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Supplemental information

Glutamate in primary afferents is required for itch transmission

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Supplemental Information – Table of Contents

3. Supplemental Figure Legends

1. Supplemental Tables

Table S1. Summary of peripheral light-evoked behavioral response percentages in six-month-old *Mrgpra3^{Cre}; Rosa$^{ChR2f/f}$* mice, related to Figure 1.

	5 mW	**10 mW**	**20 mW**	**25 mW**
Cheek				
Scratching (S)	0.0 ± 0.0%	4.0 ± 8.9%	4.0 ± 8.9%	0.0 ± 0.0%
Hind paw lifting (PL)	16.0 ± 8.9%	8.0 ± 11.0%	32.0 ± 22.8%	24.0 ± 16.7%
Head shaking (HS)	8.0 ± 11.0%	28.0 ± 22.8%	76.0 ± 35.8%	76.0 ± 8.9%
Bilateral wiping (BW)	40.0 ± 20.0%	84.0 ± 21.9%	96.0 ± 8.9%	96.0 ± 3.9%
Unilateral wiping (UW)	0.0 ± 0.0%	0.0 ± 0.0%	4.0 ± 8.9%	0.0 ± 0.0%
Nape				
Scratching (S)	52.0 ± 22.8%	64.0 ± 16.7%	60.0 ± 24.5%	64.0 ± 43.4%
Hind paw lifting (PL)	8.0 ± 17.9%	12.0 ± 17.9%	16.0 ± 16.7%	0.0 ± 0.0%
Head shaking (HS)	96.0 ± 8.9%	100 ± 0.0%	96.0 ± 8.9%	96.0 ± 8.9%
Bilateral wiping (BW)	4.0 ± 8.9%	20.0 ± 34.6%	16.0 ± 16.7%	36.0 ± 32.9%
Unilateral wiping (UW)	8.0 ± 11.0%	4.0 ± 8.9%	24.0 ± 26.1%	24.0 ± 21.9%

Table S2. Summary of pruritogen-evoked scratch bouts over 30 minutes following chemical injection in juvenile *Mrgpra3-Vglut2* CKO mice, related to Figure 2.

Numbers in parenthesis are the sample size of each cohort.

	***Mrgpra3-Vglut2* CKO**		**Control**	
	Cheek	**Nape**	**Cheek**	**Nape**
Chloroquine (CQ)	158.8 ± 25.3 (12)	293.1 ± 35.4 (7)	92.3 ± 17.2 (9)	329.8 ± 36.1 (4)
Histamine	57.3 ± 6.8 (6)	n/a	46.4 ± 9.0 (8)	n/a
BAM8-22	48.0 ± 13.8 (6)	41.9 ± 8.6 (11)	42.0 ± 16.1 (5)	39.7 ± 7.0 (12)
SLIGRL	54.4 ± 8.2 (7)	178.8 ± 35.7 (8)	47.2 ± 5.1 (9)	114.7 ± 19.6 (9)
5-HT	97.8 ± 19.2 (5)	n/a	86.6 ± 11.6 (9)	n/a

Table S3. RT-PCR primers, related to STAR Methods.

Gene	Primer 1	Primer 2
Vglut1	5'-GCAGGAGGAGTTTCGGAAG-3'	5'-ACAATGGCAAAGCCAAAGAC-3'
Vglut2 (pair 1)	5'-GGAGAAGAAGCAGGACAACC-3'	5'-TAGCCCCAGAAGAACGATCC-3'
Vglut2 (pair 2)	5'-GGGGAAAGAGGGGATAAAGA-3'	5'-CTGCAGATGGGATCAGCATA-3'
Vglut3	5'-TGTGTCATGGGTGTGAGGAT-3'	5'-TGATGGCATAGACAGGCAAG-3'
Grp	5'-TCCTGGCTAAGATGTATCCG-3'	5'-AGTCTACCAACTTAGCGGTTTG-3'
Grpr	5'-ACTGTCAGCTGACAGGTACAAAG-3'	5'-TAGGCACTCTGAATCAGATTTC-3'
Nmbr	5'-CAACCTCTCCTTTCCCACAG-3'	5'-GTCCATGGGGTTCACGATAG-3'
Gapdh	5'- GGTGAAGGTCGGTGTGAACG-3'	5'-CTCGCTCCTGGAAGATGGTG-3'

2. Supplemental Figures

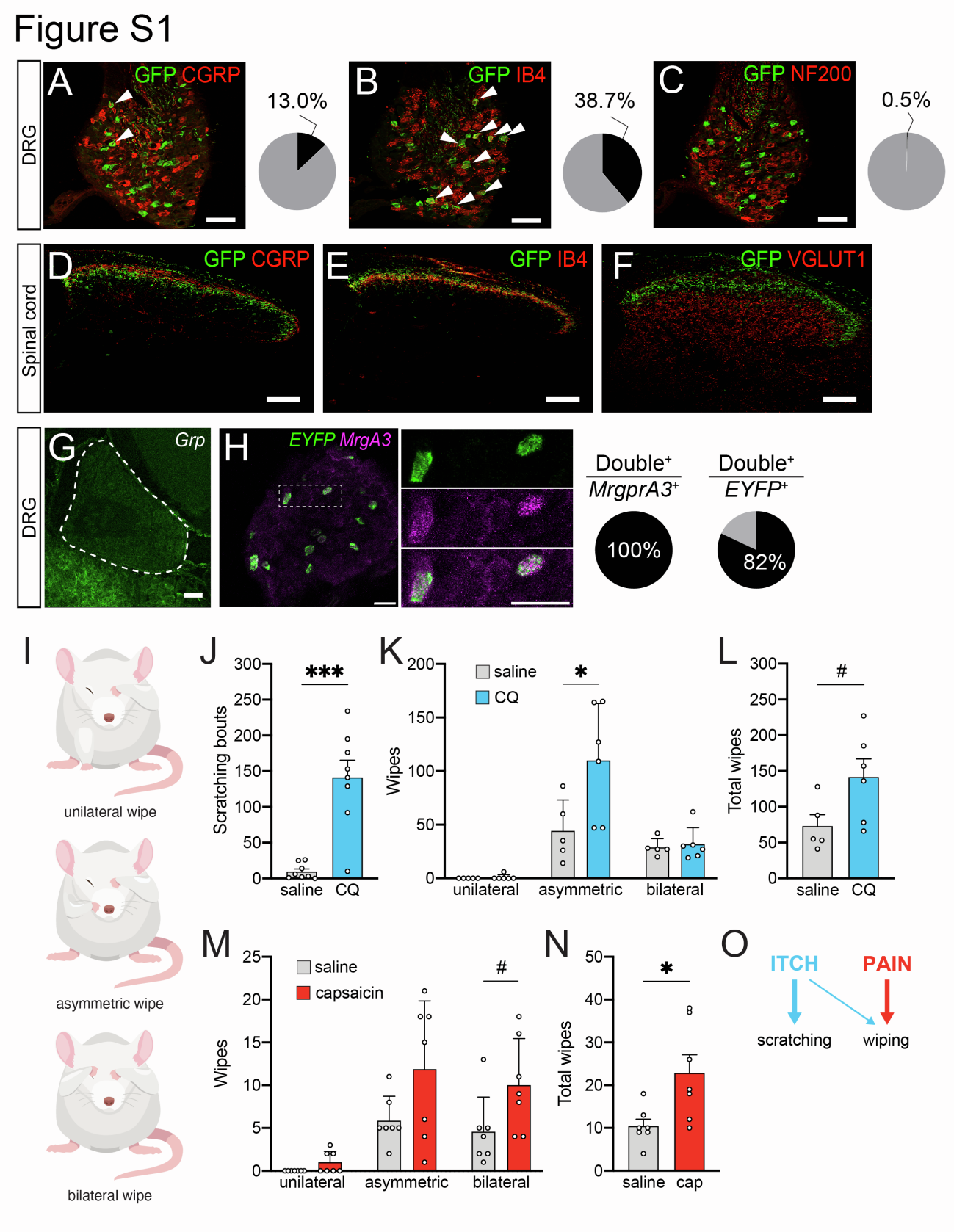


Figure S2

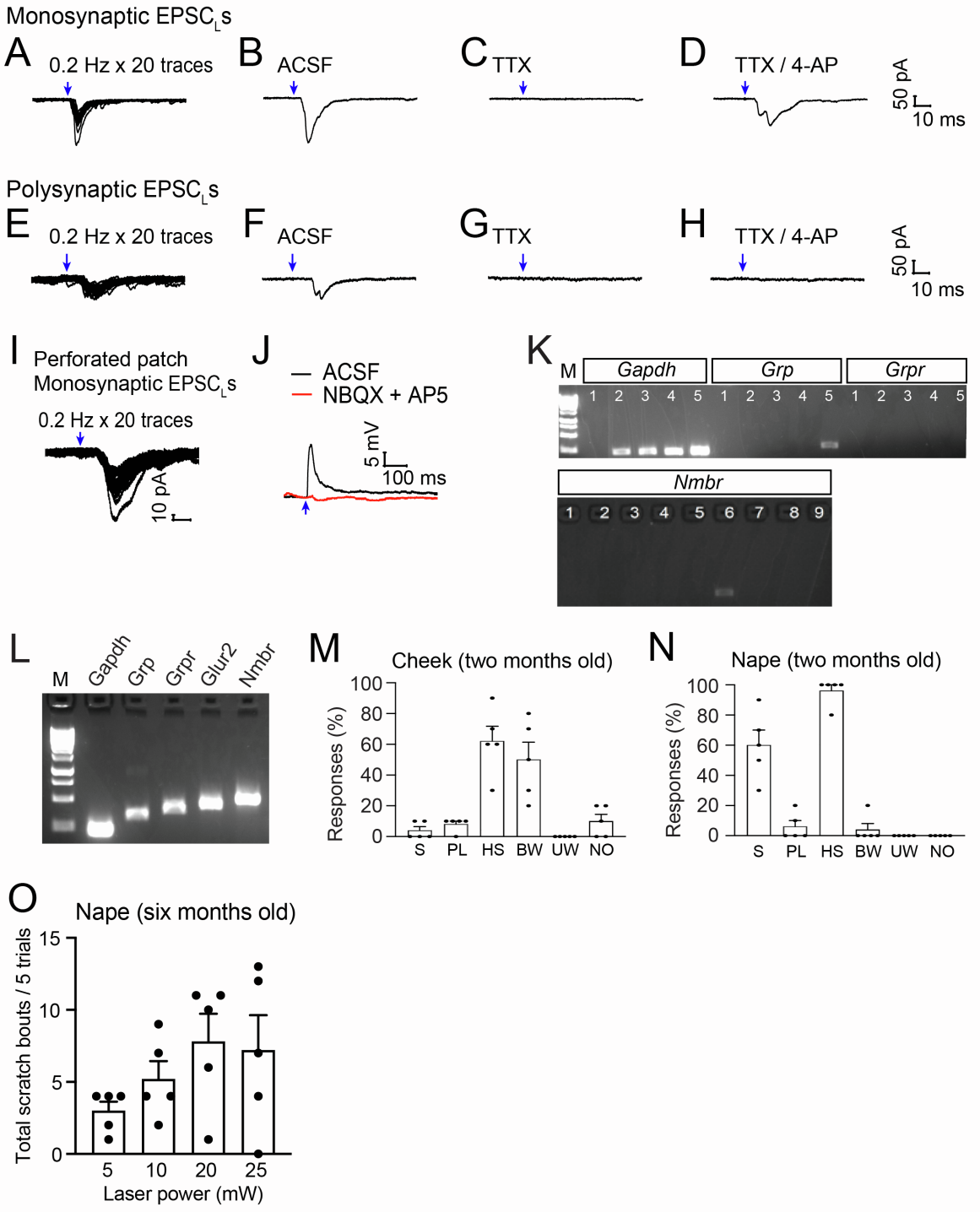

Monosynaptic EPSC$_L$s
A
0.2 Hz x 20 traces
B
ACSF
C
TTX
D
TTX / 4-AP
50 pA
10 ms
Polysynaptic EPSC$_L$s
E
0.2 Hz x 20 traces
F
ACSF
G
TTX
H
TTX / 4-AP
50 pA
10 ms
I
Perforated patch
Monosynaptic EPSC$_L$s
0.2 Hz x 20 traces
10 pA
J
ACSF
NBQX + AP5
5 mV
100 ms
K
M
Gapdh
Grp
Grpr
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
Nmbr
1 2 3 4 5 6 7 8 9
L
M
Gapdh
Grp
Grpr
Glur2
Nmbr
M
Cheek (two months old)
Responses (%)
100
80
60
40
20
0
S
PL
HS
BW
UW
NO
N
Nape (two months old)
Responses (%)
100
80
60
40
20
0
S
PL
HS
BW
UW
NO
O
Nape (six months old)
Total scratch bouts / 5 trials
15
10
5
0
5
10
20
25
Laser power (mW)

Figure S3

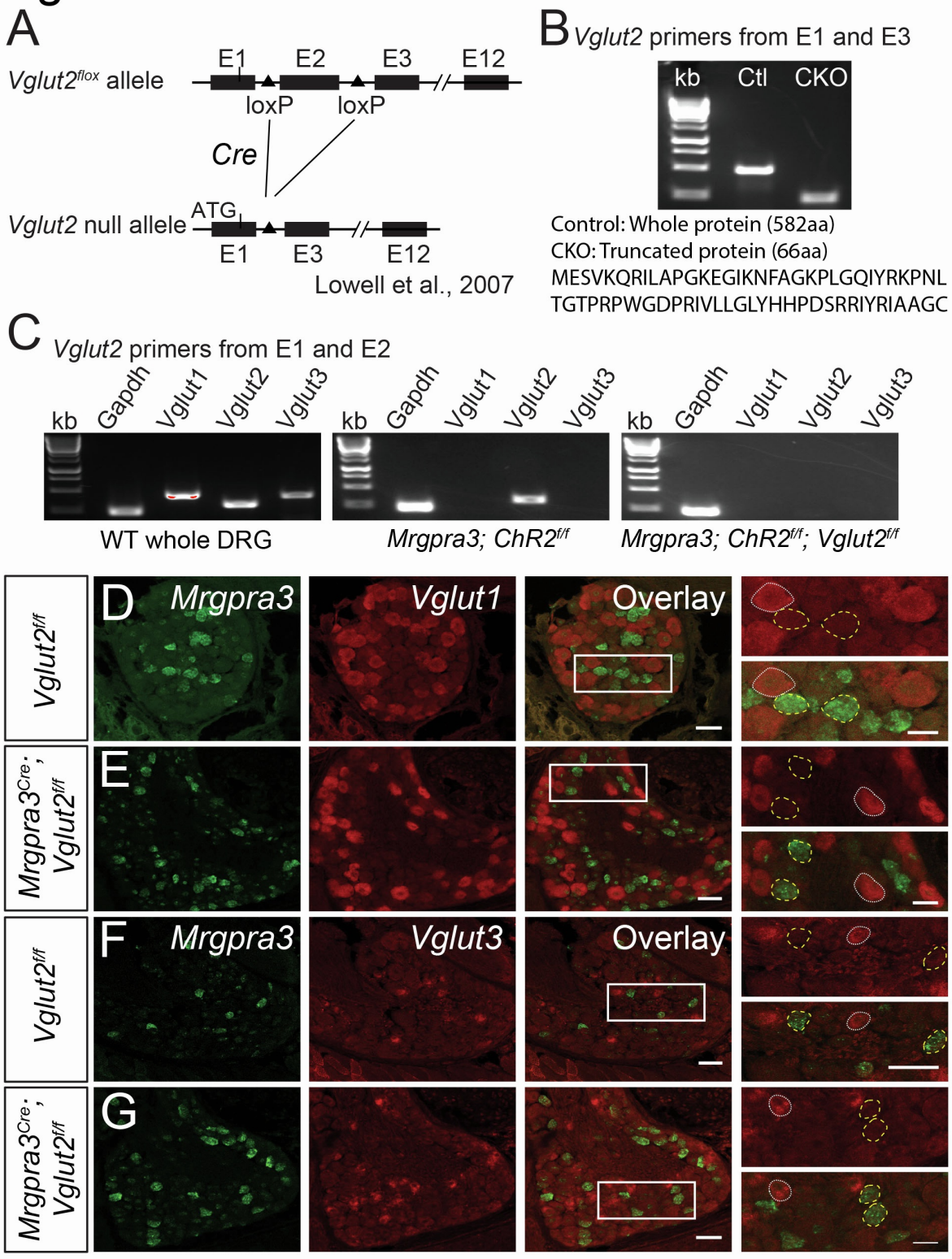

A
Vglut2flox allele
E1
E2
E3
E12
loxP
loxP
Cre
Vglut2 null allele
ATG
E1
E3
E12
Lowell et al., 2007
B Vglut2 primers from E1 and E3
kb
Ctl
CKO
Control: Whole protein (582aa)
CKO: Truncated protein (66aa)
MESVKQRILAPGKEGIKNFAGKPLGQIYRKPNL
TGTPRPWGDPRIVLLGLYHHPDSRRIYRIAAGC
C Vglut2 primers from E1 and E2
kb
Gapdh
Vglut1
Vglut2
Vglut3
WT whole DRG
Mrgpra3; ChR2f/f
Mrgpra3; ChR2f/f; Vglut2f/f
Vglut2f/f
D Mrgpra3
Vglut1
Overlay
Mrgpra3Cre; Vglut2f/f
E
Vglut2f/f
F Mrgpra3
Vglut3
Overlay
Mrgpra3Cre; Vglut2f/f
G

Figure S4

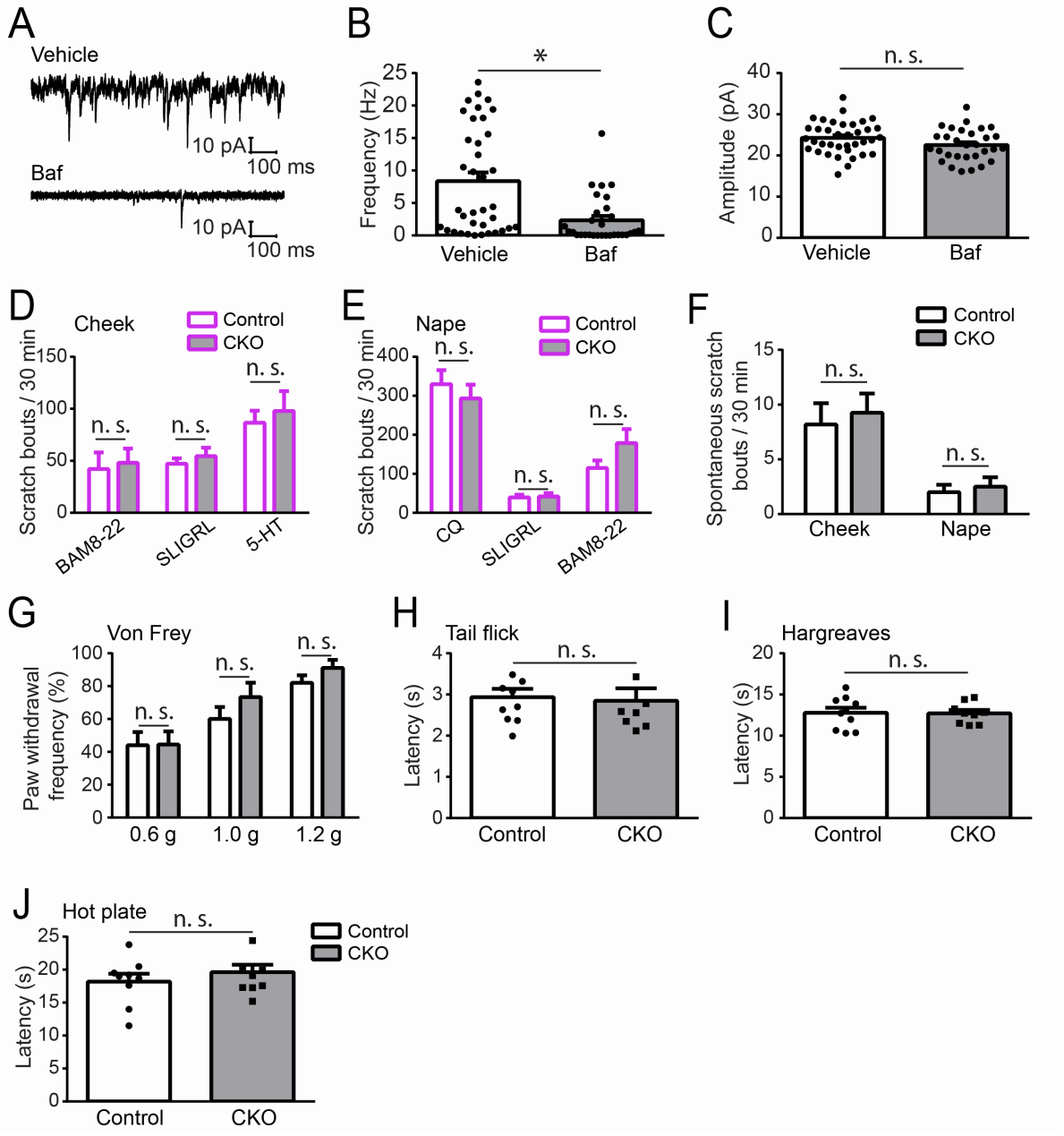

A
Vehicle
10 pA
100 ms
Baf
10 pA
100 ms
B
Frequency (Hz)
*
Vehicle
Baf
C
Amplitude (pA)
n. s.
Vehicle
Baf
D
Cheek
Control
CKO
Scratch bouts / 30 min
n. s.
n. s.
n. s.
BAM8-22
SLIGRL
5-HT
E
Nape
Control
CKO
Scratch bouts / 30 min
n. s.
n. s.
n. s.
CQ
SLIGRL
BAM8-22
F
Control
CKO
Spontaneous scratch bouts / 30 min
n. s.
n. s.
Cheek
Nape
G
Von Frey
Paw withdrawal frequency (%)
n. s.
n. s.
n. s.
0.6 g
1.0 g
1.2 g
H
Tail flick
Latency (s)
n. s.
Control
CKO
I
Hargreaves
Latency (s)
n. s.
Control
CKO
J
Hot plate
n. s.
Control
CKO
Latency (s)
Control
CKO

Figure S5

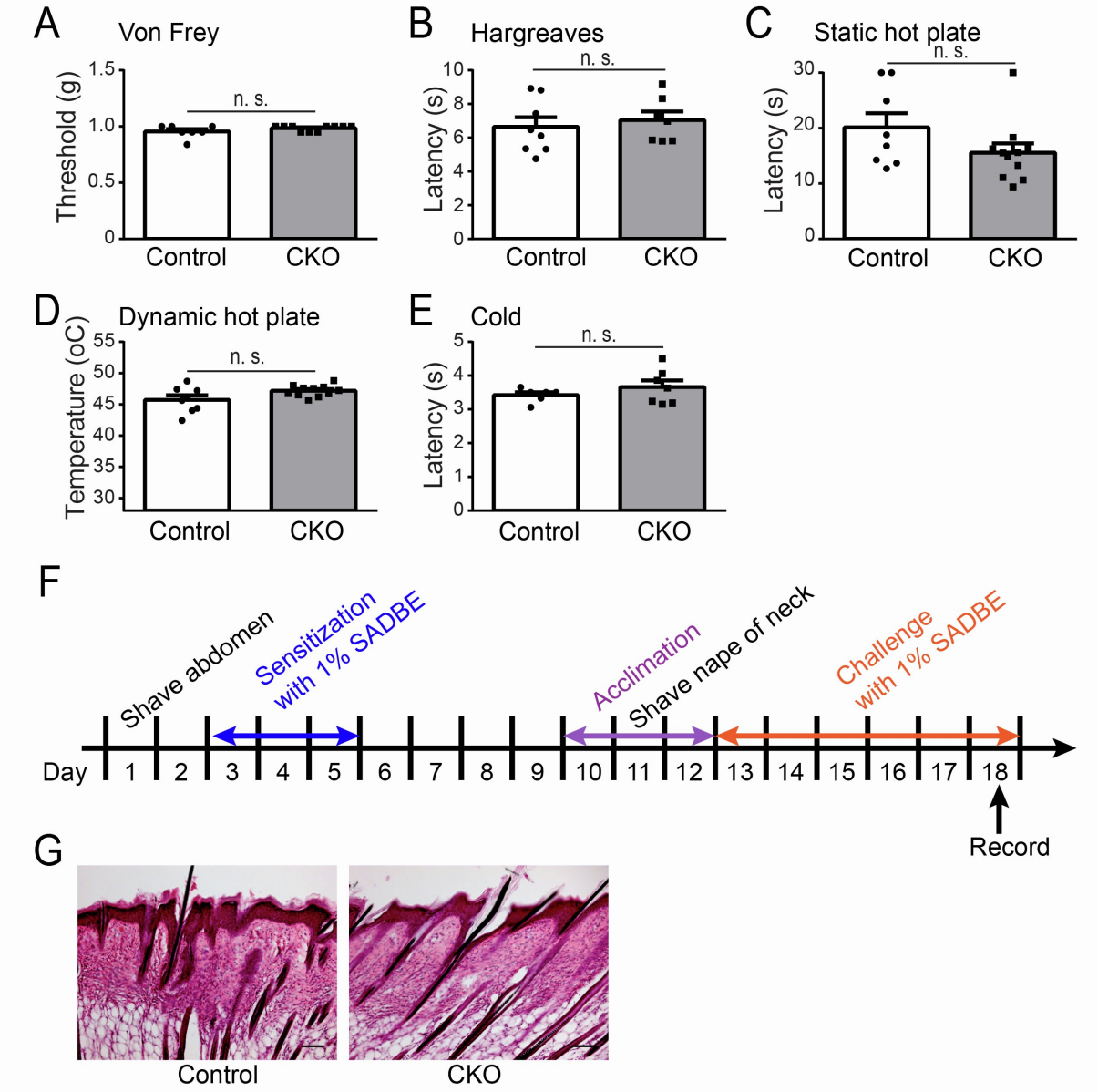

A
Von Frey
Threshold (g)
1.5
1.0
0.5
0
n. s.
Control
CKO
B
Hargreaves
Latency (s)
10
8
6
4
2
0
n. s.
Control
CKO
C
Static hot plate
Latency (s)
30
20
10
0
n. s.
Control
CKO
D
Dynamic hot plate
Temperature (oC)
55
50
45
40
35
30
n. s.
Control
CKO
E
Cold
Latency (s)
5
4
3
2
1
0
n. s.
Control
CKO
F
Shave abdomen
Sensitization with 1% SADBE
Acclimation
Shave nape of neck
Challenge with 1% SADBE
Day
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Record
G
Control
CKO

Figure S6

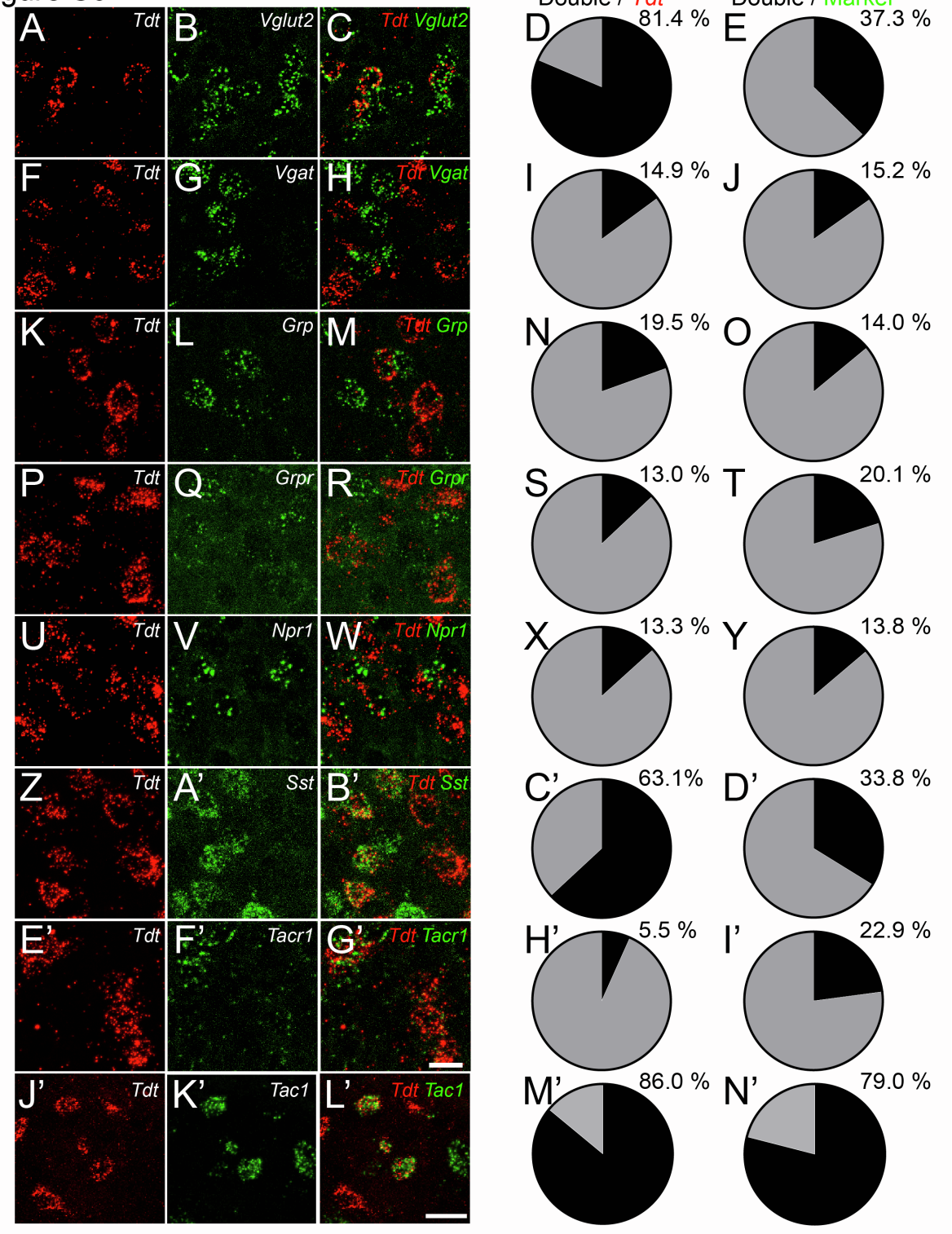
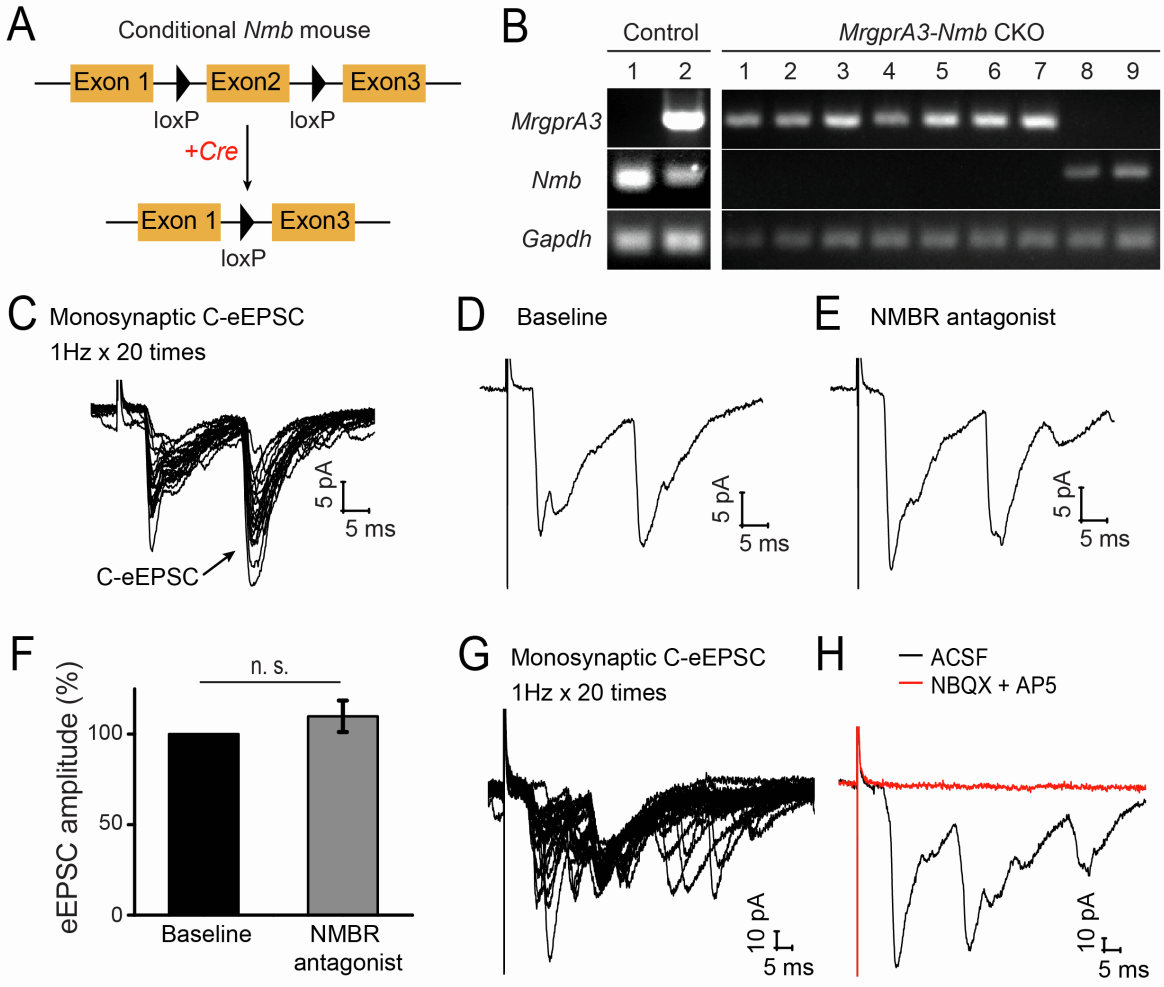

A Tdt
B Vglut2
C Tdt Vglut2
F Tdt
G Vgat
H Tdt Vgat
K Tdt
L Grp
M Tdt Grp
P Tdt
Q Grpr
R Tdt Grpr
U Tdt
V Npr1
W Tdt Npr1
Z Tdt
A' Sst
B' Tdt Sst
E' Tdt
F' Tacr1
G' Tdt Tacr1
J' Tdt
K' Tac1
L' Tdt Tac1
Double / Tdt
Double / Marker
D 81.4 %
E 37.3 %
I 14.9 %
J 15.2 %
N 19.5 %
O 14.0 %
S 13.0 %
T 20.1 %
X 13.3 %
Y 13.8 %
C' 63.1%
D' 33.8 %
H' 5.5 %
I' 22.9 %
M' 86.0 %
N' 79.0 %

Figure S7


A
Conditional Nmb mouse
Exon 1
Exon2
Exon3
loxP
loxP
+Cre
Exon 1
Exon3
loxP
B
Control
MrgprA3-Nmb CKO
1 2
1 2 3 4 5 6 7 8 9
MrgprA3
Nmb
Gapdh
C Monosynaptic C-eEPSC
1Hz x 20 times
5 pA
5 ms
C-eEPSC
D Baseline
5 pA
5 ms
E NMBR antagonist
5 pA
5 ms
F
n. s.
eEPSC amplitude (%)
100
50
0
Baseline
NMBR antagonist
G Monosynaptic C-eEPSC
1Hz x 20 times
10 pA
5 ms
H
ACSF
NBQX + AP5
10 pA
5 ms

3. Supplemental Figure Legends

Supplemental Figure 1. ChR2 expression in DRG and spinal cord of *Mrgpra3-ChR2* mice, and characterization of itch-related behavior, related to Figure 1.

(A-C) Double immunostaining of ChR2-EYFP and CGRP (A), ChR2-EYFP and IB4 (B), and ChR2-EYFP and NF200 (C) with lumbar (L4 and L5) DRG sections of three-week-old *Mrgpra3-ChR2* mice. Pie charts show the percentage of ChR2-EYFP^{+} DRG neurons that are double-positive for the indicated marker. Scale bars: 50 μm. 6-8 sections/mouse, n=3. (D-F). Double immunostaining of ChR2-EYFP and CGRP (D), ChR2-EYFP and IB4 (E), and ChR2-EYFP and VGLUT1 (F) with lumbar spinal cord sections of three-week-old *Mrgpra3-ChR2* mice. Scale bars: 50 μm. 6-8 sections/mouse, n=3. (G) RNAscope *in situ* hybridization of *Grp* with lumbar (L4 and L5) DRG sections of three-week-old wild type mice. Scale bar: 50 μm. 6-8 sections/mouse, n=3. (H) Double RNAscope *in situ* hybridization of *EYFP* and *MrgprA3* with lumbar DRG sections of six-week-old *Mrgpra3-ChR2* mice. Dashed outline indicates inset, magnified to the right. Scale bars: 50 μm. 6-8 sections/mouse, n=3. (I) Illustration of unilateral wiping (contralateral forepaw on the ground), asymmetric wiping, and bilateral (symmetric) wiping. (J) Total scratching bouts over 30 minutes in response to chloroquine (20 mM) or saline cheek intradermal injections in C57BL/6J mice. (K-N). Wiping responses to chloroquine or saline cheek injections (K and L) and capsaicin eye drops (0.06 μg/μl, 90 seconds) (M and N) in C57BL/6J mice. Each dot represents a single mouse. Data is presented as mean ± SD. Welch's t test in J, L, and N; unpaired student's t test in K and M. *$p<0.05$; ***$p<0.0001$, #$p=0.0557$, n=5-8. (O) Schematic of how scratching and wiping may perform as behavioral readouts of itch and pain sensations.

Supplemental Figure 2. Monosynaptic and polysynaptic inputs to spinal cord dorsal horn neurons from MRGPRA3$^+$ afferents, and light-evoked behavioral responses in *Mrgpra3-ChR2* mice, related to Figure 1.

(A-D) Representative traces of monosynaptic $EPSC_L$s recorded from dorsal horn neurons using spinal cord slices of 4-6w *Mrgpra3-ChR2* mice. Superimposition of monosynaptic $EPSC_L$s induced by 20 times light stimuli at 0.2 Hz (**A**). Baseline $EPSC_L$s (B) was blocked by TTX (1 μM) treatment (C), but recovered by perfusion of TTX (1 μM) and 4-AP (4 mM) (D). (E-H) Representative traces of polysynaptic $EPSC_L$s recorded from dorsal horn neurons. Superimposition of polysynaptic $EPSC_L$s induced by 20 times light stimuli at 0.2 Hz (E). Baseline $EPSC_L$s (F) was blocked by TTX (1 μM) treatment (G), but not recovered by perfusion of TTX (1 μM) and 4-AP (4 mM) (H). The blue arrows indicate laser stimuli. (I and J) In perforated patch recordings of superficial DH neurons from spinal cord slices of four- to six-week-old *Mrgpra3-ChR2* mice using amphotericin B (100 μg/mL), light stimulation-induced monosynaptic responses (black trace) were blocked by glutamate receptor antagonists (red trace). (K) RT-PCR of *Gapdh, Grp, Grpr, Glur2, and Nmbr* with RNAs from the whole spinal cord of wild type mice. (L) Single cell RT-PCR of *Gapdh, Grp, Grpr, and Nmbr* of monosynaptic responsive spinal DH neurons in spinal cord slices by light stimulation in five- to six-week-old *Mrgpra3-ChR2* mice. (M and N) The percentage of behavioral responses to 20 mW blue laser stimulation of the cheek (M) or nape (N) of two-month-old *Mrgpra3-ChR2* mice. Cheek: scratching (S) 4.0 ± 2.5%, hind paw lifting (PL) 8.0 ± 2.0%, head shaking (HS) 62.0 ± 9.7%, bilateral wiping (BW) 50.0 ± 11.4%, unilateral wiping (UW) 0.0 ± 0.0%. Nape: scratching (S) 60.0 ± 10.0%, hind paw lifting (PL) 6.0 ± 4.0%, head shaking (HS) 96.0 ± 4.0%, bilateral wiping (BW) 4.0 ± 4.0%, unilateral wiping (UW) 8.0 ± 11.0%. (O) Total scratch bouts over 5 trials of peripheral blue laser stimulation at the nape of six-month-old *Mrgpra3-ChR2* mice, as in Fig. 1C. Each dot represents an individual mouse.

Supplemental Figure 3. MRGPRA3^{+} afferent-specific *Vglut2* CKO mice, related to Figure 2.

(A) Genomic structure of conditional *Vglut2* allele and *Vglut2* null allele after Cre mediated recombination. (B) Single cell RT-PCR of *Vglut2* (PCR primers in exons 1 and 3) with dissociated EYFP^{+} DRG neurons from three- to four-week-old *Mrgpra3-ChR2* (control) and *Mrgpra3-Vglut2* (CKO) mice. The PCR products were sequenced, and the protein sequence predicted from the *Vglut2* null DRG neuron is shown. Control: 6 neurons/mice from 3 mice; CKO: 6 neurons/mice from 3 mice. (C) RT-PCR of *Gadph, Vglut1, Vglut2,* and *Vglut3* using RNA extracts from a whole DRG of a WT mouse (left) and dissociated single examples of EYFP^{+} DRG neurons of control (middle) and CKO (right) mice. Control: 14 neurons from 2 mice; CKO: 14 neurons from 2 mice. (D-G) Double fluorescent *in situ* hybridization of *Mrgpra3* (green) and transcripts of *Vglut1 or Vglut3* (red) with DRG sections of three-week-old *Vglut2$^{f/f}$* (control) and *Mrgpra3-Vglut2* (CKO) mice. Images in the rectangles are enlarged. White dashed circles indicate *Vglut1^{+} or Vglut3^{+}* but *Mrgpra3^{-}* neurons. Yellow dashed circles indicate *Mrgpra3* expressing neurons. Scale bars, 50 μm. 6-8 sections/mouse, n=3 mice.

Supplemental Figure 4. Juvenile *Mrgpra3-Vglut2* CKO mice display bafilomycin A1-dependent synaptic vesicle release and show normal somatosensory behaviors, related to Figure 2.

(A) Representative traces of sEPSCs after vehicle (0.1% DMSO) or bafilomycin A1 (4 mM) treatment of spinal cord slices of four- to six-week-old CKO mice. (B and C) Quantification of frequency and amplitude of sEPSCs under different conditions. (D) Quantification of scratching induced by cheek injections of BAM8-22, SLIGRL, and 5-HT in control and CKO mice that are around two months old. (E) Quantification of scratching in response to nape injection of CQ, SLIGRL, and BAM8-22 in control and CKO mice. (F) Quantification of spontaneous scratching of $Vglut2^{f/f}$ (control) and $Mrgpra3^{Cre}$; $Vglut2^{f/f}$ (CKO) mice that are around two months old. (G) Paw withdrawal frequency of control and CKO mice in responses to different Von Frey hairs around two months old. 0.6g: control 44.0 ± 8.0% vs CKO 44.4 ± 8.0%, $p>0.05$; 1.0g: control 60.0 ± 7.3% vs CKO 73.3 ± 8.8%, $p>0.05$; 1.2g: control 82.0 ± 4.7% vs CKO 91.1 ± 4.8%, $p>0.05$. (H) Tail withdrawal latency of control and CKO mice in tail flick test. Control 2.9 ± 0.2 s vs CKO 2.8 ± 0.3 s, $p>0.05$. (J and K) Paw withdrawal latency of control and CKO mice in Hargreaves test and hot plate test. Hargreaves test: control 12.8 ± 0.6 s vs CKO 12.7±0.4 s, $p>0.05$. Hot plate test: control 18.2 ± 1.2 s vs CKO 19.6 ± 1.1 s $p>0.05$. For the mechanical and thermal sensitivity assays: control n=9-10, CKO n=8-9. Data is presented as mean ± SEM. Unpaired student's t-test in B-J, * $p<0.05$; n.s., not significant.

Supplemental Figure 5. *Mrgpra3-Vglut2* CKO mice display normal behaviors in mechanical threshold or thermosensation tests at six months or older, related to Figure 4.

(A) Paw withdrawal threshold in Von Frey test in six-month-old or older control and CKO mice. Control 0.96 ± 0.02 g, n=7 vs CKO 0.99 ± 0.01 g, n=10, p>0.05. (B and C) Paw withdrawal latency in the Hargreaves test and static hot plate test in control and CKO mice. Hargreaves test: control 6.65 ± 0.57 s, n=8 vs CKO 7.05 ± 0.51 s, n=7, p>0.05. Static hot plate test: control 20.14 ± 2.54 s, n=8 vs CKO 15.57 ± 1.67 s, n=11, p>0.05. (D) Paw withdrawal threshold in the dynamic hot plate test in control and CKO mice. Control 45.72 ± 0.73 °C, n=8 vs CKO 47.19 ± 0.27 °C, n=11, p>0.05. (E) Paw withdrawal latency in the cold sensation test in control and CKO mice. Control 3.42 ± 0.08, n=6 vs CKO 3.66 ± 0.19, n=7, p>0.05. (F) Timeline for generating the SADBE-induced ACD mouse model. (G) H&E staining of the nape skin in control and CKO mice after SADBE treatment. Scale bars: 100 μm. Data is presented as mean ± SEM. Unpaired student's t-test in A-E, n.s., not significant.

Supplemental Figure 6. Molecular characterization of NMBR$^+$ cells in the superficial layer of the spinal cord dorsal horn, related to Figure 5.

(A-N') RNAscope *in situ* hybridizations of *Tdt* and *Vglut2, Vgat, Grp, Grpr, Npr1, Sst, Tacr1,* and *Tac1* in lumbar spinal cord sections of three-week-old *Nmbr-Tdt* mice. Scale bars: 10 μm. Pie charts summarize the quantification of double-positive cells among *Tdt*$^+$ cells or marker$^+$ cells. 6-8 sections/mouse, n=3 mice.

Supplemental Figure 7. Generation of *Mrgpra3-Nmb* CKO mice, related to Figure 7.

(A) Illustration of the genomic structure of the conditional *Nmb* allele and Cre-dependent recombination. (B) Single cell RT-PCR of *Mrgpra3, Nmb,* and *Gapdh* in dissociated DRG neurons from $Nmb^{f/f}$ (control) and *Mrgpra3-Nmb* (CKO) mice. Control n=3 mice; CKO n=3 mice. (C) Representative traces of monosynaptic EPSCs (C-eEPSCs) recorded in *Nmbr-Tdt* neurons after dorsal root electrical stimulation (C afferent stimulation). (D and E) Representative paired C-eEPSC traces at baseline (D) and after perfusion of the NMBR antagonist BIM 23042 (6 μM) (E). (F) Quantification of C-eEPSC amplitude, n=4. (G and H) Monosynaptic C-eEPSCs were blocked by glutamate receptor antagonists, NBQX (20 μM) and AP5 (50 μM) (red traces).