

1 **SARS-CoV-2 papain-like protease activates nociceptors to drive sneeze and pain**

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14 **Abstract**

15 SARS-CoV-2, the virus responsible for COVID-19, triggers symptoms such as sneezing, aches and
16 pain.¹ These symptoms are mediated by a subset of sensory neurons, known as nociceptors, that detect
17 noxious stimuli, densely innervate the airway epithelium, and interact with airway resident epithelial and
18 immune cells.²⁻⁶ However, the mechanisms by which viral infection activates these neurons to trigger
19 pain and airway reflexes are unknown. Here, we show that the coronavirus papain-like protease (PLpro)
20 directly activates airway-innervating trigeminal and vagal nociceptors in mice and human iPSC-derived
21 nociceptors. PLpro elicits sneezing and acute pain in mice and triggers the release of neuropeptide
22 calcitonin gene-related peptide (CGRP) from airway afferents. We find that PLpro-induced sneeze and
23 pain requires the host TRPA1 ion channel that has been previously demonstrated to mediate pain,
24 cough, and airway inflammation.⁷⁻⁹ Our findings are the first demonstration of a viral product that directly
25 activates sensory neurons to trigger pain and airway reflexes and highlight a new role for PLpro and
26 nociceptors in COVID-19.

27 **Main**

28 Common symptoms of viral infections including SARS-CoV-2, the virus responsible for COVID-19,
29 include sneezing, headache, and other aches and pain.^{1,10} Sneezing is a common host response to
30 SARS-CoV-2 infection that promotes transmission by generating and spreading airborne droplets that
31 contain the virus. Somatosensory and vagal sensory neurons drive protective reflexes, including
32 sneezing, and initiate pain sensations during injury or infection.^{5,11} Yet, little is known about how these
33 neurons are activated by SARS-CoV-2 infection to drive symptoms. As epithelial cells are infected,
34 they release cytokines and other inflammatory mediators that activate surrounding epithelial and
35 immune cells, as well as the sensory neurons that densely innervate the epithelia. Studies to date
36 have focused on endogenous inflammatory mediators released during viral infection that activate
37 sensory neurons.^{12–15} However, infection also generates viral proteins that may be released and serve
38 as direct host modulatory factors. Viral products are known to activate airway epithelial and immune
39 cells to drive inflammation, but whether viral molecules act directly on sensory neurons to drive pain
40 and airway reflexes is unknown.^{16,17} Among viral molecules, viral proteases have been of much
41 interest as therapeutic targets for COVID-19 due to their critical role in viral replication. While
42 proteases from plants, insects, bacteria, and immune cells have been demonstrated to activate
43 sensory neurons to trigger itch, pain and inflammation;^{18–27} viral proteases in this context have been
44 unexplored. Here, we show a new role for the SARS-CoV-2 papain-like protease (PLpro) in the
45 activation of airway-innervating sensory neurons.

46
47 PLpro plays an essential role in viral replication by processing viral polyproteins and suppressing the
48 host immune response of infected cells.^{28,29} Can active PLpro be released from infected cells and thus
49 be poised to activate surrounding uninfected cells? To test this, we infected human airway epithelial
50 cells with SARS-CoV-2 and measured PLpro activity in the supernatants of infected cells. We
51 detected PLpro activity 48 hours but not 24 hours following infection despite observing comparable
52 level of viral transcripts 24 and 48 hours post infection (Fig. 1a, b). This difference may reflect an
53 increase in protein translation and accumulation over time and/or the initiation of the mechanisms that
54 release PLpro, such as lytic cell death. We find that PLpro is released at nanomolar concentrations
55 from infected airway epithelial cells (Fig. 1c), and is therefore positioned to act on neighboring cells,
56 such as the free nerve endings of sensory neurons in the airways.

57
58 We next used functional imaging to test whether PLpro could activate the sensory neurons that
59 densely innervate the epithelial cells of the nasal mucosa and upper airways. We recorded calcium
60 responses in the trigeminal ganglion (TG) from mice expressing the calcium indicator (GCaMP6s) in
61 sensory neurons to intranasal perfusion of vehicle, PLpro (10 μ M), the TRPA1 channel agonist, allyl
62 isothiocyanate (AITC; 1 mM), and the TRPV1 channel agonist, capsaicin (100 μ M; Fig. 2a-c).

63 Strikingly, PLpro elicited rapid and robust calcium transients in 37.8% of recorded neurons (Fig. 2c-e).
64 Trigeminal sensory neurons are heterogenous and transduce a range of modalities such as pain, itch,
65 temperature, and touch. Thus, as expected, we observed both distinct and overlapping subsets of
66 these neurons that respond to intranasal perfusion of various compounds (Fig. 2b-d). Intranasal
67 perfusion of vehicle alone elicited a transient increase in calcium in a subset of recorded neurons
68 (36.4%; Fig. 2e). The subsequent perfusion of PLpro induced calcium transients in 28.6% of these
69 vehicle-sensitive neurons and recruited an additional 27.4% of neurons (Fig. 2d). Subsequent addition
70 of AITC and capsaicin activated 34.4% and 47.2% of neurons, respectively (Fig. 2e). Subsets of
71 sensory neurons that express the ion channels TRPV1 and TRPA1, known as nociceptors, trigger
72 pain and defensive reflexes in response to a variety of noxious stimuli, including mechanical stimuli,
73 chemical irritants, and inflammatory mediators. We found that PLpro (but not vehicle) activated 15.9%
74 of TRPV1-expressing and 18.4% of TRPA1-expressing neurons (defined by their capsaicin and AITC
75 responsiveness, respectively; Fig. 2f, g). In a separate control experiment, we perfused vehicle twice;
76 perfusion of this second vehicle activated only 2.7% of TRPV1-expressing and 9.1% of TRPA1-
77 expressing neurons (Fig. 2f, g). Taken together, these data demonstrate that PLpro activates a subset
78 of airway-innervating nociceptors in vivo.

79
80 Trigeminal nociceptors mediate sneezing², nasal secretion³⁰, and orofacial pain³¹, which are common
81 symptoms of viral infection. Which, if any, of these behaviors does PLpro trigger? We used high
82 speed video and audio recordings to quantify behavioral responses to intranasal treatment of vehicle
83 or PLpro (10 μ M). PLpro elicited rapid sneezing (latency: 13.6 ± 4.1 s) over two minutes in mice (Fig.
84 3a-c, Supplemental Fig. 1i). In contrast, vehicle-treated mice were slower to sneeze (latency: $30.5 \pm$
85 12.1 s) and sneezed less (Fig. 3b, c, Supplemental Fig. 1i). The sneeze reflex involves the convulsive
86 expulsion of air to clear the airways of harmful irritants and pathogens. To visualize nasal expulsion,
87 we also applied PLpro or vehicle in combination with blue dye. The number of sneezes, quantified
88 from the audio recordings, correlated with dye on the bottom of the chamber (Supplemental Fig. 1a-c).
89 We occasionally observed the expulsion of dye onto the side of the chamber and the floor during
90 these audible events (Fig. 3a, Supplemental Fig. 1b). These observations show that PLpro triggers
91 rapid sneezing and nasal secretion that could expel SARS-CoV-2 from the nasal cavity. Previous
92 studies have shown that irritants and inflammatory mediators such as capsaicin, histamine, serotonin,
93 and allergens can drive sneezing in mice.^{2,32,33} This data demonstrates for the first time that a viral
94 molecule, PLpro, is alone sufficient to trigger sneezing, a key symptom of viral infection and
95 mechanism for viral spread.

96

97

98 We also observed a nocifensive behavior, nose rubbing, characterized by a fast elliptical stroke of the
99 front paws around the nose (Fig. 3d, Supplemental Fig. 1d, e). These nose rubs are distinct from face
100 wiping (a stroke of the front paw around the face) such as those triggered by injection of algogens to
101 the cheek.³⁴ Unlike grooming behaviors, the initial nose rubs were not followed by wiping or body
102 licking (Supplemental Fig. 1e,f). Intranasal PLpro evoked the first nose rub significantly faster ($23.1 \pm$
103 3.3 s) than in vehicle-treated mice (42.3 ± 6 s); though, PLpro-treated mice did not rub any more than
104 vehicle-treated mice over two minutes (Fig. 3e, Supplemental Fig. 1g). Interestingly, we observed a
105 dose-dependence of these behaviors such that a lower dose of PLpro (100 nM) triggered fast
106 sneezing (latency: 16.1 ± 5.1 s) but not a shorter latency to nose rubbing (latency: 39.9 ± 12.0 s)
107 whereas the higher dose of PLpro (10 μ M) rapidly elicited both a sneeze and nose rub (Supplemental
108 Fig. 1i-l). Moreover, the co-occurrence of acute rubbing and sneezing in the first 30 seconds was
109 dose-dependent (vehicle: 9%, 0.1 μ M PLpro: 41.7%, 10 μ M PLpro: 55%; Fig. 3f; Supplemental Fig. 1j-
110 l).

111
112 In addition to triggering airway reflexes, activation of distinct subsets of trigeminal nociceptors also
113 promotes itch or pain behaviors. Thus, we examined the consequences of PLpro in a cheek injection
114 model, where painful stimuli promote wiping with the forelimbs and itch stimuli evoke scratching with
115 the hindlimb. Mice injected with PLpro displayed a significantly shorter latency to wipe (vehicle:
116 152.6s, PLpro (10 μ M): 56s, PLpro (50 μ M): 29.6s; Supplemental Fig. 2a) and wiped more often than
117 vehicle injection (vehicle: 0, PLpro (10 μ M): 0.56, PLpro (50 μ M): 1.3 wipes; Supplemental Fig. 2b). In
118 contrast, PLpro did not elicit itch-evoked scratching behaviors (Supplemental Fig. 2c, d). Overall, this
119 data indicates that PLpro drives acute site-directed behaviors associated with pain and irritation and,
120 interestingly, triggers a faster and more robust response in the nasal cavity than in the skin.

121
122 To elucidate the mechanisms by which PLpro activates nociceptors, we tested if the protease could
123 directly activate sensory neurons in vitro. The airways are innervated by somatosensory neurons from
124 the TG, dorsal root ganglia (DRG), and vagal sensory neurons from the nodose and jugular ganglia
125 (NJG). We found that PLpro induced calcium transients in neurons cultured from all three ganglia from
126 mice (Fig. 4a, b, f). We observed rapid and robust calcium transients and a dose-dependent increase
127 in percentage of neurons responding to PLpro in TG cultures (1 nM: 3.8%, 10 nM: 7.2%, 100 nM:
128 9.3%; Fig. 4a, f). We observed similar proportions of responses in DRG cultures (Veh: 2.9%, 1 nM:
129 4.2%, 10 nM: 6.8%, 100 nM: 11.9%; Fig. 4b). Interestingly, we observed a greater fraction of neurons
130 that responded to PLpro in cultures of NJG neurons across all PLpro concentrations (Veh: 5.5%, 1
131 nM: 21.7%, 10 nM: 23.6%, 100 nM: 33.9%; Fig. 4b). PLpro-responsive neurons were of smaller
132 diameter than vehicle-responsive neurons and the population overall (Supplemental Fig. 3a-c).

133 Consistent with the activation of a subset of nociceptors in vivo, nearly all PLpro-responsive neurons
134 also responded to capsaicin (DRG: 85.7%; NJG: 100%) and a subset also responded to AITC (DRG:
135 33.3%, NJG: 50%, Fig. 4c). As observed in mouse neurons, PLpro also evoked calcium transients in
136 a subset of human iPSC-derived nociceptors (Fig. 4d). Of the responders, 16.7% responded to
137 vehicle, 50.0% to PLpro, and 33.3% to capsaicin. PLpro activated a distinct subset than vehicle and
138 elicited significantly more calcium influx than vehicle (Fig. 4e). Taken together, we demonstrate that
139 PLpro directly activates a subset of human and mouse nociceptors that express the TRPA1 and
140 TRPV1 ion channels.

141
142 We further probed the mechanism by which PLpro activates nociceptors in mice. We observed little
143 response to PLpro in the absence of extracellular calcium (control: 5.5% vs. EGTA: 1.2%; Fig. 4i)
144 suggesting that the activation of neurons by PLpro requires a calcium-permeable ion channel. Airway
145 irritants and inflammatory mediators, including proteases, activate nociceptors via the calcium-
146 permeable ion channels, TRPV1 or TRPA1.^{7,35-38} To test the requirement of these channels, we
147 examined PLpro responses in TRPV1 or TRPA1 deficient mice and found that the percent of PLpro
148 responders was significantly reduced (WT: 5.5%, Trpv1 KO: 2.4%, Trpa1 KO: 1.9%; Fig. 4e). The
149 acute blockade of TRPA1 with the antagonist HC-030031 also reduced PLpro responders (HC: 1.7%)
150 comparable to responses observed in Trpa1 KO neurons (Fig. 4e). These data demonstrate that
151 PLpro activates sensory neurons via TRPA1 and TRPV1 ion channels. However, the mechanism by
152 which PLpro activates neurons via these channels remains unclear. Neither human nor mouse
153 TRPV1 or TRPA1 contain the cleavage sequence for PLpro, and thus, it is likely that PLpro, like other
154 proteases, may be activating a receptor that is coupled to TRPV1 and TRPA1.³⁹

155
156 PLpro is expressed by all coronaviruses including the pathogenic betacoronaviruses, SARS-CoV
157 (SARS) and MERS-CoV (MERS) that share 89% and 51% sequence similarity to SARS-CoV-2 PLpro,
158 respectively.²⁸ While PLpro from these three coronaviruses share nearly identical substrate cleavage
159 sequences, previous studies have shown differences in the structure and catalytic efficiency of MERS
160 PLpro compared to SARS PLpro and SARS-CoV-2 PLpro.^{40,41} Thus, we assessed whether the SARS
161 and MERS PLpro activate mouse neurons. SARS PLpro also activates subsets of neurons in a dose-
162 dependent manner (Fig. 4g), whereas few neurons were activated by MERS (Fig. 4h). It will be
163 interesting to assess whether PLpro from other viruses such as the common cold can also activate
164 neurons or whether this is unique to SARS and SARS-CoV-2 PLpro.

165
166

167 Recent studies have shown that SARS-CoV-2 infects sensory ganglia in human, hamster, and mouse
168 and can directly infect human iPSC-derived nociceptors. While these neurons are susceptible to
169 infection, unlike airway epithelial cells, the virus does not replicate.⁴²⁻⁴⁴ Despite this lack of replication,
170 infection may modulate neuronal physiology. Thus, we set out to assess the consequences of SARS-
171 CoV-2 infection on cultured mouse trigeminal ganglia. We detected SARS-CoV-2 viral transcripts 48
172 hours post infection in cultured mouse TG neurons including the transcript *ORF1ab* that encodes
173 PLpro (Fig. 5a). These data suggest that in addition to airway epithelial cells, neurons may be an
174 additional source for PLpro.

175

176 What are the consequences of SARS-CoV-2 infection on sensory neuron gene expression? SARS-
177 CoV-2 infection significantly upregulated 789 genes and downregulated 1664 genes with the most
178 differentially expressed genes being related to inflammation (Supplemental Fig. 4a, b). We then
179 assessed how infection and this infection-induced inflammatory response alters the transcriptional
180 signatures of distinct functional neuronal subtypes within the sensory ganglia. Nociceptor-associated
181 gene sets were significantly altered, including those corresponding to both non-peptidergic and
182 peptidergic nociceptors, while gene sets associated with mechanoreceptors, proprioceptors, and itch
183 were not significantly changed (Fig. 5b). This suggests that infection drives transcriptional changes
184 that selectively impact nociception. More so, several of the most upregulated nociceptive genes
185 encode ion channels (*P2rx3*, *Scn9a*, *Trpa1*, *Trpv1*, *Cacna1b*, *Cacna1a*, *Chrb2*, and *Grin1*) that can
186 increase the excitability of nociceptors to promote pain (Fig. 5c). Interestingly, a previous study
187 showed that human rhinovirus infection of human neuroblastoma cells also upregulated *TRPV1* and
188 *TRPA1*.⁴⁵ Here, we demonstrate that the genes for *Trpv1* and *Trpa1* channels are both upregulated
189 by SARS-CoV-2 infection and are required for acute PLpro-evoked neuronal activation (Fig. 4i, 5c).
190 SARS-CoV-2 infection in vivo also drives gene expression changes in hamster sensory ganglia that
191 are related to neuronal signaling and neuroinflammation,⁴⁶ which could reflect consequences of the
192 systemic response to infection or direct infection of the ganglia. Our in vitro data support a model in
193 which direct infection of the ganglia can drive gene expression changes to alter nociceptor function
194 and promote inflammation.

195

196 A key feature of TRPA1- and TRPV1-expressing neurons is the release of the neuropeptides
197 calcitonin gene-related peptide (CGRP) and Substance P that can drive neurogenic inflammation⁴⁷
198 and immunomodulation.^{5,48} We first tested if PLpro alone was sufficient to elicit release of CGRP and
199 Substance P from trigeminal ganglia cells in vitro. PLpro triggered acute release of CGRP (Fig. 5d).
200 However, surprisingly, unlike many allergen proteases, we did not observe the release of Substance
201 P (Fig. 5e).^{19,20} We next tested whether PLpro could stimulate the nerve endings of sensory neurons

202 to release CGRP. Using an ex vivo mouse trachea model, we found that PLpro, but not vehicle
203 treatment, induced significant release of CGRP above baseline (Fig. 5c). These data demonstrate that
204 activation of neurons by PLpro is sufficient to induce acute release of CGRP both in vitro and in the
205 airways. Interestingly, we also observed that SARS-CoV-2 infection upregulated *Calca* and *Calcb*, the
206 genes that encode CGRP- α and - β , but not *Tac1*, the gene that encodes Substance P (Supplemental
207 Fig. 4c). Taken together, these data show that SARS-CoV-2, in the absence of infected airway
208 resident cells, both promotes the transcriptional upregulation and secretion of the immunomodulatory
209 neuropeptide CGRP.

210
211 Pain is a hallmark of acute COVID-19 infection and animal models of SARS-CoV-2 infection develop
212 mechanical allodynia both during and after infection.^{1,43,46,49} Canonical algogens induce mechanical or
213 thermal sensitization via TRPV1 and TRPA1.^{7,50} Thus, we tested if, in addition to acute pain
214 behaviors (Supplemental Fig. 2a, b), PLpro could also drive pain hypersensitivity. We found that
215 PLpro promotes mechanical hypersensitivity following intradermal injection into the hindpaw. PLpro
216 significantly reduced the von Frey paw withdrawal threshold three hours after injection, unlike vehicle
217 injection (Fig. 6a). This PLpro-dependent mechanical sensitization recovered by 24 hours (Fig. 6a).
218 Unlike wild-type and TRPV1-deficient mice, TRPA1-deficient mice did not develop PLpro-induced
219 mechanical sensitization (Fig. 6b). Interestingly, we observed no difference in the thermal sensitivity
220 between vehicle- and PLpro-injected paws, consistent with TRPA1's role in mediating mechanical, but
221 not thermal sensitization (Fig. 6c).^{7,51} We next asked whether TRPA1 or TRPV1 are also required for
222 PLpro-induced sneezing. PLpro triggers robust sneezing in wild-type and TRPV1-deficient mice, but
223 not in TRPA1-deficient mice that display little sneezing, comparable to vehicle treatment (Fig. 6d).
224 Altogether these data demonstrate that PLpro activates nociceptors via TRPA1 to drive sneezing and
225 pain behaviors. While TRPA1 is well known to mediate pain and cough,^{7,8} we now also demonstrate a
226 role for TRPA1 in viral-associated sneezing and pain.

227
228 Symptoms of viral infection such as sneezing, coughing, and headache are consequences of aberrant
229 activation of somatosensory and vagal neurons, that under healthy conditions, regulate breathing,
230 promote protective airway reflexes, and initiate pain sensations. Viral infection has thus far been
231 proposed to drive these symptoms via the release of inflammatory mediators that in turn activate
232 neurons. Here, we demonstrate a new mechanism, by which SARS-CoV-2 directly activates sensory
233 neurons via PLpro to drive sneezing and pain. We show that a single administration of PLpro, in the
234 absence of viral infection or inflammation, is sufficient to activate neurons to drive these behaviors.
235 PLpro activates nociceptors from the trigeminal, dorsal root, and nodose/jugular ganglia innervate the
236 skin and viscera and thus may drive other symptoms of infection such as headache or gastrointestinal

237 pain. Viral proteases are of great interest as therapeutic targets to inhibit viral replication,⁵² and these
238 data suggest that targeting PLpro may also serve to alleviate symptoms associated with COVID-19.
239
240 More than 10% of SARS-CoV-2 patients experience chronic symptoms well after the acute infection
241 phase, known as Long COVID.^{1,53} The most prevalent Long COVID symptoms include headache
242 (40% of people who experience Long COVID), shortness of breath, (37%), persistent cough (27%),
243 sore throat (27%), and chest pain (23%). Though less common, peripheral neuropathy symptoms
244 (pins and needles and numbness, 2%) have also been reported at a greater frequency than in control
245 groups.⁴⁹ But, the mechanisms that drive these long-lasting changes, particularly in the nervous
246 system, remain unclear. A hallmark of neuropathic diseases like chronic pain and itch are
247 transcriptional changes in the sensory ganglia that drive aberrant neuronal activity.⁵⁴⁻⁵⁶ Long term
248 alterations in nociceptor function are a critical starting point to understanding the mechanisms that
249 drive Long COVID. Future studies are needed to assess whether infection-induced gene expression
250 changes persist beyond infection, and whether PLpro, like other viral proteins, lingers in the body after
251 active infection to contribute to Long COVID symptoms.^{57,58}

252 **Methods**

253 Mouse studies

254 All mice were housed in standard conditions in accordance with standards approved by the Animal
255 Care and Use Committee of the University of California Berkeley (14-hour light: 10-hour dark cycle, 21
256 °C). Wild-type C57BL/6N mice were obtained from Charles River and C57BL/6J were obtained from
257 Jackson Laboratories and raised in-house. All experiments were performed under the policies and
258 recommendations of the International Association for the Study of Pain and approved by the
259 University of California Berkeley Animal Care and Use Committee. Where appropriate, genotyping
260 was performed by Transnetyx using real-time PCR. Mouse lines used in this study included Pirt-Cre
261 (Pirttm3.1(cre)Xzd) gift from Dr Xinzhong Dong (Johns Hopkins University, Baltimore, MD) and Ai96
262 (B6J.Cg-Gt(ROSA)26Sortm96^{(CAG-GCaMP6s)Hze}/MwarJ), Trpv1 KO; Trpv1tm1Jul RRID:MGI:4417977,
263 and Trpa1 / (A1 KO; Trpa1tm1Jul, RRID:MGI:3696956) from Jackson Laboratories.

264

265 Calcium imaging

266 *In vivo calcium imaging of trigeminal ganglion*

267 In vivo calcium imaging of trigeminal neurons was conducted in anesthetized mice as previously
268 described.⁵⁹ In brief, Pirt-Cre;GCaMP6s mice were anesthetized with ketamine and xylazine (100 and
269 10 mg/kg intraperitoneal) and body temperature was maintained at 36°C throughout surgery and
270 imaging. Mice were head-fixed on a custom chamber and tracheotomized to allow normal breathing
271 and restrict intranasal perfusion to the upper airways. A unilateral craniotomy and hemispherectomy
272 was performed to expose the dorsal surface of the right trigeminal ganglion. Hemostasis was
273 achieved using Surgifoam gel sponge (Ethicon 1972). Images were collected at 5 Hz using a Sutter
274 Movable Objective Microscope (MOM) equipped with an Olympus XL-Fluor 4X/340 (NA 0.28) lens,
275 ORCA-Fusion BT Digital CMOS camera. 480nm illumination was provided by Lambda 721 (Sutter).
276 Intranasal perfusion of 10 µL of SARS-CoV-2 PLpro (R&D #E-611), vehicle (50 mM HEPES, 300 mM
277 NaCl, 1 mM TCEP, 10% (v/v) Glycerol, pH 8.0) dilution was matched to that of PLpro, AITC (Sigma)
278 or (E)- Capsaicin (Tocris) was applied evenly between both nostrils using a P20 pipette. Stimuli were
279 applied at 2-minute intervals.

280

281 *In vivo imaging analysis*

282 Motion correction and source extraction were performed using Suite2p.⁶⁰ Suite2p-generated regions
283 of interest (ROIs) were manually inspected and additional ROIs were manually identified. Neuropil
284 was subtracted with a coefficient of 0.7. F_0 is defined as the median fluorescence 150 frames prior to
285 each stimulus treatment and used to calculate F/F_0 . Normalized traces were Gaussian filtered ($s = 2$).
286 Stimulus responders were identified if the peak F/F_0 response during the 2-minute stimulation period
287 was $> 10\%$ of F_0 . Cells were identified as spontaneously active if their peak fluorescence was 5%

288 greater than the median fluorescence during the baseline window prior to any stimulus treatment. The
289 percentage of responsive cells were quantified by pooling all cells that respond to any of the applied
290 stimuli.

291

292 *Neuronal cultures*

293 TG, DRG, and NJG were cultured as previously described.^{36,61} In brief, sensory ganglia were
294 dissected and incubated in warmed Collagenase P (Roche) in Hanks calcium-free balanced salt
295 solution (HBSS) followed by incubation in 0.25% trypsin with gentle agitation. Cells were washed,
296 triturated, and plated in Minimum Essential Medium (MEM, Gibco) supplemented with 10% horse
297 serum (v/v), MEM vitamins, penicillin/streptomycin and L-glutamine. Neonatal TG cultures were plated
298 on poly-D-Lysine coated eight-well chambers for calcium imaging. Adult TG, DRG, and NJG were
299 plated on poly-D-Lysine and laminin-coated eight-well chambers for calcium imaging or 96-well plates
300 for infection and ELISA experiments. Age-matched wild-type (C57BL/6N) controls were prepared on
301 the same day for experiments on neurons from knockout mice. Human iPSC-derived neurons were
302 obtained from Axol Bioscience with informed consent obtained from the donor with ethics committee
303 approval. Neurons were differentiated per manufacturer's instructions and used in calcium imaging
304 21- and 39-days post differentiation (Axol Bioscience, ax0555).

305

306 *In vitro calcium imaging*

307 Neuronal Ca²⁺ imaging experiments were carried out as previously described.⁶¹ Neuronal cultures
308 were loaded for 45 min at room temperature with 10 μM Fura-2AM supplemented with 0.01% Pluronic
309 F-127 (wt/vol, Life Technologies) in a physiological Ringer's solution containing (in mM) 140 NaCl, 5
310 KCl, 10 HEPES, 2 CaCl₂, 2 MgCl₂ and 10 D-(+)-glucose, pH 7.4. Cells were identified as neurons by
311 eliciting depolarization with high potassium Ringer's solution (75 mM) at the end of each experiment.
312 Acquired images were displayed as the ratio of 340 nm / 380 nm. Fura-2 ratios were normalized to
313 baseline ratio. Responding neurons were defined as those having a > 10% increase from baseline
314 ratio. Human iPSC-derived neurons were defined as responding to > 5% increase from baseline ratio.
315 All calcium imaging analyses were performed using custom python scripts. Neurons were stimulated
316 with SARS-CoV-2 PLpro (R&D #E-611), SARS-CoV PLpro (R&D #E-610), MERS-CoV PLpro (R&D
317 #E-609), SARS-CoV-2 PLpro (Cayman #31817) diluted in Ringer's to the appropriate concentration.
318 Vehicle (50 mM HEPES, 300 mM NaCl, 1 mM TCEP, 10% (v/v) Glycerol, pH 8.0) dilution was
319 matched to that of PLpro. Cells were incubated in the TRPA1 antagonist (HC-030031, Tocris #2896)
320 for 15 minutes prior to imaging experiment.

321

322 Neuropeptide release

323 TG from 7-8 week old mice were cultured overnight as described above and supplemented with 50
324 ng/uL of nerve growth factor. Cultured neurons were incubated with vehicle or PLpro for 15 min in
325 Ringer's solution at room temperature. For ex vivo trachea stimulation, tracheas were dissected and
326 washed in HBSS for 30 min at 37°C. Tracheas were then incubated in DMEM for 30 min at 37° with
327 gentle rotation for baseline measurement and in DMEM with PLpro or vehicle for 1 hour at 37°C with
328 gentle rotation. After incubation, the supernatant (from in vitro experiments) and medium (from ex vivo
329 experiments) was collected and used to quantify the concentration of neuropeptide using an enzyme
330 linked immunosorbent kit (CGRP: Cayman Chemicals #589001, Substance P: Cayman Chemicals
331 #583751) according to the manufacturer's instructions.

332

333 SARS-CoV-2 infection

334 *Human airway epithelial cell and primary mouse neuron infection*

335 Human adenocarcinoma lung epithelial (Calu-3) cells (ATCC, HTB-55) were maintained with RPMI
336 (Fisher Scientific) supplemented with fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin.
337 Approximately 5×10^5 Calu-3 cells were incubated with SARS-CoV-2 (WA1) in medium (10% heat-
338 inactivated FBS, sodium pyruvate, HEPES, L-glutamine, penicillin/streptomycin) diluted in phenol-red
339 free RPMI to an MOI of 1 for 24 or 48 hours. Mouse TG were cultured as described above and plated
340 in a 96-well plate. TGs (2 from each animal) across 4 animals were pooled and plated across 8 wells.
341 TG cultures were infected with 5×10^5 SARS-CoV-2 virions or medium (mock infection) for 48 hours.

342

343 *Protease activity*

344 Supernatant from SARS-CoV-2 and mock infected Calu-3 cells was combined with the PLpro
345 substrate, Ubiquitin-Rh110 (R&D Systems, #U-555) at 670 nM in phenol-free RPMI. Fluorescence
346 was measured at 535nm on a plate reader. Samples were run in duplicates. A standard curve was
347 generated by measuring protease activity from recombinant SARS-CoV-2 PLpro (R&D) diluted in
348 mock infection medium at 1, 10, 100, 500, and 1000nM and run on the same plate as experimental
349 samples.

350

351 *RNA sequencing and analysis*

352 After removing supernatant from infected cells, RNA was isolated with DNA/RNA Shield (Zymo
353 Research) according to the manufacturer's instructions. RNA library preparation (Inc-RNA library with
354 rRNA removal) and sequencing on an Illumina NovaSeq 6000 were performed by Novogene. For
355 quantification of viral transcripts, reads were mapped to the appropriate host (human or mouse)
356 transcriptome concatenated with the SARS-CoV-2 transcriptome using *Kallisto* (0.44.0). For analysis
357 of infected mouse TG, reads were mapped to the mouse genome concatenated with the SARS-CoV-2
358 genome using *STAR* (2.7.10a). Gene counts were extracted using featureCounts in *Subread* (2.0.1).

359 All but one sample contained more than 80% mapped features; one sample contained 68% mapped
360 reads and was excluded based on the robust principal component analysis method.⁶² The uniquely
361 aligned features were used for differential expression analysis using *DESeq2* (1.36.0) and the genes
362 were determined to be significantly differentially expressed if they had a base mean > 152, log₂ fold
363 change > 0.37, and adjusted p-value < 0.001. Gene sets associated with functional neuronal subtypes
364 were generated from a previous study.⁶³ We pooled the same Gene Ontology terms Mouse Genome
365 Informatics (MGI) (updated as of November 2023) except for the itch gene list, which we used from
366 the paper. We tested if these custom gene sets were significantly differentially expressed above
367 chance. In brief, we established a null distribution by sampling random sets of genes (of the same
368 size) from all transcripts expressed across our samples 100,000 times and compared the median
369 absolute log₂ fold change for each custom gene set to the null distribution to determine the empirical
370 p-value.

371

372 Behavioral studies

373 *Intranasal behavior and scoring*

374 For intranasal behavioral experiments, mice were acclimated in a custom clear acrylic behavioral
375 chamber (4" x 2" x 6") with a filter paper bottom (VWR #28321-113) for 10 minutes prior to intranasal
376 treatment. Mice were treated intranasally with vehicle or PLpro with 10 mg/mL Evan's blue dye in
377 phosphate-buffered solution (PBS) administered 5 µL/nostril (10 uL total) with a P20 pipette. Mice
378 were then placed back in the chamber for another 10 minutes of recording. A GoPro Hero 11
379 (recording at 2.7 megapixels, 240Hz) was used to record video and ambient audio. An audio recorder
380 (aTTo digital, 16 kHz) placed inside the chambers. Experimenters were blinded to genotype or
381 treatment. Behavior was scored for 2 minutes by individuals blinded to both genotype and treatment.
382 A nose rub was defined as a singular elliptical stroke made by both forepaws simultaneously, directed
383 to the nose. A face wipe was also a singular elliptical stroke of the forelimbs but contacted the face
384 above the nose and below the ears, making a circular motion. The time of each individual nose rub
385 and face wipe was recorded. A bout of body licking was defined as an uninterrupted series of licking
386 the flank, chest, stomach, and tail. The filter paper that was on the bottom of the chamber was
387 scanned and the mean intensity was quantified using *Fiji*, and the mean intensity was inverted
388 (subtracted by 255) and scaled (by 10⁸).

389

390 *Sneeze analysis*

391 Sneeze events were identified from peaks in the audio recordings. Audio recordings were cropped to
392 the start of the experiment and a 2-3 second recording of background sound from the recording was
393 used to denoise in Audacity (Noise reduction: 30 dB, sensitivity: 6, frequency of smoothing: 6 bands).
394 Denoised audio recordings were then analyzed using a custom python script. Audio peaks were

395 identified using the SciPy “find_peaks” function (peak prominence > 1000 and a minimum distance of
396 0.2s between peaks). The audio recordings from inside the chamber were aligned to the video and
397 audio recordings from the GoPro (outside the chamber). Audio peaks recorded both inside and
398 outside the chamber were excluded as background noise. Audio peaks that correlated with other
399 behaviors such as biting the chamber and body licking were excluded.

400

401 *Cheek behavior*

402 Itch and acute pain behavioral measurements were performed as described previously.⁶¹ In brief,
403 mice were shaved and singly housed one week prior to the experiment. Mice were acclimated to the
404 chamber for an hour to the chamber both the day prior and before injection. Compounds were injected
405 intradermally (20 μ L) into the cheek. Behavior was videotaped for one hour and scored for either the
406 first 30 min (scratching) or the first 5 min (wiping) as previously described.⁶⁴

407

408 *Von Frey and radiant heat behavior*

409 Mice were lightly anesthetized with isoflurane (2.5%) and PLpro or vehicle was injected intradermally
410 into the plantar surface of the hind paw (15 μ L). For von Frey, both hind paws were injected, one with
411 treatment and the other with vehicle solution. For radiant heat, only the left hind paw was injected with
412 either treatment or vehicle solution. Mice were acclimated in behavioral chambers for 2 subsequent
413 days for at least an hour followed by an additional 30 minutes of acclimation with the investigator in
414 the room. Mechanical threshold was measured using calibrated von Frey monofilaments (Touch Test)
415 on a metal grate platform (IITC). Von Frey was performed as previously described^{65,66} using the up-
416 down method⁶⁷. Measurements were taken one day prior to injection, the 3-, 27-, and 51-hours
417 following injection. Radiant heat assay was performed using the Hargreaves test system (IITC Life
418 Science) as previously described.^{65,68} A glass platform was set at 30°C during the acclimation and
419 throughout the experiment and the radiant heat source raised the platform temperature to 41.5°C
420 within 5s and to 44.9°C within 10s, as measured by a fast temperature probe (Physitemp). Valid
421 responses for both von Frey and radiant heat included fast paw withdrawal, licking/biting/shaking of
422 the affected paw, or flinching. Experimenter was blind to treatment and genotype.

423

424 *Statistical reporting*

425 Unless otherwise noted, data are expressed as mean \pm standard error of the mean (SEM). All
426 performed statistical tests were two-tailed using Prism. Schematics were made using Biorender.
427 RNAseq analysis was performed using R. All other data were analyzed using Python.

428

429 *Data Availability*

430 RNA sequencing data were deposited into the Gene Expression Omnibus database under accession
431 number GSE252056.

432

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441

442 **Author contributions:**

443 S.S.M. and D.M.B conceived the project and designed experiments. S.S.M led and performed
444 experiments, analyzed data, and prepared figures. R.S. and C.V. performed paw and cheek behavior
445 experiments. U.V., R.S., and S.S.M. performed intranasal behavior scoring. M.C. performed and
446 J.S.C. supervised infection experiments. Z.G. performed RNAseq analysis. Y.M. performed pilot and
447 provided technical assistance for in vivo imaging experiments. S.S.M. and D.M.B wrote the
448 manuscript with input from all authors.

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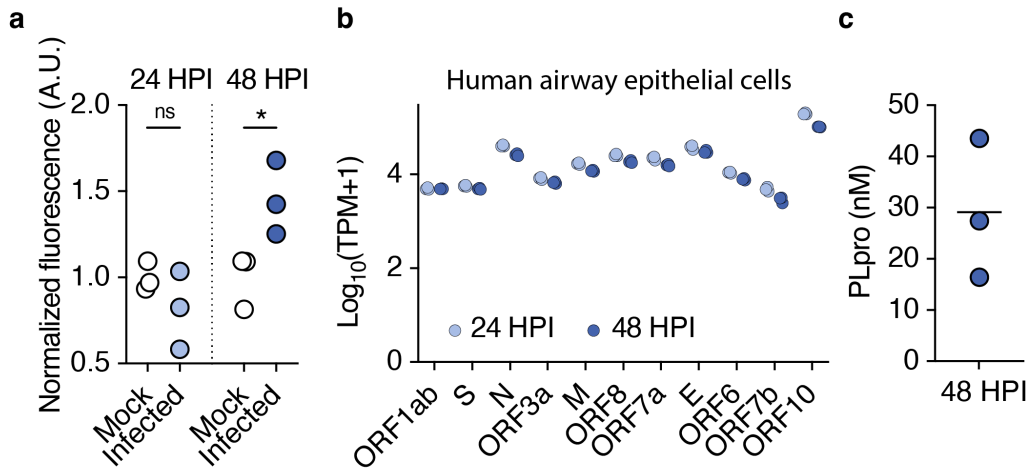


Figure 1. SARS-CoV-2 PLpro is released from infected airway epithelial cells.

a, PLpro proteolytic activity from the supernatant of SARS-CoV-2 or mock infected Calu-3 cells 24 and 48 HPI, measured by the fluorescence of cleaved substrate, normalized across samples from the same day. One-way ANOVA: $p=0.014$, $F(3,8)=6.790$; Holm-Šídák's multiple comparisons, $p_{\text{mock vs. infected 24h}}=0.244$, $p_{\text{mock vs. infected 48h}}=0.030$, $n=3$ wells per group. **b**, SARS-CoV-2 transcripts from Calu-3 cells infected with SARS-CoV-2 (multiplicity of infection = 1) for 24 and 48 hours post infection (HPI), transcripts per million (TPM), $n=3$ wells. **c**, Interpolated concentration of PLpro in the supernatant of Calu-3 cells 48 hours post infection, line represents mean.

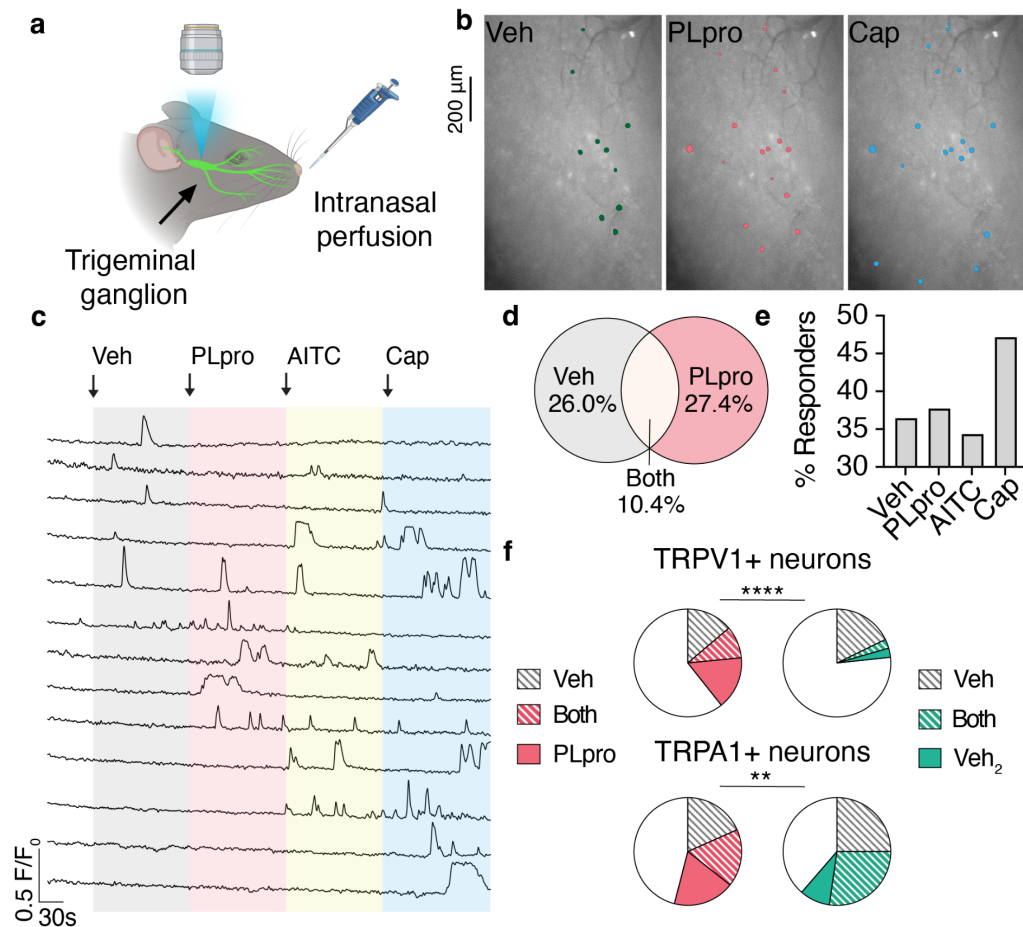


Figure 2. PLpro activates upper airway-innervating neurons in vivo.

a, Schematic of in vivo imaging preparation. **b**, Image of trigeminal ganglion overlaid with the regions of interest of neurons that respond to intranasal perfusion (10 μ L) of vehicle (Veh), 10 μ M PLpro, and 100 μ M capsaicin (Cap). **c**, Representative calcium transients in response to intranasal perfusion of Veh, 10 μ M PLpro, 1 mM AITC, 100 μ M Cap. **d**, Venn diagram of neurons responsive to vehicle (112/307) and PLpro (116/307), $n=307$ neurons from 6 mice. PLpro activates a subset of vehicle-sensitive neurons (32/112). **e**, Percent of recorded neurons that respond to each stimulus. **f**, Vehicle and PLpro activate a subset of TRPV1+ (capsaicin-responsive) neurons and TRPA1+ (AITC-responsive) neurons. Top Left, percent of TRPV1+ neurons responsive to vehicle only: 13.8%, both vehicle and PLpro: 9.7%, PLpro only: 15.9%, $n=145$ neurons from 6 mice; Top Right, percent of TRPV1+ neurons responsive to the first delivery of vehicle only: 17.8%, both first and second (Veh₂) deliveries of vehicle: 2.7%, second delivery of vehicle only: 2.7%, $n=73$ neurons from 3 mice, Chi-square test $\chi^2 = 112.5$, $df = 3$, $p < 0.0001$. Bottom Left, percent of TRPA1+ neurons responsive to vehicle only: 18.4%, both vehicle and PLpro: 17.2%, PLpro only: 18.4%, $n=87$ neurons from 4 mice; Bottom Right, percent of TRPA1+ neurons responsive to the first delivery of vehicle only: 25.0%, both deliveries of vehicle: 27.3%, second delivery of vehicle only: 9.1%, $n=44$ neurons from 3 mice, Chi-square test $\chi^2 = 14.2$, $df = 3$, $p = 0.003$.

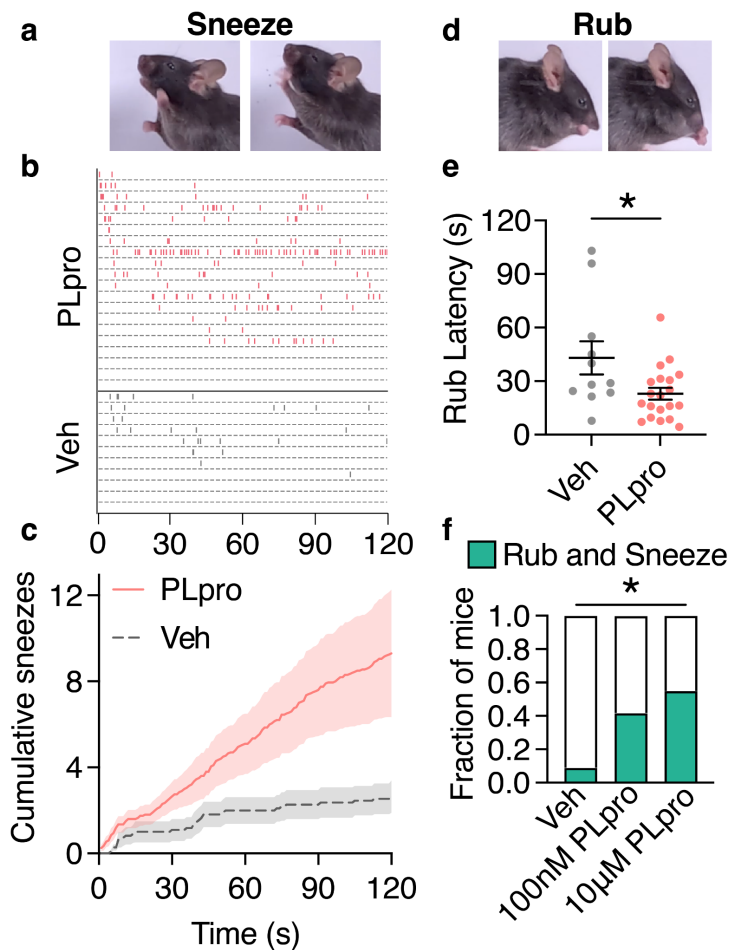


Figure 3. PLpro elicits sneezes and nose rubs in mice.

a, Sequential images of a sneeze following intranasal treatment of 10 μ M PLpro (10 μ L). **b**, Raster plot of sneezes from individual mice treated with 10 μ M PLpro or vehicle identified from audio recordings. **c**, Average cumulative sneeze count of 10 μ M PLpro and vehicle treated mice over 2 minutes **d**, Sequential images of a nose rub following intranasal treatment **e**, 10 μ M PLpro treated mice display a shorter latency to the first nose rub than vehicle treated mice, t-test: $t=2.469$, $df=29$, $p=0.020$. **f**, Fraction of mice that elicited a rub and sneeze within the first 30 seconds after treatment. (Vehicle: 9.0%, PLpro (100 nM): 41.7%, PLpro (10 μ M): 55.0%). Fisher's exact test ($p_{\text{vehicle vs. 100nM PLpro}}=0.252$, $p_{\text{vehicle vs. 10}\mu\text{M PLpro}}=0.020$). For all data in this figure, vehicle ($n=11$), 100 nM PLpro ($n=12$), and 10 μ M PLpro ($n=20$). Error bars and shading represent the mean \pm standard error of the mean (SEM), n = biological replicates (animals).

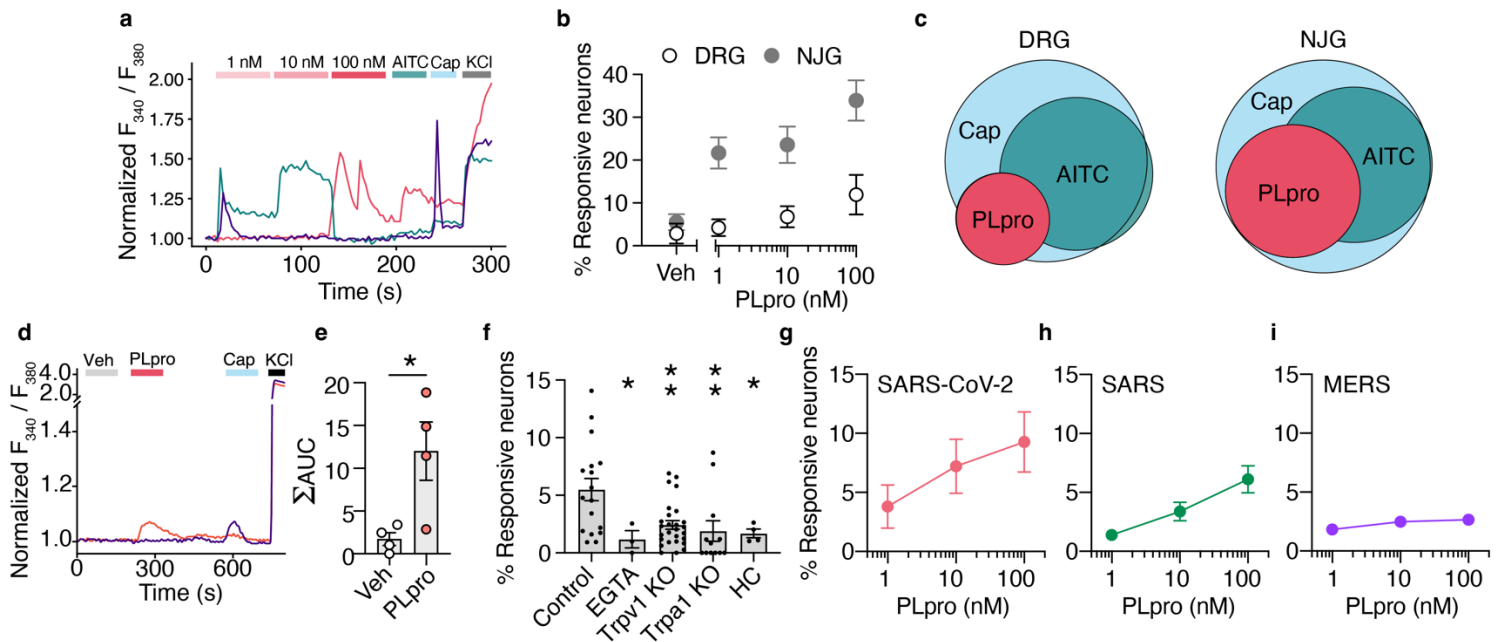


Figure 4. PLpro directly activates nociceptors.

a, Representative calcium transients from PLpro responders from cultured neonatal mouse trigeminal ganglia (TG). **b**, PLpro activates subsets of neurons from adult mouse dorsal root ganglia (DRG; Veh: 2.9%, 1 nM: 4.2%, 10 nM: 6.8%, 100 nM: 11.9%, $n=5$) and adult mouse nodose and jugular ganglia (NJG; Veh: 5.5%, 1 nM: 21.7%, 10 nM: 23.6%, 100 nM: 33.9%, $n=8$). **c**, Venn diagram of PLpro, AITC, and Cap-responsive neurons in DRG and NJG. Of the neurons that respond to these 3 stimuli, Left, in the DRG, 20% responded to PLpro, an additional 48% responded to AITC, and 32% responded to Cap alone. Right, in the NJG, 38% responded to PLpro, an additional 34% responded to AITC, and 28% responded to Cap alone. **d**, Representative calcium transients evoked by PLpro and capsaicin (Cap) in human iPSC-derived neurons. **e**, PLpro induces significantly more calcium influx (area under the curve, AUC) than vehicle, t-test: $t=2.953$, $df=6$, $p=0.026$, $n=4$ wells each. **f**, Percent of neonatal mouse TG neurons that respond to 250nM PLpro in control neurons from wild-type C57BL/6N mice in physiological extracellular calcium (2 mM Ca^{2+} , 5.5%, $n=17$) is greater than in the absence of extracellular calcium (EGTA, 1.2%, $n=3$) and in neurons from Trpv1 KO (2.4%, $n=27$) and Trpa1 KO (1.9%, $n=12$) mice, and in wild-type mice in the presence of TRPA1 antagonist (HC, 1.7%, $n=4$). One-way ANOVA: $p=0.003$, $F(4,58)=2.803$; Holm-Šidák's multiple comparisons, $p_{\text{Control vs. EGTA}}=0.035$, $p_{\text{Control vs. Trpv1 KO}}=0.004$, $p_{\text{Control vs. Trpa1 KO}}=0.004$, $p_{\text{Control vs. HC}}=0.035$. **g-i**, SARS-CoV-2 and SARS, but not MERS PLpro activate subsets of neonatal mouse TG neurons in a dose-dependent manner. **g**, SARS-CoV-2 PLpro: 1nM: 3.8%, 10 nM: 7.2%, 100 nM: 9.3%, $n=5$. **h**, SARS PLpro: 1 nM: 1.4%, 10 nM: 3.4%, 100nM: 6.1%, $n=5$. **i**, MERS PLpro: 1nM: 1.8%, 10nM: 2.5%, 100nM: 2.7%, $n=6$. * $p < 0.05$, ** $p < 0.01$. Error bars represent the mean \pm SEM, n indicates replicates (wells).

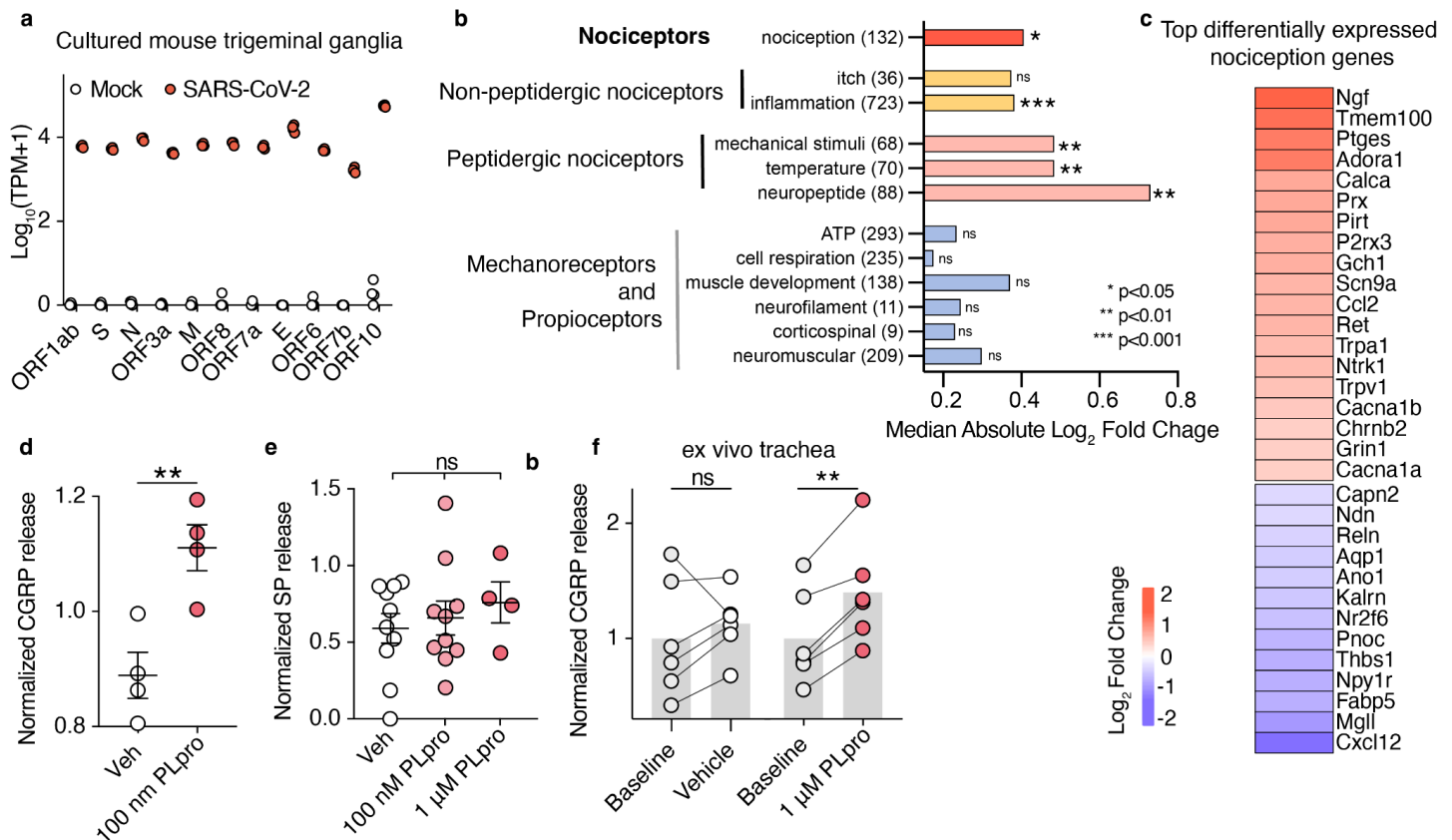


Figure 5. SARS-CoV-2 infection and PLpro activation share a key nociceptive signature.

a, SARS-CoV-2 viral transcript expression 48 hours after infection in cultured adult trigeminal ganglia, mock infected: $n=4$ wells, SARS-CoV-2 infected: $n=3$ wells. **b**, Infection alters gene sets associated with nociception but not mechanoreceptors and proprioceptors. The median absolute log₂ fold change from each custom gene set was tested against 100,000 permutations of randomly selected gene sets of the same size, size of each gene set listed in parenthesis. **c**, Heatmap of the most highly differentially expressed nociception-associated transcripts (log₂ fold change of infection vs mock). **d**, PLpro elicits CGRP release from cultured TG. t-test: $t=3.928$, $df=6$, $p=0.008$, $n=4$ per group, biological replicates (animal) **e**, PLpro does not elicit the release of Substance P from cultured TG, One-way ANOVA: $p=0.673$, $F(2,21)=0.215$, vehicle ($n=10$), 100 nM PLpro ($n=10$), and 1 μ M PLpro ($n=4$) replicates (wells from pooled animals) **f**, PLpro but not vehicle increases CGRP release from baseline measurements in an ex vivo trachea preparation. Baseline vs. vehicle: one sample t-test: $t=0.909$, $df=5$, $p=0.405$, baseline vs. 1 μ M PLpro: one sample t-test: $t=6.80$, $df=5$, $p=0.001$, $n=6$, biological replicates (animal) per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bar represents mean and error bars represent the mean \pm SEM.

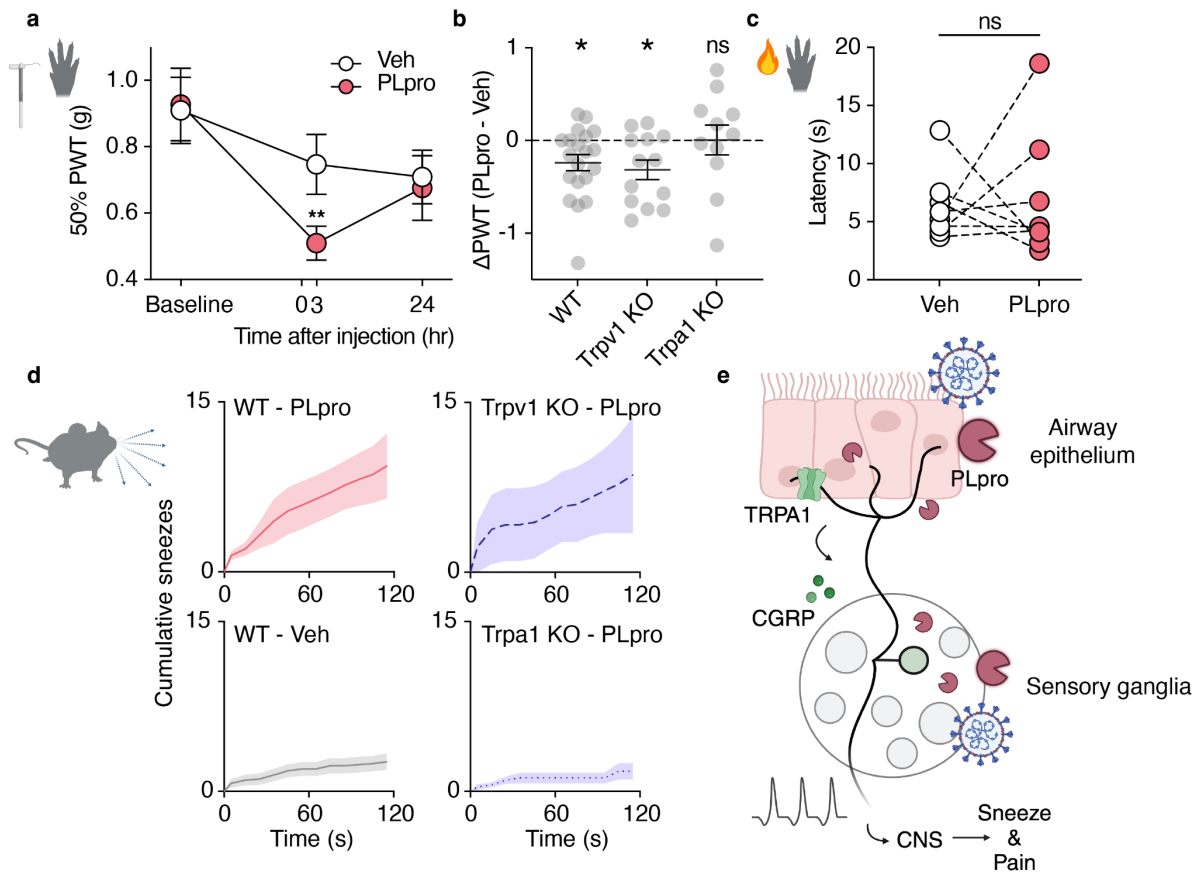
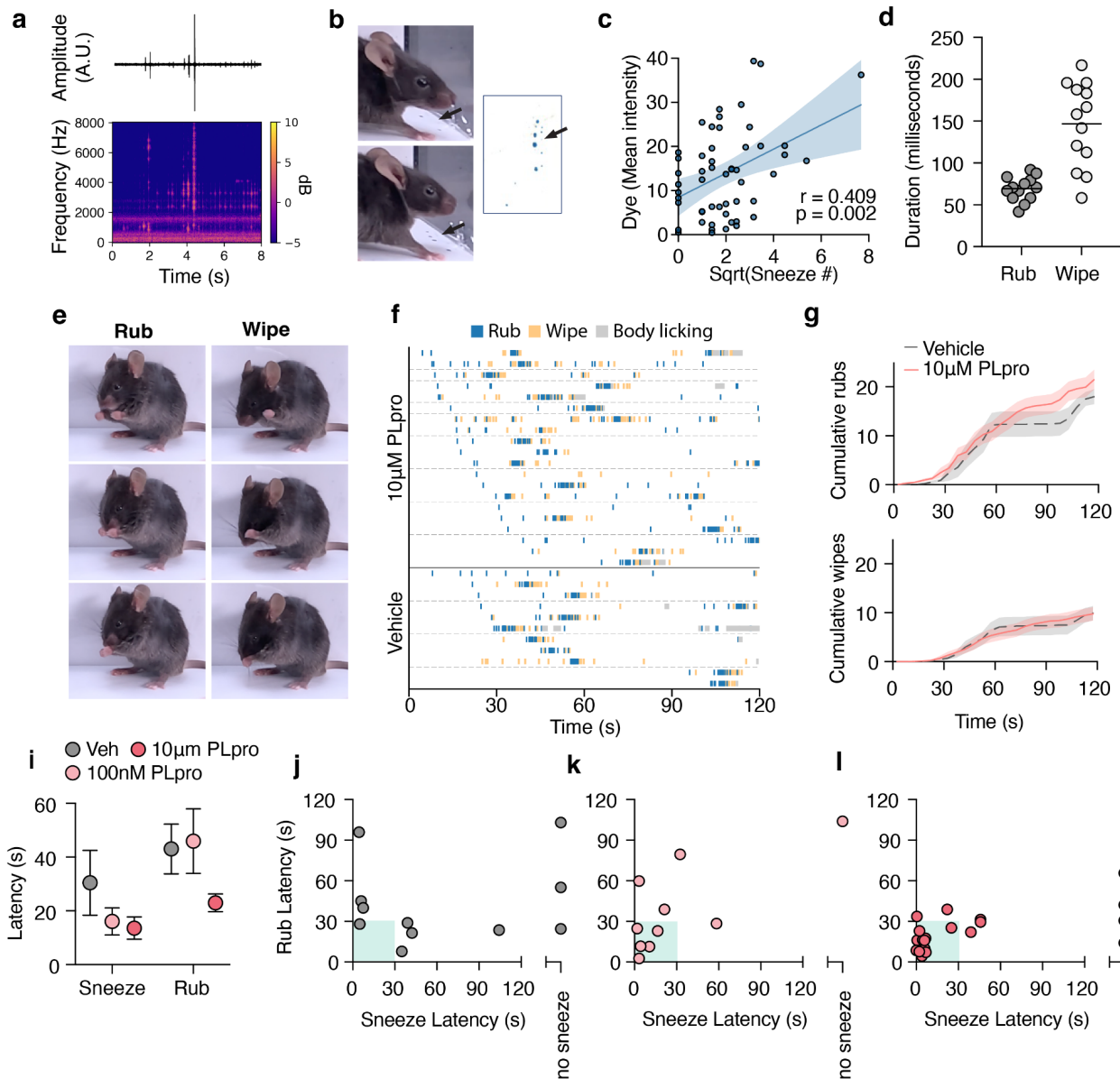


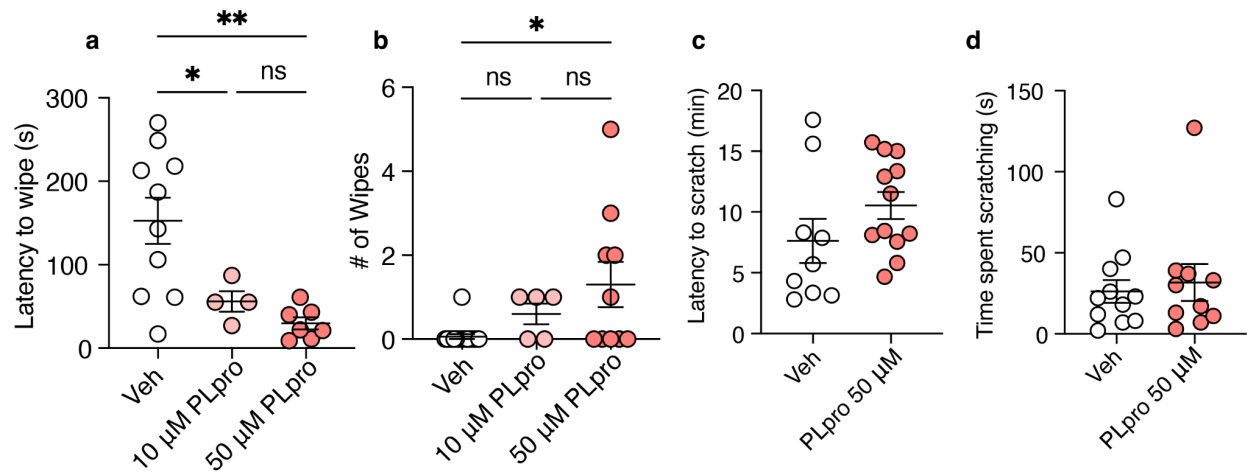
Fig 6. SARS-CoV-2 PLpro-evoked sneeze and pain require TRPA1.

a, 50 μ M PLpro induces a reduction in the paw withdrawal threshold (PWT) for force 3, but not 24 hours post injection in PLpro injected paws, but not vehicle injected paws compared to baseline. (24 hours pre-injection). Two-way ANOVA: $p_{\text{time}} = 0.008$, $F(1.793, 64.56) = 5.477$; Holm-Šidák's multiple comparisons, Veh: p-adjusted Baseline vs. 3 hours = 0.361, p-adjusted Baseline vs. 24 hours = 0.079; PLpro: p-adjusted Baseline vs. 3 hours = 0.008, p-adjusted Baseline vs. 24 hours = 0.117, $n = 19$. **b**, Wild-type C57BL/6J (WT) and Trpv1 KO mice, but not Trpa1 KO mice develop mechanical hypersensitivity 3 hours post injection. WT: one sample t-test: $t = 2.746$, $df = 19$, $p = 0.013$, $n = 11$; Trpv1 KO: one sample t-test: $t = 3.001$, $df = 20$, $p = 0.011$, $n = 13$; Trpa1 KO: one sample t-test: $t = 0.039$, $df = 10$, $p = 0.970$, $n = 11$. **c**, 50 μ M PLpro injection does not change the latency to respond to the radiant heat stimulation, paired t-test: $t = 0.233$, $df = 7$, $p = 0.822$, $n = 8$. **d**, Intranasal treatment of 10 μ M PLpro triggers more sneezing in Top Left, WT and Top Right, Trpv1 KO mice than Bottom Right, Trpa1 KO mice or Bottom Left, WT mice treated with vehicle. **e**, Proposed model: PLpro released from infected cells in the airway epithelium and in the sensory ganglia activate Trpa1-expressing neurons to drive sneeze, pain, and the release of CGRP into the airways. Error bars and shading represent the mean \pm standard error of the mean (SEM), $n =$ biological replicates (animals).



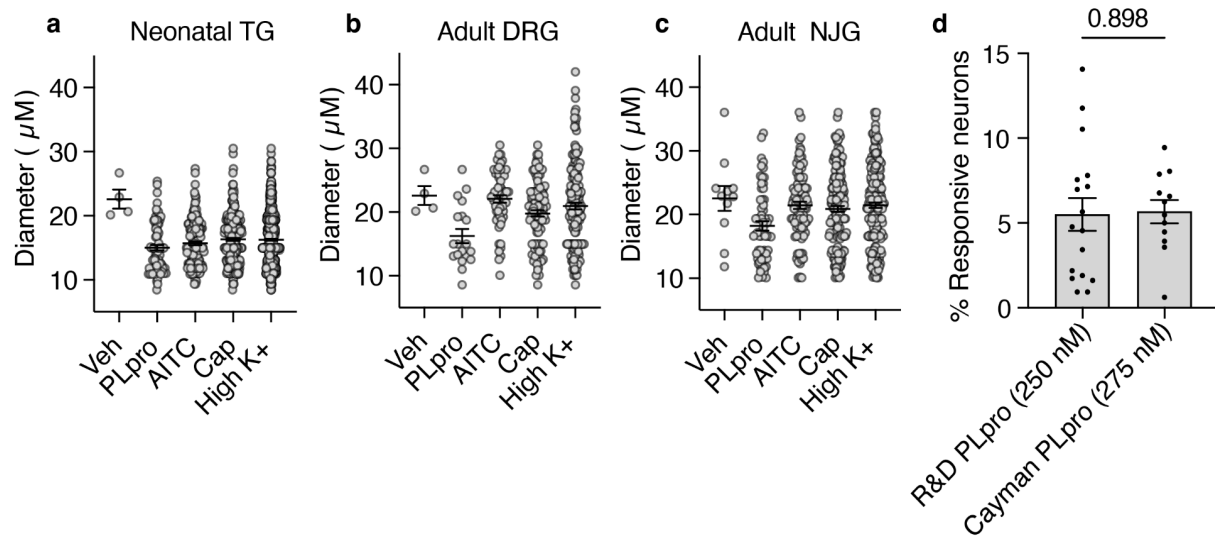
Supplemental Figure 1. Characterization of behaviors elicited by intranasal treatment.

a-c, Sneeze events are identified by audio waveforms and correlate to dye on the bottom of the chamber. **a**, Representative audio waveform during a sneeze **b**, Left, images during the same sneeze, arrows indicate dye from the sneeze on the bottom of the chamber. Right, image of dye expulsion from the sneeze event on the bottom of the chamber. **c**, The number of sneezes over 2 minutes are significantly correlated to the amount of dye at the bottom of the chamber. **d**, Duration of individual nose rub and face wipe events. **e**, Image sequence of a nose rub and face wipe. **f**, Raster plot of rubs, wipes, and body-licking events. **g**, Average cumulative rub and wipe counts from individual mice treated with 10 μ M PLpro ($n=20$) or vehicle ($n=11$). **i-l**, Mean and standard error of the sneeze and rub latency and for individual mice treated with **j**, vehicle ($n=11$), **k**, PLpro (100 nM, $n=12$), and **l**, PLpro (10 μ M, $n=20$), n = biological replicates (animals).



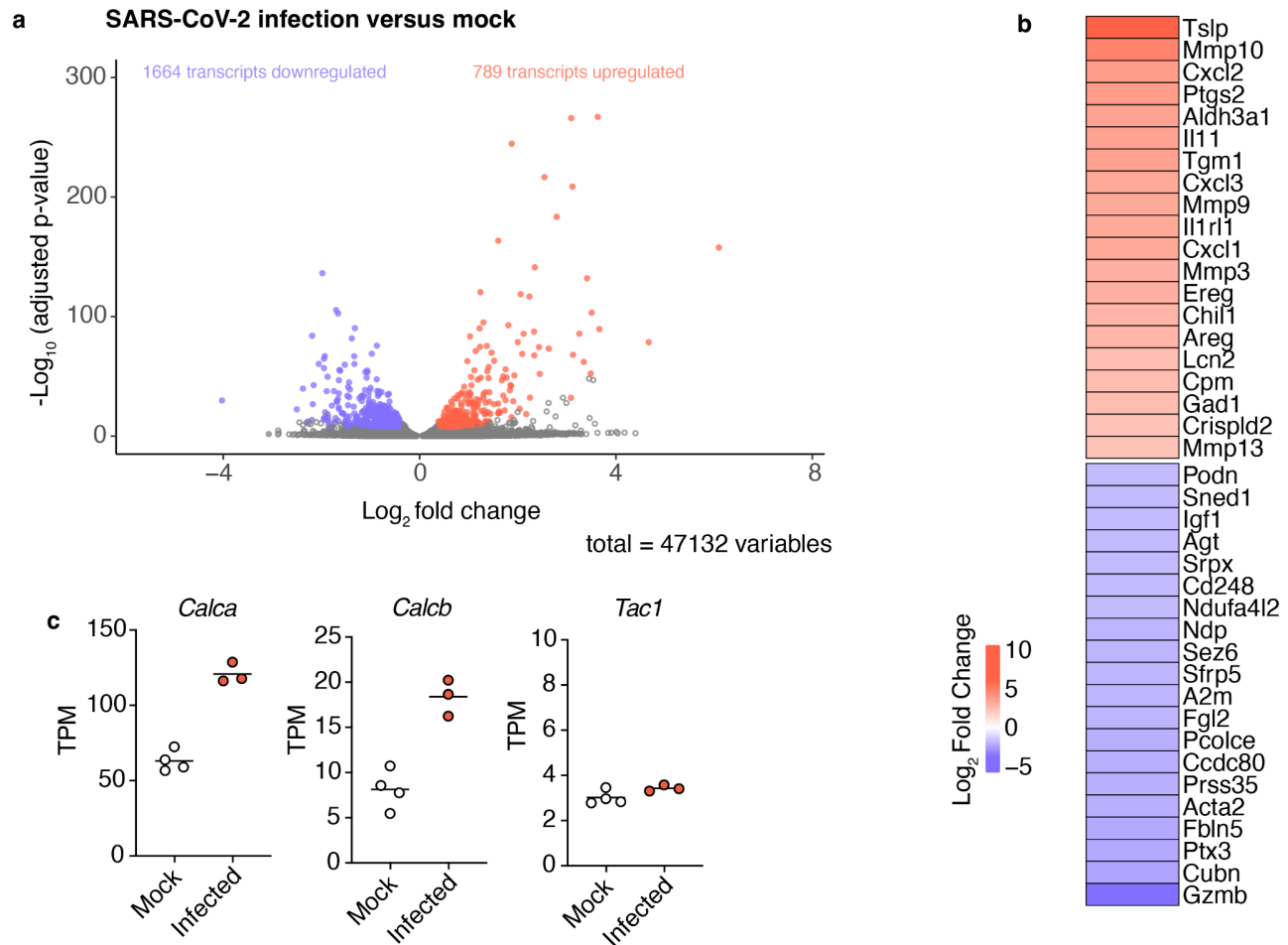
Supplemental Figure 2. PLpro triggers pain but not itch-associated behaviors.

a, PLpro elicited a shorter latency to the first wipe (One-way ANOVA: $p=0.003$, $F(2,18)=9.983$; Tukey's multiple comparisons, $p_{\text{Veh vs. } 10\mu\text{M PLpro}}=0.049$, $p_{\text{Veh vs. } 50\mu\text{M PLpro}}=0.003$, $p_{10\mu\text{M PLpro vs. } 50\mu\text{M PLpro}}=0.789$) and **b**, a dose-dependent increase in the number of wipes in the first minute. (Kruskal-Wallis: $p=0.0116$, $\chi^2=8.920$, Dunn's multiple comparisons, $p_{\text{Veh vs. } 10\mu\text{M PLpro}}=0.134$, $p_{\text{Veh vs. } 50\mu\text{M PLpro}}=0.021$, $p_{10\mu\text{M PLpro vs. } 50\mu\text{M PLpro}} > 0.999$), vehicle ($n=17$), PLpro (10 μM, $n=5$), PLpro (50 μM, $n=10$). **c-d**, PLpro does not elicit a shorter latency to scratching or any more scratching than vehicle injection, vehicle ($n=11$), PLpro (50 μM, $n=10$). Error bars represent the mean ± SEM. n = biological replicates (animals).



Supplemental Figure 3. PLpro activates small-diameter primary sensory neurons.

PLpro responders are of smaller diameter compared to vehicle responders and the population overall in **a**, neonatal mouse trigeminal ganglia (Veh: 22.6 µm, PLpro: 15.0 µm, AITC: 15.7 µm, Cap: 16.3 µm, High K+: 16.3 µm) **b**, adult DRG (Veh: 22.6 µm, PLpro: 16.2 µm, AITC: 22.1 µm, Cap: 19.8 µm, High K+: 21.0 µm) and **c**, adult NJG neurons (Veh: 22.5 µm, PLpro: 18.2 µm, AITC: 21.4 µm, Cap: 20.9 µm, High K+: 21.5 µm). **d**, PLpro from independent suppliers R&D Systems (#E-611, 5.5%, $n=17$) and Cayman (#31817, 5.7%, $n=12$) display no difference in the percent of responsive neurons in cultured TG, t-test: $t=0.13$, $df=27$, $p=0.898$. Error bars represent the mean \pm SEM.



Supplemental Figure 4. SARS-CoV-2 infection drives gene expression changes in cultured primary mouse trigeminal ganglia.

a, 48 hours of SARS-CoV-2 infection upregulates 789 and downregulates 1664 transcripts compared to mock infection in cultured adult trigeminal ganglia, mock infected: $n=4$ wells, SARS-CoV-2 infected $n=3$ wells. Significant differentially expressed genes > 152 base mean, > 0.37 \log_2 fold change and an adjusted p -value < 0.001 . **b**, Heatmap of the 20 most highly upregulated and downregulated transcripts (\log_2 fold change of infection vs mock). **c**, *Calca* and *Calcb* but not *Tac1* transcript counts (transcript per million [TPM]) are significantly upregulated by SARS-CoV-2 infection, *Calca*: adjusted $p_{\text{infection vs. mock}} = 9.05 \times 10^{-21}$, *Calcb*: adjusted $p_{\text{infection vs. mock}} = 5.52 \times 10^{-9}$, *Tac1*: adjusted $p_{\text{infection vs. mock}} = 0.991$.