SUPPLEMENTAL DATA

SUPPLEMENTAL FIGURES AND LEGENDS

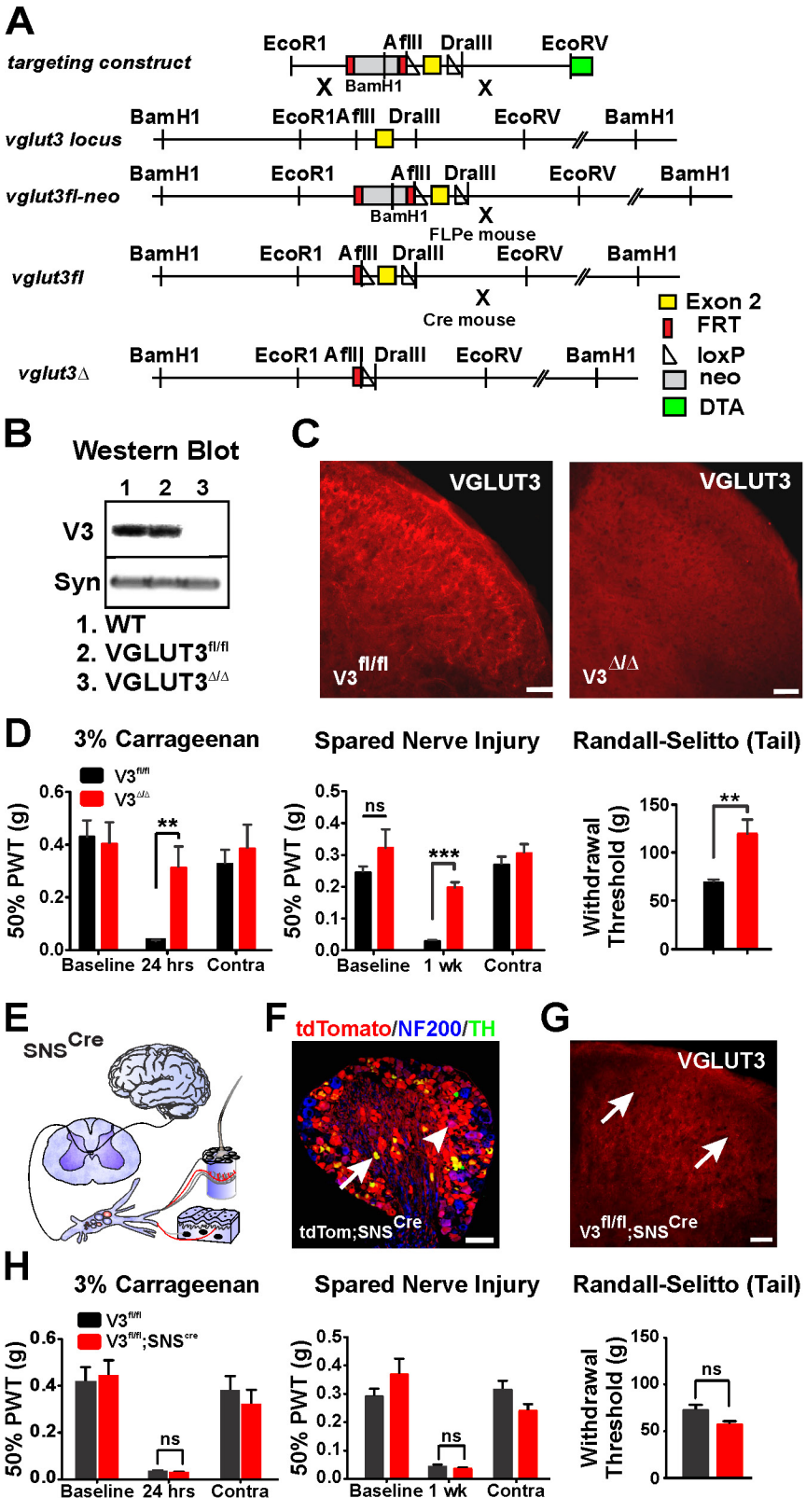
A
targeting construct
EcoR1
AflII
DraIII
EcoRV
X
BamH1
X
vglut3 locus
BamH1
EcoR1
AflII
DraIII
EcoRV
BamH1
vglut3fl-neo
BamH1
EcoR1
AflII
DraIII
EcoRV
BamH1
BamH1
X
FLPe mouse
vglut3fl
BamH1
EcoR1
AflII
DraIII
EcoRV
BamH1
X
Cre mouse
vglut3Δ
BamH1
EcoR1
AflII
DraIII
EcoRV
BamH1
Exon 2
FRT
loxP
neo
DTA
B
Western Blot
1 2 3
V3
Syn
1. WT
2. VGLUT3fl/fl
3. VGLUT3Δ/Δ
C
VGLUT3
V3fl/fl
VGLUT3
V3Δ/Δ
D
3% Carrageenan
Spared Nerve Injury
Randall-Selitto (Tail)
V3fl/fl
V3Δ/Δ
50% PWT (g)
0.6
0.4
0.2
0.0
**
Baseline
24 hrs
Contra
50% PWT (g)
0.5
0.4
0.3
0.2
0.1
0.0
ns
***
Baseline
1 wk
Contra
Withdrawal Threshold (g)
150
100
50
0
**
E
SNSCre
F
tdTomato/NF200/TH
tdTom;SNSCre
G
VGLUT3
V3fl/fl;SNSCre
H
3% Carrageenan
Spared Nerve Injury
Randall-Selitto (Tail)
V3fl/fl
V3fl/fl;SNScre
50% PWT (g)
0.6
0.4
0.2
0.0
ns
Baseline
24 hrs
Contra
50% PWT (g)
0.5
0.4
0.3
0.2
0.1
0.0
ns
Baseline
1 wk
Contra
Withdrawal Threshold (g)
150
100
50
0
ns

Figure S1, Related to Figure 1. Description of VGLUT3$^{fl/fl}$ Mice and conditional deletion of VGLUT3 from primary afferents.

(A) VGLUT3$^{fl/fl}$ targeting construct and *vglut3* gene locus before and after homologous recombination. LoxP sites flank exon 2. FRT sites flank the positive selectable marker, TK-*neomyosin*. Diphtheria toxin A (DTA) is the negative selectable marker.

(B) Western blot of brain tissue homogenate from VGLUT3^{WT}, VGLUT3$^{fl/fl}$ and VGLUT3$^{\Delta/\Delta}$ mice probed for VGLUT3 and for synaptophysin as control.

(C) VGLUT3-IR in spinal cord of adult VGLUT3$^{fl/fl}$ (left) and VGLUT3$^{\Delta/\Delta}$ (right) mice. VGLUT3-IR is absent in the VGLUT3$^{\Delta/\Delta}$ mice.

(D) Paw Withdrawal Thresholds (PWT) in the carrageenan and SNI models are normal in VGLUT3$^{fl/fl}$ mice (n=6 and 4, respectively), but are attenuated in the VGLUT3 $^{\Delta/\Delta}$ mice (n= 8 and 4, respectively). Randall-Selitto thresholds are also significantly higher in VGLUT3 $^{\Delta/\Delta}$ compared to VGLUT3$^{fl/fl}$ mice (n=4 and n=6).

(E) SNS^{Cre} mice express Cre in DRG neurons (all unmyelinated, some myelinated).

(F) In the lsl-tdTom;SNS^{Cre} mice, tomato is present in unmyelinated neurons including C-LTMRs (arrow), and some myelinated afferents (arrowhead).

(G) VGLUT3$^{fl/fl}$;SNS^{Cre} mice lack VGLUT3-IR in lamina II$_i$ (arrows) of the dorsal horn.

(H) Mechanical allodynia is normal in VGLUT3$^{fl/fl}$;SNS^{Cre} mice. PWTs did not differ from VGLUT3$^{fl/fl}$ controls after carrageenan (n=10 and n=8 respectively) or SNI (n=5 and n=4 respectively). Randall-Selitto responses also did not differ (n=8 and n=6 respectively).

All scale bars = 100 μm. Data are Mean ± SEM. **p ≤ 0.01, ***p ≤ 0.001.

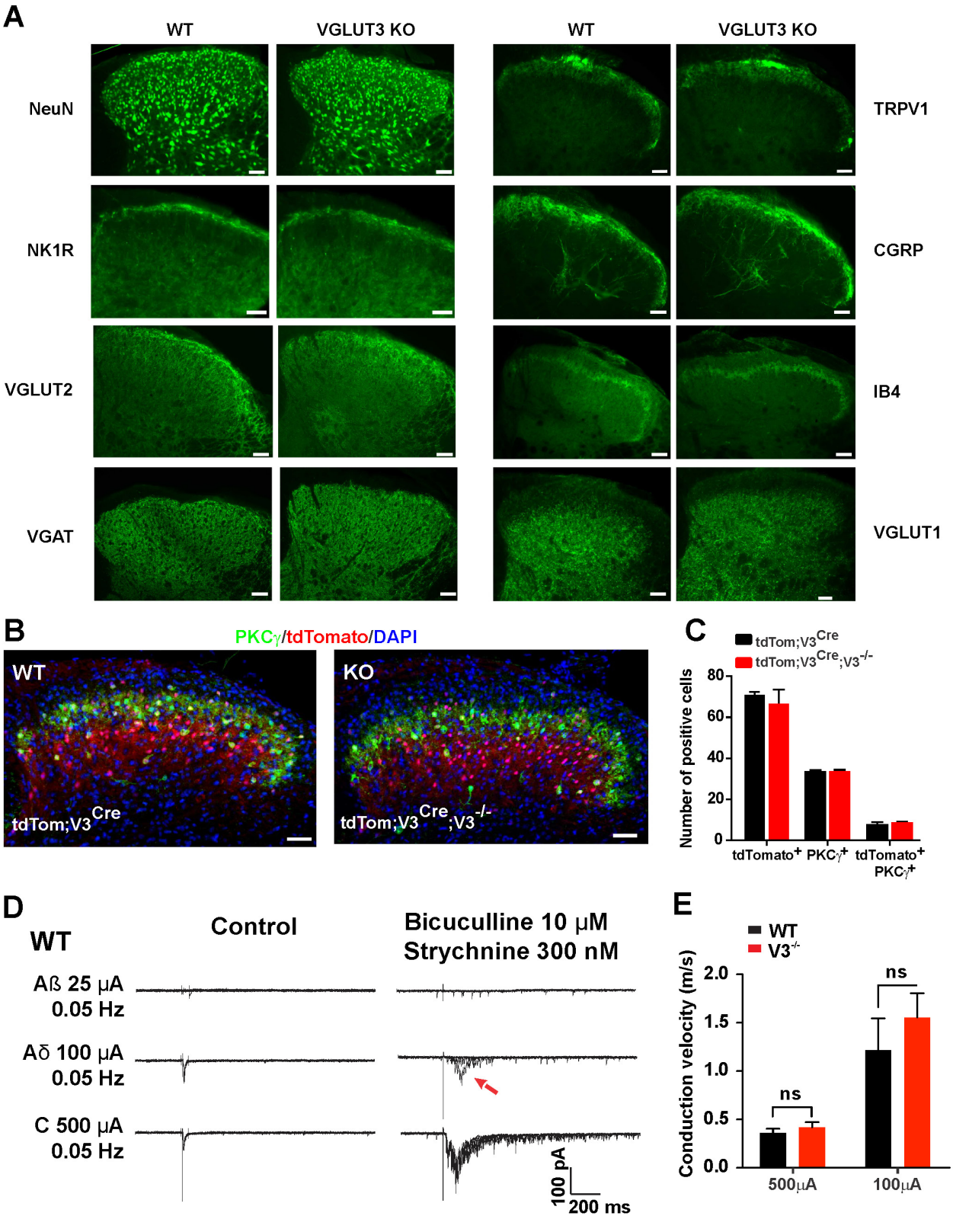
A
WT
VGLUT3 KO
WT
VGLUT3 KO
NeuN
TRPV1
NK1R
CGRP
VGLUT2
IB4
VGAT
VGLUT1
B
PKCγ/tdTomato/DAPI
WT
tdTom;V3Cre
KO
tdTom;V3Cre;V3-/-
C
tdTom;V3Cre
tdTom;V3Cre;V3-/-
Number of positive cells
80
60
40
20
0
tdTomato+
PKCγ+
tdTomato+ PKCγ+
D
WT
Control
Bicuculline 10 μM
Strychnine 300 nM
Aß 25 μA
0.05 Hz
Aδ 100 μA
0.05 Hz
C 500 μA
0.05 Hz
100 pA
200 ms
E
WT
V3-/-
Conduction velocity (m/s)
2.0
1.5
1.0
0.5
0.0
ns
ns
500μA
100μA

Figure S2, Related to Figure 4. Architecture, afferent innervation and afferent electrophysiology are normal in VGLUT3 KO dorsal horn.

(A) Spinal cords of VGLUT3 KO and WT mice stained for markers of spinal cord neurons (NeuN, VGLUT2, VGAT, NK1) and primary afferents (IB4, CGRP, TRPV1, VGLUT1) show no gross differences. Scale bars = 100 μm.

(B) Dorsal horns of lsl-tdTom;VGLUT3^{Cre} mice on a VGLUT3 KO or WT background are co-stained with PKCγ and DAPI. Scale bars = 20 μm.

(C) The number of tomato^{+} and PKCγ^{+} cells in the dorsal horn of lsl-tdTom;VGLUT3^{Cre} mice does not differ in the absence of VGLUT3.

(D) Example of polysynaptic EPSCs (red arrow) induced by stimulation of Aβ and Aδ fibers (100 μA) under pharmacological disinhibition.

(E) Conduction velocities and primary afferent thresholds are similar between VGLUT3 KO and WT (n = 7 and 8 respectively, Mann-Whitney test).

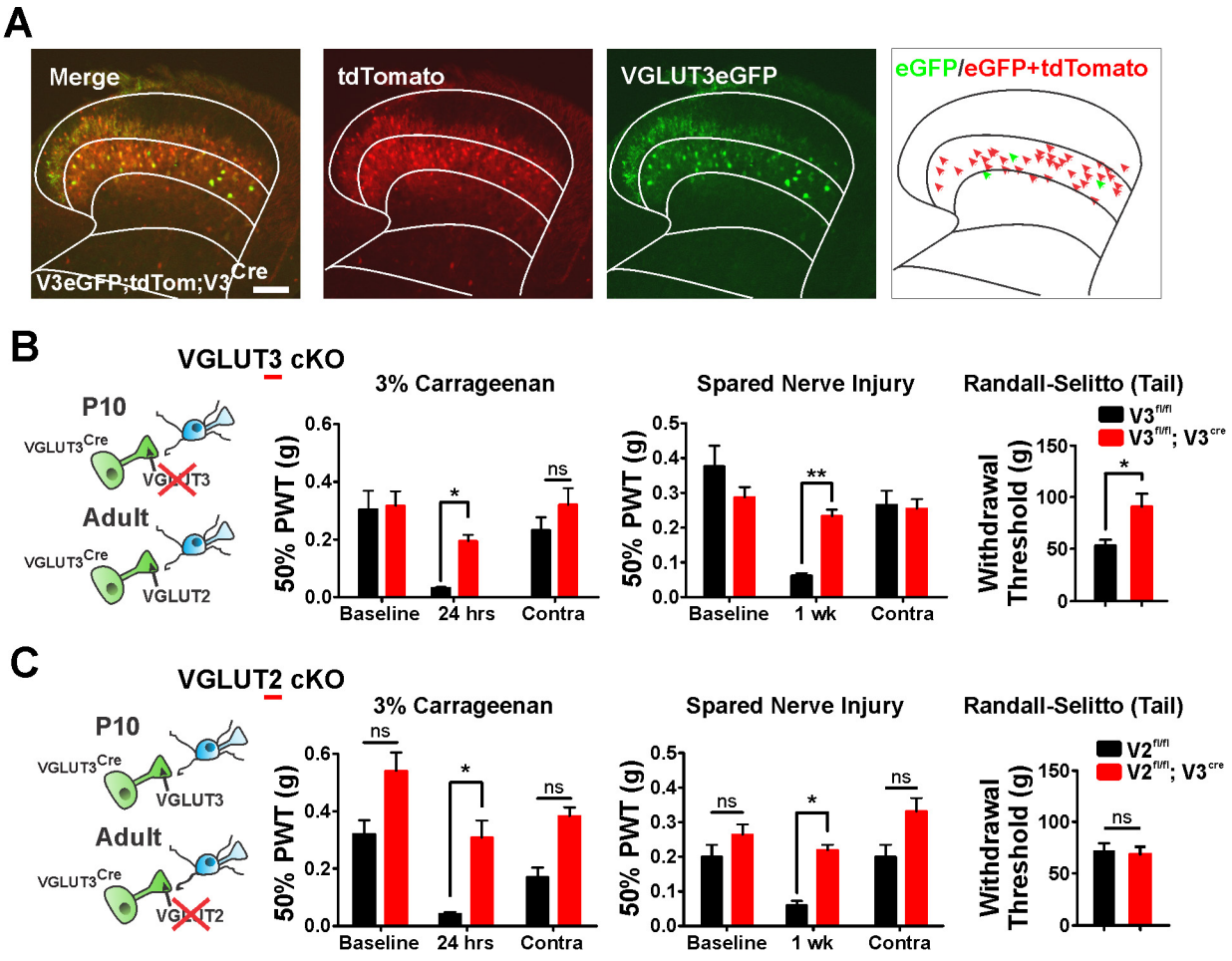

Figure S3, Related to Figure 5. Mechanical pain behavior after deletion of VGLUT2 or VGLUT3 in VGLUT3^{Cre} neurons.

(A) VGLUT3^{EGFP};lsl-tdTom;VGLUT3^{Cre} mice, EGFP co-localizes with tomato. Scale bar = 100 μm.

(B) VGLUT3$^{fl/fl}$; VGLUT3^{Cre} mice lack transient VGLUT3 expression in the dorsal horn. Baseline PWTs of VGLUT3$^{fl/fl}$;VGLUT3^{Cre} mice are similar to controls. After carrageenan or SNI, PWTs are significantly elevated compared to controls (both tests n=7 both groups). Randall-Selitto withdrawal thresholds are also significantly higher than controls (n=4 both groups).

(C) VGLUT2$^{fl/fl}$;VGLUT3^{Cre} mice lack adult VGLUT2 expression in VGLUT3 transient cells. Baseline PWTs of VGLUT2$^{fl/fl}$;VGLUT3^{Cre} mice are similar to controls. After carrageenan (n=5 both groups) or SNI (n=3 both groups), PWTs are significantly elevated compared to controls Remarkably, Randall-Selitto withdrawal thresholds of VGLUT2$^{fl/fl}$;VGLUT3^{Cre} do not differ from controls (n=4 both groups).

Data are Mean ± SEM. *$p < 0.05$, **$p \leq 0.01$.

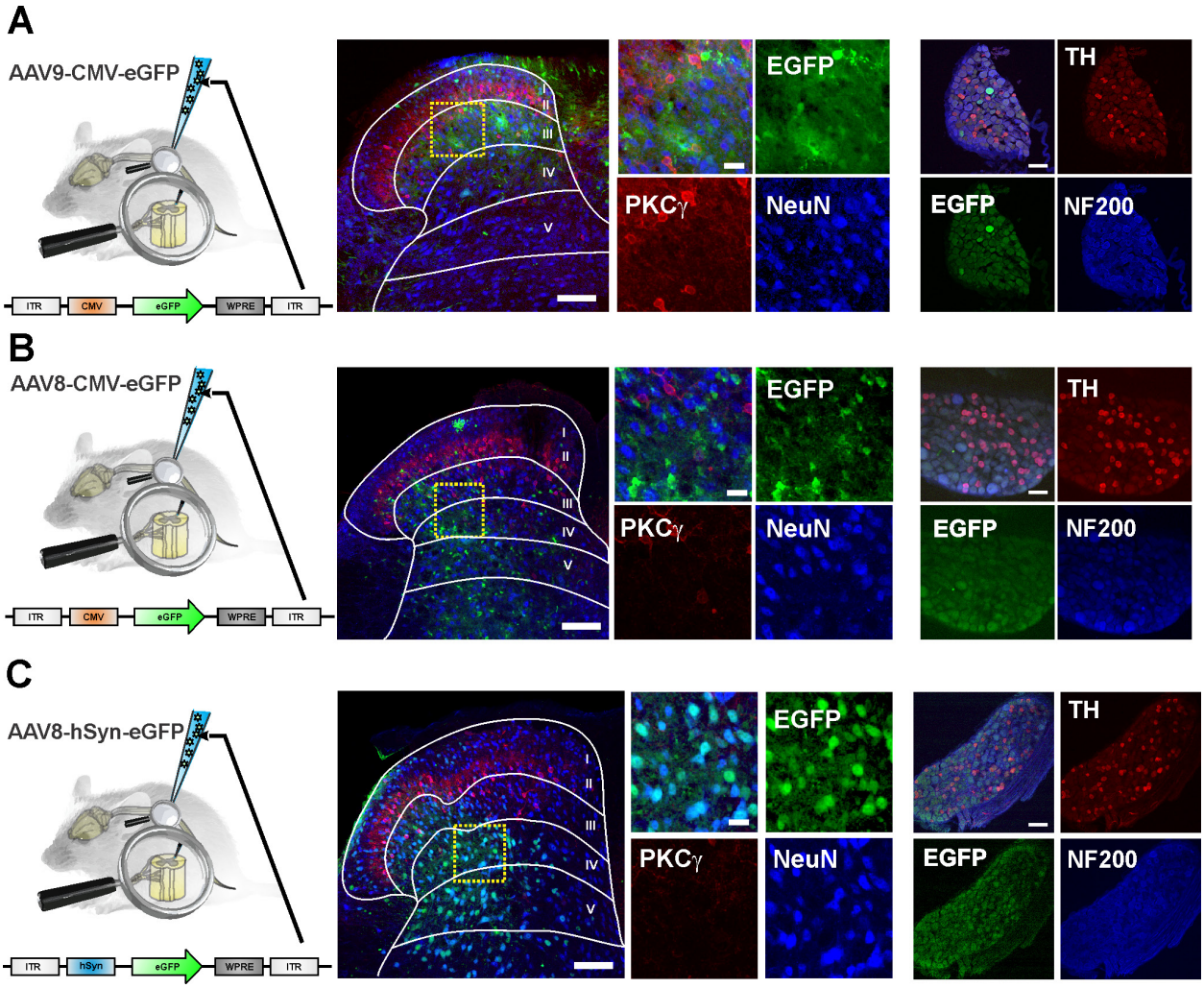

Figure S4, Related to Figure 5. Transduction of dorsal horn neurons and DRG with AAVs.

(A) AAV9-CMV-EGFP infects dorsal horn neurons and glia as well as DRG neurons. Scale bars = 100 μm except for inset (10 μm).

(B) AAV8-CMV-EGFP infects dorsal horn glia and some neurons, but not DRG. Scale bars = 100 μm except for inset (10 μm).

(C) AAV8-hSyn-EGFP exclusively infects dorsal horn neurons, not glia or DRG. Scale bars = 100 μm except for inset (10 μm).

MERGE
cFos
mCherry
NeuN
WT-DREADDs CNO-Mech
KO-DREADDs CNO-Mech
WT CNO-Mech
WT-DREADDs CNO
KO-DREADDs CNO
WT-DREADDs Saline
I
II
III
IV
V
V3Cre
V3fl/fl;V3Cre

Figure S5, Related to Figure 5. Induction of c-Fos in the dorsal horn by hM3Dq activation. Representative images of c-Fos induction in dorsal horn neurons of VGLUT3^{Cre} (labeled WT) or VGLUT3$^{fl/fl}$;VGLUT3^{Cre} (labeled KO) mice with and without injection of hM3Dq-mCherry virus, CNO or mechanical stimulation of the plantar hindpaw. All scale bars = 100 μm.

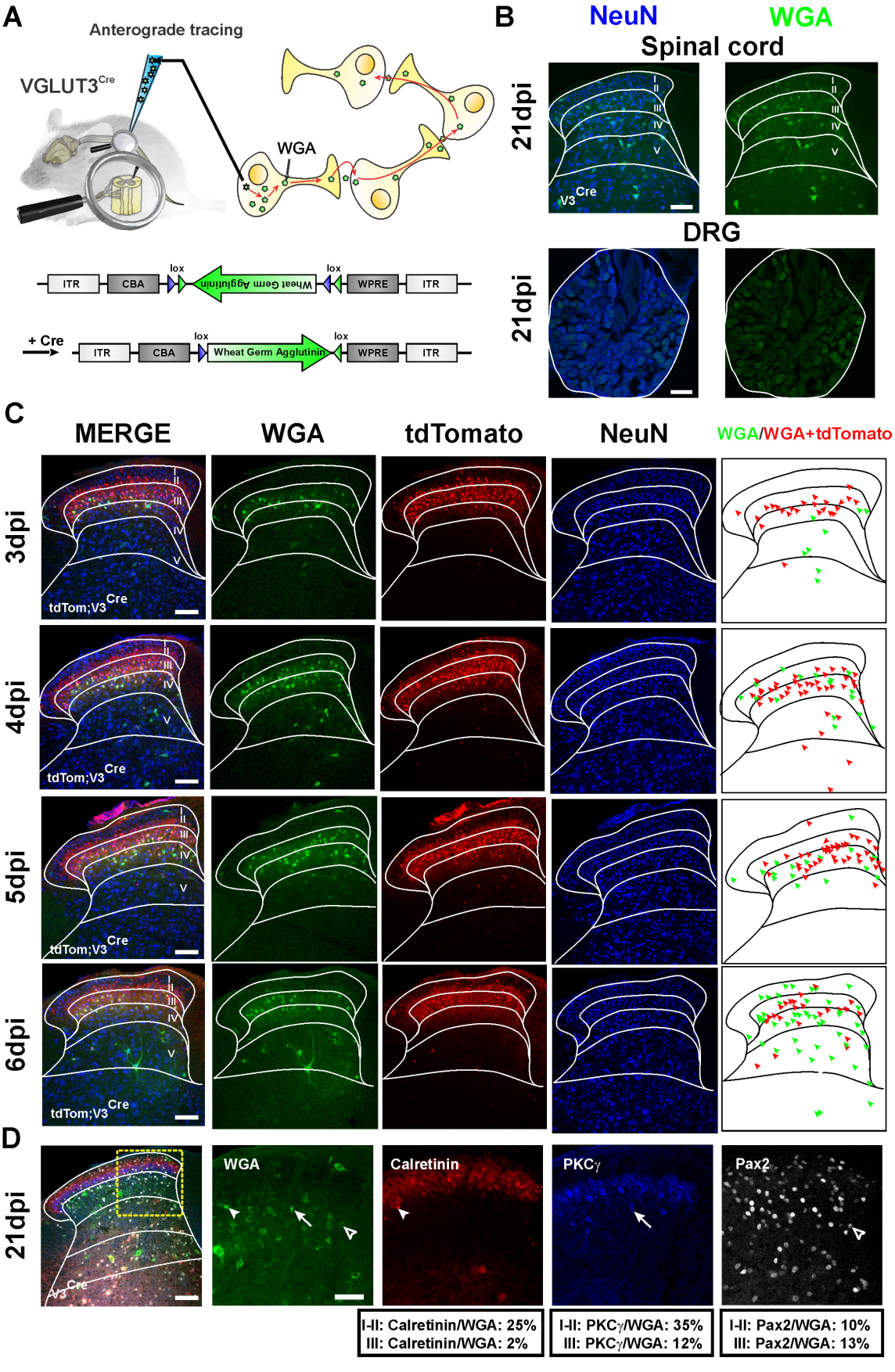
A
Anterograde tracing
VGLUT3Cre
WGA
ITR
CBA
lox
Wheat Germ Agglutinin
WPRE
+ Cre
B
NeuN
WGA
Spinal cord
21dpi
Cre
V3
DRG
C
MERGE
WGA
tdTomato
NeuN
WGA/WGA+tdTomato
3dpi
4dpi
5dpi
6dpi
tdTom;V3Cre
D
21dpi
Calretinin
PKCγ
Pax2
I-II: Calretinin/WGA: 25%
III: Calretinin/WGA: 2%
I-II: PKCγ/WGA: 35%
III: PKCγ/WGA: 12%
I-II: Pax2/WGA: 10%
III: Pax2/WGA: 13%

Figure S6, Related to Figure 5. Anterograde tracing with wheat germ agglutinin in lsl-tdTom;VGLUT3 mice.

(A) Schematic of lsl-tdTom;VGLUT3^{Cre} mice unilaterally injected with AAV2-CBA-Flex-WGA in the dorsal horn at p10.

(B) At 21 days post-injection (dpi), WGA is observed in all laminae of the dorsal horn but is not present in DRG neurons.

(C) From 3-6 dpi, WGA starts in tdTomato^{+} cells and then spreads to cells in laminae III and IV-V with time.

(D) At 21 dpi, WGA is observed in many more cells including calretinin (25%) and PKCγ (35%) excitatory interneurons and Pax2 (10%) inhibitory interneurons in lamina II. All scale bars = 100 μm except in D (50 μm).

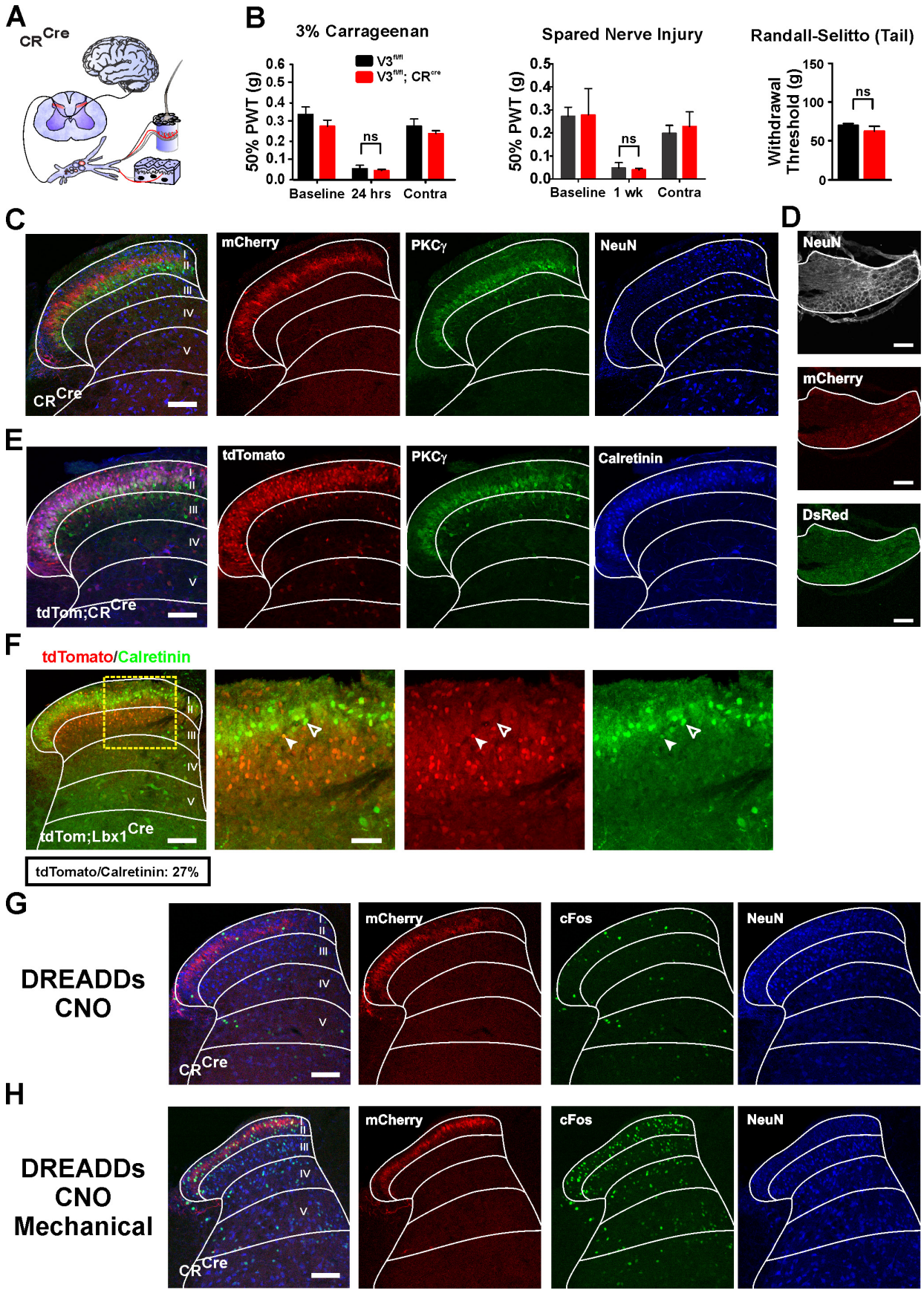
A
CR Cre
B
3% Carrageenan
V3 fl/fl
V3 fl/fl; CR cre
50% PWT (g)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ns
Baseline
24 hrs
Contra
Spared Nerve Injury
50% PWT (g)
0.5
0.4
0.3
0.2
0.1
0.0
ns
Baseline
1 wk
Contra
Randall-Selitto (Tail)
Withdrawal Threshold (g)
150
100
50
0
ns
C
I
II
III
IV
V
CR Cre
mCherry
PKCγ
NeuN
D
NeuN
mCherry
DsRed
E
tdTom;CR Cre
tdTomato
PKCγ
Calretinin
F
tdTomato/Calretinin
tdTom;Lbx1 Cre
tdTomato/Calretinin: 27%
G
DREADDs
CNO
CR Cre
mCherry
cFos
NeuN
H
DREADDs
CNO
Mechanical
CR Cre
mCherry
cFos
NeuN

Figure S7, Related to Figure 7. Behavioral characterization and cFos induction using CR^{Cre} Mice.

(A) In CR^{Cre} mice, dorsal horn excitatory neurons and some DRG neurons express Cre.

(B) Loss of VGLUT3 from calretinin neurons does not alter PWTs at baseline or after carrageenan (n=4 both groups) or SNI (n=2 both groups). Randall-Selitto thresholds are also unchanged (n=4 both groups).

(C) In CR^{Cre} mice injected unilaterally in the dorsal horn with AAV8-hSyn-DIO-hM3Dq-mCherry virus, mCherry localizes exclusively to the dorsal region of lamina II_i, and does not co-localize with PKCγ.

(D) In CR^{Cre} mice injected unilaterally in the dorsal horn with AAV8-hSyn-DIO-hM3Dq-mCherry virus, mCherry is not expressed by DRG.

(E) In lsl-tdTom;CR^{Cre} mice, tomato is expressed by neurons throughout lamina II_i and co-stains almost completely with calretinin, but very little with PKCγ.

(F) In adult lsl-tdTom;$Lbx1^{Cre}$, a few calretinin neurons in lamina II_i co-localize with tomato (closed arrowhead), but most do not (open arrowhead).

(G) Representative images of c-Fos induction in dorsal horn neurons of CR^{Cre} expressing hM3Dq-mCherry virus and injected with CNO.

(H) Representative images of c-Fos induction in dorsal horn neurons of CR^{Cre} expressing hM3Dq-mCherry virus and injected with CNO plus mechanical stimulation of the plantar hindpaw.

Data are Mean ± SEM. All scale bars = 100 μm except insets in F (20 μm).

Movie S1, Related to Figure 5. Spontaneous allodynia behavior after CNO administration in VGLUT3^{Cre} and VGLUT3$^{fl/fl}$;VGLUT3^{Cre} mice expressing hM3Dq-mCherry. After saline injection, VGLUT3^{Cre} mice expressing the hM3Dq receptor show no abnormal paw behaviors. After administration of 5 mg/kg CNO, VGLUT3^{Cre} mice show paw lifting (first arrow) and paw guarding behaviors (second and third arrow). VGLUT3$^{fl/fl}$;VGLUT3^{Cre} mice show very subdued paw lifting and guarding behavior after CNO injection (arrow).

Movie S2, Related to Figure 7. Spontaneous allodynia behavior after CNO administration in CR^{Cre} mice expressing hM3Dq-mCherry. After saline injection, CR^{Cre} mice expressing the hM3Dq receptor show no abnormal paw behaviors. After administration of 5 mg/kg CNO, CR^{Cre} mice show paw lifting and paw guarding behaviors (arrow).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals

All mice were kept on a standard 12:12 light/dark cycle in micro-isolator caging racks (Allentown Caging) with food and water provided *ad libitum* and were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and UCSF. $Rosa26^{lsl\text{-}td}$ (stock #007914), $Rosa26^{Cre}$ (stock #006054), $Rosa26^{Flpe}$ (stock #003946), CR^{Cre} (stock #010774) and $KRT14^{Cre}$ (stock #004782) mouse lines were obtained from Jackson Laboratories. Dr. Rohini Kuner provided the SNS^{Cre} mice, Dr. Fan Wang provided the $Advillin^{Cre}$ mice, Dr. Hanns-Uli Zeilhofer provided the $Hoxb8^{Cre}$ mice, Dr. Qiufu Ma provided $Tlx3^{Cre}$ mice, Dr. Carmen Birchmeier provided the $Lbx1^{Cre}$ mice and Dr. Thomas Hnasko provided the $VGLUT2^{fl/fl}$ mice.

Description of Conditional VGLUT3 knockout mouse. The mouse was generated by homologous recombination in embryonic stem (ES) cells. LoxP sites surround exon 2 in the targeting construct, which was generated from an RPCI-24 BAC clone. *Neomyocin* and *diphtheria toxin A* gene cassettes were used as positive and negative selectable markers. The targeting construct was introduced into 129/Ola ES cells. Properly targeted clones were injected into pseudopregnant C57Bl/6J mice. Chimeric founders were crossed to $Rosa26^{Flpe}$ to remove the *neomycin* gene. Germline deleted mice were generated by first crossing to $Rosa26^{Cre}$ ($VGLUT3^{\Delta/\Delta}$). Levels of VGLUT3 were assessed by Western blot of brain homogenate and immunofluorescence staining of spinal cord. VGLUT3 was present at WT levels in $VGLUT3^{fl/fl}$ mice and was absent from $VGLUT3^{\Delta/\Delta}$ mice.

Behavioral Tests

Randall-Selitto Assay

Mice were tested as previously reported (Seal et al., 2009). Briefly, mice were acclimated to a tail access restraint (StoeltingCo cat# 51338) by placing the open restraint in the home cage for 2 days prior to testing. On test day, mice were allowed to acclimate for 10 minutes to the restraint and then placed on the Ugo Basile Analgesy Meter (cat# 37215) with a blunt conical tip on the weight arm. Force was applied to the tail until the mouse struggled or flicked its tail (withdrawal threshold). The final force in grams was recorded. Each mouse was tested 3 times with a ten-minute inter-trial interval and the results of the three trials averaged.

Paw Withdrawal Threshold to von Frey Filaments

Mice were tested as previously described (Seal et al., 2009). Briefly, mice were habituated to opaque Plexiglas chambers on a wire mesh table for 1 hour the day before and 30 minutes prior to testing. Testing was performed using a set of calibrated Semmes-Weinstein monofilaments (StoeltingCo cat# 58011) using the Up-Down method (Chaplan et al., 1994). The 50% paw withdraw threshold (PWT) was determined for each mouse beginning with a 0.4 g filament. Each filament was applied to the plantar surface of the hind paw between the walking pads for 3 seconds or until a response such as a sharp withdrawal, shaking or licking of the limb was observed. Incidents of rearing or normal ambulation during filament application were not counted. Testing alternated between the ipsilateral and contralateral paws with a 5-minute interval in between each application until the thresholds were determined.

Pinprick test

Animals were first placed in a square Plexiglas chamber on top of a wire mesh table. Animals were acclimated to this testing arena for 1 hour on the day prior to testing and for an additional 30 minutes immediately before testing. A small insect pin (tip diameter = 0.03 mm) was applied with minimal pressure to the plantar surface of the left hind paw, taking care not to penetrate the skin. If the animal showed aversive behavior (lifting, shaking, licking of the paw) a positive response was recorded. A negative response was recorded if the animal showed no such reaction within 2 seconds of application. The application was repeated 10 times with a 5-minute interval between applications, and a percentage positive response determined for each animal.

Fur Clip Test

Animals were placed in an empty plastic chamber and allowed to acclimate for 15-20 minutes. A 2 cm wide alligator clip was attached to the hairs on the animal's back just above the tail, taking care not to pinch the skin. The latency (in seconds) until the animal looked towards the clip or tried to remove it was recorded. Three applications were performed per animal with a five-minute interval between applications, and the average response latency determined for each animal out of three trials.

Sticky Tape Test

Animals were placed in an empty plastic chamber and allowed to acclimate for 15-20 minutes. A ¼ inch diameter adhesive paper circle was then applied to the plantar surface of the left hind paw covering the footpads, and the animals were placed back in the cage. The latency until each animal responded to the sticky tape (by licking, looking

at or shaking of the affected paw) was then recorded and the tape circle removed. Each animal was tested 3 times with a 5-mintute interval between tests, and the three values averaged for each animal.

Dynamic Mechanical Allodynia (Cotton Swab Method)

The test was performed as described previously (Garrison and Stucky 2011). Briefly, animals were first placed in a square Plexiglas chamber on top of a wire mesh table and allowed to acclimate to this testing arena for 1 hour prior to testing. Using forceps, the head of a cotton swab was teased and puffed out until it reached approximately three times its original size. The cotton swab was lightly run across the surface of the hind paw from heel to toe. If the animal reacted (lifting, shaking, licking of the paw) a positive response was recorded. A negative response was recorded if the animal showed no such behavior. The application was repeated 10 times with a 5-minute interval between applications, and a percentage positive response determined for each animal.

Plantar Heat Test (Hargreaves' Method)

Animals were placed in a acrylic chamber on a glass top table and allowed to acclimate to the test chamber for 1 hour the day before and 30 minutes the day of testing. Using a Plantar Analgesia Meter (IITC) a radiant heat source of constant intensity was focused on the plantar surface of the hindpaw and the latency to paw withdrawal measured. The heat source was shut off upon paw withdrawal or after 20 seconds of exposure to prevent injury. The test was repeated 3 times on each hind paw with a 5 minute interval between tests and the results for each paw averaged together.

Carrageenan-induced inflammation

Mice were lightly anesthetized via inhaled 2.5% isoflurane and injected with 20 µL of 3% λ-Carrageenan (Sigma 22049) dissolved in sterile 0.9% NaCl into the plantar surface of the left hind paw. PWTs were determined before and 24 hours after injection.

Spared Nerve Injury

Surgery was performed as previously described (Seal et al., 2009; Shields et al., 2003). Briefly, Mice were deeply anesthetized using a mix of 100 mg/kg ketamine and 20 mg/kg xylazine by intraperitoneal injection. The left hind limb was shaved with trimmers and sterilized with betadine and ethanol. A small incision was made in the skin of the leg proximal to the knee and the skin and underlying muscle opened by blunt dissection to expose the three branches of the sciatic nerve. The peroneal and sural branches were tightly ligated with 6-0 nylon sutures and transected below the ligature, and a 2-3 mm section distal to the ligature was removed. Care was taken to avoid disturbing the tibial nerve, which was left intact. The muscle tissue was closed back over the nerves and the skin sutured shut with 6-0 nylon sutures. PWT to von Frey filaments were determined the day before and 1 week after surgery.

Chemogenetic Activation of hM3Dq Receptor

Von Frey and Hargreaves thresholds were performed as described above in AAV8-hSyn-DIO-hM3Dq-mCherry injected mice. Clozapine-N-Oxide (CNO) at 5 mg/kg was injected intraperitoneally 30 minutes prior to testing.

Paw Lifting/Guarding Assays

Mice were placed in a glass chamber with a smooth plastic floor and injected intraperitoneally with 5 mg/kg CNO in saline or saline only. The mouse was allowed to acclimate to the chamber for 15 minutes post-injection and then recorded by video for 10 minutes. The videos were then analyzed for incidents of the mouse visibly lifting or shaking its paw outside of ambulation (paw withdrawal), and time in which the paw was held such that the glabrous surface was not in contact with the floor or held in an abnormal posture tucked under the body (guarding).

Intraspinal AAV injections

P9-10 (for VGLUT3^{Cre}) or P15-16 (for CR^{Cre}) mice were anesthetized with 2.5% isoflurane. A midline incision along the left lumbar vertebrae was carefully performed until the spinal cord was visible from the intervertebral spaces. No laminectomy was performed to maximally avoid trauma. Glass microelectrode with around 50 µm tip was slowly inserted between L4-L5 at -250 µm from the dura using a stereotaxic frame avoiding the posterior spinal arteries. One µl of viral solution was slowly infused within a period of 5 minutes using a picospritzer and the micropipette was left in place for 2 minutes. The *lassimus dorsi* were sutured to protect the spinal cord and the skin was finally sealed with silk sutures. Animals were given an intraperitoneal injection of ketoprofen 5mg/kg before and 24h after the surgery. Behavior tests were performed 3 weeks after viral inoculation to allow maximal and stable expression. AAV8-CMV-eGFP (1.5 x 10^12 vg/ml), AAV9-CMV-eGFP (8 x 10^12 vg/ml), AAV8-hSyn-eGFP (3.7 x

10^12 vg/ml) and AAV8-hSyn-DiO-hM3Dq-mCherry (6 x 10^12 vg/ml) were purchased from UNC vector core (Chapel Hill, NC). AAV2-CBA-Flex-WGA (6.5 x 10^12 vg/ml) was a gift from Dr. Reza Sharif-Naeini.

Tissue processing for c-Fos analysis

For mice injected with the hM3Dq virus, animals were analyzed 3-5 weeks after viral injection. To investigate the expression of c-Fos after CNO in the absence of any other stimulus, animals were first anesthetized with an intraperitoneal injection of 1.5 g/kg urethane. After 30 minutes, control of proper anesthesia was assessed by the absence of reflex following a light pinch of the front paws. Then, mice were given an intraperitoneal injection of 3 mg/kg CNO or control saline and immediately placed on their back on a heating pad. Special care was taken to assure that the rear legs were not in contact to anything including the heating pad. 90 minutes after CNO injection, animals were quickly perfused as described below.

To investigate the effect of mechanical stimulation of the hindpaws following CNO injection, we used a rodent treadmill (Digigait, Mouse Specifics Inc.) as an unavoidable mechanical stimulus of the foot. To do so, mice were first placed into a Plexiglas chamber on the stationary treadmill and allowed to acclimate for 5 minutes. The speed was then slowly increased to a final rate of 10 cm/s for which lasted for a period of 5 minutes. At this final speed, animals were forced to walk but they never ran. At the end of this habituation period, mice were given an intraperitoneal injection of 3 mg/kg CNO. Animals were placed back into the chamber with the treadmill immobile and monitored for 30 minutes. The mice were then walked on the treadmill at a speed of

10 cm/s for a period of 15 minutes and finally placed back into their cage. While some mice showed discomfort, none failed to perform the task. Mice were perfused 90 minutes later as described below.

To investigate the effect of mechanical stimulation of the hindpaws following inflammation or neuropathy, mice were walked 24 hours after carrageenan injection or 1 week after SNI surgery respectively, and then perfused 90 minutes later as described above. Control mice that were not mechanically stimulated were taken from their home cages and immediately perfused.

Immunohistochemistry

Mice were deeply anesthetized with a mix of ketamine and xylazine and then transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). A laminectomy was performed and the spinal cord and dorsal root ganglia harvested. Glabrous skin from the plantar surface of the hind paw was also removed. Tissues were post-fixed in 4% PFA overnight, cryo-protected in 30% sucrose and then cut with a cryostat (Microm HM550) into 10-30 µm sections placed directly onto poly-lysine coated slides or into wells containing PBS. For fluorescent immunostaining, spinal cord slices were blocked with 5% normal donkey serum (NDS) in PBS + 1% triton-X (PBS-T) for 1 hour at room temperature (RT) then incubated in primary antibody diluted in 5% NDS in PBS-T overnight at 4°C. Sections were then washed in PBS and incubated in AlexaFluor secondary antibodies (Jackson ImmunoResearch) diluted 1:1000 in 1% NDS in PBS-T, for 1-2 hours at RT. Slices were then washed in PBS and cover-slipped with Fluoromount-G or Fluoromount-G containing DAPI (Southern Biotech).

The following primary antibodies were used for immunofluorescence staining at the following dilutions: VGLUT3 antiserum raised in guinea pig (1:500; Synaptic Systems 135204), anti-PKCγ raised in rabbit (1:1000; Santa Cruz sc-211), anti-PKCγ raised in guinea pig (1:500; Frontier Institute Af350), anti-VGLUT1 raised in guinea pig (1:5000; Chemicon AB5905), anti-VGLUT2 raised in rabbit (1:1000; Synaptic Systems 135403), anti-tyrosine hydroxylase raised in rabbit (1:500; Chemicon AB152), anti-NF200 raised in mouse (1:1000; Sigma N0142), anti-TROMA1 raised in rat (1:50; University of Iowa DSHB), anti-CGRP raised in rabbit (1:1000; Penninsula Labs 4032), anti-TRPV1 raised in rabbit (1:1000; Alomone ACC-030), anti-NK1r raised in rabbit (1:2500; Sigma SAB4502913) anti-calretinin raised in mouse (1:1000; Swant 6B3), anti-Calbindin D-28K raised in mouse (1:1000; Swant 300), anti-Pax2 raised in Goat (1:200; R&D Systems AF3364), anti-c-Fos raised in rabbit (1:10,000; Millipore PC-38), anti-lectin (WGA) raised in rabbit (1:50,000; Sigma T4144), anti-EGFP raised in rabbit (1:1000, Life Technologies A6455), anti-VGAT raised in rabbit (1:2000, Millipore AB5062), anti-DsRed raised in rabbit (1:1000, Clontech 632496) and anti-NeuN raised in mouse (1:1000; Chemicon MAB377). We also used Isolectin B4-conjugated to Alexa-488 (1:200; Life Technologies I21411).

In Situ Hybridization

Spinal cord tissue was dissected, quickly frozen in isopentane at -30°C and then cut into 30 μm cryostat sections onto slides. Slides were stored at -80°C. Two-color fluorescent *in situ* hybridization followed the protocol of Ishii *et al.* with slight modification (Ishii et al., 2004). Slices were placed in cold 4% PFA for 20 minutes, washed at RT in PBS, incubated in 0.3% H2O2 for 30 minutes at RT, washed in PBS

and then incubated with proteinase K (10 µg/ml) for 5 minutes at 37°C, washed with PBS, and then incubated in 4% PFA at RT for 10 minutes, washed in PBS and then de-acetylated for 10' at RT, washed and then dehydrated the samples in graded series of ethanol (60-100%). Slices were then incubated in prehybridization solution (50% formamide, 10 mM Tris pH8.0, yeast tRNA, 10% dextran sulfate, 1x Denhardt's solution, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, pH 8.0) at 63°C for 30 minutes. Probes were diluted in hybridization solution (100-200 ng/ml), heated to 85°C for 5', and then added to slides. Slices were incubated overnight at 63°C. Slices were washed for 30 minutes at 63°C in 2x standard saline citrate (SSC) + 50% formamide and then incubated in RNase A in buffer at 37°C for 30 minutes. Slices were then washed in a series of decreasing concentrations of SSC (2x, 0.2x, 0.1x) for 20 minutes at 65°C, blocked with Roche blocking buffer (0.5%) for 1 hour and then incubated with sheep anti-dig-AP (Roche) and rabbit anti-flu-POD (Roche) antibodies diluted in blocking buffer overnight at 4°C. Slides were washed with Tris-tween buffer and then incubated in with ABC tyramide solution (Perkin Elmer) for 10 minutes, washed and then incubated with streptavidin-conjugated to Alexa-488 at RT for one hour and incubated with HNPP/Fast Red (Roche) for 30 minutes. Slides were washed and cover-slipped with Fluoromount-G.

Fragments of genes encoding CCK, tomato, VGLUT2 and GAD67 were amplified by PCR from brain cDNA using primers taken from the Allan Brain Atlas. Each was subcloned into pBSK or pCRII (Invitrogen) and sequenced. Plasmids were linearized prior to probe synthesis. Probes were synthesized using DIG and Flu labeling kits (Roche).

Cell counting

Spinal cord sections were imaged with a Nikon A1R confocal laser-scanning microscope and Nikon Elements software using 405-, 488-, 561- and 640 nm excitation laser light. In order to suppress emission crosstalk, the microscope was configured to perform all scanning in sequential mode. Z-series were scanned at 20x magnification with an oil immersion lens and a z-step of 0.89 µm. Cells positive for tomato and/or PKCγ were scanned from three independent slices and averaged for each mouse (n = 3 per group). For cell counting, a 400 x 250 µm window was positioned so that its medial axis was perpendicular to the dorsal horn border with its medial end at the border between white and grey matter. Immunoreactive profiles were counted separately using the Fiji ImageJ 1.47 program (http://rsbweb.nih.gov/ij) cell counter plug-in. A cell was counted as immunopositive if (1) immunostaining of a single cell was clearly defined, (2) its level was at least two times that of the background levels, and (3) it had disappeared at both the top and bottom surfaces of z-stack as previously described (Peirs et al., 2014).

For c-Fos analysis, 5 slices from 1-3 animals with the highest viral expression were imaged with a z-step of 5 µm by an experimenter blind to the respective animal group. Lamina boundaries were traced based on Rexed lamination visible by NeuN staining using Fiji-ImageJ. Each image was then analyzed using only the channel corresponding to the c-Fos staining. Acquisition and analysis parameters were strictly identical during the entire experiment.

Electrophysiological recordings

Young-adult mice (P25 – P35) were deeply anesthetized with 100 mg/kg ketamine and 20 mg/kg xylazine. The spinal cord was quickly removed and transferred into an ice-cold (4°C) solution containing (in mM): 220 sucrose, 2.0 KCl, 7.0 MgCl2, 26 NaHCO3, 1.15 NaH2PO4, 11 D-glucose, and 0.5 CaCl2, (pH 7.4) bubbled with 95% O2 and 5% CO2. After removing the dura mater and ventral roots, the spinal cord was embedded into 3% low-melting agarose (Fisher Scientific BP165-25) and serial transverse slices (350 μm) were cut from the lumbar spinal cord with the dorsal roots and DRGS still attached using a vibroslice (Leica VT1000). Slices were incubated at 37^{o}C in an artificial cerebrospinal fluid (aCSF) containing (in mM): 118 NaCl, 3 KCl, 2.5 CaCl2, 1.5 MgSO4, 0.6 NaH2PO4, 25 NaHCO3, 10 glucose (290-300 mOsm), pH 7.4, bubbled with 95% O2 and 5% CO2, for a 60 min recovery period. For recordings of lamina I projection neurons, slices were incubated with aCSF containing 20 nM SP-TMR (SX 5800 Enzo Life Science) at room temperature for 20 min. After a 30 min period recovery, slices were transferred to a recording chamber (volume ≈1 ml) and held down with a pewter wire. The chamber was mounted on an upright microscope fitted with fluorescence optics (Carl Zeiss Axioskop 2) and linked to a Hamamatsu digital camera ORCA-R2. Slices were continuously perfused at 3.0 ml/min with the aCSF solution maintained at room temperature (≈ 25°C). Neurons were considered to be in lamina I within a 50 μm longitudinal area between the white matter border and the lamina II that has a distinct translucent appearance and can be easily distinguished under the microscope using a X10 objective lens. Positive fluorescent neurons were visualized using a X40 water-immersion objective lens and a Cy3 emission filter set under a 541-569 nm emission light delivered by a DG4 (Sutter Instruments) source.

Neurons were subsequently visualized using combined infrared and differential interference.

Patch pipettes were pulled from borosilicate glass tubing (Sutter Instrument) and filled with an internal solution containing (in mM): 135 K-gluconate, 4 NaCl, 2 MgCl2, 10 HEPES, 0.2 EGTA, 2.5 ATP-Na2, 0.5 GTP-Na, Dextran Alexa-488 (0.01%) (290 mOsm), pH 7.4. Pipette resistances ranged from 5–7 MΩ.

Whole-cell patch clamp recordings were acquired using an AxoPatch 200B amplifier and a Digidata 1440 digitizer (Axon laboratory) and pClamp 10.3 acquisition software. Voltage clamp data were low pass filtered at 5 kHz and digitized at 10 kHz. Junction potentials were corrected before gigaseal formation. Series resistance was monitored throughout the experiments and was not compensated. Data were discarded if series resistance varied more than ±20 MΩ. Voltage clamp data were recorded at a holding potential of -65 mV.

Dorsal roots were stimulated using a suction electrode at 25, 100 and 500 μA to activate Aβ or Aβ + Aδ or Aβ + Aδ + C fibers respectively at the low intensity of 0.05 Hz (duration 0.1 ms) using a A365 stimulus isolator (World Precision Instrument) as previously described (Torsney and MacDermott, 2006). At this intensity, both monosynaptic and polysynaptic EPSCs were observed. To address the monosynaptic nature of the response, the root was systematically stimulated five times at 0.05Hz, 1 Hz, 2Hz and 20 Hz. EPSCs were considered monosynaptic in an absence of synaptic failure or latency >2ms following a 20 Hz for Aβ fiber intensity stimulation, 2 Hz for Aδ fiber intensity stimulation and 1 Hz for C-fiber intensity stimulation as previously

described (Torsney and MacDermott, 2006). Conduction velocity was systematically measured for each root stimulation.

To address the effect of spinal disinhibition on evoked EPSCs recorded from lamina I $NK1^+$ neurons, 10 μM bicuculline and 300 nM strychnine were applied by perfusion application. Five minutes after spinal disinhibition, the root was stimulated and EPSCs recorded as described above.

To quantify the effect of spinal disinhibition on evoked EPSCs in lamina I $NK1^+$ neurons, area under the curve was measured from recordings in response to A-fiber stimulation at a 0.05 Hz frequency. Traces were filtered using a low pass Bessel filter at 1 kHz and polysynaptic EPSCs were quantified from the stimulation artifact to the end of the trace (≈ 20 s) from each trace and averaged. All data were analyzed offline using Clampfit 10.3 software (Axon instrument). Data are presented as the mean + SEM.

Neuronal morphology

Slice containing $VGLUT3^{Cre}$ neurons filled with 0.2% biocytin (Sigma Aldrich) for at least 20 minutes were used for neuron reconstruction. Tracer was revealed using Alexa488-conjugated streptavidin. Confocal images were taken with a 0.5 μm z-step and subsequently analyzed using Fiji-ImageJ. Single neurons were semi-automatically reconstructed using non-collapsed z-stacks imported in the simple neurite tracer plug-in as previously described (Alba-Delgado et al., 2015).

SUPPLEMENTAL REFERENCES

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