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Supplemental Information

Differential Coding of Itch and Pain by a Subpopulation of Primary Afferent Neurons

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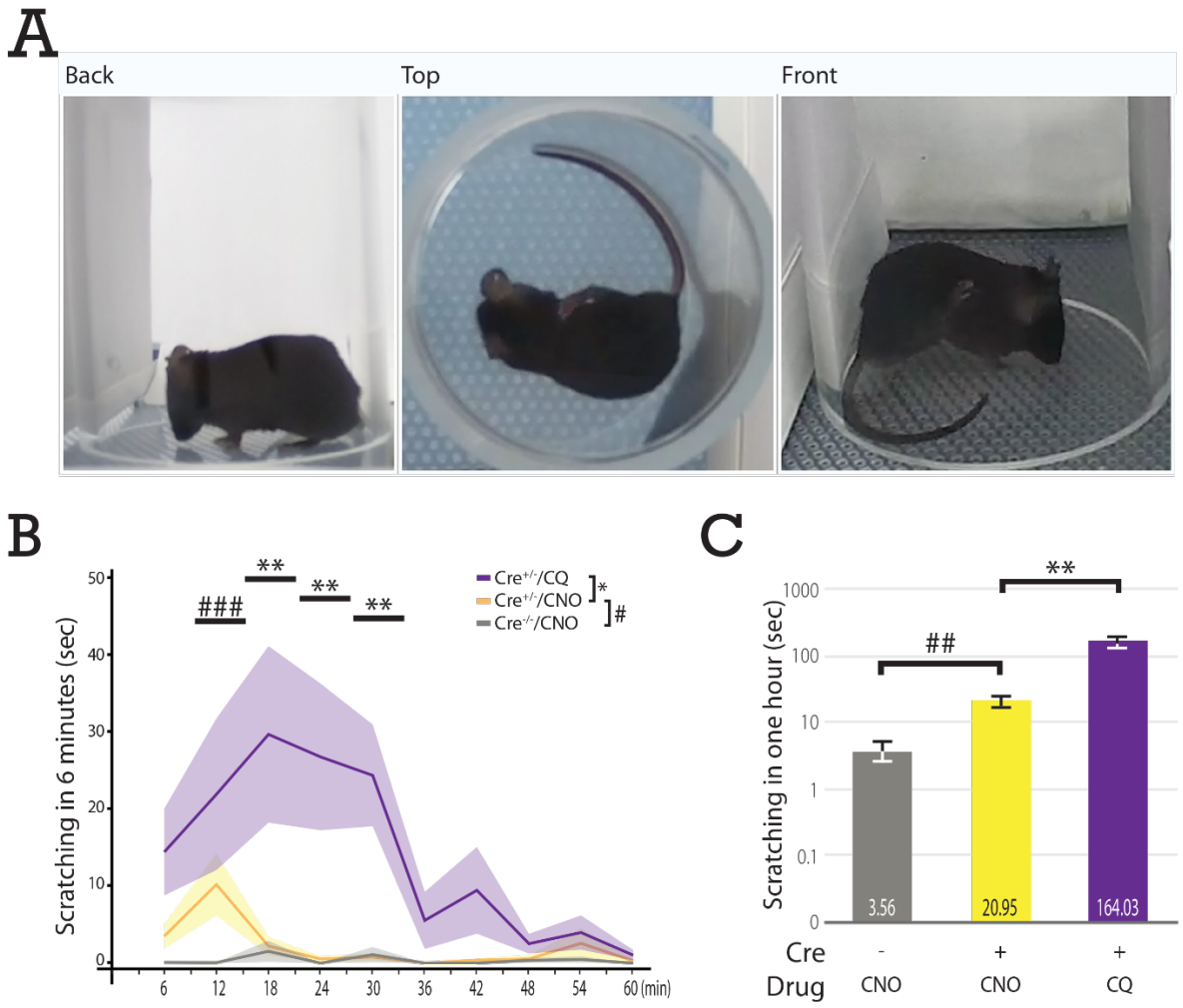
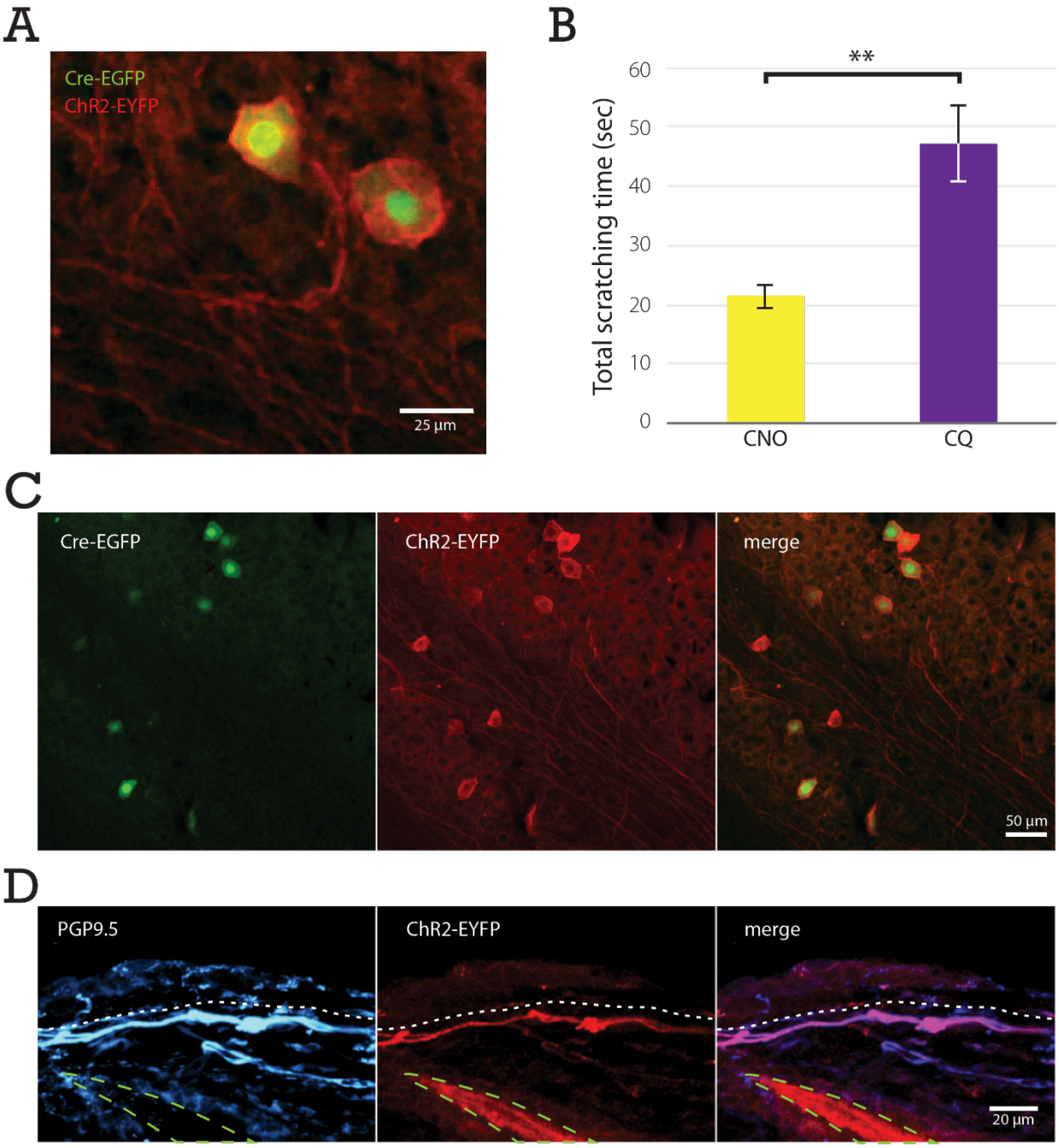


Supplemental Figure 1- Multi-angle frame-by-frame video annotation allows precise analysis of scratching behavior (related to Figure 1).

(A) Unilateral hindpaw scratching behavior can be observed and corroborated from different angles. The same mouse is shown from three different angles (Back, Top, and Front) during a scratching bout. The behavior is not detectable from the back angle camera while it can be detected in the two other angles.

(B) Timeline of time spent scratching demonstrates similar build-up and wind-down pattern of behavior in $Cre^{+/-}$/CNO and $Cre^{+/-}$/CQ groups but not in $Cre^{-/-}$/CNO, confirming induction of itch by CQ and by CNO in Cre-expressing AAV-transduced animals. Observation of sporadic and irregular scratching bouts in the $Cre^{-/-}$/CNO group, in comparison to $Cre^{+/-}$/CNO cage-/litter-mates, confirms the dependence of CNO on DREADD expression to induce itch. Similarity to figure 1e is due to individual scratching bout durations being comparable (Figure 1d). Two-way ANOVA, and Bonferroni post-hoc tests were used for comparison of Cre (#) or drug effects (*) over time. The one-hour period of observation (injection at time = 0) was divided into 10 equal 6-minute bins for statistical comparisons.

(C) DREADD-mediated metabotropic stimulation of MrgprA3 C-afferents evokes pruriception. Total time spent scratching over the 1-hour period following CNO injection indicates that scratching behavior is Cre-dependent (unpaired t-test, $p < 0.01$). Activation of endogenous MrgprA3 by CQ evokes a significantly higher intensity of pruriception (paired t-test, $p < 0.01$).



Supplemental Figure 2- Proper expression of heterologous actuators in trigeminal MrgprA3 C-afferents validates the cheek behavioral discrimination assay (related to Figure 2 and 3).

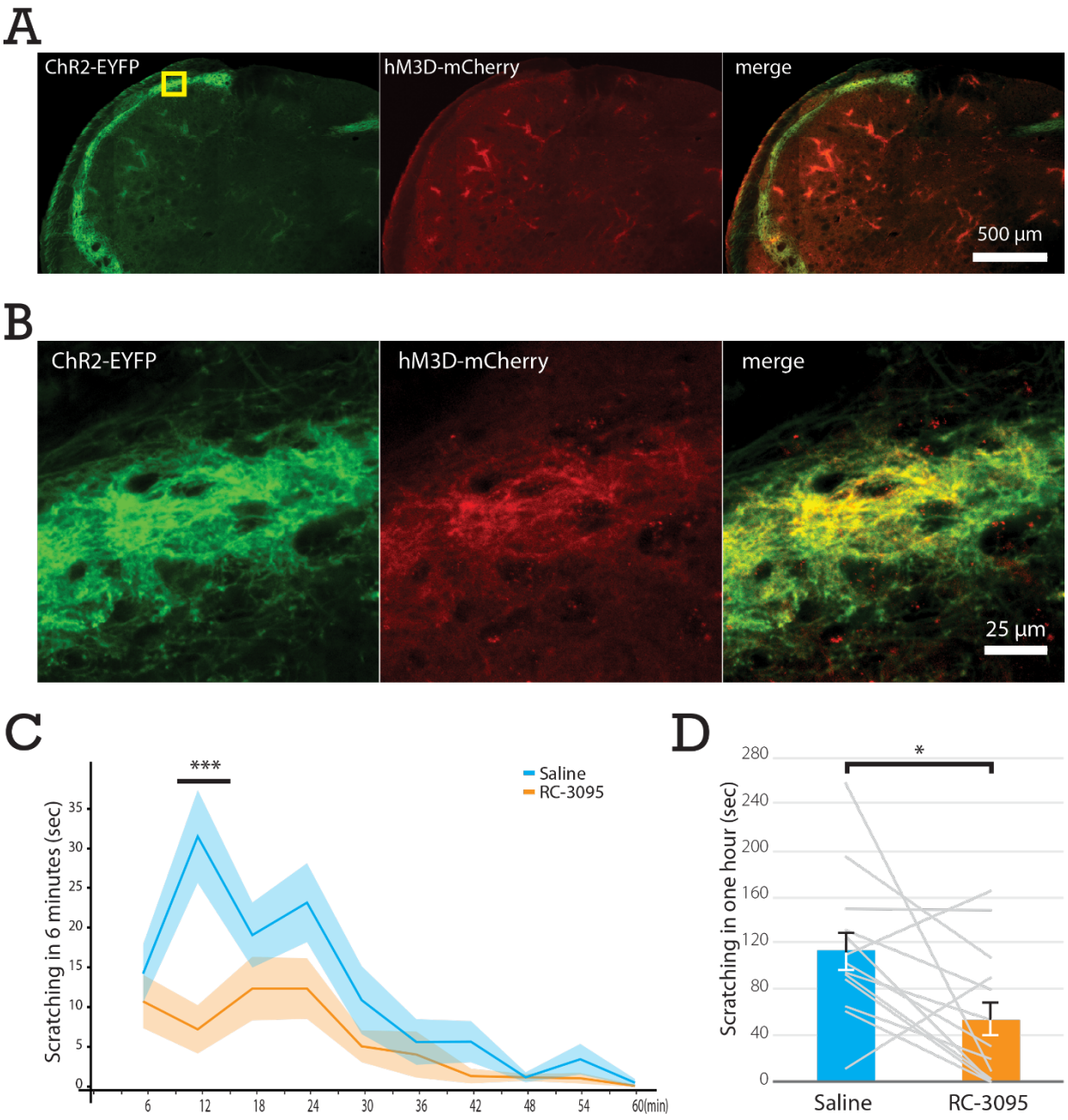
(A) Resolving EYFP and EGFP signals demonstrated by compartmentalization of Cre-EGFP mainly in the nucleus and ChR2-EYFP on the membrane of primary sensory neurons.

(B) CQ induces higher itch intensities than CNO in the cheek assay. Similar to the nape of the neck (Figure 1f), metabotropic activation of all MrgprA3 C-afferents through their endogenous GPCR induces more

total time spent scratching than activation of a subset of afferents through DREADDs (paired t-test, $p < 0.01$, n=6-7). These results are derived from the experiment represented in Figure 3.

(C) ChR2 is expressed in the trigeminal ganglia of MrgprA3$^{Cre\text{-}EGFP+/-}$:Rosa26$^{ChR2\text{-}EYFP+/-}$ animals in a Cre-dependent fashion. Similar to the cervical dorsal root ganglia (Figure 2a), membrane-bound ChR2 is expressed in Cre-expressing trigeminal afferents innervating the cheek hairy skin.

(D) ChR2-EYFP opsins are trafficked to the hairy skin of the cheek. Opsin expression in the cheek epidermis of MrgprA3$^{Cre\text{-}EGFP+/-}$:Rosa26$^{ChR2\text{-}EYFP+/-}$ mice is confirmed by the eYFP signal located in a subset of peripheral nerve endings (stained with PGP9.5). A hair shaft is indicated by dotted lines and the border between dermis and epidermis is indicated by dotted lines.



Supplemental Figure 3- MrgprA3 C-afferents innervate the substantia gelatinosa in spinal cord (related to Figure 4).

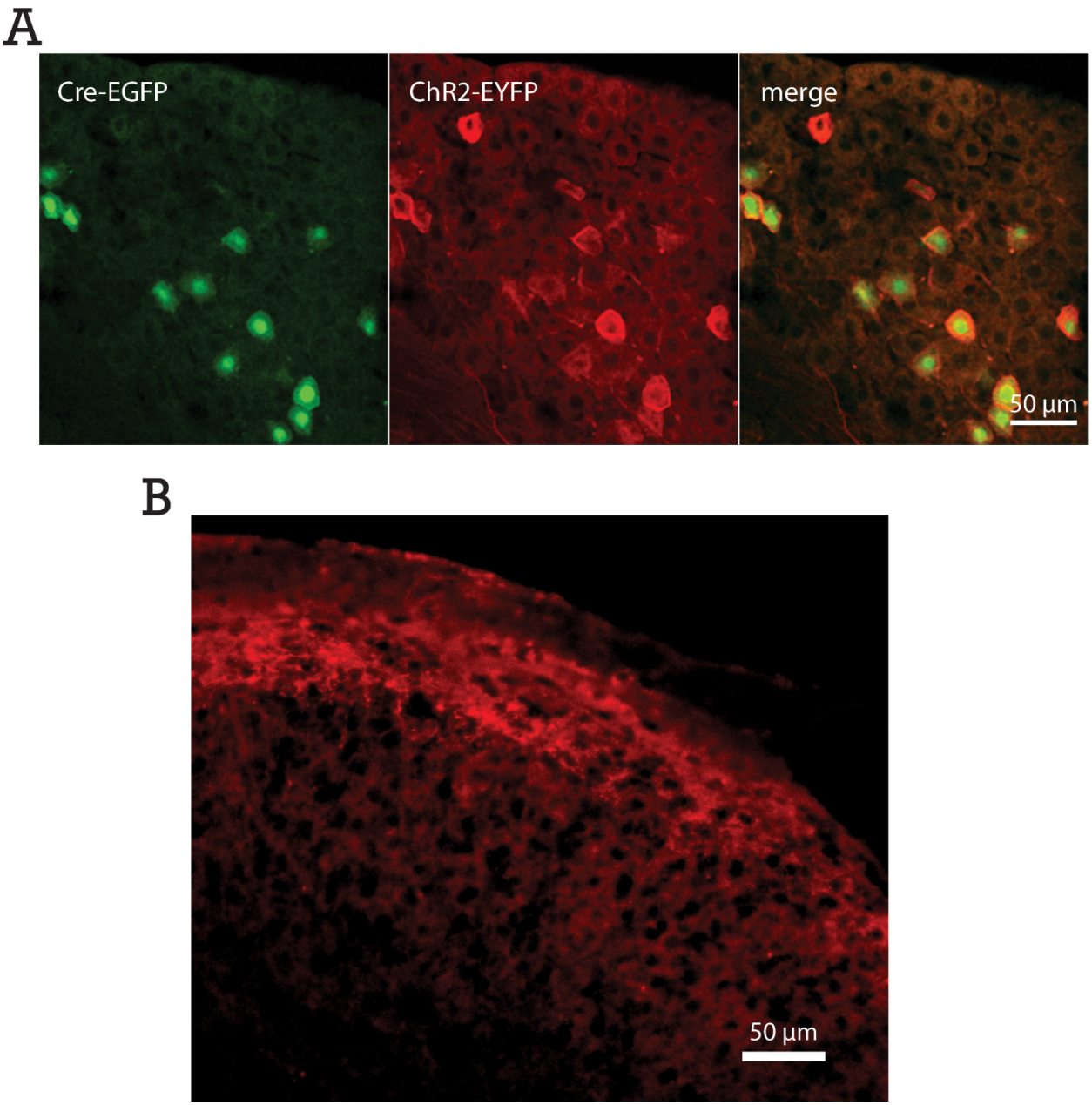
(A) Tiled coronal section of the medullary spinal cord from an AAV-transduced MrgprA3$^{Cre\text{-}EGFP+/-}$:Rosa26$^{ChR2\text{-}EYFP+/-}$ mouse shows termination of MrgprA3 afferents in lamina II.

(B) Zoomed view of the superficial laminae (yellow box in a) confirms expression of hM3Dq in EYFP-expressing terminals.

(C) i.c. administration of the GRPR blocker RC-3095 blunts CQ-induced itch. Timeline of time spent scratching, similar to the timeline of scratching bout counts (Figure 4c), indicates that GRPR blockade

alleviates pruriception more effectively at earlier time points. Average counts in 6-minute time bins are displayed with shades indicating SEMs. Two-way ANOVA test with Bonferroni post-tests was used for statistical comparison.

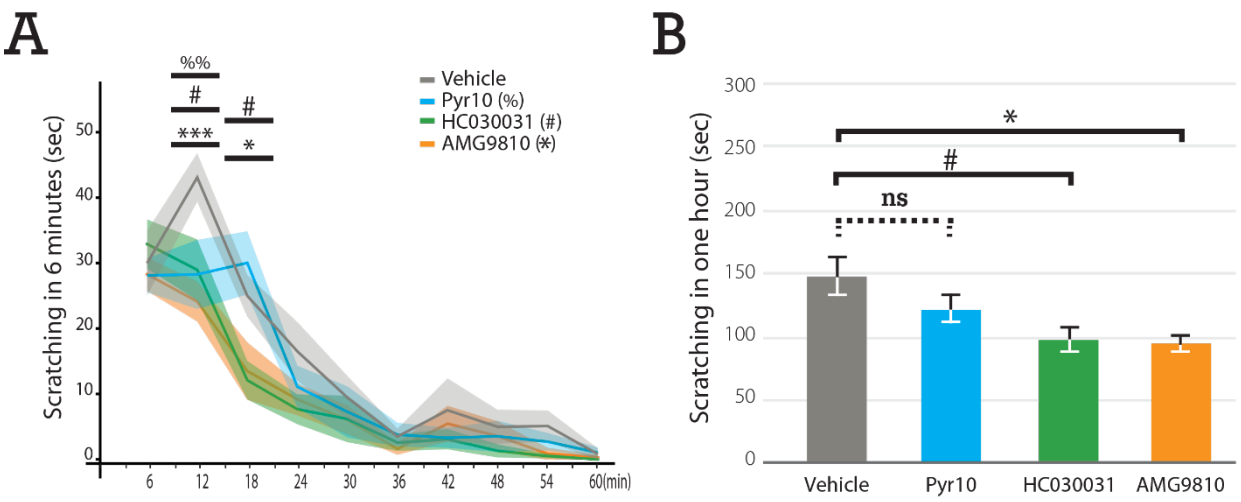
(D) Total time spent scratching also confirms the inhibitory effect of RC-3095 in the 1 hour post CQ injection in the nape of the neck ($p < 0.05$, paired t-test, n=13).



Supplemental Figure 4- ChR2 expression in MrgprA3 C-afferents innervating the hindpaw underlies light-evoked nociception by transdermal illumination of the plantar surface (related to Figure 5).

(A) MrgprA3$^{Cre\text{-}EGFP+/-}$:Rosa26$^{ChR2\text{-}EYFP+/-}$ mice express channelrhodopsin-2 in MrgprA3^{+} primary afferents in lumbar dorsal root ganglia.

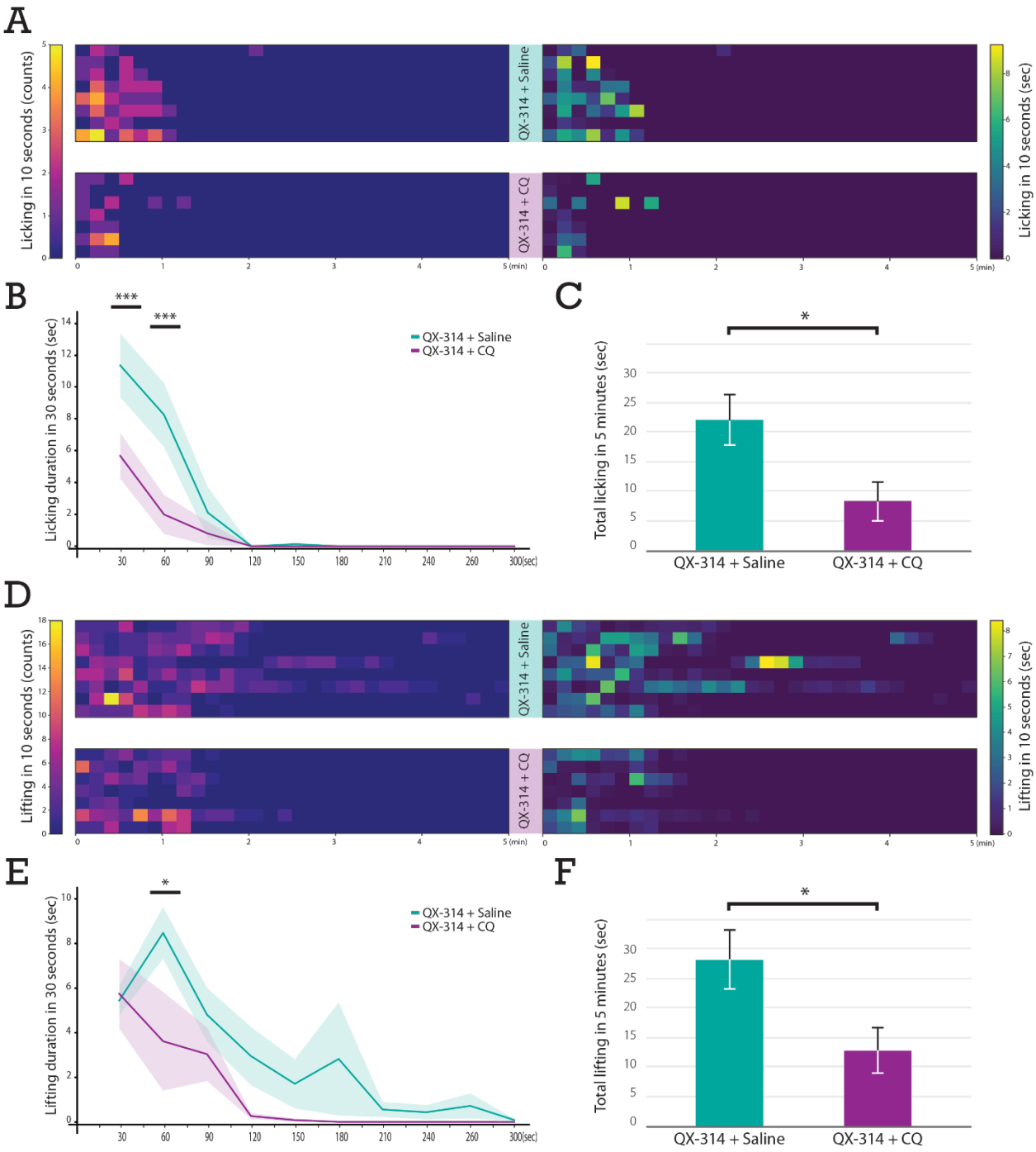
(B) The central terminals of transgenic ChR2-expressing EYFP-positive MrgprA3 C-afferents are located in the substantia gelatinosa in lumbar spinal cord.



Supplemental Figure 5- Pharmacological blockade of TRPC3, TRPA1, and TRPV1 channels reduces CQ-evoked itch mediated by MrgprA3 C-afferents (related to Figure 6).

(A) TRP channel blockers reduce CQ-induced pruriception by changing the kinetics of the scratching behavior. Similar to the timeline of scratching bout counts (Figure 6c), the timeline of time spent scratching shows that pharmacological blockade of the TRP channels reduces the itch behavior more effectively at earlier time points (Two-way ANOVA with Bonferroni post-hoc test, %, #, and * symbols depict significance levels for comparison of the vehicle vs. Pyr10, HC030031, or AMG9810 groups, respectively).

(B) Total durations of time spent scratching in the 1 hour period post CQ injection, showing a significant decrease of scratching intensity by TRPA1 and TRPV1 blockers (One-way ANOVA with Bonferroni post-Hoc test).



Supplemental Figure 6- Protective and site-directed nocifensive behaviors are reduced by specific silencing of MrgprA3 C-afferents after purinergic activation (related to Figure 7).

(A) Detailed time-course of licking behavior induced by intraplantar injection of αβmeATP (20 mM) in the hindpaw 30 minutes after conditioning with coinjection of QX-314 with saline or CQ (5 mM). Each line represents an individual wildtype C57Bl6 mouse (n = 7-8) and every block indicates the number of licking behaviors observed (left panel), or time spent behaving (right panel) in 10-second time bins, as defined by the heat maps.

(B) Timeline of the licking bouts observed, showing that QX-314-mediated silencing of CQ-responsive cells reduces this site-directed coping behavior after αβmeATP-evoked pain. Average durations of licking in 30-second time bins are displayed with shades indicating the SEM. Two-way ANOVA test with Bonferroni post-tests was used for statistical comparison ($p < 0.001$).

(C) Conditional silencing of CQ-responsive afferents reduces the total duration of purinergic licking behavior. Animals receiving QX-314 + CQ 30 minutes before αβmeATP spend significantly less time licking the site of injection in the 5 minutes period after algogen administration ($p < 0.05$, unpaired t-test).

(D-F) Similarly to licking behavior, details of lifting behavior are demonstrated in panels D, E, and F. In the time progression (E) the difference of lifting behavior in the QX-314 + CQ group is significantly less in the earlier time bins ($p < 0.05$) and this behavior is almost not observed after 2 minutes of algogen injection (D & E).