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Gene dosage of Otx2 is important for fertility in male mice ☆

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ABSTRACT

Together, the hypothalamus, pituitary and gonads direct the development and regulation of reproductive function in mammals. Gonadotropin-releasing hormone (GnRH) expression is limited to ~800 neurons that originate in the olfactory placode then migrate to the hypothalamus. Coordination of the hypothalamic–pituitary–gonadal (HPG) axis is dependent upon correct neuronal migration of GnRH neurons into the hypothalamus followed by proper synthesis and pulsatile secretion of GnRH. Defects in any one of these processes causes infertility. Otx2, the vertebrate homologue of Drosophila *orthodenticle*, is a transcription factor that has been shown to be critical for normal brain and eye development and is expressed in both the developing GnRH neurons and the pituitary, suggesting that this gene may play a critical role in development of the HPG axis. As Otx2-null mice are embryonic lethal, we have analyzed the reproductive capacity of heterozygous Otx2 mice to determine the contribution of Otx2 gene dosage to normal HPG axis function. Our data reveal that correct dosage of Otx2 is critical for normal fertility as loss of one allele of Otx2 leads to a discernible reproductive phenotype in male mice due to disruption of the migration of GnRH neurons during development.

1. Introduction

The hypothalamic–pituitary–gonadal (HPG) axis is fundamental to the endocrine control of reproduction in mammals. Dysfunction at any level of the axis leads to pathophysiologic disorders such as infertility, polycystic ovarian syndrome, and hypogonadotropic hypogonadism. Gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile pattern from a small, yet critical, population of neurons within the hypothalamus to regulate the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from gonadotrope cells within the anterior pituitary. LH and FSH are then secreted into the bloodstream where they travel to their target organs, the gonads, to regulate spermatogenesis in males and folliculogenesis and ovulation in females.

Otx2, the vertebrate homologue of Drosophila *orthodenticle*, is a transcription factor that has been shown to be critical for normal brain and eye development (Acampora et al., 1995; Simeone et al., 1993; Frantz et al., 1994; Puelles et al., 2004). During embryogenesis, Otx2 is expressed in both the developing GnRH neurons (Mallamaci et al., 1996) and presumptive pituitary at e12.5 (Simeone et al., 1993) suggesting that this gene may play a critical role in development of the HPG axis, a hypothesis supported by the identification of several heterozygous Otx2 loss-of-function mutations in patients with combined pituitary hormone deficiency (Dateki et al., 2008, 2010; Diaczok et al., 2008). Several germline and conditional knockout mice have been generated which have emphasized a role for Otx2 in head formation, postnatal survival and growth (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995; Fossat et al., 2006). However, as Otx2-null mice are embryonic lethal, due to a failure to develop the forebrain, midbrain and anterior hindbrain, analysis of the development and maintenance of the HPG axis in these mice has not been possible. Recently, Diaczok et al. (2008, 2011) established that deletion of Otx2, specifically from GnRH neurons, results in hypogonadotropic hypogonadism in mice adding *in vivo* data to previously published reports demonstrating the important role Otx2 plays as a transcriptional regulator of GnRH expression (Larder and Mellon, 2009; Kim et al., 2007; Kelley et al., 2000).

In this paper, we have analyzed the reproductive capacity of heterozygous Otx2 mice to determine the contribution of Otx2 gene dosage to normal HPG axis function. We report that male

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Otx2 heterozygotes display compromised fertility and demonstrate that, while loss of Otx2 does not affect expression of pituitary gonadotropin genes, correct gene dosage of Otx2 is critical for normal development of the GnRH neurons and expression of GnRH in adult, male mice.

2. Materials and methods

2.1. Mouse breeding and genotyping

Mouse colonies were maintained in agreement with protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. All animals were housed under a 12 h light–dark cycle and provided with food and water *ad libitum*. Otx2 flox mice were generated as previously described (Ang et al., 1996) and were a kind gift from Dr. Siew-Lan Ang (MRC NIMR, London, UK). Mice heterozygous for the Otx2 allele were generated by crossing Otx2 flox mice to ZP3-Cre mice (Lewandoski et al., 1997) to create a germ-line recombination for the deletion of Otx2. All mice were on a C57 Black6 background. Surprisingly, females were either not born or did not survive to weaning age, so studies were of male heterozygote mice only. Embryos were generated through timed-breeding with adult females with embryonic day (e) 0.5 being noon of the day the vaginal plug was detected. PCR was used to genotype the offspring for the Otx2 allele (see Table 1 for details of primer sequences).

2.2. Fertility assessments and hormone measurements

At 8 weeks of age, male mice were housed singly with a wild-type, 8-week-old, female, C57BL/6J mouse. The numbers of litters born and the number of pups per litter were recorded over a period of 180 days. For serum hormone analysis, mice were sacrificed by overdose of 5% avertin, and blood was collected by cardiac puncture. Serum was separated by centrifugation and stored at −20 °C before radioimmunoassay (RIA) analysis at the Center for Research in Reproduction Ligand Assay and Analysis Core at the University of Virginia.

2.3. Embryo collection

Plugged females were euthanized, and embryos were harvested at e13.5 and e17.5. A small amount of the tail was removed from each embryo and used to extract DNA for determination of the genotypes of the embryos. Whole embryos (e13.5) or embryo heads (e17.5) were fixed in 10% acetic acid, 30% formaldehyde, 60% ethanol, overnight at 4 °C, and dehydrated in 70% EtOH prior to embedding in paraffin. Sagittal sections (10 μm) were floated onto SuperFrost Plus slides (Fisher) and dried overnight at 37 °C. Approximately 120–200 sections were processed and stained per embryo, depending on the developmental stage analyzed.

Table 1
Quantitative RT-PCR and genotyping primer sequences.

Otx2 flox genotyping forward	5′GCACTGAAAATCAACTTGCC3′
Otx2 flox genotyping reverse	5′AGGCTAAAAGACCCTGGTC3′
Otx2 KO genotyping forward	5′TGTAGGGACTCTTGCGACCT3′
Otx2 KO genotyping reverse	5′GGGCTGAGTCTGACCACTTC3′
Q-RT-PCR GapDH forward	5′TGCACCACCAACTGCTTAG3′
Q-RT-PCR GapDH reverse	5′GGATGCAGGGATGATGTTC3′
Q-RT-PCR LHβ forward	5′CTGTCAACGCAACT3′
Q-RT-PCR LHβ reverse	5′ACAGGAGGCAAAGC3′
Q-RT-PCR FSHβ forward	5′GCCGTTTCTGCATAAGC3′
Q-RT-PCR FSHβ reverse	5′CAATCTTACGGTCTCGTATACC3′
Q-RT-PCR αGSU forward	5′CGAGGTAATAATCTTTGGAAC3′
Q-RT-PCR αGSU reverse	5′GTCATTCTGGTCATGCTGTCC3′
Q-RT-PCR GnRHR forward	5′GCCCCTTGCTGTACAAAGC3′
Q-RT-PCR GnRHR reverse	5′CCGTCTGCTAGGTAGATCATCC3′
Q-RT-PCR GnRH forward	5′TGCTGACTGTGTGTTTGGAAGGCT3′
Q-RT-PCR GnRH reverse	5′TTTGATCCACCTCCTTGCGACTCA3′

2.4. GnRH immunohistochemistry

Immunohistochemistry was performed as previously described (Larder et al., 2011). The primary antibody used was anti-GnRH antibody (Affinity BioReagents PA1-121; 1:1000 dilution). Biotinylated goat-anti-rabbit IgG (Vector Laboratories, 1:300 dilution) was used as a secondary antibody and GnRH peptide was then visualized using the Vectastain ABC elite kit and VIP peroxidase kit (Vector Labs). Sections from embryos were counterstained using methyl green (Vector Labs). Every section was visualized at 40× magnification using a Nikon Eclipse E800 microscope with a Nikon DS Fi1 camera and using NIS elements imaging software and the numbers of GnRH neurons present in each section, and their position along the migratory path (nasal, cribriform plate or brain) recorded. All slides were blinded before counting so the genotype was not known.

2.5. Quantitative RT-PCR

Hypothalami and pituitaries were dissected from 3–6 month-old male mice, snap frozen, and stored at −80 °C until processed. RNA was extracted using Trizol (Invitrogen) according to manufacturer's instructions and reverse transcribed using First-Strand cDNA Synthesis Kit (GE Healthcare) according to the manufacturer's instructions. Q-RT-PCR was performed as previously described (Larder and Mellon, 2009). For primer sequences, see Table 1.

2.6. Gonadal histology

Testes were dissected and weighed from animals of each of the three genotypes. Testes were fixed for 8 h in Bouins fixative (Sigma) at room temperature. Gonads were paraffin embedded, serially sectioned at 10 μm and stained with hematoxylin and eosin (H&E, Sigma).

2.7. Cell culture and transient transfections for luciferase reporter assays

Cell lines used were GT1-7 and LβT2. GT1-7 cells represent a fully differentiated GnRH neuron that secretes high levels of GnRH in a pulsatile manner (Wetsel et al., 1992) and were used to assess the effect of Otx2 on GnRH promoter activity. LβT2 cells represent a mature gonadotrope cell that expresses both luteinizing and follicle stimulating hormone (Alarid et al., 1996) and were used to assess the effect of Otx2 on gonadotropin promoter activity. All cells were cultured in DMEM (Mediatech) containing 10% fetal calf serum (Gemini Bio-Products), and 1% penicillin/streptomycin (Invitrogen) in a humidified 5% CO_2 incubator at 37 °C. Cells were seeded into 24-well plates and incubated overnight at 37 °C before being transiently transfected using FuGENE reagent (Roche Applied Science). Luciferase reporters were pGL3-1800-αGSU (−1.8 kb of the human alpha GSU regulatory region), pGL3-1800-LH (−1800 bp of the mouse LH-Beta regulatory region), pGL3-398-FSH (−398 bp of the mouse FSH-Beta regulatory region, pGL3-1200-GnRHr (−1200 bp of the mouse GnRH receptor regulatory region) and pGL3-5 kb-GnRH (5 kb of the rat GnRH regulatory region). Cells were transfected with 200 ng of expression plasmid (pSG5-Otx2), 400 ng of luciferase-reporter plasmid and 100 ng of the internal-control TK-109 bp promoter on β-galactosidase. Cells were harvested after

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