Supplemental Figure 1.

(A) The localization of Cre DNA recombinase in the testis of Cyp19a1-Cre mice was detected by immunohistchemical analyses using an anti-Cre antibody; testes at 1 week (left panel), testes at 12 weeks (right panel). The positive signals were only detected in round interstitial cells (newly formed adult Leydig cells) in testes at 1 week. Scale bars correspond to 100 μm.

(B) Cross-sections of mouse testes were stained with antibodies to visualize either Cre DNA recombinase (green) or Cyp19 (red), at postnatal 1 week. White lines delineate the border of the seminiferous tubules. Scale bars correspond to 100 μm.

(C) Expression of RFP in the testes of Cyp19-Cre; RFP knock in mice.

(D) Expression of the EGF domain of NRG1 in testes of WT and Nrg1fl/fl; Cyp19a1-Cre mice. NRG1 was detected by immunofluorescence using an anti-EGF domain of NRG1 antiserum (Kawashima et al., 2014). Red signals are the EGF domain positive cells and blue signals are nuclei. The white line delineates the border of the seminiferous tubules. Scale bars correspond to 100 μm. Positive signals were not detected in interstitial cells of Nrg1fl/fl; Cyp19a1-Cre mice, indicating that the functional NRG1 was deleted in ALCs in the mice. NRG1 staining also occurs in spermatogenic cells as reported previously (29) but is not altered in the Nrg1fl/fl; Cyp19a1-Cre mice. N.C.; negative controls without primary antibody.
Supplemental Figure 1

(A) 1 week 12 week

: CRE Positive cell

(B) 1 week

CRE / CYP19/ DAPI
Supplemental Figure 1
Supplemental Figure 2.

(A) The expression of type II Nrg1 was not detected in testis. Type II of Nrg1 mRNA was analyzed by real-time PCR using specific primer set to recognize the specific region of type II Nrg1. Values are represented as the mean ± SEM of three replicates.

(B) The expression of NRG1 in hCG-stimulated mouse testes following GnRH-antagonist treatment. Red signals are NRG1 positive cells; blue signals are nuclei (DAPI). Immature mice at 21 days of age were treated daily for 2 days with a GnRH antagonist (25 μg/day) followed by an i.p. injections of saline or 10 I.U. hCG. After 4 hours, testes was collected and immune-stained. White lines show the localization of seminiferous tubule. Scale bars correspond to 100 μm. The percent of NRG1 positive cells per total number of the interstitial cells was significantly increased by hCG injection. N.C.; negative controls without primary antibody.
Supplemental Figure 2

(A) Type II Nrg1 mRNA

<table>
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<tr>
<th>Time</th>
<th>0week</th>
<th>1week</th>
<th>3week</th>
<th>5week</th>
<th>Adult</th>
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<tbody>
<tr>
<td>Value</td>
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<td>0.0004</td>
<td>0.0006</td>
<td>0.0008</td>
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(B) The percent of NRG1 positive cell per interstitial cells

- Antagonist
- Antagonist+hCG
- N.C

NRG1/DAPI

The percent of NRG1 positive cell per interstitial cells

- Anta
- Anta+hCG

* Significant difference
Supplemental Figure 3.
ErbB3 that is one of the NRG1 receptors is expressed in HSD17B3 positive-Leydig cells (ALCs) in mouse testes.
Cross-sections of mouse testes were stained with antibodies to visualize either ERBB3 (green) or HSD17B3 (red) at postnatal 1 week (infant) and 12 weeks (adult). White lines delineate the seminiferous tubules. Scale bars correspond to 100 μm. Arrows indicate ERBB3 and/or HSD17B3 positive cells in the interstitial tissue. N.C.; negative controls without primary antibody.
Supplemental Figure 4
The photographic image shows the gross morphology of testes at 12 weeks of age in WT and LeyNrg1KO (KO) mice. Scale bars correspond to 5 mm.
Supplemental Figure 5.
(A) Phosphorylation of ERK1/2 in testis of WT but not LeyNrg1KO (KO) mice at 3 and 12 weeks of age. Red signals are phosphorylated ERK1/2 positive cells. Blue signals are nuclei (DAPI). The white lines delineate the seminiferous tubules. Scale bars correspond to 100 µm.
(B) Phosphorylation of AKT is detected in testes of WT but not LeyNrg1KO (KO) mice at 3 and 12 weeks of age. Red signals are phosphorylated AKT positive cells. Blue signals are nuclei (DAPI). The white line delineate the seminiferous tubules. Scale bars correspond to 100 µm.
Supplemental Figure 6.

The phenotype of seminiferous tubules in *LeyNrgIKO*.

(A) The percent of each stage of spermatogenesis in the seminiferous tubules of 3-month-old WT and *LeyNrgIKO* (KO) mice. Paraffin sections of each testis were stained in periodic acid-Shiff and the stages of spermatogenesis in the tubules were determined according to Oakberg et al (1956). Values are presented as the mean +/- SEM of three different testes in each genotype.

(B) The number of Sertoli cells per seminiferous tubule in testes of WT and *LeyNrgIKO* (KO) mice at 1 and 12 weeks of age. Values are mean +/- SEM of three different testes in each genotype.

(C) The morphology of seminiferous tubules in testes collected from 3-month-old WT or *LeyNrgIKO* (KO) mice. Scale bars correspond to 100 µm in low magnification (×100) and 25 µm in high magnification (×400). Approximately half of the elongated spermatids were detached (black arrow) from Sertoli cells into the lumen of the tubule at stage VI in the *LeyNrgIKO* (KO) mice. However, most of elongated spermatids remained attached to Sertoli cells at the same stage in testes of WT mice (white arrow).

(D) Cross sections of testes collected from 3-month-old WT and *LeyNrgIKO* (KO) mice were stained by the TUNEL method to detect apoptotic cells (DNA fragmentation). TUNEL-positive signals (green) and nuclei (blue; DAPI) indicate that there are more apoptotic cells in the mutant compared to WT testes. The white line delineates the border of the seminiferous tubule. Scale bars correspond to 100 µm.

(E) The percent of TUNEL-positive cells in the lumen of testes in 3-month-old WT and *LeyNrgIKO* (KO) mice. Values are mean +/- SEM of three different testes in each genotype. *denotes a significant difference observed between genotypes.
The number of Sertoli cells per seminiferous tubes

(A) The percent of seminiferous tubes stage

(B) The number of Sertoli cells per seminiferous tubes

Supplemental Figure 6
Supplemental Figure 6

(C)

Low magnification

WT

KO

High magnification

WT

KO

Supplemental Figure 6
The percent of TUNEL-positive lumen

(D) WT KO

(E) The percent of TUNEL-positive lumen

Supplemental Figure 6
Supplemental Table 1. List of primers employed for RT-PCR and the expected size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size</th>
<th>Anneling temperature</th>
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<td>Hsd3b6</td>
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<td>5′-GGGACTCCCGTCGTATGTA-3′</td>
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