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Title

Differential medication overuse risk of novel anti-migraine therapeutics

Running Title

Medication overuse risk, migraine

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Abstract

Medication overuse headache is estimated to affect two percent of the population, and is ranked in the top 20 most disabling disorders, due to its high level of disability. Several therapies used in the treatment of acute migraine are thought to be associated with medication overuse headache, including opioids and triptans. With limited treatment options, it is critical to determine the risk profile of novel therapies prior to their widespread use. The current study explores the potential medication overuse risk of two novel therapeutic drug classes, namely the ditans: 5-HT_{1F} receptor agonists, and the gepants: calcitonin gene-related peptide receptor antagonists, in a preclinical model of medication overuse. Persistent exposure of mice to the 5-HT_{1F} agonist LY344864, but not olcegepant produced a significant reduction in hindpaw and orofacial mechanical withdrawal thresholds as a surrogate readout of allodynia. In agreement, only LY344864 induced neuroplastic changes in trigeminal sensory afferents, increasing calcitonin gene-related peptide expression and basal trigeminal nociception. Our data highlight a differential medication overuse headache risk profile for the ditan and gepant classes of drugs that has important implications for their clinical use and patient education to help reduce the burden of medication overuse headache.

Keywords

Migraine, Headache: experimental models, Headache: drug treatment, Secondary headache, Trigeminal ganglion.

Introduction

Headache disorders are common causes of disability, particularly chronic migraine, chronic cluster headache and medication overuse headache (MOH). These complex conditions represent a major challenge for healthcare services, with 30-50% of all tertiary headache clinic patients suffering from MOH (Bigal *et al.*, 2008). Migraine is the most common disabling headache disorder, with over 1 billion sufferers globally (Collaborators, 2018a), 2.5% of whom transition to a chronic state annually (Bigal *et al.*, 2008). Patients with headache disorder biology appear particularly susceptible to the development of MOH (Bahra *et al.*, 2003), whereby persistent overuse of their acute anti-migraine therapies for between 10-15 days per month significantly increases the risk of developing MOH (Headache Classification Committee of the International Headache Society, 2018). MOH, despite only affecting approximately 2% of the population (Bigal *et al.*, 2008) is considered one of the most disabling disorders (Global Burden of Disease Study, 2015), resulting in a total of 9.5 million years lived with disability (Collaborators, 2018b) and a socioeconomic cost of €37 billion annually in the EU (Linde *et al.*, 2012).

Several headache medications increase the potential risk for MOH when used to excess, including triptans (5-$HT_{1B/1D}$ receptor agonists), some nonsteroidal-anti-inflammatory-drugs (NSAIDs) and opioids (Bigal *et al.*, 2008). Thus, MOH appears to develop in genetically susceptible individuals (Cargnin *et al.*, 2018) in response to a diverse array of agents. While there are no specific treatments, withdrawal of the causative agent normally improves the symptoms (Engelstoft *et al.*, 2019); however, approximately 40% relapse within 12 months. Given the broad spectrum of agents that can induce MOH it is critical to determine the relative MOH-risk of novel anti-migraine therapies.

In the past six months lasmiditan (5-HT_{1F} receptor agonist, ditan), ubrogepant and rimegepant (CGRP receptor antagonists, gepants) received FDA approval. Lasmiditan, shares partial receptor affinity with selected triptans (Goadsby and Classey, 2003) that have an established MOH-risk profile. Whereas, data from CGRP monoclonal antibodies (Tepper *et al.*, 2019), and preliminary data on preventive action of gepants (Goadsby *et al.*, 2019), suggest lower MOH-risk profiles. Interestingly, several of the established and novel therapies share similar mechanisms, via the modulation of CGRP signaling (Durham and Russo, 2003; Labastida-Ramirez *et al.*, 2020), a key neuropeptide in the pathophysiology of headache, making it difficult to predict potential MOH-risk.

Therefore, the aim of the current study was to determine the potential MOH-risk of ditans and gepants in an established preclinical model of MOH, whereby persistent exposure of rodents to specific therapeutic agents induces a state of mechanical hypersensitivity (De Felice *et al.*, 2010) as a surrogate readout of allodynia observed in MOH patients (Lipton *et al.*, 2019). This information is essential to inform clinical practice and permit patient education as to the potential risks of overuse of these novel compounds.

Materials and Methods

Animals

Adult male C57Bl6/J mice (*n* = 78; Charles River, UK) aged eight weeks, were maintained under standard animal husbandry conditions with food and water available *ad-libitum*. All studies were ethically approved and conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 and are reported in agreement with the ARRIVE guidelines.

Drugs

LY344864-hydrochloride, a selective 5-HT_{1F} receptor agonist and olcegepant, a CGRP receptor antagonist were selected due to their commercial availability (Tocris, UK) and prior *in-vivo* use. Both compounds were dissolved in 2% dimethyl sulfoxide in saline and sumatriptan in saline. Drug doses were based on available literature demonstrating biological effects in rodents at these doses. Olcegepant and LY344864 were injected at a dose of 1mg/kg and sumatriptan at 0.6mg/kg. Drugs were administered intraperitoneally daily for 11 days for hindpaw assessment of olcegepant and LY344864 (n = 10 per group), or for 17 days for orofacial sensory testing in response to LY344864 (n = 8 per group). A second study, established to assess orofacial sensory thresholds in response to olcegepant (n = 8 per group), was terminated early at 7 days due to the immediate suspension of all research during the COVID-19 pandemic.

Sensory testing

Mechanical withdrawal thresholds were assessed using von Frey filaments, as previously described using the up down method in separate groups of mice (Moye *et al.*, 2019). For the hindpaw, following habituation, mice were tested to establish reliable baseline responses and then every second day following the onset of drug administration. Due to potential sensitization from excessive orofacial stimuli, this was conducted every four to seven days following the establishment of stable baseline responses and the onset of drug administration. All testing was conducted at the same time of day under dim light (30-50 lux) to minimise variability. Graduated von Frey filaments (0.008 - 2g) were then applied to the hindpaw or periorbital region using the up-down method to calculate mechanical withdrawal thresholds (Chaplan *et al.*, 1994).

Tissue processing

At the conclusion of testing, mice were perfused with heparinized phosphate buffered saline, followed by 4% paraformaldehyde under terminal anaesthesia. The spinal cord was dissected, post-fixed for one hour and cryoprotected, prior to sectioning at 30µm on a cryostat. To determine the expression of CGRP or the immediate-early gene c-Fos in the trigeminocervical complex (TCC), the primary interface of peripheral trigeminal sensory afferents and the central nervous system, sections were then processed using standard immunohistochemical approaches and incubated with either anti-CGRP (Abcam, UK; 1:2000) or anti-c-Fos (Millipore, UK; 1:10000) primary antibodies. Specific staining was confirmed by the omission of primary antibodies and the specificity of each antibody has been previously confirmed. The expression of CGRP immunoreactive fibres in the TCC was visualized using an appropriate secondary antibody, conjugated to Alexa Fluor 568 and the number of c-Fos positive nuclei in the TCC was visualized via 3, 3'diaminobenzidine (DAB), following appropriate amplification. Sections were then cover slipped prior to undergoing fluorescent or light microscopic analysis (Zeiss Axio Imager).

Statistical analysis

Sample sizes, calculated in G.Power, are based on previous studies (Moye *et al.*, 2019), combined with a medium to high effect size (0.25 - 0.5), a probability of 0.05 and power of 0.8 - 0.9, resulting in an *n* of 8 - 10 for behavioural analysis, depending on the number of repeated measures. This additionally provided sufficient tissue for immunohistochemical analysis. Mice were initially counterbalanced into groups that were subsequently randomly assigned to an experimental grouping. All analysis was conducted blind to the experimental group, and all data is presented as the mean ± standard error of the mean or median and [interquartile ranges]. All mice tested were included in the behavioural analysis and all analysis and graphs were generated in GraphPad Prism v.7. To assess hindpaw and orofacial mechanical withdrawal thresholds the 50% withdrawal

thresholds were compared across time via a two-way repeated measures ANOVA, comparing to vehicle control treated mice, followed by Sidak's multiple comparison test exploring group differences at different time points where appropriate. Additionally, the integrated area under the curve (AUC) for each intervention across time was calculated for graphical representation. To assess CGRP and c-Fos expression $n = 8$ and 7 mice per group, respectively were included in the final analysis, based on those mice that had all appropriate tissue samples available for analysis following processing. The percentage area stained for CGRP (six sections across the TCC) in lamina I and II or the total number of c-Fos positive nuclei (nine sections across the TCC) in lamina I-V of the TCC were calculated. The groups were then compared via the Kruskal-Wallis test, followed by Dunn's multiple comparison test where appropriate.

Data availability

All data are available upon reasonable request.

Results

Differential impact of persistent exposure to two novel anti-migraine therapies in the hindpaw.

Persistent exposure of mice to sumatriptan ($F_{(6, 108)} = 9.79$, $P \leq 0.0001$) and LY344864 ($F_{(6, 108)} = 13.08$, $P \leq 0.0001$), but not olcegepant ($F_{(6, 108)} = 0.84$, $P = 0.54$) for 11 days induced a time-dependent reduction in mechanical withdrawal thresholds as compared to vehicle control treated mice (fig. 1A, $n = 10$ per group). The AUC across the 11 days was 4.80 ± 0.33 and 5.17 ± 0.32 for

olcegepant and vehicle control, respectively; however, this was reduced to 3.0 ± 0.33 for LY344864 (fig. 1B).

Differential impact of persistent exposure to two novel anti-migraine therapies in the orofacial dermatome.

Having determined a differential MOH-risk for LY344864 and olcegepant, we next sought to confirm these results in the orofacial dermatome (n = 8 per group). Given the longer interval between sensory testing in the face, mice were exposed to sumatriptan (0.6mg/kg), LY344864 (1mg/kg), or vehicle control for 17 days following the establishment of basal sensory thresholds; however for olcegepant the study was terminated early after 7 days due to the coronavirus pandemic. Persistent exposure of mice to sumatriptan ($F_{(5, 70)} = 17.86$, $P \leq 0.0001$) and LY344864 ($F_{(5, 70)} = 17.15$, $P \leq 0.0001$) for 17 days induced a time-dependent reduction in orofacial mechanical withdrawal thresholds as compared to vehicle control treated mice (fig. 2A). The AUC across the 17 days was 4.3 ± 0.3 for vehicle control treated mice and 2.3 ± 0.30 and 3.10 ± 0.28 for the sumatriptan and LY344864 groups, respectively (fig 2B). In a separate cohort, persistent exposure of mice to daily olcegepant for 7 days did not alter orofacial mechanical withdrawal thresholds when compared to vehicle treated mice ($F_{(2, 28)} = 1.38$, $P = 0.27$; fig. 3A-B), despite a clear reduction in response to sumatriptan ($F_{(2, 28)} = 21.94$, $P \leq 0.0001$; fig. 3A-B). Due to the reduced duration, no AUC calculations were conducted.

Calcitonin-gene-related peptide expression in the trigeminocervical complex

CGRP is a key neuropeptide involved in the pathophysiology of headache and a potential biomarker for chronic migraine and MOH. Preclinically, increased CGRP expression in these

trigeminal sensory afferents is a reliable marker of neuroplastic changes following the induction of MOH (De Felice *et al.*, 2010). Following 11 days of drug exposure there was a significant increase in the percentage area stained with CGRP in the TCC ($H(2) = 7.22$, $P \leq 0.05$, $n = 8$ per group). Mice persistently exposed to LY344864 had significantly increased CGRP expression when compared to vehicle control mice (130 [110 - 170] v's 99 [87 - 112], $Z = 2.62$, $P \leq 0.05$; fig. 4A). There was no significant difference between vehicle control and olcegepant treated mice (99 [87 - 112] v's 112 [81 - 127], $Z = 0.78$, $P = 0.99$).

C-Fos neuronal activation in the trigeminocervical complex

Trigeminal sensory afferents expressing CGRP synapse on second order neurons in the TCC, giving rise to the trigeminothalamic tract that conveys nociceptive information from the head. Increased expression of the immediate early gene c-Fos in the TCC is an established readout of increased trigeminal nociception (Harriott *et al.*, 2019). Following 11 days of drug exposure there was a significant increase in the number of c-Fos positive cells in the TCC ($H(2) = 7.40$, $P \leq 0.05$, $n = 7$ per group). Mice persistently exposed to LY344864 had a significantly increased number of c-Fos positive cells when compared to vehicle control (33 [32 - 36] v's 23 [15 - 26], $Z = 2.59$, $P \leq 0.05$; fig. 4B). There was no significant difference between vehicle control and olcegepant treated mice (23 [15 - 26] v's 22 [22 - 29], $Z = 0.78$, $P = 0.99$; fig. 4B).

Discussion

The results demonstrate a differential potential MOH-risk profile for gepants: CGRP receptor antagonists, and ditans: 5-HT_{1F} receptor agonists. While the 5-HT_{1F} receptor agonist LY344864 induced a significant reduction in mechanical withdrawal thresholds, olcegepant showed no reduction. This selective MOH-risk of a ditan is supported by increased expression of CGRP in trigeminal sensory afferents and neuronal activation (c-Fos) in the TCC.

Our data is in agreement with an established MOH-risk for the 5-$HT_{1B/1D}$ receptor agonists: triptans. It is further supported by a potential beneficial effect of blocking CGRP signaling via monoclonal antibodies (Tepper *et al.*, 2019) and preliminary data for the gepants (Goadsby *et al.*, 2019), with no evidence of MOH. While the pathophysiology of MOH remains to be fully characterized it is interesting that several drugs, including NSAIDs (Vellani *et al.*, 2017) and triptans (Durham and Russo, 2003), which are known to block CGRP release, increase MOH-risk. Herein our data suggests that the ditans (Labastida-Ramirez *et al.*, 2020) may have a similar impact, a common effect of which being increased CGRP expression in trigeminal sensory afferents. While our results, and that from patients (Goadsby *et al.*, 2019; Tepper *et al.*, 2019), suggest that blockade of the CGRP receptor does not. Interestingly, CGRP expressing trigeminal afferents consistently express Nav1.9 (Bonnet *et al.*, 2019), with known roles in orofacial neuropathic pain (Luiz *et al.*, 2015), and recently linked to MOH (Bonnet *et al.*, 2019). As such, persistent exposure to drugs that can act presynaptically to alter primary sensory afferent neuropeptide expression and receptor function, may lead to a state of increased evoked activity and neurotransmitter/neuropeptide release. Clinically, this is supported by the ability of CGRP-targeted antibodies to reduce attack frequency and acute medication use in MOH patients (Tepper *et al.*, 2019).

Given the need for clinical confirmation, our study is strengthened by the use of sumatriptan. It is well established that the triptans induce mechanical hypersensitivity in rodents and increase the risk of progression to MOH in people with an underlying headache condition, when used to excess. As such, the behavioral effects and underlying mechanistic actions of LY344864 parallel sumatriptan, resulting in comparative sensitization and neuroplastic changes. These neuroplastic changes, including increased CGRP expression, can outlast sumatriptan withdrawal in rodents and persist after normalization of sensory thresholds, creating a state of "latent sensitization". It is a limitation of our current study that we did not explore potential latent sensitization. Further, having identified that olcegepant did not induce MOH-like phenotypes in mice it would have been interesting to test the ability of gepants to block MOH induction. A recent report demonstrated that ubrogepant could prevent bright light-induced mechanical hypersensitivity is sumatriptan-induced latently sensitized mice (Navratilova *et al.*, 2020), suggesting that at the very least gepants may be effective for established MOH.

As the 5-HT_{1F} receptor agonist lasmiditan, and the CGRP antagonists ubrogepant and rimegepant have been approved by the FDA in the past six months, these molecules will shortly join the anti-migraine therapeutic toolkit. While it is clear that lasmiditan has specific advantages over the triptans with respect to cardiovascular risk factors (Shapiro *et al.*, 2019), our data suggests that both classes of drugs confer a comparable MOH-risk. It further suggests, that gepants may demonstrate a more favorable MOH-risk profile.

Understanding the MOH-risk is critical, since MOH places a severe burden on healthcare services (Westergaard *et al.*, 2014), individuals (Collaborators, 2018b) and the wider economy (Linde *et al.*, 2012). Our data now provides a rationale for understanding the potential risk of their overuse

and should inform patient education to avoid such excessive exposure and potential increased risk of developing chronic headache and MOH.

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Competing Interests

C, Saengjaroentham and L.C. Strother and MR. Sultan Jabir declare no competing financial interests. A.A. Pradhan, reports research funding from Amgen. P.J. Goadsby reports grants and personal fees from Amgen and Eli-Lilly and Company, grant from Celgene, and personal fees from Alder Biopharmaceuticals, Allergan, Autonomic Technologies Inc., Biohaven Pharmaceuticals Inc., Clexio, Electrocore LLC, eNeura, Impel Neuropharma, MundiPharma, Novartis, Teva Pharmaceuticals, Trigemina Inc., WL Gore, and personal fees from MedicoLegal work, Massachusetts Medical Society, Up-to-Date, Oxford University Press, and Wolters Kluwer; and a patent magnetic stimulation for headache assigned to eNeura without fee. P.R. Holland reports honoraria for educational and advisory purposes from Allergan, Eli-Lilly, Novartis and TEVA as well as research funding from Amgen and Eli-Lilly.

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Figure legends

Figure 1. Persistent exposure to the 5-HT$_{1F}$ receptor agonist LY344864, but not olcegepant reduces hindpaw mechanical withdrawal thresholds in mice.
Repeated daily exposure of mice to LY344864 (1 mg/kg; ($F_{(6, 108)} = 13.08$, $P \leq 0.0001$)), but not olcegepant (1 mg/kg; ($F_{(6, 108)} = 0.84$, $P = 0.54$)) induced a temporal reduction in hindpaw mechanical withdrawal thresholds when compared to vehicle treated mice (**A**) as a preclinical readout of medication overuse-induced cutaneous allodynia. LY344864 reduced mechanical withdrawal thresholds from day 3 ($t_{16.4} = 3.21$, $P \leq 0.05$) that remained significantly reduced across the 11 days, maximally at day 11 ($t_{12.2} = 11.10$, $P \leq 0.0001$). The integrated area under the curve (AUC) was similar between vehicle (5.17 ± 0.32) and olcegepant (4.80 ± 0.33) groups, but reduced following persistent LY344864 exposure 3.0 ± 0.33 (**B**). Highlighting a potential medication overuse headache risk profile for the 5-HT$_{1F}$ agonist ditan class of drugs. *$P < 0.05$, $n = 10$ mice per group.

Figure 2. Persistent exposure to the 5-HT$_{1F}$ receptor agonist LY344864 reduces orofacial mechanical withdrawal thresholds in mice.
Repeated daily exposure of mice to LY344864 ($F_{(5, 70)} = 17.15$, $P \leq 0.0001$) and sumatriptan ($F_{(5, 70)} = 17.86$, $P \leq 0.0001$) induced a temporal reduction in orofacial mechanical withdrawal thresholds (**A**) as a preclinical readout of medication overuse-induced cephalic allodynia, when compared to vehicle treated mice. LY344864 reduced mechanical withdrawal thresholds from day 14 ($t_{84} = 6.93$, $P \leq 0.0001$) that remained significantly reduced across the 17 days, maximally at day 17 ($t_{84} = 9.02$, $P \leq 0.0001$). Sumatriptan reduced mechanical withdrawal thresholds from day 7 ($t_{84} = 4.09$, $P \leq 0.001$) that remained significantly reduced across the 17 days, maximally at day 17 ($t_{84} = 9.28$, $P \leq 0.0001$). The integrated area under the curve (AUC) for vehicle treated mice was 4.3 ± 0.32, compared to 3.10 ± 0.28 for LY344864 and 2.3 ± 0.30 for sumatriptan (**B**). Highlighting a potential medication overuse headache risk profile for the 5-HT$_{1F}$ agonist ditan class of drugs that is similar to the related triptans that are known to increase the risk of medication overuse headache in migraineurs. *$P < 0.05$, $n = 8$ mice per group.

Figure 3. Persistent exposure to the CGRP receptor antagonist olcegepant has no effect on orofacial mechanical withdrawal thresholds in mice. In an additional cohort of mice that had to be terminated early (day 7) due to the COVID-19 pandemic, repeated daily exposure of mice to sumatriptan ($F_{(2, 28)} = 21.94$, $P \leq 0.0001$) but not olcegepant ($F_{(2, 28)} = 1.38$, $P = 0.27$) reduced orofacial mechanical withdrawal thresholds (**A**) out to day 7 (**B**). $*P < 0.05$, $n = 8$ mice per group.

Figure 4. Persistent exposure to the 5-HT$_{1F}$ receptor agonist LY344864, but not olcegepant induces neuroplastic changes in trigeminal sensory afferents.
A. Repeated daily exposure of mice to LY344864, but not olcegepant induced an increased expression of calcitonin gene-related peptide (CGRP) in the trigeminal sensory afferents synapsing on the superficial dorsal horn of the trigeminocervical complex. The % area stained for CGRP in lamina I and II was significantly increased across all groups ($H(2) = 7.22$, $P < 0.05$). There was no significant increase following olcegepant administration when compared to vehicle control treated mice (99 [87 - 112] v's 112 [81 - 127], $Z = 0.78$, $P = 0.99$). LY344864 significantly increased the AUC when compared to vehicle control mice (130 [110 - 70] v's 99 [87 - 112], $Z = 2.62$, $P < 0.05$).
B. Repeated daily exposure of mice to LY344864, but not olcegepant induced an increase in the number of c-Fos positive neuronal nuclei in lamina I to V of the trigeminocervical complex. The number of c-Fos positive nuclei was significantly increased across all groups ($H(2) = 7.40$, $P < 0.05$). There was no significant increase following olcegepant administration when compared to vehicle control treated mice (22 [22 - 29] v's 23 [15 - 26], $Z = 0.78$, $P = 0.99$). LY344864 significantly increased the number of c-Fos positive nuclei when compared to vehicle control (33 [32 - 36] v's 23 [15 - 26], $Z = 2.59$, $P < 0.05$). Highlighting underlying neuroplastic changes in trigeminal sensory afferents and their increased basal activity as a surrogate readout of increased trigeminal nociception. *$P < 0.05$, $n = 8$ mice per group for A and $n = 7$ mice per group for B. Scale bar = 100 μm and 50 μm for A and B, respectively.

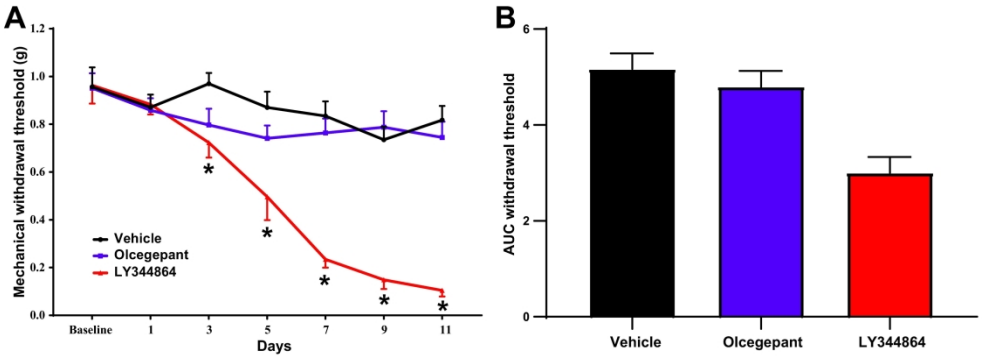

Figure 1. Persistent exposure to the 5-HT1F receptor agonist LY344864, but not olcegepant reduces hindpaw mechanical withdrawal thresholds in mice.
Repeated daily exposure of mice to LY344864 (1 mg/kg; ($F(6, 108) = 13.08$, $P \leq 0.0001$)), but not olcegepant (1 mg/kg; ($F(6, 108) = 0.84$, $P = 0.54$)) induced a temporal reduction in hindpaw mechanical withdrawal thresholds when compared to vehicle treated mice (A) as a preclinical readout of medication overuse-induced cutaneous allodynia. LY344864 reduced mechanical withdrawal thresholds from day 3 ($t16.4 = 3.21$, $P \leq 0.05$) that remained significantly reduced across the 11 days, maximally at day 11 ($t12.2 = 11.10$, $P \leq 0.0001$). The integrated area under the curve (AUC) was similar between vehicle (5.17 ± 0.32) and olcegepant (4.80 ± 0.33) groups, but reduced following persistent LY344864 exposure 3.0 ± 0.33 (B). Highlighting a potential medication overuse headache risk profile for the 5-HT1F agonist ditan class of drugs.
*$P < 0.05$, $n = 10$ mice per group.

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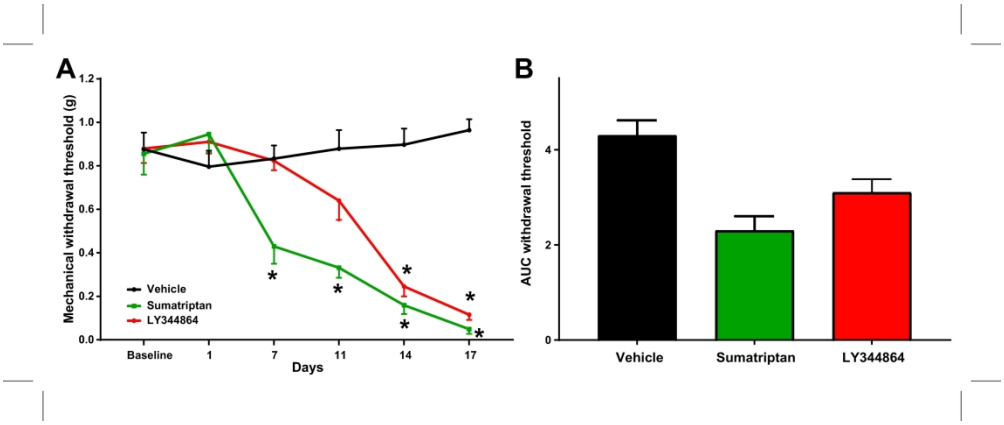

Figure 2. Persistent exposure to the 5-HT1F receptor agonist LY344864 reduces orofacial mechanical withdrawal thresholds in mice.
Repeated daily exposure of mice to LY344864 ($F(5, 70) = 17.15$, $P \leq 0.0001$) and sumatriptan ($F(5, 70) = 17.86$, $P \leq 0.0001$) induced a temporal reduction in orofacial mechanical withdrawal thresholds (A) as a preclinical readout of medication overuse-induced cephalic allodynia, when compared to vehicle treated mice. LY344864 reduced mechanical withdrawal thresholds from day 14 ($t84 = 6.93$, $P \leq 0.0001$) that remained significantly reduced across the 17 days, maximally at day 17 ($t84 = 9.02$, $P \leq 0.0001$). Sumatriptan reduced mechanical withdrawal thresholds from day 7 ($t84 = 4.09$, $P \leq 0.001$) that remained significantly reduced across the 17 days, maximally at day 17 ($t84 = 9.28$, $P \leq 0.0001$). The integrated area under the curve (AUC) for vehicle treated mice was 4.3 ± 0.32, compared to 3.10 ± 0.28 for LY344864 and 2.3 ± 0.30 for sumatriptan (B). Highlighting a potential medication overuse headache risk profile for the 5-HT1F agonist ditan class of drugs that is similar to the related triptans that are known to increase the risk of medication overuse headache in migraineurs. *$P < 0.05$, $n = 8$ mice per group.

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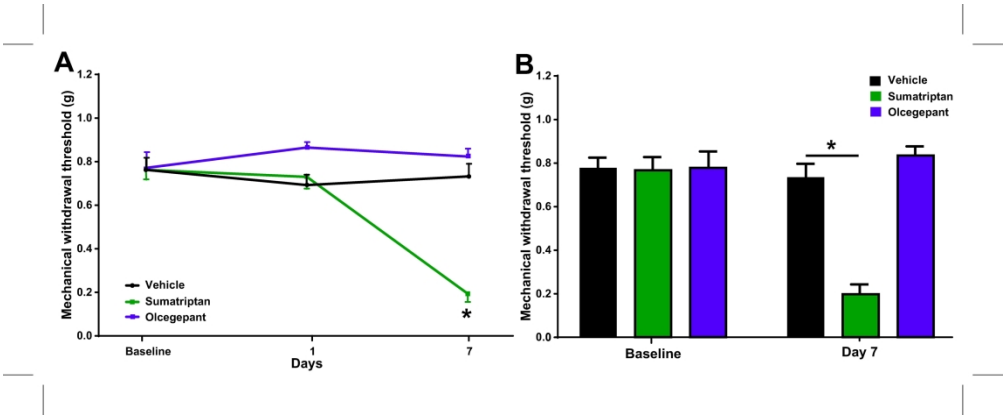

Figure 3. Persistent exposure to the CGRP receptor antagonist olcegepant has no effect on orofacial mechanical withdrawal thresholds in mice. In an additional cohort of mice that had to be terminated early (day 7) due to the COVID-19 pandemic, repeated daily exposure of mice to sumatriptan ($F(2, 28) = 21.94$, $P \leq 0.0001$) but not olcegepant ($F(2, 28) = 1.38$, $P = 0.27$) reduced orofacial mechanical withdrawal thresholds (A) out to day 7 (B). *$P < 0.05$, $n = 8$ mice per group.

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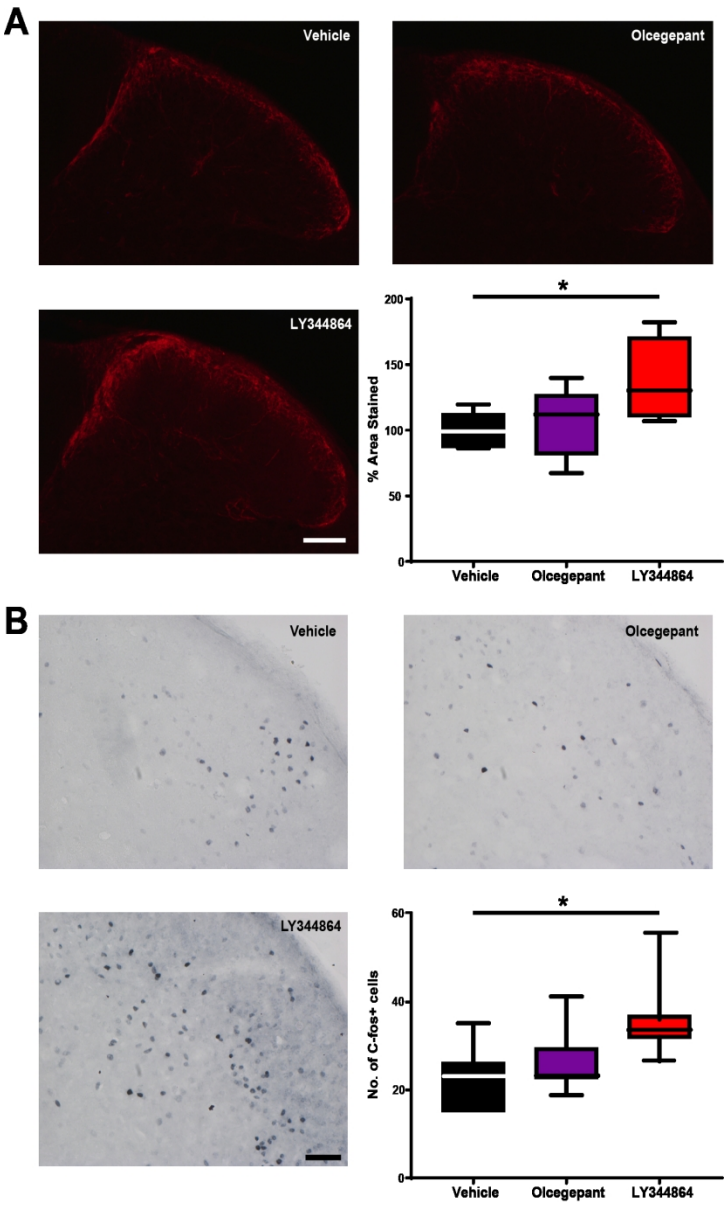

Figure 4. Persistent exposure to the 5-HT1F receptor agonist LY344864, but not olcegepant induces neuroplastic changes in trigeminal sensory afferents.

A. Repeated daily exposure of mice to LY344864, but not olcegepant induced an increased expression of calcitonin gene-related peptide (CGRP) in the trigeminal sensory afferents synapsing on the superficial dorsal horn of the trigeminocervical complex. The % area stained for CGRP in lamina I and II was significantly increased across all groups ($H(2) = 7.22$, $P < 0.05$). There was no significant increase following olcegepant administration when compared to vehicle control treated mice (99 [87 - 112] v's 112 [81 - 127], $Z = 0.78$, $P = 0.99$). LY344864 significantly increased the AUC when compared to vehicle control mice (130 [110 - 70] v's 99 [87 - 112], $Z = 2.62$, $P < 0.05$). B. Repeated daily exposure of mice to LY344864, but not olcegepant induced an increase in the number of c-Fos positive neuronal nuclei in lamina I to V of the trigeminocervical complex. The number of c-Fos positive nuclei was significantly increased across all groups ($H(2) = 7.40$, $P < 0.05$). There was no significant increase following olcegepant administration when compared to vehicle control treated mice (22 [22 - 29] v's 23 [15 - 26], $Z = 0.78$, $P = 0.99$). LY344864 significantly increased the number of c-Fos positive nuclei when compared to vehicle control (33 [32 - 36]

v's 23 [15 - 26], Z = 2.59, P < 0.05). Highlighting underlying neuroplastic changes in trigeminal sensory afferents and their increased basal activity as a surrogate readout of increased trigeminal nociception. *P < 0.05, n = 8 mice per group for A and n = 7 mice per group for B. Scale bar = 100 μm and 50 μm for A and B, respectively.

ARRIVE

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny[1], William J Browne[2], Innes C Cuthill[3], Michael Emerson[4] and Douglas G Altman[5]

[1]The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, [2]School of Veterinary Science, University of Bristol, Bristol, UK, [3]School of Biological Sciences, University of Bristol, Bristol, UK, [4]National Heart and Lung Institute, Imperial College London, UK, [5]Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Title page, top.
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Abstract, pg 2.
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	Introduction, pg 3 Intro, pg 4, last paragraph &
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Introduction, pg 4, last
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Materials & methods, pg. 5, first
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	Materials & methods, pg. 5-7. To include Statistical Analysis.
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Materials & methods, pg. 5-7.
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Materials & methods, pg. 5, first paragraph.

The ARRIVE guidelines. Originally published in *PLoS Biology*, June 2010[1]

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	Materials & methods, pg. 5, first paragraph.
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	Materials & methods, pg. 5, first paragraph. Also in figure legends.
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	Statistical analysis section, starting pg. 7
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Statistical analysis
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	Statistical analysis section, starting pg. 7 1st
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Materials & methods, pg. 5, 1st
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	Materials & methods, pg. 5, 1st
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Results section, pg.
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	N/A
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	Discussion, pg. 10 onward.
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Discussion, pg. 10 onward
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Funding section, pg

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.