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Hypothalamic but not pituitary or ovarian defects underlie the reproductive abnormalities in *Axl/Tyro3* null mice☆

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ABSTRACT

AXL and TYRO3, members of the TYRO3, AXL and MER (TAM) family of tyrosine kinase receptors, modulate GnRH neuronal cell migration, survival and gene expression. *Axl/Tyro3* null mice exhibit a selective loss of GnRH neurons, delayed sexual maturation and irregular estrous cycles. Here we determined whether the defects were due to direct ovarian defects, altered pituitary sensitivity to GnRH and/or an impaired LH surge mechanism. Ovarian histology and markers of folliculogenesis and atresia as well as corpora luteal development and ovarian response to superovulation were not impaired. *Axl/Tryo3* null mice exhibited a robust LH response to exogenous GnRH, suggesting no altered pituitary sensitivity. Ovariectomized *Axl/Tyro3* null mice, however, demonstrated an impaired ability to mount a steroid-induced LH surge. Loss of GnRH neurons in *Axl/Tyro3* null mice impairs the sex hormone-induced gonadotropin surge resulting in estrous cycle abnormalities confirming that TAM family members contribute to normal female reproductive function.

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1. Introduction

Members of the TAM family of receptor tyrosine kinases (TYRO3, AXL and MER) contain an intracellular kinase and an extracellular ligand binding domain with characteristics of a neural cell adhesion molecule including fibronectin and immunoglobulin repeats (Lai and Lemke, 1991; Lemke and Rothlin, 2008; Linger et al., 2007). Growth arrest specific gene 6 (GAS6), and in some tissues the closely related Protein S (ProS1), are ligands for the TAM family members (Manfioletti et al., 1993; Hafizi and Dahlback, 2006). Analysis of TAM expression in immortalized GnRH neuronal cells showed that *Axl* and *Tyro3* (but not *Mer*) mRNA and protein are expressed in NLT cells, a model of migrating GnRH neurons, while *Tyro3* and *Mer* are expressed in post-migratory GT1-7 GnRH neuronal cells (Fang et al., 1998). We hypothesized that AXL and TYRO3 may mediate GnRH neuron migration and/or survival and impact on the ontogeny of the GnRH neuronal network during development (Allen et al., 2002; Allen et al., 1999).

Previously, we determined the reproductive consequences of disrupting both the *Axl* and *Tyro3* genes in female mice (Pierce et al., 2008). Overall fertility, birth rates, and age at vaginal opening of the mutant mice were similar to WT, but *Axl/Tyro3* null mice showed delayed first estrous and consistently impaired estrous cycles. Cycles were irregular and prolonged with an increased percentage of days in proestrous. Immunoblots of homogenates from brain regions dissected from WT embryos at embryonic day 15 (E15) revealed that AXL and TYRO3 were expressed in the developing hypothalamus. In contrast, TYRO3 and MER, but not AXL were expressed in adult WT hypothalamus, consistent with a role for AXL and TYRO3 in the early development of GnRH neurons. Compared with WT, *Axl/Tyro3* null adults exhibited a 34% loss in the number of immunoreactive GnRH neurons in the region of the organum vasculosum of the lateral terminalis (OVLT), previously shown to be important for the GnRH-induced LH surge in rodent species (Adachi et al., 2007; Herbison, 2008; Mayer et al., 2010). Together these data supported a central defect in GnRH neuronal development contributing to the reproductive phenotype.

In the current study we sought to determine whether the reproductive abnormalities were due to concomitant peripheral as well

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as central defects. Because other studies suggested TAM family members were expressed in rodent and human gonads (Lu et al., 1999; Schulz et al., 1995; Wang et al., 2005; Wu et al., 2008), we examined markers of folliculogenesis and luteal function, ovarian TAM expression and the response to a standard superovulation protocol. We assessed pituitary function and also determined if the localized loss of GnRH neurons in *Axl/Tyro3* null mice impaired the LH surge mechanism in response to sex steroids.

2. Materials and methods

2.1. Mice

Axl and *Tyro3* null mice established in a C57BL/6 × 129sv background (Lu et al., 1999) were bred to create *Axl/Tyro3* null mice. Control C57BL/6 × 129sv mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Animal care and experimental procedures were performed in accordance with the guidelines established by the Denver Veterans Affairs IACUC. Female and male mice were housed in microisolator cages in the same room under a 12 h light cycle with food and water *ad libitum*. All mice were genotyped by PCR analysis of tail DNA as previously described (Pierce et al., 2008). Mice were anesthetized with isoflurane from Webster Veterinary Supply (Sterling, MA) and sacrificed by cervical dislocation for tissue and blood collection. For major surgeries, anesthesia was induced with a cocktail of ketamine (80 mg/kg body weight) (Hospira, Inc., Lake Forest, IL,) and xylazine (10 mg/kg body weight) (Lloyd Labs, Shenandoah, IO) injected intraperitoneally and maintained with isoflurane as needed.

2.2. Reagents

Axl (M-20) antibody and horseradish peroxidase (HRP)-linked anti-goat antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tyro3 antiserum was a generous gift from Cary Lai at The Scripps Research Institute. Mer antibody was purchased from R&D Systems (Minneapolis, MN). Gas6 antibody was obtained from Bryan Varnum at Amgen (Thousand Oaks, CA). GAPDH antibody was purchased from Millipore (Billerica, MA). HRP-conjugated secondary antibodies (Donkey anti-rabbit IgG and sheep anti-mouse IgG) were purchased from GE Healthcare Bio-Sciences Corp (Piscataway, NJ).

2.3. RT-PCR

Ovaries and pituitaries from adult mice were collected in RNAlater from Ambion (Austin, TX). RNA was purified using TRIzol Reagent from Invitrogen (Carlsbad, CA) or Qiagen RNeasy mini kit (Valencia, CA). RNA (1.0 μg) was reverse transcribed using Thermo Verso cDNA kit from Fisher (Pittsburgh, PA). RT-PCR was performed under the following conditions: 94 °C for 3 min; 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 1 min for 35 cycles; 72 °C for 10 min. Primer sequences for amplifying *Axl* were: 5′-CCCCTGAGAACGTTAGCG-3′ and 3′-TGCTCTGCAGTACCATCTAGC-5′. The primer sequences for amplifying *Tyro3* were: 5′-CGATCTCCAGCTACAACGC-3′ and 3′-GCATGGCTGAGTCCGGAAT-5′. The primer sequences for amplifying *Mer* were: 5′-CTGCACAGTGAGAATCGCG-3′ and 3′-GCCTGGCTCAGATGTGTTCG-5′. Primer sequences for amplifying *Gas6* were: 5′-CCAGACCTGCCAAGATATCG-3′ and 3′-GCCATGTTACCGCAACCC-5′. The quantity of *Axl, Tyro3, Mer*, and *Gas6* mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. The primer sequences for amplifying *GAPDH* were: 5′-CGACCCCTTCATTGACCTCA-3′ and 3′-GCCACGACTCATACAGCACC-5′. The primer sequences for amplifying ovarian RNA are: *Foxo1*-F: 5′-GTGAACACCATGCCTCACAC-3′, *Foxo1*-R: 5′-TGGACTGCTCCTCAGTTCCT-3′; *Sfrp4*-F: 5′ATGCTCCGCTCCATCCTGGTG-3′, *Sfrp4*-R: 5′-TGGCCAGGATGGCGTTCTCC-3′; *Cyp11a1*-F: 5′-GTACTTGGGCTTTGGCTGGG-3′ *Cyp11a1*-R: 5′-CAGGTCCTGCTTGAGAGGCT-3′. Ovarian RNA was normalized to the ribosomal protein L19 using specific primer pairs: *Rpl19*-F 5′-GGTGACCTGGATGAGAAGGA and *Rpl19*-R 5′-TTCAGCTTGTGGATGTGCTC.

2.4. Immunoblots

Tissues were homogenized in 1× RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 59 mM Tris, pH 8.0) from Upstate (Lake Placid, NY) with freshly added 0.5 mM PMSF (Sigma–Aldrich, St. Louis, MO), 1× protease inhibitor (Sigma–Aldrich, St. Louis, MO), 20 mM Na_3VO_4 and 25 mM NaF (Fisher, Pittsburgh, PA). Protein lysates were quantified using BCA assay (Pierce, Rockford, IL). An aliquot of 50 μg of total protein was resolved by 7.5% SDS/PAGE using a Bio-Rad mini-gel system (Bio-Rad, Hercules, CA). Proteins were transferred to Hybond polyvinylidene difluoride (Amersham, Arlington Heights, IL) using a Bio-Rad mini transblotter system. The membranes were blocked in 3% BSA in TBS-T buffer (20 mM Tris–Cl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1 h at room temperature. Primary antibodies were diluted to 1:500 to 1:2000 in 0.5% BSA/0.1% NaN_3 in TBS-T and incubated with the membranes at 4 °C overnight. The membranes were washed in TBS-T. The HRP-linked secondary antibodies were diluted to 1:3000 in TBS-T and incubated with the membranes for 1 h at room temperature. The membranes were washed and visualized using Enhanced Chemiluminescence (ECL) immunodetection reagents from Thermo Fisher Scientific, Inc. (Rockford, IL).

2.5. Ovarian histology and analysis of corpora lutea

Ovaries from adult mice were collected in 10% formalin, sectioned and stained with hematoxylin and eosin (H&E). For estimation of average number of corpora lutea per cross-section, 3–7 mid-ovary sections from one ovary per mouse from 5 WT and 5 *Axl/Tyro3* null random cycling females were counted. For *in situ* hybridization analyses, ovaries of adult wild type ($n = 2$) and *Axl/Tyro3* null mice ($n = 3$) were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (7 μm) were cut and processed for *in situ* hybridization using specific probes for genes expressed in luteal cells (*Cyp11a1, Sfrp4*) and granulosa cells and oocytes (*Foxo1*) as described previously (Robker and Richards, 1998; Richards et al., 2002; Hsieh et al., 2003). cDNA fragments of the relevant genes were amplified by RT-PCR from mouse ovarian total cDNA and sub-cloned into the pCR-TOPO4 vector (Invitrogen, Carlsbad, CA) (1–3). *In situ* hybridization was performed as previously reported. Tissue histology and the radioactive probes were visualized under light- and dark-field illumination, respectively, using a Zeiss AxioPlan2 microscope equipped with a 2.5× objective.

2.6. TUNEL protocol

Paraffin-embedded ovary sections were deparaffinized and fixed in formaldehyde. Apoptotic follicles were identified using the DeadEnd™ Fluorometric TUNEL System (Promega Corp, Madison, WI). The percentage of growing follicles that stained positive for TUNEL was determined using a Nikon Eclipse E600 fluorescent microscope; images were acquired using Image ProPlus software.

2.7. Superovulation protocol

Female mice 28–34 days old were intraperitoneally injected with 5 IU Pregnant Mares Serum Gonadotropin (PMSG, Sigma–Aldrich, St. Louis, MO) on Day 1 and 5 IU or 2.5 IU human chorionic gonadotropin (hCG, Sigma–Aldrich) on Day 3. On Day 4, mice were anesthetized with isoflurane and sacrificed; both oviducts were dissected out onto a glass slide and ova were flushed from the ampulla with PBS and coverslipped. Ova were counted on a Nikon Diaphot microscope (Nikon Inc., Melville, NY) using a 10× or 20× objective.

2.8. Hormone assays

Blood was obtained by terminal cardiac puncture from animals deeply anesthetized with isoflurane. Samples were allowed to clot at room temperature; serum was stored at −20 °C until analysis. LH, FSH, estradiol and testosterone levels were assessed by radioimmunoassay performed by the Animal Reproduction and Biotechnology Laboratory at Colorado State University. Assay variations were as follows: estradiol intra-assay CV = 10.59%; inter-assay CV = 21.19%. LH intra-assay CV = 4.74%; inter-assay CV = 16.91%. Testosterone intra-assay CV = 6.62%; inter-assay CV = 14.19%. FSH intra-assay CV = 3.94%.

2.9. Pituitary (GnRH) stimulation protocol

Female mice between 2 and 3 months of age were injected subcutaneously with 200 ng/kg GnRH peptide (Sigma–Aldrich) in 100 μl saline. Blood was collected 10 min later by terminal cardiac puncture under anesthesia with isoflurane. Serum LH levels were measured and compared with LH in serum obtained from untreated controls; all blood was collected between 0800 and 1000 h.

2.10. LH surge protocol

Mice between 2 and 3 months of age were bilaterally ovariectomized and implanted with Silastic® RX-50 Medical Grade Tubing (Dow Corning Corp, Midland, MI) filled with 17-β estradiol (Sigma–Aldrich) dissolved in ethanol and mixed with Silastic® Medical adhesive Silicon, Type A (Dow Corning) at 0.1 mg/ml for final estrogen dosage of 1 μg/cm/20 g body weight. Mice were subcutaneously injected on Day 6 after implant with 1 μg estradiol benzoate (Sigma–Aldrich) dissolved in 100 μl of sesame oil (Sigma–Aldrich) and on Day 7 with 500 μg progesterone (Sigma–Aldrich) dissolved in 100 μl of sesame oil (Sigma–Aldrich). Mice were anesthetized with isoflurane (Webster Veterinary Supply) and blood collected by terminal cardiac puncture on Day 7.

2.11. Statistical analyses

All data are expressed as a mean ± SEM for each group. Statistical differences between the means of two groups were tested using unpaired *t*-test. *P*-values were reported as "*<0.05", "**<0.01" or "NS" (not significant). Statistical analyses were performed using GraphPad Software QuickCalcs Online Calculators for Scientists (www.graphpad.com, GraphPad Software, Inc., La Jolla, CA), Microsoft Office Excel 2003 v. 11 (Microsoft Corp, Redmond, WA) and SPSS Software (SPSS Inc., Chicago, IL).