

Evoked and spontaneous pain assessment during tooth pulp injury

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Abstract

Injury of the tooth pulp is excruciatingly painful and yet the receptors and neural circuit mechanisms that transmit this form of pain remain poorly defined in both the clinic and preclinical rodent models. Easily quantifiable behavioral assessment in the rodent orofacial area remains a major bottleneck in uncovering molecular mechanisms that govern inflammatory pain in the tooth. Here we use a dental pulp injury model in the mouse and expose the tooth pulp to the outside environment, a procedure we have previously shown produces pulpal inflammation. We demonstrate here with RNAscope technology in the trigeminal ganglion of injured mice, an upregulation of genes that contribute to the inflammatory pain state. Using both evoked and spontaneous measures of pain in the orofacial area, including application of von Frey Hair filaments and pain feature detection with the mouse grimace scale, we reveal a differential timeline of induction of spontaneous pain versus mechanical allodynia following pulpal injury. This work demonstrates that tooth pain can be easily assessed in freely behaving mice using approaches common for other types of pain assessment. Harnessing these assays in the orofacial area during gene manipulation should assist in uncovering mechanisms for tooth pulp inflammation and other forms of trigeminal pain.

1 **Introduction**

2 Pain from the infected tooth pulp (pulpitis) can be unrelenting and many patients report
3 this form of pain as the most intense type of pain they have ever experienced¹. Mechanical
4 hypersensitivity of the tooth is associated with greater pain intensity ratings overall². Prevailing
5 treatment options for painful pulpitis consists of pulp or tooth removal, which can have lasting
6 consequences for dental function and in some patients there may still be lingering pain^{3,4}.
7 Therefore, there is a critical need for development of new therapeutic approaches that alleviate
8 tooth pain while leaving pulpal issue intact and avoiding complex dental procedures. Moreover,
9 untreated ongoing inflammation of the pulp can lead to more widespread nociceptive
10 hypersensitivity in trigeminal tissues, an issue further compounded in individuals who cannot
11 afford proper dental treatment⁵. The era of new innovative approaches to treat tooth pain will be
12 driven by an increase in our fundamental understanding of the genes and neural circuit pathways
13 that drive tooth pain states. However, we first need to establish feasible and objective behavioral
14 paradigms that measure pain in the orofacial area in preclinical rodent models.

15
16 Clinically, mechanical hypersensitivity and spontaneous pain are particularly problematic
17 for patients²⁻⁴, and there are behavioral assessment tools for these in rodent models. To date,
18 only a handful of studies using the tooth pulp injury model have examined mouse behavior, and
19 these studies have not incorporated some of the common assays used to measure pain
20 hypersensitivity⁶⁻⁸. The predominant assessment tool for mechanical pain measurement in
21 rodents are reflexive withdrawal assays in which calibrated von Frey hair filaments (VFHs) are
22 applied to the hind paw and the experimenter decides which filaments evoke an animal's
23 withdrawal⁹. We have recently improved on this method by incorporating high-speed
24 videography, statistical modeling, and machine learning to more objectively assess the mouse
25 pain state following hind paw stimulation¹⁰. VFHs can also be applied to the face, but this
26 presents more challenges because the animal's attention is more engaged with the stimulus, as
27 we have previously experienced¹¹. However, recent elegant work in freely behaving mice used
28 both VFH stimulation of the whisker pad and optogenetic activation of trigeminal nociceptors to
29 uncover a craniofacial neural circuit for pain¹², so it is not impossible.

30
31 Another approach to measure spontaneous pain in rodents is a paradigm called the Mouse
32 Grimace Scale (MGS), that interrogates facial expressions including the positioning of the mouse
33 nose, cheek, ear, eye, and whiskers^{13,14}. An advantage of the MGS over reflexive assays is that
34 spontaneous pain resembles pain reports in the clinic and facial expressions are used in the clinic
35 to measure pain in infants, although these assays are currently not as high-throughput in rodents
36 as delivering stimuli and recording immediate responses. Together however, both reflexive and
37 spontaneous measurements of pain provide advantages in that they can be performed without
38 anesthesia, invasive implants, or time intensive tasks performed by the animal that require long
39 term learning and memory, which may mean that interpretation of the behavior may be
40 confounded by factors outside of the animal's pain level.

41
42 Here we take advantage of existing pain assays, with some custom modifications, and
43 adapt them for behavioral analyses during tooth pulp inflammation. After morphologically
44 confirming our tooth pulp injury model, we used RNAscope technology to determine the time
45 course of changes in molecular mediators of nociception relative to behavioral changes. To the
46 best of our knowledge, this is the first study using the MGS to evaluate pain following dental

47 injury, and our results revealed the occurrence of spontaneous pain within the first day following
48 dental injury. We also adapted previous facial Von Frey methods¹⁵ to evaluate mechanical
49 sensitivity, relying on the published scoring scheme, as well as the animals' willingness to put its
50 head through a custom designed chamber with an adjustable opening for stimulation. Because
51 the mice can decide if they want to expose their faces to the stimuli, we were also able to record
52 the threshold in which mice are no longer inquisitive enough to tolerate facial stimuli, and this
53 pain threshold was able to segregate injured versus sham mice. Interestingly, these two assays
54 present a different time course following injury, indicating spontaneous pain early and
55 throughout the 6 day observation period, while mechanical allodynia is delayed. Taken together,
56 the behavioral assays we have defined here to assess tooth pain should make it easier for
57 researchers to adopt these approaches to aid in uncovering mechanisms for tooth pain.

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60 Results

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62 Morphological and gene expression changes twenty-four hours after tooth pulp exposure

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64 In order to study inflammatory tooth pain, we used the dental pulp injury (DPI) model that we
65 have previously described⁶ in which the dental pulp of one maxillary molar tooth is mechanically
66 exposed using a dental drill, producing pulpitis. We began our first analyses 24 hours post-
67 injury, and confirmed controlled removal of enamel and dentin and exposure of the pulp

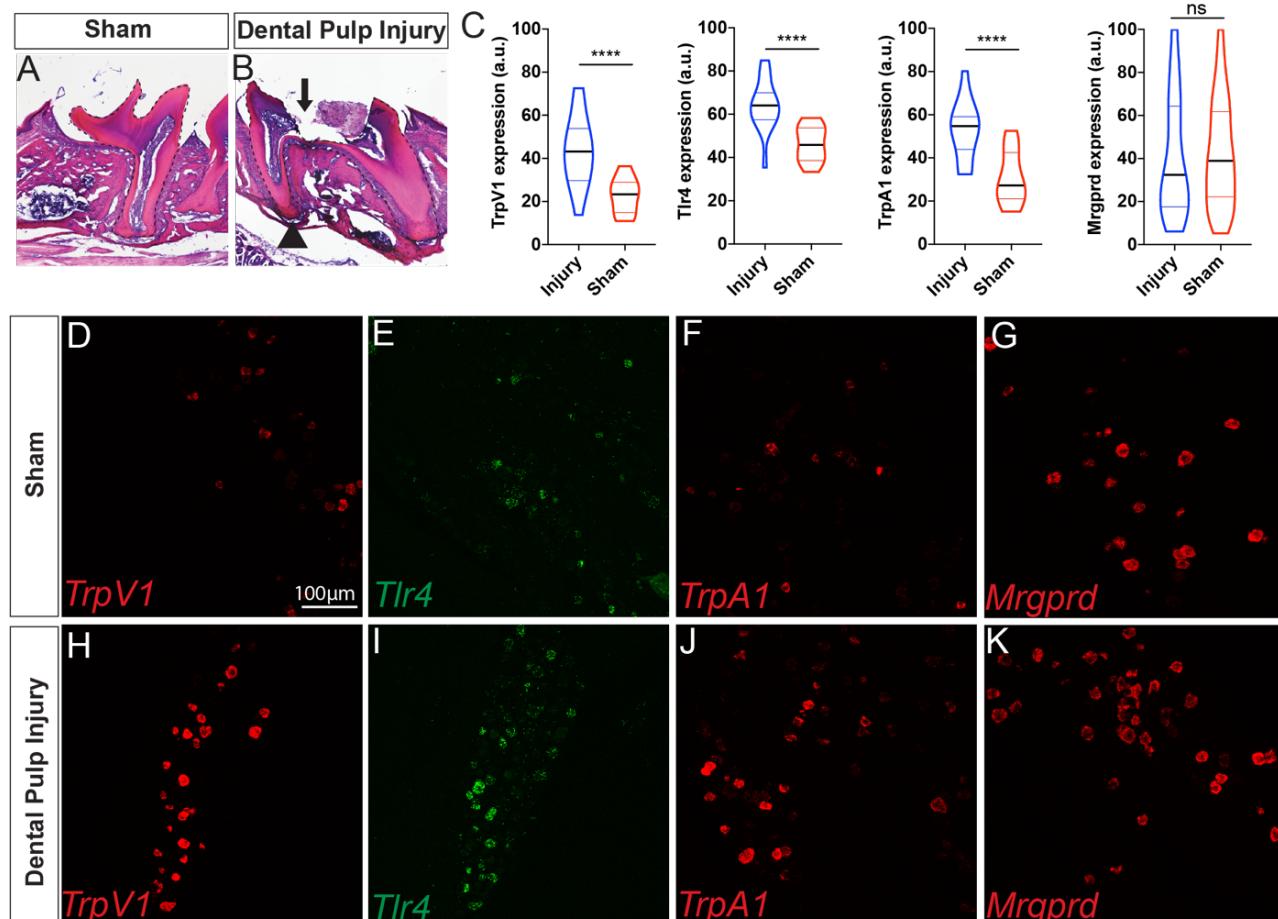


Figure 1. Changes in tooth morphology and trigeminal ganglia transcript levels 1 day following pulp injury. (A) Intact first maxillary molar from a sham animal and (B) injured maxillary molar with pulp exposure evident. Dotted outline marks the molar and the arrow indicates the exposure site. Healthy pulp is still evident on either side of the opening, and compacted foreign material (next to arrow) was present in the cavity. (C) Violin plot of quantified fluorescence intensity measured in arbitrary units (a.u.) defined in ImageJ for TrpV1, Tlr4, TrpA1, and Mrgprd in the trigeminal ganglia of injured (blue, n = 3 mice) or sham/naive (red, n = 3 mice) mice. (D-G) Representative images of RNAscope in situ hybridization following sham and (H-K) 1 day after dental pulp injury. *** is p<0.0001 for an unpaired T-test.

68 occurred in the molars of DPI mice (Fig.1A,B). Foreign material was microscopically present in
69 the tooth cavity of all 3 DPI mice, sometimes in contact with the pulp (material next to arrow in
70 Fig.1B), demonstrating that an exposed pulp collects materials from the mouse's outside
71 environment. Importantly, the pulp was still present and clearly exposed, and not yet necrotic,
72 within the injury site at 24 hours (arrow Fig.1B).
73

74 Next, we utilized RNAscope technology for a sensitive read-out of RNA levels in the
75 trigeminal ganglion of genes implicated in both nociceptive and inflammatory responses. The
76 cell bodies of the primary afferent neurons that innervate the dental pulp reside in the trigeminal
77 ganglion²⁴. We chose to assess the Toll-like Receptor 4 (Tlr4), transient receptor potential
78 channels vanilloid 1 and ankyrin 1 (Trpv1 and Trpa1), and the mas-related G protein coupled
79 receptor D (Mrgprd), because all are found in neurons that innervate the dental pulp^{7,20,22,25-27}
80 and could be involved in the development of either spontaneous or mechanical pain in the
81 context of infection and injury. In particular, TLR4 is part of a larger class of receptors that
82 recognize pathogen- and damage- associated molecular patterns (PAMPs and DAMPs)¹⁹, and
83 has known interactions with both TRPV1 and TRPA1 in the context of dental injury²⁰⁻²³. A
84 direct role for Mrgprd in dental injury-related pain has not been established, but is possible given
85 its expression in dental afferents²⁵ and its role in cutaneous mechanical pain perception²⁸. We
86 found that the PAMP/DAMP family member Tlr4 was upregulated in DPI versus sham mice
87 (Fig. 1C,E,I), as were the associated nociceptive channels TrpV1 (Fig. 1C,D,H), and TrpA1 (Fig.
88 1C,F,J). However, we did not observe an increase in the mechanosensitive nociceptor marker
89 Mrgprd at 24-hours following DPI, suggesting that gene expression changes of this nociceptive
90 membrane protein may not be driving the earliest phases of pain in the DPI model (Fig. 1C,G,K).
91

92 **Mouse grimace scale reveals presence of spontaneous pain beginning one day following 93 pulp exposure**

94 To assess spontaneous pain in freely behaving DPI and sham mice, we moved mice into clear
95 custom-made chambers and recorded video of their faces. Still images were selected from these
96 videos for assessment with the MGS (Fig. 2A). All mice, regardless of treatment exhibited very
97 low MGS scores at baseline, which was not different between the assigned treatment groups and
98 was not significantly affected by the sham treatment (Fig.2). We found a significant increase in
99 the MGS at all post-exposure time points captured (Fig.2C, there was a significant effect of time,
100 F_{3,30} = 5.776 p=0.0031, treatment F_{1,10} = 18 p=0.0017, and a significant interaction, F_{3,30} = 12.75
101 p<0.0001). The MGS features that differed in the DPI group versus sham were the pulling back
102 of the ears, nose and cheek bulging, as well as orbital tightening (Fig. 2B). These results are
103

104 consistent with the previous report showing that MGS scores are highest for pain emanating from
105 internal organs¹³, demonstrating that this assessment tool can be successfully co-opted for
106 painful pulpitis. This finding also indicates that mice experience ongoing pain within the first
107 day following pulp exposure that persists throughout the observation period.

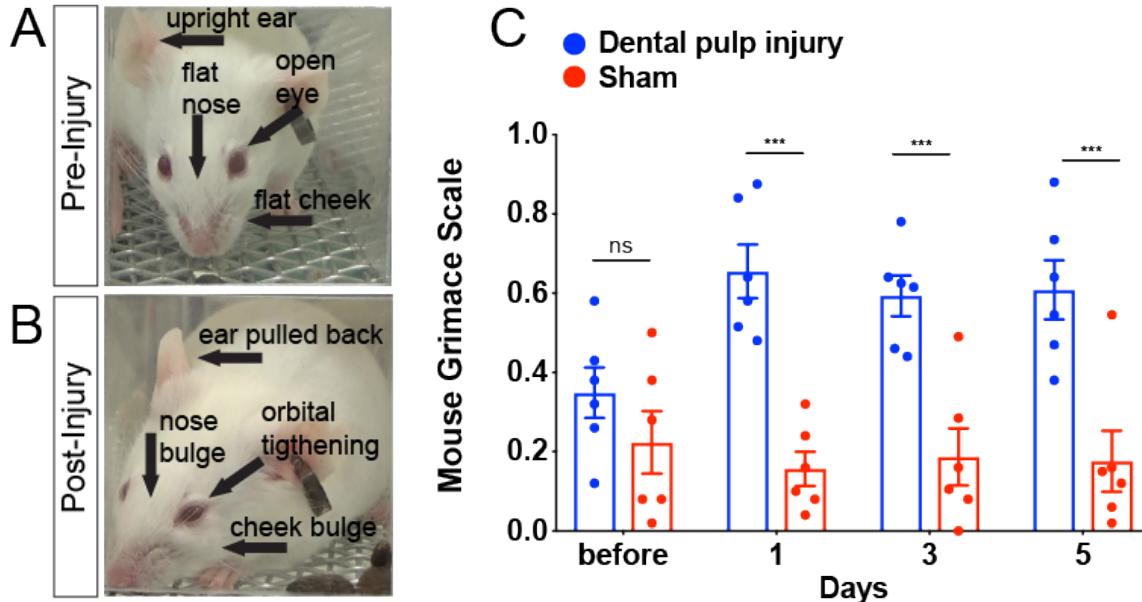


Figure 2. Mouse Grimace Scale following pulp exposure. (A) Before injury mice have low or no score for each of the action units, while after injury (B) prominent presence of the action units, as labeled on the example images from the same mouse. (C) We found a significant increase in the Mouse Grimace Score at all post-exposure time points captured, n = 6/treatment, Dental Pulp Injury (blue) and sham (red). * indicates p<0.0001 within the pulp exposed group before vs. after (Dunnett's post-hoc).

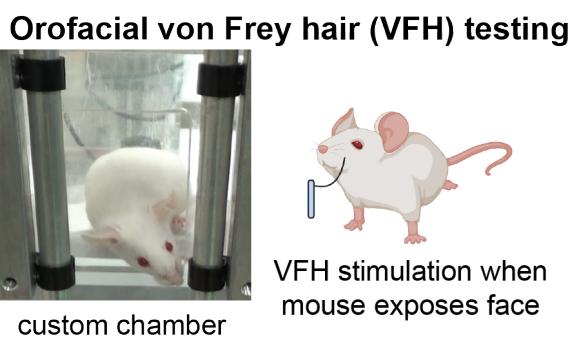
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109 **Mechanical allodynia in the face is fully developed by day 4 post pulp exposure and**
110 **worsens**
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112 To determine how mice respond to evoked stimuli, we applied von Frey hair filaments to the
113 skin between the whisker pad and eye in DPI and sham mice and recorded nocifensive
114 behavioral responses associated with withdrawal from the stimulus. Although our stimulus did
115 not touch the tooth directly, we hypothesized that we might observe hypersensitivity in the
116 orofacial skin surrounding teeth, which would indicate a more widespread trigeminal
117 sensitization, reminiscent of findings in the clinic when treatment complications arise^{29,30}.
118 Additionally, mice had to poke their heads through our custom-made chambers to allow the
119 VFHs to make contact with the facial skin. Using this paradigm, we observed that unilateral
120 exposure of tooth pulp on one molar changes response scores and thresholds for Von Frey
121 stimulation on both sides of the face suggestive of mechanical allodynia (Figs.3-5). The earliest
122 significant change was an increase in response scores across all VFHs averaged together, at day
123 2 on the contralateral side (Fig.3B, Contralateral: a significant effect of time: $F_{3,30} = 6.43$ p
124 = 0.0017, a significant interaction: $F_{3,30} = 7.69$ p=0.0006, but no significant effect of treatment
125 alone: $F_{1,10} = 2.272$ p =0.1626). By day 4, response scores are significantly increased in pulp-

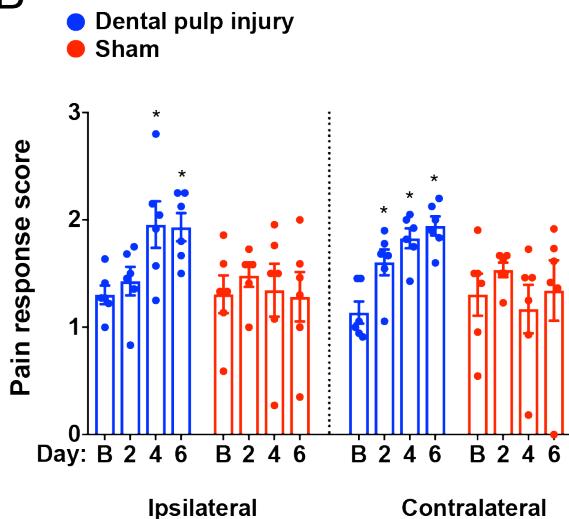
126 exposed mice on both sides, which persists on day 6 (Fig. 3B, Ipsilateral: significant effect of
127 time: $F_{3,30} = 3.302$ p =0.0336, no significant effect of treatment alone: $F_{1,10} = 2.34$ p =0.1571, but
128 significant interaction: $F_{3,30} = 4.836$ p=0.0073). Overall this indicates that injured mice, but not
129 shams, exhibit a gradual increase in response scores that is most evident on day 4 and maximal
130 on day 6.

131 We also examined the threshold where animals either scored a 3 or refused stimulation.
132 Raw thresholds (Fig. 3C) were log-transformed to better conform to normality (Fig. 3D) and
133 statistical analysis of transformed data indicated a significant decrease from baseline threshold
134 on both ipsilateral and contralateral sides beginning at day 4 post pulp exposure. On day 6, the
135 thresholds of pulp exposed mice were significantly lower than shams (a significant effect of
136 time: ipsilateral - $F_{3,30} = 6.981$ p =0.0011, contralateral - $F_{3,30} = 6.842$ p=0.0012, of treatment
137 ipsilateral - $F_{1,10} = 7.424$ p =0.0214, contralateral - $F_{1,10} = 4.981$ p = 0.0497, and interaction
138 ipsilateral - $F_{3,30} = 5.599$ p =0.0036, contralateral - $F_{3,30} = 4.01$ p=0.0164).

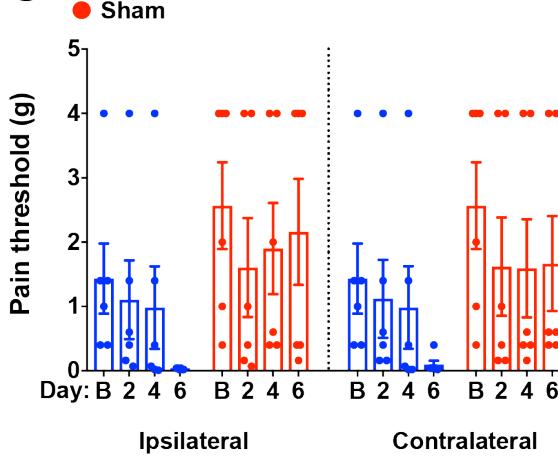
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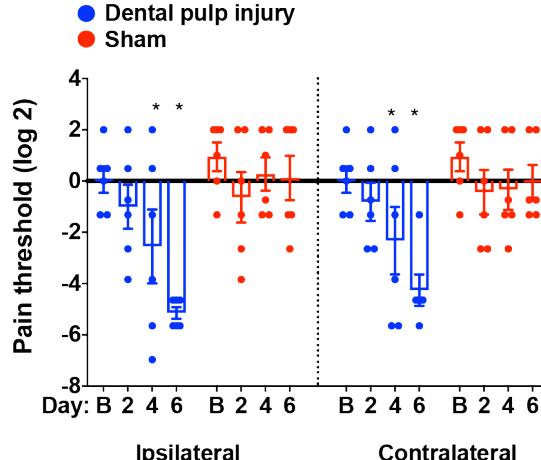
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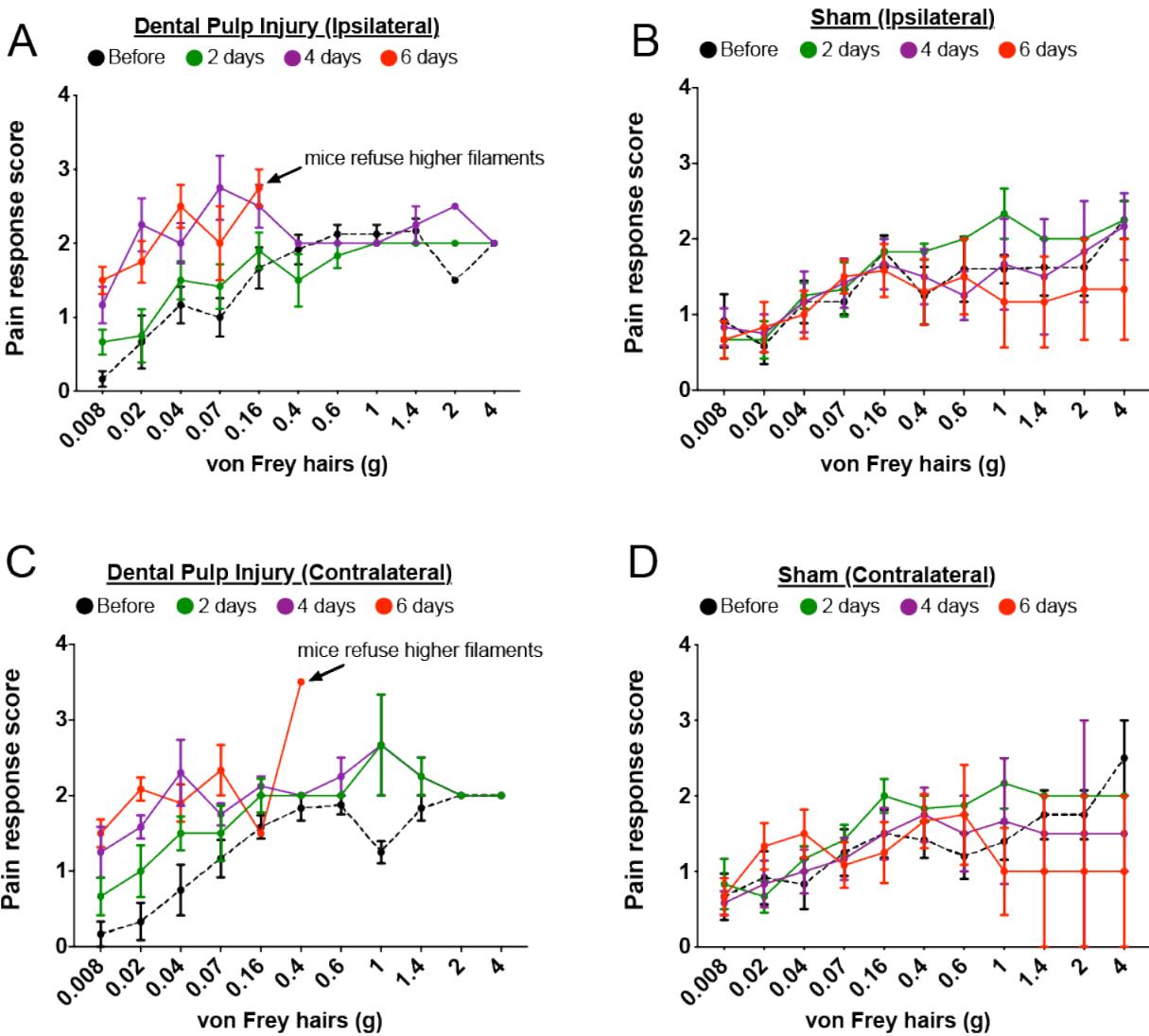
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Figure 3. Facial Von Frey apparatus, response scores, and threshold changes following pulp exposure. (A) The mice were placed in the chamber for testing, which has adjustable openings that can be closed between tests and allows mice to put their head past the bars if they choose. When mice choose to expose their faces they are stimulated as depicted with a von Frey hair. (B) After dental pulp injury (blue) there is a significant increase in pain response scores across all of the filaments tested, which is not observed in sham-treated mice (red). This is evident from day 4 onward on the ipsilateral side, and on day 2 onward on the contralateral side. (C) Raw threshold are significantly different from normality according to the Shapiro-Wilks Test, but a decrease with time is apparent in the injured group not observed in sham mice. (C) After log transformation of threshold data to conform to normality, we find a significant decrease in threshold following pulp exposure on both sides, evident from day 4 and significantly lower than shams at day 6. * indicates $p < 0.05$ for the indicated time point versus baseline (Dunnett's post-hoc).

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Next, we analyzed our Von Frey data in two different ways guided by the response score and the "break point" built into the assay design. The apparatus allows the mouse to learn over time that their natural urge to explore may result in mechanical stimulation to a hyperalgesic area, at which point they might choose not to expose their face through the opening and no stimulation would occur, i.e. their "break point". This may occur in the sham group also at the higher stimulus intensities as they are repeatedly tested. There is a large degree of disagreement in the field regarding what filament ranges constitute normally "painful" vs "non-painful" stimulation in the absence of injury or damage, which we have attempted to address for hind paw stimulation with VFHs¹⁰. Often this determination is made arbitrarily by the investigators based on human perception. Here, we use the Von Frey response scores and the break point to determine what range of filament weights correspond to a "non-painful" versus "painful" range. First, we examined the response scores by the weight of each VFH filament, to determine if they were higher across all intensities, which would suggest the presence of allodynia and hyperalgesia. Pulp exposed mice exhibit increasing response scores over time, particularly at lower filament weights (0.008-0.16g) on both sides (Fig.4A,C), which is not exhibited by the sham mice (Fig.4B,D). This seems to indicate mechanical allodynia in the injured group.

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Figure 4. Von Frey pain response score by filament intensity and day post-injury or sham. (A) Ipsilateral and (C) contralateral response scores for low intensity filaments are increased at day 4 (purple) following injury, and by day 6 (red) mice met threshold criteria or refused filaments higher than 0.16 or 0.4g. In contrast, (B) ipsilateral and (D) contralateral scores were similar across days post-sham procedure and low filament scores did not increase over time.

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Second, we determined the weight of filaments that correspond to the break point (when the mouse takes more than 5 minutes to pass its face out of the opening) for both DPI and sham mice for each testing day. As time following pulp exposure increases, the intensity of the filaments where the mice indicate stimulation should stop becomes lower on both sides (Fig.5A,C), such that by day 6 the break point occurs at 0.4g ipsilateral and 0.6g contralateral. In

172 contrast, although there is some change in the number of sham mice that tolerate stimulation with
173 filaments above 0.4g after 7 tests, at least two mice tolerated the entire range of filaments during
174 every test (Fig.5B,D). Taken together this indicates that exposure of one tooth pulp results in a
175 progressive development of mechanical allodynia, which is fully realized on day 4 post-exposure
176 and increases in severity by day 6.
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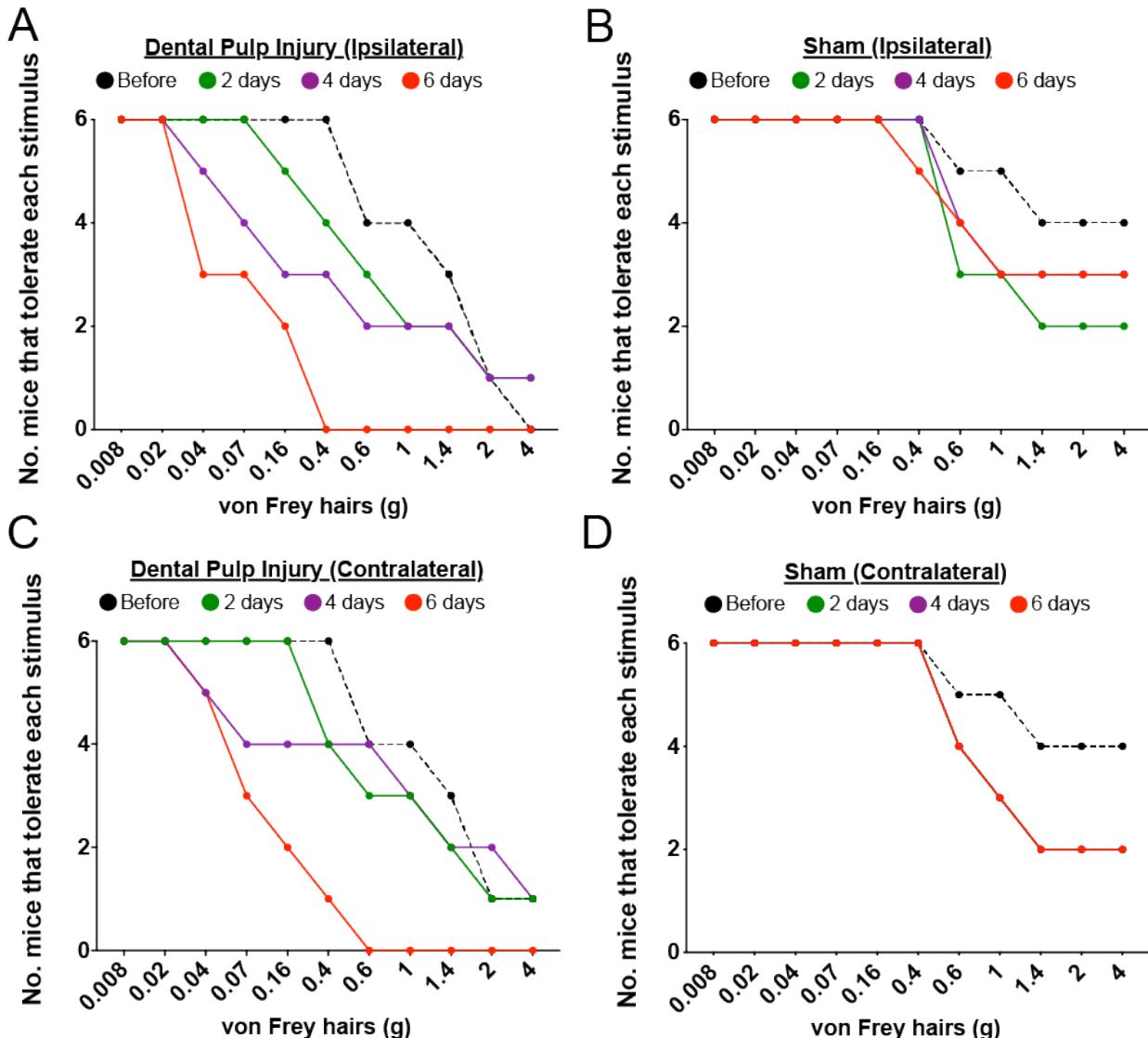


Figure 5. Loss of mouse participation by stimulus intensity (the break point) before and after injury or sham. On the (A) Ipsilateral and (C) contralateral sides, there is a progressive decrease in the number of injured mice willing to tolerate filaments higher than 0.4g, such that by day 6 none of them progressed further than 0.16g (ipsilateral) or 0.4g (contralateral). In contrast, on the (B) ipsilateral and (D) contralateral sides the sham cohort consistently tolerated testing with lower intensity filaments and at least two mice tolerated all the filaments across all testing days.

179 **Discussion**

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181 In this study, we found that unilateral pulp exposure injury in mice to the first maxillary molar
182 resulted in a statistically significant increase in MGS from the first 24 hours onward, which
183 indicates increased spontaneous pain. The pulp was still present at this time, but clearly exposed
184 when examined histologically, supporting that the behavior could capture pain originating from
185 the dental pulp, modeling pulpitis. Surprisingly, mechanical allodynia, as assessed by Von Frey
186 filament testing, progressed more gradually, with initial changes in scores observed only on the
187 contralateral side on day 2 post-injury, significant increases seen on both sides at day 4, and
188 unwillingness to tolerate filaments above 0.6g by day 6 post-injury. This work demonstrates that
189 we have clear easily identifiable behavioral readouts for tooth pain in the mouse. Associated with
190 these behavioral changes, we observed significant increases in transcript levels of Tlr4, Trpv1,
191 and Trpa1, but not Mrgprd, in the ipsilateral TG of injured mice as compared to controls at 24
192 hours post-injury. Taken together and in support of previous literature, we believe these findings
193 may suggest that Tlr4, Trpv1, and Trpa1, may contribute to early changes resulting in the
194 presentation of spontaneous pain, as indicated by the MGS. However, this does not rule out a
195 role for Mrgprd in the progressive development of mechanical allodynia that seems to worsen
196 around 4 days following pulp-exposure injury. Based on existing literature from other body
197 regions, TRPA1 could serve as a bridge between the early signaling indicated here and a
198 hypothesized later process involving MRGPRD, which we will discuss in further detail below.
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200 Part of our objective in this study was to establish a time course of behavioral changes
201 associated with tooth pulp exposure injury, which is considered by many to be a translationally
202 relevant model for pulpitis^{6,31}. To our knowledge, this is the first assessment of MGS following
203 tooth pulp exposure injury, and somewhat surprisingly the first for facial Von Frey in mouse
204 with this model as well. MGS and the rat equivalent RGS are significantly elevated following
205 other types of dental pain, including tooth movement³² and mechanical load injury to the
206 temporomandibular joint (TMJ)³³, but in both of these cases, the elevation in score is transient,
207 likely only corresponding with the presence of acute mechanical load. Our elevation in score
208 does not subside, possibly due to the more invasive nature of the injury and the fact that ongoing
209 inflammation is not being treated. In agreement with this idea, in a chronic model of trigeminal
210 neuropathic pain, significant change in MGS is observed 10 days following the constriction
211 injury³⁴. It is possible, however, that there may be site or model specific differences. MGS was
212 only transiently elevated following Complete Freund's Adjuvant inflammation of the TMJ³⁵,
213 which does not have ongoing infection occurring in the model. In terms of change in the score,
214 our data reflect a similar to slightly greater increase in MGS as compared to tooth movement³²,
215 and potentially within the lower end of ranges reported for an exogenous-CGRP migraine
216 model³⁶ and neuropathic injury of the infraorbital trigeminal nerve³⁴. This indicates that the
217 mice are likely in a level of discomfort or pain similar to other experimental pain states.
218 Spontaneous pain is diagnostically associated with irreversible pulpitis, supporting the
219 translational relevance of our findings²⁹.
220

221 In addition to spontaneous pain, greater than 50% of patients with irreversible pulpitis
222 also have mechanical allodynia with percussion of the tooth, and these patients have higher
223 ratings of spontaneous pain than those without allodynia²⁹. Facial Von Frey, an equivalent
224 means of testing mechanical sensitivity in rodents is challenging, but not impossible in the

225 mouse. Mechanical allodynia in the face has been examined in other experimental paradigms,
226 but has not been published following tooth pulp exposure injury. Most studies have used rats as
227 the model animal, and only one of these used the exact model we use here, where the pulp is left
228 exposed and not treated with exogenous substances³¹. Our findings are in agreement with this
229 previous work in rats. Tsuboi and colleagues also observed a reduction in threshold both
230 ipsilateral and contralateral to the injury, first detectable at day 3, which worsened at day 5 and
231 persisted at least 3 weeks later³¹. This period of time around day 3 or day 4 seems to mark a
232 transitional state between the acute inflammatory response and development of pathological pain
233 states often associated with chronic or ongoing pain. We speculate that the early change in MGS
234 may be established by either the same or different mechanisms than those that produce
235 mechanical allodynia later.
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237 To begin to address this question, we examined the mRNA expression of Tlr4, trpv1,
238 Trpa1, and Mrgprd using *in situ* hybridization at 24 hours following pulp exposure injury. A
239 great deal of attention has been paid to TLR4 as a possible drug target for the treatment of
240 inflammatory pain in various parts of the body¹⁹, but particularly in pulpitis given its role in
241 recognizing molecular signals of bacterial presence and mechanical injury and upregulation in
242 human pulpitis samples²⁰. Furthermore, antagonism of TLR4 is associated with reversal of
243 pain-associated behaviors in two different rat models of pulpitis^{7,37}, including mechanical
244 hypersensitivity in lightly anesthetized rats³⁷. Our findings of increased Tlr4 in the trigeminal
245 ganglia 24 hours following pulp injury suggest an association between the function of this
246 receptor and at least increased malaise or spontaneous pain associated with increased MGS. We
247 need to directly antagonize TLR4 in the context of pulp-exposure injury to verify causality for
248 increased MGS and determine if early intervention might prevent the delayed presentation of
249 mechanical allodynia. It is possible that TLR4 upregulation begins a cascade of molecular
250 events, as of yet not clearly identified, that establish a change in mechanical sensitivity.
251

252 Coinciding with the increase in TLR4 we also observed an increase in Trpv1 mRNA
253 expression at 24 hours post injury, similar to increased protein expression found in rats with pulp
254 exposure or CFA-induced pulpitis models^{7,38}. Upregulation of the nociceptive channel TRPV1
255 has been demonstrated within 24 hours of LPS application to the tooth pulp, but returned to
256 control levels 3 and 5 days later²⁶. Furthermore, LPS can directly act on TRPV1+ trigeminal
257 nociceptors via TLR4 signaling²¹. Antagonism of TRPV1 in the CFA model blocks mechanical
258 hypersensitivity in lightly anesthetized rats³⁸, suggesting that TRPV1 could be involved in the
259 development of mechanical allodynia in our pulpitis model. However, given the delayed
260 progression of mechanical allodynia reported here, it is likely that other events downstream of
261 the increased TRPV1 expression in the ganglia are also involved in the pulp exposure model.
262

263 We also observed an increase in the expression of Trpa1 in the ipsilateral TG at 24 hours
264 post-injury. TRPA1 is also of interest in the pathology of painful pulpitis, but only one other
265 study has examined protein expression following pulp exposure injury in rat molar³⁹. They also
266 observed increased expression of TRPA1, but it was not significant until Day 4³⁹. Our differing
267 results may be due to species differences, or may reflect a disconnect between the time to peak
268 mRNA levels versus protein levels. Like TRPV1, there is also evidence for an interaction
269 between TLR4 or LPS and TRPA1-related activity. In the DRG, there is evidence that TRPA1 is
270 required for direct nociceptor responses to LPS, even in the absence of TLR4²². LPS increases

271 the percentage of trigeminal neurons responding to the TRPA1 agonist acyl-isothiocyanate
272 (AITC) as demonstrated by calcium imaging²³. TRPA1 has been implicated in the development
273 of mechanical allodynia in the lower body⁴⁰, thus could be involved in the mechanical allodynia
274 reported here.

275
276 While we observed increased expression of Tlr4, Trpv1, and Trpa1 24 hours post pulp
277 exposure, we did not observe an increase in Mrgprd, also found in the pulp²⁵ and directly
278 implicate in cutaneous mechanical nociception^{28,41}. However, this does not completely rule
279 involvement of Mrgprd+ trigeminal neurons in the development of delayed mechanical
280 allodynia. Future studies will evaluate the expression of Mrgprd closer in time to the
281 manifestation of mechanical allodynia around day 3 or 4. It is also possible that TRPA1 could
282 serve as a mechanistic bridge between the early upregulation of TLR4 and TRPV1 and a
283 currently unverified but likely later involvement of Mrgprd following tooth pulp exposure injury.
284 In the DRG recent studies suggest that TRPA1 exists as part of two populations, one that is co-
285 localized with TRPV1 and/or TLR4 in a primarily peptidergic population, which has been the
286 dogma for this channel until very recently, and the other non-peptidergic population containing
287 MRGPRD⁴². Functional studies suggest that at baseline functional TRPA1 protein is actually
288 more frequent in the IB4 positive "non-peptidergic" cell population, which contains MRGPRD,
289 than in the CGRP+ population⁴². The authors suggest that the interaction between TRPA1 and
290 Mrgprd may be broadly important for the development of mechanical allodynia⁴². Very recent
291 evidence suggests that Mrgprd activation by its agonist beta-alanine results in phosphorylation of
292 TRPA1 via Protein Kinase A⁴³, but it is not clear if TRPA1 may also influence any aspect of
293 Mrgprd expression or function. The overlap of TRPA1 and Mrgprd in the tooth pulp afferents
294 has not been explored. Alternatively, paracrine signaling in the trigeminal ganglia via gap
295 junction connections with satellite glia^{37,38,44} could allow for recruitment of the non-peptidergic
296 TLR4 negative MRGPRD population by the peptidergic TLR4+/TRPV1 and/or TRPA1+ cell
297 populations to produce the delayed mechanical allodynia we observed.
298

299 Although Tlr4 has been heavily examined for its ability to recognize LPS produced by
300 gram-negative bacteria and to influence nociceptor function, it should be cautioned that this is
301 likely not the only potential therapeutic target. Gram negative bacteria are likely to be more
302 involved in the early stages of infection⁴⁵, which may occur before patients make it to the clinic.
303 Bacteria most highly associated with cold and heat sensitive irreversible pulpitis infections are
304 actually gram positive⁴⁶, and may influence pulp nociceptors by different mechanisms than LPS-
305 mediated activation of TLR4 or other TLRs. Other bacterial products⁴⁷ or aspects of
306 inflammation, such as oxidative stress may also recruit both TRP channel expressing
307 nociceptors⁴⁸ and Mrgprd⁴⁹. Clearly, more work is needed on our path to improving the care of
308 endodontic patients.
309

310 **Authors Competing Interests Statement**

311
312 No competing interests to declare.
313

314 **Author Contributions**

315

316 HLR, LPS, JG, BS, CHM, and IAS designed experiments. HLR and LPS carried out
317 experiments, and WF and SM scored florescent intensity of RNA scope images. All authors
318 contributed to the writing and editing of the manuscript.

319

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321

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327

328 **Methods**

329

330 **Animals**

331

332 For these studies we used male and female adult wildtype consisting of a mixed CD1 and
333 C57BL6/J background. Mice were 17-21 weeks old at the time of testing. Mice were maintained
334 in a standard 12:12 light dark cycle (lights on at 07:00) tested within a time range of 08:30 –
335 13:00. Mice had access to food and water ad libitum when not being tested. All procedures
336 were approved by the University of Pennsylvania Institutional Animal Care and Use Committee
337 and follow the guidelines established by the National Institutes of Health.

338

339 **Dental Pulp Injury**

340

341 Mice were anesthetized with ketamine/xylazine (i.p. 100 mg/kg and 12.5mg/kg respectively) and
342 positioned under a dissecting microscope and warming pad on their back, with their head
343 supported at an angle, and their mouth propped open with forceps. After trimming the oral
344 whiskers, the upper first maxillary molar was drilled on one side using ¼ round carbide burr until
345 the enamel and dentin layers were breached and the pulp was exposed. This process took about
346 5 minutes. The enamel is hard and white, the dentin is gray, and when the pulp is visible
347 vasculature and white to pink tissue can be seen in the hole in the enamel under the microscope.
348 Sham animals underwent the same anesthesia, positioning and oral manipulation, but their teeth
349 were not drilled. We provided moist food and monitored body weight following the procedure.
350 Weight loss did not exceed 10%. Mice were either used for behavioral testing on days 1-6 post
351 procedure (n = 6/ treatment group), or were immediately euthanized on day 1 (n =3 injured) to
352 collect tissue. The same set of mice was used for Mouse Grimace Scale and Von Frey testing,
353 performed on alternating days.

354

355 **Mouse Grimace Scale**

356

357 The Mouse Grimace Scale is a scoring system developed in the laboratory of Jeff Mogil to
358 objectively evaluate pain-like facial expressions following experimental procedures¹³, which has
359 been adopted for many trigeminal pain models^{33,34,36}, but not yet used to evaluate tooth pain in
360 rodents. Mice (6/treatment group) were acclimated in the chambers at least twice prior to
361 baseline testing, and were in the chamber for 10 minutes before recording began each day.

362 Before the procedure and on days 1, 3, and 5 after injury, we video recorded mice for 10 minutes
363 in clear acrylic chambers (4.3 W x 4.3 H x 11 L cm) on a mesh platform from the small end of
364 the chamber with a camcorder (Sony, HVC) with digital zoom. A 3-way mirror was placed at
365 the back of one end to facilitate assessment of unilateral grooming and to prevent the mouse
366 from viewing the next acclimating mouse. From the 10-minute video, one still image for every
367 second of video was extracted using Video to Picture Converter Software (Hootech). From these
368 ~600 images, 10 were selected that contained a clear view of the animal's face. All of the 480
369 selected baseline, sham, and post-pulp exposure images were cropped to show only the face and
370 randomized for scoring in a Power Point file. Scoring was performed blind to day and treatment,
371 as indicated in the original method for 5 action units (orbital tightening, nose bulge, cheek bulge,
372 ear position and whisker change), from 0 (not present) to 2 (very visible), and action units were
373 averaged to arrive at the score for each image¹³. In some cases, the whiskers could not be
374 viewed, so this unit was omitted for the score of that image. Performing the statistical analysis
375 with or without the whisker change action unit did not affect the overall statistical results. For
376 example images of a mouse before and after pulp exposure, see Fig. 2 A,B.
377

378 **Mechanical Allodynia Assessment by Von Frey**

379

380 For these studies we placed the animals in confined chambers with adjustable openings (Fig. 3).
381 The mice were contained chamber about 7cm in all directions, with an opening as wide as 2.5
382 cm. The animals were acclimated to the chambers once for 30 minutes the day prior to baseline
383 testing. Their natural tendency is to put their face out of the opening when it is wide enough, but
384 they are elevated from the floor, which prevents immediate escape. In this way, we can prompt
385 the animal to present its face for stimulation. We then stimulated twice on either side of the face,
386 alternating between sides, aiming for the region including the vibrissae to the point in front of the
387 eyes. The animal's response was scored from 0 to 4 based on early work in rats with neuropathic
388 injury^{15,50} (see Table 1 for score description). We considered "threshold" to be the filament that
389 either produced a score of 3 followed by a response of 2 or more, or the point the animal was no
390 longer willing to pass its face out of the opening after about 5 minutes. The animals were tested
391 on days 2, 4, and 6 post injury with the full filament series (Baseline Tactile Sensory Evaluators,
392 consisting of 11 graded filaments from 0.008g to 4g).
393

394

Table 1. Score for Responses to Facial Von Frey

Score	Response
0	no response
1	orientation to the stimulus or a slower head turn away from the stimulus
2	a rapid withdrawal that may or may not be followed by a single facial wipe
3	attacking or biting the filament or rapid withdrawal followed by 2 facial wipes
4	a rapid withdrawal with multiple facial wipes

395

396

Preparation of Tissue for Histology

397

398

399 At 1 day post-injury, sham and naive, mice were deeply anesthetized with ketamine/xylazine and
400 perfused with cold Phosphate Buffered Saline followed by 4% paraformaldehyde through the
401 heart. We removed their trigeminal ganglia and either the remaining cranium or just the portion
of the mouth containing the teeth and nerve roots. Trigeminal ganglia and mandible regions

402 were post fixed for up to 4 hours and overnight, respectively. Trigeminal ganglia were placed in
403 30% sucrose until they sunk (overnight), and then frozen in Neg50 media for cryosectioning
404 (20 μ m). Teeth were placed in 10% EDTA for approximately two weeks to decalcify,
405 cryoprotected in 30% sucrose, embedded and cryosectioned (20 μ m). All tissues were sectioned
406 on a Leica cryostat onto superfrost plus slides, in a series of 16 (TGs) or 10 (teeth). Adjacent
407 series of TG sections were selected for *in situ* hybridization using the RNAscope system for 2
408 probes. Four sections per left and right TGs from 3 animals were mounted on one slide. One
409 series from the teeth underwent standard hematoxylin and eosin staining to visualize injury
410 related alterations in the tissue.

411

412 ***In Situ* Hybridization using RNAscope**

413

414 Trigeminal ganglia were prepared using a modified version of the manufacturer's
415 recommendations for fixed frozen tissues used for fluorescence visualization. Briefly, slides
416 were dehydrated in a graded series of alcohol, peroxidase activity was blocked with hydrogen
417 peroxide, and protease IV was applied to the tissue for 30 minutes at room temperature before
418 undergoing the RNAscope Multiplex Fluorescent v2 assay (ACD). The assay was performed
419 according to the manufacturer's protocol using two probes. TG sections were assessed for
420 overlap between Tlr4 (channel 1) and either Trpa1, Trpv1, or Mrgprd (channel 2). Channel 1
421 was visualized using opal dye 520 and channel 2 was visualized with opal dye 570 (1:1500 for
422 both dyes). Tissues were imaged on a Leica SPE TCM using the same laser power and gain
423 settings for all slides. Because we did not know the time course of pain changes in our DPI
424 model, we opted to leave the pulp exposed, rather than applying a dye after exposure and sealing
425 it and the injury site. Thus, we could not be fully certain that the neurons we visualized in the
426 trigeminal ganglion came from the tooth pulp versus other trigeminal tissues. However, our own
427 preliminary studies and others²⁴ have shown that maxillary molar labeling with the Dil paste
428 Neurotrace (Invitrogen) results in positive cells in all branches of the trigeminal nerve, therefore
429 we imaged cell clusters observed in both the region where V3 and V2 meet, as well as the region
430 where V1 and V2 meet, resulting in 2 images per section with the 20x objective. All cells with
431 detectable signal were selected for quantification and the signal intensity of mRNA clusters
432 observed within each cell was analyzed by drawing a region of interest around each cell and
433 mean signal intensity in arbitrary units generated by ImageJ software was noted. The dimensions
434 of the region of interest were kept constant throughout the analysis to avoid bias. This process
435 was repeated for each channel including overlay images. The entire quantification was
436 performed by an observer in a manner blinded to mRNA probes and channel assignments.

437

438 **Statistical Analysis**

439

440 Data were assessed for normality using the Shapiro-Wilkes test. Raw Von Frey thresholds were
441 log-transformed to achieve normality so that parametric statistical tests could be used. For Von
442 Frey and Grimace behaviors, two-way ANOVA with repeated measures and between subjects
443 effects were used to determine if there were any significant effects of time, treatment, or a
444 significant interaction, followed by with post-hoc Dunnett's and Sidak tests where appropriate.
445 For fluorescence intensity, an independent sample t-test was used. We consider p <0.05
446 significant, and used Graph Pad Prism (v8).

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