UPREGULATION OF THE MU-OPIOID RECEPTOR IN THE CORNEA AND TRIGEMINAL GANGLION FOLLOWING CORNEAL INFLAMMATORY PAIN

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Background and aim

Ocular pain following corneal injury is frequently observed in clinic but the management of this debilitation condition remains today a therapeutic challenge in ophthalmology. The neural pathway implicated in corneal pain experience starts in the cornea, the most densely innervated epithelium by nociceptors in human body (Müller, 2003) . The corneal innervation is provided by sensitive neurons located in the ophthalmic branch (V1) of trigeminal ganglion (TG, Fig. 1) (Marfurt, 1987; Launay, 2015). Somatic pain can be modulated by exogenous and endogenous opioid peptides, which bind to mu-(MOR), delta-(DOR) and kappa-(KOR) opioid receptors widely expressed by the peripheral and central nervous system (Mansour, 1994). Morphine, opioid receptor agonist, remains as the gold standard for treating somatic pain, however, their systemic application give rise to unwanted secondary effects. To overcome these limitations arranging from its action in the central nervous system, topical morphine have been proposed for ocular pain (Peyman, 1994; Stiles, 2003; Faktorovich, 2010). These studies have clearly highlighted that targeting of peripheral opioid receptors promote significant analgesic effects. In this context, we recently reported that increasing endogenous enkephalin levels reduced ocular pain in a opioid receptor-mediated manner (Reaux-Le Goazigo, 2019). However, there are no study evaluating the expression of MOR in cornea and ophthalmic branch of the trigeminal ganglion as well as the potential analgesic effects of specific MOR selective agonists, such as DAMGO, on corneal pain relief. Therefore, the objective of the present work is the study of **MOR distribution in cornea and TG both in naïve and under corneal inflammatory pain conditions and test the effects of DAMGO in corneal pain relief.**

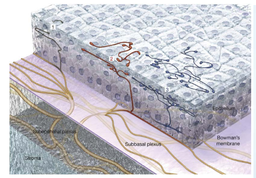
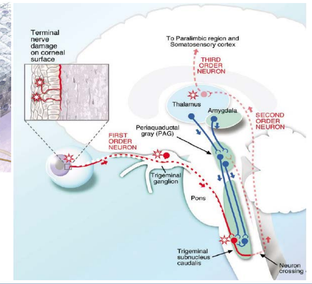


Figure 1

Rosenthal, 2009.
Belmonte, 2017.

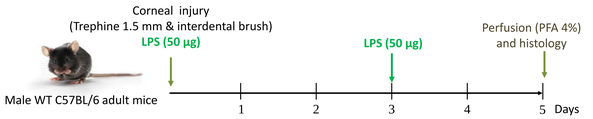


Methodology:

All animal procedures were performed in strict accordance with institutional guidelines for the care and use of experimental animals.

Animals: Male WT C57BL/6 mouse and cynomolgus macaque (*Macaca fascicularis*)

Preclinical model of corneal inflammatory pain: Corneal scraping/LPS Model: Mice were submitted to a corneal scraping followed by of lipopolysaccharide (LPS) at day 1 and day 3.



Samples: TG and cornea were obtained from mice and macaque and used for histology.

Immunolabeling: Samples were incubated with antibodies against:

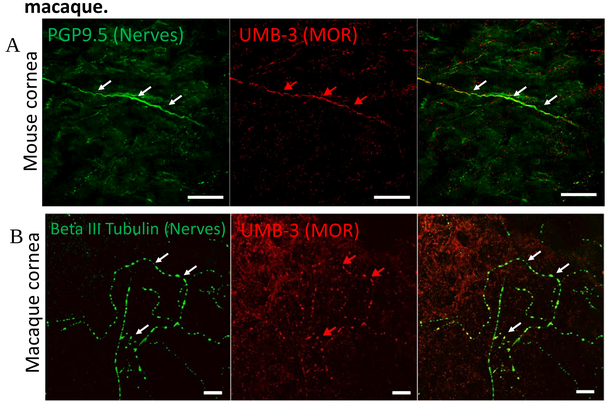
- Neuronal marker: PGP9.5 (ab8189, Abcam), Beta III tubulin (ab78078, Abcam) or Pan neuronal marker (Millipore, MAB2300)
- MOR (ab134054, Abcam) characterized by Lupp et al., 2011.

***In situ* hybridization:** RNAscope® method, MOR and TRPV1 mRNA probe, https://acdbio.com/.

Image acquisition: Confocal, epifluorescence and Nanozoomer microscopes.

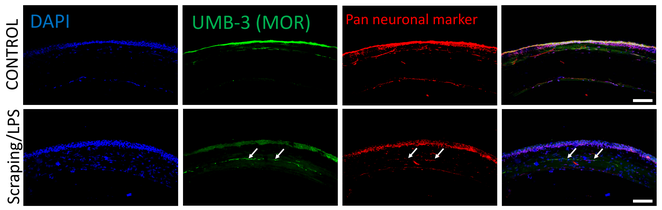
Image analysis: ImageJ (Fiji) software, **statistical analysis**: Prism software

Result 1: MOR is detected in corneal nerves in WT mice and macaque.



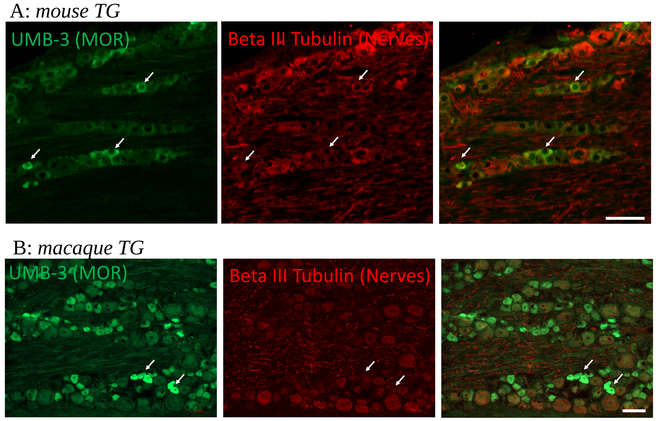
(A) Double-immunofluorescence against PGP9.5 (nerves, green) and MOR (red) . The technique was performed in whole-mount corneas from WT mice.
(B) MOR (red) and beta III tubulin (green) neuronal marker detection by immunolabeling on free floating sections from cynomolgus macaque cornea. Scale bars : 25 µm

Result 2: Corneal MOR expression is increased during inflammatory pain



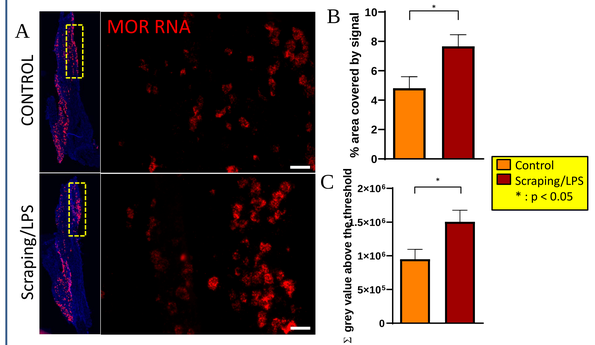
Immunostaining against MOR (red) and Pan neuronal marker (green) in frozen cornea sections of control mice and scraping/LPS pain model . DAPI staining (blue) shows the complete disorganization of the corneal layers and cellular infiltration in the stroma of scraping/LPS cornea. MOR labeling was higher in corneal nerves of scraping/LPS animals (arrows). Scale bars: 50 µm

Result 3: MOR is expressed in primary afferent neurons of the ophthalmic branch (V1) of trigeminal ganglion in WT mice and macaque.



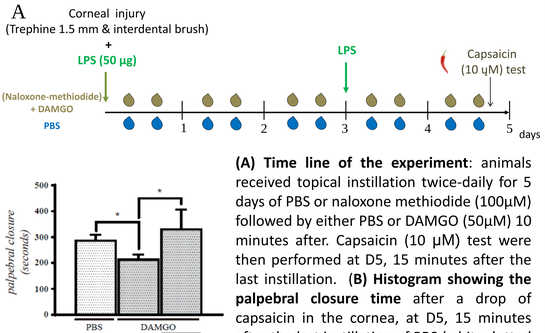
Immunostaining of MOR (red) and beta III tubulin (green) in frozen sections from mouse and macaque TG. Images show the presence of the MOR in primary afferent neurons at the level of ophthalmic branch (V1) of TG in both mice (A) and primates (B). Arrows show the of MOR-immunoreactivity in beta tubulin-positive neurons. Scale bars: 100 µm

Result 4: MOR expression is upregulated under painful conditions in the trigeminal ganglion ophthalmic branch



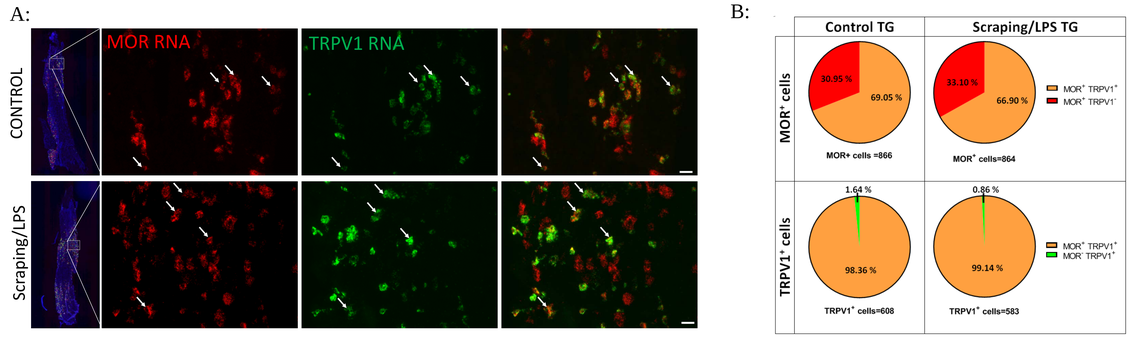
(A) Left: **Overview of the MOR expression** (red) and nuclei (DAPI, blue) in the TG from control vs scraping/LPS mouse revealed by RNAScope method. Dashed-line rectangle represents the ophthalmic branch of the trigeminal ganglion (V1). Right: Higher magnification showing the expression of MOR mRNA in the ophthalmic branch (V1) of trigeminal ganglion. Scale bars: 50 µm. **(B, C) Quantitative analysis of MOR RNA levels**. **(B) % area covered by signal.** Area with pixels whose grey level is above the threshold. **(C) Summation of all grey values** above the threshold. Both parameters showed an increase in MOR RNA expression in Scraping/LPS animals. n=4 in each group. Unpaired t-test was applied for comparison between groups.

Result 5: Repeated topical instillations of DAMGO decrease capsaicin-induced corneal pain



(A) Time line of the experiment: animals received topical instillation twice-daily for 5 days of PBS or naloxone methiodide (100µM) followed by either PBS or DAMGO (50µM) 10 minutes after. Capsaicin (10 µM) test were then performed at D5, 15 minutes after the last instillation. **(B) Histogram showing the palpebral closure time** after a drop of capsaicin in the cornea, at D5, 15 minutes after the last instillation of PBS (white dotted bar) or DAMGO or naloxone methiodide followed by DAMGO (gray dotted bar). n=5-10 mice per group. Differences between groups were analyzed using 1-way ANOVA test. * : $p < 0.05$

Result 6: MOR is expressed in TRPV1⁺ cells of the trigeminal ganglion .



(A) Left: **Overview of the MOR (red) and TRPV1 (green) RNA expression** in the TG revealed by RNAScope Method. Right: Extension of the zone delimited by a square in the left image, showing the expression of MOR and TRPV1 RNA in the ophthalmic branch of trigeminal ganglion. Notice the co-expression between MOR and TRPV1 (arrows). Scale bars: 50 µm. **(B) Quantification of the co-expression between MOR and TRPV1.** Almost all the TRPV1⁺ cells were also MOR positive. There were no significative differences between Scraping/LPS and control animals. Chi square was applied for comparison between groups.

Conclusions:

The present study shows for the first time the presence of MOR in corneal nerve fivers in mice and primates and reveal it as reliable target for ocular pain treatment with topical drugs. An innovative *in situ* hybridization method for RNA detection, RNAScope, was used in a pioneer way for the detection of MOR expression in the trigeminal ganglion. The experiments revealed an increase of MOR expression in the cornea and the TG during inflammatory pain, enhancing the availability of MOR at corneal sensory neurons during inflammatory conditions. Topical application of MOR agonist DAMGO showed analgesic effects in inflammatory corneal pain, confirming it as reliable topical treatment for corneal pain. Almost all TRPV1 expressing neurons in trigeminal ganglion were also MOR positives. Activation of MOR in these cells could be blunting nociceptive activation from the capsaicin receptor TRPV1.
This study provides novel information about the distribution of MOR in the cornea and TG in naïve and under corneal pain condition and highlights its key role in the treatment for ocular inflammatory pain.

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Conflict of interest:
The authors declare no conflict of interest.