

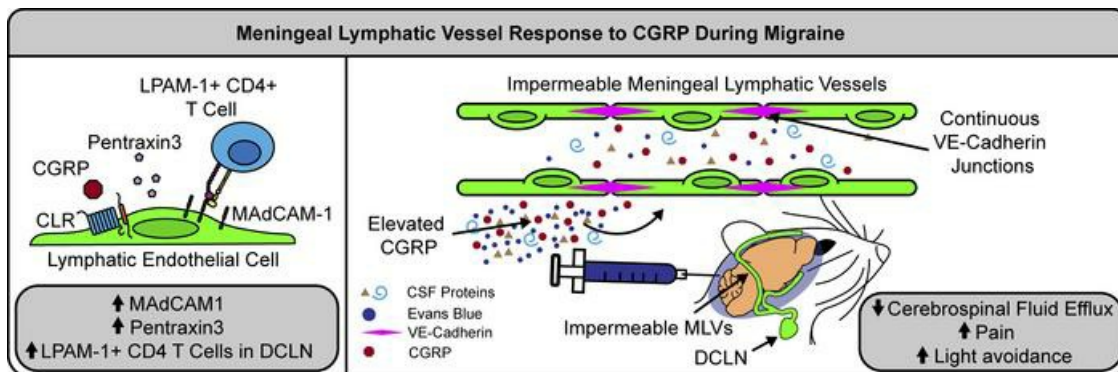
# Meningeal lymphatic CGRP signaling governs pain via cerebrospinal fluid efflux and neuroinflammation in migraine models

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## Graphical abstract



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1 **Meningeal lymphatic CGRP signaling governs pain via cerebrospinal fluid efflux**  
2 **and neuroinflammation in migraine models**

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10

11 The authors have declared that no conflict of interest exists.

## 12 **Abstract**

13 Recently developed anti-migraine therapeutics targeting calcitonin gene-related peptide (CGRP)  
14 signaling are effective, though their sites of activity remain elusive. Notably, the lymphatic  
15 vasculature is responsive to CGRP signaling, but whether meningeal lymphatic vessels (MLVs)  
16 contribute to migraine pathophysiology is unknown. Mice with lymphatic vasculature deficient  
17 in the CGRP receptor (*Calcr<sup>fl<sup>LEC</sup></sup>* mice) treated with nitroglycerin (NTG)-mediated chronic  
18 migraine exhibit reduced pain and light avoidance compared to NTG-treated littermate  
19 controls. Gene expression profiles of lymphatic endothelial cells (LECs) isolated from the  
20 meninges of *Rpl22<sup>HA/+</sup>;Lyve1<sup>Cre</sup>* RiboTag mice treated with NTG revealed increased MLV-immune  
21 interactions compared to cells from untreated mice. Interestingly, the relative abundance of  
22 mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1)-interacting CD4<sup>+</sup> T cells was  
23 increased in the deep cervical lymph nodes of NTG-treated control mice but not in NTG-  
24 treated *Calcr<sup>fl<sup>LEC</sup></sup>* mice. Treatment of cultured hLECs with CGRP peptide in vitro induced vascular  
25 endothelial (VE)-cadherin rearrangement and reduced functional permeability. Likewise, intra  
26 cisterna magna injection of CGRP caused rearrangement of VE-Cadherin, decreased MLV  
27 uptake of cerebrospinal fluid (CSF), and impaired CSF drainage in control mice, but not  
28 in *Calcr<sup>fl<sup>LEC</sup></sup>* mice. Collectively, these findings reveal a previously unrecognized role for  
29 lymphatics in chronic migraine, whereby CGRP signaling primes MLVs-immune interactions and  
30 reduces CSF efflux.

31

## 32 **Introduction**

33 The meninges have traditionally been considered a protective and physical barrier to the brain,  
34 shielding the central nervous system from external trauma or systemic insults such as infection  
35 and inflammation. More recently, a complex network of meningeal lymphatic vessels (MLVs)  
36 within the dura mater layer of the meninges have been studied intensely, revealing unexpected  
37 and critical functions for the meninges in normal cerebrospinal fluid (CSF) turnover (1, 2) and in  
38 pathological conditions such as Alzheimer's disease, Parkinson's disease, aging, and  
39 neuroinflammation (3-6). Mice also have MLVs that course along the superior sagittal sinus and  
40 transverse sinus on the dorsal aspect of the cranium and along the petrosquamosal and sigmoid  
41 sinuses including localizations near the retroglenoid vein and pterygopalatine artery on the  
42 basal aspect of the cranium (1, 2, 7). Drainage of CSF to the deep cervical lymph nodes (DCLN)  
43 can be visualized by intra cisterna magna (ICM) injection of tracers into the CSF (3, 7, 8),  
44 validating the physiological function of MLVs in CSF drainage. The lymphatic system also  
45 functions as a conduit for immune cells and antigens from peripheral tissues to the tissue  
46 draining lymph nodes using well-established chemotactic gradients such as CCL21 and CCL27 to  
47 attract CCR7 and/or CCR10 expressing immune cells (9). This CCR7-CCL21 immune cell  
48 trafficking was observed to occur in a mouse model of multiple sclerosis, thus validating that  
49 this well-established paracrine signaling is present in the MLVs as well. Therefore, it is  
50 surprising that only a few studies have begun to explore the involvement of MLVs in the highly-  
51 prevalent condition of chronic migraine (10).

52 Chronic migraine is a pain syndrome that has neuronal, vascular, and immunologic components  
53 (11) and is classically mediated by elevated levels of the neuropeptide calcitonin gene-related

54 peptide (CGRP) in both plasma (12-14) and CSF (15-17). CGRP is a potent vasodilatory  
55 neuropeptide that is released from trigeminal C-fibers during migraine(18, 19) and is  
56 hypothesized to act upon dural and cerebral vessels, including the superior sagittal sinus (an  
57 anatomical location of the dorsal MLVs) to cause vasodilation and consequent stimulation of  
58 immune cells (20). This meningeal neurogenic inflammation impacts cells from both innate and  
59 adaptive immune responses, provoking inflammatory responses including  $IL-1\beta$ ,  $IL-6$ ,  $TNF-\alpha$ ,  
60 leukotrienes, prostaglandins, soluble VCAM-1, and soluble ICAM-1, as well as transient blood  
61 brain barrier breakdown allowing for immune cell transmigration into the CNS (21-24).  
62 Meningeal neurogenic inflammation is also hypothesized to support a feed-forward pain  
63 pathway, contributing to the overall pathophysiology of migraine with the trigeminal ganglion  
64 serving as critical component of the ascending CNS pain pathways to the thalamus and  
65 hypothalamus and the dorsal horn of the spinal cord, playing a central role in the onset of  
66 ensuing headache pain (25). Non-steroidal anti-inflammatory drugs (NSAIDS) are effective as a  
67 migraine abortive medication and are often first choice medications to treat migraine acutely.  
68 However, NSAIDS are ineffective in a large proportion of migraine patients, are not  
69 recommended for chronic use, and migraine therapeutics generally do not target inflammation  
70 (11). Consequently, several new medications recently approved by the FDA target the CGRP  
71 signaling axis and are effective at reducing both the duration and intensity of migraine pain:  
72 monoclonal antibodies to the CGRP receptor (erenumab), monoclonal antibodies to plasma  
73 CGRP (galcanezumab, fremanezumab and eptinezumab), and small molecule antagonists for  
74 the CGRP receptor (26).

75 Though these novel antibody medications targeting CGRP and the CGRP receptor have shown  
76 promise in migraine treatment, their precise sites of action—which according to the  
77 pharmacodynamics of monoclonal antibodies must reside outside the blood brain barrier—  
78 remain poorly understood. As a potent vasodilator and vascular permeability agent CGRP  
79 targets the smooth muscle and endothelial cells of large cerebral blood vessels of the  
80 meninges, suggesting that some of the therapeutic effectiveness of anti-CGRP medications is  
81 imparted through modulation of the vasculature (21). The CGRP receptor is a heterodimer  
82 formed from Calcitonin-receptor like-receptor (gene: *Calcrl*, protein: CLR) and receptor activity  
83 modifying protein 1 (gene: *Ramp1*, protein: Ramp1) and interestingly, is expressed at higher  
84 levels in lymphatic endothelial cells (LECs) compared to blood endothelial cells (27, 28).  
85 Consistently, mice and humans with homozygous loss-of-function mutations in the gene that  
86 encodes CLR die in utero from non-immune hydrops fetalis and arrested developmental  
87 lymphangiogenesis (29, 30). Lymphatic-specific deletion of *Calcrl* in mice recapitulates the  
88 edematous hydrops fetalis phenotype of global *Calcrl* deletion, demonstrating the requisite role  
89 for CLR signaling in lymphatic development. In adult animals, tamoxifen-mediated deletion of  
90 *Calcrl* in lymphatics causes impaired intestinal lymphatic vessel (LV) lipid uptake and intestinal  
91 lymphangiectasia (31, 32), illustrating the necessity of sustained CLR signaling in maintaining  
92 lymphatic structure and function during adulthood. Moreover, global loss of the *Ramp1* gene,  
93 which encodes a requisite chaperone protein required for CGRP signaling (33-36), also causes  
94 impaired lymphatic growth and function (37). Collectively, these data support a principal role  
95 for CGRP receptor signaling in lymphatics during development and adulthood.

96

97 At the cellular level, activation of the CLR G protein-coupled receptor pathway results in  
98 increased MAPK/ERK and pAKT downstream signaling in LECs, culminating in cellular  
99 proliferation (38). CLR signaling can also support the transactivation of other important,  
100 growth-promoting lymphatic signaling pathways, such as vascular endothelial growth factor-C  
101 (VEGFC)/VEGFR3 signaling, both in cultured cells and in vivo (39). Finally, activation of CLR  
102 stimulates the rapid and robust reorganization of LEC junctional and gap proteins, like vascular  
103 endothelial (VE)-cadherin, zonula occludens-1, and connexins, to tighten intercellular barriers  
104 and reduce lymphatic permeability (30, 40, 41). Most of these cellular effects of CLR signaling  
105 have been attributed to the activation of the CLR-RAMP2 heterodimer by adrenomedullin (AM)  
106 ligand, leaving open the possibility that migraine-induced CGRP ligand may elicit similar effects  
107 through CLR-RAMP1 heterodimers in LECs of meningeal lymphatic vessels.

108

109 There are several animal models of chronic migraine that faithfully recapitulate many aspects of  
110 human chronic migraine pathophysiology, including elevations in CGRP (42-44). Some migraine  
111 models, including direct stimulus of cortical spreading depression require invasive procedures  
112 including burrhole craniotomy or skull thinning, potentially damaging the delicate underlying  
113 meninges or MLVs or provoking trauma-induced inflammatory responses (12, 42, 43). Peripheral  
114 or central administration of CGRP remains a widely-used and translationally-relevant model of  
115 migraine in multiple species with resultant systemic vasodilation, peripheral allodynia,  
116 hyperalgesia, periorbital hypersensitivity, and immobile behaviors (42, 43). Finally, i.p. injection  
117 of nitroglycerin (NTG), a potent NO donor, sensitizes the trigeminovascular system to cause the  
118 release of endogenous CGRP from trigeminal ganglion neurons, evoking migraine-like pain and

119 associated behaviors in rodents including aversion to light and hyperalgesia in response to light  
120 touch (44-46). Humans taking NTG for clinical use have reported migraine-like episodes similar  
121 to spontaneous migraines, mimicking neural activity in a spontaneous migraine, though it is still  
122 unclear how much this model depends on CGRP in humans (46). Therefore, in this study, we  
123 chose to use cell culture models, non-surgical NTG injections, and intrathecal CGRP injections in  
124 genetic mouse models to evaluate whether and to what extent chronic migraine-induced  
125 elevations in CGRP influence MLVs and subsequent migraine pathophysiology.

126

## 127 **Results**

### 128 **Lymphatic-specific deletion of the *Calcrl* gene, encoding the CGRP receptor, alleviates facial** 129 **expression of chronic migraine pain**

130 To test whether meningeal lymphatic vessels (MLVs) contribute to the pathophysiology of  
131 chronic migraine pain, *Calcrl<sup>fl/fl</sup>;Vegfr3<sup>CreERT2/+</sup>* (*Calcrl<sup>LEC</sup>*) mice with lymphatic-specific, temporal  
132 genetic deletion of the CGRP receptor, were evaluated for facial pain following nitroglycerin  
133 (NTG) induction of chronic migraine. *Vegfr3<sup>CreERT2</sup>* mice have been previously described and  
134 are commonly used as a tool to modify genes in lymphatics and, depending on timing,  
135 copy number and dosage, in some capillary blood endothelial cells of some organs (47). To  
136 assess the extent of the tamoxifen mediated deletion, expression of *Calcrl* 2-weeks post  
137 tamoxifen injection was significantly reduced by 60% in meningeal lymphatic endothelial cells  
138 isolated from *Calcrl<sup>LEC</sup>* mice compared to those of *Calcrl<sup>fl/fl</sup>* mice ( $0.413 \pm 0.19$  SD,  $p = 0.0063$ ,  
139 unpaired student's t test). *Calcrl* mRNA levels were unchanged in Lyve1-, CD68- cells isolated



140 from cardiac tissue of *Calcr<sup>l<sup>LEC</sup></sup>* mice compared to those of *Calcr<sup>fl/fl</sup>* mice ( $1.8 \pm 1.6$  SD, not  
141 significant by student's t test).

142 In this study, chronic migraine was induced by i.p. injection of NTG every other day for 8  
143 days (5 total injections) and compared to animals with 0.9% normal saline injections (Figure 1 A,  
144 B). This model has been demonstrated to be primarily mediated by CGRP (44-46). Pain behavior  
145 was quantified 30 minutes after each injection using the well-established murine grimace scale  
146 (48), which incorporates changes in ear, eye, nose, and cheek positions as a surrogate measure  
147 of pain (Figure 1 B). Mean grimace scores (MGS) were calculated from video recordings once  
148 every minute for 20 minutes and averaged to generate a mean grimace score (Figure 1 A, B). As  
149 we assigned 0-2 total points to 4 clinical categories, mice could score between 0 points (no pain  
150 at all) and 8 points (most possible pain detectable, such as surgical pain without analgesia).

151 There were no significant differences in facial characteristics of pain between *Calcr<sup>fl/fl</sup>*  
152 and *Calcr<sup>l<sup>LEC</sup></sup>* mice at baseline 1 day before NTG injection (Figure 1 C, D). As expected, NTG-  
153 treated *Calcr<sup>fl/fl</sup>* mice exhibited significantly higher MGS compared to vehicle treated  
154 littermates (Figure 1 B, C), with peak values at Day 3 (Figure 1 C, E) that remained chronically  
155 elevated through Day 9 (final day of testing) (Figure 1 C, F). Interestingly, *Calcr<sup>l<sup>LEC</sup></sup>* mice with  
156 lymphatic loss of the CGRP receptor exhibited significantly attenuated MGS after treatment  
157 with NTG compared to *Calcr<sup>fl/fl</sup>* littermates treated with NTG (Figure 1 C). Throughout the  
158 testing period, *Calcr<sup>l<sup>LEC</sup></sup>* mice treated with NTG exhibited average MGS ranging from 1.8 to 2.2,  
159 which did not differ significantly at any point from vehicle injected *Calcr<sup>l<sup>LEC</sup></sup>* mice (Figure 1 D, E,  
160 F). Success of the lymphatic specific deletion of *Calcr* was confirmed via immunoprecipitation  
161 and qPCR, which indicated approximately 70% deletion of *Calcr* in meningeal lymphatic vessels

162 (Supplemental Figure 1 A). Changes to the lymphatic vascular network in the meninges 3 weeks  
163 following tamoxifen induced deletion was assessed using immunofluorescence microscopy.  
164 Using AngioTool, it was determined that there was no difference in vessel area, vessel length,  
165 vessel branchpoints, or vessel endpoints (Supplemental Figure 1 B, C, D, E, F).

166

167 To confirm whether the observations made in *Calcr<sup>lLEC</sup>* mice were mediated by CGRP, chronic  
168 migraine-like pain was also assessed in *Ramp1<sup>-/-</sup>* mice using the NTG mediated model of chronic  
169 migraine following the same protocol as above. These animals were only assessed to day 7, due  
170 to experimental endpoint constraints. There were no significant differences in facial  
171 characteristics of pain between wild type control and *Ramp1<sup>-/-</sup>* mice at baseline 1 day before  
172 NTG injection (Figure 1 G, H). Consistent with our findings in NTG-treated *Calcr<sup>fl/fl</sup>* mice, NTG  
173 treated wild type controls exhibited significantly higher MGS compared to vehicle treated  
174 littermates (Figure 1 G, I, J), with peak values at Day 7 that remained chronically elevated  
175 through the extent of testing (Figure 1 G, J). As expected, mice that are systemically deficient in  
176 *Ramp1* exhibited completely normal MGS after treatment with NTG compared to wild type  
177 controls treated with NTG (Figure 1 G), as these animals have no tissues that express *Ramp1*, a  
178 required component for the highest affinity CGRP receptor. Throughout the testing period,  
179 *Ramp1<sup>-/-</sup>* mice treated with NTG exhibited average MGS ranging from 1.4 to 1.7, which did not  
180 differ significantly at any point from vehicle injected mice (Figure 1 G, H, I, J).

181 Together these data indicate that expression of the CGRP receptor *Calcr1* in lymphatic vessels  
182 contributes to the manifestation of chronic migraine pain induced by NTG injection in mice.

183 These data also indicate that the NTG model of chronic migraine is primarily mediated through  
184 CGRP signaling through its primary receptor complex, CLR in complex with Ramp1.

185

186 **Light avoidance and anxiety-like behavior is abrogated in *Calcr<sup>flEC</sup>* mice treated with chronic**  
187 **migraine-inducing NTG**

188 Another clinical hallmark of migraine is aversion to sensory stimuli such as bright lights or  
189 strong odors. To assess the involvement of lymphatic CGRP signaling on migraine behavior in a  
190 second assay complementary to pain, we assessed light aversion and movement behavior. Mice  
191 undergoing the chronic NTG-induced chronic migraine protocol described above were placed in  
192 a VersaMax light aversion/activity recording apparatus, consisting of a bright and dark  
193 chamber, with free access between chambers (Figure 2 A). Daytime video recordings during the  
194 natural nocturnal period were evaluated to quantify the percent of time that mice were moving  
195 in the dark chamber, as indicated by breaks within a grid of laser beams. Light avoidance  
196 behavior was assessed two days prior to the first injection on day -1, and then following NTG  
197 injection on days 1, 3, and 5 of the experimental protocol (Figure 2 B). On each testing day,  
198 animals were allowed to acclimate to the testing room for 30 minutes prior to injection.  
199 Animals were then injected with 10mg/kg NTG and allowed to rest for an additional 30  
200 minutes. Light avoidance behavior was recorded for 30 minutes (Figure 2 B). Due to limitations  
201 of the darkened chamber's construction, we were unable to assess rearing behaviors. Typical of  
202 mouse behavior during the nocturnal period, we found that all mice spent a majority of the test  
203 time in the dark chamber (Figure 2 C).

204

205 There were no significant differences in light avoidance or movement between *Calcr<sup>fl/fl</sup>* and  
206 *Calcr<sup>i<sup>LEC</sup></sup>* mice at baseline 1 day before NTG injection (Figure 2 C). Significant increases in time  
207 spent in the dark were observed on days 3 and 5 of testing between saline *Calcr<sup>fl/fl</sup>* and NTG  
208 treated *Calcr<sup>fl/fl</sup>* mice. Correlating with our facial expression of pain observations, NTG treated  
209 *Calcr<sup>i<sup>LEC</sup></sup>* mice demonstrated reduced light avoidance behaviors when compared to NTG treated  
210 *Calcr<sup>fl/fl</sup>* mice on days 3 and 5 (Figure 2 C, D, E, F).

211  
212 Activity level while in the dark is an additional appropriate measure that integrates light  
213 aversion, movement, and anxiety behavioral measurements for assessing pain intensity (49).  
214 Chronic migraine induced by NTG injection caused a significant attenuation of relative  
215 movement in the dark chamber in NTG treated *Calcr<sup>fl/fl</sup>* mice compared to vehicle treated  
216 *Calcr<sup>fl/fl</sup>* mice on day 3 and 5. Importantly, throughout the testing period no significant  
217 difference in light avoidance or relative time moving was observed between *Calcr<sup>i<sup>LEC</sup></sup>* mice  
218 injected with NTG and *Calcr<sup>fl/fl</sup>* or *Calcr<sup>i<sup>LEC</sup></sup>* mice on days 1, 3, and 5 (Figure 2 G, H), indicating  
219 that loss of CGRP signaling in lymphatics attenuates chronic migraine-associated behaviors to a  
220 level observed in saline injected animals. *Calcr<sup>i<sup>LEC</sup></sup>* mice exhibited significantly different  
221 movement in the dark behaviors compared to NTG treated *Calcr<sup>fl/fl</sup>* mice on day 1 and on day 5,  
222 though the p value was calculated to be 0.056 (Figure 2 G) indicating that *Calcr<sup>i<sup>LEC</sup></sup>* mice do not  
223 experience chronic migraine-associated light aversion or anxiety. The converse phenotype,  
224 relative amount of time spent moving in the light, was not found to be significantly different  
225 between any of the genotypes or treatments (Figure 2 H). These findings are consistent with  
226 the results of similar studies of wild type mice (50, 51).

227

228 Thus, using 2 independent behavioral metrics of chronic migraine pain, facial grimace and light  
229 avoidance/movement, these data support the conclusion that CGRP signaling in lymphatics  
230 contributes significantly to the pathophysiological presentation of chronic migraine pain.

231

232 **Meningeal lymphatic vessels exhibit transcriptional changes in response to NTG-mediated**  
233 **chronic migraine**

234 To query and to characterize the genome-wide transcriptional and translational response of  
235 meningeal lymphatic vasculature during NTG-mediated chronic migraine we utilized the  
236 RiboTag genetic model (Figure 3 A) (52). We generated and used *Rpl22<sup>HA/+</sup>;Lyve1<sup>Cre</sup>* mice, which  
237 enables the HA-tagging and immunoprecipitation of actively-translating mRNAs from Lyve1-  
238 Cre+ lymphatic endothelial cells of dissected meninges (Figure 3 A). The *Rpl22<sup>HA/+</sup>;Lyve1<sup>Cre</sup>* mice  
239 were treated with either vehicle or NTG to induce a state of CGRP elevation and chronic  
240 migraine. Prior to meninges dissection, we confirmed that mice receiving NTG injections  
241 exhibited significant increases in pain behavior compared to vehicle controls, as quantified by  
242 their mean grimace score (Figure 3 B).

243

244 Clariom S Pico microarray analysis of *Lyve1-Cre+* ribosome-associated mRNAs revealed 2  
245 distinct groups of 700 differentially expressed genes between NTG and vehicle treated mice,  
246 calculated using one-way ANOVA with a false discovery rate of 0.05 (Figure 3 C, Supplemental  
247 Table 1). Ingenuity Pathway Analysis (IPA, Qiagen) of differentially expressed genes identified  
248 NTG-induced increases in the expression of genes associated with phagocytosis, cholesterol

249 biosynthesis, G protein-coupled receptor (GPCR) signaling, cAMP response element-binding  
250 protein signaling, and actin cytoskeletal signaling along with NTG-induced decreases in several  
251 Th1 and Th2 activation pathways (Figure 3 D).

252

253 Consistent with the effectiveness of RiboTag to isolate actively translated lymphatic mRNAs  
254 from meninges, a high number of genes with previously described roles in lymphatics were  
255 identified through bibliometric analysis (Figure 3 E, labeled genes), including *Gjc2* and *Cxcl12*,  
256 which have been linked to *Calcr1* signaling in lymphatics (40, 53, 54). Interestingly, the data  
257 revealed enrichment of several established serum biomarkers of chronic migraine, *Crp* (55) and  
258 *Ptx3* (56-58), again suggesting the involvement of meningeal lymphatics in chronic migraine  
259 pathophysiology. In addition, many chemokine/cytokine and immune signaling genes were  
260 identified, including *Ptx3*, *Madcam1*, *Cxcl10*, *Cd74*, *Il4ra*, *Ccr2*, *Cd36*, *Ccl20*, *Cxcl12*, and *Ccr8*.  
261 This gene signature is indicative of an immune response within the meninges following NTG  
262 treatment and broadly supporting neuro-vascular-immune crosstalk during chronic migraine  
263 responses (11, 23, 59). Because MLVs play significant roles in both immune cell trafficking and  
264 the homeostatic efflux of cerebrospinal fluid (CSF) from the CNS, we elected to validate several  
265 candidate genes that might play functional roles in either neuro-immuno-vascular crosstalk or  
266 fluid homeostasis of migraine pathophysiology.

267

268 **CGRP treatment of cultured hLECs is sufficient to induce transcriptional changes in**  
269 **differentially translated genes observed in MLVs during NTG-induced chronic migraine**

270 Since the RiboTag gene expression analysis reflects translational changes in response to  
271 systemically-administered NTG, we elected to validate that CGRP peptide itself was sufficient to  
272 induce changes in gene expression of 3 identified genes using cultured human dermal LECs  
273 treated with 100 nM CGRP: Connexin-47 (gene: *Gjc2*, protein: Connexin-47), Pentraxin3 (gene:  
274 *Ptx3*, protein: PTX3), and Mucosal vascular addressin cell adhesion molecule 1 (gene:  
275 *Madcam1*, protein: MADCAM1) (Figure 3 E, red text).

276

277 Mutations in the gene encoding for the gap junction protein Cx47, *GJC2*, have been described  
278 as causal variants in primary lymphedema (60, 61), however the expression of *GJC2* mRNA was  
279 not significantly changed in cultured hLECs upon treatment with CGRP for 8 hours (Figure 4 A).  
280 Cx47 protein levels were significantly, though modestly, upregulated by approximately 1.5 fold  
281 8 hours after CGRP treatment as quantified by immunofluorescence (Figure 4 B, C).

282 Interestingly, we noted that Cx47 appeared to co-localize along continuous VE-Cadherin  
283 adherens junctions between LECs—a pattern that was more apparent in the CGRP-treated  
284 condition (Figure 4 B, white arrowheads).

285

286 Pentraxin3 (PTX3) is a secreted, acute phase reactant expressed by many cell types, including  
287 within blunt end terminals of lymphatic capillaries (62) and in paracortical and cortical sinus  
288 LECs of lymph nodes (63). Moreover, PTX3 is associated with endothelial dysfunction and  
289 vascular inflammation and serves as a biomarker of endothelial damage during chronic  
290 migraine (57). *PTX3* mRNA expression in human LECs was significantly elevated 2.5-fold  
291 following 8 hour CGRP treatment in cultured hLECs (Figure 4 D). Additionally, PTX3 protein was

292 significantly upregulated approximately 2-fold 8 hours following CGRP treatment as quantified  
293 by immunofluorescence (Figure 4 E, F).

294

295 MADCAM1 is an endothelial cell adhesion molecule that interacts with  $\alpha4/\beta7$  integrins  
296 expressed on immune cells, facilitating immune cell adhesion to endothelial cells (64).

297 *MADCAM1* mRNA was robustly enriched 9-fold in hLECs following 8 hour CGRP treatment  
298 (Figure 4 G). MADCAM1 protein was also significantly upregulated 3-fold in hLECs after 8 hours  
299 of CGRP treatment (Figure 4 H, I).

300

301 Collectively, these data demonstrate that meningeal LECs in mice with NTG-induced chronic  
302 migraine as well as cultured hLECs cells exposed to CGRP peptide exhibit changes in gene and  
303 protein expression that are suggestive of lymphatic vascular-immune interactions during  
304 chronic migraine pathophysiology.

305

306 **NTG-induced CGRP signaling primes MLV capillary endpoints and is required for egress of**  
307 **LPAM1+ CD4+ T cells to draining cervical lymph nodes**

308

309 Meningeal lymphatic vessels have capillary endpoints that are hypothesized to be sites of CSF  
310 and immune cell egress from the CNS (3). Interestingly, *Ptx3* expression is increased at these  
311 blunt endpoints during macrophage-associated lymphatic malformation (62). To assess the  
312 expression of *Ptx3* during chronic migraine, we performed whole mount immunofluorescence  
313 microscopy on decalcified dorsal skull with adherent meninges from vehicle or NTG-treated



314 mice. Ptx3 protein expression was higher in the MLV capillary endpoints of NTG-treated mice  
315 compared to vehicle treated mice (Figure 5 A, B). These Ptx3 positive endpoints may represent  
316 lymphatic vascular endothelial damage or priming of MLVs for immune cell interactions during  
317 chronic migraine.

318

319 Therefore, to assess whether there were differences in immune cell egress during NTG-induced  
320 chronic migraine we performed multispectral flow cytometry of the deep cervical lymph nodes  
321 (DCLN) to which the MLVs drain (Figure 5 C). Peripherally located inguinal lymph nodes (ILN),  
322 subjected to systemic NTG-injections but not implicated in chronic migraine pathophysiology,  
323 were included as controls. Because the RiboTag IPA analysis identified significant changes in  
324 both Th1 and Th2 responses, and because MADCAM1 is a potent adhesion molecule for  $\alpha 4/\beta 7$   
325 integrin (LPAM1) positive T cells, we elected to profile a broad range of T cell populations (see  
326 Supplemental Table 2). No differences were detected in  $Ifn\gamma^+$  CD4+ Th1 cells, Gata3+ CD4+ Th2  
327 cells, CCR7+ CD4+ T Cells,  $Ifn\gamma^+$  CD8+ Tc1 cells, Gata3+ CD8+ Tc2 cells, CCR7+ CD8+ T Cells in  
328 DCLNs, nor in ILNs between NTG-treated or vehicle-treated *Calcr<sup>fl/fl</sup>* and *Calcr<sup>iLEC</sup>* mice  
329 (Supplemental Figure 4). However notably, the only significant change was an increase in the  
330 relative abundance of LPAM1 ( $\alpha 4/\beta 7$  integrin) positive CD4 T cells in NTG-treated *Calcr<sup>fl/fl</sup>* mice.  
331 This increase was not detected in NTG-treated *Calcr<sup>iLEC</sup>* mice (Figure 5 D), nor was it detected in  
332 peripheral ILNs (Figure 5 E).  $\alpha 4/\beta 7$  integrin positive CD4 T cells are capable of interacting with  
333 endothelial cells expressing MADCAM1 (Figure 5 F), which is significantly increased in  
334 lymphatics by CGRP stimulation (Figures 3 E, 4 G-I). These findings indicate that CGRP signaling

335 in the lymphatic endothelium is required for the entry of LPAM1+ CD4+ T cells into MADCAM1-  
336 enriched MLVs, leading to egress to the DCLN during chronic migraine (Figure 5 F).

337

338 **CGRP induces formation of continuous, non-permeable VE-Cadherin junctions in cultured**  
339 **hLECs**

340 In addition to functioning as conduits for immune cell trafficking from peripheral tissues to  
341 secondary lymphoid organs, lymphatic vessels maintain fluid homeostasis, including CSF  
342 turnover and efflux from the CNS through MLVs (1, 5, 8). Thus, changes in lymphatic endothelial  
343 cell junctions influence the permeability and function of lymphatic vessels.

344

345 To assess the role of CGRP on LEC junctions, hLECs were cultured in media or treated with 100  
346 nM CGRP, or 100 nM adrenomedullin (AM, non-permeabilizing agent) for 20 minutes with or  
347 without treatment with 3 nM olcegepant (a.k.a. BIBN4096), a small molecule receptor  
348 antagonist for CLR/Ramp1 – the canonical CGRP receptor. The organization of inter-endothelial  
349 cell junctions was quantified using VE-cadherin staining and characterized as percent of total  
350 cell-cell junctional borders in the impermeable confirmation (Figure 6 A). This measurement  
351 reflects a physiologic, protein localization response, as it is assessed 20 minutes after  
352 treatment.

353

354 When compared to untreated cells the positive control AM peptide stimulated a robust  
355 increase in the linear organization of VE-cadherin (Figure 6 A, white arrows), from 60% to  
356 approximately 85% continuous junctions (Figure 6 B). As expected, olcegepant did not reverse

357 the observed AM mediated increase in linear junction because AM signals through CLR/Ramp2  
358 (Figure 6 B, C). Treatment with CGRP was equivalently potent in converting VE-cadherin  
359 arrangement to linear, continuous junctions (Figure 6 A, white arrows), with a statistically  
360 significant increase to approximately 80% continuous junctions compared to untreated cells  
361 (Figure 6 B). Treatment with 3 nM olcegepant was sufficient to prevent the CGRP mediated  
362 linearization of VE-Cadherin junctional proteins, yielding a statistically significant reduction to  
363 approximately 60% continuous VE-Cadherin junctions compared to CGRP treatment. There was  
364 no significant difference between CGRP plus olcegepant treated LECs and olcegepant alone  
365 treated LECs.

366

367 Furthermore, the functional permeability of LEC monolayers to protein solutes was also  
368 assessed using similar treatment conditions. Confluent monolayers of hLECs were grown on  
369 biotinylated-fibronectin coated coverslips. Following 20 minutes of treatment conditions,  
370 fluorescent streptavidin (MW 60 kDa) was added to the cells for 3 minutes and the cells were  
371 fixed and stained for streptavidin. Permeability was quantified as mean fluorescence intensity  
372 (MFI) of streptavidin that passed between endothelial cell junctions (Figure 6 C). Considering  
373 the inherent permeability of LEC junctions, the dynamic range of the assay is maximal under  
374 both untreated and histamine-treated conditions, with high streptavidin staining demarcating  
375 nearly all cell borders and abundant streptavidin puncta at multicellular junctions (Figure 6 C,  
376 gray arrows). In contrast, MFI of hLECs treated with 100 nM CGRP was equivalent to the low  
377 permeability control of 100 nM AM, with a statistically significant reduction of 40% compared  
378 to media alone conditions (Figure 6 D). The very low levels of streptavidin staining between cell

379 junctions (Figure 6 D, white arrows) is consistent with the increased linear arrangement of VE-  
380 cadherin under CGRP and AM treatment conditions.

381  
382 Protein quantification by western blot illustrated ERK phosphorylation in cultured LECs as early  
383 as 5 minutes post treatment with CGRP persisting until 20 minutes post treatment and these  
384 changes were not observed in AKT phosphorylation (Figure 6 E, F, G). Similarly, CREB  
385 phosphorylation was detected as early as 5 minutes post CGRP treatment, persisting until 10  
386 minutes post treatment (Figure 6 E, H). Taken together, increased VE-Cadherin linearization and  
387 reduced protein transit through a monolayer indicates that CGRP structurally and functionally  
388 reduces LEC permeability in vitro, likely through ERK or CREB mediated effects.

389

### 390 **NTG-induced CGRP signaling is required for formation of continuous VE-Cadherin junctions**

#### 391 **MLV capillary endpoints**

392 To determine whether endogenously elevated CGRP during chronic migraine affects the cellular  
393 junctions of MLVs in vivo, we quantified the arrangement of VE-cadherin within capillary  
394 endpoints of meningeal lymphatics of *Calcr<sup>fl/fl</sup>* and *Calcr<sup>iLEC</sup>* mice treated with vehicle or NTG, as  
395 described in Figure 1. All cell-cell junctions within 500  $\mu\text{m}$  of lymphatic capillary end-points  
396 were assessed. The relative proportion of continuous VE-Cadherin junctions (Figure 7 A, white  
397 arrows) to discontinuous junctions (Figure 7 A, gray arrows) in LYVE1-positive MLV endpoints  
398 was significantly increased to 80% in *Calcr<sup>fl/fl</sup>* mice treated with NTG compared to vehicle-  
399 treated *Calcr<sup>fl/fl</sup>* mice and vehicle-treated *Calcr<sup>iLEC</sup>* mice (Figure 7 B). Importantly, *Calcr<sup>iLEC</sup>* mice  
400 treated with NTG had approximately 55% continuous VE-Cadherin LEC junctions that were

401 indistinguishable from the proportions observed in vehicle-treated *Calcr<sup>fl/fl</sup>* and *Calcr<sup>i<sup>LEC</sup></sup>*  
402 animals (Figure 7 A, B).  
403  
404 Cell-cell junctions within 500  $\mu\text{m}$  of lymphatic capillary end-points were also in systemically  
405 deficient *Ramp1<sup>-/-</sup>* mice. Vehicle treated wild type control animals exhibited similar phenotypes  
406 to *Calcr<sup>fl/fl</sup>* mice (approximately 55% continuous junctions) as well as NTG treated wild type  
407 controls (approximately 75% continuous junctions) (Figure 7 C). The proportion of continuous  
408 junctions in *Ramp1<sup>-/-</sup>* mice treated with vehicle was found to be approximately 45%, not  
409 significantly different from wild type controls (Figure 7 C). As expected, *Ramp1<sup>-/-</sup>* mice treated  
410 with NTG had approximately 50% continuous junctions, significantly less than wild type NTG  
411 treated animals and not significantly different from *Ramp1<sup>-/-</sup>* mice treated with vehicle (Figure 7  
412 C). Taken together, these data indicate that chronic migraine-associated rearrangement of MLV  
413 VE-cadherin cell junctions requires CGRP signaling.

414

#### 415 **CGRP reduces MLV permeability and CSF drainage to DCLN in vivo**

416 To directly test the effect of CGRP on meningeal lymphatic vascular permeability in vivo, 5  $\mu\text{l}$  of  
417 1% Evans blue (EB) in normal saline with or without 5  $\mu\text{g}$  CGRP ([CGRP] = 1  $\mu\text{g}/\mu\text{L}$ ) was injected  
418 into the cisterna magna of *Calcr<sup>fl/fl</sup>* and *Calcr<sup>i<sup>LEC</sup></sup>* mice. EB is a traceable dye that can be  
419 detected colorimetrically by light microscopy or fluorescently after bound to albumin by  
420 fluorescent emission in the red and far-red wavelengths. Thus, intracisterna magna (ICM)  
421 injections of EB can be utilized to quantify CSF uptake by MLVs and relative efflux of CSF to the  
422 deep cervical lymph nodes (8).

423  
424 EB solution diluted in normal saline was readily observed within the LYVE1+ lymphatic vessels  
425 of the superior sagittal sinus of both *Calcr<sup>fl/fl</sup>* and *Calcr<sup>iLEC</sup>* mice, visualized by confocal and  
426 epifluorescence microscopy of decalcified meninges (Figure 8 A, B). Using fluorescence  
427 intensity plot profiles across the vessel, the area under the EB curve between the borders of the  
428 LYVE1+ meningeal lymphatic vessels was calculated and adjusted to vessel width for an overall  
429 quantitation of MLV permeability (Figure 8 C, D). With this quantitation method, there was no  
430 significant difference in MLV permeability or dye uptake between vehicle-injected *Calcr<sup>fl/fl</sup>* and  
431 *Calcr<sup>iLEC</sup>* animals (Figure 8 B-D). However, inclusion of CGRP in the EB injection caused a marked  
432 and significant decline in MLV dye uptake in *Calcr<sup>fl/fl</sup>* mice (Figure 8 B-D), consistent with the  
433 robust non-permeabilizing effects of CGRP observed in vitro and in vivo (Figures 5 and 6).  
434 As expected, *Calcr<sup>iLEC</sup>* mice with loss of the CGRP receptor in lymphatics were unresponsive to  
435 CGRP/EB injection and did not differ significantly from vehicle-treated *Calcr<sup>fl/fl</sup>* and *Calcr<sup>iLEC</sup>*  
436 animals.

437  
438 As a secondary measure of CSF efflux, EB transit from the MLVs to the draining deep cervical  
439 lymph nodes (DCLN) located below the sternocleidomastoid muscle was quantified. DCLNs  
440 were visualized and dissected bilaterally (Figure 9 A) and mean fluorescence intensity (MFI) of  
441 bilateral DCLNs was recorded using epifluorescence microscopy (Figure 9 A) and averaged to  
442 represent net MLV drainage by animal (Figure 9 B). Robust amounts of EB dye were visually  
443 present and quantified by epifluorescence in the DCLNs of both *Calcr<sup>fl/fl</sup>* and *Calcr<sup>iLEC</sup>* mice  
444 injected with vehicle diluted in EB 5 minutes after ICM injection (Figure 9 A, B). Remarkably,

445 inclusion of CGRP in the EB dye robustly reduced EB MFI in the DCLNs of *Calcr<sup>fl/fl</sup>* mice, with no  
446 visible EB dye present in the DCLNs and with dramatically reduced DCLN fluorescence in the red  
447 channel 5 minutes after ICM injection (Figure 9 A, B). This attenuation of MLV drainage to the  
448 DCLN was specific to CGRP peptide and EB fluorescence in *Calcr<sup>fl<sup>LEC</sup></sup>* mice injected ICM with  
449 CGRP/EB did not differ significantly from vehicle treated *Calcr<sup>fl/fl</sup>* and *Calcr<sup>fl<sup>LEC</sup></sup>* animals.

450

451 CGRP mediated reduction of EB dye was also assessed in systemically deficient *Ramp1<sup>-/-</sup>* mice.  
452 Consistent with *Calcr<sup>fl/fl</sup>* mice, wild type control mice demonstrated robust EB fluorescence in  
453 DCNLs bilaterally, corresponding reduction in EB fluorescence with CGRP co-injection (Figure 9  
454 C, D). As expected, *Ramp1<sup>-/-</sup>* mice exhibited EB fluorescence consistent with wild type baseline  
455 (Figure 9 D). Inclusion of CGRP in the EB dye injected in *Ramp1<sup>-/-</sup>* mice did not impact EB  
456 fluorescence in the DCLNs, again suggesting that CGRP signaling through CLR/Ramp1 is  
457 sufficient to change CSF efflux through the meningeal lymphatic vasculature.

458

459 Collectively, these data demonstrate that lymphatic-CGRP signaling through CLR/Ramp1  
460 markedly reduces CSF outflow to the DCLNs by rearranging lymphatic VE-cadherin endothelial  
461 junctions and thereby reducing MLV permeability (Figure 9 E).

462

## 463 **Discussion**

464 Despite its notoriety as the third most prevalent illness globally, affecting more than 10% of the  
465 worldwide population (10, 65), the etiology and pathophysiology of migraine remains poorly  
466 understood. Here we used a well-established murine model of chronic migraine coupled with

467 two genetic models of CGRP signaling insufficiency, *Ramp1* systemic deficiency and lymphatic-  
468 specific *Calcr1* insufficiency to discover a previously unrecognized role of meningeal lymphatics  
469 in chronic migraine pain and pathophysiology. Loss of CGRP signaling in lymphatics significantly  
470 ameliorated chronic migraine pain in mice, whereas direct injection of CGRP into the cisterna  
471 magna reduced MLV permeability and abrogated CSF drainage. These findings were consistent  
472 with our findings in *Ramp1* systemically deficient animals that have no tissues capable of  
473 robustly responding to CGRP. Overall, these data support that elevated levels of CGRP during  
474 chronic migraine can act on MLVs to reduce their permeability and CSF drainage functions. This  
475 reduced permeability may culminate in an increase in CSF pressure (66, 67) or in the retention  
476 of vascular-immune pro-migraine factors within the dura (68), exacerbating the propagation of  
477 chronic migraine pain.

478

479 Monoclonal antibodies targeting either the CGRP receptor (erenumab) or plasma CGRP  
480 (fremanezumab, galcanezumab, eptinezumab), and small molecule antagonists of the CGRP  
481 hetero-receptor, CLR/RAMP1 are effective antimigraine therapeutics (25, 26). However, the  
482 cellular targets of these medications remain unknown (21). I-125 labeled galcanezumab has  
483 been measured in meningeal tissue at approximately 10% of its plasma concentration (69), and  
484 fluorescently labeled fremanezumab was detected in the dura, dural blood vessels and  
485 trigeminal ganglion, but not in the central nervous system (70). These data support the  
486 plausibility that MLVs represent a previously unappreciated CGRP effector target within the  
487 trigeminovascular system. Although considered revolutionary, only 50% of patients on the  
488 highest dose of erenumab experience a reduction in monthly migraine days greater than 50%



489 (71). This partial reduction of chronic migraine symptoms correlates with our observations of  
490 approximately 50% attenuation of migraine symptoms in mice with impaired lymphatic  
491 response to CGRP, compared to the total prevention as observed in *Ramp1*<sup>-/-</sup> mice. Because our  
492 data demonstrates a full protection in systemic Ramp1 deficient animals and partial protection  
493 in lymphatic *Calcr1* deficient animals, our findings indicate that migraine pathophysiology is  
494 multifactorial and that the MLVs are one of many CGRP target cells.

495  
496 Reduced meningeal lymphatic and glymphatic CSF efflux has so far been observed in patients  
497 with spontaneous chronic migraine by MRI and reduced flow correlated negatively with  
498 worsened migraine symptoms (72). These changes were observed both during and between  
499 migraine episodes. Altered CSF efflux through the MLVs has been implicated in a variety of  
500 additional neurodegenerative and neuroinflammatory diseases including Alzheimer's disease  
501 (AD), Parkinson's disease, multiple sclerosis, traumatic brain injury, brain tumors, as well as in  
502 aging. Disruption of CSF efflux through MLVs caused increased amyloid  $\beta$  deposition in the  
503 meninges and increased perivascular plaque formation in an AD mouse model (3). Interestingly,  
504 the incidence of AD in patients with migraine is nearly twice the rate of AD in healthy controls  
505 (73). Additionally,  $\alpha$ -synuclein pathology correlates with delayed MLV drainage and blocked  
506 MLV flow exacerbates motor and memory deficits in a mouse model of Parkinson's disease (6).  
507 Moreover, increased CSF pressures are causally associated with another chronic headache  
508 disorder, idiopathic intracranial hypertension, and reduction of elevated CSF pressure alleviates  
509 pain (74). If, as suggested by our data, chronic migraine-induced CGRP triggers transient  
510 changes in CSF efflux, then these acute changes may propagate migraine pain, while chronic

511 alterations to CSF efflux over decades of migraine episodes may permanently damage the  
512 MLVs, potentially contributing to the pathogenesis or pathophysiology of Alzheimer's dementia  
513 other neurodegenerative or neuroinflammatory conditions. Promisingly, our data strongly  
514 suggests that CGRP inhibitor therapeutics may act to restore MLV drainage during a migraine  
515 attack which is important for the prevention of long-term CSF efflux related diseases.

516

517 We leveraged lymphatic-specific RiboTag mice to develop a deeper mechanistic understanding  
518 of how the cellular transcription of meningeal LECs changes in response to migraine. We  
519 identified 3 promising translationally altered immunovascular proteins associated with chronic  
520 migraine and CGRP signaling: *GJC2*, *PTX3*, and *MADCAM1*.

521

522 *GJC2* encodes for Connexin-47 (Cx47) and is a causally mutated in some forms of human  
523 primary lymphedema (60). Cx47 forms homotypic gap junctions as well as heterotypic gap  
524 junctions with Cx43, which is also causally associated with lymphedema in humans (75). Using  
525 cultured hLECs, we noticed that CGRP promoted the localization of Cx47 along continuous, low  
526 permeability VE-cadherin adherens junctions. Interestingly, tonabersat, a Cx43 gap junction  
527 signaling inhibitor is an effective prophylaxis for migraine attacks with aura, though it was  
528 found to be ineffective for migraine attacks without aura (76). These data suggest that under  
529 migraine conditions of elevated CGRP, lymphatics may exhibit increased inter-cellular gap  
530 junction communication that may be therapeutically targeted.

531

532 Pentraxin3 (*PTX3*) is a member of the pentraxin family and, alongside C-reactive peptide, is an  
533 acute phase reactant in the humoral immune response. *PTX3*, which is expressed by LECs, is a  
534 newly characterized serum biomarker of migraine attacks (56-58). Additionally, *Ptx3* defines a  
535 novel subset of immune-interacting, capillary lymphatic endothelial cells that promote  
536 pathologic lymphatic vessel remodeling (62). Our current results suggest that *PTX3*  
537 upregulation in MLV endpoints during migraine may indicate a state of endothelial damage  
538 and/or function as a chemoattractant for egressing immune cells.

539

540 *MADCAM1* typically interacts with  $\alpha4\beta7$  integrins to recruit immune cells to mucosal tissues  
541 such as the gut, but it is also expressed by some lymphatic endothelial cells (77-79). Likely  
542 correlated with the upregulation of *Madcam* expression by CGRP, we also discovered a  
543 significant increase in the relative abundance of CD4+ T cells expressing LPAM1 ( $\alpha4\beta7$  integrin)  
544 in the DCLNs of mice with NTG-induced migraine. These LPAM1+ ( $\alpha4\beta7$  integrin) CD4+ T cells  
545 are critical for the initiation and development of inflammatory bowel disease (IBD) (80, 81), and  
546 are being targeted by monoclonal antibodies etrolizumab ( $\beta7$ ) and natalizumab ( $\alpha4$ ). While the  
547 current study is the first to implicate these T cells with migraine, it may be worth noting that  
548 patients with IBD have an increased prevalence of migraine headache compared to the general  
549 population (82, 83), hinting at a potential link between CGRP-mediated gut immunity and  
550 migraine.

551

552 Taken together, this work provides the first demonstration of the importance of the lymphatic  
553 vascular system in the pathophysiology of migraine along with several plausible mechanistic

554 frameworks for understanding the complex CGRP-mediated neuro-vascular-immune  
555 interactions in chronic migraine pathophysiology. The impact of migraine-related reductions of  
556 CSF efflux and of potential chronic MLV dysfunction on the development of neurodegenerative  
557 diseases remains to be fully characterized. Future studies to evaluate the contributions of MLV  
558 CSF drainage in humans during migraine, with and without CGRP-targeted therapies, is  
559 warranted.

560 **Methods**

561 Additional Experimental Details are provided in Supplementary Methods.

562 **Animals**

563 Sex as a Biological Variable: 3-6 month old female and male *C57BL/6*, and *Calcr<sup>fl<sup>LEC</sup></sup>* mice were  
564 used to characterize the role of lymphatic CGRP signaling in chronic migraine. Female mice  
565 were used for all facial expression of pain and light avoidance behavioral assays. This study  
566 primarily used female mice because the human disease condition is predominant in females,  
567 with 3-5 times higher incidence in women than men.

568 *C57BL/6* mice were obtained from Jackson Laboratories or bred in house. *Calcr<sup>fl/fl</sup>* mice  
569 were previously described and were generated as previously described (38). *Vegfr3-Cre<sup>ERT2</sup>* mice  
570 were generated and described previously (47). *Ramp1<sup>-/-</sup>* mice were generated as previously  
571 described (84). To establish deletion of *Calcr*, *Calcr<sup>fl/fl</sup>;Vegfr3<sup>CreERT2</sup>* (*Calcr<sup>fl<sup>LEC</sup></sup>*) and control  
572 *Calcr<sup>fl/fl</sup>* mice were injected intraperitoneally with 100 µg/g body weight tamoxifen (T5648,  
573 Sigma-Aldrich) diluted in corn oil for 5 consecutive days. Experimental protocols were initiated  
574 one week after the termination of tamoxifen administration. Deletion of *Calcr* was determined  
575 by qPCR of mRNA isolated from immunoprecipitated LECs from meninges and non-meningeal  
576 tissue. For translational analysis, *C57BL/6* mice were obtained from Jackson Labs. For  
577 translational analysis *B6J.129(Cg)-Rpl22<sup>tm1.1Psam</sup>/J* (RiboTag) mice were obtained from Jackson  
578 Labs. All mice were bred and maintained in a specific pathogen-free facility at the University of  
579 North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina.

580 **Lymphatic endothelial cell immunoprecipitation from tissue**

581 Single cell suspension of meningeal tissue was negatively selected with CD68  
582 immunoprecipitation and positively selected with Lyve1+ immunoprecipitated to generate  
583 enriched LECs.

584 **Nitroglycerin chronic migraine model**

585 Mice were injected intraperitoneally (IP) with 10 mg/Kg body weight of sterile  
586 nitroglycerin (T-021, Sigma-Aldrich) diluted in 0.9% saline (PAA128035, Hospira) with vehicle  
587 consisting of 0.9% normal saline containing 10% propylene glycol or with 0.9% normal saline  
588 every other day for up to 5 injections. Vehicle injections consisting of 0.9% normal saline with  
589 10% propylene glycol (the solvent for commercially available NTG) did not provoke significantly  
590 different pain behavior compared to 0.9% normal saline alone injections (data not shown).

591 Pain assessment was performed using the murine grimace scale, as previously described  
592 (48, 85), and using light aversion and movement behavior, as described in Supplementary  
593 Methods.

594 **Intra-cisterna magna injections and CSF drainage quantification**

595 Animals were anesthetized using 20  $\mu$ L/10 g body weight of 1.25% avertin (T48402, Millipore-  
596 Sigma). After a sagittal skin incision, muscle layers were retracted and 5  $\mu$ L of 0.9% saline  
597 containing 1  $\mu$ g/ $\mu$ L CGRP (015-02, Phoenix Pharmaceuticals) and 1% Evans Blue (E2129,  
598 Millipore-Sigma) or 1% Evans Blue vehicle control solution was injected intra cisterna magna  
599 over 60 seconds. The needle was removed after 5 minutes. Deep cervical lymph nodes, inguinal

600 lymph nodes, cranial meninges were dissected 2 minutes following retraction of needle.  
601 Collected tissues were fixed in 4% paraformaldehyde for up to 24 hours. Lymph nodes were  
602 imaged using an I83 Olympus inverted fluorescence microscope with 10x objective lens,  
603 connected to a Hamamatsu camera. Mean fluorescence index was quantified using ImageJ (86).

#### 604 **Whole mount immunofluorescence**

605 Dorsal skull bones with adherent meninges were fixed in 4% paraformaldehyde for 24 hours  
606 and decalcified using 0.5M EDTA (02793, Fisher Scientific) in PBS for 5-7 days at 4°C on a rocking  
607 platform in the dark. Tissues were blocked and permeabilized in PBS containing 5% normal  
608 donkey serum (017-000-121, Jackson ImmunoResearch), and 0.5% Tween-20 (BP337 Fisher  
609 Scientific) for 24 hours at 4°C and then incubated with antibodies detailed in the  
610 immunofluorescence antibody table at the indicated dilution for 24-48 hours at 4°C and then  
611 washed three times in PBS containing 5% normal donkey serum, and 0.5% Tween-20.  
612 Secondary antibodies were diluted in PBS containing 5% normal donkey serum for 4 hours at  
613 room temperature or 24 hours at 4°C. Decalcified dorsal skull and meninges were mounted in  
614 50% glycerol (BP2291, Fisher Scientific) and imaging was performed using a Zeiss 800 upright  
615 confocal microscope.

#### 616 **Flow cytometry**

617 Multispectral flow cytometry was performed on single cell suspensions of bilateral deep  
618 cervical lymph nodes or inguinal lymph nodes, as further described in Supplementary Methods.

#### 619 **Lymphatic Endothelial Cell Culture and Analyses**

620 Lymphatic endothelial cells (LECs) (c-12217, PromoCell Inc.) were cultured in endothelial  
621 supplemental media MV2 (C-22121, PromoCell Inc.) to 3-5 passages from primary sample  
622 collection and were grown at 37°C with 5% CO<sub>2</sub>. Confluent monolayers of LECs were used for  
623 immunocytochemistry, permeability assays and Western Blot analysis as described in  
624 Supplementary Methods.

625

### 626 **RiboTag Gene Expression Analysis**

627 *Rpl22<sup>HA/+</sup>;Lyve1<sup>Cre</sup>* (52) animals were induced with chronic migraine following the protocol listed  
628 above. 60 minutes after the last injection, animals were euthanized using CO<sub>2</sub> asphyxiation.  
629 After preparation of the dorsal skull bone as described above, cranial meninges were dissected  
630 using fine tipped forceps and the whole meningeal tissue was lysed using Lysing Matrix D beads  
631 (116913100, MP Biomedicals) in 50 mM Tris, 10 mM KCl (51343180, Mettler-Toledo), 12 mM  
632 MgCl<sub>2</sub> (AM9530G, Thermo Fisher Scientific), 1% nonidet-p40 (198596, MP Biomedical)  
633 “Homogenization Buffer”. Hemagglutinin-tagged ribosomes were immunoprecipitated using  
634 Pierce anti-HA conjugated magnetic beads (88837, Thermo Scientific) in supplemented  
635 homogenization buffer, containing the above reagents with 0.5 mM DTT (7016L, Cell Signaling),  
636 100 µg/mL cycloheximide (01810, Sigma-Aldrich), 1 mg/mL Heparin (BP2524100, Thermo  
637 Scientific), 200 units/mL RNase out (10-777-019, Thermo Fisher Scientific), and 1.5x Protease  
638 inhibitor cocktail (11697498001, Sigma Aldrich). Anti-HA beads were incubated with lysed  
639 meninges samples for 24 hours on a rotator at 4°C. HA-Tagged ribosomes were eluted from  
640 beads using a modified homogenization buffer with 300 mM KCl. RNA was isolated using  
641 RNeasy Kits (13997, Qiagen). Clariom S Pico microarray targeting mouse (902932, Thermo



642 Fisher Scientific) was used in collaboration with the UNC Functional Genomics Core to measure  
643 expression changes. Data analysis was completed using Partek Genomics Suite (Partek) and  
644 Ingenuity Pathway Analysis (Qiagen) software programs.

#### 645 **Statistics**

646 Data were analyzed using either two-tailed Student's *t*-test with or without Welch's correction,  
647 one-way ANOVA with multiple comparisons and Tukey's post hoc test, or two-way ANOVA with  
648 multiple comparisons and Tukey's post hoc test. Statistical test performed is noted in  
649 corresponding figure legends. P values <0.05 were considered significant and precise P value is  
650 depicted on figures where appropriate. The persons performing the analyses were blinded to  
651 the genotype of treatment of the animals or cells until the end of analysis.

#### 652 **Study Approval**

653 All protocols involving animals were approved by the UNC-CH's Institutional Animal Care and  
654 Use Committee.

#### 655 **Data Availability**

656 Microarray data is provided in the following MIAME-compliant GEO database: GSE266558. All  
657 data in the manuscript is included in the Supporting Data Values file.

#### 658 **Online Supplemental Material**

659 Expanded Methods are available in the Supplementary Material. Table S1 provides raw data for  
660 all genes queried using the Clariom S microarray. Table S2 provides a list of antibodies used for  
661 immunostaining and for flow cytometry.

662

663 **Author Contributions**

664 N.P.N.M. designed research studies, acquired and analyzed data, prepared figures, wrote and  
665 edited the manuscript, and acquired funding. L.B. conducted experiments, acquired and  
666 analyzed data, and edited the manuscript. A.L.S.B conducted experiments and acquired and  
667 analyzed data. B.K. acquired data, analyzed blinded behavioral data, and analyzed data. D.S.S  
668 performed western blot and acquired data. L.S.D. analyzed data. A.H.S. conducted experiments  
669 and acquired data. A.M.T. conducted experiments and acquired data. K.M.C. designed research  
670 studies, supervised the research, acquired funding, and wrote, and edited the manuscript.

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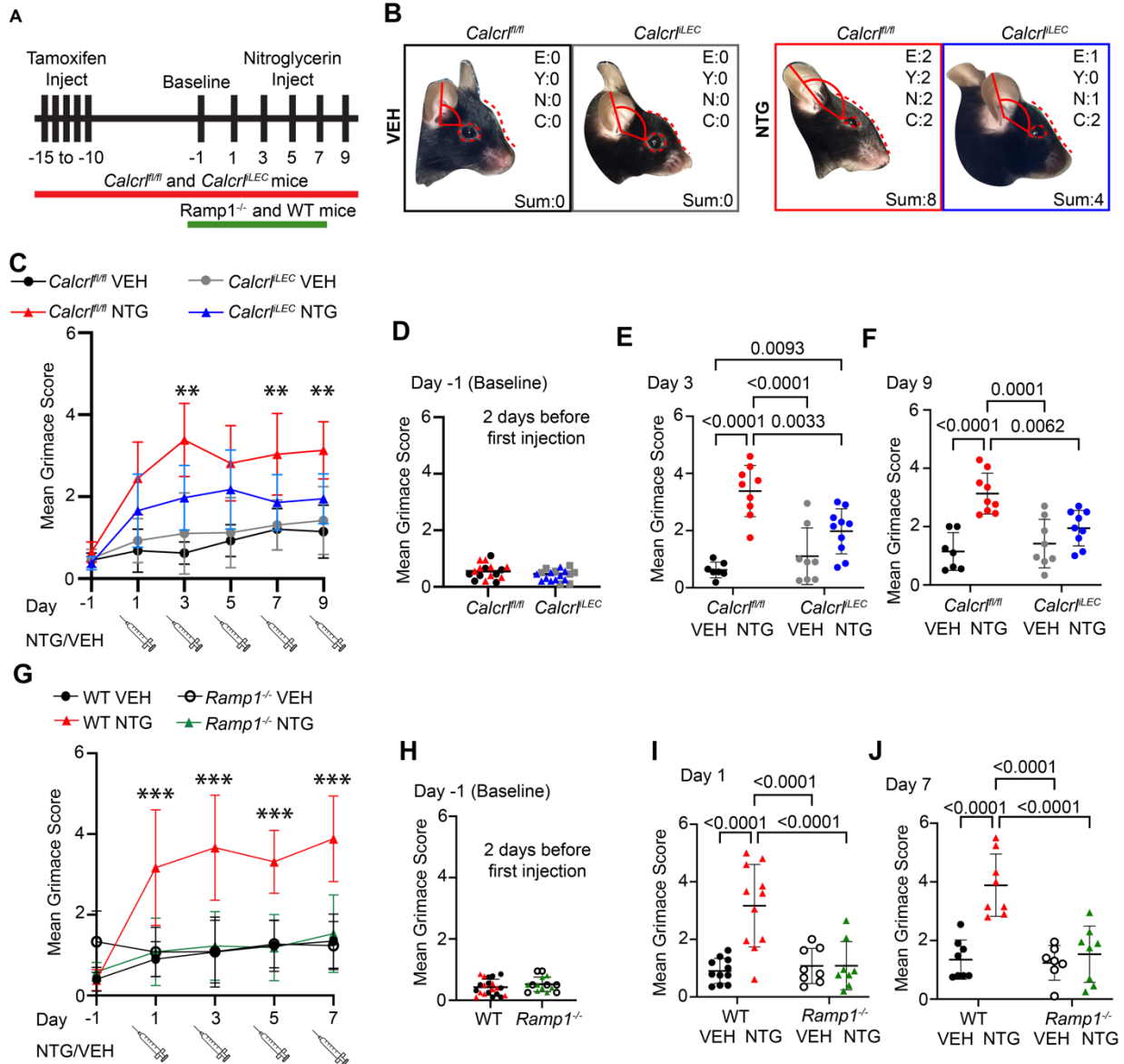
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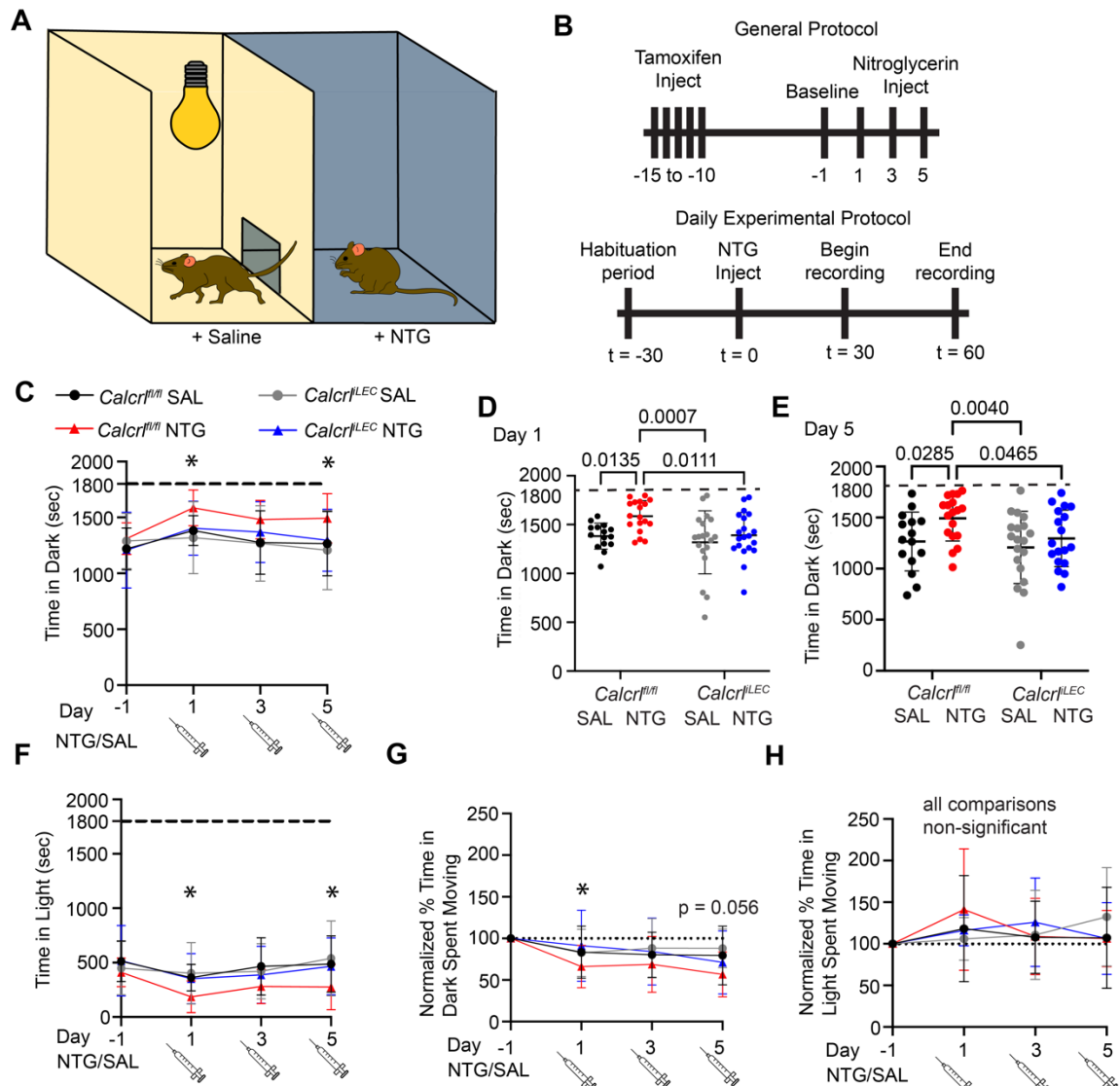
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883 **Figures:**



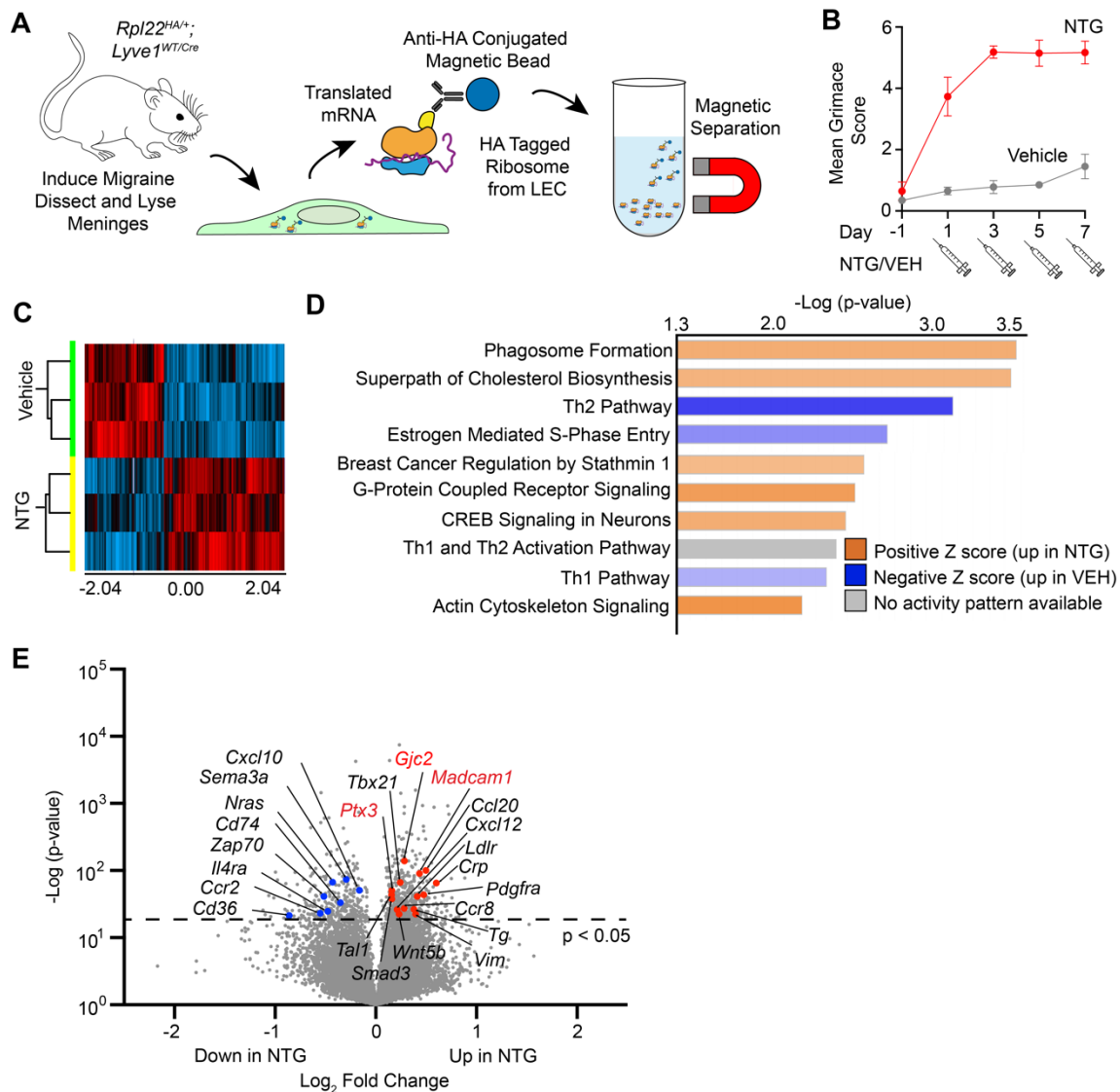
884 **Figure 1. *Calcr<sup>LEC</sup>* mice treated with NTG exhibit partially ameliorated chronic migraine pain.** (A) Experimental protocol representation. (B) Images of mouse facial expression of pain on Day 6, minute 42 following injection. Unit scores are depicted. E = ears, Y = eyes, N = nose, C = cheek. Sum of scores tallied bottom right. (C) Facial expression of pain measured 30 minutes after NTG injection and recorded for 20 minutes. Facial expression of pain is scored once per minute for the 20-minute recording duration and averaged. Mice were allowed to acclimate to the chamber 4 days before first injection (day 1) and 2 days before baseline measurement (day -1). \*\* = p<0.01 between *Calcr<sup>fl/fl</sup>* and *Calcr<sup>LEC</sup>* treated with NTG. Mean Grimace Score on (D) day -1 (Pre-injection baseline) grouped by genotype. Colors indicate injection given later in experimental protocol, matching panel B. Mean Grimace Score on (E) day 3, and (F) day 9 of chronic migraine model. For C-F, n = 7-10 animals per group representing 4 independent cohorts. Significance calculated using two-way ANOVA with Tukey's multiple comparisons test. Graphs = mean ± standard deviation. (G) Facial expression of pain for *Ramp1<sup>-/-</sup>* animals and controls. \*\*\* = p<0.001 between *Ramp1<sup>-/-</sup>* and WT treated with NTG. Mean Grimace Score on (H) day -1 (Pre-injection baseline) grouped by genotype. Colors indicate injection given later in experimental protocol, matching key in panel G. Not all animals were recorded for baseline. Mean Grimace Score on (I) day 1, and (J) day 7 of chronic migraine model. For G-J, n = 8-11 animals per group

899 representing 3 independent cohorts. Significance calculated using two-way ANOVA with Tukey's multiple  
900 comparisons test. Graphs = mean  $\pm$  standard deviation.

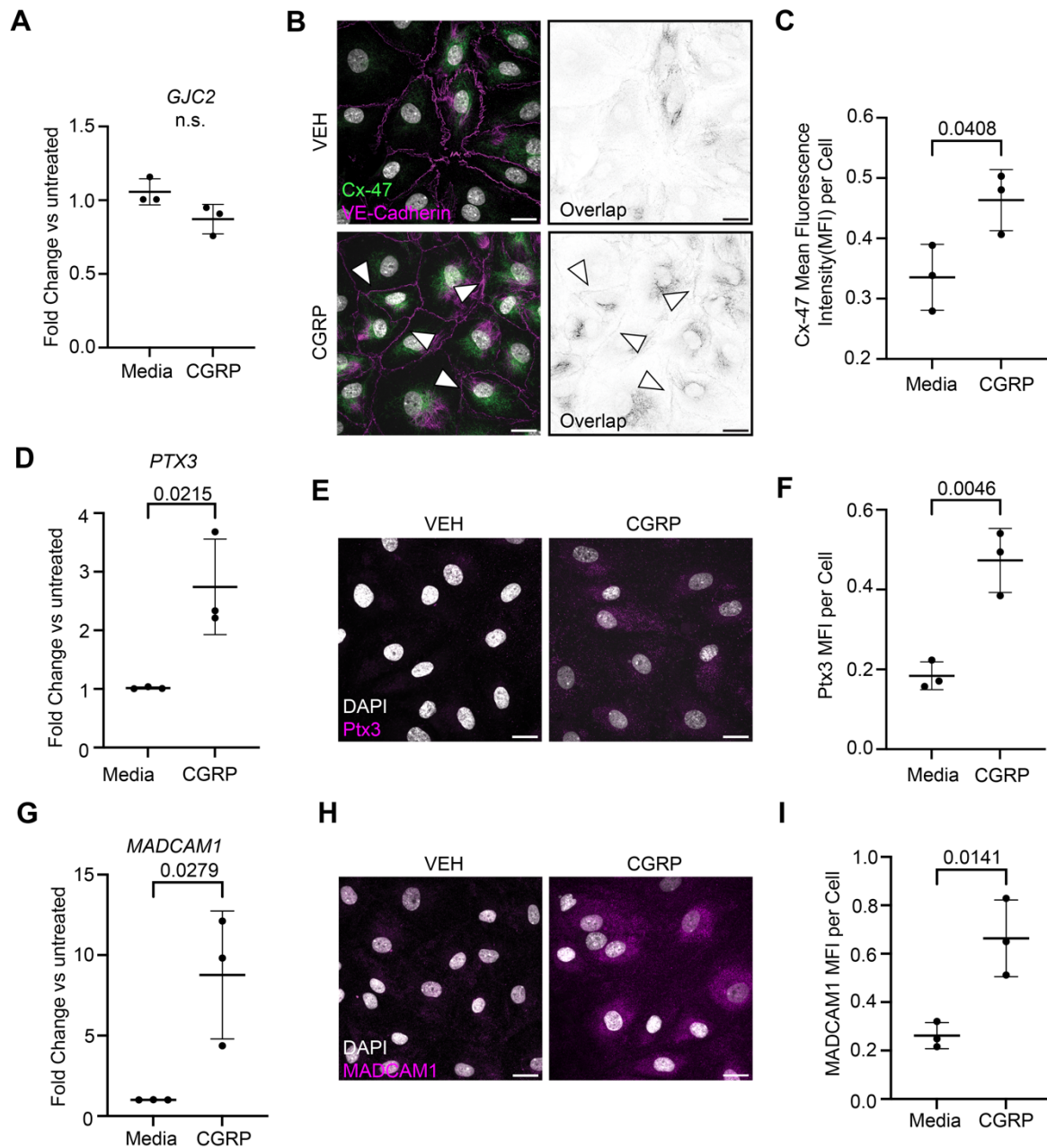


901  
 902 **Figure 2. *Calcr<sup>fl/LEC</sup>* mice treated with NTG exhibit ameliorated light aversion and anxiety behavior.** (A) Schematic  
 903 of light aversion and movement assay. (B) Schematic of general (top) and daily (bottom) experimental protocol.  
 904 Mice were allowed to acclimate to the chamber 4 days before first injection and 2 days before baseline  
 905 measurement (baseline measurement recorded on day -1) Light avoidance and movement behavior are measured  
 906 30 minutes after NTG injection and were recorded for 30 minutes. (C) Time spent in dark chamber reported for all  
 907 test days and on day 1 (D) and day 5 (E). Dashed line = total test time (1800 seconds = 30 minutes). (F) Time spent  
 908 in light chamber reported for all test days. (G) Normalized percent time spent moving in the dark chamber and (H)  
 909 normalized percent time spent in moving in the light chamber for all experimental days. Percent of time spent  
 910 moving in the respective chamber were moving normalized to baseline movement in the same chamber data.  
 911 Data are normalized to the baseline measurement on day -1 by individual calculated as percent time moving on  
 912 test day divided by baseline percent time moving (day -1) multiplied by 100. \* = p < 0.05, \*\* = p < 0.01 reported  
 913 between *Calcr<sup>fl/fl</sup>* and *Calcr<sup>fl/LEC</sup>* treated with NTG. Dashed line = baseline. C-H, n = 14-20 animals per group. Data  
 914 represents 12 independent cohorts. Significance for all data calculated using two-way ANOVA with Tukey's  
 915 multiple comparisons test. P values depicted if < 0.1. Graphs = mean ± standard deviation.

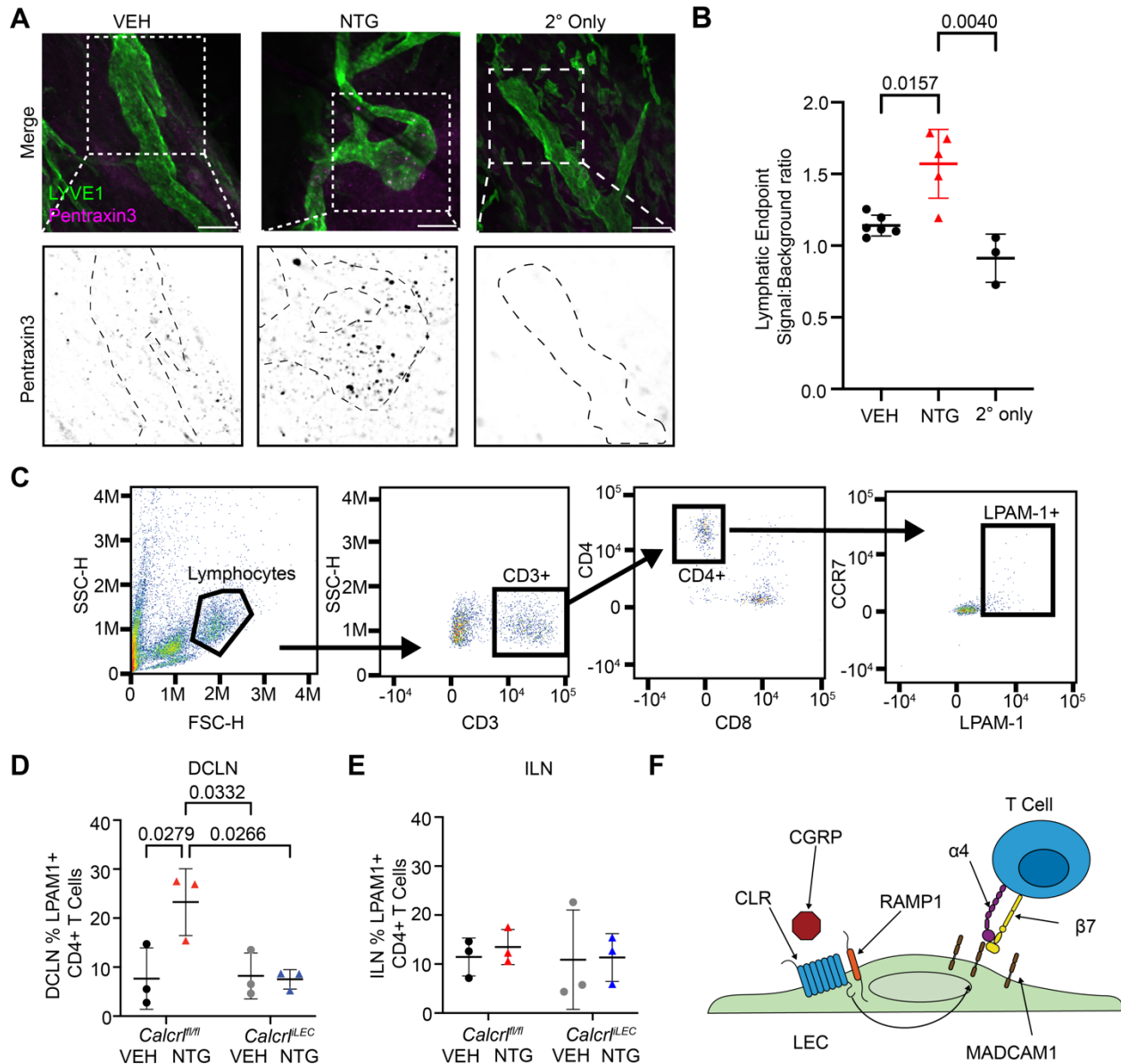




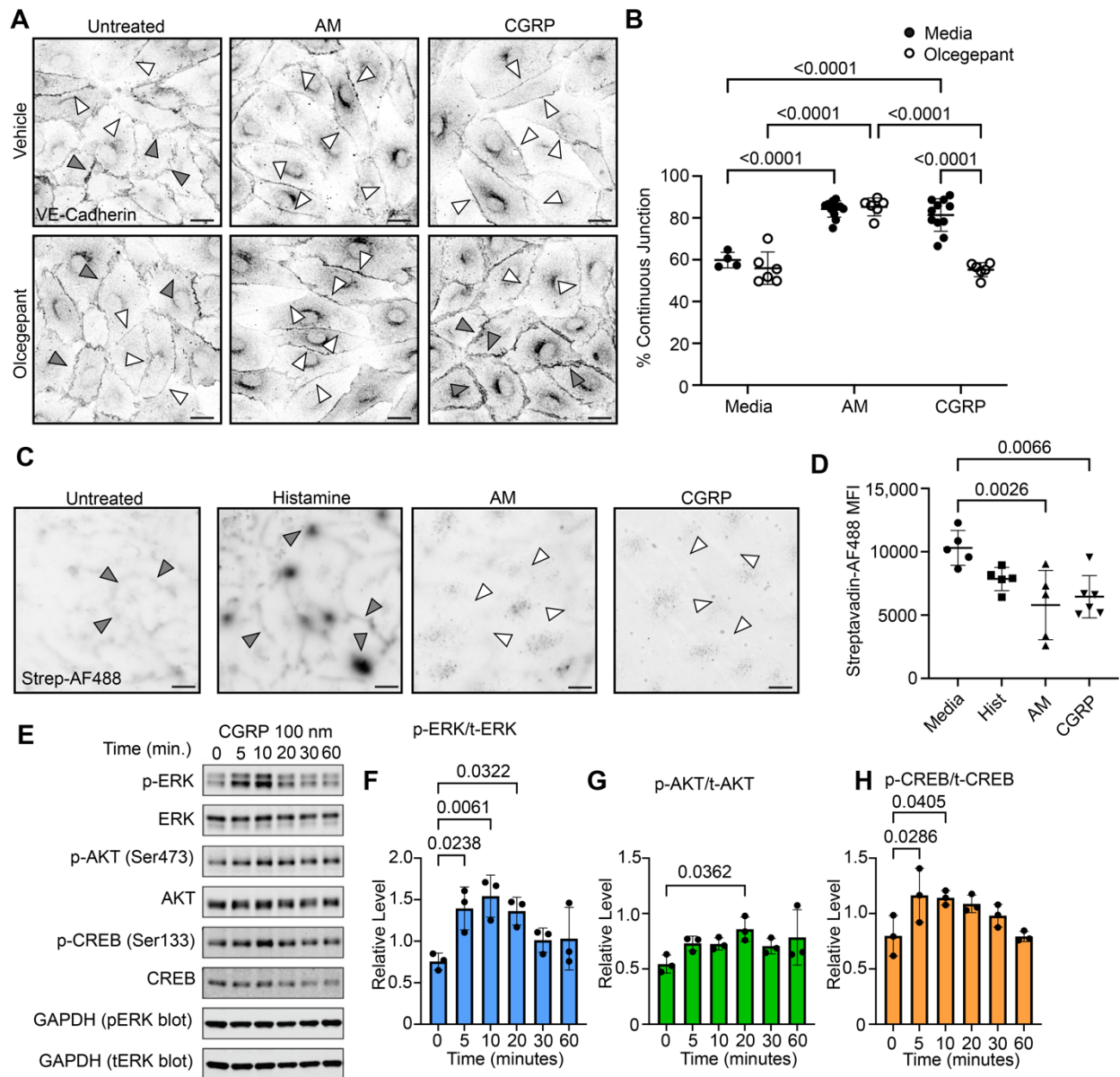
917  
 918 **Figure 3. Mice treated with nitroglycerin model of chronic migraine exhibit unique MLV translational profiles. (A)**  
 919 **RiboTag Lyve1-Cre schematic depicting experimental protocol. (B)** Mean Grimace Score for RiboTag mice,  
 920 **confirming initiation of chronic migraine model. N = 3 animals per group. Data not recorded from final injection day**  
 921 **(Day 8). Graph = mean ± Standard Deviation. (C)** Heatmap of differentially translated genes. Red = up with NTG, Blue  
 922 **= down with NTG. N = 3 animals per group from one cohort. (D)** IPA analysis of differentially expressed genes from  
 923 **microarray. Top 10 pathways with >4 identified transcripts per pathway displayed. (E)** Volcano plot of significant  
 924 **differentially genes in meningeal lymphatic vessels. Genes depicted were identified from PubMed searches for**  
 925 **(Lymphatics) and (Gene Symbol), had >7 papers and the topic trend was lymphatic biology. Red text = upregulated**  
 926 **and investigated. Significance determined by one-way ANOVA. Significant p values <0.05 (above dashed line).**



927  
 928 **Figure 4. CGRP induces protein level changes in LECs.** (A) qPCR analysis of *GJC2* in LECs. (B) Confocal microscopy of  
 929 Connexin-47 (green) and VE-Cadherin (magenta) in vehicle (VEH) and CGRP treated LECs in vitro. Right: Image of  
 930 overlapping Connexin-47 and VE-Cadherin signal. Black represents Connexin-47 and VE-Cadherin colocalization.  
 931 Pixels have signal only if there is signal for both VE-Cadherin and Cx-47. Arrows = Connexin-47 at continuous VE-  
 932 Cadherin borders. (C) Quantification of Mean fluorescence intensity (MFI) of Connexin-47. (D) qPCR analysis of *PTX3*  
 933 in CGRP treated LECs. (E) Immunofluorescence of Pentraxin3 in LECs. (F) Quantification of MFI of Pentraxin3 in LECs.  
 934 (G) qPCR analysis of *MADCAM1*. (H) Immunofluorescence of *MADCAM1* in CGRP treated LECs. (I) Quantification of  
 935 MFI of *MADCAM1* in LECs. For all qPCR analysis (A, D, G), N = 3 biological replicates with N = 3 technical replicates.  
 936 For all immunofluorescence experiments (C, F, I), N = 3 biological replicates with 3 randomly selected fields of view  
 937 averaged for each biological replicate. Significance for all data presented calculated using two tailed, unpaired  
 938 student's t-test. Scale bar = 20  $\mu$ m. Graphs = mean  $\pm$  standard deviation.

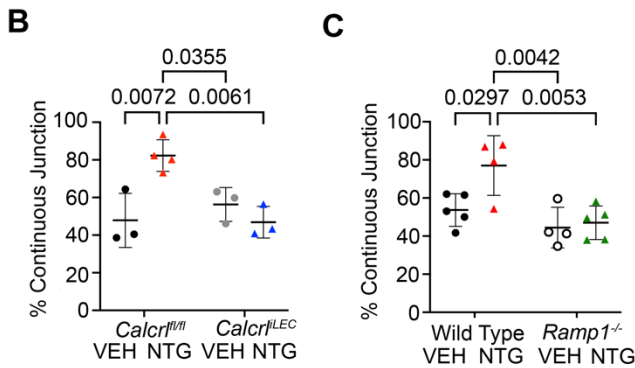
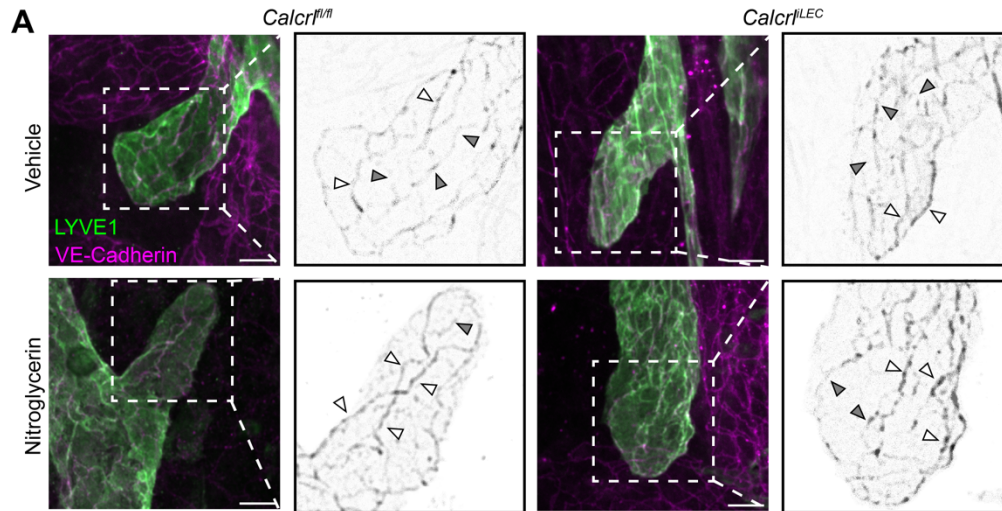


939  
 940 **Figure 5. RiboTag and in vitro changes are recapitulated in NTG mediated chronic migraine. (A)** Whole-mount  
 941 microscopy of decalcified meninges from mice treated with vehicle or NTG. Secondary only immunofluorescence  
 942 included as negative control. Top = costaining of LYVE-1 (green) and Pentraxin3 (magenta). Bottom = increased  
 943 magnification images of the white dashed square in top row. Black = Pentraxin3. Black dashed lines = MLV outlines.  
 944 Scale bar = 20  $\mu$ m. **(B)** Quantification of PTX3 fluorescence relative to background fluorescence in MLV endpoints.  
 945 Significance calculated using one-way ANOVA with N = 3 -6 animals with at least 2 endpoints assessed per animal.  
 946 Two independent cohorts were assessed. **(C)** Flow cytometry gating strategy. Quantification of flow cytometric  
 947 analysis of LPAM1+ ( $\alpha$ 4/ $\beta$ 7 integrin+) CD4+ T cells in **(D)** DCLNs (draining meninges) and **(E)** inguinal lymph nodes  
 948 (distal lymph nodes) of NTG treated chronic migraine *Calcr<sup>flLEC</sup>* mice. N = 3 animals per group, pooled left and right  
 949 DCLN from two independent cohorts, performed in duplicate. **(F)** Schematic indicating proposed relationship  
 950 between CGRP, MADCAM1, and  $\alpha$ 4/ $\beta$ 7 integrin+ (LPAM1+) CD4+ T cell interaction with LECs. Significance for all  
 951 graphs calculated using two-way ANOVA with Tukey's multiple comparisons test. P value shown if less than 0.05.  
 952 Graphs = mean  $\pm$  standard deviation.

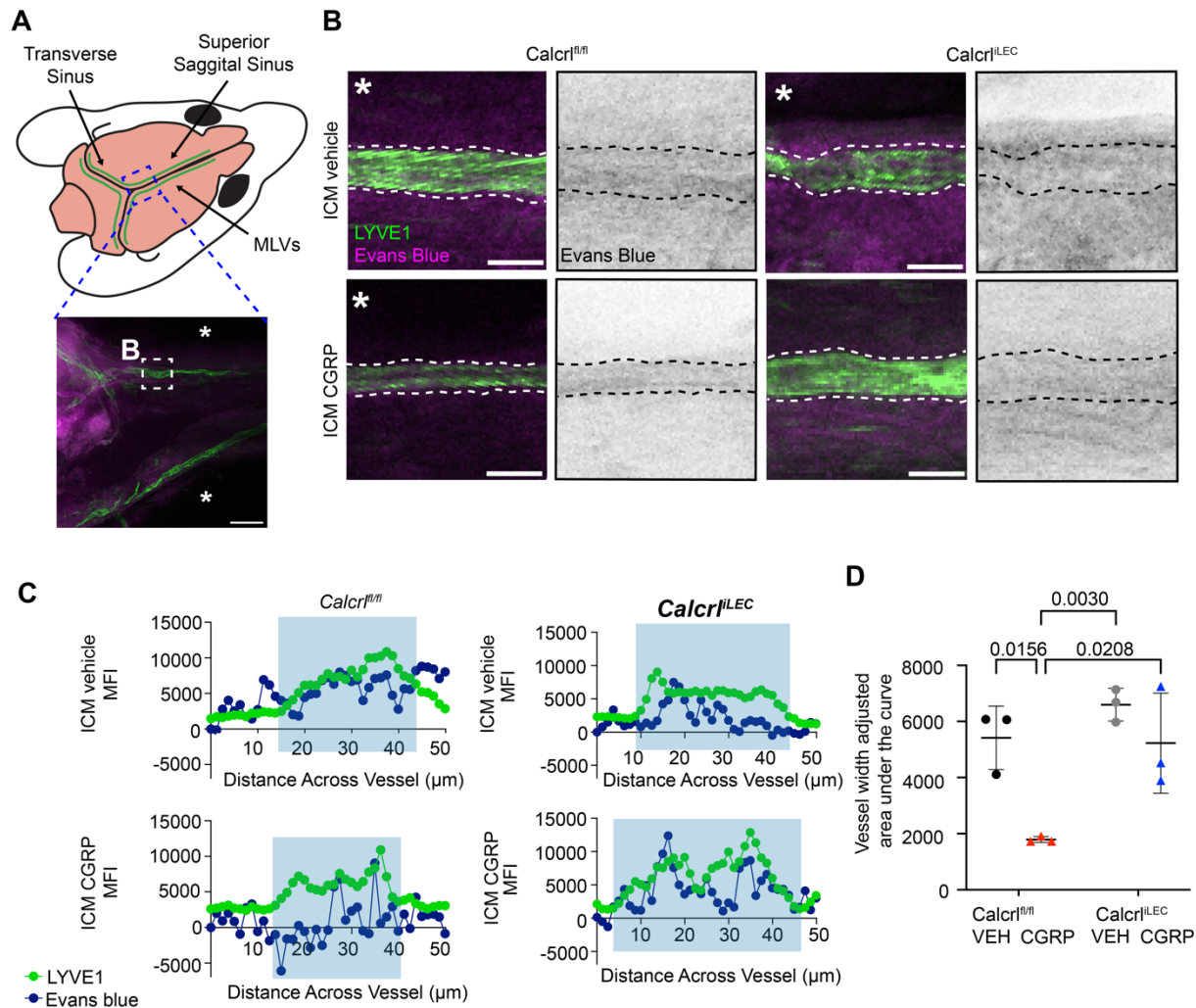


953  
 954 **Figure 6. CGRP induces formation of continuous, non-permeable VE-Cadherin LEC junctions in cultured hLECs. (A)**  
 955 LECs treated with media, 100 nM adrenomedullin (low-permeability control), or 100 nM CGRP and treated with or  
 956 without CGRP receptor antagonist olcegepant and incubated with antibodies targeting VE-Cadherin. White arrows  
 957 indicate continuous VE-Cadherin arrangement, gray arrows indicate discontinuous VE-Cadherin arrangement. Scale  
 958 bar = 10  $\mu$ m **(B)** Quantification of proportion of LEC continuous junctions treated with media, CGRP, or  
 959 adrenomedullin. Significance calculated using one-way ANOVA with Tukey's multiple comparison's test. N = 4-11  
 960 biological replicates with 2-3 randomly selected fields quantified per coverslip from 5 independent assays. All visible  
 961 adherens junctions were counted and scored as either continuous or discontinuous by a blinded scorer. Percent  
 962 continuous junctions is calculated as number of continuous VE-Cadherin junctions divided by total number of  
 963 observed junctions multiplied by 100. **(C)** Fluorescence microscopy of LECs grown on biotinylated-fibronectin coated  
 964 coverslip and treated with 10  $\mu$ M histamine (high permeability control), 100 nM adrenomedullin, or 100 nM CGRP  
 965 or media alone then treated with Alexa Fluor-488 labeled streptavidin. Dark signal indicates increased Alexa Fluor-  
 966 488 streptavidin permeability between LECs. White arrows indicate low permeability cell borders, gray arrows  
 967 indicate highly permeable cell borders. Scale bar = 20  $\mu$ m. **(D)** Permeability between LECs quantified as mean  
 968 fluorescence intensity of labeled streptavidin bound. N = 5-6 biological replicates with 3-4 randomly selected fields  
 969 quantified from 3 independent assays. Mean fluorescence intensity of each field assessed is averaged together to

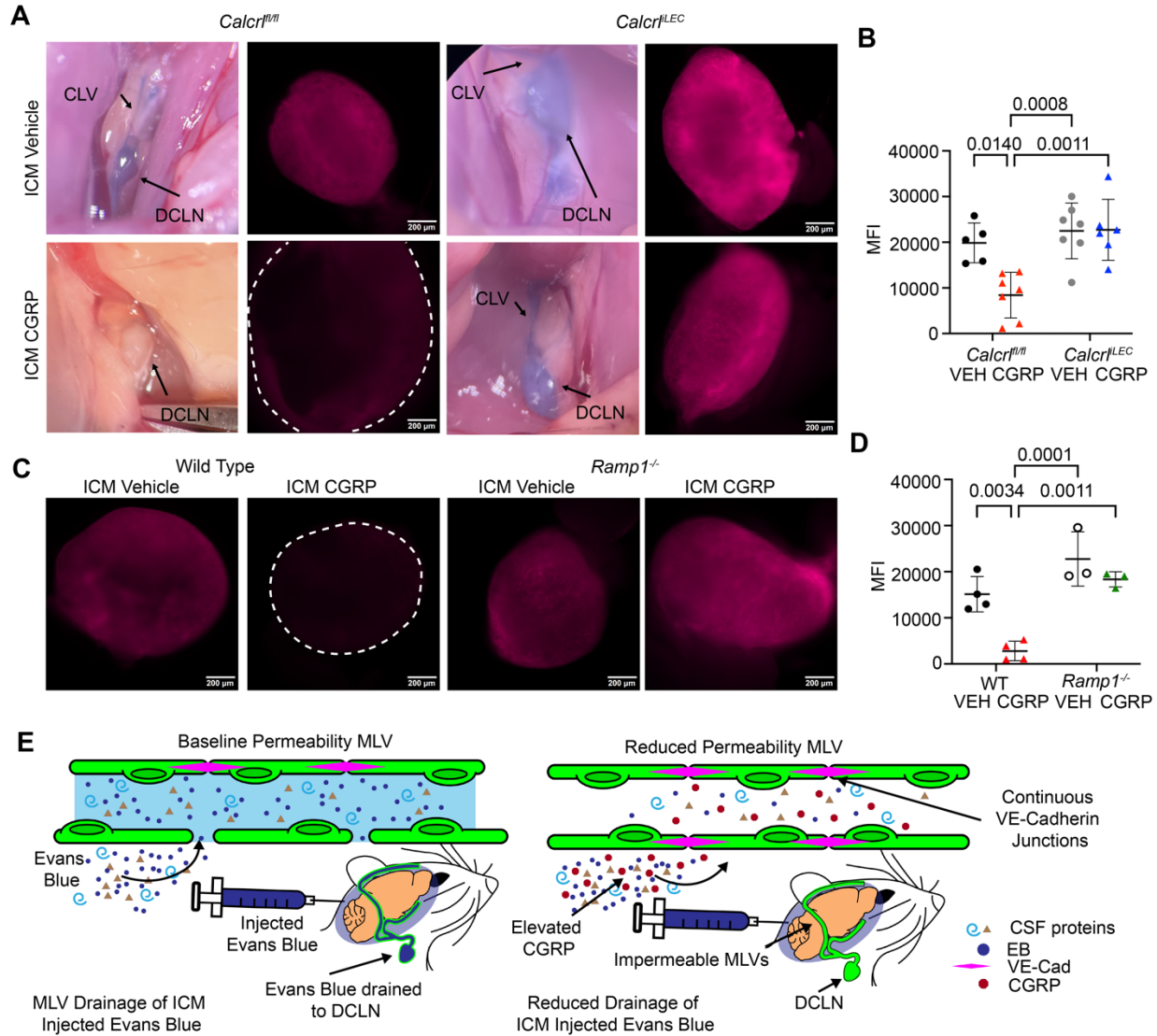
970 represent one biological replicate. Significance for B and D calculated using one-way ANOVA with Tukey's multiple  
971 comparisons test. Graphs = mean  $\pm$  standard deviation. **(E)** Representative western blot assessing LEC intracellular  
972 signaling response to CGRP over 60 minutes. Quantification of phosphor-ERK (p-ERK) to total ERK (t-ERK) (F) p-AKT  
973 to t-AKT (G) and p-CREB to t-CREB (H) response to CGRP over 60 minutes. **F, G, H** significance was determined using  
974 an ordinary one-way ANOVA with Dunnett's multiple comparisons. Three independent experiments were  
975 conducted.



976  
 977 **Figure 7. NTG induced CGRP stimulus is required for formation of continuous VE-Cadherin junctions in vivo. (A)**  
 978 Whole-mount immunofluorescence microscopy targeting LYVE1-1 and VE-Cadherin in NTG induced chronic migraine  
 979 in *Calcr*<sup>fLEC</sup> mice. White arrows = continuous VE-Cadherin junctions, gray arrows = discontinuous VE-Cadherin  
 980 junctions. Scale bar = 10  $\mu$ m. Black and white inset image are increased magnification of white dashed square. **(B)**  
 981 Quantification of proportion of MLV endpoint linear VE-Cadherin junctions in NTG treated chronic migraine *Calcr*<sup>fLEC</sup>  
 982 mice. All visible VE-Cadherin positive adherens junctions were counted and scored as either continuous or  
 983 discontinuous by a blinded scorer. Percent continuous junctions is calculated as number of continuous VE-Cadherin  
 984 junctions divided by total number of observed junctions multiplied by 100. N = 3-4 animals with percent continuous  
 985 junctions scored from 2-5 meningeal lymphatic vessel endpoints assessed per animal from 2 independent cohorts.  
 986 **(C)** Quantification of proportion of MLV endpoint linear VE-Cadherin junctions in NTG treated chronic migraine  
 987 *Ramp1*<sup>-/-</sup> mice, calculated as in **(B)**. N = 4-5 animals per group scored from 2-5 meningeal lymphatic endpoints per  
 988 animal from 3 independent cohorts. Graph = mean  $\pm$  standard deviation. Significance for all graphs calculated using  
 989 two-way ANOVA with Tukey's multiple comparisons test. P value shown if less than 0.05.



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 991 **Figure 8. CGRP reduces CSF uptake into meningeal lymphatic vessels.** (A) Schematic of MLV anatomy. Blue dashed  
 992 square = location assessed White dashed square = location for dye measurement, as visualized in B. \* = space outside  
 993 sinus. Scale bar = 200  $\mu\text{m}$ . (B) Left: representative image of LYVE1+ (Green) MLVs outlined with white dashed line.  
 994 Right: EB fluorescence in black. Black dashed line = MLV border. Scale bar = 50  $\mu\text{m}$ . (C) Representative integrated  
 995 fluorescence intensity across MLV measured using plot profile feature in ImageJ. An average background  
 996 fluorescence intensity for each field was measured and subtracted from raw EB fluorescence intensity values. Green  
 997 line = LYVE1 MFI, Blue line = EB MFI, Blue box = margins of MLV. (D) Quantification of Background adjusted integral  
 998 of EB intensity across MLV. N = 3 animals one drainage site assessed per animal from 3 independent cohorts.  
 999 Graph = mean  $\pm$  standard deviation. Significance calculated using two-way ANOVA with Tukey's multiple comparisons test.  
 1000 P value shown if less than 0.05.



**Figure 9: Intra cisterna magna injection of CGRP reduces CSF efflux to the DCLNs by EB dye transport. (A)** Photograph and whole mount fluorescence microscopy of DCLN from *Calcr<sup>flLEC</sup>* mice injected intra cisterna magna (ICM) with 1  $\mu\text{g}/\mu\text{L}$  CGRP or vehicle diluted in 1% EB. Scale bar = 200  $\mu\text{m}$ . CLV = cervical lymphatic vessel, DCLN = deep cervical lymph node. **(B)** Quantification of mean fluorescence intensity of DCLN of *Calcr<sup>flLEC</sup>* mice injected ICM with 1  $\mu\text{g}/\mu\text{L}$  CGRP or vehicle diluted in 1% EB. N = 5-7 animals, average MFI of left and right DCLNs graphed from 4 independent cohorts. Graph = mean  $\pm$  standard deviation. Significance for calculated using two-way ANOVA with Tukey's multiple comparisons test. P value shown if less than 0.05. **(C)** Whole mount fluorescence microscopy of DCLN from *Ramp1<sup>-/-</sup>* or Wild Type mice injected intra cisterna magna (ICM) with 1  $\mu\text{g}/\mu\text{L}$  CGRP or vehicle diluted in 1% EB. Scale bar = 200  $\mu\text{m}$ . **(D)** Quantification of mean fluorescence intensity of DCLN of *Ramp1<sup>-/-</sup>* or Wild Type mice injected ICM with 1  $\mu\text{g}/\mu\text{L}$  CGRP or vehicle diluted in 1% EB. N = 3-4 animals, average MFI of left and right DCLNs graphed from 3 independent cohorts. Graph = mean  $\pm$  standard deviation. Significance for calculated using two-way ANOVA with Tukey's multiple comparisons test. P value shown if less than 0.05. **(E)** Representative schematic of CGRP impact on MLVs demonstrating reduced MLV permeability and CSF efflux to the DCLN.

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