Pain Relief Foundation Research Grant

Project:

Mechanisms of inflammatory joint pain: The role of TRPM3 in knee nociceptors and its interaction with fibroblast-like synoviocytes;

Award:

£20,556;

Awardees:

Dr. Javier Aguilera-Lizarraga (Principal Investigator), Department of Pharmacology, University of Cambridge, Cambridge, United Kingdom.

Prof. Ewan St. John Smith (Co-investigator), Department of Pharmacology, University of Cambridge, Cambridge, United Kingdom.

Background and objectives:

Rheumatoid arthritis is a debilitating inflammatory condition affecting around 0.46-1% of the global population, including >400 individuals per 100,000 inhabitants in the UK[1]. The hallmarks of rheumatoid arthritis include joint damage and swelling, which lead to the development of chronic pain and the subsequent decrease in patients' quality of life[2]. Indeed, patients with rheumatoid arthritis identify pain as their most important and disabling symptom[3], which may persist despite proper control of the inflammation[4]. Rheumatoid arthritis is a complex condition, but it is becoming increasingly clear that fibroblast-like synoviocytes, a cell type that lines synovial joints, such as the knee, play an important role in disease progression. Recent research in our laboratory showed that molecules produced by these cells during inflammation can increase the sensitivity of sensory neurons that transmit pain signals[5]. This process results in stronger and/or longer-lasting activation of sensory neurons and can lead to the development of chronic pain. Therefore, the interaction between fibroblast-like synoviocytes and sensory neurons is of great scientific and clinical interest, as might be a crucial factor in the development of inflammatory joint pain.

We previously showed that the Transient Receptor Potential (TRP) cation channel subfamily Vanilloid member 1 (TRPV1 channel), an important receptor for the transmission of pain signals in sensory neurons, plays a key role in inflammatory joint pain[6] and that its function is modulated by fibroblast-like synoviocytes[5]. However, the clinical success of treatments targeting TRPV1 has been hindered due to negative side effects[7], emphasising the need of identifying alternative disease targets for inflammatory joint pain.

In this study, we aim to establish new mechanisms in inflammatory joint pain to identify novel markers of potential therapeutic interest. We have focused our attention on another TRP channel, the TRP channel subfamily Melastatin member 3 (TRPM3). TRPM3 is expressed by both mouse[8] and human[9] sensory neurons and, like TRPV1, TRPM3 functionality has been shown to increase during inflammation[8,10]. However, the role of TRPM3 in inflammatory joint pain has not been investigated. Furthermore, which mediators contribute to TRPM3 sensitisation, and thus the potentiation of its function, in inflammatory pain remains to be investigated.

We hypothesised that activation of fibroblast-like synoviocytes during inflammatory joint leads to the release of pro-nociceptive mediators which in turn sensitise TRPM3 and drive inflammatory joint pain. To answer this question, we designed the following objectives:

1. Evaluate TRPM3's involvement in inflammatory joint pain using a mouse model of knee joint inflammation.
2. Assess how inflammatory molecules affect the activity of TRPM3 in sensory neurons cultured in a laboratory setting.

3. Investigate whether fibroblast-like synoviocytes release inflammatory mediators that can sensitise or activate TRPM3.

Results:

Firstly, we explored the expression of TRPM3 channel in sensory neurons innervating the knee. To this end, a fluorescent dye (Fast Blue) was injected into the knee joint of mice. The nerve endings innervating this structure transport the fluorescent dye from the tip of the axons to the cell body, situated in clusters called dorsal root ganglia (DRG) next to the spinal cord. Thus, a few days after dye injection, knee-projecting neurons can be identified using conventional microscopy techniques. In agreement with previous reports[11], we found that ~5% of neurons in the DRG evaluated (lumbar (L)2, L3, L4 and L5 levels of the spinal cord), innervated the knee joint (Fig. 1a, b). Moreover, we found that the expression of TRPM3 and TRPV1 was comparable between knee-innervating neurons (Fast Blue^{+}) and total DRG neurons (including sensory neurons innervating other structures, Fast Blue^{-}) (Fig. 1a-f).

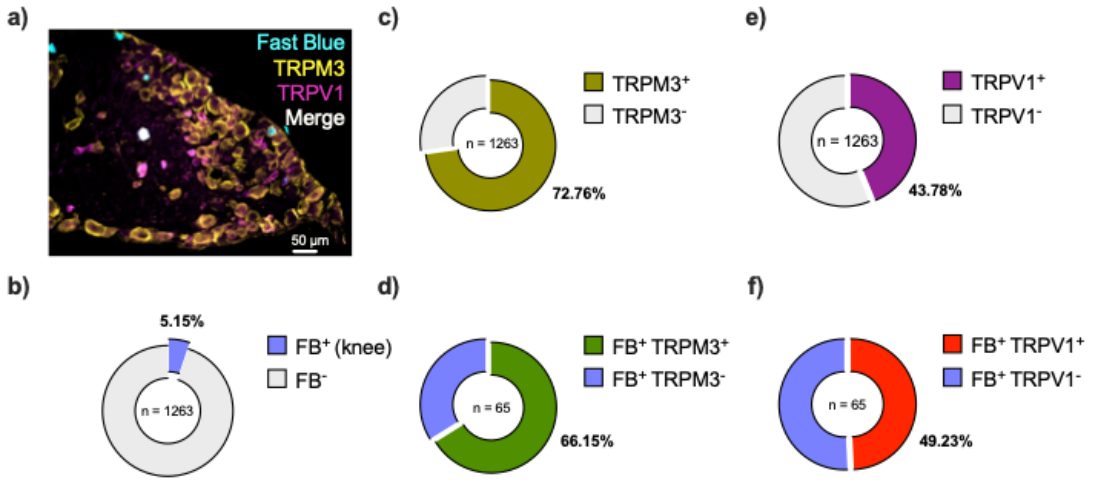

Figure 1. ***Expression of TRPM3 in knee-innervating sensory neurons***. **a**) Representative fluorescent image of DRG sensory neurons, with markers for Fast Blue (cyan), TRPM3 (yellow) and TRPV1 (magenta). **b**) % of total DRG neurons expressing Fast Blue (i.e., innervating the knee joint). **c**) % of total DRG neurons expressing TRPM3. **d**) % of knee-innervating DRG neurons expressing TRPM3. **e**) % of total DRG neurons expressing TRPV1. **f**) % of knee-innervating DRG neurons expressing TRPV1.

We next used the well-characterised complete Freund's adjuvant (CFA) model[6,12,13] to study inflammatory joint pain. Similar to the administration of Fast Blue, animals were injected intra-articularly with CFA (100 µg) through the patellar tendon of one knee, the contralateral knee being used as control. We confirmed that animals receiving CFA developed significant inflammation in the injected knee, compared to mice that received saline (Fig. 2a). To evaluate inflammatory knee pain, we assessed the digging behaviour in these animals, as we previously showed that this is a representative and reliable assay to measure spontaneous pain in laboratory rodents[14]. Indeed, mice injected with CFA showed a remarkable impairment in digging activity, compared to animals that received knee injections of saline (Fig. 2b-d). Thereafter, we assessed the expression of TRPM3 in DRG sensory neurons innervating the knee in these animals. In agreement with previous reports[6] we observed an increased in the relative expression of TRPV1 in knee-projecting sensory neurons in mice injected with CFA compared with those injected with saline (Fig. 2e), however, the expression of TRPM3 in knee-projecting neurons was comparable between knees injected with CFA or saline (Fig. 2f).

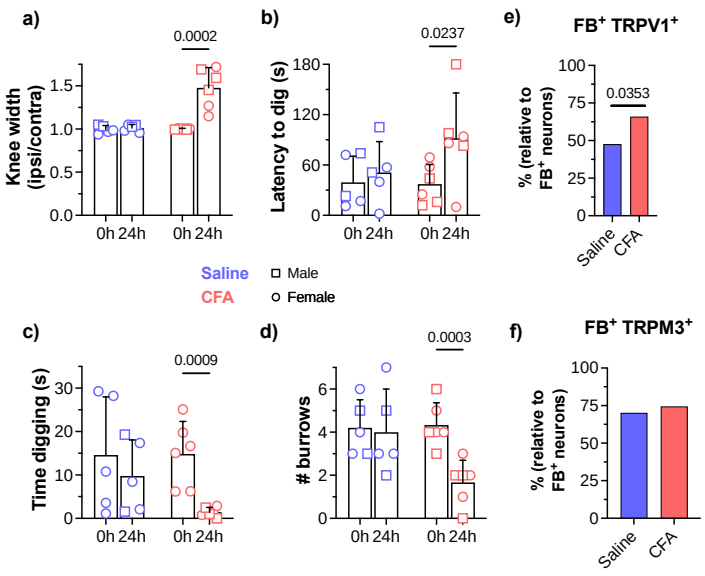

Figure 2. ***CFA-induced inflammatory knee joint pain and expression of pain markers in knee sensory neurons***. **a**) Ratio of ipsilateral knee width to contralateral knee width on the day of injections and 24 hours after. **b**) Latency of mice to dig. **c**) Time spent digging before and 24 hours after CFA injection. **d**) Number of visible burrows at the conclusion of the 3-minute digging test. **e**) % of knee-innervating DRG neurons expressing TRPV1 in mice receiving saline and CFA. **f**) % of knee-innervating DRG neurons expressing TRPM3 in mice receiving saline and mice CFA. For digging behaviour: n saline group: 5 mice (3 females, denoted by open circles; and 2 males, denoted by open squares); n CFA group: 6 mice (3 females, denoted by open circles; and 3 males, denoted by open squares). For DRG immunostaining: saline group, 951 neurons evaluated, 4.42% of knee-projecting neurons, Fast Blue^{+}; CFA group, 998 neurons evaluated, 3.91% of knee-projecting neurons, Fast Blue^{+}. **a**, **b**, **c** and **d**, 2-way ANOVA and Sidak's multiple comparison test; **e** and **f**, Fisher's exact test.

Although the number of TRPM3^{+} positive knee-innervating neurons supplying inflamed knees was not altered, previous studies have shown that the activity of this channel is augmented under inflammatory conditions[8]. Hence, to evaluate whether an increased TRPM3 activity is involved in the CFA-induced knee joint pain, we used isosakuranetin, as this has been shown to inhibit the activity of TRPM3 *in vivo*[15]. To this end, mice subjected to CFA-induced knee inflammation were treated intraperitoneally with isosakuranetin, and their digging behaviour has evaluated. Treatment with the TRPM3 inhibitor did not affect the level of inflammation following the administration of CFA (Fig. 3a). Although animals treated with isosakuranetin did not show a delay in the latency to dig (Fig. 3b), this treatment did not improve the deficits in digging behaviour (time digging or number of burrows) caused by CFA, compared to animals treated with the vehicle (Fig. 3c, d). Altogether, our results indicate that CFA-induced knee inflammation does not increase TRPM3 expression in sensory neurons innervating the knees, and that treatment with the TRPM3-inhibitor isosakuranetin does not alleviate acute inflammatory knee joint pain.

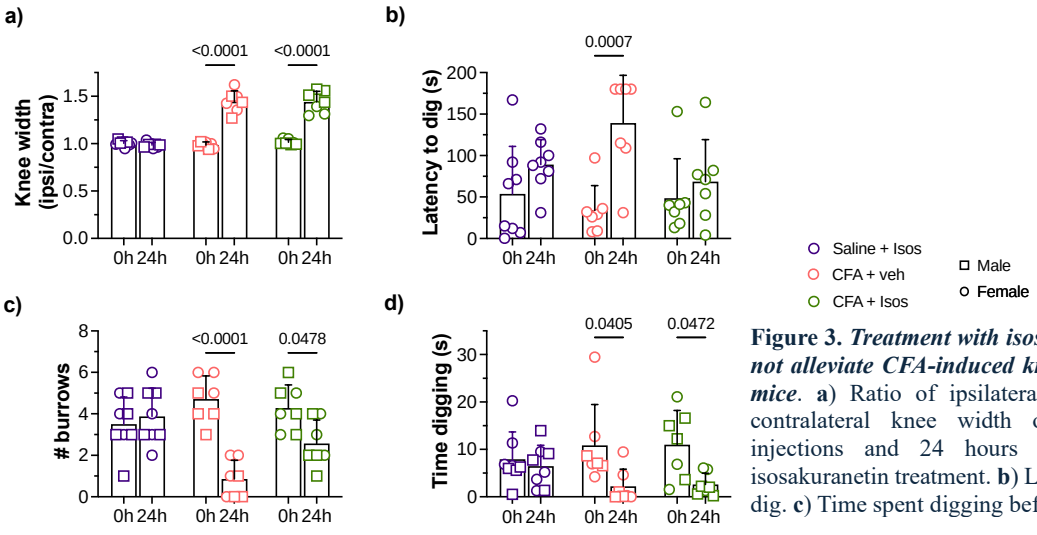

Figure 3. ***Treatment with isosakuranetin does not alleviate CFA-induced knee joint pain in mice***. **a**) Ratio of ipsilateral knee width to contralateral knee width on the day of injections and 24 hours after and after isosakuranetin treatment. **b**) Latency of mice to dig. **c**) Time spent digging before and 24 hours after CFA injection and after isosakuranetin treatment. **d**) Number of visible burrows at the conclusion of the 3-minute digging test. Saline + isos (control group): 8 mice (4 females, denoted by open circles; and 4 males, denoted by open squares); n CFA + veh (control group): 7 (4 females, denoted by open circles; and 3 males, denoted by open squares); n CFA + isos (experimental group): 7 (4 females, denoted by open circles; and 3 males, denoted by open squares). 2-way ANOVA and Sidak's multiple comparison test was used to analyse all data. *Veh* = vehicle; *isos* = isosakuranetin.

Despite the lack of efficacy of isosakuranetin in our inflammatory joint pain model, it has previously been shown that TRPM3 activity is potentiated during inflammation[8]. However, which inflammatory molecules are responsible for this process remains poorly characterised. Thus, our next aim was to answer this question. To this end, we used primary cell cultures of DRG sensory neurons and Ca^{2+} imaging. TRPM3 channels are activated by the neurosteroid pregnenolone sulfate[16] (PregS) (Fig. 4a). In agreement with our previous immunochemistry analysis (Fig. 1c), we found that ~76% of DRG neurons responded to PregS (Fig. 2b). Moreover, in line with previous studies[8,9], most neurons responding to the TRPV1 agonist capsaicin also responded to PregS (87.32%, Fig. 2c, d), thus confirming the high co-expression of TRPM3 in $TRPV1^+$ DRG sensory neurons. This was confirmed using immunohistochemistry, as we found that 82.1% of DRG neurons expressing TRPV1 also stained positive for TRPM3 channel.

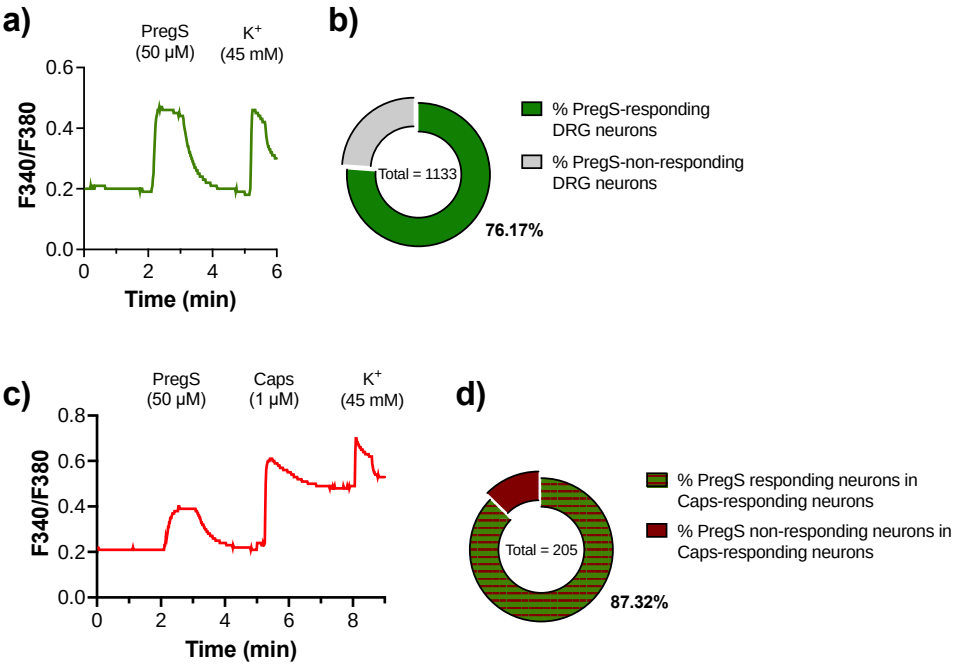

Figure 4. ***TRPM3 activation in DRG sensory neurons.*** **a**) Representative trace of [Ca^{2+}] changes in DRG sensory neurons responding to PregS. **b**) % of DRG neurons that responded to PregS. **c**) Representative trace of calcium changes in DRG sensory neurons responding to PregS and capsaicin. **d**) % of PregS-responding DRG neurons that also responded to capsaicin.

Next, we sought to determine whether neurons responding to PregS, also express receptors for inflammatory cytokines, whose activation could lead to the sensitisation/potentiation of this channel. Thus, we focused on tumour necrosis factor α (TNFα), and interleukins (IL) 1β and after application of PregS, DRG neurons were challenged with these inflammatory molecules. We found that TNFα induced Ca^{2+} responses in around 30% of DRG neurons (Fig. 5a, b), and that ~38% PregS responding neurons also responded to TNFα (Fig. 5c). In contrast, IL-6 only triggered Ca^{2+} responses in ~7% of DRG neurons (Fig. 5d, e), and less than 4.5% of those that also responded to PregS (Fig. 5f). By contrast, IL-1β caused Ca^{2+} increases in ~31% of DRG neurons (Fig. 5g, h) and almost 34% of neurons that responded to PregS. Taken together, these results indicate that both TNFα and IL-1β may be potential candidates to the cause sensitisation/potentiation of the TRPM3 channel. Future experiments of this project will evaluate this.

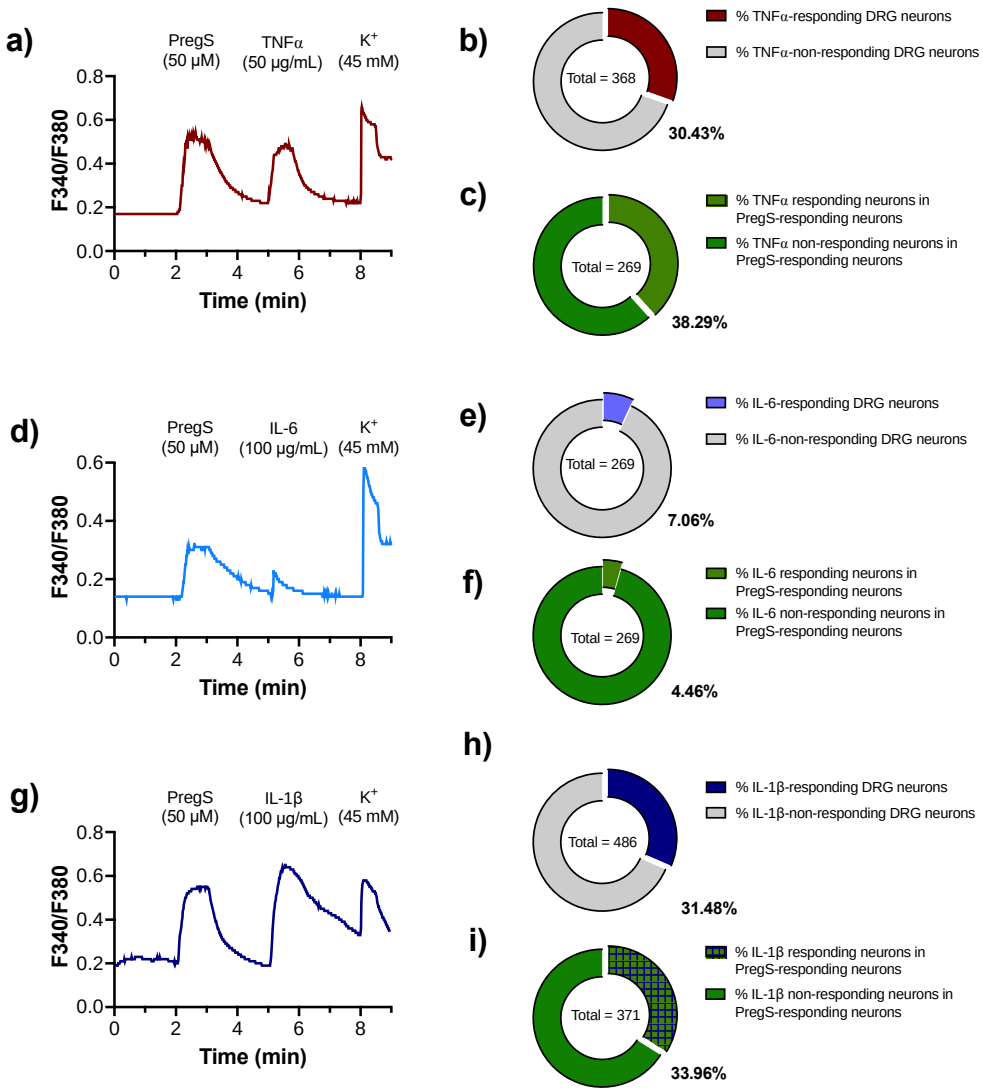

Figure 5. ***Cytokine activation of TRPM3⁺ DRG sensory neurons.*** **a**) Representative trace of calcium changes in DRG sensory neurons responding to PregS and TNFα. **b**) % of DRG neurons that responded to TNFα. **c**) % of PregS-responding DRG neurons that responded to TNFα. **d**) Representative trace of calcium changes in DRG sensory neurons responding to PregS and IL-6. **e**) % of DRG neurons that responded to IL-6. **f**) % of PregS-responding DRG neurons that responded to IL-6. **g**) Representative trace of calcium changes in DRG sensory neurons responding to PregS and IL-1β. **h**) % of DRG neurons that responded to IL-1β. **i**) % of PregS-responding DRG neurons that responded to IL-1β.

Conclusion and future plans:

Our results indicate that despite the high expression of TRPM3 in knee-innervating sensory neurons, pharmacological inhibition of TRPM3 is not sufficient to alleviate inflammatory knee joint pain in mice. However, we found that >1/3 of neurons responding to the TRPM3-agonist pregnenolone sulphate (PregS) are also activated by TNFα and IL-1β. Although we also found that some neurons responding to PregS activation are activated by IL-6, this occurrence was very small (>5%). Therefore, we will next use both TNFα and IL-1β to evaluate whether these inflammatory cytokines can potentiate TRPM3 function. Moreover, we will also evaluate the potential role of fibroblast-like synoviocytes as a source of inflammatory mediators and assess whether activated fibroblast-like synoviocytes can modulate TRPM3 function in sensory neurons.

References:

1. Finckh A, Gilbert B, Hodkinson B, et al. Global epidemiology of rheumatoid arthritis. *Nat Rev Rheumatol*. 2022;18(10):591-602. doi:10.1038/s41584-022-00827-y
2. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *New England Journal of Medicine*. 2011;23(365):2205-2219.
3. Heiberg T, Kvien TK. Preferences for improved health examined in 1,024 patients with rheumatoid arthritis: Pain has highest priority. *Arthritis Care Res (Hoboken)*. 2002;47(4). doi:10.1002/art.10515
4. Lee YC, Cui J, Lu B, et al. Pain persists in DAS28 rheumatoid arthritis remission but not in ACR/EULAR remission: A longitudinal observational study. *Arthritis Res Ther*. 2011;13(3):R83. doi:10.1186/ar3353
5. Chakrabarti S, Hore Z, Pattison LA, et al. Sensitization of knee-innervating sensory neurons by tumor necrosis factor-α-activated fibroblast-like synoviocytes: an in vitro, coculture model of inflammatory pain. *Pain*. 2020;161(9):2129-2141. doi:10.1097/j.pain.0000000000001890
6. Chakrabarti S, Pattison LA, Singhal K, Hockley JRF, Callejo G, Smith ESJ. Acute inflammation sensitizes knee-innervating sensory neurons and decreases mouse digging behavior in a TRPV1-dependent manner. *Neuropharmacology*. 2018;143:49-62. doi:10.1016/j.neuropharm.2018.09.014
7. Koivisto AP, Belvisi MG, Gaudet R, Szallasi A. Advances in TRP channel drug discovery: from target validation to clinical studies. *Nat Rev Drug Discov*. 2022;21(1):41-59. doi:10.1038/s41573-021-00268-4
8. Mulier M, Van Ranst N, Corthout N, et al. Upregulation of TRPM3 in nociceptors innervating inflamed tissue. *Elife*. 2020;9:e61103. doi:10.7554/ELIFE.61103
9. Vangeel L, Benoit M, Miron Y, et al. Functional expression and pharmacological modulation of TRPM3 in human sensory neurons. *Br J Pharmacol*. 2020;177(12):2683-2695. doi:10.1111/bph.14994
10. Vanneste M, Mulier M, Freitas ACN, et al. TRPM3 is expressed in afferent bladder neurons and is upregulated during bladder inflammation. *Int J Mol Sci*. 2022;23(1):107. doi:10.3390/ijms23010107
11. Bohic M, Pattison LA, Jhumka ZA, et al. Mapping the neuroethological signatures of pain, analgesia, and recovery in mice. *Neuron*. 2023;111(18):2811-2830.e8. doi:10.1016/j.neuron.2023.06.008
12. Chakrabarti S, Pattison LA, Doleschall B, et al. Intraarticular Adeno-Associated Virus Serotype AAV-PHP.S–Mediated Chemogenetic Targeting of Knee-Innervating Dorsal Root Ganglion Neurons Alleviates Inflammatory Pain in Mice. *Arthritis and Rheumatology*. 2020;72(10):1749-1758. doi:10.1002/art.41314
13. Keeble J, Russell F, Curtis B, Starr A, Pinter E, Brain SD. Involvement of transient receptor potential vanilloid 1 in the vascular and hyperalgesic components of joint inflammation. *Arthritis Rheum*. 2005;52(10):3248-3256. doi:10.1002/art.21297
14. Pattison LA, Cloake A, Chakrabarti S, et al. Digging deeper into pain – an ethological behavior assay correlating well-being in mice with human pain experience. *Pain*. 2024;(doi: 10.1097/j.pain.0000000000003190). doi:10.1101/2023.08.18.553862
15. Straub I, Krügel U, Mohr F, et al. Flavanones that selectively inhibit TRPM3 attenuate thermal nociception in vivo. *Mol Pharmacol*. 2013;84(5):736-750. doi:10.1124/mol.113.086843
16. Wagner TFJ, Loch S, Lambert S, et al. Transient receptor potential M3 channels are ionotropic steroid receptors in pancreatic β cells. *Nat Cell Biol*. 2008;10(12):1421-1430. doi:10.1038/ncb1801