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Supplementary Information for

Neural crest-derived neurons invade the ovary but not the testis during mouse gonad development

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Supplementary methods

Real-time quantitative PCR. Total RNA was extracted from gonad pairs with the RNeasy RNA Isolation kit (Qiagen). Reverse transcription was performed using the Verso cDNA synthesis kit (Thermo Scientific) and RT-qPCR was performed using QuantStudio 6 (Applied Biosystems). For the list of primers used in this study, see SI Appendix, Table S3. Each reaction was done in triplicate. Expression levels were determined with Applied Biosystems analysis software relative to standard curves. Data are represented as the mean level of gene expression relative to the expression of the reference gene *Gapdh*. Relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method and converted into fold changes. Statistical analysis was performed using GraphPad Prism 7.

Confocal image acquisition. Gonads were imaged in the longitudinal plane with an LSM710 Meta confocal microscope and the affiliated Zen software (Carl Zeiss, Inc.). Acquired confocal Z-Stacks were imported into FIJI software for minor processing, including cropping and rotations. All images of individual gonads are oriented with the anterior end of the gonad to the left and posterior end to the right. Unless otherwise stated, all images presented in this study are maximum intensity projections of confocal Z-Stacks generated with FIJI. Final processing, such as channel overlays, brightness and contrast adjustments and figure compositions were made in Adobe Photoshop CC. For blow-up panels, we either zoomed and cropped lower magnification images (referenced in the figures with a ‘ next to the image letter) or imaged the same sample with a higher magnification objective (referenced in the figures with a new letter).

Immunostaining and clearing for entire gonad imaging using Light Sheet Microscopy. To visualize the entire gonads and confirm that the absence of innervation in the testis was not due to lack of visualization, entire mouse gonads were stained and cleared using the iDISCO+ method (1). After fixation for 30min in 4% PFA and dehydration into methanol, samples were incubated overnight in 33%methanol, 66% dichloromethane at room temperature. The samples were then treated with a solution of 7% hydrogen peroxide in methanol overnight a 4ºC. After progressive rehydration to PBS 0.2% Triton X-100, (PTx.2) samples were permeabilized overnight at 37ºC in PTx.2 2.3% glycine, 20% DMSO and blocked for 6h at 37ºC in PTx.2 10% DMSO, 3% horse serum solution. Tissues were then incubated in primary antibodies diluted in PTwH (PTx.2 with 0.001% heparin) 3% horse serum, 10% DMSO overnight at 37 ºC. The following day, samples were washed 3 times in PTwH and incubated overnight in secondary antibodies diluted in PTwH 3% horse serum at 37ºC. After washing 3 times for 1h in PtWH, samples were progressively dehydrated into methanol and incubated for 3h in 33%methanol, 66% dichloromethane at room temperature. Following 2 washes in dichloromethane, samples were cleared in dibenzylether. Samples were left overnight in DBE to allow for sufficient clearing. After dibenzylether clearing, samples were progressively rehydrated to PBS and re-cleared following the CUBIC clearing protocol (2). Once sufficiently cleared, samples were mounted for imaging in glass capillaries in 1.8% agar/PBS. Samples were imaged in

CUBIC reagent-2 using the Z1 Zeiss Light sheet microscope (Carl Zeiss, Inc.). Videos of full stacks, 3D projections and montages of image stacks were rendered in Imaris imaging software (Bitplane, Inc.).

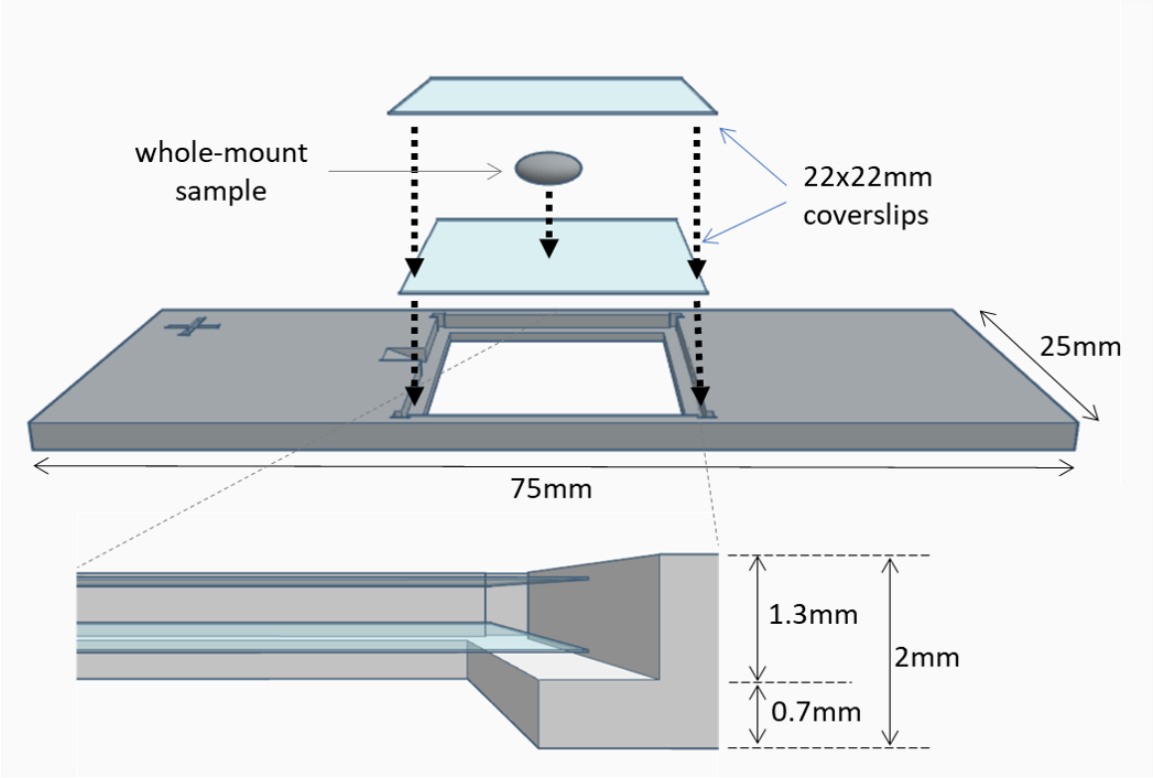

Figure S1. Technical specifications of the 3D-printable reversible slide. A slide (2x25x75mm) with an inset sized for 22x22mm coverslips to mount tissue samples for confocal imaging from both sides. STL file (download file at https://3dprint.nih.gov/discover/3DPX-009765) rendered in Autodesk Tinkercad. Clay posts adapted to the size of the sample can be used to support the top coverslip. The chamber is sealed with nail polish.

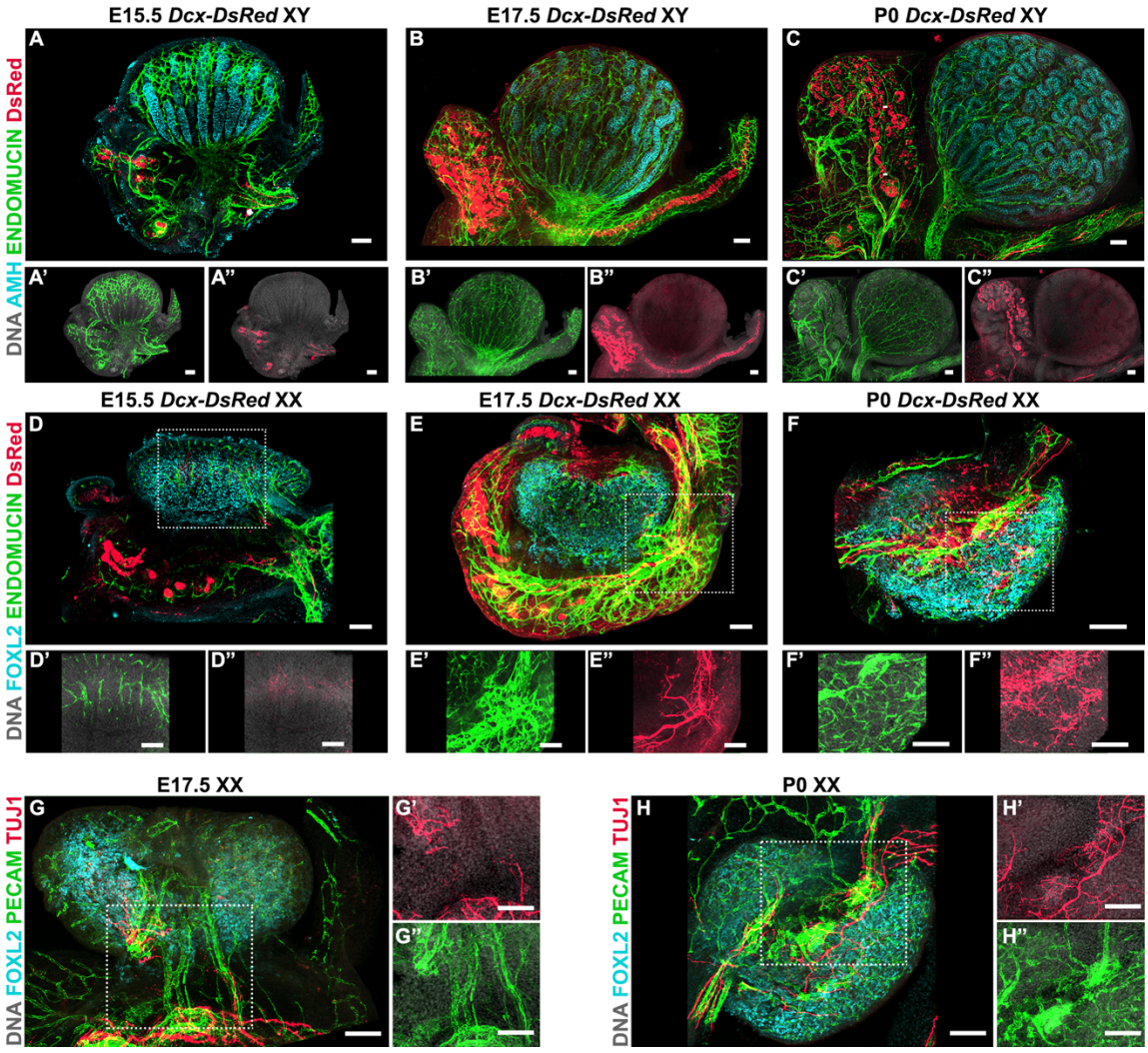

Figure S2. Innervation reaches the gonad/mesonephros complex after vasculature and follows a distinct pattern within the ovary. (*A-H*) Whole-mount fluorescent immunostaining of XY (*A-C*) or XX (*D-F*) gonads from *Dcx-DsRed* embryos at E15.5 (*A*, *D*), E17.5 (*B*, *E*) or P0 (*C*, *F*). Samples were stained for RFP to visualize DsRed expression (red, *A-F*) or for the neuronal marker TUJ1 (*G*, H). Vasculature was visualized with staining for the venous marker ENDOMUCIN (*A-F*) or the pan-endothelial marker PECAM (*G*, *H*). XY samples were stained for the Sertoli cell marker AMH to label the testis (*A-C*). XX samples were stained for the granulosa cell marker FOXL2 to label the ovary (*D-H*). All samples were counterstained with Hoechst nuclear dye. *A'-C'*, *A''-C''*, *D'-F'* and *D''* to *F''* are single channel views of *A-F* showing the vascular (ENDOMUCIN – Green) and neural patterns (DsRed – red) in the testis and ovary. *G'-H'* and *G''-H''* are single channel magnified views of *G*, *H* showing the vascular (PECAM – Green) and neural patterns (TUJ1 – red) in the ovary. Scale bars: 100µm.

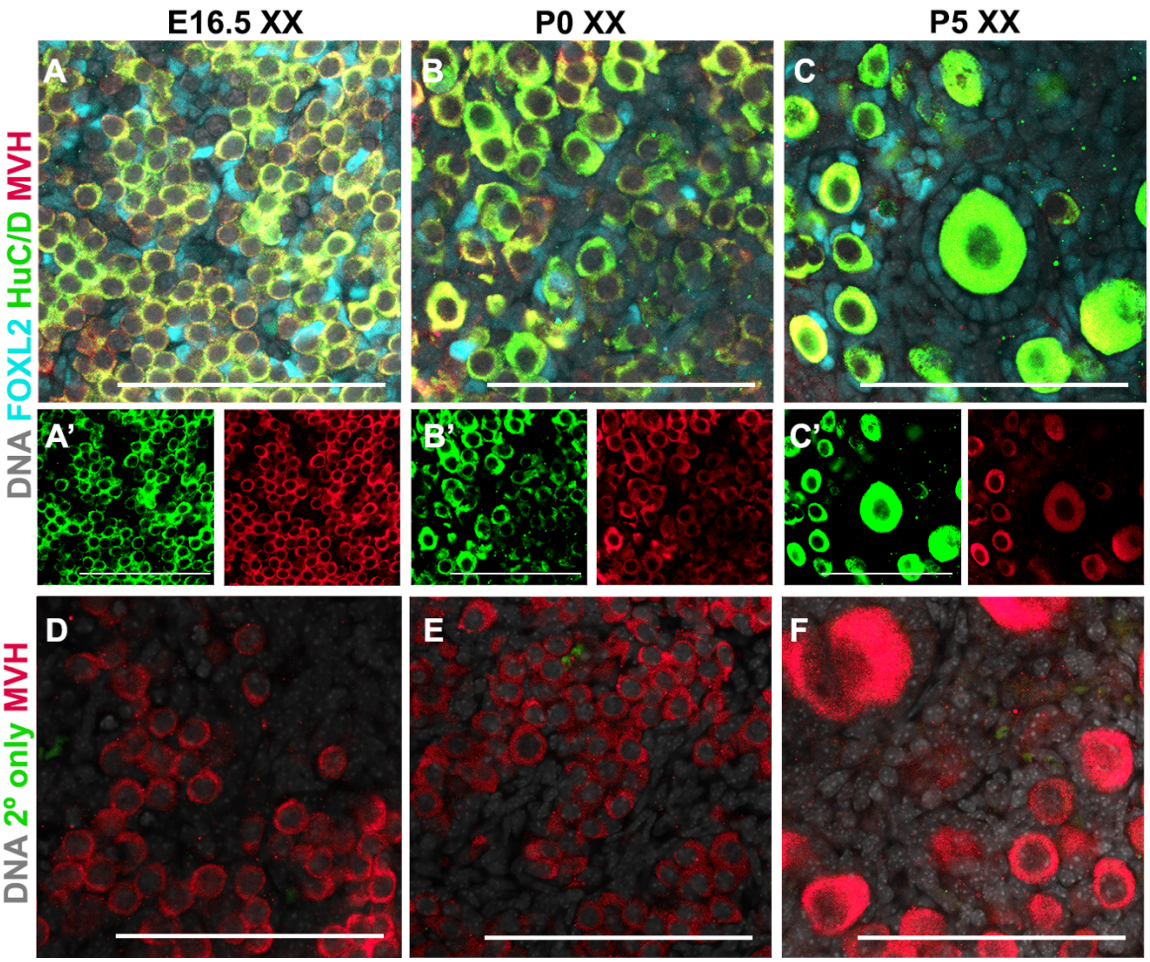

Figure S3. The HuC/D antibody used in this study labels oocytes in XX gonads. (*A-F*) Whole-mount fluorescent immunostaining of gonads from XX embryos at E16.5 (*A*, *D*), P0 (*B*, *E*) or P5 (*C*, *F*) pups. *A'-C'* are single channel views of *A-C.* Samples were stained for the granulosa cell marker FOXL2 (cyan, *A-C*), HuC/D (green, *A-C*) and the germ cell marker MVH (red, *A-F*). *D-F* are no-primary controls to test the specificity of the HuC/D staining, meaning the samples were incubated only with the anti-MVH primary antibody but the secondary antibodies against both MVH and HuC/D primary antibodies and imaged with the same settings as *A-C*. All samples were counterstained with Hoechst nuclear dye (grayscale). All images are maximum intensity projections from confocal Z-Stacks. Scale bars: 100µm.

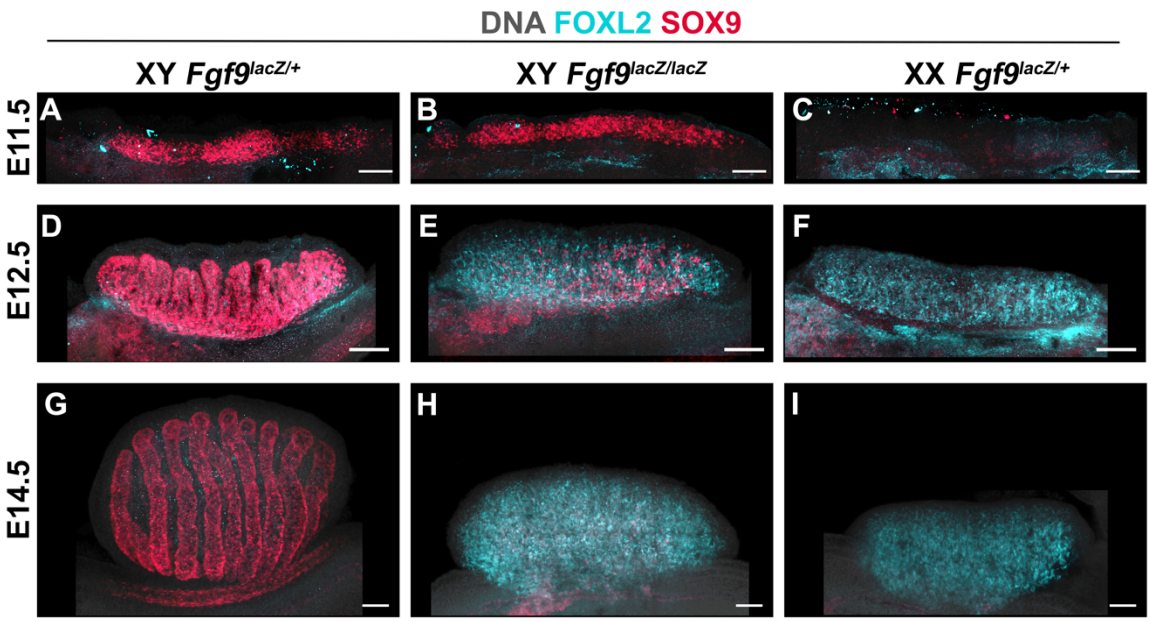

Figure S4. *Fgf9*$^{lacZ/lacZ}$ XY gonads are sex-reversed by E14.5. (*A-I*) Whole-mount fluorescent immunostaining of gonads from *Fgf9*$^{lacz/+}$ XY (*A*, *D*, *G*), *Fgf9*$^{lacz/lacZ}$ XY (*B*, *E*, *H*) and *Fgf9*$^{lacz/+}$ XX (*C*, *F*, *I*) embryos at E11.5 (*A-C*), E12.5 (*D-F*) or E14.5 (*G-I*). All samples were stained for the Sertoli cell marker SOX9 (red) and the granulosa cell marker FOXL2 (cyan). All samples were counterstained with DAPI nuclear dye (grayscale). All images are maximum intensity projections from confocal Z-Stacks. Scale bars: 100µm.

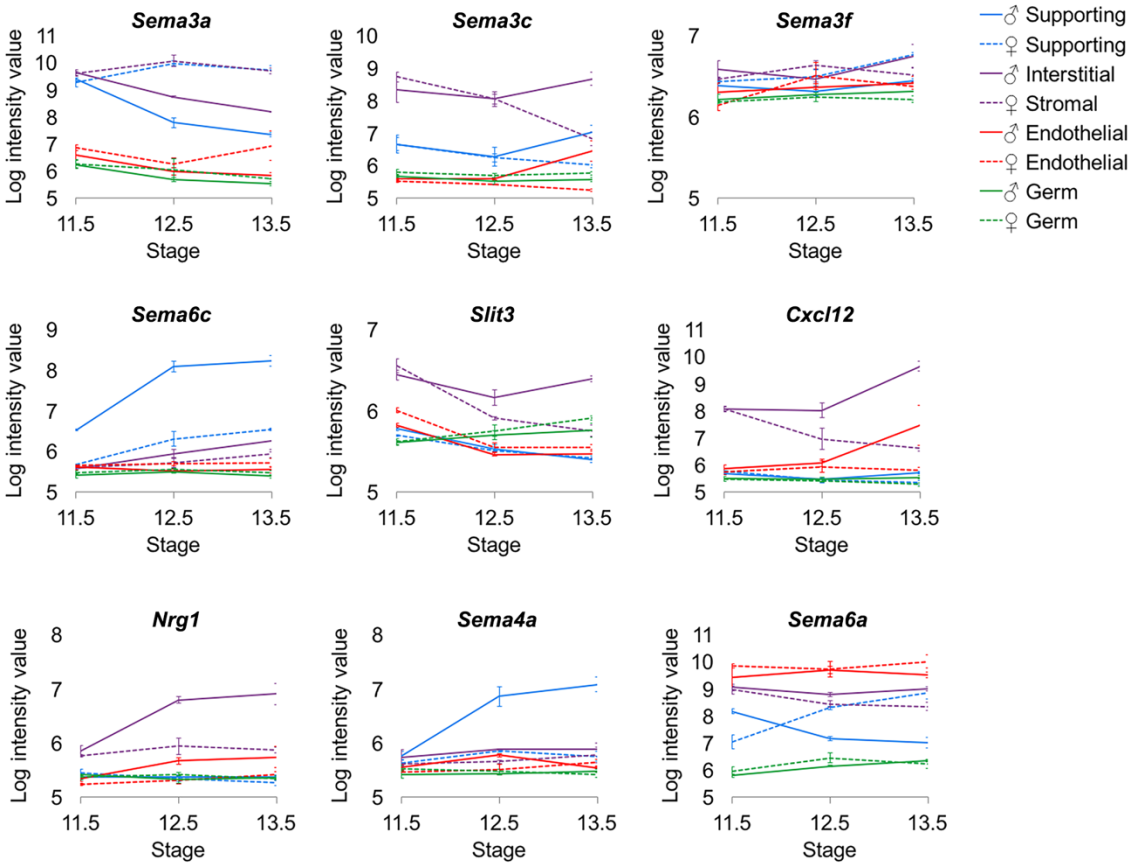

Figure S5. Neural crest guidance cues are differentially expressed in developing mouse gonads. Transcriptome analysis of neural crest guidance cue expression in sorted cell populations from male and female gonads at E11.5, E12.5 and E13.5. Graphs were generated using the previously published dataset (3).

Table S1. List of primary antibodies used in this study

Primary Antibody	Host Species	Dilution	Source	Product #
AMH/MIS	Goat	1:500	Santa Cruz Biotechnology	sc-6886 (discontinued)
DCX	Guinea Pig	1:1000	Millipore	AB2253
DDX4/MVH	Rabbit	1:250	Abcam	ab13840
ENDOMUCIN	Rat	1:250	Santa Cruz Biotechnology	sc-65495
FOXL2	Goat	1:250	Novus Biologicals	NB100-1277
GATA4	Goat	1:500	Santa Cruz Biotechnology	sc-1237 (discontinued)
HuC/D	Human	1:10000	Gift from V. Lennon (Mayo Clinic)*	N/A
mCHERRY	Chicken	1:1000	EnCor Biotechnology	CPCA-mCherry
PECAM1	Rat	1:1000	BD Pharmingen	553370
RFP	Rabbit	1:2000	Rockland	600-401-379
S100b	Rabbit	1:500	Abcam	ab52642
SOX9	Rabbit	1:1000	Millipore	AB5535
TH	Sheep (anti-Goat secondary used)	1:1000	Novus Biologicals	NB300-110
TUJ1	Rabbit	1:1000	Abcam	ab18207

* HuC/D / ANNA-1 antisera pooled from 3 patients, first reported in Lennon *et al*, 1991(4)

Table S2. List of secondary antibodies used in this study

Secondary Antibody	Dilution	Source	Product #
Cy3 Donkey anti-**Rabbit**	1:1000	Jackson ImmunoResearch	711-165-152
AF488 Donkey anti-**Rabbit**	1:1000	Life Technologies	A-21206
Cy3 Donkey anti-**Goat**	1:1000	Jackson ImmunoResearch	705-165-147
AF647 Donkey anti-**Goat**	1:1000	Life Technologies	A-21447
Cy5 Donkey anti-**Guinea Pig**	1:500	Jackson ImmunoResearch	706-175-148
AF488 Donkey anti-**Chicken**	1:1000	Jackson ImmunoResearch	703-545-155
Cy5 Donkey anti-**Human**	1:400	Jackson ImmunoResearch	709-175-149
AF488 Donkey anti-**Rat**	1:1000	Life Technologies	A-21208

Table S3. List of RT-qPCR primers used in this study

Target	Forward Primer	Reverse Primer
mCxcl12	CCGCTGCCGCACTTTCACTCT	GCTTGACGTTGGCTCTGGCGA
mGapdh	GGTGAAGGTCGGTGTGAACG	CTCGCTCCTGGAAGATGGTG
mFoxl2	GCAAGGGAGGCGGGACAACAC	GAACGGGAACTTGGCTATGATGT
mNrg1	ATGGAGATTTATCCCCCAGACA	GTTGAGGCACCCTCTGAGAC
mSema3a	GCCTGCAGAAGAAGGATTCA	TCAGGTTGGGGTGGTTAATG
mSema3c	GGGTTCAATCTGAAAGCATACA	TGTCTTTCTGCAGCAACCAC
mSema3f	CATCTGCCTCAACGATGACG	AGAGCCTGAAGAGGTAAAGACA
mSema4a	ATCACTGCCACCTGCTTCTGGGACTGGT	AGAGGTGAGTAGCATTGTAAGAGACCA
mSema4a	ATGGAGTCTCCTGCGTGTTT	GAAGCAGGTGGCAGTGATG
mSema6a	ACAGCCTGCCCCCTAAAGT	AGCTCCTCTTATATTCGAGCCC
mSema6c	CTGGACACTGAGGGTCACAG	CGCACGCCATAAGCAGAATC
mSlit3	CTCAAGGAGATTCCCATCCA	GGGCACTGCTGTTAAATGGT

Movie S1. Innervation is absent from the neonatal testis. Animation of images taken with the Zeiss Z1 light sheet microscope of a cleared neonatal XY gonad stained with antibodies against the pan-neuronal marker TUJ1 (red), the gonadal marker GATA4 (cyan) and counterstained with Hoechst nuclear dye (grayscale). The video shows an animation of the optical sections and rotating views of 3D renders of the light sheet Z-stack.

Movie S2. A dense neural network is present within the neonatal ovary. Animation of images taken with the Zeiss Z1 light sheet microscope of a cleared neonatal XX gonad stained with antibodies against the pan-neuronal marker TUJ1 (red) and the gonadal marker GATA4 (cyan). The video shows an animation of the optical sections and rotating views of 3D renders of the light sheet Z-stack.

References

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4. Lennon VA, et al. (1991) Enteric neuronal autoantibodies in pseudoobstruction with small-cell lung carcinoma. *Gastroenterology* 100(1):137–142.