

**Supplemental information**

**c-Maf-positive spinal cord neurons  
are critical elements of a dorsal horn circuit  
for mechanical hypersensitivity in neuropathy**

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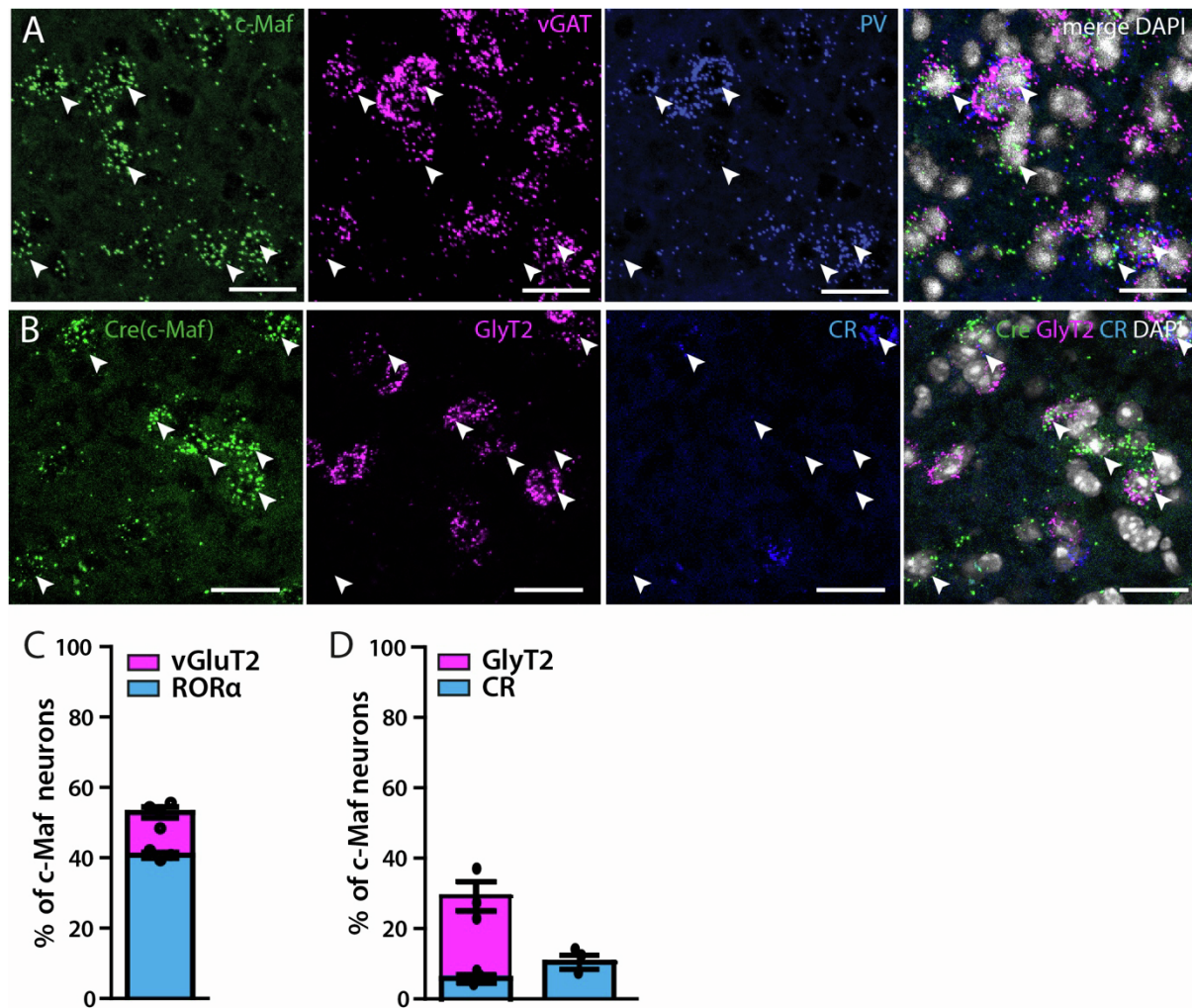
## Supplemental information

**Table S1. (related to Fig. 4-6, S6 and S8): ANOVA results and P values for behavioral analysis**

Fig.	mouse line	Viral transgene	test	n (TG)	n (control)	ANOVA
4B	c-Maf <sup>fEX</sup>	hM4Di	von Frey	9	9	F(4,64)=3.499; P=0.012
4C			Hargreaves			F(2.32,37.18)=0.183; P=0.86
4D			Cold			F(4,64)=1.284; P=0.286
4E			Pin prick			F(4,64)=3.189; P=0.025
4F			Brush			F(4,64)=2.467; P=0.054
4G			Rotarod			<i>t test</i> : BL: P=0.768; post CNO: P=0.094
4H	c-Maf <sup>fEX</sup>	hM3Dq	von Frey	8	5	F(1.47,44)=6.023; P=0.017
4I			Hargreaves			F(4,32)=0.943; P=0.452
4J			Cold			F(4,44)=9.318; P≤0.000
4K			Pin prick			F(1.87,20.565)=0.189; P=0.815
4L			Brush			F(4,44)=0.551; P=0.699
4M			Biting/liking	8	8	<i>t test</i> : P = 0.0032
4M			Flinching	8	8	<i>t test</i> : P = 0.0157
5C	c-Maf <sup>fEX</sup>	hM4Di+CCI	von Frey	8	7	F(5,65)=5.194; P≤0.000
5D			Pin prick			F(5,65)=4.982; P=0.001
5E			Brush			F(5,65)=0.554; P=0.735
5F	c-Maf <sup>fIN</sup>	Hm3Dq+CCI	von Frey	8	8	F(2.6,36.5)= 1.28; P=0.292
5G			Pin prick			F(5,70)= 3.98; P=0.004
5H			Brush			F(5,70)= 2.5; P=0.038
6F	PV <sup>IN</sup>	iDTR	von Frey	12	16	F(8,208)=26.469; P<0.000
6G			Hargreaves	11	16	F(8,200)=0.303; P=0.964
6H			Cold	12	16	F(8,208)=2.274; P=0.024
6I			Pin prick	5	4	F(8,56)=1.557; P=0.159
6J			Brush	12	16	F(4.47, 116.2)= 1.274; P=0.282
6K			Biting/liking	8	11	<i>t test</i> : P = 0.0014
6K			Flinching	8	11	<i>t test</i> : P < 0.0001
S6B	c-Maf <sup>fIN</sup>	hM4Di	von Frey	5	5	F(4,32)=5.266; P=0.002
S6C			Hargreaves			F(4,32)=0.772; P=0.551
S6D			Cold			F(4,32)=0.508; P=0.730
S6E			Pin prick			F(4,32)=0.277; P=0.891
S6F			Brush			F(4,32)=0.294; P=0.880
S6G	c-Maf <sup>fIN</sup>	hM3Dq	von Frey	5	8	F(2.43,28.8)=0.812; P=0.510
S6h			Hargreaves			F(4,44)=2.55; P=0.053

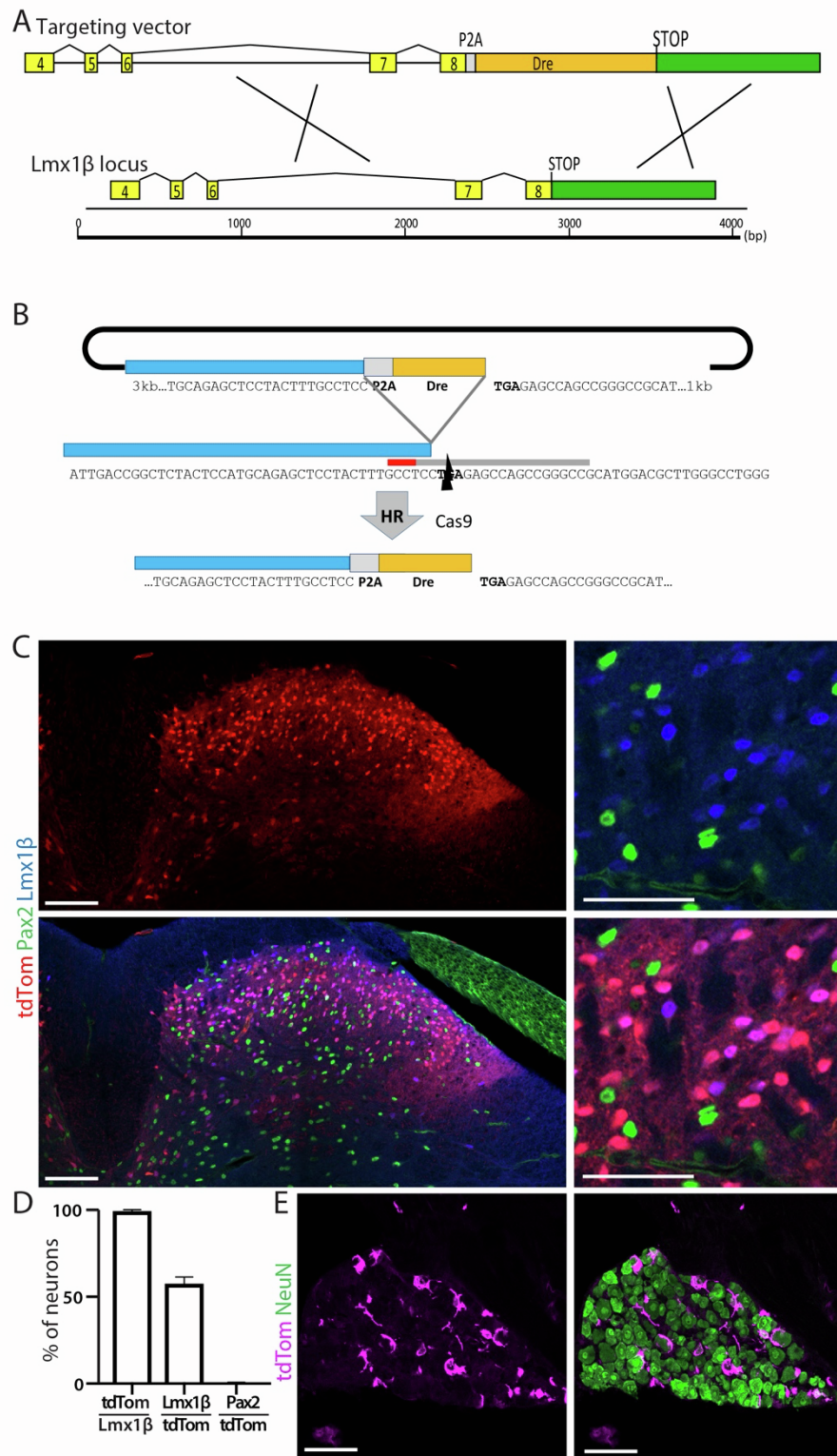
S6I			Cold			F(2.26,24.9)=1.904; P=0.166
S6J			Pin prick			F(4,44)=8.20; P≤0.001
S6K			Brush			F(4,44)=3.21; P=0.04
S8C	c-Maf <sup>EX</sup>	hM4Di+	von Frey	6	6	F(5,65)=0.762; P=0.581
S8D		ZymosanA	Pin prick			F(0.486,2.243)=2.38; P=0.105
S8E			Brush			F(5,65)=0.271; P=0.927

## Supplemental figures



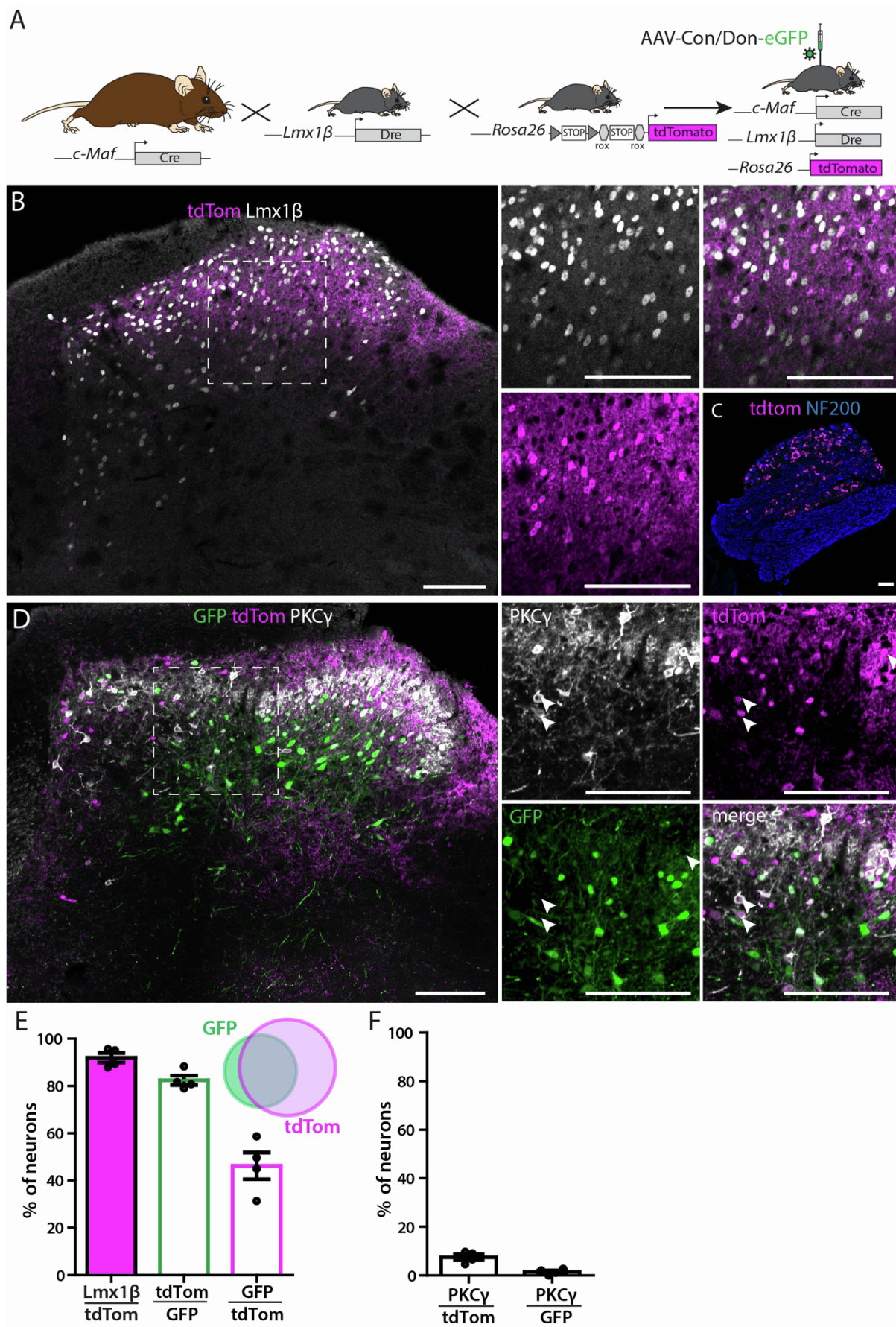
**Fig. S1 (related to Fig. 1): ISH showing the proportion c-Maf<sup>+</sup> neurons expressing other markers of deep dorsal horn neurons. A. Triple ISH showing overlap between c-Maf<sup>flN</sup>, vGAT- and PV-expressing neurons. c-Maf<sup>flN</sup> neurons represent about a third ( $31.28 \pm 1.6\%$ ) of all c-Maf neurons and they overlap with inhibitory PV<sup>+</sup> interneurons ( $12.75\% \pm 0.6\%$  of all c-Maf neurons are positive for vGAT, i.e. half of the c-Maf<sup>+</sup>PV<sup>+</sup> neurons). B. Triple ISH showing overlap of Cre (c-Maf)-, GlyT2- and CR-expressing neurons. C. Quantification of the overlap between Cre (c-Maf)-, vGluT2- and RORα -expressing neurons (ISH,  $n = 3$  c-Maf<sup>Cre</sup> mice; 302 Cre<sup>+</sup> neurons). D. Quantification of (C) ( $n = 3$  c-Maf<sup>Cre</sup> mice; 302 Cre<sup>+</sup> neurons). E. Quantification of the number of c-Maf neurons that are positive for both CCK and PV or both CCK and RORα ( $n = 4$ ; 878 neurons, and  $n = 3$ ; 317 neurons respectively). Arrowheads: examples of Cre (c-Maf) positive neurons. Error bars:  $\pm$  SEM. Scale bars: A: 100 μm, B-C: 20 μm.**





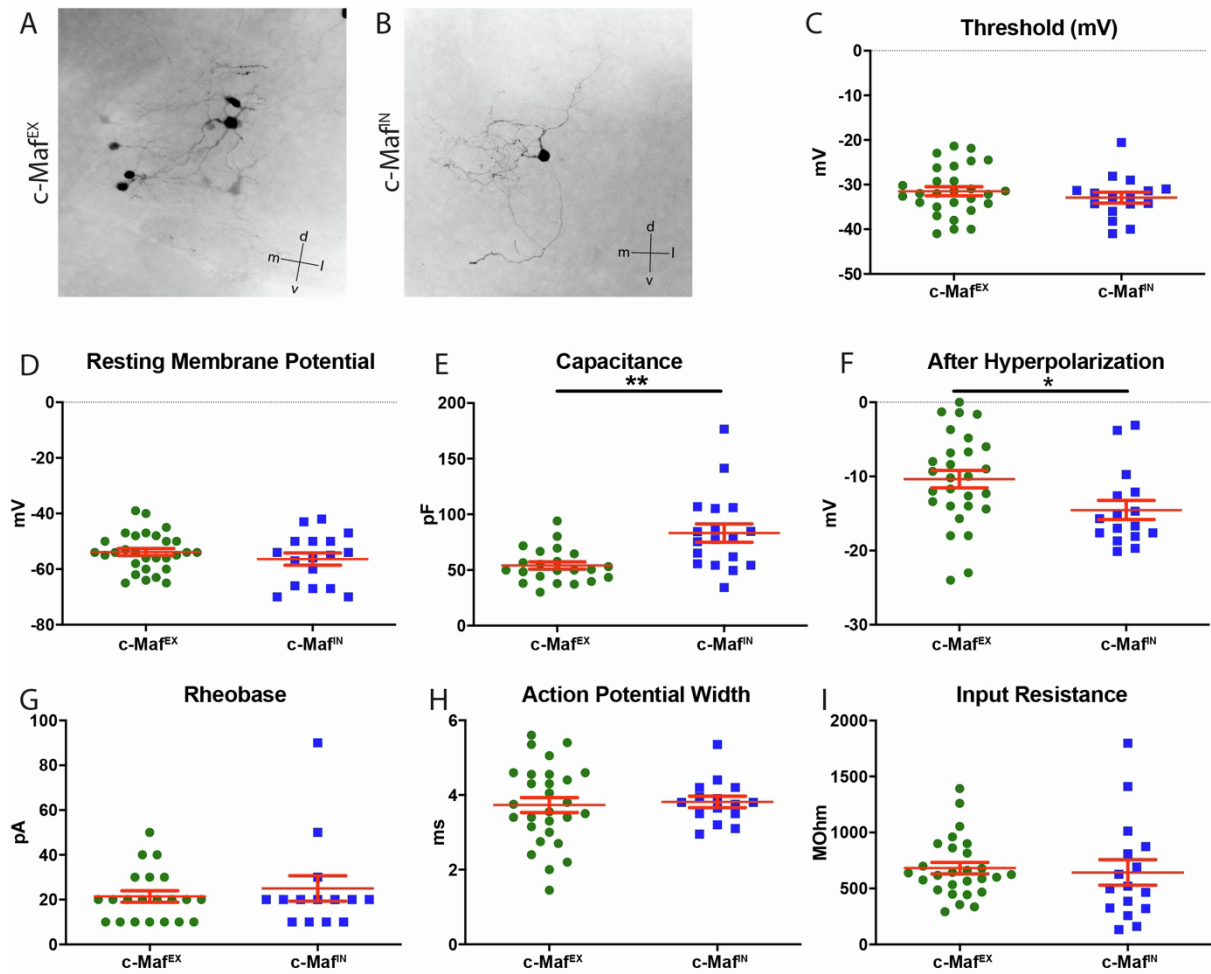
**Fig. S2 (related to Fig. 2): Generation of the *Lmx1b*<sup>Dre</sup> mouse line.** **A.** Schematic representation of the generation of the *Lmx1b*<sup>Dre</sup> allele. A *P2A-Dre* coding sequence was inserted into the STOP codon of the *Lmx1b* gene. **B.** The *Lmx1b* sequence proximal to the STOP codon was scanned using the CRISPOR software (<http://crispor.tefor.net/>) for the presence of optimal sgRNA target sequences. The target sequence attgtaggagaagactcaagagg

residing on the opposite strand and encompassing the *Lmx1b* STOP codon (in bold) was selected and the sgRNA targeting the sequence was transcribed *in vitro* using T7 polymerase (NEB) from a gBlock (IDT) linear DNA template containing the T7 promoter, the Cas9 target sequence and the sgRNA F+E backbone <sup>[S1]</sup>. An applicable targeting vector was designed to insert the P2A-Dre cassette just upstream of the *Lmx1b* STOP codon with the help of 2.6kb 5' and 1kb 3' homology arms. **C.** IHC showing the overlap between tdTomato, Pax2 and Lmx1b in Lmx1b<sup>Dre</sup> mice crossed with tdTomato (Rosa26<sup>roxStopTom/wt</sup>) reporter mice. **D.** Quantification of the overlap between tdTomato, Lmx1b and Pax2 in (C) **E.** IHC showing no expression of tdTomato in sensory neurons in Lmx1b<sup>Dre</sup> mice crossed with tdTomato (Rosa26<sup>roxStopTom/wt</sup>) reporter mice. tdTomato expression in DRGs is due to recombination in satellite glia. Error bars:  $\pm$  SEM. Scale bars: B: 200  $\mu$ m, C: 20  $\mu$ m, E: 100  $\mu$ m .



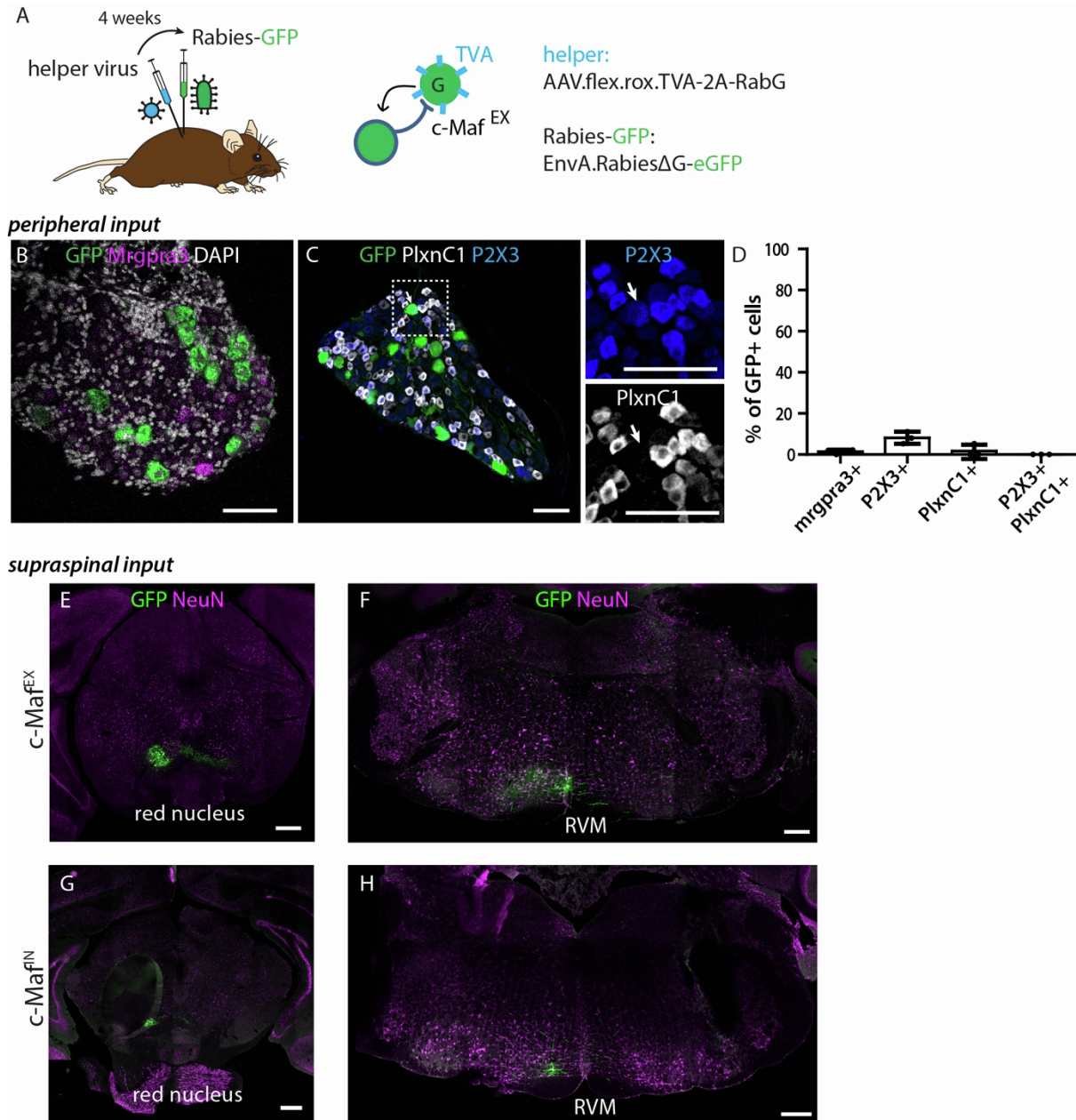


**Fig. S3 (related to Fig. 2): Characterization of c-Maf<sup>Cre</sup>; Lmx1b<sup>Dre</sup>; Rosa26<sup>dstdT<sub>Tom</sub>/wt</sup> (c-Maf<sup>EX</sup>; Rosa26<sup>dstdT<sub>Tom</sub>/wt</sup> mice).** **A.** Crossing of c-Maf<sup>Cre</sup> mice to Lmx1b<sup>Dre</sup> mice, followed by crossing of the double transgenic line to tdTomato (Rosa26<sup>dstdT<sub>Tom</sub>/wt</sup>) reporter mice, and intraspinal injection of rAAV9.CAG.C<sub>on</sub>/D<sub>on</sub>.eGFP. **B.** Immunofluorescence staining on a transverse section of lumbar spinal cord of c-Maf<sup>EX</sup>; Rosa26<sup>dstdT<sub>Tom</sub>/wt</sup> *reporter* mice, showing the overlap between tdTom<sup>+</sup> and Lmx1b<sup>+</sup> neurons. The general localization of tdTom<sup>+</sup> neurons in adult mice spinal cord was very similar to that of eGFP<sup>+</sup> neurons in c-Maf<sup>EX</sup> mice after viral injection. **C.** Immunofluorescence staining of DRG sections in the same experiment, showing no expression of tdTomato in sensory neurons (n = 4 mice). **D.** Immunofluorescence staining on a transverse section of lumbar spinal cord of c-Maf<sup>EX</sup>; Rosa26<sup>dstdT<sub>Tom</sub>/wt</sup> *reporter* mice injected with rAAV9.CAG.C<sub>on</sub>/D<sub>on</sub>.eGFP showing the overlap between eGFP and tdTom and the location of labelled neurons relative to the PKC $\gamma$  plexus. **E.** Quantification of the number of tdTom<sup>+</sup> neurons positive for Lmx1b<sup>+</sup> in (B) (n = 4; 1523 neurons) and between eGFP<sup>+</sup> and tdTom<sup>+</sup> neurons (same samples as in Fig.3C; n = 4 mice; 1523 tdTom<sup>+</sup> and 853 eGFP<sup>+</sup> neurons). *The vast majority of tdTom<sup>+</sup> neurons expressed Lmx1b* ( $92.03 \pm 2.0$  % of tdTom<sup>+</sup> neurons). *82.45  $\pm$  2.03 % of eGFP<sup>+</sup> neurons expressed tdTom.* Conversely,  $46.20 \pm 5.7$ % of tdTom<sup>+</sup> neurons expressed eGFP. **F.** Quantification of the overlap between eGFP<sup>+</sup> and tdTom<sup>+</sup> neurons with PKC $\gamma$  in (D) (n = 4 mice, 571 eGFP<sup>+</sup> and 857 tdTom<sup>+</sup> cells). In contrast to eGFP, tdTom was expressed in a few PKC $\gamma$ <sup>+</sup> neurons ( $7.49 \pm 1.16$ %) that had transiently expressed Cre and Dre during development. Arrowheads: examples of PKC $\gamma$ <sup>+</sup> tdTom<sup>+</sup> neurons. Error bars:  $\pm$  SEM. Scale bars: 100  $\mu$ m.



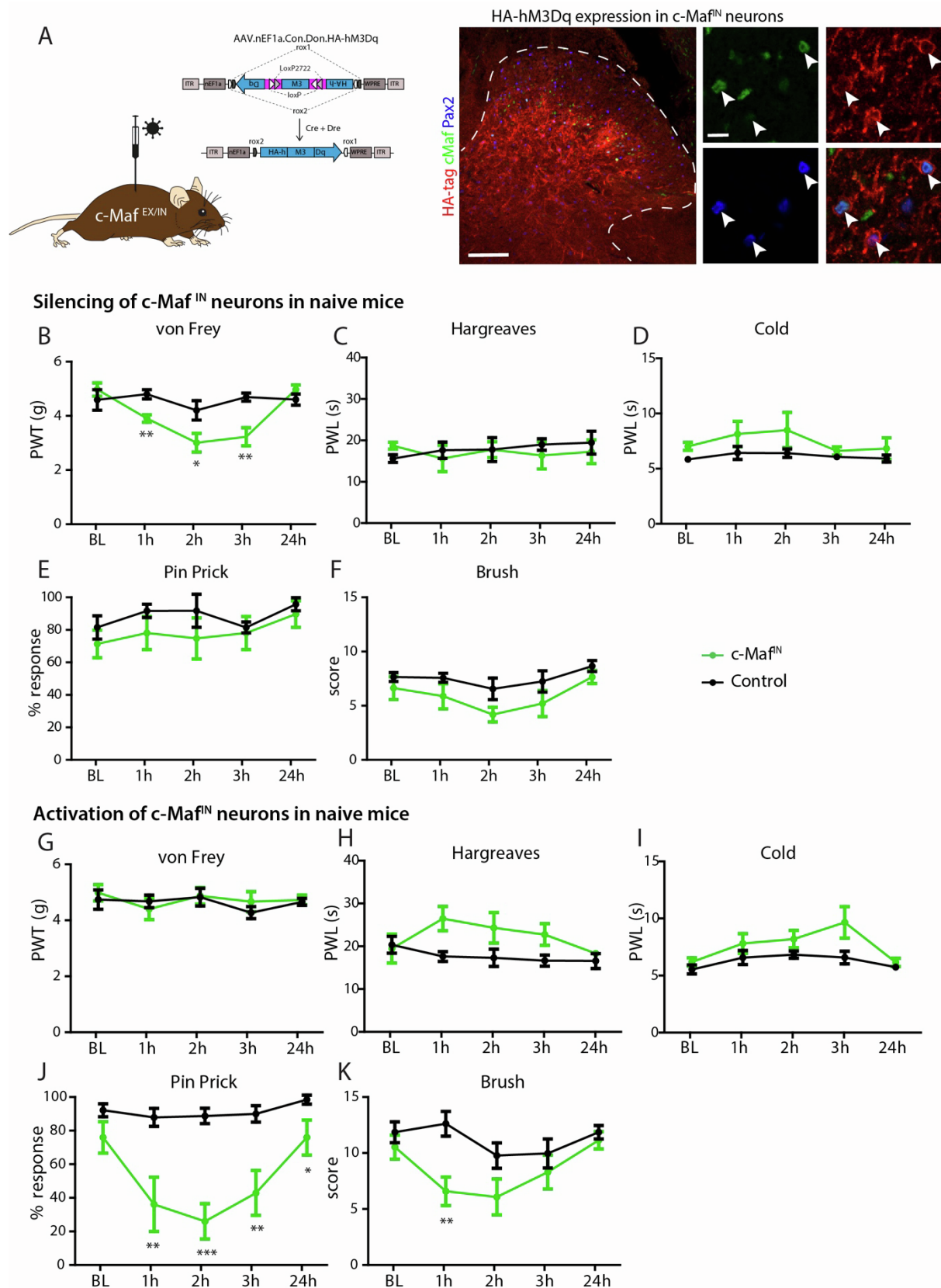
**Fig. S4 (related to Fig. 2): Morphological and biophysical characterization of c-Maf<sup>EX</sup> and c-Maf<sup>fN</sup> neurons.**

**A, B.** Neuronal morphology of c-Maf neurons revealed by sparse virus mediated labelling. **C-I.** Biophysical characterization of c-Maf<sup>EX</sup> and c-Maf<sup>fN</sup> neurons. **C.** Threshold potential, c-Maf<sup>EX</sup> =  $-31.46 \pm 1.028$  mV (n = 28), c-Maf<sup>fN</sup> =  $-32.9 \pm 1.2$  mV (n = 16), unpaired t-test p = 0.4. **D.** Resting membrane potential, c-Maf<sup>EX</sup> =  $-53.9 \pm 1.3$  mV (n = 29), c-Maf<sup>fN</sup> =  $-56.4 \pm 2.2$  mV (n = 17), unpaired t-test p = 0.3. **E.** Capacitance, c-Maf<sup>EX</sup> =  $54 \pm 3.3$  pF (n = 22), c-Maf<sup>fN</sup> =  $83.2 \pm 8.2$  pF (n = 18), unpaired t-test p = 0.001. **F.** After hyperpolarization, c-Maf<sup>EX</sup> =  $-10.4 \pm 1.2$  mV (n = 28), c-Maf<sup>fN</sup> =  $-14.5 \pm 1.3$  mV (n = 16), unpaired t-test p = 0.03. **G.** Rheobase, c-Maf<sup>EX</sup> =  $21.4 \pm 2.5$  pA (n = 21), c-Maf<sup>fN</sup> =  $25.0 \pm 5.7$  pA (n = 14), unpaired t-test p = 0.5. **H.** Action potential width, c-Maf<sup>EX</sup> =  $3.7 \pm 0.2$  ms (n = 28), c-Maf<sup>fN</sup> =  $3.8 \pm 0.2$  ms (n = 15), unpaired t-test p = 0.8. **I.** Input resistance, c-Maf<sup>EX</sup> =  $681.9 \pm 51.3$  MOhm (n = 27), c-Maf<sup>fN</sup> =  $642.9 \pm 114.1$  MOhm (n = 16), unpaired t-test p = 0.7. Error bars:  $\pm$  SEM.



**Fig. S5 (related to Fig. 3): Retrogradely labelled sensory neurons after rabies virus-based monosynaptic tracing from c-Maf<sup>EX</sup> and c-Maf<sup>IN</sup> neurons.** **A.** A helper virus (TVA, RabG) was injected in the spinal cord of c-Maf<sup>EX</sup> mice, followed by injection of the EnvA-pseudotyped rabies virus (EnvA.RV.ΔG.eGFP). **B.** Multiplex *in situ* hybridization on DRG sections showing overlap between *eGFP* and *Mrgpra3*. **C.** IHC on DRG sections showing the overlap between *eGFP*, PlxnC1 and P2X3. Arrow points to a GFP<sup>+</sup>; P2X3<sup>+</sup>; PlxnC1<sup>-</sup> neuron. **D.** Quantification of the number of *eGFP*<sup>+</sup> DRG neurons positive for *Mrgpra3* (n = 3, 374 *eGFP*<sup>+</sup> neurons), P2X3 and PlxnC1 (n = 3 mice, 152 *eGFP*<sup>+</sup> neurons). P2X3<sup>+</sup>PlxnC1<sup>+</sup> neurons = NP1-2-3 populations [S2]. **E-H.** Immunofluorescence staining showing *eGFP* labelled neurons in supraspinal sites retrogradely traced from c-Maf<sup>EX</sup> (I-K) or c-Maf<sup>IN</sup> (L-N) neurons. Neurons

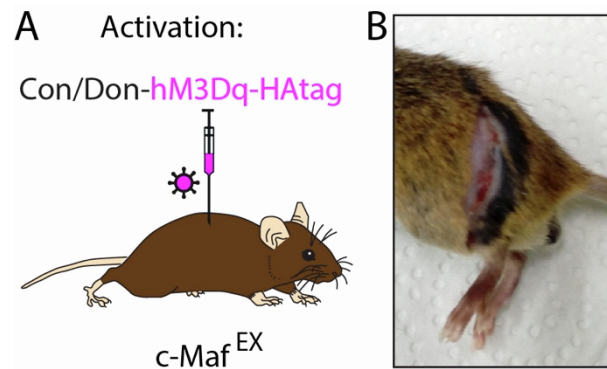
were found in the red nucleus (RN), and in the rostroventral medulla (RVM) ( $n = 4$ ). Error bars:  $\pm$  SEM. Scale bars: 30  $\mu\text{m}$  (B,C) and 100  $\mu\text{m}$  (E-H).



**Fig. S6 (related to Fig. 4): Pharmacogenetic silencing and activation of c-Maf<sup>flN</sup> spinal interneurons in naïve mice.** **A.** Schematic representation of the viral construct used for encoding of Cre-and-Dre dependent transgenes [53]. Representative image displays DREADD

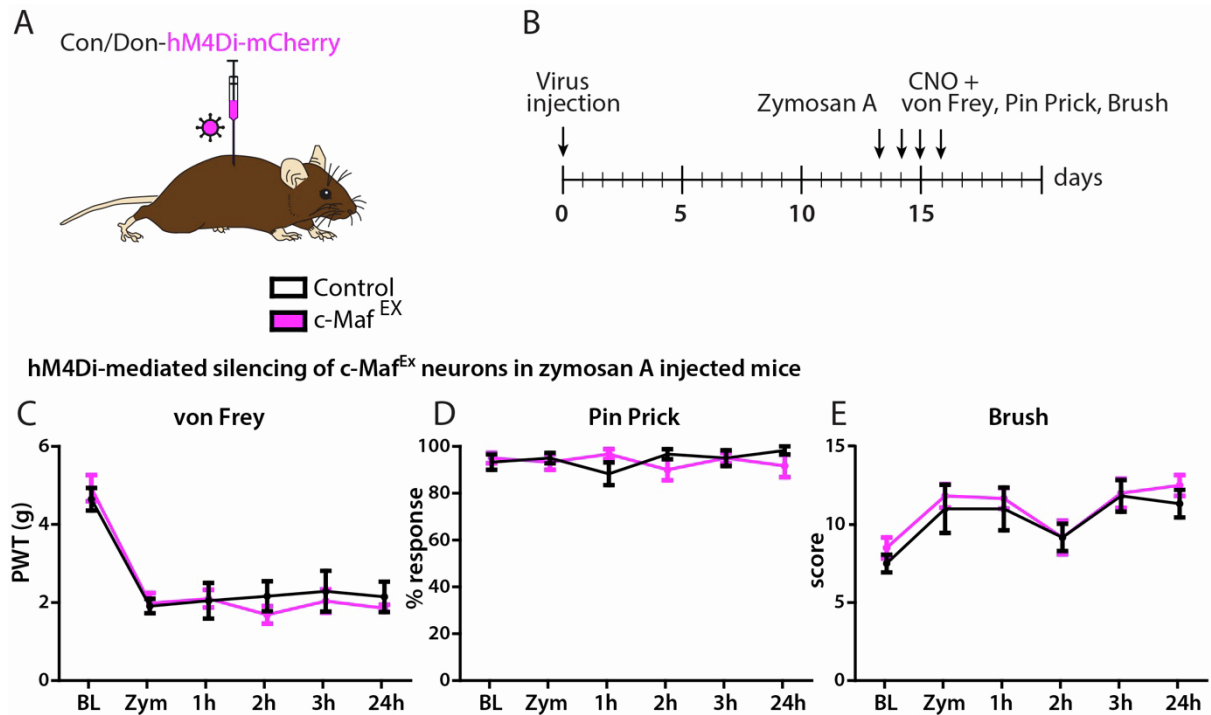


expression (HA-tag) driven by the AAV.EF1 $\alpha$ .C<sub>on</sub>/D<sub>on</sub>.HA-hM3Dq injected into the lumbar spinal cord of c-Maf<sup>f<sup>N</sup></sup> mice. **B-F.** Behavioral responses after hM4Di-mediated silencing of c-Maf<sup>f<sup>N</sup></sup> neurons (hM4Di: c-Maf<sup>f<sup>N</sup></sup>: n = 5; control: n = 5, Table S1). **G-K.** Behavioral responses after hM3Dq-mediated activation of c-Maf<sup>f<sup>N</sup></sup> neurons (hM3Dq: c-Maf<sup>EX</sup>: n = 5; control: n = 8; Table S1). Activation of c-Maf<sup>f<sup>N</sup></sup> neurons increased sensitivity to mechanical (Pin Prick and brush) stimulation. PWT: paw withdrawal threshold; PWL: paw withdrawal latency; BL: baseline (pre-CNO); 1h to 24h refers to time post CNO injection. Error bars:  $\pm$  SEM. Scale bars: 100  $\mu$ m (overview image) and 10  $\mu$ m (higher magnification images). Number of mice and statistics are shown in Table S1. In brief: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$  (ANOVA, followed by pairwise comparisons)



**Fig. S7 (related to Fig. 4): Skin lesion induced by scratching and biting of the left flank after repeated CNO injection on mice expressing hM3Dq in c-Maf<sup>EX</sup>.**

**A.** rAAV.EF1 $\alpha$ .C<sub>on</sub>/D<sub>on</sub>.hM3Dq was injected into the lumbar spinal cord of c-Maf<sup>EX</sup> and control mice. The mice received CNO injections from 14 days after virus injection. **B.** c-Maf<sup>EX</sup> mice receiving repeated CNO injections also developed skin lesions on the flank ipsilateral to the hM3Dq virus injection over the course of 8-15 days.



**Fig. S8 (related to Fig. 5): Pharmacogenetic Activation of c-Maf<sup>EX</sup> spinal interneurons in Zymosan A-induced inflammatory pain.** **A.** DREADD expression was driven by injection of rAAV.EF1 $\alpha$ .C<sub>on</sub>/D<sub>on</sub>.hM3Dq into the lumbar spinal cord of c-Maf<sup>EX</sup> and control mice. **B.** Virus injection was followed by intraplantar injection of zymosan A to induce inflammatory pain. **C-E:** Responses to mechanical stimulation using the von Frey (C), Pin prick (D) or light brush (E) tests before and after induction of inflammatory pain with zymosan A injection (c-Maf<sup>EX</sup>; n = 6; control: n = 6, Table 2). PWT: paw withdrawal threshold; BL: baseline before injury; Zym: BL 24 hours after intraplantar injection of zymosan A and before CNO injection; 1h to 24h refers to time post CNO injection. Error bars:  $\pm$  SEM. Number of mice and statistics are shown in Table 2.



subpopulation is excitatory ( $14.99 \pm 1.2\%$  express *vGluT2*). **D.** PV<sup>IN</sup> neurons were ablated by injection of a virus carrying a transgene for a Cre-and-Dre dependent iDTR (AAV1.EF1 $\alpha$ -flex-rox.iDTR(HB-EGF).hGH) followed by i.p. injection of DTX in GlyT2::Cre; Pvalb<sup>Dre</sup> double transgenic mice. **E.** IHC showing the ablation of PV<sup>+</sup> neurons in the dorsal horn. The majority of remaining PV<sup>+</sup> neurons are excitatory (Lmx1b<sup>+</sup>Pax2<sup>-</sup>, white arrows in bottom insets). **F.** Quantification of (E) in laminae I-II and III-IV of the dorsal horn (DH) (n = 3 control mice; 608 Pax2<sup>+</sup> neurons; n = 4 ablated mice; 679 Pax2<sup>+</sup> neurons). Error bars:  $\pm$  SEM. Scale bars: 100  $\mu$ m.

## References

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