Figures and figure supplements

BNP facilitates NMB-encoded histaminergic itch via NPRC-NMBR crosstalk

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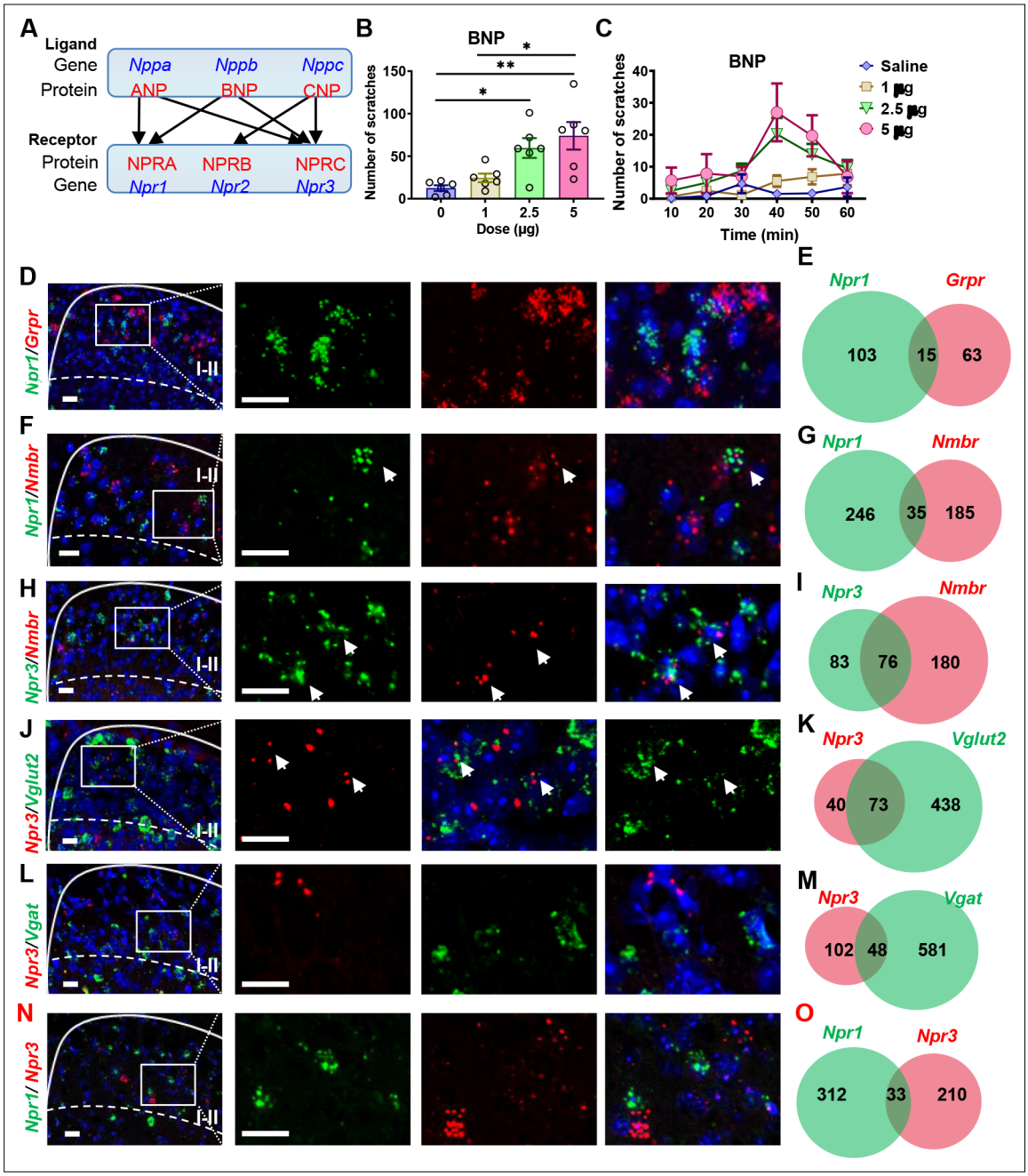

Figure 1. Expression of *Npr1*, 2,and *3* and other molecular markers in the spinal cord. (**A**) Diagram shows crosstalk between NPs and NP receptors. BNP can bind NPRA and NPRC. (**B**) BNP dose-dependently evoked scratching behaviors 60 min after i.t. injection. n = 6. *p < 0.05, **p < 0.01, one-way ANOVA followed by Tukey's test. (**C**) Time-course of scratching behaviors induced by different doses of BNP shows a delayed onset of scratching responses. (**D, F, H, J, L, N**) Images of double RNAscope ISH showing that the overlapping expression of *Npr1* (green) with *Grpr* (red) (**D**), *Nmbr* (**F**), of *Npr3* (green) with *Nmbr* (red) (**H**), *Npr3* (red) with *Vglut2* (green) (**J**), *Vgat* (green) (**L**), or *Npr1* (green) (**N**) in laminae I-II of the dorsal horn. Dashed white lines divide laminae I-II from III. White boxes are shown at higher magnification in the right panel. Arrows indicate double-positive neurons. E, G, I, K, M, O, Venn diagrams showing the overlap between *Npr1* and *Grpr* (**E**), *Nmbr* (**G**), between *Npr3* and *Nmbr* (**I**), *Vglut2* (**K**), *Vgat* (**M**) or *Npr1* (**O**). n = 10–15 sections from 3 mice. Scale bar, 20 μm in **D – N**.

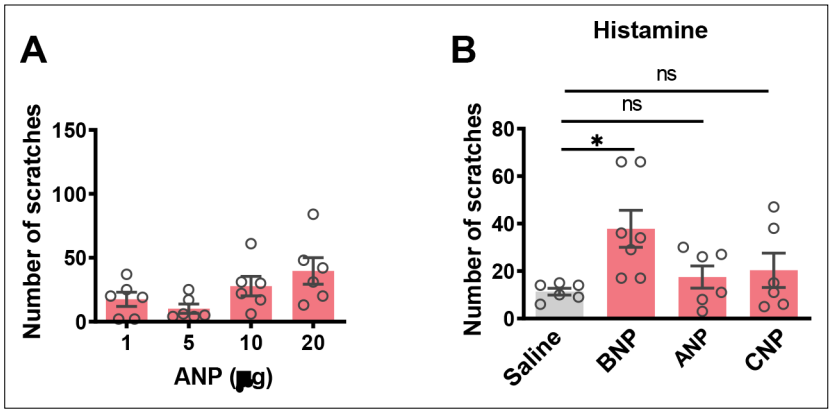

Figure 1—figure supplement 1. Failure of ANP and CNP in facilitating histamine itch. (**A**) ANP 1 ~ 20 μg, (equivalent to 6–120 μM, i.t.) failed to induce robust scratching behaviors in mice. n = 6. (**B**) Only BNP (30 μM, i.t.) facilitated histamine itch. Note that neither ANP (60 μM, i.t.) nor CNP (60 μM, i.t.) exhibited facilitatory effect. n = 6–7. *p < 0.05, ***p < 0.001, one-way ANOVA followed by Dunnett's test. Values are presented as mean ± SEM.

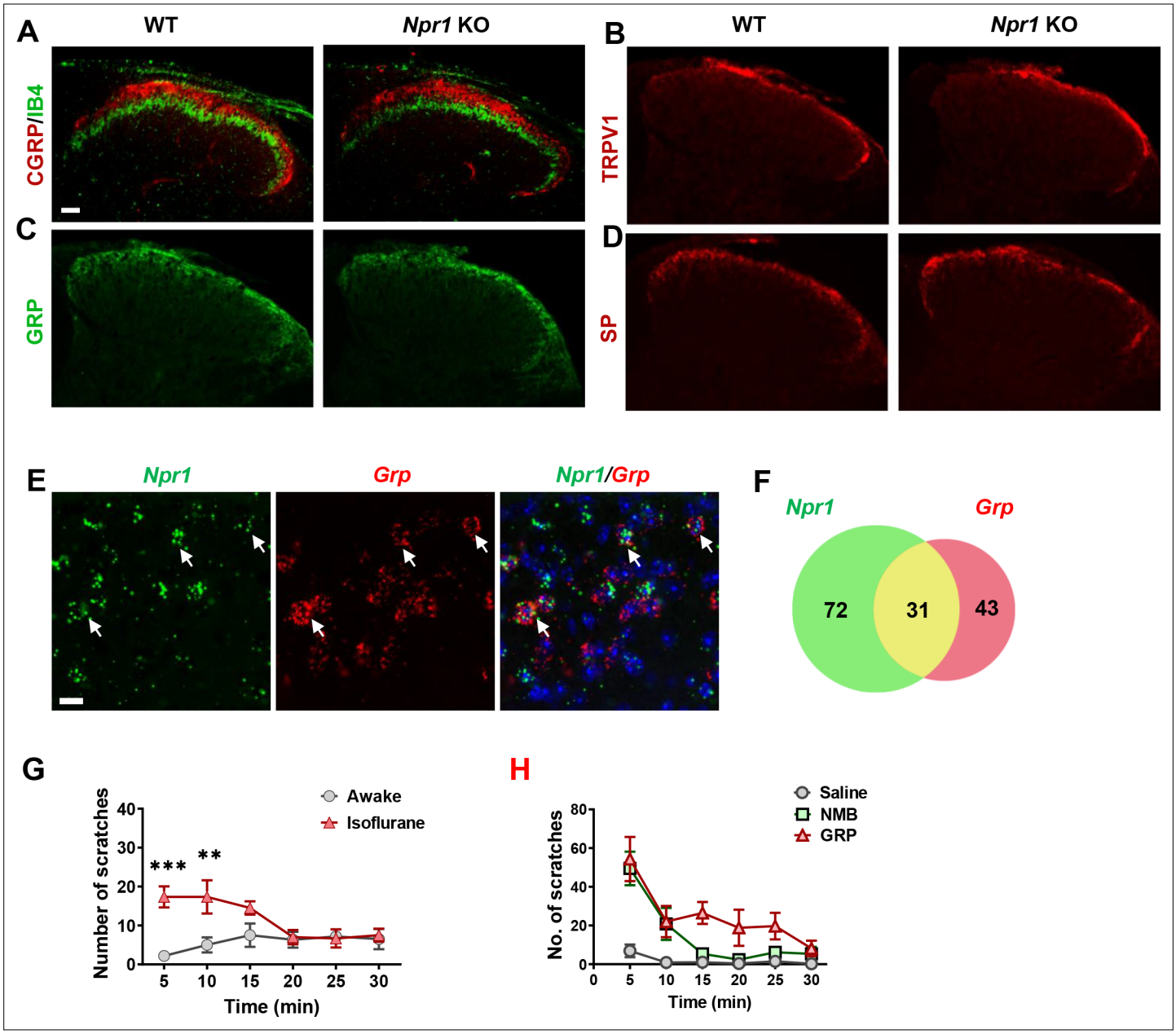

Figure 1—figure supplement 2. Normal innervation of primary afferents in *Npr1* KO mice and WT mice. (**A-D**) Comparable expression of CGRP (red) and IB4 staining (green), TRPV1, GRP, and SP in the superficial dorsal horn of WT and *Npr1* KO mice. Scale bar, 50 μm. (**E**) Images of double RNAscope ISH showing that *Npr1* (green) is partially co-expressed with *Grp* (red) in the dorsal horn. Arrows indicate double-positive neurons. Scale bar, 20 μm. (**F**) Venn diagram showing partial overlapping of *Npr1* and *Grp* expression. (**G**) Scratching behaviors elicited by i.t. BNP (150 μM) were significantly enhanced by isoflurane. (**H**) Time course of i.t. NMB (1 nmol) and GRP (1 nmol) evoked scratching behavior. n = 6. **p < 0.01, ***p < 0.001, two-way ANOVA followed by Bonferroni's test. Values are presented as mean ± SEM. Scale bar, 50 μm in A-D, 20 μm in E.

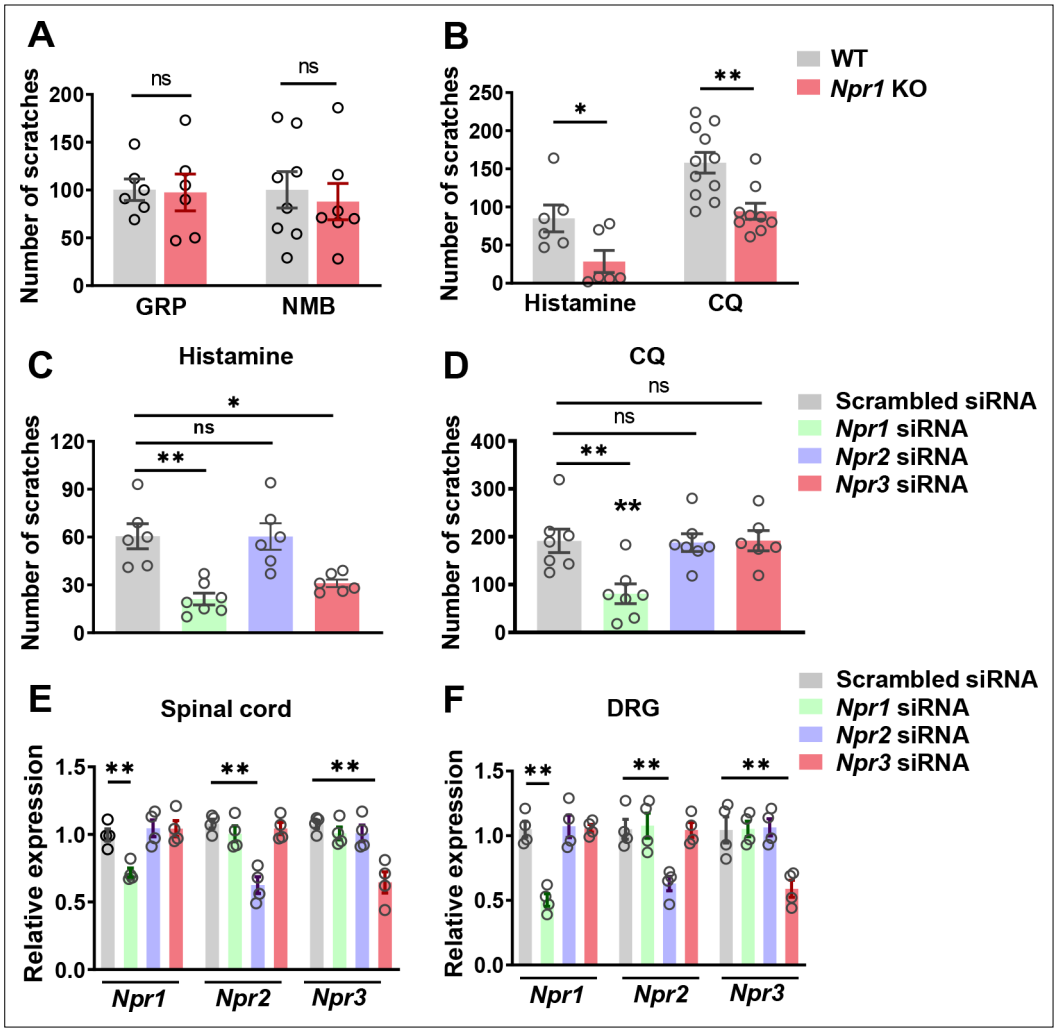

Figure 2. NPRA and NPRC are involved in acute itch. (**A**) *Npr1* KO mice and their WT littermates showed comparable scratching behaviors in response to GRP (0.05 nmol, i.t.) and NMB (0.5 nmol, i.t.). n = 6–8. (**B**) *Npr1* KO mice showed significantly reduced scratching behavior elicited by histamine (200 μg, i.d.) and CQ (200 μg, i.d.). n = 9–11. *p < 0.05, **p < 0.01, unpaired t test. (**C, D**) Mice treated with *Npr1* siRNA showed significantly reduced scratching responses to histamine (**C**), CQ (**D**), wherea mice treated with *Npr3* siRNA displayed deficits only in histamine (**C**) but not CQ itch (**D**). n = 6–7. *p < 0.05, **p < 0.01, one-way ANOVA followed by Dunnett's test. (**E, F**) Real-time PCR confirmed the reduced *Npr1-3* expression by *Npr1*, *Npr2*, and *Npr3* siRNA knockdown in the spinal cord (**E**) and DRG (**F**). n = 4. **p < 0.01, one-way ANOVA followed by Dunnett's test. Values are presented as mean ± SEM.

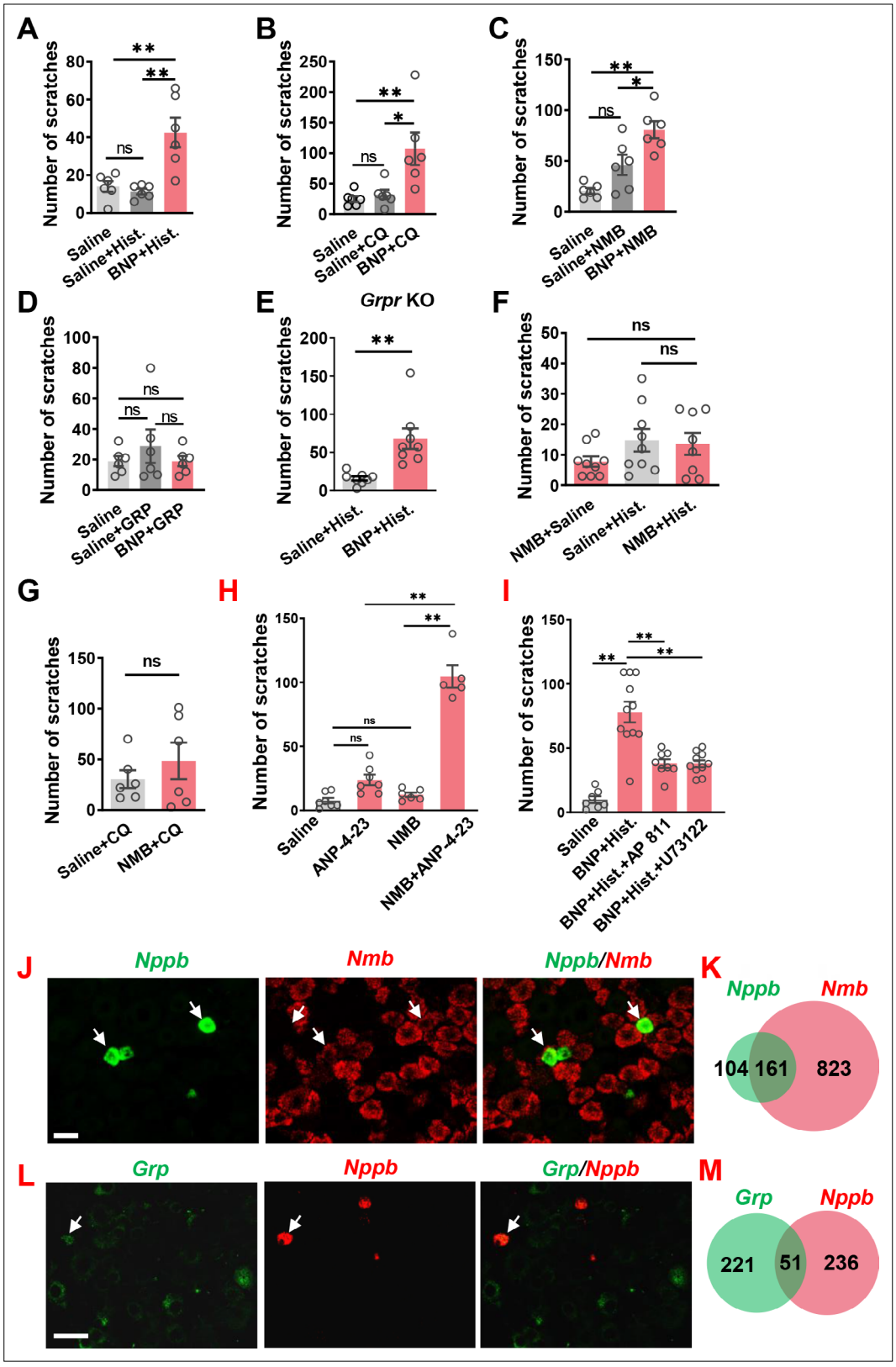

Figure 3. BNP facilitates histamine itch. (**A**) Pre-injection of BNP (30 μM, i.t.) for 1 min significantly enhanced scratching behavior evoked by i.d. injection of histamine (Hist.) (100 μg). n = 6. (**B**) Scratching behavior evoked by i.d. injection of CQ (50 μg, i.d.) was significantly enhanced by pre-injection of BNP for 1 min. n = 6. (**C, D**) Co-injection of 1 μg BNP (30 μM, i.t.) facilitated scratching behaviors evoked by NMB (0.05 nmol, i.t.) (**C**) but not GRP

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(0.01 nmol) (**D**). n = 6. (**E**) Pre-injection of 1 μg BNP (30 μM, i.t.) for 1 min significantly enhanced scratching behavior evoked by i.d. injection of histamine (100 μg) in *Grpr* KO mice. n = 8. (**F, G**) Pre-injection of NMB (0.05 nmol, i.t.) had no effect on scratching behavior induced by histamine (**F**) or CQ (**G**). Note that NMB barely evoked scratching bouts. n = 6. (**H**), NPRC agonist ANP-4–23 (6 nmol, i.t.) facilitates NMB (0.005 nmol, i.t.) induced scratching behavior. n = 5–9. (**I**), Histamine (25 μg, i.d.)-induced scratching behavior facilitated by BNP (30 μM, i.t.) was attenuated with AP 811 (10 μM, i.t.) or U 73122 (13.5 nmol, i.t.) treatment. n = 6–11. (**I–K**) Double RNAScope ISH images (**J and L**) and Venn diagrams (**K and M**) showing 60% of *Nppb* neurons co-express *Nmb* (**J and K**), but little *Grp* in DRGs (**L and M**). Values are presented as mean ± SEM, *p < 0.05, **p < 0.01, unpaired t test in (**A–E**), one-way ANOVA in (**F and G**). Scale bar, 20 μm in **J**, 50 μm in **L**.

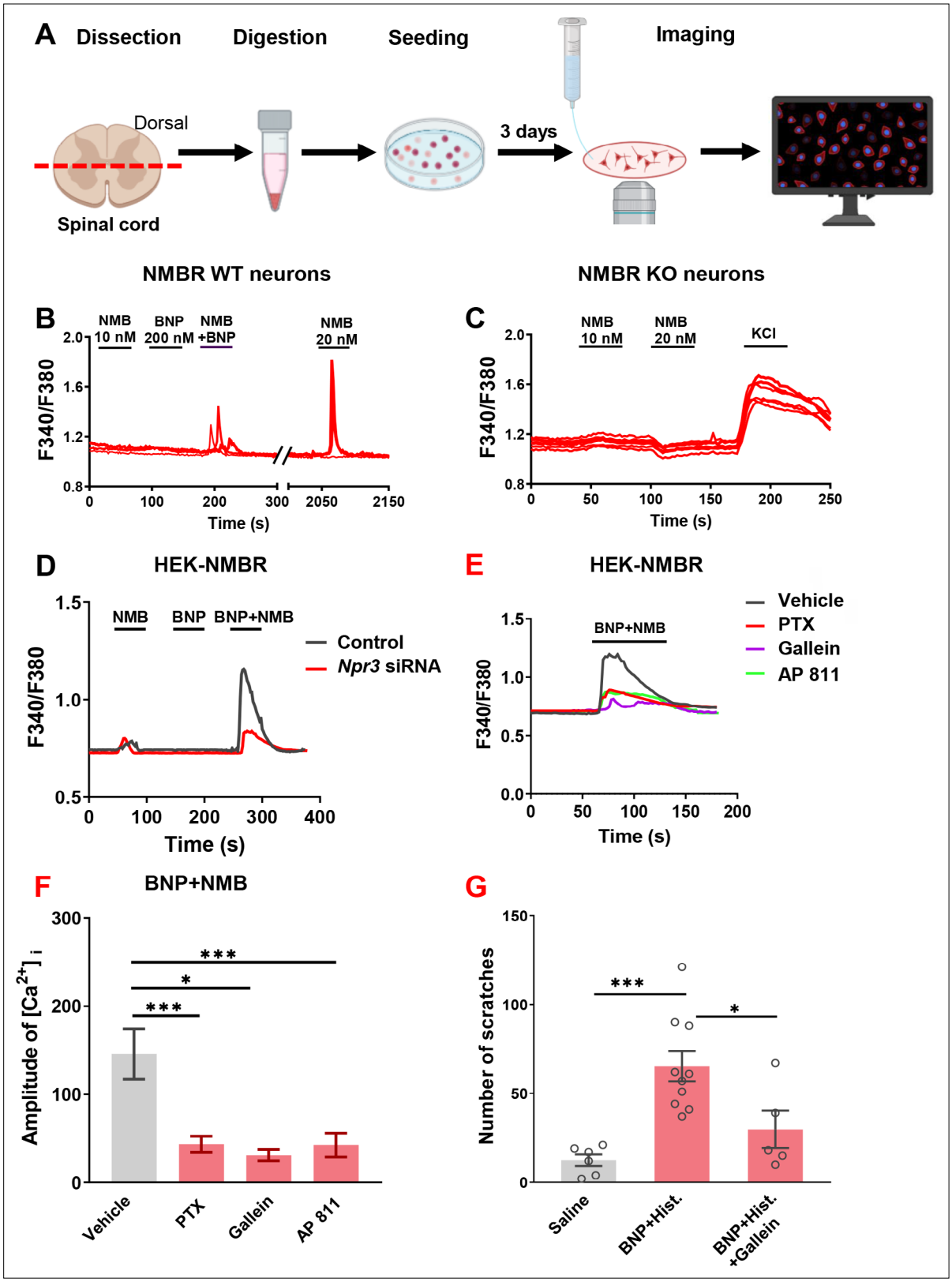

Figure 4. Potentiation of NMB-evoked calcium and scratching responses by BNP requires G_i-G_q crosstalk between NPRC-NMBR. (**A**) A diagram showing the procedure for calcium imaging on dissociated spinal cord dorsal horn neurons. (**B**) Sample traces showing that co-application of BNP and NMB at low doses evoked Ca^{2+} transients in WT dorsal horn neurons (n = 8 neurons from 33 NMBR neurons analyzed, n = 10 pups). These neurons responded to both BNP/NMB at the low doses responded to NMB at 20 nM robustly, indicating that they are healthy neurons. (**C**) No dorsal horn neurons responded

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to NMB (20 nM) isolated from the spinal cord of *Nmbr* KO mice (n = 2 mice), whereas they responded to KCl, indicating that they were healthy neurons. (**D**) Co-application of BNP (1 μM) with subthreshold of NMB (1 pM) evoked robust calcium response in HEK 293 cells co-expressing NMBR, which was significantly attenuated by *Npr3* siRNA treatment. (**E**) Calcium transients induced by BNP and NMB were attenuated by pre-incubation of PTX (200 ng/ml), gallein or AP 811 (0.1 μM) for 30 min. n = 6 slides per group with at least 50 cells imaged on each slide. (**F**) Quantification of calcium concentration ([Ca2+]i) of **E**. (**G**) I.t. gallein (20 nmol) significantly reduced scratching behavior evoked by histamine (25 μg, i.d.) facilitated with BNP (30 μM, i.t.). Values are presented as mean ± SEM, n = 6–10. *p < 0.05, ***p < 0.001, one-way ANOVA followed by Tukey's test.

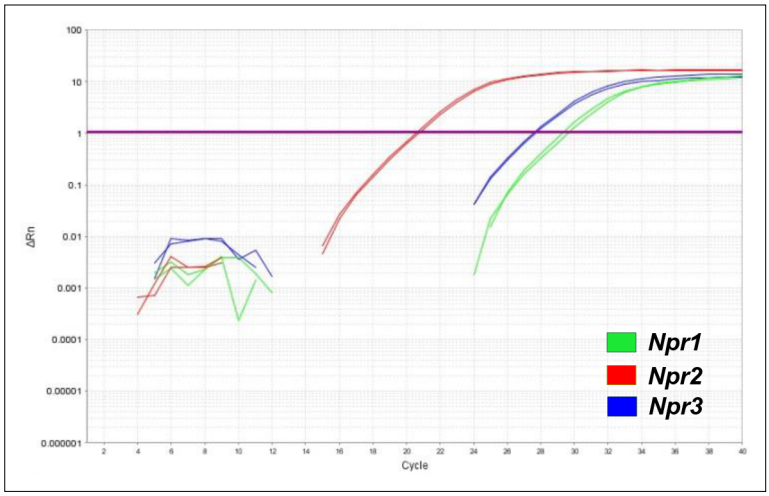

Figure 4—figure supplement 1. real-time RT-PCR detected endogenous expression of *Npr1*, *Npr2*, and *Npr3* in HEK 293 cells.

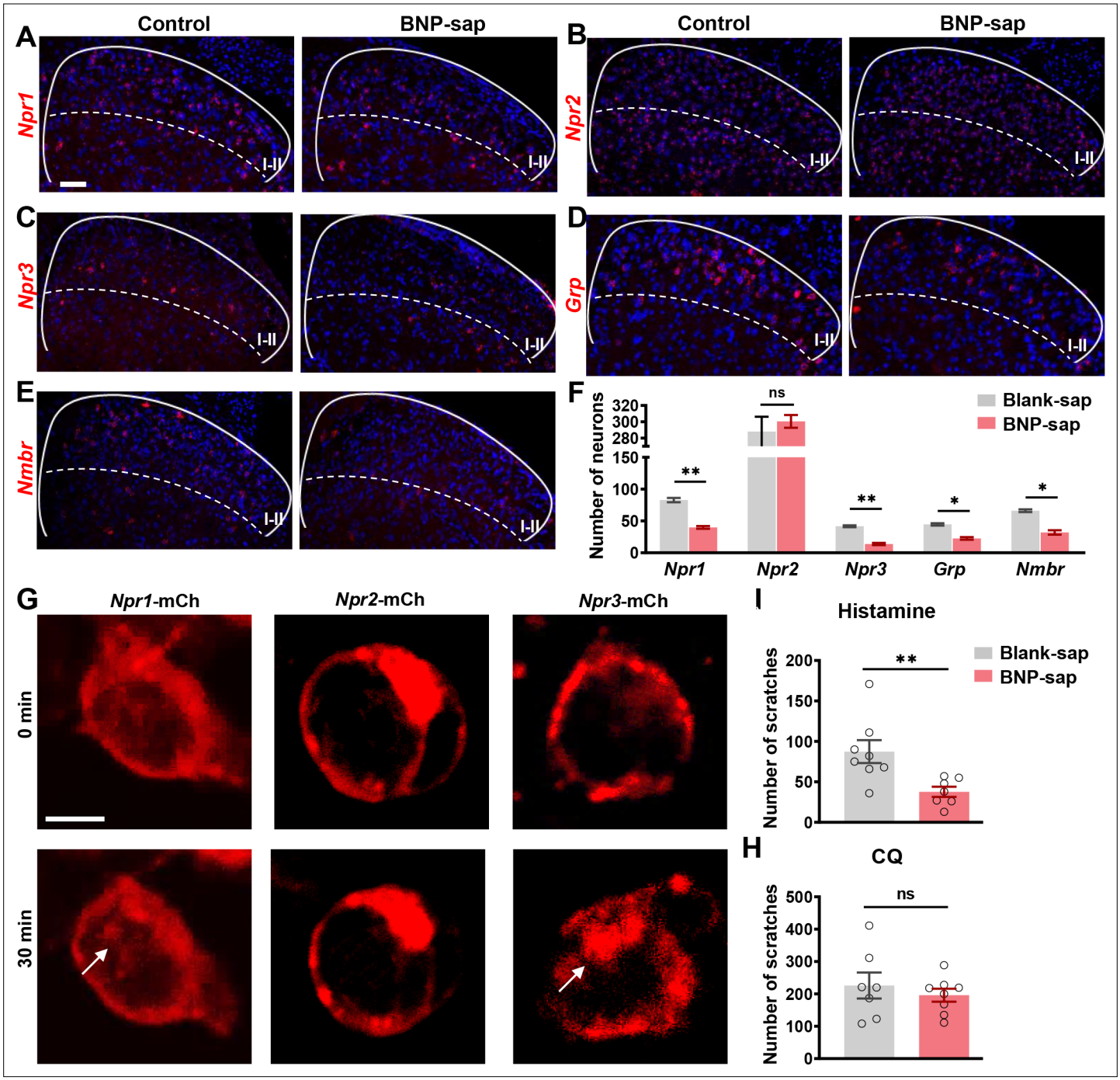

Figure 5. BNP-sap ablates spinal cord neurons expressing *Npr1* and *Npr3*. (**A-F**) RNAscope ISH images (**A and C**) and quantified data (**F**) showing that BNP-sap ablated *Npr1*+ (**A**), *Npr3*+ (**C**), *Grp*+ (**D**), and *Nmbr*+ (**E**) neurons (red) in the dorsal horn of the spinal cord, while *Npr2*+ (**B**) neurons (red) were not affected. n = 4. (**G**) Incubation of BNP (10 μM) for 30 min caused internalization of *Npr1*-mCh and *Npr3*-mCh in HEK 293 cells transfected with NMBR cDNA as indicated by arrows. No internalization of *Npr2*-mCh was observed. Scale bar, 20 μm. mCh: mCherry. (**H, I**) Scratching behaviors induced by histamine (**H**), but not CQ (**I**) were significantly reduced in BNP-sap treated mice. n = 7–8. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01, unpaired t test. Scale bar, 50 μm in **A–F**, 10 μm in **G**.

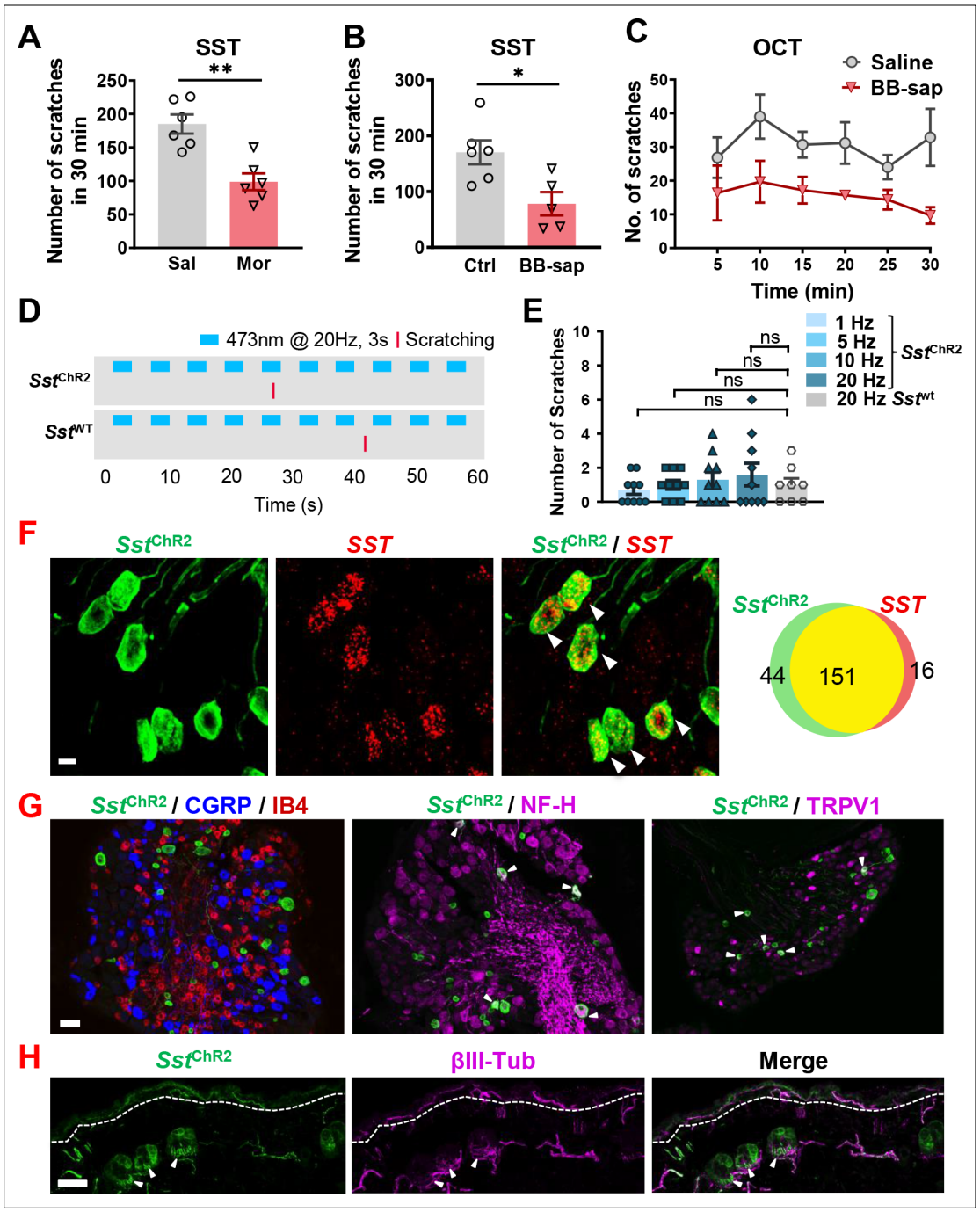

Figure 6. SST evoked both pain and itch responses in mice. (**A**) Pre-injection of morphine (10 mg/kg, i.p.) for 30 min attenuated scratching behaviors induced by i.t. injection of SST (5 nmol). n = 6 mice per group. Sal, saline; Mor, morphine. (**B, C**) SST (5 nmol, i.t.)(**B**) and OCT (**C**) -evoked scratching behaviors were significantly reduced in bombesin-saporin-treated mice comparing with control mice that were treated with blank saporin. n = 5–6 mice per group. Ctrl, control; BB-sap, bombesin-saporin. (**D**) Raster plot of scratching behavior induced by light stimulation of skin in Sst-ChR2 and *Sst-cre* mice. (**E**) Number of scratches in 5 min induced by 3 s – 1, 5, 10, or 20 Hz light stimulation of nape skin in Sst-ChR2 and *Sst-cre* mice. n = 8–10 mice. ns – not significant, one-way ANOVA with Tukey *post hoc*. (**F**) IHC images of Sst-ChR2/*Sst* co-expression in DRG of Sst-ChR2 mice (Left). Arrowheads indicate co-expression. Scale bar, 10 μm. Venn diagram showing overlapping expression of Sst-ChR2 and *Sst* in DRG neurons (Right). (**G**) IHC images of Sst-ChR2/CGRP/IB4 (left), Sst-ChR2/NF-H (middle), and Sst-ChR2/TRPV1 (right) in DRG of Sst-ChR2 mice. Arrowheads indicate co-expression. (**H**) IHC image of Sst-ChR2/βIII-Tubulin in hairy nape skin. Dashed line marks epidermal/dermal boundary. Arrowheads indicate ChR2 expression in lanceolate endings of hair follicles. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01, unpaired t test. Scale bars, 10 μm in **F**, 100 μm in **G** and **H**.

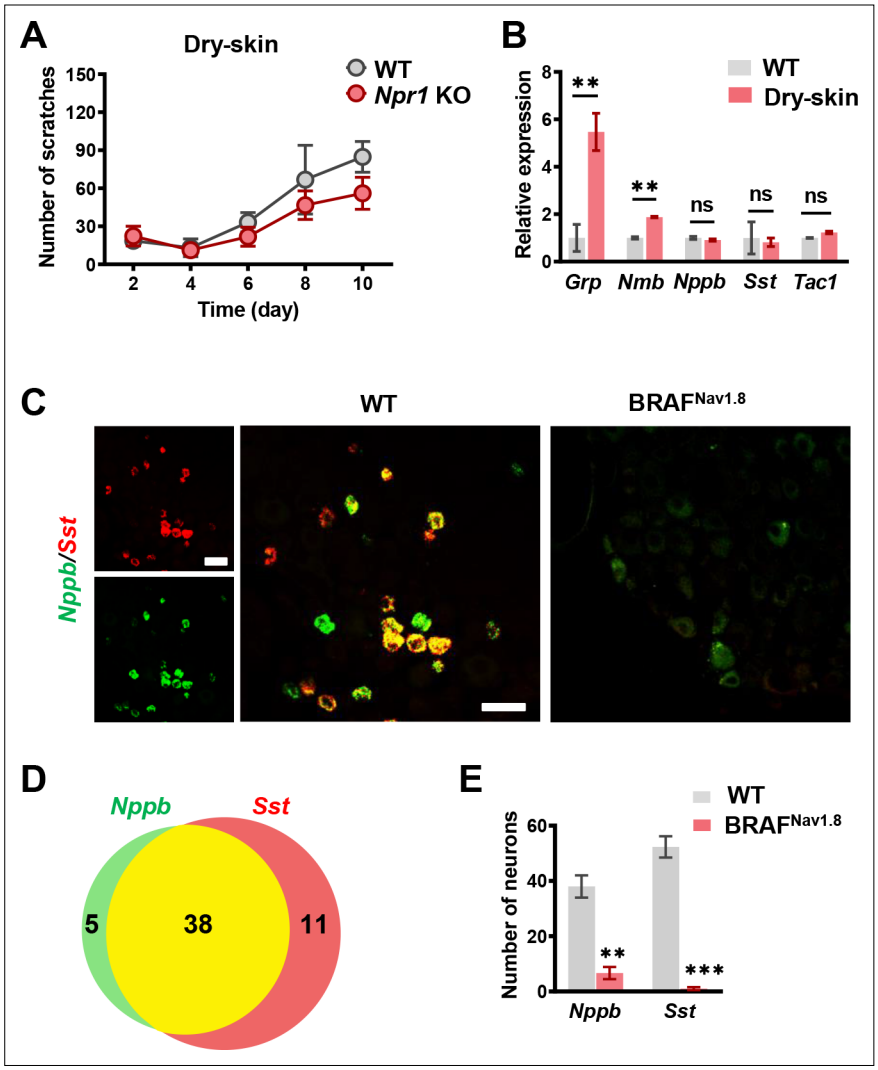

Figure 6—figure supplement 1. BNP-NPRA signaling is dispensable for nonhistaminergic itch and neuropathic itch. (**A**) *Npr1* KO mice and WT littermates showed comparable spontaneous scratching behaviors in the dry skin model. n = 6, p = 0.1283, *F*1,50 = 2.392, repeated measures Two-way ANOVA. (**B**) Real-time RT PCR showing significantly reduced levels of *Grp*, *Nmb*, *Nppb*, *Sst*, and *Tac1* in DRGs of dry skin mice relative to WT mice. n = 4. (**C**), RNA scope ISH images showing that *Nppb* and *Sst* were largely co-expressed in WT DRG neurons. *Nppb* and *Sst* signals were dramatically reduced in DRGs of BRAF$^{Nav1.8}$ mice. (**D**) Venn diagram showing overlapping expression of *Nppb* and *Sst*. (**E**) Quantified data of RNA scope showing that the numbers of *Nppb* neurons and *Sst* neurons were significantly reduced in the DRGs of BRAF$^{Nav1.8}$ mice. n = 4. Values are presented as mean ± SEM. **p < 0.01, ***p < 0.001, unpaired t test. Scale bars, 50 μm.

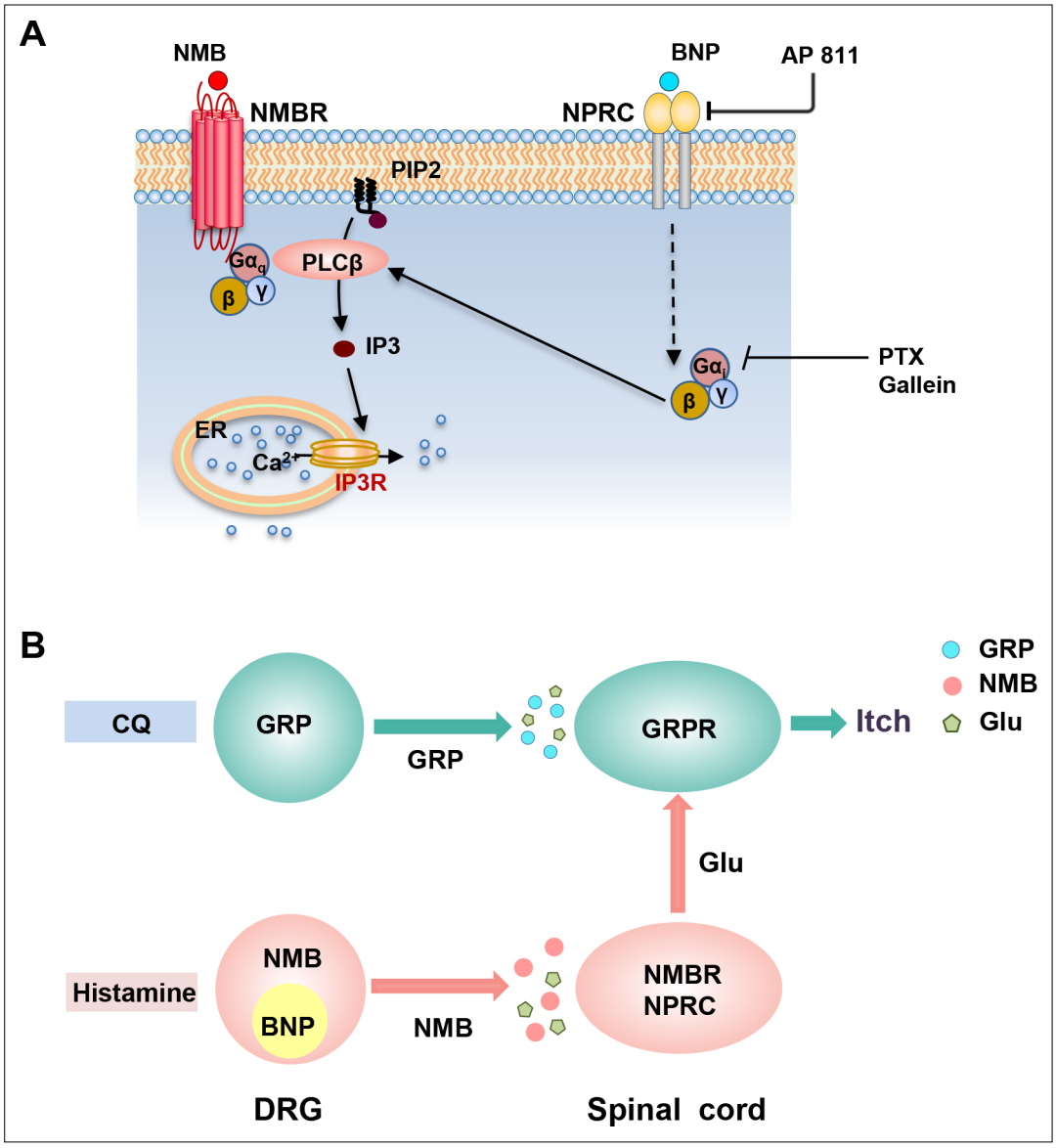

Figure 7. Schematics for the BNP-NPRC facilitated signaling pathway and distinct neuropeptide pathways for histamine-dependent and -independent itch. (**A**) A schematic showing a model for NMBR-NPRC cross-signaling facilitated by BNP via the NMB-NMBR pathway. In response to histamine, NMB and BNP are released from primary afferents to activate NMBR and NPRC concurrently. Activation of NMBR by NMB at a low concentration may prime PLCβ signaling, whereas activation of NPRC by BNP stimulates Gai signaling, which in turn stimulates PLCβ to activate downstream Ca2+ signaling. (**B**) A hypothetic model depicting the respective roles of neuropeptides and glutamate in itch transmission. CQ itch is mediated in part by GRP-GRPR signaling independent of glutamatergic transmission. In contrast, histamine itch is mediated by NMB-NMBR signaling from primary afferents to NMBR neurons and by glutamatergic transmission from NMBR neurons to GRPR neurons. BNP facilitates NMB-NMBR signaling via NPRC independent of GRP-GRPR signaling but dependent on GRPR neurons. Glu: glutamate; GRP: gastrin-releasing peptide; BNP: B-type natriuretic peptide; NMB: neuromedin B.

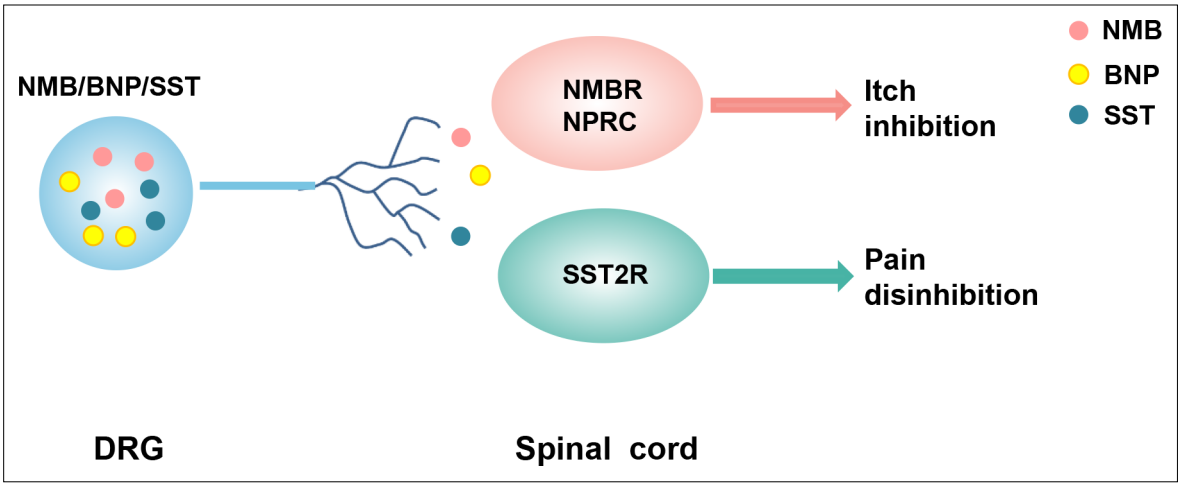

Figure 7—figure supplement 1. A hypothetic model depicting the role of BNP, NMB, and SST in facilitation of itch and disinhibition of pain, respectively. In response to histamine injection, NMB is released from primary afferents to activate NMBR neurons, while BNP is released to activate NPRC to facilitate NMBR signaling in NMBR neurons. Note that NMB and BNP do not have to be released from the same sensory neurons since NMB is also expressed in non-BNP neurons that may also innervate NMBR/NPRC neurons. During itch transmission, SST is not released. However, in response to certain types of noxious stimuli, SST may be released due to more intense firing of primary afferents to inhibit SST2R neurons, contributing to nociceptive transmission as a result of disinhibition.