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Muscle Pain in Models of Chemotherapy- and Alcohol-induced Peripheral Neuropathy

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Abstract

Objective—While inflammatory pain is well described in skeletal muscle, neuropathic muscle pain remains to be clarified. We used three well-established rodent models of peripheral neuropathy to evaluate for muscle pain.

Methods—In rats exposed to either of two neurotoxic cancer chemotherapies, paclitaxel or oxaliplatin, or to alcohol consumption, we assessed the evolution of mechanical hyperalgesia in skeletal muscle and skin, in the same animal. To explore the involvement of protein kinase C epsilon (PKCε), a second messenger implicated in some forms of neuropathic pain, oligodeoxynucleotides (ODN) antisense (AS) or mismatch (MM) for PKCε were administered intrathecally.

Results—Rats submitted to models of chemotherapy- and alcohol-induced neuropathy developed persistent muscle hyperalgesia, which evolved in parallel in muscle and skin. The administration of PKCε AS, which has been shown to mediate cutaneous hyperalgesia in paclitaxel and ethanol models of neuropathic pain, also inhibited muscle hyperalgesia induced by these agents. Stopping AS-ODN was associated with the reappearance of hyperalgesia at both sites. The AS-ODN to PKCε treatment was devoid of effect in both muscle and skin in the oxaliplatin neuropathy model.

Interpretation—Our results support the suggestion that neuropathic muscle pain may be a greater clinical problem than generally appreciated.

Introduction

Acute and chronic pain syndromes are usually categorized into inflammatory and neuropathic based on etiology 1. For musculoskeletal pain, however, this distinction has not been so clearly established, even though muscle pain is observed in neuropathic as well as inflammatory diseases 2. Thus, despite the fact that the concept of neuropathic muscle pain has been largely neglected, a number of reports have communicated muscle pain as a prominent symptom in patients with peripheral neuropathies 2–8. Muscle pain observed in patients with peripheral neuropathies is particularly incapacitating and commonly resistant to pharmacologic interventions 9. Unfortunately, most of the basic and clinical research on neuropathic pain has focused on cutaneous symptoms 1–10. Although this approach has revealed much about mechanisms related to cutaneous pain their possible role in muscle pain remains largely unknown. Such lack of knowledge results in part from the absence of

examination of nociception in muscle under conditions of well-established peripheral neuropathies.

In the present study we evaluated skin and muscle nociception in well-established models of neuropathic pain. We also determined if those symptoms share an underlying mechanism that has been implicated in the same models of neuropathic pain, dependence on protein kinase C epsilon (PKC ϵ) signaling in nociceptors 11–14.

Materials and Methods

Animals

Experiments were performed on adult male Sprague Dawley rats weighting 250–400 g (Charles River, Hollister, CA). They were housed in the Laboratory Animal Resource Center of the University of California, San Francisco, under a 12 h light/dark cycle and environmentally controlled conditions (7 am–7 pm light cycles; 21°–23°C) with food and water available *ad libitum*. Animal care and use conformed to National Institutes of Health guidelines. The University of California, San Francisco, Institutional Animal Care and Use Committee approved experimental protocols.

Drugs

Unless specifically stated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Pharmacologic induction of neuropathy

Paclitaxel treatment—Paclitaxel was administered as previously described 12, 15. Briefly, it was formulated at a concentration of 1 mg/ml in a vehicle composed of absolute ethanol and Cremophor EL[®] (1:1). A final paclitaxel concentration of 1 mg/ml was made by adding sterile NaCl (0.9%), at the time of injection. After habituation to the test environment and baseline measurements of pain sensitivity (see below), two groups of rats were injected intraperitoneally with paclitaxel (1 mg/kg) on the days 0, 2, 4 and 6.

Oxaliplatin treatment—Briefly, oxaliplatin was dissolved in normal saline at a concentration of 2 mg/ml, for intravenous administration (1 ml/kg). After habituation to the test environment and determination of baseline mechanical nociceptive threshold (see below), rats were injected intravenously with a single dose of oxaliplatin (2 mg/kg) 16.

Chronic alcohol consumption—Alcohol-induced painful neuropathy was produced by feeding experimental rats with Lieber-DeCarli liquid diet (Dyets Inc., Bethlehem, PA) 17, 18 containing 6.5% ethanol, for 3 weeks. Rats were exposed to 6.5% ethanol-containing diet in weekly cycles of 4 days on ethanol-containing liquid diet and 3 days of standard laboratory rat chow 19.

Nociceptive testing

Mechanical threshold in the gastrocnemius muscle—Mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a digital force transducer (Chatillon DF12; Amtek Inc., Largo, FL) with a custom-made 7-mm-diameter probe. It has been shown that the use of a probe with a tip diameter ≥ 2.6 mm allows reliable measurements of mechanical nociceptive threshold in muscle, even when overlying cutaneous hyperalgesia is present 20–22. Indeed, readings of mechanical muscle nociceptive threshold obtained with this method are not affected by cutaneous topical anesthesia but significantly enhanced by intramuscular injection of lidocaine 21. Rats were lightly restrained in a plexiglas holder that allows for easy access to the hind limb and application,

including application of the transducer probe to the belly of the gastrocnemius muscle. The nociceptive threshold was defined as the force, in Newtons, required to produce a flexion reflex in the hind leg. Baseline limb-withdrawal threshold was defined as the mean of 3 readings taken at 5-minute intervals. Each hind limb was treated as an independent measure and each experiment performed on a separate group of rats.

Mechanical threshold in the skin—The nociceptive flexion reflex was quantified using the Randall–Selitto paw pressure test 23, which produces a linearly increasing mechanical force (Analgesymeter; Stoelting, Chicago, IL) that is applied to the dorsum of the rat's hind paw 11. In this procedure, animals were lightly restrained using cylindrical restrainers designed to minimize restraint stress, and also accommodate the size variations of individual rats. All experimental animals used in this study were acclimated to the testing procedure such that restraint and test techniques were parallel across groups. Briefly, animals were placed in individual restrainers for 1 h before the commencement of each study, and for 30 min before testing on each study day. The nociceptive threshold was defined as the force in grams at which the rat withdrew its paw. The baseline paw-withdrawal threshold was defined as the mean of three readings taken before test agents were injected. Each paw was treated as an independent measure and each experiment performed on a separate group of rats. All behavioral testing was performed between 10.00–16.00 h.

Oligodeoxynucleotide (ODN) antisense to PKC ϵ

ODNs antisense to PKC ϵ , shown previously to decrease PKC ϵ in dorsal root ganglion neurons 24, were synthesized by Invitrogen (San Francisco, CA). The antisense ODN, 5'-GCC AGC TCG ATC TTG CGC CC-3', was directed against a unique sequence of PKC ϵ . The mismatch ODN, 5'-GCC AGC **GCG** ATC TTT CGC CC-3', is the antisense sequence with two bases, denoted in bold face, switched. A search of the EMBL and NCBI GeneBank *Rattus norvegicus* databases identified no homologous sequences. Before use, the ODNs were lyophilized and reconstituted in 0.9% NaCl to a concentration of 2 μ g/ μ l. For each injection, the rat was briefly anaesthetized with 2.5% isoflurane in 95% O₂. A 30-gauge hypodermic needle was inserted into the subarachnoid space on the midline, between the L4 and L5 vertebrae. A total of 40 μ g ODN in a volume of 20 μ l per rat was slowly injected 25. Proper intrathecal injections were systematically confirmed by checking for a sudden flicking of the tail, a reflex that is evoked by subarachnoid space access and bolus injection 26. This method of injecting into the intrathecal space has proven to be very accurate and reproducible, as revealed by intrathecal injections of vital dyes and radioligands such as methylene blue and [³H] morphine 26· 27. The animals regained consciousness approximately 1 min after the injection. The use of antisense ODNs to manipulate the expression of proteins in nociceptors important for their sensitization is well supported by previous studies 19· 24· 28· 29.

Statistical analysis

One-way or two-way repeated measures ANOVAs with appropriate post hoc analyses to determine if significant differences between experimental groups were observed. If there was a significant group $\times$ time interaction, multivariate analyses (i.e., one-way ANOVAs) were performed for all time points in order to determine which points accounted for the interaction. In these cases, a Bonferroni correction was applied in order to account for multiple comparisons. For within-subjects effects the Mauchly criterion was used to determine if the assumption of sphericity was met; if not, Greenhouse-Geisser *p*-values are presented. Statistical significance (i.e. the α -level) was set at $p < 0.05$.

Results

Paclitaxel treatment

Intraperitoneally administered paclitaxel induced persistent cutaneous and muscle hyperalgesia. A two-way repeated measures ANOVA with one within-subjects factor (days with 11 levels) and one between subjects factor (group with two levels, muscle hyperalgesia and skin hyperalgesia) showed a significant main effect of time ($F_{10,220} = 37.264$; $p < 0.001$) but not a significant time by group interaction ($F_{10,220} = 0.771$; $p = 0.550$) nor a significant main effect of group ($F_{1,22} = 0.235$; $p = 0.632$), indicating that although the groups did not differ significantly from each other, they did vary significantly over time, in parallel (Fig. 1A). These results suggest that cutaneous and muscle hyperalgesia are similarly induced by paclitaxel.

To investigate the role of PKC ϵ in paclitaxel-induced cutaneous and muscle hyperalgesia, rats received daily intrathecal injection of PKC ϵ ODN antisense (40 μ g) for 3 days starting on day six following paclitaxel administration; a separate group of rats received ODN mismatch as a control. Antisense, but not mismatch, ODN-treated rats exhibited a significant attenuation of both the cutaneous (Fig. 1B) and muscle (Fig. 1C). hyperalgesia, indicating dependence on PKC ϵ .

Oxaliplatin treatment

A single intravenous injection of oxaliplatin induced persistent cutaneous and muscle hyperalgesia. A two-way repeated measures ANOVA with one within-subjects factor (days with 11 levels) and one between subjects factor (group with two levels, muscle hyperalgesia and skin hyperalgesia) showed a significant days by group interaction ($F_{10,220} = 22.882$; $p < 0.001$), a significant main effect of group ($F_{1,22} = 14.524$; $p < 0.001$), and a significant main effect of time ($F_{10,220} = 185.316$; $p < 0.001$), indicating that the two groups differed significantly in both the time course and the overall magnitude of hyperalgesia (Fig. 2A). However, separate one-way repeated measures ANOVAs with simple contrasts showed significant cutaneous and muscle hyperalgesia ($p < 0.001$) on all test days (2 through 21) following injection.

To investigate the role of PKC ϵ in both cutaneous and muscle hyperalgesia induced by oxaliplatin, rats received three daily intrathecal injections of PKC ϵ ODN antisense (40 μ g) for 3 days starting either on day one (early phase hyperalgesia, Fig. 2A) or day 21 (late phase hyperalgesia, Fig. 2B) following oxaliplatin administration; separate groups of rats received ODN mismatch as controls. In contrast to paclitaxel, ODN antisense to PKC ϵ did not significantly attenuate either early or late phase oxaliplatin muscle or cutaneous hyperalgesia.

Chronic alcohol consumption

Exposure to binge alcohol consumption induced both cutaneous and muscle hyperalgesia. A two-way repeated measures ANOVA with one within-subjects factor (days with 11 levels) and one between subjects factor (group with two levels, muscle hyperalgesia and skin hyperalgesia) showed a significant days by group interaction ($F_{10,220} = 14.329$; $p < 0.001$), a significant main effect of group ($F_{1,22} = 22.133$; $p < 0.001$), and a significant main effect of time ($F_{10,220} = 69.797$; $p < 0.001$), indicating that the two groups differed significantly in both the time course and the overall magnitude of hyperalgesia (Fig. 3A). One-way repeated measures ANOVAs with pairwise contrasts showed that the onset of significant cutaneous hyperalgesia was on day 8 ($p < 0.001$), and the onset of significant muscle hyperalgesia was on day 15 ($p < 0.001$), indicating that the onset of muscle hyperalgesia is delayed by about one week with respect to that of cutaneous hyperalgesia in this model of alcoholic

neuropathy. Once established, however, both forms of hyperalgesia persisted for the duration of the experiment.

To investigate the role of PKC ϵ in alcohol-induced cutaneous and muscle hyperalgesia, rats received daily intrathecal injection of PKC ϵ ODN antisense (40 μ g) for 3 days beginning 30 days after the start of the ethanol diet; a separate group of rats received ODN mismatch as a control. Antisense, but not mismatch, ODN-treated rats exhibited a significant attenuation of the both cutaneous (Fig. 1B) and muscle (Fig. 1C). hyperalgesia, indicating dependence on PKC ϵ .

Discussion

Many clinical conditions that produce painful peripheral neuropathies, have been reported to produce, in addition to classic cutaneous symptoms, persistent and sometimes severe muscle pain. However, as a clinical entity, neuropathic muscle pain has been largely neglected. As preclinical studies of neuropathic muscle pain are lacking, the mechanisms that underlie this type of pain remain unexplored. Our results provide experimental evidence for the presence of persistent muscle hyperalgesia in three well-established rat models of painful peripheral neuropathy.

Muscle hyperalgesia in models of peripheral neuropathy

The chemotherapeutic agent paclitaxel causes a dose-limiting, predominantly sensory, painful peripheral neuropathy 7, 30. Cutaneous sensory symptoms include numbness, tingling, burning pain and cold allodynia of the hands and feet, in a distal stocking-and-glove pattern 7, 30. In addition, chemotherapy based on paclitaxel often results in a subacute pain syndrome characterized by intense myalgias 5, 9, which can persist for several months 5. We extend these observations, reporting a persistent mechanical hyperalgesia in muscle comparable in intensity, duration and time course to cutaneous mechanical hyperalgesia. The rapid onset and time to peak of such muscle hyperalgesia is consistent with the paclitaxel-induced myalgia reported in humans, which usually begins a few days after therapy, rapidly reaching an intensity that requires opioid treatment 9, 31.

Oxaliplatin also induces a dose-dependent painful peripheral neuropathy characterized by cold allodynia, dysesthesias and paresthesias of rapid onset. Accompanying cutaneous symptoms, prominent muscle pain and cramps have been reported after oxaliplatin treatment 7, 32, 33. Our group has previously reported cutaneous mechanical hyperalgesia, induced by systemically administered oxaliplatin 16. Muscle pain observed after oxaliplatin treatment appears to result, at least in part, from abnormal spontaneous motor activation induced by neuromyotonic-type motor discharges 34, 35. Although we cannot exclude that this phenomenon contributes to our results, it must be stressed that muscle hyperalgesia was still prominent during the late phase of neuropathy when motor symptoms and neuromyotonic discharges are absent 34, 35. Thus, it is unlikely to contribute to the chronic muscle hyperalgesia induced by oxaliplatin.

The peripheral neuropathy produced by alcohol is thought to be caused by a direct neurotoxic action involving mainly small-diameter fibers 36–38. The clinical picture of alcohol-induced neuropathy usually includes symmetric, dysaesthetic sensory disorders, described as cramp-like, burning or stabbing pain 4, 6, 36, 37. In addition, pain evoked by deep palpation of muscles and tendons is often observed in patients with established alcohol-induced polyneuropathy 6. Such symptoms persist, or even become worse, after sustained alcohol withdrawal 4, 19, a characteristic of neuropathic pain referred to as coasting 39, 40. Available rodent models of alcohol-induced neuropathy have previously revealed cutaneous mechanical, thermal and chemical hyperalgesia, as well as primary afferent hypersensitivity

11, 19, 41, 42. Extending these reports we observed a marked muscle mechanical hyperalgesia in muscle paralleling that in the skin, in intensity and duration, but showing a somewhat different time course. We observed a delayed appearance of muscle hyperalgesia with respect to cutaneous hyperalgesia, which fits with clinical reports of late onset muscle pain in alcohol-induced neuropathy 6. After the second cycle of ethanol diet consumption an intense muscle hyperalgesia was observed which persisted along with cutaneous hyperalgesia until the end of the experiment 11, 19. In spite of the fact that we cannot rule out a contribution of ethanol-induced myopathies to muscle hyperalgesia observed here it seems unlikely since no other symptoms indicative of myopathy are present. Indeed, alcohol-induced acute necrotizing myopathy, a sporadic condition, usually occurs during a particularly heavy bout of drinking which manifests as tightly swollen and tender limbs 43. Chronic forms of alcoholic myopathy typically induce a marked muscle weakness and evident proximal atrophy or swelling, which are improved after a diminution of alcohol consumption 43. None of these were apparent in our experimental series.

Effects of PKC ϵ down-regulation

PKC ϵ has been implicated in the cutaneous hyperalgesia observed in preclinical models of painful neuropathies, including those induced by alcohol and paclitaxel 11, 12, 19, 41. Regarding muscle pain, PKC ϵ has also been shown to contribute to muscle hyperalgesia in a model of vibration-induced muscle pain 44, which produces changes in sensory neuron function in muscle afferents more characteristic of neuropathy than inflammation 45. Here we report that muscle hyperalgesia induced by paclitaxel and alcohol consumption, but not that induced by oxaliplatin, are dependent on PKC ϵ . Such dependence on PKC ϵ occurs, or not, in parallel for muscle and cutaneous hyperalgesia.

Paclitaxel induces intense release of the proinflammatory cytokine IL-6 46, 47 which produces a PKC ϵ -dependent enhanced nociceptive responsiveness of skeletal muscle 48. In addition, paclitaxel induces microtubule stabilization which results in activation of PKC ϵ 49, 50. And, intradermal injection of selective second messenger antagonists of PKC ϵ or its down-regulation decreased the paclitaxel-induced mechanical hyperalgesia in rats 12.

Chronic alcohol exposure up-regulates PKC ϵ expression in dorsal root ganglion neurons 11, whereas acute inhibition or down-regulation of PKC ϵ decreased alcohol-induced allodynia and hyperalgesia 11, 19, 41. In agreement with these studies we observed a marked attenuation of paclitaxel and alcohol-induced muscle hyperalgesia in rats treated with an ODN antisense to PKC ϵ .

Of note, PKC ϵ , which does not contribute to cutaneous mechanical hyperalgesia in oxaliplatin-induced neuropathy did not contribute to mechanical hyperalgesia in muscle, induced by this chemotherapeutic agent. Importantly, animals were randomly assigned to each experimental group and submitted to the same procedure of ODN administration as rats submitted to the other two models of neuropathy, using the same batches of ODN (MM and AS). Thus, the lack of efficacy observed in rats submitted to the oxaliplatin-neuropathy model cannot be attributed to an experimental mishap. Furthermore, present results are consistent with previous results showing that PKC inhibitors do not modify the cutaneous mechanical hyperalgesia induced by oxaliplatin 16.

Clinical implications

There is a growing interest in the use of PKC modulators as a part of chemotherapy 51. Indeed many PKC modulators, such as Bryostatin-1, have been studied in Phase I and II trials 52–55. Interestingly, this agent produces alone or combined with other antineoplastic drugs an intense muscle hyperalgesia, which represents the main dose-limiting side effect

observed in approximately 90% of treated patients 52. In spite of the fact that the rationale for using Bryostatin-1, sustained inhibition of most PKC isoenzymes, this agent can act as a selective PKC ϵ activator 56. Thus, our results might help explain the origin of such intense muscle pain induced by Bryostatin-1, as well as the risk to develop certain forms of chemotherapy-induced neuropathic muscle pain. Finally, given that both opioid tolerance 57 and paclitaxel-induced mechanical hyperalgesia 12 are PKC ϵ -dependent phenomena, one might expect a poor outcome for an opioid-based analgesic therapy in patients suffering from paclitaxel-induced neuropathic muscle pain.

In conclusion, we have shown that persistent muscle hyperalgesia can be demonstrated in different models of painful peripheral neuropathy in the rat. We have also established that PKC ϵ contributes in parallel to both muscle and cutaneous neuropathic hyperalgesia. These findings provide insight into muscle pain as a part of painful neuropathies.

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Figure 1. Cutaneous and muscle hyperalgesia in paclitaxel-induced peripheral neuropathy

(A) Time course of nociceptive mechanical threshold in rat hind paw skin (open bars) and gastrocnemius muscle (solid bars) after i.p. paclitaxel (ptxl) injections (arrows). Separate one way repeated measures ANOVAs followed by pairwise contrasts (10 levels) were used to reveal time points significantly different from baseline for cutaneous and muscle nociceptive thresholds. Cutaneous and muscle nociceptive thresholds decreased significantly by day 4 ($p < 0.001$), and remained significantly decreased until day 16 ($p < 0.001$) and day 23 ($p < 0.001$), respectively.

(B) Effect of ODN AS to PKCε on paclitaxel-induced cutaneous hyperalgesia. The two way repeated measures ANOVA showed a significant group $\times$ time interaction ($F_{6,132} = 19.135$; $p < 0.001$) and a significant main effect of group ($F_{1,22} = 13.221$; $p = 0.001$), indicating that the two groups differed significantly in both time course and magnitude. Multivariate analysis showed that cutaneous hyperalgesia was significantly attenuated by the AS-treatment (solid bars) compared to the MM-treatment (open bars) on days 2 through 7 ($p < 0.003$ at days 2, 3 and 7), with recovery observed by day 11.

(C) Effect of ODN AS to PKCε on paclitaxel-induced muscle hyperalgesia. The two way repeated measures ANOVA showed a significant group $\times$ time interaction ($F_{6,132} = 53.465$; $p < 0.001$) and a significant main effect of group ($F_{1,22} = 04.964$; $p < 0.001$), indicating that the two groups differed significantly in both time course and magnitude. Multivariate analysis showed that muscle hyperalgesia was significantly attenuated by the AS-treatment (solid bars) compared to the MM treatment (open bars) on days 1 through 7 ($p < 0.001$) and also on day 11 ($p = 0.001$), with recovery observed on day 13.

Figure 2. Cutaneous and muscle hyperalgesia in oxaliplatin-induced peripheral neuropathy

(A) Time course of cutaneous and muscle hyperalgesia induced by intravenous oxaliplatin administration. One-way repeated measures ANOVA followed by simple contrasts revealed significant cutaneous (open squares) and muscle (solid triangles) hyperalgesia beginning on day 1 and lasting through day 21, the last day of testing ($p < 0.001$ on all days for both groups).

(B) Effect of ODN AS to PKC ϵ (solid bars) compared to MM (open bars) on cutaneous (left panel) or muscle (right panel) hyperalgesia during early phase oxaliplatin-induced neuropathy (days 1 to 7 after oxaliplatin injection). The two-way repeated measures ANOVA for cutaneous testing was not significant for either the time $\times$ group interaction ($F_{5,100} = 1.192$; $p = 0.321$) or the main effect of group ($F_{1,20} = 3.098$; $p = 0.094$); a similar analysis for muscle hyperalgesia showed a significant time $\times$ group interaction ($F_{5,100} = 4.382$; $p = 0.003$) but not a significant main effect of group ($F_{1,20} = 0.741$; $p = 0.399$). Based on the significant interaction, a multivariate analysis failed to reveal any time points at which the AS and MM groups differed significantly.

(C) Effect of ODN AS to PKC ϵ (solid bars) compared to MM (open bars) on cutaneous (left panel) or muscle (right panel) hyperalgesia during late phase oxaliplatin-induced neuropathy (days 21 to 28 after oxaliplatin injection). The two-way repeated measures ANOVA for cutaneous testing was not significant for either the time $\times$ group interaction ($F_{4,88} = 1.567$; $p = 0.190$) or the main effect of group ($F_{1,22} = 1.132$; $p = 0.299$); a similar analysis for muscle hyperalgesia was also not significant for either the time $\times$ group interaction ($F_{4,88} = 0.847$; $p = 0.467$) or the main effect of group ($F_{1,22} = 2.342$; $p = 0.140$).

Figure 3. Effect of binge alcohol consumption on cutaneous and muscle hyperalgesia

(A) Time course of cutaneous and muscle hyperalgesia induced by chronic alcohol consumption. Three cycles of ethanol diet (ED; 4 days on 3 days off) were administered. Although both cutaneous and muscle hyperalgesia were induced in this model, muscle hyperalgesia was delayed about one week with respect to cutaneous hyperalgesia.

(B) Effect of ODN AS to PKC ϵ on alcohol-induced cutaneous hyperalgesia. The two way repeated measures ANOVA showed a significant group $\times$ time interaction ($F_{7,154} = 23.864$; $p < 0.001$) and a significant main effect of group ($F_{1,22} = 16.886$; $p = 0.001$), indicating that the two groups differed significantly in both time course and magnitude. Multivariate analysis showed that cutaneous hyperalgesia was significantly attenuated by the AS-treatment (open bars) compared to the MM treatment (solid bars) on days 2 through 8 ($p = 0.007$ at days 2, 3 and 8), with recovery observed by day 10.

(C) Effect of ODN AS to PKC ϵ on alcohol-induced muscle hyperalgesia. The two way repeated measures ANOVA showed a significant group $\times$ time interaction ($F_{7,154} = 40.363$; $p < 0.001$) and a significant main effect of group ($F_{1,22} = 84.242$; $p < 0.001$), indicating that the two groups differed significantly in both time course and magnitude. Multivariate analysis showed that cutaneous hyperalgesia was significantly attenuated by the AS-treatment (open bars) compared to the MM treatment (solid bars) on days 1 through 12 ($p < 0.001$ at days 1, 2, 3, 8, 10, and 12), with recovery observed by day 15.