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Nociceptor Interleukin 33 Receptor/ST2 Signaling in Vibration-Induced Muscle Pain in the Rat

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Abstract

Occupational exposure to mechanical vibration can produce the Hand-Arm Vibration Syndrome (HAVS), whose most disabling symptom is persistent muscle pain. Unfortunately, the pathophysiology of HAVS pain is still poorly understood, precluding the development of mechanism-based therapies. Since interleukin 33 (IL-33) is essential for inflammation and recovery that follows skeletal muscle injury, we explored its role in muscle pain in a model of HAVS, in adult male rats. Concomitant to mechanical hyperalgesia, an increase in IL-33 in the ipsilateral gastrocnemius muscle was observed 24 h after vibration. A similar hyperalgesia was produced by intramuscular injection of recombinant rat IL-33 (rrIL-33, 10-300 ng). Intrathecal administration of an oligodeoxynucleotide antisense to IL-33R/ST2 mRNA decreased the expression of ST2 in DRG and attenuated both rrIL-33 and vibration-induced mechanical hyperalgesia. Together these data support the suggestion that IL-33 plays a central role in vibration-induced muscle pain by action, at least in part, on skeletal muscle nociceptors.

Perspective: Our findings provide evidence of the contribution of IL-33, acting on its canonical receptor, in nociceptors, to muscle pain induced by ergonomic vibration. This suggests that targeting IL-33/ST2 signaling may be a useful strategy for the treatment of muscle pain in hand-arm vibration syndrome.

Keywords

Ischemia; Muscle Pain; Nociceptor Hyperexcitability; Vibration White Finger; Muscle Strain

Introduction

Occupational vibration exposure produces a condition characterized by musculoskeletal, neurological and vascular pathology, referred to as hand-arm vibration syndrome (HAVS)[31]. This condition affects up to 50% of workers who use handheld power tools such as

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jackhammers, pneumatic chipping and grinding mechanical tools [36]. One of the most problematic symptoms of HAVS is persistent muscle pain, which can be extremely disabling and resistant to treatment[13, 14, 31]. We have developed a preclinical model of HAVS in the rat that displays persistent mechanical hyperalgesia in the gastrocnemius muscle, mediated by pro-inflammatory cytokines, interleukin (IL) 6 and tumor necrosis alpha (TNFα)[2, 6, 8]. This model is based on exposure to relevant frequencies of vibration (60-80 Hz) that are well within the range produced by hand-held vibrating tools that produce HAVS [28]. We have provided behavioral and electrophysiological evidence that our model of HAVS reproduces the most prominent symptom of this clinical entity, namely persistent muscle pain. In this model long-lasting mechanical hyperalgesia in the vibrated muscle, persisting for at least 2 weeks, has been observed [4, 8]. We have also observed the induction of hyperalgesic priming (i.e., enhanced and/or prolonged latent hyperalgesia to subsequent nociceptive stimulus) after recovery from vibration, to either additional exposure to vibration or to proalgesic mediators such as prostaglandin E_2 [8]. Of note, HAVS patients display a progressive aggravation of pain upon repeated exposure to ergonomic vibration, which could be due to hyperalgesic priming. Our *in vivo* recordings from Aδ (group III) and C (group IV) nerve fibers innervating the vibrated gastrocnemius muscle show marked increases in nociceptor responsiveness, as revealed by decrease in mechanical threshold and long-lasting firing upon supra-threshold mechanical stimulation [6].

Recent evidence indicates that IL-6, TNFα and other cytokines are part of a critically important immune response that contributes to skeletal muscle regeneration (for a review, see Tidball[34]). For instance, IL-33 (also called Interleukin-1 Family, Member 11 [IL-1F11] and Nuclear Factor From High Endothelial Venules [NF-HEV]) contributes to both the initial pro-inflammatory response after muscle damage, and also to the subsequent anti-inflammatory response needed for optimal muscle regeneration [20]. IL-33 has also been identified as an important pro-algesic mediator in preclinical models of joint, visceral and cutaneous pain [12, 24, 35, 37]. Although the role of IL-33 in muscle pain is unknown, evidence indicates that nociceptors dynamically sense local levels of cytokines after muscle injury, modifying their responses to mechanical stimulation, in a time-dependent manner [1, 2, 6, 8]. Therefore, by sensing local IL-33 levels, nociceptors could also provide important inputs for adaptive behaviors after muscle injury. Such a functional arrangement is possible, since the canonical receptor for IL-33, IL-33R (also known as suppression of tumorigenicity 2 [ST2] or interleukin 1 receptor-like 1 [IL1RL1]), and its co-partner for IL-33 signaling, the interleukin 1 receptor accessory protein (IL1RAcP, also known as IL1RAP), are expressed in human and mouse DRG neurons [23, 33]. And, IL-33R/ST2 knockout mice display intact mechanical and thermal sensitivity but decreased responses to chemical and inflammatory stimuli known to release IL-33 [12, 24, 37], suggesting a pivotal role for IL-33/ST2 in nociceptor responses associated to peripheral sensitization.

Importantly, IL-33 is released after several types of cellular stress, including ischemia-reperfusion[10, 17] and mechanical strain [9, 16, 29], both of which contribute to HAVS[27, 32]. In the present study we evaluated the hypothesis that exposure to vibration up-regulates skeletal muscle IL-33, which is detected by nociceptors, producing persistent muscle hyperalgesia.

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Materials and Methods

Animals

Experiments were performed on adult male Sprague-Dawley rats (Crl:CD[SD], Charles River Laboratories, Hollister, CA) weighing 250 to 350 g. Since the vast majority of HAVS patients are males, likely due to specific occupational gender/sex bias for the use of power tools, we performed this study in male rats. Rats were housed three per cage in an AAALAC International accredited animal facility, the Laboratory Animal Resource Center of the University of California, San Lrancisco, under a 12-h light/dark cycle (lights on 7 AM-7 PM; room temperature 21-23°C), with water and food available *ad libitum*. Upon completion of experiments, rats were euthanized using CO_2 induced asphyxia followed by bilateral thoracotomy. Animal care and use adhered to the guidelines set by the National Institutes of Health, and all studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals. The University of California, San Francisco Institutional Animal Care and Use Committee approved all experimental protocols. Concerted effort was made to reduce the suffering and number of animals used.

The Hand-Arm Vibration Syndrome (HAVS) model

After assessment of baseline nociceptive threshold (see below), rats were anesthetized with isoflurane and their right hind limbs subjected to mechanical vibration as previously described [2–4, 6–8]. Rats were anesthetized with 2.5% isoflurane in oxygen and their right hind limb affixed to the platform of a vortex mixer (Digital Vortex Genie II; Thermo Fisher Scientific, Waltham, MA) with Micropore surgical tape (3M Health Care, St. Paul, MN) so that the knee and ankle joint were both at 90°, without rotational torque on the leg. Each hind limb was vibrated at a frequency of 60 to 80 Hz with a 5-mm peak-to-peak displacement amplitude for 15 min. These vibration frequencies are within the range produced by hand-held power tools (35-150 Hz)[28].

Measurement of hyperalgesia

Mechanical nociceptive threshold was assessed as previously reported [1–3, 6–8] Briefly, mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a Chatillon loadcell sensor transducer (model SLC-0002; AMETEK Inc., Largo, FL), attached to a digital force gauge (model DFS2-R-ND, AMETEK Inc.). Rats were lightly restrained in a cylindrical acrylic restrainer that allows for easy access to the hind limb for mechanical nociceptive threshold testing. A 7-mm diameter probe was used to stimulate the belly of the gastrocnemius muscle with an increasing compression force. The nociceptive threshold was defined as the force (mN) at which the rat withdrew its hind leg. Baseline nociceptive withdrawal threshold was defined as the mean of 3 readings taken at 5-min intervals. Behavioral experiments were performed blinded to the treatment group.

Drugs and Reagents

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant rat IL-33 (rrIL-33; amino acid Ser109-Met264, accession# NP_001014188,

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expressed in *Escherichia coli*, cat. # 766404, BioLegend, San Diego, CA), was diluted in 0.1% Bovine Serum Albumin-Dulbecco's Phosphate Buffer Saline (BSA-DPBS) and stored in 10 μl aliquots at −80°C. Immediately before injection the rrIL-33 aliquots were further diluted in 0.1% BSA-DPBS. The final rrIL-33 doses (10-300 ng in 20 μl), or vehicle (0.1% BSA-DPBS 20 μl), were injected into the right gastrocnemius muscle using a 29G × 1/2" needle-attached insulin syringe.

An antisense (AS) oligodeoxynucleotide (ODN) was designed to knockdown IL-33R/ST2. The AS ODN sequence, 5'-TGC TGT CTG ATG TAG GGT CC -3', was directed against a unique sequence in rat IL-33R/ST2 mRNA (NCBI GenBank accession number and ODN position within the cDNA sequence are NM_001127689.1 and 174-193, respectively). The mismatch (MM) ODN sequence, 5′- **C**GC T**TG** CTG ATG T**GA** GGT C**T** -3′, corresponds to the IL-33/ST2 AS sequence with 6 mismatched bases (bold letters). The ODNs were synthesized by Invitrogen (San Francisco, CA), dissolved in sterile 0.9% NaCl (B. Braun Medical Inc., Irvine, CA) to a concentration of 10 μg/μl, and stored in 60 μl aliquots at −20°C until use. Immediately before injection, AS/MM ODN aliquots were further diluted in sterile 0.9% NaCl and injected i.t. (120 μg/20 μl) daily for 3 consecutive days under brief anesthesia with 2.5% isoflurane. Tail-flick was systematically checked to ensure proper i.t. injections[25].

Tissue harvesting and Western blot analysis

Changes in protein expression of IL-33 in skeletal muscle and IL-33R/ST2 in L4-L5 DRGs were evaluated by Western blot as previously described [1, 2, 6]. Briefly, rats were killed by exsanguination, while under deep isoflurane anesthesia, 24 h after vibration or last i.t. ODN injection. The ipsilateral gastrocnemius muscle was dissected out and homogenized in buffer solution supplemented with a 2x protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Muscle homogenates were centrifuged for 15 min at 4°C and 3,750 rpm and supernatants were stored at −80°C. Ipsilateral L4 and L5 DRGs were quickly dissected out and immediately placed on dry ice and then stored at −80 °C until use. Thereafter, they were transferred into cold homogenization buffer (150 mM NaCl, 10 mMEDTA, 2% sodium dodecyl sulfate, 50 mM Tris-HCl, pH 7.4), supplemented with a 2x protease inhibitor cocktail and homogenized manually. Proteins were solubilized by incubating the DRG extracts at 37°C and 1,400 rpm for 2 h in an Eppendorf ThermoMixer (Eppendorf AG, Hamburg, Germany). Proteins were then extracted by a 15-minute centrifugation at 14,000 rpm. The protein concentration of muscle and DRG extracts was determined using the Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with BSA as standard. Protein samples (40 μg) from muscle and DRGs were separated by SDS-PAGE on a 4–15% gradient gel and transferred onto nitrocellulose membranes (10 V for 1 h). Membranes were incubated with primary antibodies: anti-IL-33 (rabbit, 1:500; ab187060, Abcam, Cambridge, MA), IL-33R/ST2 (rabbit, 1:500; ab228543, Abcam), Grk2 (rabbit, 1:500; sc562, Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (rabbit, 1:500; ab8227, Abcam). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit (donkey, 1:2500; na934v, GE Healthcare Life Sciences, Piscataway, NJ) and anti-mouse (goat, 1:2500; 1858413, Thermo Fisher Scientific). Immunoreactivity was visualized by chemiluminescence (SuperSignal

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West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific) and analyzed by computer-assisted densitometry.

Statistical analysis

Group data are expressed as mean ± SEM of n independent observations. Statistical comparisons were made using GraphPad Prism 8.1 statistical software (GraphPad Software Inc., La Jolla, CA). Rats were assigned randomly to each experimental group. Comparisons between groups were made by means of unpaired Student *t* test or 2-way repeated-measures ANOVA, followed by Dunnett or Bonferroni multiple comparisons tests. A *P*-value < 0.05 was considered statistically significant.

Results

Vibration induces mechanical hyperalgesia and increases IL-33 expression in skeletal muscle

Consistent with previous reports[2, 3, 6–8], exposure to vibration produced a significant decrease in the nociceptive mechanical threshold in the ipsilateral gastrocnemius muscle. This hyperalgesia was already present 1 day after vibration injury (−33.5 ± 0.6% vibrated group [1678.2 ± 18.9 mN] *vs* 0 ± 0.8% control group [2513.2 ± 20.5 mN], n = 6/group, $P < 0.001$, Fig. 1A), reached a peak by day 10 (−43.5 ± 0.8% vibrated group [1426.5 ± 22.3 mN] *vs* 1.6 ± 0.3% control group [2553.5 ± 9.5 mN], n = 6/group, $P < 0.001$, Fig. 1A), and lasted for at least 14 days (−22.7 ± 1.5% vibrated group [1952.5 ± 41.4 mN] *vs* 1.4 ± 0.2% control group [2548 ± 4.5 mN], n = 6/group, $P < 0.001$, Fig. 1A). Compared to controls, rats submitted to ergonomic vibration 24 h prior exhibited significant up-regulation of IL-33 in the ipsilateral gastrocnemius muscle (42.6 ± 6%, n=6/group, $P < 0.05$, Fig. 1B,C).

Intramuscular IL-33 produces mechanical hyperalgesia

To evaluate whether increased levels of IL-33 observed in rats submitted to vibration injury could contribute to the mechanical hyperalgesia displayed by these animals, rrIL-33 was injected into the gastrocnemius muscle. Compared to vehicle (n = 6), i.m. rrIL-33 produced a dose-dependent ($r^2 = 0.718$, $P < 0.0005$) decrease in mechanical nociceptive threshold, peaking ~1 h after injection (n = 5/6 group, $P < 0.001$; Fig. 2A,B). For the highest dose tested (300 ng), hyperalgesia peaked at a 38.5 ± 1.4% decrease in nociceptive threshold (rrIL-33 group [1541.6 ± 23 mN] *vs* −5 ± 1.4% vehicle group [2409.7 ± 35.6 mN], n = 6, $P < 0.0012$, Fig. 2A,B); significant hyperalgesia persisted for at least 3 days (−24 ± 2.7% rrIL-33 group [1906.4 ± 82.8 mN] *vs* −2.7 ± 1.3% vehicle group [2469 ± 44.4 mN], $P < 0.05$, Fig. 2B).

IL-33R/ST2 antisense

To assess the potential role of nociceptor IL-33R/ST2 in IL-33-induced hyperalgesia, we designed an AS ODN sequence against the IL-33R/ST2 mRNA to knockdown the protein expression of ST2 in nociceptors. Western blot analysis of L4-L5 DRG extracts from rats submitted to 3 consecutive days of i.t. ODN treatment demonstrated that, compared to MM, AS produced a significant decrease in the expression of ST2 (−25.5 ± 5.4%, n = 6/group, $P < 0.05$, Fig. 3A,B).

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IL-33R/ST2 antisense inhibits IL-33-induced hyperalgesia

Next, in order to evaluate *in vivo* the efficacy of AS ODN treatment, the effect of IL-33R/ST2 knockdown on rrIL-33-induced muscle hyperalgesia was assessed. While both, MM and AS ODN, treatments were devoid of effect on baseline nociceptive threshold (−0.5 ± 0.3% MM group [2528.7 ± 6.7 mN] *vs* −0.4 ± 0.5% AS group [2526.5 ± 12.6 mN], n = 6/group, $P > 0.05$; Fig. 4A), compared to MM, the AS treatment prevented the mechanical hyperalgesia induced by 100 ng of rrIL-33 (−39 ± 0.6% MM group [1550 ± 15.9 mN] *vs* −8.2 ± 1.7% AS group [2328.2 ± 45.3 mN], n = 6/group, $P < 0.0012$, Fig. 4A).

IL-33R/ST2 antisense inhibits vibration-induced hyperalgesia

Finally, we explored the effect of AS ODN knockdown of IL-33R/ST2 on vibration-induced mechanical hyperalgesia in the ipsilateral gastrocnemius muscle. In a preventive protocol, compared to MM controls, AS treatment reversibly attenuated the vibration-induced hyperalgesia (Fig. 4B). This effect was observed on days 1 (−26.6 ± 1.8% MM group [1878.7 ± 46.4 mN] *vs* −9.5 ± 1.5% AS group [2330 ± 41.6 mN], n = 6/group, $P < 0.0012$, $P < 0.001$, Fig. 4B) through 3 (−37.6 ± 2.1% MM group [1597 ± 55.3 mN] *vs* −22.4 ± 1.9% AS group [1996.5 ± 46.6 mN], n = 6/group, $P < 0.0012$, $P < 0.001$, Fig. 4B) after exposure to vibration. This antihyperalgesic effect was fully reversed by day 5 (−41.6 ± 1% MM group [1496.3 ± 25.3 mN] *vs* −41.7 ± 0.5% AS group [1498.8 ± 11.7 mN], n = 6/group, $P > 0.05$, Fig. 4B).

Discussion

While severe musculoskeletal pain is a hallmark symptom in HAVS [13, 14, 31], its pathophysiology remains poorly understood. The finding that IL-33, acting on muscle nociceptors, contributes to the pain observed in a rat model of HAVS provides insight into this condition, unraveling a promising diagnostic and therapeutic target.

In line with studies of inflammatory pain [35, 37], we observed enhanced expression of IL-33, in the skeletal muscle, concomitant with mechanical hyperalgesia following hind limb exposure to vibration. Indeed, IL-33 transcripts and protein are locally up-regulated after injection of inflammogens such as carrageenan[37] or immune sensitization to antigens such as ovalbumin or methylated-bovine serum albumin[35]. Evidence suggests that in injured skeletal muscle IL-33 is produced by several cell types that display markers for fibro/adipogenic progenitor cells, which are associated with neural structures such as nerve fibers, nerve bundles, and muscle spindles [20] Which of these cell types contribute to our model of HAVS remains to be established. Of note in this regard, muscle spindles are extremely sensitive to vibration, which may contribute to the pathophysiology of HAVS[5](see below).

While we did not explore the mechanism underlying enhanced IL-33 expression, vibration can induce muscle damage by mechanisms such as persistent muscle contraction due to tonic vibration-reflex triggered by activation of muscle spindles[5] and ischemia-reperfusion injury secondary to reflex vasoconstriction [11, 15, 32]. Indeed, others have observed that either single or multiple exposures to comparable vibration produce vascular abnormalities, including vasoconstriction, vasospasm and enhanced reactivity to vasoconstrictor agents

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[18, 19]. Additionally, IL-33 is released by mechanical strain [16], well-established to contribute to HAVS[19]. Of note, both ischemia-reperfusion injury and mechanical strain increase local expression and release of IL-33 in several pathological settings, including mechanical ventilation-induced lung injury [9], kidney ischemia-reperfusion injury [10], cerebral stroke [17], and mechanical overload induced-cardiac hypertrophy [29]. While these reports and clinical observations [31, 32] support the suggestion that vascular changes contribute to HAVS pathophysiology, further studies to establish the nature of the actual injury induced by exposure to vibration are needed.

Intramuscular injection of rrIL-33 produced a time- and dose-dependent mechanical hyperalgesia, consistent with its reported proalgesic effect after intra-articular [35] or cutaneous [35, 37] administration. It has been shown that the nociceptive effect of IL-33 depends on activation of immune cells and subsequent release of proalgesic mediators [35, 37]. Furthermore, serine proteases from neutrophils (which are readily recruited locally upon muscle injury) and mast cells (which are resident in skeletal muscle) are reported to process the N-terminus of the full-length IL-33 protein to several active protein fragments, which display enhanced activity on the ST2 receptor [21, 22, 30] Thus, significant hyperalgesia could be still present in the absence of full-length native IL-33.

Our results are in line with previous evidence showing that a number of pro-inflammatory cytokines and proalgesic mediators are involved in the mechanical hyperalgesia and nociceptor sensitization induced by ergonomic vibration [2, 3, 8, 26] Indeed, enhanced levels of IL-6 [2] and the proalgesic neurotrophin Glial Cell-Derived-Neurotrophic-Factor (GDNF) [3] have been observed in the muscle tissue exposed to vibration. Moreover, administration of intrathecal antisense oligodeoxynucleotides to knockdown receptors to pro-inflammatory cytokines, such as TNFR1 [8], gp130 [2, 6] or the canonical receptor for GDNF, GFRα1 [3], markedly attenuate the hyperalgesia induced by vibration. Indeed, pro-inflammatory cytokines such as IL-6 and TNFα that are involved in vibration-induced muscle pain [2, 6, 8], also contribute to IL-33-mediated hyperalgesia [35]. Furthermore, we observed that antisense knock down of ST2 produced a marked, but incomplete, decrease in vibration-induced hyperalgesia. In contrast, an almost complete inhibition of IL-33-induced muscle hyperalgesia was observed after the same antisense treatment. Thus, although an indirect effect on immune cells cannot be ruled out, our results indicate that IL-33 acts as a direct peripheral sensitizer to induce mechanical hyperalgesia.

Increased levels of IL-33 are critical for local expansion of T regulatory (Treg) cells, a limiting step in the induction of tolerogenic and anti-inflammatory responses after muscular damage [20]. Indeed, age-related deficits in IL-33 are related to poor muscle regeneration, whereas IL-33 administration to old mice ameliorates Treg cell accumulation and muscle regeneration [20]. These observations support the suggestion that IL-33 up-regulation not only contributes to skeletal muscle regeneration but is also sensed by nociceptors. Of note, ST2 antisense also prevented the acute mechanical hyperalgesia induced by intramuscular rrIL-33. Given that sensory neurons are the only cells in the gastrocnemius muscle exposed to both rrIL-33 and intrathecal ST2 antisense, the observed antihyperalgesia suggests that IL-33 directly acts on these neurons. Similarly, ST2 antisense prevented vibration-induced mechanical hyperalgesia. However, in this case we observed residual hyperalgesia,

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consistent with our previous observations on the involvement of other proalgesic mediators in vibration-induced muscle pain [2, 3, 6, 8].

In line with these findings, studies of ST2 expression in DRG neurons innervating skin revealed co-localization with neuronal markers (PGP9.5) and a nociceptor phenotype (TRPV1) in small and medium-sized neurons [23], whereas transcript profiling revealed ST2 mRNA expression in lung-innervating $Na_V1.8(+)$ nodose ganglion nociceptors [33]. Finally, *in vitro* studies showed that up to 85% of the DRG neurons displaying calcium responses to IL-33 also responded to two noxious agents, mustard oil and capsaicin, indicating that ST2 expressing sensory neurons likely mediate pain responses [23].

In summary, IL-33 plays a critical role in the musculoskeletal pain induced by mechanical vibration. Moreover, nociceptor expression of IL-33R/ST2 receptor is necessary for the induction of hyperalgesia. Together these observations support the suggestion that strategies targeting IL-33/ST2 signaling may be valuable for the treatment of vibration-induced muscle pain.

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Highlights

- Chronic muscle pain is a prominent feature of Hand-Arm Vibration Syndrome (HAVS).
- Muscle pain and IL-33 up-regulation were observed in a rat model of HAVS.
- Intramuscular injection of IL-33 produced dose-dependent persistent muscle pain.
- Antisense knockdown of IL-33 receptor attenuated IL-33 and HAVS-induced pain.
- Thus, IL-33 contributes to HAVS pain by acting on muscle nociceptors.

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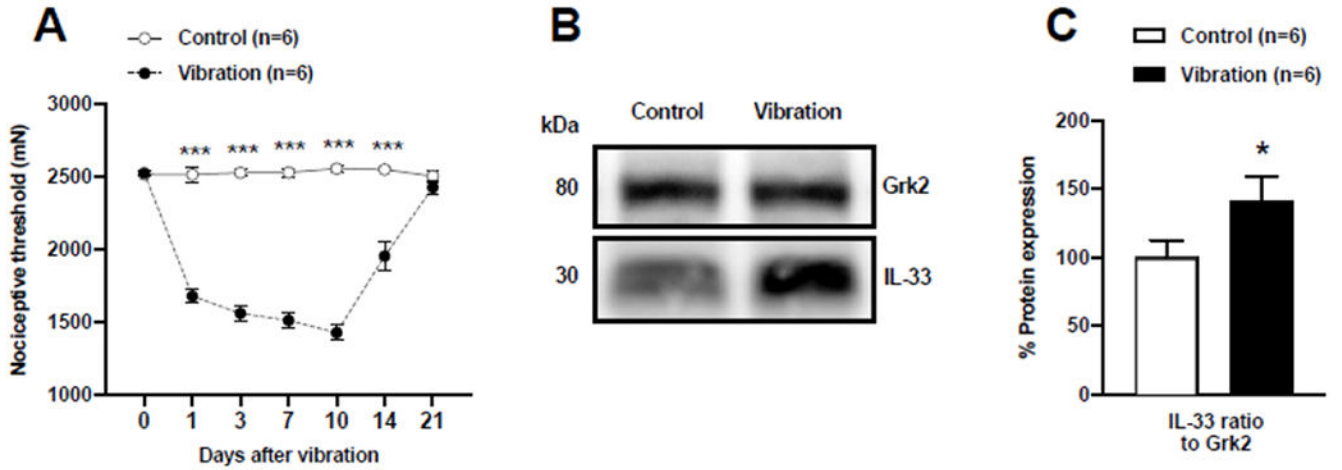

Figure 1: Upregulation of gastrocnemius muscle IL-33 in a model of HAVS, and intramuscular rrIL-3-induced muscle hyperalgesia.

A. Time course of mechanical hyperalgesia induced by exposure to vibration (HAVS model); repeated measures two-way ANOVA showed significant effects for treatment ($F_{1,10} = 1.030$, $P < 0.0001$), time ($F_{6,60} = 270.1$; $P < 0.0001$), and their interaction ($F_{6,60} = 297.7$; $P < 0.0001$). Bonferroni post hoc analysis revealed significant differences between control and HAVS rats between 1 and 14 days after vibration (***$P < 0.001$). **B.** Representative Western blots showing IL-33 immunoreactivity in extracts from gastrocnemius muscle of a rat exposed to vibration 24 h prior and a control rat. **C.** Ratio of reference protein Grk2 to IL-33 showed that IL-33 levels were significantly increased in muscle from HAVS rats (unpaired Student *t*-test, t = 1.923, df = 10; *$P < 0.05$).

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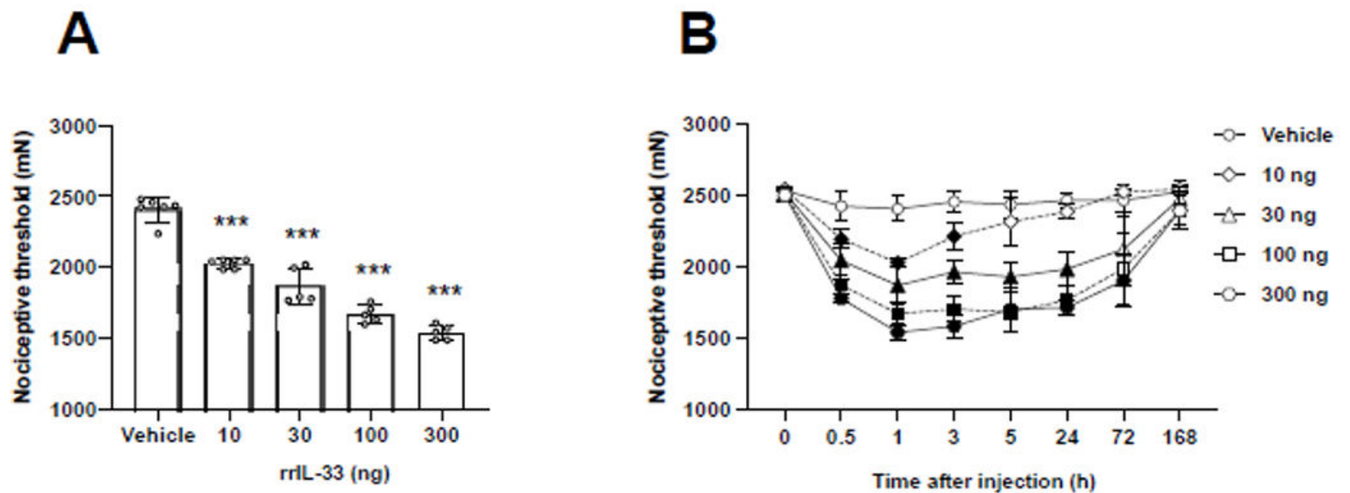

Figure 2: Local IL-33 produces long-lasting muscle mechanical hyperalgesia.

A. Injection of rrIL-33 into the right gastrocnemius muscle produced a dose-dependent decrease in mechanical nociceptive threshold as measured 1 hour after injection; one-way ANOVA showed significant effects for treatment ($F_{4,22} = 106.1$, $P < 0.001$). Dunnett post hoc analysis revealed significant differences between vehicle and rrIL-33 doses (***$P <$ 0.001). **B.** Time course of hyperalgesia induced by different doses of rrIL-33; repeated measures two-way ANOVA showed significant effects for treatment ($F_{4,22} = 104.8$, $P <$ 0.0001), time ($F_{7,154} = 167.9$; $P < 0.0001$), and their interaction ($F_{28,154} = 13.75$; $P <$ 0.0001). Bonferroni post hoc analysis revealed significant differences between vehicle and rrIL-33 treated rats over the period 0.5 to 72 h after injection (solid symbols, $P < 0.05$).

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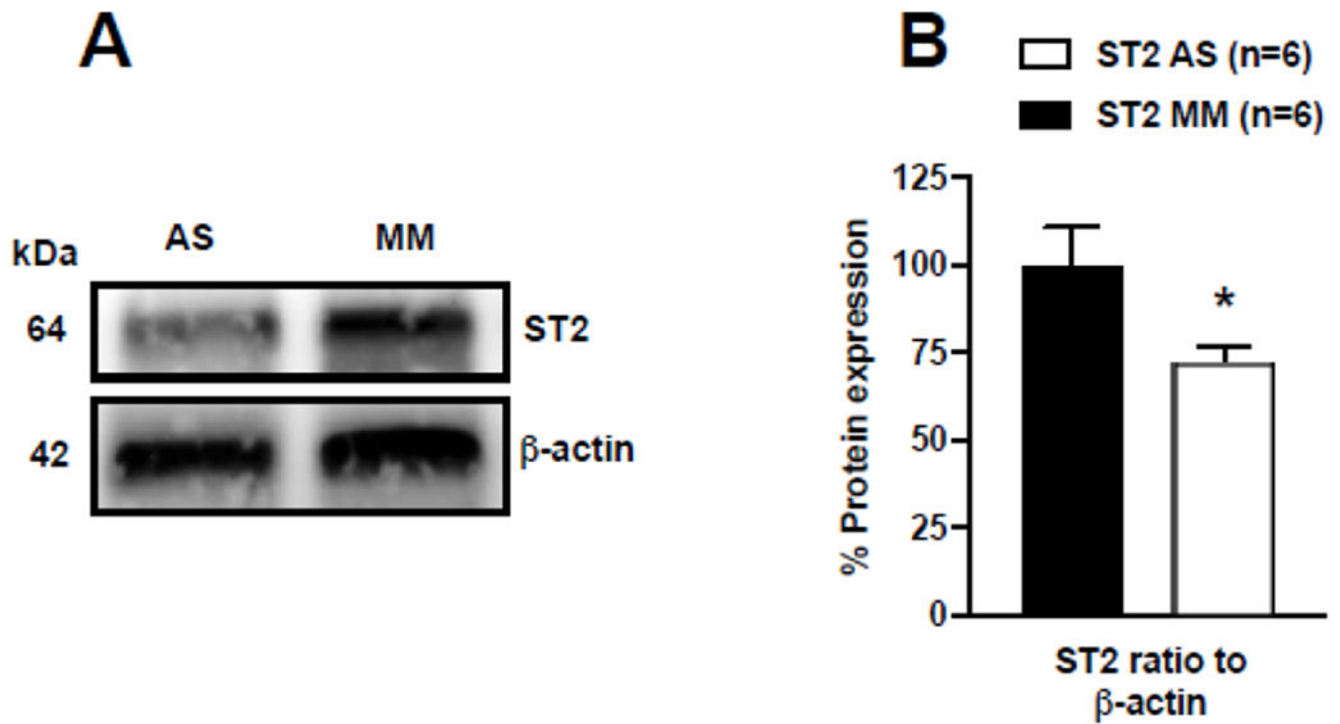

Figure 3: Effect of antisense (AS)/mismatch (MM) oligodeoxynucleotides (ODN) treatment directed against IL-33R/ST2 mRNA on protein expression of IL-33R/ST2 in L4-L5 DRG homogenates.

A. Representative Western blots showing expression of IL-33R/ST2 in extracts from L4-L5 DRG from rats treated with AS or MM ODN. **B.** Quantitative analysis (ratio to β-actin reference protein) showed that, compared to MM, AS treatment significantly attenuated the expression of IL-33R/ST2 (unpaired Student *t* test, $t = 2.296$, $df = 10$, $^*P < 0.05$).

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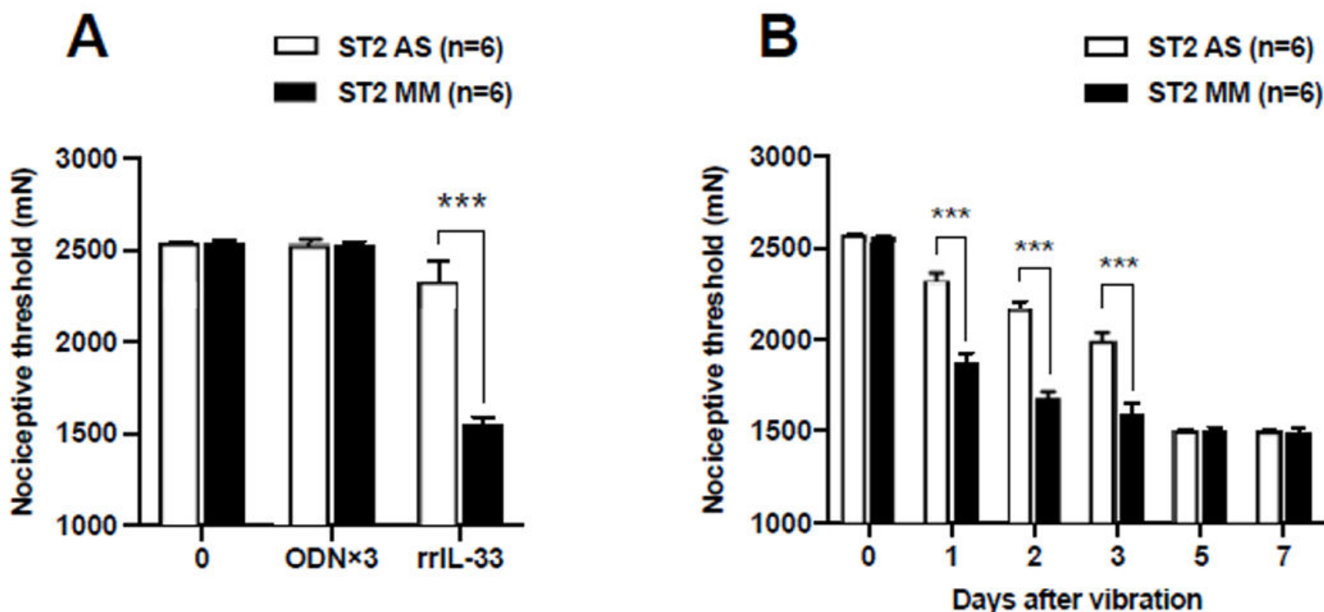

Figure 4: Intrathecal ODN antisense to IL-33R/ST2 attenuates both rrIL-33 and HAVS hyperalgesia.

A. One day after the last of 3 daily i.t. injections the ODN treatment did not produce significant differences in nociceptive threshold but significantly attenuated the hyperalgesia induced by i.m. rrIL-33; repeated measures two-way ANOVA showed significant effects for treatment ($F_{1,10} = 164.6$; $P < 0.0001$), time ($F_{2,20} = 693.8$; $P < 0.0001$), and their interaction ($F_{2,20} = 299.7$; $P < 0.0001$). Bonferroni post hoc analysis revealed significant differences between AS and MM treated rats after rrIL-33 injection (***$P < 0.001$). **B.** Compared to MM ODN, AS directed against ST2 significantly attenuated vibration-induced mechanical hyperalgesia. Two-way ANOVA showed significant effects for treatment ($F_{1,10} = 62.91$; $P < 0.0001$), time ($F_{5,50} = 411.4$; $P < 0.0001$) and their interaction ($F_{5,50} = 37.04$; $P < 0.0001$). Post hoc analysis revealed significant differences between AS and MM treated rats on days 1 to 3 after exposure to vibration (***$P < 0.001$).