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Stomatin-like protein 3 modulates the responses of Aδ, but not C fiber bone afferent neurons to noxious mechanical stimulation in an animal model of acute experimental bone pain

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Abstract

STOML3 is a membrane bound scaffolding protein that has been shown to facilitate the opening of mechanically sensitive ion channels and contribute to noxious mechanical sensation, allodynia and hyperalgesia. In this study, we aimed to determine the role of STOML3 in noxious mechanical sensitivity of bone afferent neurons and carrageenan-induced acute inflammation in the bone. An *in vivo,* electrophysiological bone-nerve preparation was used to make recordings of the activity and sensitivity of bone afferent neurons that innervate the tibial marrow cavity in anaesthetised rats, in response to noxious mechanical stimuli delivered to the marrow cavity, before and after injection of either the STOML3 oligomerisation inhibitor OB-1 or vehicle, in either naïve animals or animals with carrageenan-induced inflammation of the marrow cavity. A dynamic weight-bearing apparatus was used to measure weight bearing in response to inflammatory pain before and after injection of OB-1 or saline into the tibial marrow cavity in the presence of carrageenan-induced inflammation. Electrophysiological recordings revealed that Aδ, but not C bone afferent neurons have a reduced discharge frequency in response to mechanical stimulation, and that carrageenan-induced sensitisation of Aδ, but not C bone afferent neurons was attenuated by inhibition of STOML3 oligomerisation with OB-1. Animals treated with OB-1 spent a significantly greater amount of time on the limb injected with carrageenan than animals treated with saline. Our findings demonstrate that inhibition of STOML3 oligomerisation reduces inflammatory bone pain by reducing the sensitivity of Aδ bone afferent neurons to mechanical stimulation. Targeting STOML3 may be an effective approach to reduce pain from noxious pressure and/or painful inflammatory pathology in bone.

Keywords

STOML3, stomatin-like protein 3, bone pain, electrophysiology

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Introduction

Inflammation and mechanical disturbances of the bone are features common to a number of painful musculoskeletal pathologies, including bone cancers, fractures, intra-osseous engorgement syndrome, osteoarthritis and osteomyelitis.[1–7] They can contribute to pain by activating and/or sensitising peripheral sensory nerve terminals that innervate bone through the release of inflammatory mediators and/or by mechanical compression or distortion.[1,2,5–9] Whilst it is now

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clear that the majority of bone afferent neurons have properties consistent with a role in nociception, including conduction velocities confined to the Aδ and C fiber ranges,[4,5,10–12] little is known about the mechanisms by which noxious mechanical stimuli activate and/or sensitise sensory neurons that innervate bone.

Mechanical stimuli are transduced into electrical signals via the gating of mechanically-activated ion channels, and the gating properties of these channels can be influenced by scaffolding proteins that tether the channel to its surrounding membrane and/or external matrix.[13] Stomatin-like protein 3 (STOML3) is one such scaffolding protein that is required for normal mechanoreceptor function.[14] STOML3 depletion in mutant mice results in reduced mechanical sensitivity in peripheral sensory neurons, including nociceptors.[14–16] It also affects the sensitivity of animals to touch and noxious mechanical stimulation.[14–17] These effects are predicted to result from functional interactions of STOML3 with a number of mechanically gated ion-channels, including Piezo1, Piezo2 and ASIC channels.[16–20]

Small molecules that interact with STOML3 have been identified from a primary screen of approximately 35,000 small molecules obtained from the central compound collection of the Leibniz Institute for Molecular Pharmacology screening unit[21] and are now commercially available (OB-1 and OB-2; ChemDiv, San Diego, CA). OB-1 in particular blocks STOML3 oligomerisation, a characteristic that is important for the functional interaction of STOML3 with mechanically-gated ion channels.[22,23] It reduces the sensitivity of mechanically gated currents in sensory neurons *in vitro,*[21] silences Aβ and Aδ neurons in the skin-nerve preparation,[17] and reverses mechanical hypersensitivity in animal models of diabetic neuropathy and chronic constriction injury.[15–17]

In the present study, we use OB-1 to identify roles for STOML3 in tuning the response of Aδ bone afferent neurons to noxious mechanical stimulation, and in an animal model of acute experimental inflammatory bone pain.

Materials and methods

Male Sprague-Dawley rats weighing between 200 and 250 g were used in this study. Animals were housed in pairs in a 12 h light/dark cycle and were provided with food and water *ad libitum*. All experiments conformed to the Australian National Health and Medical Research Council code of practice for the use of animals in research and were approved by the University of Melbourne Animal Experimentation Ethics Committee.

Electrophysiological recordings using an in vivo bone-nerve preparation

Recordings of the activity and sensitivity of bone afferent neurons were made using an *in vivo* bone-nerve electrophysiological preparation, described in detail in our previous publications.[12,24–28] In brief, urethane anesthetized rats (50% w/v, 1.5 g/kg i.p.) were prepared for recording. A fine branch of the tibial nerve that innervates the marrow cavity of the tibia was isolated and placed over a platinum hook electrode for extracellular recording. Whole-nerve electrical activity was amplified (1000×) and filtered (high pass 100 Hz, low pass 3 kHz) (DP-311 differential amplifier, Warner Instruments), sampled at 20 kHz (PowerLab, ADInstruments, Australia) and stored to PC using LabChart recording software (ADInstruments). A ramp-and-hold mechanical stimulus was delivered to the endings of bone afferent neurons by raising intra-osseous pressure with an injection of heparinised physiological saline (0.9% sodium chloride), delivered through a needle implanted into the marrow cavity. The ramp-and hold stimulus was applied with an initial flow rate of 7 mL/min (ramp phase), followed by a constant 300 mmHg of pressure delivered for 15-s duration (hold phase). The threshold for mechanical activation of bone afferent neurons was calculated from the rising phase of the pressure stimulus. Discharge frequency was reported over the ramp phase, the hold phase, and the entire ramp-and-hold pressure stimulus, and was expressed as the number of action potentials/second (Hz).

All action potentials (spikes) with positive and/or negative peaks clearly above noise were sampled from the whole-nerve recordings. We were unable to routinely record conduction velocities in each experiment because we could not electrically stimulate the receptive fields of individual bone afferent neurons buried deep inside the marrow cavity. Instead, we classified spikes as originating from C, Aδ or Aβ units on the basis of previously published experiments, using the same recording configuration, in which we demonstrated a linear relationship between conduction velocity and peak-to-peak action potential amplitude for units activated with mechanical stimulation from within the bone marrow.[12,24] On the basis of this relationship, impulses with amplitudes <40 μV were defined as originating from C-fibres (conduction velocities <2.5 m/s) and those with amplitudes between 40 and 145 μV were defined as originating from Aδ fibres (conduction velocities between 2.5 and 12.5 m/s). For a thorough discussion of how this division was selected, see Nencini et al.[24] Further analyses were performed on data derived from spikes with amplitudes consistent with either Aδ or C fiber conduction velocities.

Nerve impulses arising from single, Aδ mechanically sensitive bone afferent neurons were discriminated from the whole nerve recordings by their similar amplitude and duration. We were not able to isolate individual C fiber bone afferent neurons because they did not have clearly discriminable amplitudes and/or durations. However, we report data for all spikes with amplitudes consistent with C fiber activity in whole-nerve recordings of nerve to the rat tibia. This allows us to assess if there are changes in the discharge frequency of C fiber bone afferent neurons at the whole nerve level.

To determine if inhibition of STOML3 oligomerisation affected the responses of bone afferent neurons to noxious mechanical stimuli, we compared their threshold for activation or discharge frequency in response to the ramp-and-hold pressure stimulus, before and after injection of OB-1 (STOML3 oligomerisation inhibitor; 250 pmol in 10 µl saline; ID 3570-0107; ChemDiv, San Diego, CA, US) or vehicle, in naïve animals. This concentration of OB-1 has been shown to inhibit mechanical hypersensitivity in mice with diabetic neuropathy.[21] To further determine if inhibition of STOML3 oligomerisation reverses acute inflammation-induced sensitisation of bone afferent neurons, we also compared their threshold for activation or discharge frequency in response to OB-1 (250 pmol in 10 µl saline) or vehicle, in animals injected with carrageenan (2% in 10 µl saline; λ-carrageenan, Sigma-Aldrich, Australia). We have previously demonstrated this concentration of carrageenan causes rapid sensitisation of bone afferent neurons to mechanical stimulation, and an accompanying change in weight bearing behaviour when applied to bone.[28] In these latter experiments, the OB-1 or vehicle was delivered directly to the marrow cavity at the same time as the carrageenan.

Dynamic weight bearing to assay pain behaviour

Weight bearing was evaluated using the advanced dynamic weight-bearing (ADWB) device (Bioseb; Boulogne, France). This apparatus consists of a clear Plexiglas chamber (22 x 22 x 30 cm), and a sensory pad composed of 1936 pressure transducers, which is synchronised to a video camera mounted to the top of the enclosure. This allows for simultaneous and automated exploration of weight bearing on each paw during movement of the animals through the chamber, and requires minimal handling. Animals were habituated to the device 1 day prior to surgery. Behavioural testing was performed to generate a baseline metric on the day of carrageenan injection (Day 0, baseline), then again at 15, 30, 60, 120, 300 min and 24 h after carrageenan injection.

Animals were anesthetised with isoflurane (4% induction; 2.5% maintenance; O2: 2 mL/min). A skin incision was made on the medial aspect of the tibia and a hole was placed through the underlying cortical bone using a sterile 26-gauge needle. A Hamilton syringe was used to apply either carrageenan (2% in 10 µl of saline) + saline (10 µl), carrageenan (2% in 10 µl of saline) + OB-1 (250 pmol in 10 µl saline), or saline (10 µl) + saline (10 µl) volume control, directly into the marrow cavity through the hole. The hole was sealed with bone wax and the skin was closed with stainless steel autoclips. Recovery from isoflurane anaesthesia occurred within a few minutes from the end of the surgery, allowing behavioural testing to be performed within 15 min of surgery.

Testing was always performed blinded to the experimental condition, by the same experimenter, and at the same times each day. The animals were allowed to move freely within the chamber for 4 min at each testing time-point. The data were analysed, by a blinded investigator, using the ADWB software (module version 1.4.3.98; Bioseb; Boulogne, France). This involved assigning pressure zones to the corresponding paws (front left, front right, rear left, rear right) for each time segment of the data. A zone was considered valid when one pressure transducer recorded a weight of ≥4 g and at least two adjacent pressure transducers recorded a weight of ≥2 g. A time segment was considered valid if the activated zones were stable for ≥0.5 s. Animals were excluded if there was less than 60 s of total validated time available, if there was excessive grooming behaviour during the recording period, or if animals exhibited freezing behaviour. We calculated weight-bearing on the injected limb as a percentage of weight borne on both hindlimbs, and time spent on the injected limb as a percentage of total time.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software). In datasets for which multiple units were isolated from a single recording, N = number of action potential units and n = number of animals/recordings, and the discharge frequencies of single units were evaluated using a Two-way nested ANOVA. This was followed by a Dunnett's post hoc analysis when there were more than two groups. A mixed model design was used when possible, to avoid potential errors related to pseudo-replication for electrophysiological data that included multiple cells derived from a single recording preparation. In datasets for which only one unit was isolated or sampled from each recording, comparisons of discharge frequency were made using an unpaired t test, and comparisons of threshold for mechanical activation were made with a Mann-Whitney test. For analyses of the response of C fiber activity in whole-nerve recordings, comparisons between treatment groups were made with a Kruskal-Wallis test followed by Dunn's post hoc analysis or a Mann-Whitney test. For behavioural experiments, a Two-way ANOVA with repeated measures followed by Dunnett's post hoc analysis was used to compare treatment groups. Area under the curve (AUC) was determined using the trapezoid rule between 0 and 30 min for individual animals to determine the overall effect of each treatment between timepoints on pain behaviour.[29] For analyses of the differences in AUC, a One-way ANOVA followed by Dunnett's post hoc analysis was used to compare treatment groups. Post hoc analyses were performed only if the ANOVA reported significant effects. Data are represented as mean ± SEM. In all cases, $p < 0.05$ was used to define statistical significance.

Results

Inhibition of STOML3 oligomerisation decreases the response of Aδ bone afferent neurons to mechanical stimulation

There are no known ligands or chemical activators of STOML3 so instead, we first tested whether inhibition of STOML3

oligomerisation with OB-1 altered the sensitivity of bone afferent neurons to mechanical stimuli, presented at interstimulus intervals (ISIs) of 15 min, in naïve animals. We have previously shown that bone afferent neurons recorded in naïve animals have recovered from stimulus-evoked fatigue well before this timepoint.[12] Figure 1a and b show a typical response to the mechanical stimulus before (a) and after (b) application of OB-1. Inhibition of STOML3 with OB-1 caused a significant reduction in the activity (entire stimulus discharge frequency) of Aδ bone afferent neurons relative to saline control (Two-way nested ANOVA, F [12.3], DFn [1], DFd [26], $p = 0.0017$; Figure 1c). Reduced activity was observed during both the ramp (Two-way nested ANOVA, F [7.405], DFn [1], DFd [24], $p = 0.0058$; Figure 1d) and the hold phase of the pressure stimulus (Two-way nested ANOVA, F [5.537], DFn [1], DFd [24], $p = 0.0272$; Figure 1e). There was no difference in the threshold for mechanical activation of Aδ bone afferent neurons recorded from animals injected with OB-1 relative to those injected with saline (Two-way nested ANOVA, F [3.370], DFn [1], DFd [9], $p = 0.0996$; Figure 1f). There was no difference in the entire stimulus discharge frequency of C bone afferent neurons recorded from animals injected with OB-1 relative to those injected with saline (Mann-Whitney test, $p > 0.05$) (Figure 1g).

Inhibition of STOML3 oligomerisation prevents carrageenan-induced sensitisation of Aδ bone afferent neurons to mechanical stimulation

To further investigate whether STOML3 contributes to sensitisation of bone afferent neurons, we then tested whether carrageenan-induced sensitisation of bone afferent neurons was affected by inhibition of STOML3 oligomerisation with OB-1. Figure 2 shows typical responses to the mechanical stimulus before and after application of carrageenan + saline (a) or carrageenan + OB-1 (b). For analysis of the discharge frequency of single Aδ bone afferent neurons, Two-way nested ANOVA with Dunnett post hoc testing (F [6.098,] DFn [2], DFd [31], $p < 0.05$) revealed an increase in discharge frequency in animals administered carrageenan + saline relative to those administered saline alone, and a reduction in

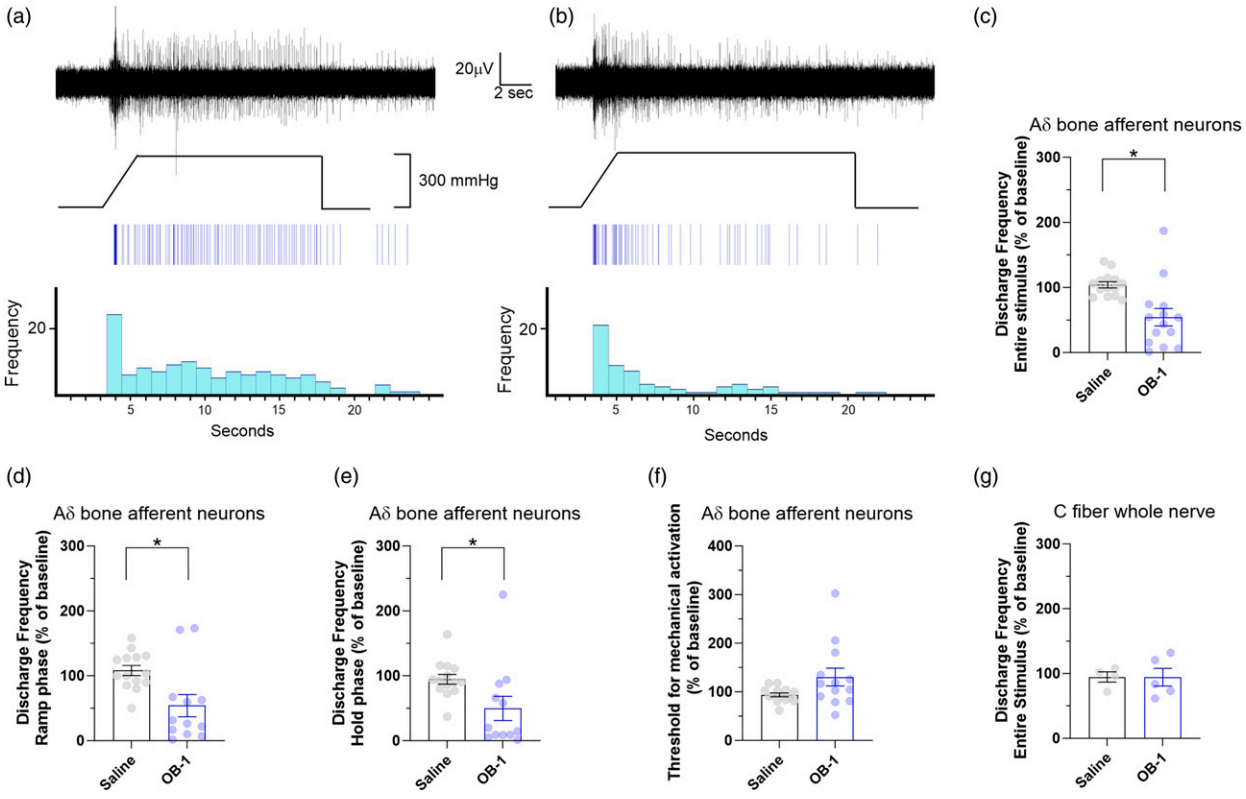

Figure 1. The STOML3 oligomerisation inhibitor OB-1 reduces the response of Aδ bone afferent neuron to noxious mechanical stimuli. a-b are examples of a whole-nerve recording, raster and frequency histogram of a single Aδ bone afferent neuron activity in response to 300 mmHg ramp-and-hold pressure stimuli, before (a) and after injection with OB-1 (b). There was a significant reduction in the entire stimulus discharge frequency of Aδ bone afferent neurons, in animals injected with OB-1 compared to those injected with saline ($n = 14$ saline/14 OB-1, $N = 6$ saline/6 OB-1) (c). The reductions in discharge frequency occurred during both the ramp ($n = 14$ saline/12 OB-1, $N = 6$ saline/6 OB-1) (d) and hold ($n = 14$ saline/12 OB-1, $N = 6$ saline/6 OB-1) (e) phases of the pressure stimulus. Treatment with OB-1, compared to saline, did not affect the threshold for activation of Aδ bone afferent neurons ($n = 14$ saline/13 OB-1, $N = 6$ saline/6 OB-1) (f). There were no differences in the entire stimulus discharge frequency of C bone afferent neurons isolated from whole-nerve recordings, in animals injected with OB-1 compared to saline ($N = 4$ saline, $N = 4$ OB-1) (g). Asterisks denote $p < 0.05$.

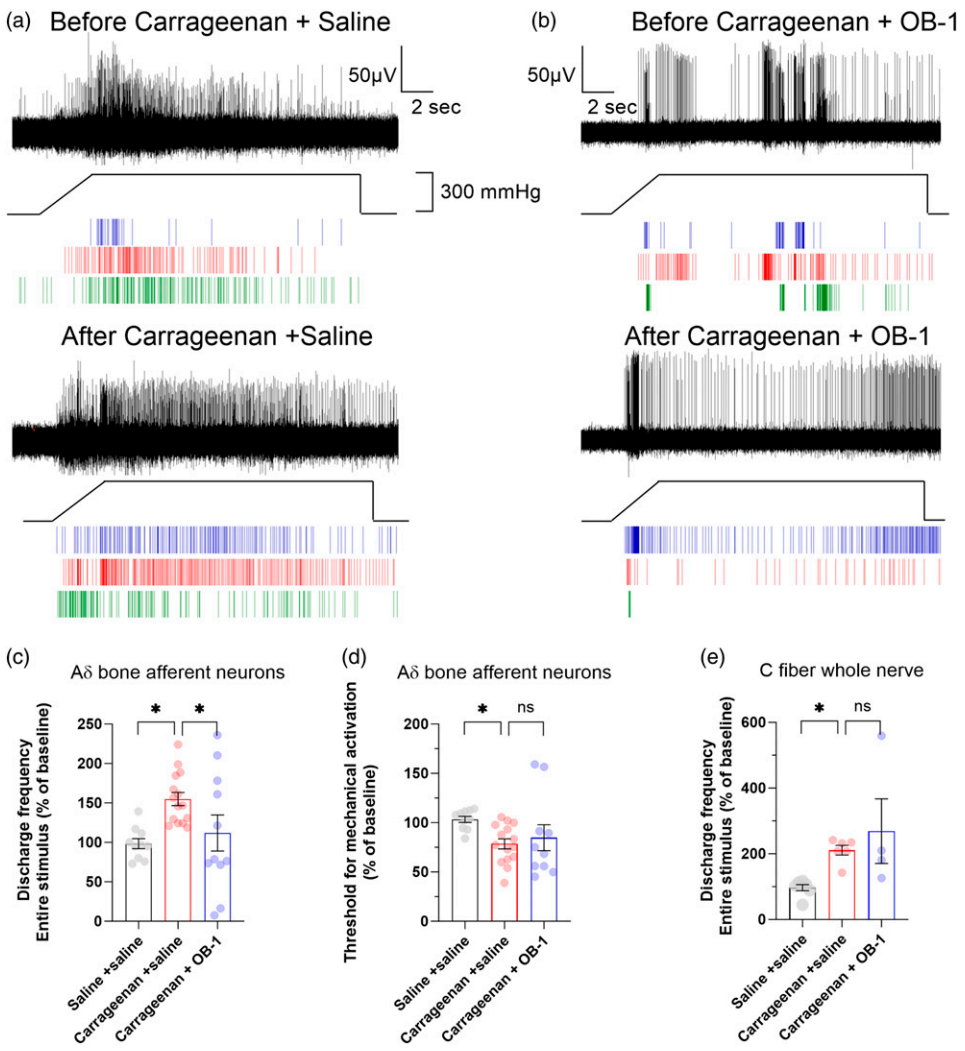

Figure 2. The STOML3 oligomerisation inhibitor OB-1 reverses carrageenan-induced increases to Aδ, but not C fiber bone afferent neuron discharge frequency. a-b are examples of a whole-nerve recording and rasters of single Aδ fiber neuronal activity in response to a 300 mmHg ramp-and-hold pressure stimulus, in an animal before (baseline) and after injection with carrageenan + saline (a) or carrageenan + OB-1 (b). There was a significant reduction in the discharge frequency of single Aδ fiber units in animals injected with carrageenan + OB-1 compared to those treated with carrageenan + saline (n = 10, N = 7, saline + saline, n = 15, N = 6, carrageenan + saline, n = 9, N = 5, carrageenan + OB-1) (c), but no change to carrageenan-induced decreases in threshold for activation (n = 10, N = 7, saline + saline, n = 15, N = 6, carrageenan + saline, n = 9, N = 5, carrageenan + OB-1) (d). There were no changes to the entire stimulus discharge frequency of spikes derived from all C bone afferent neurons isolated from whole-nerve recordings, between animals injected with carrageenan + OB-1 and carrageenan + saline (N = 7 saline + saline, N = 6 carrageenan + saline, N = 4 carrageenan + OB-1) (e). Asterisks denote $p < 0.05$.

discharge frequency in animals administered carrageenan + OB-1 relative to those administered carrageenan + saline (Figure 2c). Two-way nested ANOVA with Dunnett's post hoc testing (F [4.747], DFn [2], DFd [15], $p < 0.05$) also revealed a reduction in the threshold for activation in animals administered carrageenan + saline relative to those administered saline alone, but no change to the threshold for activation in animals administered carrageenan + OB-1 relative to those administered carrageenan + saline (Figure 2d). For analysis of the whole-nerve C fiber activity, a Kruskal Wallis test with Dunn's post hoc testing (KW ANOVA; entire stimulus discharge frequency H(2) = 11.73, $p < 0.05$) revealed significantly increased C fiber discharge in animals treated with carrageenan + saline relative to animals treated with saline alone, but no alterations in discharge frequency in animals treated with carrageenan + OB-1 relative to animals treated with carrageenan + saline (Figure 2e).

Inhibition of STOML3 oligomerisation with OB-1 attenuates carrageenan-induced pain behaviour

We have previously demonstrated that application of carrageenan to the marrow cavity rapidly induces pain

behaviour measured as a reduction in static weight-bearing in rats.[28] Here we used a dynamic weight-bearing apparatus to confirm freely moving animals also have increased pain behaviour in response to intra-osseous application of carrageenan, and to determine whether inhibition of STOML3 oligomerisation with OB-1 can attenuate that pain behaviour. Two-way ANOVA with repeated measures revealed a significant treatment effect for time spent on injected limb (Figure 3a and c [2.165], DFn [2], DFd [29], $p = 0.022$) and total weight-bearing on the injected limb (Figure 3b and d [7.937] DFn [2], DFd [28], $p = 0.022$). Post-hoc testing revealed that animals treated with carrageenan + saline spent significantly less time on the injected limb at 15 and 30 min (Dunnett's $p < 0.05$), and had significantly reduced weight-bearing on the injected limb at 15 and 30 min (Dunnett's $p < 0.05$), in comparison to animals treated with saline alone.

Animals treated with carrageenan + OB-1 spent significantly greater time on their injected limb at the 30 min timepoint compared to animals treated with carrageenan + saline (Figure 3a, Dunnett's $p < 0.05$). Analysis of AUC revealed a significant increase in the time spent on injected

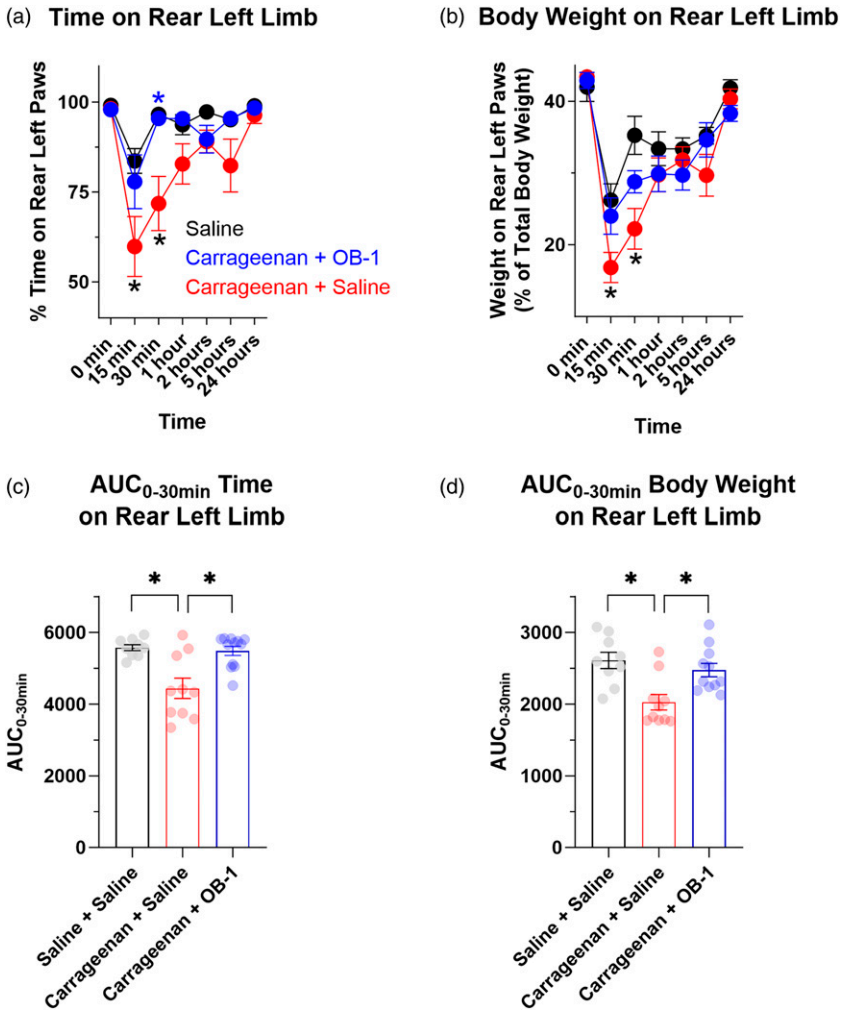

Figure 3. The STOML3 oligomerisation inhibitor OB-1 reduces pain behaviour induced by carrageenan injection into the tibial marrow cavity. Intraosseous injection of carrageenan + saline resulted in pain behaviour characterised by a significant decrease in time spent on the injected limb (a) and weight-bearing on the injected limb (b), compared to animals injected with saline + saline, at both 15 and 30 min (saline + saline $n = 9$, carrageenan + saline $n = 11$, carrageenan + OB-1 $n = 12$). Significantly greater time was spent on the injected limb when carrageenan + OB-1 was administered to animals, compared to when carrageenan + saline was administered to animals, at 30 min (saline + saline $n = 9$, carrageenan + saline $n = 11$, carrageenan + OB-1 $n = 12$) (a). Analysis of $AUC_{0\text{-}30\ mins}$ also revealed a significant reduction in time spent (c) and weight-bearing (d) on the injected limb in animals treated with carrageenan + saline compared to animals injected with saline + saline, and significantly greater time spent (c) and weight-bearing (d) on the injected limb in animals treated with carrageenan + OB-1 compared to carrageenan + saline. Asterisks denote $p < 0.05$. In time course experiments (a) and (b) black asterisks denote significantly different post hoc comparisons between carrageenan + saline and saline + saline, while blue asterisks denote significantly different post hoc comparisons between carrageenan + saline and carrageenan + OB-1.

limb $AUC_{0\text{-}30min}$ (Figure 3c, Dunnett's $p < 0.05$) and weight bearing on the injected limb $AUC_{0\text{-}30min}$ (Figure 3d, Dunnett's $p < 0.05$), in animals treated with carrageenan + OB-1 compared to those treated with carrageenan + saline.

Discussion

In this study, we have shown that interfering with STOML3 oligomerisation using OB-1 reduces the response of Aδ, but not C bone afferent neurons to noxious mechanical stimuli. It also reverses carrageenan-induced increases in the response of Aδ, but not C bone afferent neurons, and attenuates carrageenan-induced pain behaviour measured using the dynamic weight bearing apparatus. Taken together, our findings suggest that STOML3 regulates acute inflammatory bone pain by affecting the activity and/or sensitivity of Aδ bone afferent neurons to mechanical stimulation.

The bone marrow is innervated by neurons with morphological and physiological characteristics of Aδ and C fiber nociceptors.[4,10–12,25,26,30–34] Our data show that STOML3 regulates the function of Aδ, but not C fiber bone afferent neurons *in vivo*. This is consistent with previous reports of loss of mechano-sensitivity in Aδ, but not C fiber cutaneous afferent neurons recorded from STOML3 mutant mice, compared to wildtype controls, using the skin-nerve preparation.[14] We have previously shown that Aδ bone afferent neurons can be activated by noxious mechanical stimuli and be sensitised by carrageenan.[12,27,28] Here we also show that interfering with STOML3 function by preventing oligomerisation can affect the mechanical sensitivity of Aδ bone afferent neurons in both normal conditions, and in the context of inflammation-induced sensitisation.

It is thought that the primary function of STOML3 is to facilitate the transfer of force to mechanically gated ion channels, including Piezo2 and some of the ASICs, to alter their channel opening kinetics.[14,15] Piezo2 proteins co-precipitate with STOML3 after overexpression in HEK-293 cells.[15] STOML3 is a potent modulator of Piezo2 channels in this system, where it may be necessary to maintain the sensitivity of Piezo2 channels to physiologically relevant membrane displacement.[15] Nociceptors acutely dissociated from the DRG of STOML3 knockout mice produced significantly smaller currents on mechanical deflection of neurites than nociceptors isolated from control mice, and STOML3 oligomerisation appears to be important for these effects.[15] Interestingly, Piezo2 is expressed in myelinated, small to medium sized (likely Aδ) bone afferent neurons, but not unmyelinated C bone afferent neurons.[12] Knockdown of Piezo2 inhibits the response of Aδ but not C bone afferent neurons to noxious mechanical stimuli, and affects discharge frequency but not threshold for mechanical activation of Aδ bone afferent neurons, in a manner similar to what we have reported after blocking STOML3 oligomerisation in the present study.[35] Thus, it is likely that STOML3 and Piezo2 are functionally coupled in mechanically sensitive Aδ bone afferent neurons, and that interfering with STOML3 function may reduce the mechanical sensitivity of Piezo2 channels in Aδ bone afferent neurons.

STOML3 can also physically interact with and modulate the gating of ASIC3, another channel implicated in the transduction of mechanical stimuli by sensory neurons. ASIC3 co-precipitates with STOML3 after overexpression in HEK-293 cells,[14] and STOML3 expression in this context is associated with reduced proton gating of endogenous ASICs.[14] We have previously shown that a substantial proportion of bone afferent neurons, including those likely to be Aδ nociceptors, express ASIC3.[27] Furthermore, electrophysiological recordings in the same study revealed that application of a selective inhibitor of ASIC3, APETx2, to the marrow cavity inhibited carrageenan-induced spontaneous activity, and sensitisation of Aδ bone afferent neurons to mechanical stimulation,[27] in a manner similar to what we have reported after blocking STOML3 oligomerisation in the present study. Thus, it is also likely that STOML3 and ASIC3 are functionally coupled in mechanically sensitive Aδ bone afferent neurons, and that interfering with STOML3 function may reduce the mechanical sensitivity of ASIC3 channels in Aδ bone afferent neurons.

Carrageenan results in an acute inflammation that sensitises bone afferent neurons.[27,28] Here we showed that preventing STOML3 oligomerisation reverses carrageenan-induced increases in the response of Aδ, but not C bone afferent neurons to mechanical stimulation. To determine if this effect of interfering with STOML3 oligomerisation on Aδ bone afferent neurons translated to altered pain behaviour, we further assayed dynamic weight bearing in a model of carrageenan-induced bone pain. We found altered weight bearing behaviour in the hindlimbs of animals injected with carrageenan compared to those injected with saline alone. This effect was limited to the 15 and 30 min timepoints, thus we only examined the AUC over this time. While we didn't see a significant effect of OB-1 on behaviours at all the individual time points we assayed, we did see an overall increase in weight bearing $AUC_{0\text{-}30\ min}$ and time spent $AUC_{0\text{-}30\ min}$ on the injected limb, suggesting that interfering with STOML3 function may be a useful tool in attenuating mechanically-induced bone pain. Our findings provide evidence that interfering with STOML3 oligomerisation reduces mechanical sensitivity and pain behaviour driven by carrageenan-induced inflammation. This is in contrast to a previous study that showed inhibition of STOML3 oligomerisation by intraplantar application of OB-1 reversed nerve injury-induced mechanical hypersensitivity, but had no effect on NGF-induced inflammatory pain.[17] The lack of effect on NGF-induced inflammation suggests that OB-1 may not be interacting with NGF dependant neurons, and highlights a potential interaction with other populations of peripheral sensory neurons in carrageenan-induced inflammation. Regardless, sensitisation of bone afferent neurons play a role in a number of painful musculoskeletal conditions, including

bone cancer, intra-osseous engorgement syndrome, osteoarthritis and osteomyelitis.[1–7,30,32] Interfering with STOML3 oligomerisation with OB-1 may provide a way to treat pain in these musculoskeletal pathologies.

Taken together, the findings of the present study suggest that STOML3 regulates acute inflammatory bone pain by affecting the activity and/or sensitivity of Aδ bone afferent neurons to mechanical stimulation, and that interfering with STOML3 oligomerisation using OB-1 may provide a way to treat pain in musculoskeletal pathologies.

Author contributions

J.J.I., M.M., J.T., S.N., J.X. made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; drafted the article or revised it critically for important intellectual content; and approved the final of the version of the manuscript.

Declaration of conflicting interests

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