). Because of this dual transcriptional control, it has been hypothesized that TTF1 belongs to an upstream hierarchy of genes that control the pubertal process by differentially regulating the transcription of subordinate genes expressed in different hypothalamic cellular subsets involved in the control of reproductive function (Ojeda et al., 2006). The

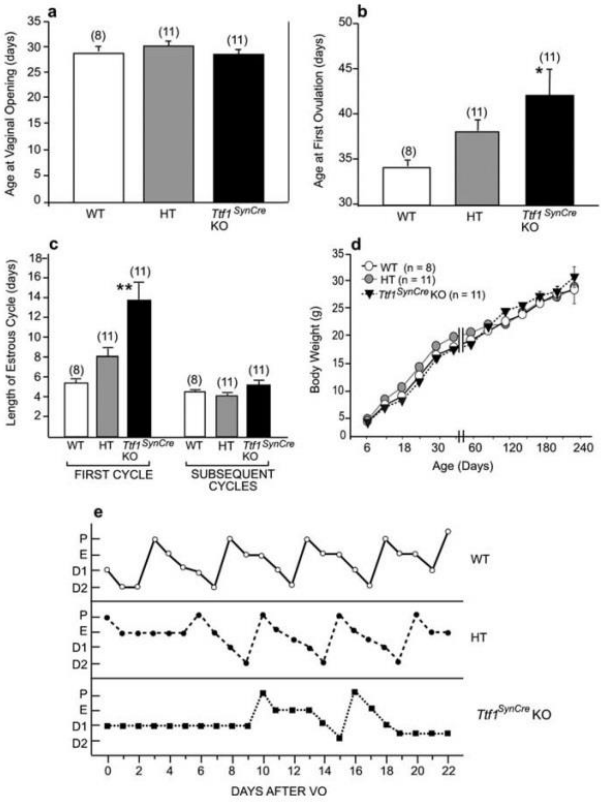

Figure 6. Neuronal deletion of the *Ttf1* gene delays the onset of female puberty. ***a–c***, Although the age at vaginal opening occurred at similar ages in WT, HT, and $Ttf1^{SynCre}$ KO mice (***a***), the age at first ovulation was significantly delayed in $Ttf1^{SynCre}$ KO animals (***b***), and the first estrous cycle was longer in $Ttf1^{SynCre}$ KO than WT and HT mice (***c***). ***d***, These changes were not secondary to differences in body weight gain. ***e***, Representative estrous cycle profiles illustrating the delayed onset of normal cyclicity and the slight lengthening of subsequent estrous cycles in $Ttf1^{SynCre}$ KO mice compared with HT and WT mice. P, Proestrus; E, estrus; D1, diestrous day 1; D2, diestrous day 2; VO, vaginal opening. $*p < 0.05$, $**p < 0.01$ versus WT controls.

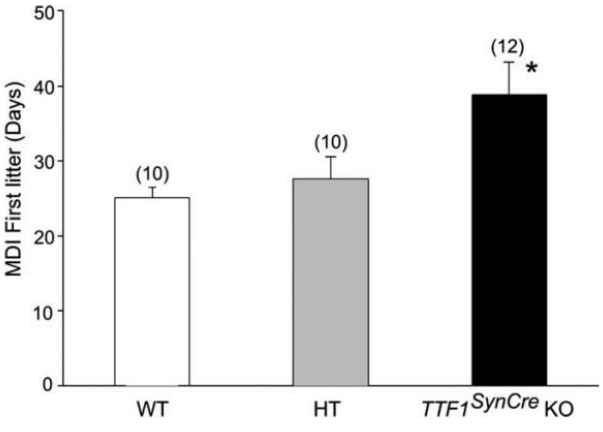

Figure 7. Mating–delivery interval for the first litter born to WT, HT, and $Ttf1^{SynCre}$ KO female mice. The KO mice delivered their first litter significantly later than both the HT and WT groups, indicating delayed first ovulation. $*p < 0.05$.

present results support this view. Not only was puberty delayed in TTF1-deficient mice, but we also demonstrate that the *KiSS1* gene [recently shown to play a pivotal role in the hypothalamic control of the pubertal process in humans and mice (de Roux et al., 2003; Seminara et al., 2003)] is subjected to TTF1 transcriptional control. Because kisspeptin, the peptide product of the *KiSS1* gene, stimulates LHRH secretion (Gottsch et al., 2004; Navarro et al., 2004), our results add credence to the concept that TTF1 acts as an upstream regulator of the pubertal process by transactivating genes involved in the facilitatory control of puberty and repressing genes that restrain sexual development (Ojeda et al., 2006).

We recently identified a novel gene termed EAP1 (enhanced at puberty) as another upstream regulator of mammalian puberty (S. Heger, C. Mastronardi, G. A. Dissen, A. Lomniczi, R. Cabrera, C. Roth, H. Jung, F. Galimi, and S. R. Ojeda, unpublished results). EAP1 appears to be placed at a higher hierarchical level than TTF1, because it controls TTF1 expression via direct transactivation of the TTF1 promoter and is expressed in a wider spectrum of neuronal subsets involved in the control of puberty than TTF1 (A. Lomniczi, C. Mastronardi, and S. R. Ojeda, unpublished results). In addition to LHRH, preproenkephalinergic, and KiSS1 neurons, EAP1 is expressed in glutamatergic and GABAergic neurons, the two major neuronal networks controlling female sexual development (Ojeda and Skinner, 2006). As predicted by the emerging features of gene networks controlling complex biological systems (Davidson et al., 2002; Basso et al., 2005), TTF1 and EAP1 are not only expressed in separate (but functionally connected) domains of the neuronal network controlling LHRH secretion, but they also share discrete functional domains, because both can transactivate the LHRH promoter and repress the preproenkephalin gene (this study) (Heger, Mastronardi, Dissen, Lomniczi, Cabrera, Roth, Jung, Galimi, and Ojeda, unpublished results).

In addition, the pubertal increase in hypothalamic TTF1 expression (and that of EAP1) (Heger, Mastronardi, Dissen, Lomniczi, Cabrera, Roth, Jung, Galimi, and Ojeda) appears to occur in an estrogen-independent manner, because the transcriptional activity of the TTF1 gene is not affected by the stimulation of ERα or ERβ receptors with either physiological or supraphysiological doses of the steroid. Reinforcing this notion are the following: (1) the absence of recognizable EREs in the TTF1 promoter of rats, mice, and humans, and (2) the finding that in rats, hypothalamic TTF1 mRNA increases during juvenile development (i.e., between days 20–30), when ovarian steroid levels are low, and decreases at the time when plasma estrogen levels increase, preceding the first preovulatory surge of gonadotropins (Lee et al., 2001). We believe that, because of this independence from estrogen, TTF1 can be considered as a component of the centrally driven, gonad-independent machinery that initiates female puberty in mammals.

A surprising finding was that the TTF1 deficiency did not affect LHRH mRNA abundance in young mice but abolished the age-related increase observed in older WT mice. We do not know the reasons for this delayed effect. Perhaps SynCre-mediated recombination is inefficient and/or occurs late in LHRH neurons. Alternatively, LHRH gene transcription might be redundantly controlled by other transcriptional regulators that become less influential in relation to TTF1 as the animal ages. It is noteworthy that the POA (in which LHRH neurons are located) derives from the ventral telencephalon but develops independently of TTF1 control (Sussel et al., 1999). Adding complexity to this issue, LHRH neurons express TTF1, but they originate in either the olfactory placode (Schwanzel-Fukuda and Pfaff, 1989) or elsewhere in the brain (Whitlock, 2005), and thus, they cannot be considered to have a telencephalic origin.

The $Ttf1^{SynCre}$ KO mice did not show impairments in locomo-

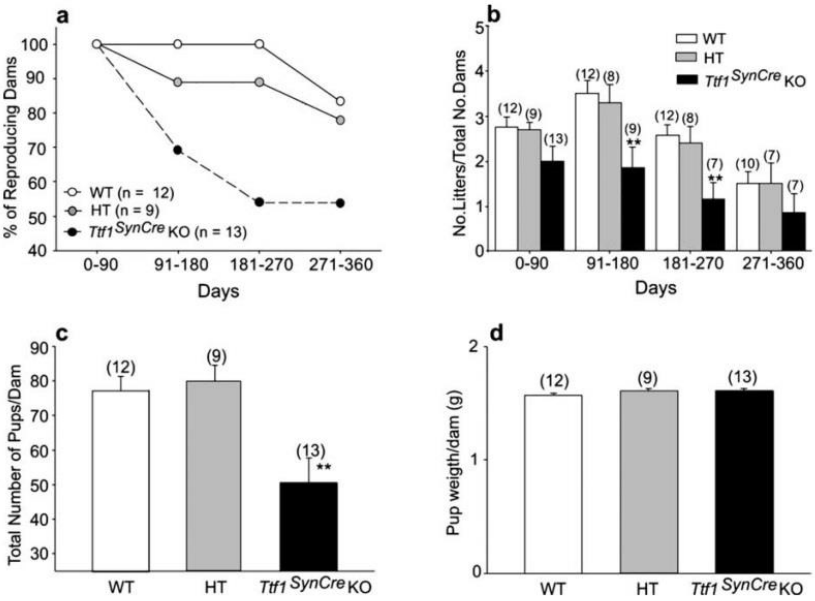

Figure 8. Reproductive capacity is diminished in *Ttf1*^{SynCre} KO female mice. ***a***, Fifty percent of *Ttf1*^{SynCre} KO dams ceased delivering pups by 6–9 months of age, a time during which all WT females and 90% of HT mice were reproducing normally. ***b***, The number of litters produced by each dam every 90 d significantly declined in *Ttf1*^{SynCre} KO females by 3 months of age. ***c***, *Ttf1*^{SynCre} KO females produce fewer pups than WT or HT dams in a 1 year period. ***d***, The body weight of the pups at birth was similar in all three experimental groups. $^{**}p < 0.01$ versus WT controls. No., Number.

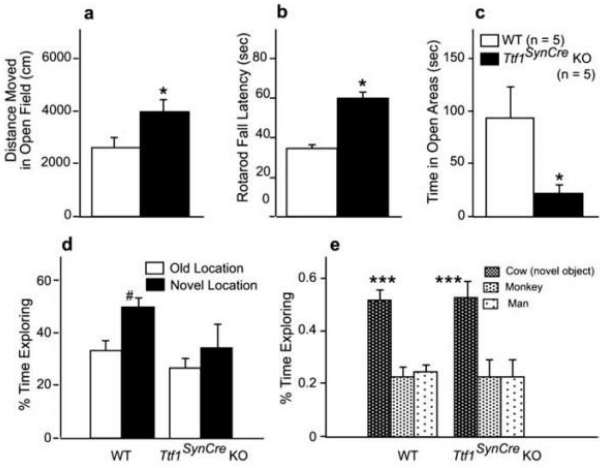

Figure 9. Functions directly or indirectly related to basal ganglia activity are not negatively impacted in *Ttf1*^{SynCre} KO mice. ***a–e***, Locomotor activity in the open field is increased (***a***), rotarod performance is increased (***b***), anxiety is increased as assessed by the elevated zero maze (***c***), and novel location recognition is reduced (***d***), but novel object recognition is not affected (***e***). The tests were performed on 14-month-old WT and *Ttf1*^{SynCre} KO mice. $n = 5$ mice per genotype. $^{*}p < 0.05$ versus WT; $^{\#}p < 0.05$ versus old location; $^{***}p < 0.001$ versus the two other objects.

tor activity in the open field or sensorimotor function on the rotarod. These data indicate that basal ganglia function is not diminished when TTF1 is deleted from postmitotic differentiated neurons and thus support the concept that continuous expression of TTF1 is not required for postnatal basal ganglia function. A note of caution must be introduced here, because the persistent expression of TTF1 in some neurons of the striatum and pallidum after birth (Marin et al., 2000) suggests that loss of expression in *Ttf1*^{SynCre} KO mice may have indeed resulted in subtle defects not detected with the methodology we used.

The increased anxiety observed in *Ttf1*^{SynCre} KO mice cannot be a result of a lack of GABAergic interneuron migration from the pallidum into the cerebral cortex, because such a migratory deficiency, which has been observed in conventional *Ttf1* KO mice (Sussel et al., 1999), would not be expected to increase anxiety (Siniscalchi et al., 2003). Instead, the increased anxiety displayed by *Ttf1*^{SynCre} KO mice in the elevated zero maze might be related to loss of TTF1 from neuronal subsets located in the bed nucleus of the stria terminalis and parts of the septum and the amygdala. TTF1 is expressed in postmitotic neurons of these regions (Sussel et al., 1999), which are implicated in the regulation of anxiety (Gray and Bingaman, 1996; Lee and Davis, 1997).

Dysfunction of cholinergic basal forebrain neurons, a well established defect in Alzheimer's disease, is associated with cognitive impairment (Ginsberg et al., 2006). Although *Ttf1*^{SynCre} KO mice appeared to have some impairment of novel location recognition, they displayed an intact novel object recognition, as well as normal learning and memory, as assessed by the water maze and passive avoidance tests. Because all of these parameters are affected in aging rats or animals bearing basal forebrain lesions (Paban et al., 2005), our results indicate that basal forebrain cholinergic neuronal function is essentially intact in conditional TTF1-deficient mice. Major behavioral defects measured by the tests we used would have been expected to occur if these neurons were functionally impaired or absent, because it occurs in conventional *Ttf1* KOs (Sussel et al., 1999).

In conclusion, our results suggest that beyond its role in regional specification of the ventral forebrain, the continuous presence of TTF1 is required only for the maintenance of postnatal functions inherent to a diencephalic derivative (the hypothalamus) but not those performed by a telencephalic derivative (the basal ganglia). In a broader context, these results also raise the possibility that a similar functional dichotomy may exist in other embryologically distinct brain regions whose developmental fate is controlled by the same homeodomain gene(s).

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3. Diskussion und aktueller Bezug

In dieser Dissertation wurde die transkriptionelle Kontrolle des *KiSS1*-Gens und seine Verknüpfung mit regulierenden, übergeordneten Genen des von Roth et al. beschriebenen TSG-Netzwerkes näher untersucht *(Roth et al., 2007)*.

Für eine präzise Analyse der regulierenden Elemente der *KiSS1*-Transkription ist die Identifikation der genauen TSS wichtig. Wir konnten experimentell die TSS -153 bis -156 bp upstream des Startkodons ATG festlegen, was sich mit der in silico Vorhersage deckt (NM_002256). Die 5´ Region von dieser Stelle enthält promotortypische Elemente wie eine TATA-Box, GC-reiche Regionen und zahlreiche Transkriptionsfaktor-Bindungsmotive. Die Abwesenheit von anderen Transkriptionsprodukten lässt die Aussagen zu, dass zumindest im menschlichen Plazentagewebe nur eine TSS vorhanden ist.

Zum Zeitpunkt unserer Untersuchungen war nicht bekannt, ob es eventuell spezies- oder gewebeabhängig alternative TSS gibt. Es konnte nachgewiesen werden, dass im MBH von Mäusen und Affen, im Hypothalamus von Menschen und in der Area praeoptica (POA) von Ratten eine einzige TSS genutzt wird, wobei sich die Länge des nicht translatierten, ersten Exons speziesabhängig unterscheidet *(Castellano et al., 2014)*. Dies deckt sich mit unserem Ergebnis und dem anderer Studien *(Tomikawa et al., 2012; West et al., 1998; Huijbregts und de Roux, 2010; Ota et al., 2004).*

Interessanterweise wurde im Gebiet des MBH (ARC) von Ratten eine zusätzliche, alternative TSS2 nachgewiesen, deren Promotor andere Eigenschaften hat als der der Haupt-TSS1. Die *Kiss1*-Expressionsmenge ausgehend von TSS2 ist insgesamt niedrig und nicht durch Estrogen beeinflussbar. Die funktionelle Bedeutung dieser alternativen TSS2 ist aktuell noch ungeklärt *(Castellano et al., 2014)*.

Die höchste Basalaktivität konnten wir im kürzesten der von uns klonierten Promotorkonstrukte nachweisen. Dies könnte dafür sprechen, dass transkriptionsverstärkende Elemente eher proximal zur TSS liegen, während hemmende distaler lokalisiert sind.

Die funktionelle Analyse der Promotorregion in Anwesenheit verschiedener, wahrscheinlich übergeordneter Transkriptionsfaktoren ergab ein sehr komplexes, teilweise scheinbar widersprüchliches Bild. Es verdeutlicht jedoch erneut, dass die Expression von Kisspeptin einer empfindlichen Balance aus aktivierenden und inhibierenden Einflüssen unterliegt.

EAP1 zeigte in unseren Versuchen einen moderat inhibierenden Effekt auf den *KiSS1*-Promotor, was im Hinblick auf die Tatsache, dass EAP1 zu Beginn der Pubertät verstärkt exprimiert wird, interessant ist *(Heger et al., 2007).* Die Bedeutung dieser inhibierenden Wirkung bleibt spekulativ, ebenso ob sich EAP1 womöglich in Gegenwart von Bindungspartnern, die spezifisch in KiSS1-Neuronen exprimiert werden, anders verhält. Auch die funktionellen Unterschiede der beiden KiSS1-Neuronengruppen sollten bei weiterführenden Studien Beachtung finden. Es konnte für EAP1 gezeigt werden, dass es in manchen Zellen Teil eines Repressionskomplexes ist, der die Transkription nachgeschalteter Gene supprimiert. So stellt EAP1 womöglich einen Inhibitor untergeordneter Repressoren dar *(Yeung et al., 2011; Pinilla et al., 2012).*

In einer neueren Studie wurde der Einfluss von Eap1 auf *Kiss1* in weiblichen Ratten untersucht *(Li und Li, 2017). Eap1* wurde mittels lentiviral-vermittelter RNA-Interferenz in seiner Expression gestört und die Versuchstiere in drei Entwicklungsstadien auf Pubertätszeichen und

die Expression von *Gnrh* mRNA und *Kiss1* mRNA untersucht. Die Tiere zeigten eine verzögerte Pubertät und eine verminderte Expression von *Gnrh* mRNA zu Beginn der Pubertät. Die Expression von *Kiss1* zeigte währenddessen keinen Unterschied zwischen einzelnen Gruppen und stieg im Laufe der Entwicklung bei allen Tieren an. Li et al. schlossen daraus, dass das KiSS1/KiSS1R-System nicht an der regulatorischen Wirkung von EAP1 beteiligt ist.

Welche Bedeutung die Ergebnisse von Li et al. im Gesamtkontext haben, ist noch nicht endgültig erklärt. Zum einem nutzen sie in dieser Studie lediglich AVPV-KiSS1-Neurone, welche sich in ihrer Physiologie deutlich von ARC-KiSS1-Neuronen unterscheiden. Dies lässt die Möglichkeit offen, dass EAP1 in diesem Kontext eine regulatorische Wirkung auf *KISS1* haben könnte *(Li und Li, 2017).* Zum anderen konnten wir in unserer Arbeit sowohl eine Ko-Expression von Eap1 in KiSS1-Neuronen im MBH (ARC) von Ratten nachweisen, als auch die Bindung von EAP1 an die Promotorregion von *KiSS1*. Es ist offensichtlich, dass weitere Untersuchungen nötig sind, um diesen Punkt suffizient zu klären.

TTF1 zeigte einen stimulierenden Effekt auf die *KiSS1*-Promotoraktivät. Damit konnten wir Teilergebnisse aus Arbeit 2 bestätigen. Dort wurde außerdem durch gerichtete Mutagenese gezeigt, dass für die transaktivierende Wirkung von TTF1 ein Bindungsmotiv -100bp bis -109bp von der TSS nötig ist. Es wurde auch die zweite TTF1-Bindungsstelle -1010bp bis -1019bp deletiert, ohne dass sich das Ergebnis signifikant unterschied. Die Tatsache, dass die distal gelegenere Bindungsstelle von untergeordneter Bedeutung zu sein scheint, unterstützt die oben erwähnte These, dass die transaktivierenden Elemente des *KiSS1*-Promotors eher proximal zur TSS liegen.

Überraschenderweise konnte dieser stimulierende Effekt nur in der nicht-neuronalen Zelllinie nachgewiesen werden und blieb in GT1-7 Zellen aus. Obwohl es sich bei diesen um eine murine Hypothalamuszelllinie handelt (genauer GnRH-Neurone), bleibt zu diskutieren, ob für die transaktivierende Wirkung auf den *KiSS1*-Promotor Co-Aktivatoren nötig sind, welche in GripTite^{MSR} 293 Zellen (einer humanen, embryonalen Nierenzelllinie) und womöglich auch in KiSS1-Neuronen exprimiert werden. Die physiologische Bedeutung der Interaktion von TTF1 und dem *KiSS1*-Promotor wird durch folgende Ergebnisse untermauert: i. Konditionelles *Ttf1*^{SynCre} Knock-out führt bei Mäusen zu einer verminderten Expression von *Ttf1* mRNA im MBH. Als Folge davon ist auch die *Kiss1*-Expressionsmenge im MBH reduziert und die Versuchstiere zeigen eine verzögerte Pubertät (Arbeit 2), ii. Durch in situ Hybridisierung konnte die Expression von *Ttf1* in KiSS1-Neuronen nachgewiesen werden (Arbeit 2), iii. TTF1 bindet an den *KiSS1*-Promotor (Arbeit 1).

Obwohl wir bei unseren Betrachtungen davon ausgingen, dass es sich bei *TTF1* und *EAP1* um nicht-TSGs handelt, wurde nachfolgend gezeigt, dass beide Gene an der Tumorbiologie beteiligt sind *(Winslow et al., 2011; Yeung et al., 2011)*. In Anbetracht dieser Informationen gehen wir, bei aktuellem Wissensstand davon aus, dass diese Gene ein eigenständiges Modul innerhalb des TSG Netzwerks bilden *(Lomniczi et al., 2013a)*.

Für CUX1 konnten wir eine divergente, transkriptionelle Aktivität der beiden Isoformen bestätigen *(Goulet et al., 2002; Sansregret und Nepveu, 2008)*. Während p-200 im nicht-neuronalen Zellmodell aktivierend wirkt, zeigt sie in GT 1-7 Zellen eine eingeschränkte inhibierende Wirkung. Die p-110 Form agiert in beiden Zelllinien als Repressor. Zwar konnten wir zeigen, dass KiSS1-Neurone CUX1 exprimieren, jedoch ist, aufgrund fehlender

Isoformenspezifität der Antikörper, eine Diskriminierung zwischen p-200 und p-110 immunhistochemisch nicht möglich. Auch die Frage wie CUX1 die Expression von Kisspeptin in KiSS1-Neuronen beeinflusst, bleibt offen.
Die Ergebnisse für YY1 waren ebenfalls zellmodellabhängig. In GripTite^{TM}293 MSR hatte YY1 einen repressiven Einfluss auf die Promotoraktivität, in GT 1-7 blieb diese unbeeinflusst. Auch hier stellt sich die Frage, welchen transregulatorischen Effekt YY1 in KiSS1-Neuronen bietet, da auch für diesen Transkriptionsfaktor eine duale, zellspezifische Wirkung beschrieben wurde *(Gordon et al., 2006).*
Zusammenfassend kann festgestellt werden, dass wir in unserer Arbeit den vermuteten transregulatorischen Einfluss der von uns ausgewählten Kandidatengene auf den *KiSS1*- Promotor experimentell nachweisen konnten. Die genaue Interaktion in der Kontrolle einzelner Gene innerhalb des TSG-Netzwerkes ist nicht vollständig geklärt und womöglich auch abhängig vom Entwicklungsstadium *(Lomniczi et al., 2013a)*.
In späteren Untersuchungen wurde beobachtet, dass der *EAP1*-Promotor der gleichen dualen Transkriptionskontrolle unterliegt, wobei TTF1 aktivierend und YY1 und CUX1 supprimierend wirken. Außerdem scheint EAP1 sich via negativer Auto-Feedback-Schleife selbst zu hemmen *(Mueller et al., 2012).* Dies weist erneut auf die hohe Interkonnektivität in der Regulation dieser zentralen transregulierenden Gene hin.
Es kann in diesem Rahmen nur erwähnt werden, dass sich die aktuellen wissenschaftlichen Bemühungen zunehmend mit den übergeordneten Regulatoren der Pubertät beschäftigen. Auf Grundlage des oben beschriebenen TSG Netzwerkes wird von einem Regulationskomplex mit drei Ebenen, bestehend aus Repression-Repression-Aktivierung, ausgegangen (Abb.3, S.54). Pubertätsaktivierende Gene wie *Kiss1* oder *Tac3* (codiert für Neurokinin B) stellen dabei die unterste Stufe der Kaskade dar. Auf diese nehmen in der nächsten Stufe diverse Aktivatoren wie TTF1 und CUX1 oder Repressoren wie der Polycomb-Silencing-Komplex Einfluss. Die Repressoren haben scheinbar nicht nur einen regulierenden Effekt auf die pubertätsaktivierenden Gene, sondern auch auf deren Aktivatoren. Die dritte Ebene besteht aus Genen, die hemmend auf die Repressoren wirken. Nach aktueller Meinung nimmt ihr Einfluss zu Beginn der Pubertät zu, wodurch der auf den pubertätsaktivierenden Genen lastende inhibierende Tonus aufgehoben wird. Zu diesen gehört unter anderem die Familie der *POK* Gene. Es handelt es sich hierbei um transkriptionelle Repressoren mit einer N-terminalen BTB/POZ-Domäne und einer C-terminalen Zinkfinger-Domäne vom Krüppel-Typ.
Die Gene der verschiedenen Regulationsstufen sind stark miteinander verknüpft. Zusätzlich finden auf allen Ebenen dieses Regulationskomplexes Veränderungen durch epigenetische Mechanismen statt *(rez. in Lomniczi et al., 2013a,b; rez. in Lomniczi und Ojeda, 2016a,b)*.
Die Fortschritte im Verständnis der genetischen und epigenetischen Hintergründe, die zur Regulation der Pubertät beitragen, bieten zum einen ein besseres Verständnis der Wirkweise von endokrin wirksamen Substanzen aus der Umwelt (sogenannten endokrinen Disruptoren), welche die pubertäre Entwicklung verändern können *(Mueller und Heger, 2014; Franssen et al., 2014).* Zum anderen ergeben sich daraus möglicherweise Behandlungskonzepte für Patienten mit Pubertätsstörungen.

4. Zusammenfassung

Dissertation zur Erlangung des akademischen Grades Dr. med.

„Charakterisierung und funktionelle Analyse des humanen *KiSS1*-Promotors“

eingereicht von Anja Dietzel

angefertigt an der Universität Leipzig
Klinik und Poliklinik für Kinder und Jugendliche der Universität Leipzig

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Pubertät ist ein Prozess, bei dem der Körper eine Reihe anatomischer, physiologischer und emotionaler Veränderungen erlebt, die zur sexuellen Reifung und somit zur Fortpflanzungsfähigkeit führen. Der zeitlich koordinierte Ablauf und die Kontrolle darüber sind entscheidend für die Erhaltung einer Art.
Voraussetzung für eine normale pubertäre Entwicklung ist die pulsatile Sekretion von GnRH aus den gleichnamigen Neuronen im Hypothalamus. Diese untersteht einer Vielzahl regulatorischer Einflüsse auf hormoneller, transsynaptischer, glialer und genetischer Ebene. Eine entscheidende Schlüsselrolle hat hierbei Kisspeptin, das prozessierte Genprodukt des *KiSS1*-Gens. Es stimuliert, über Bindung an seinen zugehörigen Rezeptor KiSS1R, die Freisetzung von GnRH. Loss-of-function-Mutationen im *KiSS1*- oder im *KiSS1R*-Gen führen zum klinischen Bild des hypogonadotropen Hypogonadismus. Die neuroendokrine Funktion des Kisspeptins und die Neuroanatomie der KiSS1-Neurone waren in den letzten Jahren Gegenstand intensiver Forschungen.
Über die transkriptionelle Kontrolle des *KiSS1*-Gens ist indes wenig bekannt. Es wurde postuliert, dass *KiSS1* Teil eines hierarchisch angeordneten Netzwerkes aus tumorassoziierten Genen ist, welches maßgeblich an der Regulation der Pubertät und dem Erhalt der Fortpflanzungsfähigkeit beteiligt ist. Diese Gene zeichnen sich durch eine hohe Interkonnektivität aus. Durch in silico Computeranalysen konnten Kandidatengene, die einen möglichen transregulatorischen Einfluss auf *KiSS1* haben, identifiziert werden. Von besonderem Interesse für diese Arbeit waren die Transkriptionsfaktoren TTF1, EAP1, CUX1 und YY1.
Ziel dieser Arbeit war es, die 5´-flankierende Region des *KiSS1*-Gens genauer zu charakterisieren und auf die Funktionalität des *KiSS1*-Promotors und dessen Regulierbarkeit durch übergeordnete Transkriptionsfaktoren zu untersuchen.

Nach experimenteller Verifizierung der Transkriptionsstartstelle mittels 5´-RACE-PCR und 5´-nested-PCR aus humanem Plazentagewebe wurden *KiSS1*-Promotorkonstrukte verschiedener Längen in einen Reportervektor kloniert. Es folgte die transiente Transfektion in eine neuronale (GT1-7) und eine nicht-neuronale Zelllinie (GripTite^{TM}293 MSR). Anschließend wurden sowohl die Promotorbasalaktivitäten in den beiden Zelllinien, als auch die Promotoraktivität in Anwesenheit der einzelnen Transkriptionsfaktoren mittels Dual-Luciferase-Reporter-Assays analysiert.
Um die Bedeutung der Bindung von TTF1 an die putative Promotorregion nachzuweisen, wurden konsekutiv die proximale und distale Bindungsstelle durch gerichtete Mutagenese deletiert und funktionell analysiert.
Ob für den transregulatorischen Effekt eine Bindung der Transkriptionsfaktoren an den Promotor nötig ist, wurde mittels Chromatin-Immunpräzipitation-Assay untersucht.
Durch Immunohistofluoreszenzfärbungen sollte gezeigt werden, ob KiSS1-Neurone EAP1, CUX1 und YY1 exprimieren.
Die Ergebnisse dieser Dissertation lassen sich wie folgt zusammenfassen:

- *KiSS1* besitzt, zumindest in menschlichem Plazentagewebe, eine einzelne TSS -153bp bis -156bp upstream des ATG-Startcodons. Dies deckt sich mit der in silico Vorhersage (NM_002256).
- Die Promotorbasalaktivität ist im kürzesten Promotorfragment am höchsten und nimmt mit zunehmender Länge ab. Dies deutet darauf hin, dass aktivierende Elemente eher proximal zur TSS und inhibierende eher distal lokalisiert sind.
- Der *KiSS1*-Promotor wird von TTF1 und der p200-Isoform des CUX1 aktiviert.
- Zur Transaktivierung des *KiSS1*-Promotors durch TTF1 wird dessen proximale Bindungsstelle benötigt.
- Der *KiSS1*-Promotor wird vom EAP1, YY1 und CUX-p110 inhibiert.
- Der Einfluss der Transkriptionsfaktoren scheint zellkontextabhängig zu sein.
- Für die vier Transkriptionsfaktoren konnte eine Bindung am *KiSS1*-Promotor mittels ChIP-Assay nachgewiesen werden.
- Die In-situ-Hybridisierung erbrachte eine Ko-Expression der Transkriptionsfaktoren in KiSS1-Neuronen des MBH.

Die hier vorgelegte Arbeit stützt auf experimentelle Weise die postulierte Annahme, dass *KiSS1* als Teil eines pubertätsregulierenden Netzwerkes durch übergeordnete Gene kontrolliert wird. Es konnte gezeigt werden, dass diese Regulation durch direkte Beeinflussung der Promotoraktivität stattfindet.
Es zeigte sich aber auch, dass die Expression von Kisspeptin in vivo durch eine empfindliche Balance zwischen aktivierenden und inhibierenden Elementen gesteuert wird. Die inkongruenten Ergebnisse der Reporter-Assays in den beiden unterschiedlichen Zellmodellen, weisen auf einen zellkontextabhängigen Einfluss der Transkriptionsfaktoren auf den *KiSS1*-Promotor hin. Weitere Analysen in Bezug auf noch nicht identifizierte coregulatorische Elemente erscheinen sinnvoll.

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www.ensembl.org Gene-ID: 3814

II. Anlagen

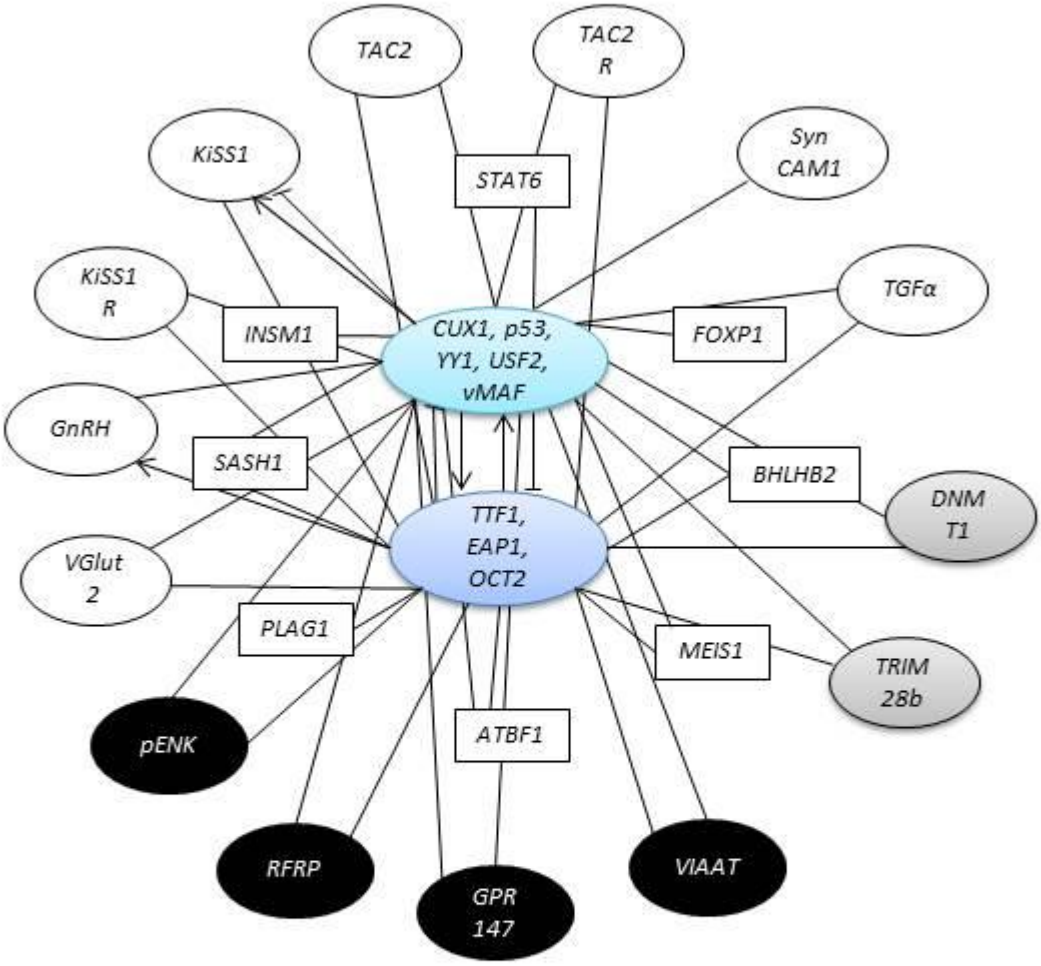

Abb. 1: Das Netzwerk aus Tumorsuppressor-Gene, welche an der Regulation der Pubertät beteiligt sind. Aufgrund des Vernetzungsgrades der Transkriptionsfaktoren (TF) *CUX1, p53, YY1, USF2* und *vMAF* wird davon ausgegangen, dass diese zentrale Knotenpunkte des Netzwerkes darstellen. Es besteht eine enge funktionelle Verbindung mit anderen, die Pubertät regulierenden TFs (*TTF1, EAP1* und *OCT2).* Untergeordnete Gene, die eine aktivierende Wirkung auf die GnRH-Sekretion und damit die pubertäre Entwicklung haben, sind in der Peripherie des Netzwerkes in *weißen*, Repressoren in *schwarzen Ovalen* dargestellt. Gene in *grauen Ovalen*, kodieren für Proteine, die an der epigenetischen Repression beteiligt sind. Zwischengeschaltete Gene wie die Transkriptionsfaktoren *SASH1, FOXP1* und *STAT6* sind in *weißen Rechtecken* dargestellt. *KiSS1* stellt in diesem Netzwerk, trotz seiner zentralen Rolle bei der transsynaptischen Stimulation der GnRH-Sekretion, ein untergeordnetes Gen dar *(*nach *Lomniczi und Ojeda, 2016a).* Die Darstellung dieses Netzwerkes beruht auf Computeranalysen aus high-throughput DNA-Arrays *(Roth et al., 2007).*

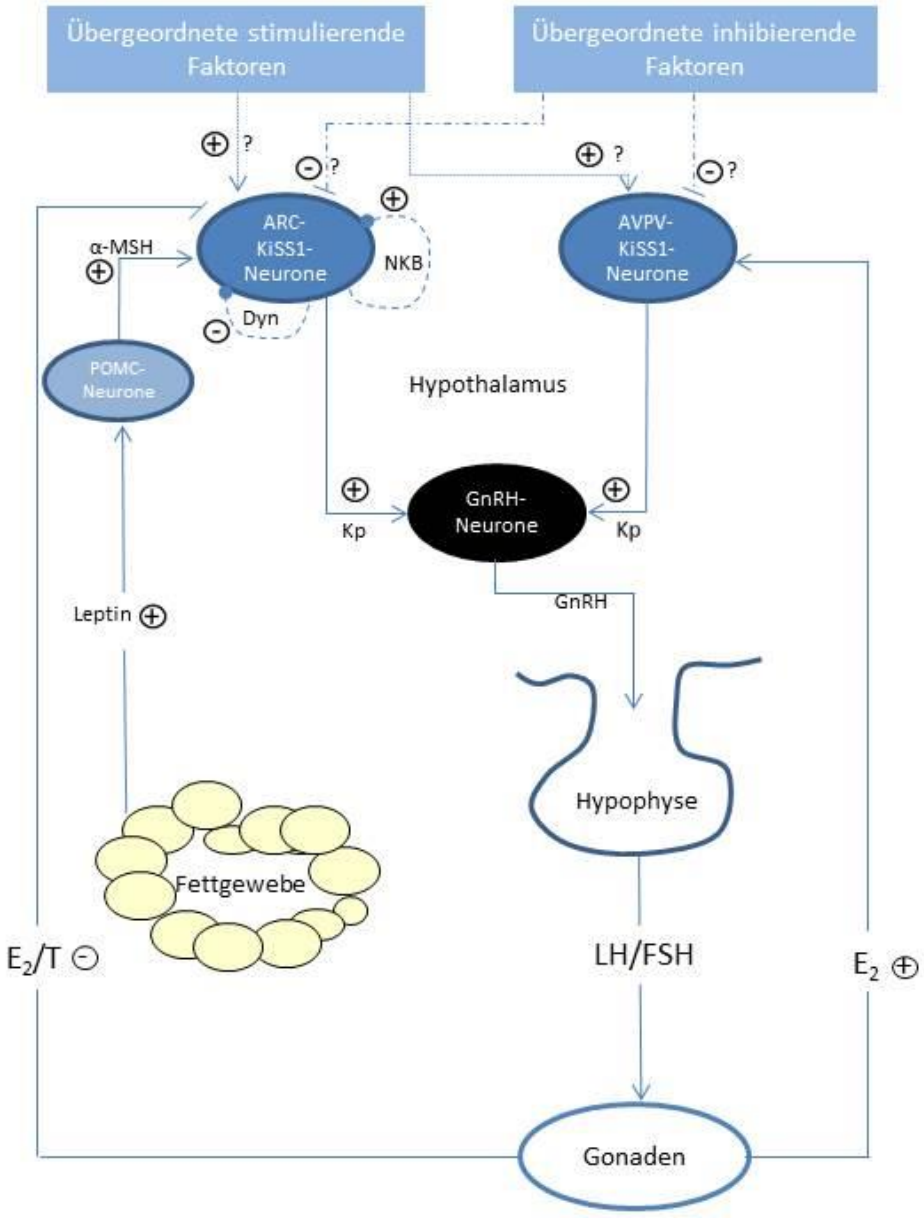

Abb. 2: Vereinfachtes Modell der Rolle der KiSS1-Neurone in der Steuerung der GnRH Sekretion. Andere regulierende Einflüsse auf die GnRH-Neurone wurden aus didaktischen Gründen weggelassen. T = Testosteron, E_2 = Estrogen, Dyn = Dynorphin, NKB = Neurokinin B, Kp = Kisspeptin, POMC = Proopiomelanocortin, α-MSH = α-Melanozyten stimulierendes Hormon (nach *Tena-Sempere, 2006; Pinilla et al., 2012).*

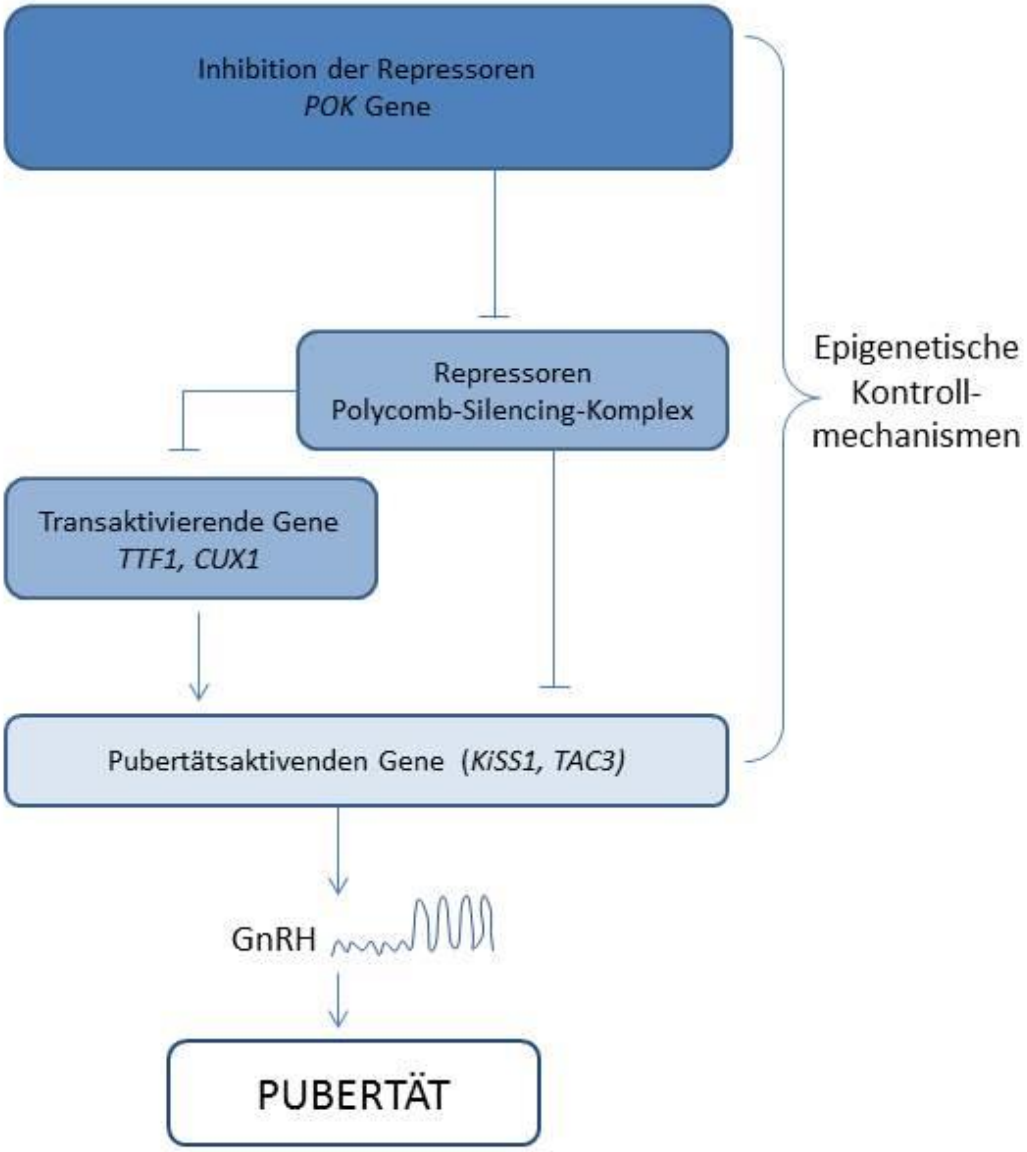

Abb. 3: Vereinfachtes Modell des Regulationskomplexes, bestehend aus den an der transkriptionellen Kontrolle der Pubertät beteiligten Genen. Details im Text S.41.
POK = Transkriptioneller Repressor mit einer N-terminalen BTB/POZ-Domäne und einer C-terminalen Zinkfinger-Domäne vom **K**rüppel-Typ. Die hier erwähnten Gene, stehen stellvertretend für die große Vielzahl derer, die an der Pubertätsregulation beteiligt sind *(vereinfacht nach Lomniczi und Ojeda, 2016a)*.

III. Spezifikation des Eigenanteils

Arbeit 1:

Mueller JK*, Dietzel A*, Lomniczi A, Loche A, Tefs K, Kiess W, Danne T, Ojeda SR, Heger S. Transcriptional regulation of the human *KiSS1* gene. Mol Cell Endocrinology 2011; 342(1-2): 8-19

* these two authors contributed equally to this work

Anja Dietzel trug zu oben genannter Arbeit folgendermaßen bei:

- Planung, Durchführung und Auswertung der experimentellen Arbeiten zur Bestimmung der Transkriptionsstartseite
- Design und Klonierung der Promotorfragmente
- Funktionelle Analyse der Promotorfragmente in den beiden Zelllinien GT 1-7 und GripTite™ 293 MSR bezgl. der Promotorbasalaktivität und des Promotorverhaltens in Zusammenhang mit TTF1 und EAP1 (inkludiert die transiente Transfektion der beiden Zelllinien und die Etablierung, Durchführung und Auswertung des Dual Luciferase Reporter Assays)
- Anteil am veröffentlichen Manuskript:
  - Unterpunkte 2.1 und 2.2 sowie 3.1, 3.2 und 3.3
  - Bereitstellung von Tabelle 1 und anteilige Bereitstellung von Ergebnissen zu Fig.1, Fig.2, Fig.6 A und B, Fig.7

(Dr. rer. nat. J. K. Müller)

(Prof. Dr. med. S. Heger)

(Prof. Dr. med. W. Kiess)

(Dr. rer. nat. K. Tefs)

Arbeit 2:

Mastronardi C, Smiley GG, Raber J, Kusakabe T, Kawaguchi A, Matagne V, Dietzel A, Heger S, Mungenast AE, Cabrera R, Kimura S, Ojeda SR. Deletion of the *Ttf1* gene in differentiated neurons disrupts female reproduction without impairing basal ganglia function. J Neurosci 2006; 26(51): 13167-79

Anja Dietzel trug zu oben genannter Arbeit folgendermaßen bei/ Anja Dietzel contributed as follows:

- Gerichtete Mutagenese der TTF1 Bindungsstellen im *KiSS1*-Promotorkonstrukt/ site-directed mutagenesis of the TTF1 binding sites in the *KISS1* promoter construct
-
- Anteil am veröffentlichen Manuskript/ contribution to the published manuscript: Abschnitt/ Section „Site-directed mutagenesis of the TTF-1 binding sites"

Sergio R. Ojeda

(S. R. Ojeda, D.V.M., Head,
Neuroscience Division, ONPRC)

(Prof. Dr. med. S. Heger)

IV. Eigenständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

_____ _____

Datum Unterschrift

V. Curriculum Vitae

Anja Dietzel, geboren am 12.08.1983 in Rudolstadt

Schulische Ausbildung:

1990 - 1994	Grundschule Dittrichshütte
1994 - 2002	Friedrich-Fröbel-Gymnasium, Bad Blankenburg (Abitur, Note 1,7)
2002 - 2003	Freiwilliges Soziales Jahr, Universitätsklinikum Heidelberg

Medizinische Ausbildung:

10/2003	Beginn Studium Humanmedizin an der Universität Leipzig
08/2005	Erster Abschnitt der Ärztlichen Prüfung (Note - gut -)
05/2010	Zweiter Abschnitt der Ärztlichen Prüfung (Note - gut -) Approbation als Ärztin
2010 - 2013	Assistenzärztin Klinik für Anästhesie und Intensivmedizin, Park-Krankenhaus Leipzig-Südost GmbH
2013 - 2016	Assistenzärztin Klinik für Anästhesie, Intensivmedizin, Schmerztherapie und Palliativmedizin, Sana Kliniken Leipziger Land
seit 2016	Fachärztin für Anästhesiologie in der Klinik für Anästhesie, Intensivmedizin, Schmerztherapie und Palliativmedizin, Sana Kliniken Leipziger Land

Dissertation:

10/2005	Beginn der experimentellen Doktorarbeit im Forschungslabor der Universitätsklinik für Kinder und Jugendliche der Universität Leipzig
2005/2006	Forschungssemester
12/2008	Abschluss der experimentellen Arbeiten

VI. Liste der Publikationen und wissenschaftlicher Beiträge

Mastronardi C, Smiley GG, Raber J, Kusakabe T, Kawaguchi A, Matagne V, **Dietzel A**, Heger S, Mungenast AE, Cabrera R, Kimura S, Ojeda SR.Deletion of the *Ttf1* gene in differentiated neurons disrupts female reproduction without impairing basal ganglia function. J Neurosci 2006; 26(51):13167-79

Dietzel A, Lomnizci A, Schulz A, Ojeda SR, Klammt J, Kiess W, Heger S. Klonierung und funktionnelle Analyse des humanen KiSS1-Promotors. Posterpräsentation, Jahrestagung AGPD, 2006 Dresden

Mueller JK*, **Dietzel A***, Lomniczi A, Loche A, Tefs K, Kiess W, Danne T, Ojeda SR, Heger S. Transcriptional regulation of the human *KiSS1* gene. Mol Cell Endocrinology 2011; 342(1-2):8-19
* these two authors contributed equally to this work

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