Figure 1

1) What was the rationale for doing this experiment/including this data?

The researchers wanted to prove that morphine tolerance and opioid-induced hyperalgesia was independent of microglial activation as there are controversial hypotheses regarding the role of microglial activation in opioid tolerance. Through this experiment, the researchers sought to understand the consequences of morphine administration on microglial activation in both wild-type and μ-opioid receptor knockout mice.

2) What, specifically, did the researchers do?

The researchers administered chronic morphine treatment to wild-type control mice and global μ-opioid receptor knockout mice. On Day 1, all mice were given a morphine injection of 10 mg/kg, following which they were divided into three groups based on saline, fixed-dose morphine, or escalating-dose morphine administration. The treatments were given for 6 days, and on the 8^{th} day, sensory levels were tested to determine opioid-induced hyperalgesia. Analgesic tolerance was tested by giving a 10 mg/kg morphine injection to all mice. Densitometric analysis was carried out in the spinal cord dorsal horn to test for microglial activation. In order to establish which cellular populations expressed μ-opioid receptors, the researchers performed immunohistochemistry, *in situ* hybridization, and knock-in mice expressing fluorescent tagged receptors. In order to confirm the results of these experiments, RNA-sequencing of the transcriptome of spinal microglia was performed.

3) What are the results?

The researchers found that in wild-type mice, morphine treatment resulted in opioid-induced hyperalgesia, antinociceptive tolerance, and microglial activation, whereas in the global μ-opioid receptor knockout mice, there was microglial activation but no opioid-induced hyperalgesia. Through the immunohistochemical and *in situ* hybridization assays, the researchers determined that μ-opioid receptors were expressed in nociceptive neurons in the spinal cord dorsal horn. However, this result could not be confirmed by anti-μ-opioid receptor immunoreactive assays and RNA sequencing.

4) What did these results contribute to the author's conclusions?

Opioid signaling through μ-opioid receptors takes place through nociceptive neural circuits and not through spinal microglia resulting in opioid-induced hyperalgesia. Cells and neural circuits expressing μ-opioid receptors may show altered properties following chronic opioid treatment. Thus, upon morphine administration, morphine binds to μ-opioid receptors expressed by nociceptors inducing tolerance and hyperalgesia.

5) What do you think is the strength of the evidence, and why?

Chronic use of opioids for pain management has resulted in an increase in analgesic tolerance and reduction in the efficacy of pain management medications. The knowledge of the pathway where the drug binds and induces pain management pathways can help prevent the development of tolerance and hyperalgesia in patients.

Figure 2

1) What was the rationale for doing this experiment/including this data?

After understanding that nociceptive neurons contributed to pain management pathways, the researchers wanted to identify the specific sub-group of neurons expressing μ-opioid receptors that are responsible for initiating tolerance and opioid-induced hyperalgesia.

2) What, specifically, did the researchers do?

The researchers applied genetic engineering techniques to mice that lacked μ-opioid receptors in neurons of the TRPV1 lineage. This lineage was specifically used because earlier studies have shown that control of nociceptors belonging to this lineage reduces opioid-induced tolerance and hyperalgesia. The *Oprm1* transcript is absent in microglia and so, mice carrying exons of the *Oprm1* gene flanked by *loxP* sites were crossed with mice in which Cre recombinase was present downstream to the *Trpv1* promoter. The *Oprm1* gene was selectively excised in the dorsal root ganglia and fluorescence immunohistochemistry and *in situ* hybridization was carried out to identify the expression profile of OPRM1. These mice were used for testing morphine antinociception by monitoring sensory-reflexive and affective-motivational behaviours.

3) What are the results?

The researchers found that acute intrathecal injection of morphine did not produce reflexive or affective-motivational antinociception in the knockout mice as compared to the control mice, which indicated that μ-opioid receptor signaling in nociceptors is important for spinal opioid antinociception. On the other hand, subcutaneous injection of morphine produced significant antinociception in knockout mice.

4) What did these results contribute to the author's conclusions?

The researchers could conclude that the knockout mice showed normal microglial activation but did not develop opioid-induced hyperalgesia. Hence, μ-opioid receptors expressed by nociceptors and not by microglia are responsible for downstream pain pathways.

5) What do you think is the strength of the evidence, and why?

This result helps researchers point the exact cell-type bearing μ-opioid receptors that are responsible for pain management pathways. It also helps them dissociate microglia from the induction of hyperalgesia.

Figure 3

1) What was the rationale for doing this experiment/including this data?

After identifying that nociceptors, and not microglia, were responsible for pain management pathways, this experiment was performed to evaluate the development of antinociceptive tolerance and opioid-induced hyperalgesia in the knockout mice.

2) What, specifically, did the researchers do?

The researchers provided chronic morphine treatment to the knockout mice by injecting fixed-dose morphine every day for 10 days. Hyperalgesia and tolerance were monitored every day by evaluating responses to thermal and mechanical stimuli.

3) What are the results?

The researchers found that the knockout mice showed full antinociceptive efficacy for all the days of the treatment whereas the antinociception was found to gradually decrease in control mice. Also, significantly lesser hyperalgesia was developed in the knockout mice as compared to the control mice for thermal and mechanical stimuli.

4) What did these results contribute to the author's conclusions?

Through this experiment, the researchers concluded that μ-opioid receptors expressed by nociceptors play a significant role in triggering analgesic tolerance and opioid-induced hyperalgesia via pronociceptive maladaptive plasticity.

5) What do you think is the strength of the evidence, and why?

This data provides strong evidence for the role of nociceptor-expressed μ-opioid receptors in downstream pain management pathways and the subsequent induction of tolerance and hyperalgesia.

Figure 4

1) What was the rationale for doing this experiment/including this data?

Opioids normally function by repressing synaptic transmission pathways in the spinal cord and triggering excitatory plasticity mechanisms such as long-term potentiation. The researchers

wanted to understand if this long-term potentiation was triggered by MOR signaling in the pre-synaptic or post-synaptic pathways.

2) What, specifically, did the researchers do?

The researchers studied spinal cord slices of the control and knockout mice expressing a light-activated channel protein in TRPV1 nociceptors for analyzing the synaptic transmission between spinal neurons and nociceptors. Immunohistochemistry was used to confirm expression of the channel protein in the cell population of interest. Whole-cell recordings of the neurons were prepared using monosynaptic input from the channel protein-expressing nociceptors and a μ-opioid receptor agonist was applied to the cells.

3) What are the results?

Application of the μ-opioid receptor agonist to the nociceptors of the knockout mice showed an immediate repression of light-evoked excitatory postsynaptic currents in the neurons. After washing out the μ-opioid receptor agonist, the repression and the long-term potentiation were completely lost in the knockout mice.

4) What did these results contribute to the author's conclusions?

Through this experiment, the researchers concluded that presynaptic signaling of μ-opioid receptors in nociceptors is responsible for inducing long-term potentiation.

5) What do you think is the strength of the evidence, and why?

Long-term potentiation is a major contributor to opioid-induced hyperalgesia and tolerance; however, until now, it was unclear if it was triggered by presynaptic or postsynaptic events. This experiment helped conclude that deletion of presynaptic μ-opioid receptors eliminated repression of synaptic transmission and long-term potentiation.

Figure 5

1) What was the rationale for doing this experiment/including this data?

This experiment was performed to test the hypothesis that pharmacological blockage of peripheral μ-opioid receptors should prevent the onset of tolerance and hyperalgesia.

2) What, specifically, did the researchers do?

In order to test this hypothesis, the researchers administered morphine along with a μ-opioid receptor antagonist in wild-type mice and assessed their antinociceptive effects using mechanical and thermal stimuli.

3) What are the results?

The researchers found that in control mice, morphine administration reduced nociceptive reflexes, whereas in the presence of the antagonist, the antinociceptive effects of morphine were not altered. Additionally, mice treated with morphine and μ-opioid receptor antagonist showed a significant reduction in the onset of tolerance and hyperalgesia.

4) What did these results contribute to the author's conclusions?

From these results, the authors could prove their hypothesis that blocking μ-opioid receptors in nociceptors using an antagonist can prevent tolerance to the medication.

5) What do you think is the strength of the evidence, and why?

Due to the increased use of morphine among patients, there has been a marked increase in the reports of tolerance to the opioid. The efficacy of the opioid in humans may be retained by using a μ-opioid receptor antagonist along with the medication to reduce opioid analgesic tolerance.

Figure 6

1) What was the rationale for doing this experiment/including this data?

Based on the previous results, the researchers hypothesized that administration of a combination of morphine and μ-opioid receptor antagonist could provide relief from chronic pain in humans.

2) What, specifically, did the researchers do?

The researchers decided to test the above hypothesis on a bone pinning model of orthotrauma inflammatory pain to evaluate the antinociceptive effects of morphine alone and in combination with a μ-opioid receptor antagonist.

3) What are the results?

The researchers found that morphine alone produced considerable antinociception initially; however, it could not reduce pain to a significant extent after chronic treatment for 7 days. On the other hand, combination therapy with morphine and μ-opioid receptor antagonist produced significant antinociception when tested for pain sensitivity responses. Furthermore, no tolerance or hyperalgesia was noticed when combination therapy was used on the model. Repitition of the experiment with a chronic constriction injury model of neuropathic chronic pain gave similar results.

4) What did these results contribute to the author's conclusions?

These results prove the researchers' hypothesis that a combinational therapy is beneficial in maintaining the antinociceptive properties of morphine in pain models and can prevent the development of tolerance and hyperalgesia.

5) What do you think is the strength of the evidence, and why?

Based on these results, μ-opioid receptor antagonists have the potential to be used pharmacologically to limit the side effects that occur due to prolonged opioid use.

What may be done to improve or extend the scope of the study?

The given results have been proved using mice models and two human pain models, and both have shown promising results. However, in order to make their use in pharmacology mainstream, the choice and dosage of μ-opioid receptor antagonist needs to be standardized and it needs to be ensured that this antagonist will not produce harmful side effects in patients.

Clinical trials need to be designed using various combinations of opioids and μ-opioid receptor antagonists to analyze pain responses and determine the best combination and dosage for pain management strategies.