SUPPLEMENTARY MATERIAL

Fig. S1. NPR2 is dephosphorylated in response to LH signaling, and remains dephosphorylated for several hours even when LH is washed out after a brief exposure. Experiments were done using follicles from rats, using methods as described by Egbert et al. (2014). Preovulatory follicles without LH treatment were compared with follicles that were treated with LH for 30 or 240 min, or treated with LH for 30 min, then washed 5 times in medium without LH for a total of 20 min, and incubated without LH until 240 min after the initial treatment. The western blot shows NPR2 that was immunoprecipitated from crude follicle membranes and run on a gel containing Phos-tag, which retards the migration of phosphorylated proteins. Without LH, most of the NPR2 signal is represented by several slower-migrating bands, indicating phosphorylation. Upon treatment with LH for 30 or 240 min, the upper bands collapse into a single lower band, indicating NPR2 dephosphorylation. NPR2 remains dephosphorylated for several hours after removal from a 30-min LH treatment. These results are representative of 2 similar experiments. The findings indicate that a brief exposure to LH may induce persistent signaling (Reizel et al., 2010), perhaps as a consequence of receptor endocytosis (Calebiro et al., 2009). It is also possible that since LH binds with high affinity to its receptor (Lawrence et al., 1979), it remains bound despite extensive washing.

Additional references


**Fig. S2. Generation of knock-in mice with phosphomimetic glutamate mutations in NPR2.** (A) Outline of the strategy. A sequence encoding the 7E point mutations (red asterisks), located in exon 8 and 9, was introduced into the endogenous Npr2 gene by homologous recombination. (B) Sequencing chromatographs from an Npr2-7E/+ mouse, confirming the point mutations in the seven serine and threonine sites of the Npr2 gene.
Fig. S3. Similar CNP concentration-dependence of the activity of wild-type and 7E-mutated NPR2. HEK-293T cells transiently transfected with either wild-type or 7E-mutated NPR2 were seeded in 48-well plates and serum starved for 4 hours. Medium containing 1 mM of the cyclic nucleotide phosphodiesterase inhibitor isobutylmethylxanthine was added to the cells for 10 min and then this medium was aspirated and replaced with the same medium containing the indicated concentrations of CNP. After 1 min, the medium was aspirated and the reaction was stopped by adding 80% ice-cold ethanol. Cyclic GMP concentrations in the ethanolic extracts were determined by radioimmunoassay. Vertical bars represent SEM (n = 18).
Supplementary materials and methods

Generation of knock-in mice with phosphomimetic glutamate mutations in NPR2

A sequence encoding the 7E point mutations, located in exon 8 and 9, was introduced into the endogenous Npr2 gene by homologous recombination (Fig. S2A). The targeting vector was prepared by recombineering (Lee et al., 2001). Briefly, we first retrieved approximately 9.2 kb of the Npr2 genomic sequence spanning introns 2 to 12 from the BAC vector, RP24-306K11 (Children’s Hospital Oakland Research Institute), into a vector containing the negative selectable marker PGKdta, by gap repair. We then inserted a single LoxP site into exon 8 together with a unique restriction site, PmeI. An 800 bp genomic fragment spanning intron 7 to 9 with the 7E mutations S489E, S513E, T516E, S518E, S523E, S526E, and T529E, was prepared by PCR and inserted into the targeting vector to replace the single LoxP. Unique site elimination, by PmeI digestion, was used to remove plasmids that did not undergo recombineering to generate the pNpr2-7E.dta vector (Noll et al., 2009). We then inserted a single LoxP with two unique restriction sites, SalI and AscI, in the 3’ end into intron 8. A fragment containing a wild-type Npr2 minigene with a unique SalI site and 200 bp of intron 8 and cDNA from exons 8 to 22 was prepared by overlapping PCR using the BAC and a full length Npr2 cDNA as initial templates. The mini-cDNA was then inserted into pSK+ and sequenced to confirm its identity. A DNA fragment containing IRES-eGFP followed by Npr2 3’UTR and the Frt-PGK-Neo-Frt-LoxP cassette was prepared by a combination of PCR and conventional cloning. This fragment, which contains a unique AscI in the 3’ end, was inserted 3’ to the Npr2 mini-cDNA plasmid. The final DNA fragment was then released from pSK+ and inserted into 3’ to the single LoxP site in the
pNpr2-7E.dta vector by restriction digestion and ligation to generate the final targeting vector.

This vector, which contains approximately 5.3 kb and 4 kb of 5’ and 3’ arms, respectively, was linearized and electroporated into mouse ES cells derived from F1 (129Sv/C57BL6/J) blastocysts. Electroporated ES cells were cultured for 48 hour prior to drug selection using G418. Drug resistant colonies were picked and screened by long range PCR using primers corresponding to sequences outside the arms and specific to the 5’LoxP site, and eGFP fluorescence to identify targeted ES clones. These targeted ES clones were then further analyzed by PCR and sequencing to confirm the presence of the 7E mutations prior to using them for the generation of chimeric animals by ES cell-morula aggregation (Behringer et al., 2014). Chimeric animals were then bred with Hprt-Cre mice (Tang et al., 2002) to generate global knock-in mice. Cre recombinase excises the Npr2 wild-type mini-gene and IRES-eGFP sequences, leaving a single LoxP site in intron 7, and inducing expression of the Npr2 gene with the 7E mutations (Npr2-7E). We performed PCR and sequencing of positive F1 pups to confirm the 7E knock-in mutation (Fig. S2B).

The single LoxP located approximately 900 bp 5’ of exon 9 serves as a convenient marker for PCR genotyping. All subsequent PCR-based genotyping was performed using genomic DNA isolated from ear biopsies with the following primers, located as shown by the purple arrows.

LoxF(forward): 5'- CATCCTAGGTATTTATCTTGC
LoxF(reverse): 5'- TAAAAACTACTTCTTTTTTAAAAATCCTTA

These primers amplify a 399-bp fragment of the wildtype sequence, and a 496-bp fragment of the 7E-mutated sequence.
**Additional references**


