Published in final edited form as:

Eur J Neurosci. 2010 September ; 32(5): 811–818. doi:10.1111/j.1460-9568.2010.07355.x.

Alcohol consumption enhances antiretroviral painful peripheral neuropathy by mitochondrial mechanisms

Luiz F. Ferrari* and Jon D. Levine*

*NIH Pain Center (UCSF), Division of Neuroscience and Biomedical Sciences Program, University of California at San Francisco 521 Parnassus Avenue, San Francisco, CA 94143, USA

Abstract

A major dose-limiting side effect of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) chemotherapies, such as the nucleoside reverse transcriptase inhibitors (NRTIs), is a small-fiber painful peripheral neuropathy, mediated by its mitochondrial toxicity. Co-morbid conditions may also contribute to this dose-limiting effect of HIV/AIDS treatment. Alcohol abuse, which alone also produces painful neuropathy, is one of the most important co-morbid risk factors for peripheral neuropathy in patients with HIV/AIDS. Despite the prevalence of this problem and its serious impact on the quality of life and continued therapy in HIV/AIDS patients, the mechanisms by which alcohol abuse exacerbates highly active antiretroviral therapy (HAART)-induced neuropathic pain has not been demonstrated. In this study, performed in rats, we investigated the cellular mechanism by which consumed alcohol impacts antiretroviral-induced neuropathic pain. NRTI 2',3'-dideoxycytidine (ddC) (50 mg/kg) neuropathy was mitochondrial dependent and PKC ϵ independent, and alcohol-induced painful neuropathy, PKC ϵ dependent and mitochondrial independent. At low doses, ddC (5 mg/kg) and alcohol (6.5% ethanol diet for one week), which alone do not affect nociception, together produce profound mechanical hyperalgesia. This hyperalgesia is mitochondrial dependent but PKC ϵ independent. These experiments, which provide the first model for studying the impact of co-morbidity in painful neuropathy, support the clinical impression that alcohol consumption enhances HIV/AIDS therapy neuropathy, and provide evidence for a role of mitochondrial mechanisms underlying this interaction.

Keywords

alcoholic neuropathy; HAART; NRTI neuropathy; hyperalgesia; rat

Introduction

Alcohol abuse is one of the most important co-morbid risk factors for peripheral neuropathy in patients being treated for human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (Moyle & Sadler, 1998; Nath *et al.*, 2002; Lopez *et al.*, 2004; Nicholas *et al.*, 2007). Despite the prevalence of this problem and its serious impact on quality of life and ability to continue treatment, the mechanisms by which alcohol abuse exacerbates highly active antiretroviral therapy (HAART)-induced neuropathic pain has not been investigated. To create a foundation for the development of rational therapeutic strategies to treat alcohol-exacerbated neuropathic pain in HIV/AIDS patients, we investigated the cellular mechanisms by which consumed alcohol aggravates antiretroviral-induced neuropathic pain. We employed well-established, clinically relevant, rodent models

of HIV/AIDS therapy-induced painful peripheral neuropathy (Joseph *et al.*, 2004; Joseph & Levine, 2004; 2006), and neuropathic effects of alcohol abuse and withdrawal (Dina *et al.*, 2000; Dina *et al.*, 2006) to create a model for their co-morbidity, and to evaluate the underlying mechanism.

Materials and Methods

Animals

The experiments were performed on adult male Sprague–Dawley rats (200–220 g, Charles River, Hollister, CA, USA). Animals were housed in the Laboratory Animal Resource Center of the University of California, San Francisco, under a 12-h light/dark cycle. All experimental protocols were approved by the UCSF Institutional Animal Care and Use Committee (IACUC), and conformed to NIH guidelines for the care and use of experimental animals. Effort was made to limit the numbers of animals used and their discomfort.

Drugs

The chemicals used in this study were: the broad spectrum caspase inhibitor Z-Val-Ala-Asp(OMe)-fluoro methyl ester (Z-VAD-FMK, R&D Systems, Minneapolis, MN); the antioxidant α -lipoic acid, the mitochondrial respiratory complex (mETC) selective inhibitors rotenone (complex I) and oligomycin (complex V), the nucleotide antagonist of ATP-dependent mechanisms P^1, P^4 -di(adenosine-5') tetraphosphate (Ap4A) (Sigma, St. Louis, MO), and PKC ϵ_{V1-2} a PKC ϵ specific translocation inhibitor peptide (PKC ϵ -I, Calbiochem, La Jolla, CA) (Johnson *et al.*, 1996; Khasar *et al.*, 1999). Stock solution (1 μ g/ μ l) of PKC ϵ -I (in 0.9% saline) was stored at -20°C and the injections [1 μ g/2.5 μ l, using a 10 μ l microsyringe (Hamilton, Reno, NV)] were preceded by injection of distilled water (2.5 μ l) in the same syringe, separated by a small air bubble, to produce hypo-osmotic shock, thereby enhancing cell membrane permeability to these cell agents (Tsapis & Kepes, 1977; West & Huang, 1980; Taiwo & Levine, 1989; Khasar *et al.*, 1995; Widdicombe *et al.*, 1996). Drug dose selection was based either on the results of previous studies (Dina *et al.*, 2000; Joseph *et al.*, 2004; Joseph & Levine, 2004; 2006) or on preliminary experiments carried out for this study. All inhibitors were diluted with distilled water before intradermal injection into a hind paw. The mETC inhibitors, Z-VAD-FMK, α -lipoic acid and Ap4A (each 5 μ g), were administered intradermally (i.d.) on the dorsum of the hind paw, in a volume of 5 μ l, via a 30-gauge hypodermic needle. Rotenone, oligomycin and Ap4A were dissolved in 10% DMSO. All the other drugs were dissolved in saline. Paw withdrawal threshold was determined before and 30 minutes after inhibitor administration. The effect of each chemical was determined on different groups of rats.

Measurement of mechanical nociceptive threshold

Mechanical nociceptive threshold was quantified using the Randall–Selitto paw pressure test (Randall & Selitto, 1957), in which a force that increases linearly over time is applied to the dorsum of the rat's hind paw (Taiwo *et al.*, 1989; Taiwo & Levine, 1989), using an Ugo Basile Algesymeter® (Stoelting, Chicago, IL, USA). Rats were placed in cylindrical acrylic restrainers designed to provide adequate comfort and ventilation, to allow extension of the hind leg from the cylinder, and to minimize restraint stress. All rats were acclimatized to the testing procedure, and testing was performed in parallel across groups. Rats were placed in individual restrainers for 1 h prior to starting each study and for 30 min prior to experimental manipulations. Nociceptive threshold was defined as the force at which the rat withdrew its paw. The baseline paw-withdrawal threshold was defined as the mean of three readings. Each paw was treated as an independent measure and each experiment performed on a separate group of rats. All behavioral testing was done between 10:00 and 17:00 h.

Experimental protocols

The protocol used to study the effects of the interaction between chronic ethanol consumption and nucleoside reverse transcriptase inhibitor (NRTI) was based on two models of neuropathic pain described previously (Dina *et al.*, 2000; Joseph *et al.*, 2004) and used as controls in the current experiments:

ddC neuropathy model—Previous studies from our laboratory have shown that a single intravenous (i.v.) injection (50 mg/kg) of the NRTI 2',3'-dideoxycytidine (ddC, Sigma, St Louis, MO) produces an ~25% reduction in paw withdrawal threshold (Joseph *et al.*, 2004) 1 day after administration, with maximum intensity (~35%) on the fifth day. The ddC was dissolved in normal saline, and the volume adjusted to 1 ml/kg for i.v. administration. Before removal of the injection needle, administration of this drug was followed by a bolus injection of an equal volume of saline.

Ethanol neuropathy model—Previous studies from our laboratory have established a model of alcoholic painful peripheral neuropathy in the rat (Dina *et al.*, 2000). Male Sprague Dawley rats (200–220 g), individually caged and maintained under a 12 hr light/dark cycle, were fed Lieber–DeCarli liquid diet (Dyets Inc., Bethlehem, PA) (Lieber & DeCarli, 1982; 1989; Lieber *et al.*, 1989) with ethanol (6.5%) for 3 weeks, in a regimen of 4 days of diet with ethanol/3 days normal diet. After the second week the mechanical nociceptive threshold was significantly lower in the rats on the ethanol diet (ED) than in the control group. After the third week of ED, the animals showed persistent hyperalgesia that lasted for at least five weeks.

Co-morbidity neuropathy model—The protocol used to study the effects of the interaction between ethanol consumption and NRTI therapy consisted in the administration of a low dose of ddC (5 mg/kg), which does not induce changes in mechanical threshold (Joseph *et al.*, 2004) in rats submitted to ED (6.5%) for 4 days, which also does not produce a change in nociceptive threshold. The ddC was intravenously injected on the 4th day of ED.

Test of pharmacological inhibitors—The effect of pharmacological inhibitors on the hyperalgesia induced by the neuropathic pain models was determined in three different groups of rats, i.e., in ddC-treated rats, in rats on ED for 3 weeks, and in the co-morbidity neuropathy model (4 days ED + low-dose ddC). Mechanical paw withdrawal threshold was measured immediately before the administration of the pharmacological inhibitors and again 30 min afterwards. For the groups that received intravenous ddC, the inhibitors were tested five days post-ddC injection. The tests with the inhibitors in the ethanol-fed groups were performed on the fourth week after the ED has started (one week after finishing ED). The inhibitors were tested, in the animals submitted to the combination protocol (ED/ddC), one day after the ddC injection.

Antisense and mismatch oligodeoxynucleotide

Oligodeoxynucleotide (ODN) antisense (AS) and mismatch (MM) to PKC ϵ mRNA were prepared as described previously (Parada *et al.*, 2003a). The AS ODN, 5'-GCC AGC TCG ATC TTG CGC CC-3', was directed against a unique sequence of rat PKC ϵ mRNA. The corresponding GeneBank (National Institute of Health, Bethesda, MD) accession number and oligodeoxynucleotide position within the cDNA sequence are XM345631 and 226–245, respectively. We have previously shown that spinal intrathecal administration of AS ODN with this sequence decreases PKC ϵ protein in dorsal root ganglia (Parada *et al.*, 2003b; Parada *et al.*, 2003a). The sequence of the MM ODN, 5'-GCC AGC **GCG** ATC TTT CGC CC-3', corresponds to the PKC ϵ AS sequence with 2 bases mismatched (in bold typeface).

Prior to use, lyophilized ODN was reconstituted in nuclease-free 0.9% NaCl to a concentration of 10 µg/µl and stored at -20°C until use. A dose of 40 µg of AA or MM oligodeoxynucleotide was administered intrathecally once daily in a volume of 20 µl. For this study, the animals were treated for 3 consecutive days before the ED was started, and daily until the 4th day, when the ddC was administered. Prior to each injection, rats were anesthetized with 2.5% isoflurane in oxygen. ODN was injected using a 30-gauge hypodermic needle inserted between the fifth and sixth lumbar vertebrae, at the level of the cauda equina; intrathecal location of the injection needle was confirmed by a flicking of the rat's tail (Papir-Kricheli *et al.*, 1987).

Statistics

In all experiments, the dependent variable was paw withdrawal threshold expressed as percent change from baseline. One-way ANOVA of the pre-intervention (baseline) paw withdrawal threshold values of all groups (N=198) showed no significant difference ($F_{32,165}=1.058$; $p=0.395$). Average baseline paw withdrawal threshold was 103.4 ± 0.59 g (standard error of the mean - SEM). For the data presented in figure 1, a three-way repeated measures ANOVA with two between-subjects factors (diet with two levels and drug with two levels) and one within subjects factor (time with five levels) was performed. Because there was a significant three-way interaction, separate two-way repeated measures ANOVAs were performed for each of the between subjects factors, diet and drug, in order to determine the basis of the three-way interaction. For the data presented in figure 2, one-way ANOVAs with one between-subjects factor (drug with seven levels) were performed, followed by Scheffé post-hoc analyses to identify the significant differences. Because there was a significant interaction, separate one-way ANOVAs were performed for each of the drug groups to determine the basis of the difference. For data presented in figure 3, a two-way ANOVA with two between-subjects factors (drug group with two levels) and ODN treatment group (two levels) was performed. For the data presented in figure 4, two-way repeated measures ANOVAs with one between-subjects factor (drug with two levels) and one within-subjects factor (time with 10 levels) were performed. For all repeated measures ANOVAs, the Mauchly criterion was tested to determine if the assumption of sphericity for the within-subjects effects was met; if the Mauchly criterion was not satisfied, Greenhouse-Geisser adjusted p-values are presented. Data are presented in figures as mean $\pm$ SEM.

Results

Experimental models to study co-morbidity

We developed an experimental model to test the changes in mechanical threshold induced by ethanol consumption and NRTI therapy in the same animals, using doses (ddC) or duration of administration (ethanol) that alone do not cause sensory changes. Rats submitted to ED (6.5% of ethanol) for four days did not show changes in pain threshold. However, when a low dose of ddC was administered (5 mg/kg, i.v.) on day 4, the mechanical threshold decreased precipitously by ~30% (Figure 1), thus demonstrating an interaction between ethanol consumption and the NRTI in the induction of a painful peripheral neuropathy. To evaluate mechanisms mediating this hyperalgesia, we used this model to test the effect of drugs that affect each type of neuropathic model separately and when administered to the animals submitted to the combination.

Involvement of mitochondria in co-morbidity neuropathy

We first confirmed that inhibitors of the mitochondrial electron transport chain, rotenone (complex I) and oligomycin (complex V) and the antioxidant α -lipoic acid, as well as the ATP-dependent mechanism antagonist P^1, P^4 -di(adenosine-5') tetrphosphate (Ap4A), inhibited the hyperalgesia induced by ddC (50 mg/kg, i.v.) (rotenone 76% inhibition,

oligomycin 72%, α -lipoic acid 76%, and Ap4A 79%) (Figure 2A). In addition, the non-specific caspase inhibitor Z-VAD-FMK also inhibited ddC hyperalgesia (94%). However, the PKC ϵ translocation inhibitor (PKC ϵ -I) had no effect in this model.

In the ethanol-induced neuropathy model drugs that inhibit mitochondrial processes (rotenone, oligomycin, α -lipoic acid and Ap4A, and non-selective caspase inhibitor Z-VAD-FMK) did not affect the ED (6.5% ethanol for 3 weeks, in a regimen of 4 days ED/3 days normal diet)-induced decrease in mechanical nociceptive threshold, while PKC ϵ -I decreased hyperalgesia (70%, Figure 2B).

In the painful peripheral neuropathy model induced by low doses of ddC plus short duration ED, when we administered the same pathway inhibitors, we observed a profile more similar to that observed in ddC- than ethanol-induced neuropathy, as hyperalgesia was decreased by rotenone (98% of inhibition), oligomycin (74%), α -lipoic acid (63%) and Ap4A (76%), and PKC ϵ -I had no effect (Figure 2C). However, Z-VAD-FMK, effective in the ddC painful peripheral neuropathy model, had no effect in the co-morbidity model.

We also confirmed the lack of a role of PKC ϵ in the co-morbidity model by spinal administration of oligodeoxynucleotides antisense or mismatch to PKC ϵ . In the ED model of painful peripheral neuropathy, as previously reported (Dina *et al.*, 2006), ODN AS but not MM to PKC ϵ markedly inhibited hyperalgesia (Figure 3, ED for 2 weeks, two right bars). However, in the co-morbidity model PKC ϵ AS did not significantly affect hyperalgesia (Figure 3, two left bars).

Effect of repeated ethanol exposure in co-morbidity neuropathy

Finally, we examined the impact of repeated exposure to ED in the co-morbidity model. We found that repeated cycles of ethanol exposure further enhanced hyperalgesia in the co-morbidity model (Figure 4), as ethanol diet in ddC-treated animals that received ED for 2 weeks still showed significant decrease in mechanical nociceptive threshold, at least 2 weeks after the interruption of the ED, when compared to rats treated only with ED for 2 weeks (Figure 4B). Animals submitted to ED plus ddC that received ED for only one week, still showed decreased mechanical threshold (~15%) 20 days after interruption of ED, when compared to control animals fed ED for 1 week (Figure 4A).

Discussion

The most effective treatment for HIV/AIDS is “highly active anti-retroviral therapy” (HAART), which consists of combinations of nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors and protease inhibitors. While it is a highly effective therapy for the treatment of HIV/AIDS, HAART can induce a painful peripheral neuropathy, a distal symmetric small fiber dying back axonal neuropathy (Dubinsky *et al.*, 1989; Simpson & Tagliati, 1995; Dalakas, 2001; Pardo *et al.*, 2001; Reliquet *et al.*, 2001), also known as antiretroviral toxic neuropathy, a cause of significant morbidity in HIV/AIDS patients (Berger *et al.*, 1993; Simpson & Tagliati, 1995; Dalakas, 2001; Cohen *et al.*, 2002; Quasthoff & Hartung, 2002). This therapy-induced peripheral neuropathy compromises adherence to treatment and may alter a clinically effective regimen or even necessitate its discontinuation (Dieterich, 2003). Among the drugs that comprise HAART, the NRTIs (*e.g.*, zidovudine (AZT), zalcitabine (ddC), didanosine (ddI), and stavudine (d4T)) clearly play a role in HAART-induced painful peripheral neuropathy (Brinkman *et al.*, 1998; Moyle & Sadler, 1998; Dalakas, 2001; Dalakas *et al.*, 2001; Simpson, 2002; Gerschenson & Brinkman, 2004; Hulan *et al.*, 2005), being associated with a three-fold increase in the incidence of peripheral neuropathy in AIDS patients (Moore *et al.*, 2000). This type of neuropathy occurs in up to two-thirds of patients taking NRTIs

(Simpson & Tagliati, 1995; Oh *et al.*, 2001) and limits the amount of time HAART can be administered (Sharma *et al.*, 2004). It produces clinically significant morbidity in 10–35% of HIV-positive individuals (Hall *et al.*, 1991; Kiebert *et al.*, 1998; Sacktor, 2002).

The toxic effects of chronic ethanol consumption on the peripheral nervous system are also well-documented (Juntunen *et al.*, 1978; Bosch *et al.*, 1979; Scott & Edwards, 1980; Oakes & Pozos, 1982; Juntunen *et al.*, 1983; Massarotti, 1983; Riopelle *et al.*, 1984; Scott *et al.*, 1986; McLane, 1987; 1990; Diamond & Messing, 1994; Hundle *et al.*, 1997; Wu & Kendig, 1998; Dina *et al.*, 2000). Neuropathic pain syndromes occur as a result of ethanol-induced peripheral neuropathy (Foster *et al.*, 1999). Importantly, abuse of ethanol is one of the most important co-morbid risk factors for peripheral neuropathy in patients with HIV/AIDS, and the painful peripheral neuropathy induced by HAART (Moyle & Sadler, 1998; Nath *et al.*, 2002; Lopez *et al.*, 2004; Nicholas *et al.*, 2007). Of note, alcohol abuse is especially prevalent in the population of HIV patients; for example, most HIV patients (53–63%) regularly drink ethanol (Galvan *et al.*, 2002; Miguez *et al.*, 2003), and it has been reported that 4–41% of HIV patients in various cohorts are alcoholic (Atkinson *et al.*, 1988; Brown *et al.*, 1992; Rosenberger *et al.*, 1993; Zenilman *et al.*, 1994; Lefevre *et al.*, 1995; Dew *et al.*, 1997; Cook *et al.*, 2001; Galvan *et al.*, 2002; Samet *et al.*, 2004). This condition led to a number of studies involving the effects of ethanol consumption on HIV-positive patients, including how it affects the progression of the infection (Cook *et al.*, 1997; Cook *et al.*, 2001; Liu *et al.*, 2003; Brailoiu *et al.*, 2006) and the negative impact upon the patient's response to NRTI therapies (Giancola *et al.*, 2006). However, few studies have focused on the effects of the interaction of ethanol consumption and NRTI therapy in sensory systems.

We have previously established a model of ethanol-induced painful peripheral neuropathy in rats by feeding them a Lieber-DeCarli diet, which simulates human chronic alcohol consumption while assuring normal micronutrient intake (Dina *et al.*, 2000; Dina *et al.*, 2006). In protocols in which rats underwent intermittent withdrawal, painful peripheral neuropathy developed much more rapidly (Dina *et al.*, 2006); the C-fiber mechanical threshold was lowered and the number of action potentials elicited during sustained mechanical stimulation increased in ethanol fed rats. In our current study, ethanol-containing diet with normal micronutrient levels was used to study the interaction of ethanol consumption and NRTIs on the function of the peripheral nervous system. We first tested the hypothesis that exposure to ethanol enhances the neuropathic impact of HAART by characterizing the effect of NRTI in the setting of ethanol consumption. We then determined the second messengers mediating the hyperalgesia induced by ethanol- and nucleoside-induced hyperalgesia, and if ethanol exacerbation of nucleoside-induced hyperalgesia involves second messenger pathways implicated in ddC- and/or ethanol-induced painful peripheral neuropathy.

To address the question, what is the mechanism underlying the co-morbid effects of NRTI-induced painful peripheral neuropathy and alcohol consumption, we developed a model of co-morbid painful peripheral neuropathy. After establishing a model system for studying co-morbidity in painful peripheral neuropathy, we focused our attention on the mechanisms in sensory neurons that underlie the interaction between the hyperalgesia induced by ethanol and antiretroviral therapy. Given that the mechanical hyperalgesia induced by giving both low dose ddC and short duration ethanol consumption is mitochondria but not PKC ϵ dependent, we suggest that ethanol consumption enhances ddC effects, rather than vice versa. Since the painful peripheral neuropathy produced by longer term consumption of ethanol is mitochondria independent, we assume ethanol acts by an indirect mechanism to enhance neuropathic effects of ddC. Of note in this regard, we have previously shown that physiological activation of neuroendocrine stress pathways play a crucial role in the neurological effect of ethanol (Dina *et al.*, 2008). In fact, it is well established that ethanol

abuse activates the neuroendocrine stress axes and its withdrawal further exacerbates neuroendocrine stress axis activation (Linnoila *et al.*, 1987; Koob, 1999; Sofuoglu *et al.*, 2001; Errico *et al.*, 2002; Bruijnzeel *et al.*, 2004; Devaud *et al.*, 2006; Koob, 2006; Rasmussen *et al.*, 2006). Since alcoholics consume ethanol intermittently, they may enter early withdrawal before they are able to re-administer ethanol, and recovering alcoholics report increased stress (Lamon & Alonzo, 1997; Koob, 2003; Poage *et al.*, 2004). Importantly, recent evidence has shown that stress hormones modulate mitochondrial function (Du *et al.*, 2009a; Du *et al.*, 2009b; Fujita *et al.*, 2009). Additional studies will be needed to assess the indirect mechanism by which ethanol affects the painful peripheral neuropathy induced by ddC, by impacting mitochondria-dependent mechanisms.

Finally, while mitochondrial mechanisms appear to underlie the painful peripheral neuropathy produced by sub neuropathic doses of ddC and ethanol, there was one difference between the mechanism of ddC painful peripheral neuropathy and that induced by low dose ddC and ethanol. Thus, in ddC peripheral neuropathy inhibitors of three mitochondrial functions – the mitochondrial electron transport chain, oxidative stress and caspase signaling – attenuate mechanical hyperalgesia while in the co-morbidity model the caspase inhibitor was without effect. What underlies this difference in the role of this one mitochondrial mechanism in the two peripheral neuropathies is currently unknown.

In summary, the impact of co-morbid risk factors in peripheral neuropathies is poorly understood, in large part due to lack of model systems in which to evaluate this clinically important problem. We have developed an animal model of co-morbid neuropathic insults for two common neurotoxic exposures in patients with HIV/AIDS, a nucleoside reverse transcriptase inhibitor used to treat HIV/AIDS and ethanol consumption, a common co-morbid factor in patients with HIV/AIDS. This is, to our knowledge, the first model system for studying the mechanism by which co-morbid risk factors induce painful peripheral neuropathy. Based on our studies, we suggest that ethanol consumption enhances, by an indirect mechanism, the mitochondrial-dependence underlying ddC-induced painful peripheral neuropathy. Our ultimate goal is to use this type of study to improve the medical management of painful peripheral neuropathy in patients with HIV/AIDS.

Acknowledgments

This study was funded by the National Institutes of Health (NIH). We thank Dr. Robert Gear for assistance with statistical analysis.

Abbreviations

Ap4A	P ¹ ,P ⁴ -di(adenosine-5') tetraphosphate
AS	antisense
ddC	2',3'-dideoxycytidine
ED	ethanol diet
HAART	highly active anti-retroviral therapy
HIV/AIDS	human immunodeficiency virus/acquired immunodeficiency syndrome
mETC	mitochondrial respiratory complexes
MM	mismatch
NRTIs	nucleoside reverse transcriptase inhibitors
ODN	<u>oligodeoxynucleotide</u>

PKCϵ	protein kinase C epsilon isoform
PKCϵ-I	PKC ϵ specific translocation inhibitor peptide (PKC ϵ_{V1-2})
SEM	<u>standard error of the mean</u>
Z-VAD-FMK	ZVal-Ala-Asp(OMe)-fluoro methyl ester

REFERENCES

- Atkinson JH Jr, Grant I, Kennedy CJ, Richman DD, Spector SA, McCutchan JA. Prevalence of psychiatric disorders among men infected with human immunodeficiency virus. A controlled study. *Arch Gen Psychiatry*. 1988; 45:859–864. [PubMed: 3415427]
- Berger AR, Arezzo JC, Schaumburg HH, Skowron G, Merigan T, Bozzette S, Richman D, Soo W. 2', 3'-dideoxycytidine (ddC) toxic neuropathy: a study of 52 patients. *Neurology*. 1993; 43:358–362. [PubMed: 8382349]
- Bosch EP, Pelham RW, Rasool CG, Chatterjee A, Lash RW, Brown L, Munsat TL, Bradley WG. Animal models of alcoholic neuropathy: morphologic, electrophysiologic, and biochemical findings. *Muscle Nerve*. 1979; 2:133–144. [PubMed: 232540]
- Brailoiu E, Brailoiu GC, Mameli G, Dolei A, Sawaya BE, Dun NJ. Acute exposure to ethanol potentiates human immunodeficiency virus type 1 Tat-induced Ca(2+) overload and neuronal death in cultured rat cortical neurons. *J Neurovirol*. 2006; 12:17–24. [PubMed: 16595370]
- Brinkman K, ter Hofstede HJ, Burger DM, Smeitink JA, Koopmans PP. Adverse effects of reverse transcriptase inhibitors: mitochondrial toxicity as common pathway. *Aids*. 1998; 12:1735–1744. [PubMed: 9792373]
- Brown GR, Rundell JR, McManis SE, Kendall SN, Zachary R, Temoshok L. Prevalence of psychiatric disorders in early stages of HIV infection. *Psychosom Med*. 1992; 54:588–601. [PubMed: 1438661]
- Bruijnzeel AW, Repetto M, Gold MS. Neurobiological mechanisms in addictive and psychiatric disorders. *Psychiatr Clin North*. 2004; 27:661–674.
- Cohen C, Elion RA, Frank I, Kloser P, Sherer R, Squires KE, Steinhart C, Tebas P. Once-daily antiretroviral therapies for HIV infection: Consensus Statement of an Advisory Committee of the International Association of Physicians in AIDS Care. *J Int Assoc Physicians AIDS Care (Chic Ill)*. 2002; 1:141–145. [PubMed: 12942673]
- Cook RL, Sereika SM, Hunt SC, Woodward WC, Erlen JA, Conigliaro J. Problem drinking and medication adherence among persons with HIV infection. *J Gen Intern Med*. 2001; 16:83–88. [PubMed: 11251758]
- Cook RT, Stapleton JT, Ballas ZK, Klinzman D. Effect of a single ethanol exposure on HIV replication in human lymphocytes. *J Investig Med*. 1997; 45:265–271.
- Dalakas MC. Peripheral neuropathy and antiretroviral drugs. *J Peripher Nerv Syst*. 2001; 6:14–20. [PubMed: 11293802]
- Dalakas MC, Semino-Mora C, Leon-Monzon M. Mitochondrial alterations with mitochondrial DNA depletion in the nerves of AIDS patients with peripheral neuropathy induced by 2'3'-dideoxycytidine (ddC). *Lab Invest*. 2001; 81:1537–1544. [PubMed: 11706061]
- Devaud LL, Risinger FO, Selvage D. Impact of the hormonal milieu on the neurobiology of alcohol dependence and withdrawal. *J Gen Psychol*. 2006; 133:337–356. [PubMed: 17128955]
- Dew MA, Becker JT, Sanchez J, Caldararo R, Lopez OL, Wess J, Dorst SK, Banks G. Prevalence and predictors of depressive, anxiety and substance use disorders in HIV-infected and uninfected men: a longitudinal evaluation. *Psychol Med*. 1997; 27:395–409. [PubMed: 9089832]
- Diamond I, Messing RO. Neurologic effects of alcoholism. *West J Med*. 1994; 161:279–287. [PubMed: 7975567]
- Dieterich DT. Long-term complications of nucleoside reverse transcriptase inhibitor therapy. *AIDS Read*. 2003; 13:176–184. 187. [PubMed: 12741368]

- Dina OA, Barletta J, Chen X, Mutero A, Martin A, Messing RO, Levine JD. Key role for the epsilon isoform of protein kinase C in painful alcoholic neuropathy in the rat. *J Neurosci*. 2000; 20:8614–8619. [PubMed: 11069970]
- Dina OA, Khasar SG, Alessandri-Haber N, Bogen O, Chen X, Green PG, Reichling DB, Messing RO, Levine JD. Neurotoxic catecholamine metabolite in nociceptors contributes to painful peripheral neuropathy. *Eur J Neurosci*. 2008; 28:1180–1190. [PubMed: 18783367]
- Dina OA, Messing RO, Levine JD. Ethanol withdrawal induces hyperalgesia mediated by PKCepsilon. *Eur J Neurosci*. 2006; 24:197–204. [PubMed: 16800864]
- Du J, McEwen B, Manji HK. Glucocorticoid receptors modulate mitochondrial function: A novel mechanism for neuroprotection. *Commun Integr Biol*. 2009; 2:350–352. [PubMed: 19721888]
- Du J, Wang Y, Hunter R, Wei Y, Blumenthal R, Falke C, Khairova R, Zhou R, Yuan P, Machado-Vieira R, McEwen BS, Manji HK. Dynamic regulation of mitochondrial function by glucocorticoids. *Proc Natl Acad Sci U S A*. 2009; 106:3543–3548. [PubMed: 19202080]
- Dubinsky RM, Yarchoan R, Dalakas M, Broder S. Reversible axonal neuropathy from the treatment of AIDS and related disorders with 2',3'-dideoxycytidine (ddC). *Muscle Nerve*. 1989; 12:856–860. [PubMed: 2558314]
- Errico AL, King AC, Lavallo WR, Parsons OA. Cortisol dysregulation and cognitive impairment in abstinent male alcoholics. *Alcohol Clin Exp Res*. 2002; 26:1198–1204. [PubMed: 12198394]
- Foster JH, Powell JE, Marshall EJ, Peters TJ. Quality of life in alcohol-dependent subjects--a review. *Qual Life Res*. 1999; 8:255–261. [PubMed: 10472156]
- Fujita C, Ichikawa F, Teratani T, Murakami G, Okada T, Shinohara M, Kawato S, Ohta Y. Direct effects of corticosterone on ATP production by mitochondria from immortalized hypothalamic GT1-7 neurons. *J Steroid Biochem Mol Biol*. 2009; 117:50–55. [PubMed: 19631743]
- Galvan FH, Bing EG, Fleishman JA, London AS, Caetano R, Burnam MA, Longshore D, Morton SC, Orlando M, Shapiro M. The prevalence of alcohol consumption and heavy drinking among people with HIV in the United States: results from the HIV Cost and Services Utilization Study. *J Stud Alcohol*. 2002; 63:179–186. [PubMed: 12033694]
- Gerschenson M, Brinkman K. Mitochondrial dysfunction in AIDS and its treatment. *Mitochondrion*. 2004; 4:763–777. [PubMed: 16120431]
- Giancola ML, Lorenzini P, Balestra P, Larussa D, Baldini F, Corpolongo A, Narciso P, Bellagamba R, Tozzi V, Antinori A. Neuroactive antiretroviral drugs do not influence neurocognitive performance in less advanced HIV-infected patients responding to highly active antiretroviral therapy. *J Acquir Immune Defic Syndr*. 2006; 41:332–337. [PubMed: 16540934]
- Hall CD, Snyder CR, Messenheimer JA, Wilkins JW, Robertson WT, Whaley RA, Robertson KR. Peripheral neuropathy in a cohort of human immunodeficiency virus-infected patients. Incidence and relationship to other nervous system dysfunction. *Arch Neurol*. 1991; 48:1273–1274. [PubMed: 1668978]
- Hulgan T, Haas DW, Haines JL, Ritchie MD, Robbins GK, Shafer RW, Clifford DB, Kallianpur AR, Summar M, Canter JA. Mitochondrial haplogroups and peripheral neuropathy during antiretroviral therapy: an adult AIDS clinical trials group study. *Aids*. 2005; 19:1341–1349. [PubMed: 16103764]
- Hundle B, McMahon T, Dadgar J, Chen CH, Mochly-Rosen D, Messing RO. An inhibitory fragment derived from protein kinase Cepsilon prevents enhancement of nerve growth factor responses by ethanol and phorbol esters. *J Biol Chem*. 1997; 272:15028–15035. [PubMed: 9169479]
- Johnson JA, Gray MO, Chen CH, Mochly-Rosen D. A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. *J Biol Chem*. 1996; 271:24962–24966. [PubMed: 8798776]
- Joseph EK, Chen X, Khasar SG, Levine JD. Novel mechanism of enhanced nociception in a model of AIDS therapy-induced painful peripheral neuropathy in the rat. *Pain*. 2004; 107:147–158. [PubMed: 14715401]
- Joseph EK, Levine JD. Caspase signalling in neuropathic and inflammatory pain in the rat. *Eur J Neurosci*. 2004; 20:2896–2902. [PubMed: 15579143]
- Joseph EK, Levine JD. Mitochondrial electron transport in models of neuropathic and inflammatory pain. *Pain*. 2006; 121:105–114. [PubMed: 16472913]

- Juntunen J, Matikainen E, Nickels J, Ylikahri R, Sarviharju M. Alcoholic neuropathy and hepatopathy in mice. An experimental study. *Acta Pathol Microbiol Immunol Scand A*. 1983; 91:137–144. [PubMed: 6303042]
- Juntunen J, Teravainen H, Eriksson K, Panula P, Larsen A. Experimental alcoholic neuropathy in the rat: histological and electrophysiological study on the myoneural junctions and the peripheral nerves. *Acta Neuropathol*. 1978; 41:131–137. [PubMed: 205081]
- Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, Green PG, Hodge CW, Levine JD, Messing RO. A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. *Neuron*. 1999; 24:253–260. [PubMed: 10677042]
- Khasar SG, Wang JF, Taiwo YO, Heller PH, Green PG, Levine JD. Muopioid agonist enhancement of prostaglandin-induced hyperalgesia in the rat: a G-protein beta gamma subunit-mediated effect? *Neuroscience*. 1995; 67:189–195. [PubMed: 7477899]
- Kieburts K, Simpson D, Yiannoutsos C, Max MB, Hall CD, Ellis RJ, Marra CM, McKendall R, Singer E, Dal Pan GJ, Clifford DB, Tucker T, Cohen B. A randomized trial of amitriptyline and mexiletine for painful neuropathy in HIV infection. AIDS Clinical Trial Group 242 Protocol Team. *Neurology*. 1998; 51:1682–1688. [PubMed: 9855523]
- Koob GF. Stress, corticotropin-releasing factor, and drug addiction. *Ann N Y Acad Sci*. 1999; 897:27–45. [PubMed: 10676433]
- Koob GF. Alcoholism: allostasis and beyond. *Alcohol Clin Exp Res*. 2003; 27:232–243. [PubMed: 12605072]
- Koob GF. The neurobiology of addiction: a neuroadaptational view relevant for diagnosis. *Addiction*. 2006; 101(Suppl 1):23–30. [PubMed: 16930158]
- Lamon BC, Alonzo A. Stress among males recovering from substance abuse. *Addict Behav*. 1997; 22:195–205. [PubMed: 9113214]
- Lefevre F, O'Leary B, Moran M, Mossar M, Yarnold PR, Martin GJ, Glassroth J. Alcohol consumption among HIV-infected patients. *J Gen Intern Med*. 1995; 10:458–460. [PubMed: 7472704]
- Lieber CS, DeCarli LM. The feeding of alcohol in liquid diets: two decades of applications and 1982 update. *Alcohol Clin Exp Res*. 1982; 6:523–531. [PubMed: 6758624]
- Lieber CS, DeCarli LM. Liquid diet technique of ethanol administration: 1989 update. *Alcohol Alcohol*. 1989; 24:197–211. [PubMed: 2667528]
- Lieber CS, DeCarli LM, Sorrell MF. Experimental methods of ethanol administration. *Hepatology*. 1989; 10:501–510. [PubMed: 2673971]
- Linnoila M, Mefford I, Nutt D, Adinoff B. NIH conference. Alcohol withdrawal and noradrenergic function. *Ann Intern Med*. 1987; 107:875–889. [PubMed: 2825572]
- Liu X, Zha J, Nishitani J, Chen H, Zack JA. HIV-1 infection in peripheral blood lymphocytes (PBLs) exposed to alcohol. *Virology*. 2003; 307:37–44. [PubMed: 12667812]
- Lopez OL, Becker JT, Dew MA, Caldararo R. Risk modifiers for peripheral sensory neuropathy in HIV infection/AIDS. *Eur J Neurol*. 2004; 11:97–102. [PubMed: 14748769]
- Massarotti M. Ganglioside treatment of alcoholic neuropathies: experimental and clinical aspects. *Pharmacol Biochem Behav*. 1983; 18(Suppl 1):51–54. [PubMed: 6314385]
- McLane JA. Decreased axonal transport in rat nerve following acute and chronic ethanol exposure. *Alcohol*. 1987; 4:385–389. [PubMed: 2445363]
- McLane JA. Retrograde axonal transport in chronic ethanol-fed and thiamine-deficient rats. *Alcohol*. 1990; 7:103–106. [PubMed: 1691645]
- Miguez MJ, Shor-Posner G, Morales G, Rodriguez A, Burbano X. HIV treatment in drug abusers: impact of alcohol use. *Addict Biol*. 2003; 8:33–37. [PubMed: 12745413]
- Moore RD, Wong WM, Keruly JC, McArthur JC. Incidence of neuropathy in HIV-infected patients on monotherapy versus those on combination therapy with didanosine, stavudine and hydroxyurea. *Aids*. 2000; 14:273–278. [PubMed: 10716503]
- Moyle GJ, Sadler M. Peripheral neuropathy with nucleoside antiretrovirals: risk factors, incidence and management. *Drug Saf*. 1998; 19:481–494. [PubMed: 9880091]

- Nath A, Hauser KF, Wojna V, Booze RM, Maragos W, Prendergast M, Cass W, Turchan JT. Molecular basis for interactions of HIV and drugs of abuse. *J Acquir Immune Defic Syndr*. 2002; 31(Suppl 2):S62–69. [PubMed: 12394784]
- Nicholas PK, Kemppainen JK, Canaval GE, Corless IB, Sefcik EF, Nokes KM, Bain CA, Kirksey KM, Eller LS, Dole PJ, Hamilton MJ, Coleman CL, Holzemer WL, Reynolds NR, Portillo CJ, Bunch EH, Wantland DJ, Voss J, Phillips R, Tsai YF, Mendez MR, Lindgren TG, Davis SM, Gallagher DM. Symptom management and self-care for peripheral neuropathy in HIV/AIDS. *AIDS Care*. 2007; 19:179–189. [PubMed: 17364396]
- Oakes SG, Pozos RS. Electrophysiologic effects of acute ethanol exposure. I. Alterations in the action potentials of dorsal root ganglia neurons in dissociated culture. *Brain Res*. 1982; 281:243–249. [PubMed: 7150977]
- Oh SB, Tran PB, Gillard SE, Hurley RW, Hammond DL, Miller RJ. Chemokines and glycoprotein120 produce pain hypersensitivity by directly exciting primary nociceptive neurons. *J Neurosci*. 2001; 21:5027–5035. [PubMed: 11438578]
- Papir-Kricheli D, Frey J, Laufer R, Gilon C, Chorev M, Selinger Z, Devor M. Behavioural effects of receptor-specific substance P agonists. *Pain*. 1987; 31:263–276. [PubMed: 2448728]
- Parada CA, Yeh JJ, Joseph EK, Levine JD. Tumor necrosis factor receptor type-1 in sensory neurons contributes to induction of chronic enhancement of inflammatory hyperalgesia in rat. *Eur J Neurosci*. 2003b; 17:1847–1852. [PubMed: 12752784]
- Parada CA, Yeh JJ, Reichling DB, Levine JD. Transient attenuation of protein kinase Cepsilon can terminate a chronic hyperalgesic state in the rat. *Neuroscience*. 2003a; 120:219–226. [PubMed: 12849754]
- Pardo CA, McArthur JC, Griffin JW. HIV neuropathy: insights in the pathology of HIV peripheral nerve disease. *J Peripher Nerv Syst*. 2001; 6:21–27. [PubMed: 11293804]
- Poage ED, Ketzenberger KE, Olson J. Spirituality, contentment, and stress in recovering alcoholics. *Addict Behav*. 2004; 29:1857–1862. [PubMed: 15530729]
- Quasthoff S, Hartung HP. Chemotherapy-induced peripheral neuropathy. *J Neurol*. 2002; 249:9–17. [PubMed: 11954874]
- Randall LO, Selitto JJ. A method for measurement of analgesic activity on inflamed tissue. *Arch Int Pharmacodyn Ther*. 1957; 111:409–419. [PubMed: 13471093]
- Rasmussen DD, Wilkinson CW, Raskind MA. Chronic daily ethanol and withdrawal: 6. Effects on rat sympathoadrenal activity during “abstinence”. *Alcohol*. 2006; 38:173–177. [PubMed: 16905443]
- Reliquet V, Mussini JM, Chennebault JM, Lafeuillade A, Raffi F. Peripheral neuropathy during stavudine-didanosine antiretroviral therapy. *HIV Med*. 2001; 2:92–96. [PubMed: 11737385]
- Riopelle RJ, Dow KE, Eisenhauer E. Some observations on vinca alkaloid and ethanol neurotoxicity using dissociated neuronal cultures. *Can J Physiol Pharmacol*. 1984; 62:1032–1036. [PubMed: 6488080]
- Rosenberger PH, Bornstein RA, Nasrallah HA, Para MF, Whitaker CC, Fass RJ, Rice RR Jr. Psychopathology in human immunodeficiency virus infection: lifetime and current assessment. *Compr Psychiatry*. 1993; 34:150–158. [PubMed: 8339532]
- Sacktor N. The epidemiology of human immunodeficiency virus-associated neurological disease in the era of highly active antiretroviral therapy. *J Neurovirol*. 2002; 8(Suppl 2):115–121. [PubMed: 12491162]
- Samet JH, Phillips SJ, Horton NJ, Traphagen ET, Freedberg KA. Detecting alcohol problems in HIV-infected patients: use of the CAGE questionnaire. *AIDS Res Hum Retroviruses*. 2004; 20:151–155. [PubMed: 15018702]
- Scott B, Petit TL, Lew J. Differential survival of fetal and adult neurons and non-neuronal cells exposed chronically to ethanol in cell culture. *Neurotoxicology*. 1986; 7:81–90. [PubMed: 3714128]
- Scott BS, Edwards BA. Electric membrane properties of adult mouse DRG neurons and the effect of culture duration. *J Neurobiol*. 1980; 11:291–301. [PubMed: 7391831]
- Sharma PL, Nurpeisov V, Hernandez-Santiago B, Beltran T, Schinazi RF. Nucleoside inhibitors of human immunodeficiency virus type 1 reverse transcriptase. *Curr Top Med Chem*. 2004; 4:895–919. [PubMed: 15134548]

- Simpson DM. Selected peripheral neuropathies associated with human immunodeficiency virus infection and antiretroviral therapy. *J Neurovirol.* 2002; 8(Suppl 2):33–41. [PubMed: 12491149]
- Simpson DM, Tagliati M. Nucleoside analogue-associated peripheral neuropathy in human immunodeficiency virus infection. *J Acquir Immune Defic Syndr Hum Retrovirol.* 1995; 9:153–161. [PubMed: 7749792]
- Sofuoglu M, Nelson D, Babb DA, Hatsukami DK. Intravenous cocaine increases plasma epinephrine and norepinephrine in humans. *Pharmacol Biochem Behav.* 2001; 68:455–459. [PubMed: 11325399]
- Taiwo YO,Coderre TJ, Levine JD. The contribution of training to sensitivity in the nociceptive paw-withdrawal test. *Brain Res.* 1989; 487:148–151. [PubMed: 2752281]
- Taiwo YO, Levine JD. Contribution of guanine nucleotide regulatory proteins to prostaglandin hyperalgesia in the rat. *Brain Res.* 1989; 492:400–403. [PubMed: 2502301]
- Tsapis A, Kepes A. Transient breakdown of the permeability barrier of the membrane of *Escherichia coli* upon hypoosmotic shock. *Biochim Biophys Acta.* 1977; 469:1–12. [PubMed: 329877]
- West LK, Huang L. Transient permeabilization induced osmotically in membrane vesicles from Torpedo electroplax: a mild procedure for trapping small molecules. *Biochemistry.* 1980; 19:4418–4423. [PubMed: 6996718]
- Widdicombe JH, Azizi F, Kang T, Pittet JF. Transient permeabilization of airway epithelium by mucosal water. *J Appl Physiol.* 1996; 81:491–499. [PubMed: 8828701]
- Wu JV, Kendig JJ. Differential sensitivities of TTX-resistant and TTX-sensitive sodium channels to anesthetic concentrations of ethanol in rat sensory neurons. *J Neurosci Res.* 1998; 54:433–443. [PubMed: 9822154]
- Zenilman JM, Hook EW 3rd, Shepherd M, Smith P, Rompalo AM, Celentano DD. Alcohol and other substance use in STD clinic patients: relationships with STDs and prevalent HIV infection. *Sex Transm Dis.* 1994; 21:220–225. [PubMed: 7974074]

Figure 1. Mechanical hyperalgesia induced by the combination of ddC and ethanol diet (ED)
 Animals were fed ethanol (■; △) or control (▼; ◇) diet for four days. Low dose of ddC (5 mg/kg; i.v.; ■; ▼) or vehicle (△; ◇) was injected on the fourth day. Rats treated with ddC (▼) or ED (△) alone did not show significant changes in mechanical nociceptive threshold. However, ED rats that received a single injection of ddC (■) showed rapid onset mechanical hyperalgesia that was still present, without attenuation, on the 8th day. The three-way repeated measures ANOVA showed a significant time × diet × drug group interaction ($F_{4,80}=2.509$, $p=0.048$). The follow-up 2-way repeated measures ANOVA comparing ddC with vehicle in animals with ED showed a significant drug × time interaction ($F_{4,40}=4.496$; $p<0.001$), as well as a significant main effect of drug ($F_{1,10}=14.016$; $p=0.004$). The follow-up 2-way repeated measures ANOVA comparing ddC with vehicle in animals with the control diet showed no significant interactions of main effects. $N=6$ paws for all groups.

Figure 2. Antagonism of hyperalgesia induced by ddC, ethanol diet (ED) or the combination of ddC and ED

The effect of the non-specific caspase inhibitor Z-VAD-FMK (ZVAD, 5μg/5μl), the mitochondrial electron transport complex (mETC) selective inhibitors rotenone (complex I, 5μg/5μl) and oligomycin (complex V, 5μg/5μl), the antioxidant α-lipoic acid (5μg/5μl), the ATP-dependent mechanisms antagonist (Ap4A, 5μg/5μl) or the PKCε-specific translocation inhibitor peptide (PKCε-I, 1μg/5μl) on mechanical hyperalgesia was tested in the three experimental models. All inhibitors were injected intradermally into the hind paw at the site of nociceptive testing and the mechanical withdrawal threshold evaluated 30 minutes after their injection. (A) Rats were treated with a single intravenous injection of ddC (50 mg/kg). The inhibitors were tested five days later. The one-way ANOVA was significant ($F_{6,35}=20.122$; $p<0.001$). Scheffé post-hoc test showed that all groups except PKCε-I were significantly different from the vehicle control group (all $*p<0.001$); (B) Rats were fed ED during three weeks in a regimen of 4 days with ED/3 days normal diet. The inhibitors were tested at the end of the third week. The one-way ANOVA was significant ($F_{6,35}=11.024$; $p<0.001$). Scheffé post-hocs showed that only the $C_{\epsilon} \sim I$ group was significantly different from the vehicle control group ($*p<0.001$); (C) Rats were fed ED for four days and, on the fourth day a low dose of ddC (5 mg/kg; i.v.) was administered. The inhibitors were tested 24 hours later. The one-way ANOVA was significant ($F_{6,35}=30.772$; $p<0.001$). Scheffé post-hocs showed that the vehicle control was significantly different from all groups ($*p<0.001$) except the ZVAD and the $C_{\epsilon} \sim I$ groups ($p=0.709$ and $p=0.612$, respectively). Paw withdrawal threshold was evaluated by the Randall-Selitto paw withdrawal test. All groups N=6.

Figure 3. PKC ϵ independence of hyperalgesia induced by the combination of ddC and ED

Treatment with ODN antisense for PKC ϵ mRNA (AS) or mismatch (MM), started 3 days before ethanol diet (ED) and continued until the last day of ED (4th day). ddC was intravenously injected into the tail on the last day of ED; the hind paw mechanical withdrawal threshold was evaluated 24 hours later. Control experiment (two right bars) was performed in rats submitted to ED for 2 weeks (4 days with ED/3 days normal diet) and treated with AS for PKC ϵ mRNA or MM for 3 days before the evaluation for the presence of hyperalgesia. Hind paw mechanical withdrawal threshold was evaluated by the Randall Selitto paw withdrawal test. Two-way ANOVA demonstrated a significant interaction ($F_{1,20}=12.431$; $p=0.002$). In order to determine the basis of this interaction the responses to the AS and MM treatments were compared separately for the ED+ddC group and for the control (ED, 2 weeks) group. For the control group, the AS treatment differed significantly from the MM treatment ($F_{1,10}=34.967$; $*p<0.001$), but for the ED+ddC group, the AS and MM treatments did not differ significantly ($F_{1,10}=1.687$; $p=0.223$). $N=6$ paws for all groups.

Figure 4. Interruption of ethanol diet (ED) does not reverse low-dose-ddC-induced mechanical hyperalgesia

Animals were submitted to ED for one (panel A) or two (panel B) weeks, in a regimen of 4 days with ED/3 days normal diet. Single low dose of ddC (5 mg/kg; ■) or vehicle (o) was injected intravenously into the tail four days after ED was begun. Twenty-four hours later, the ED+ddC group showed decreased hind paw mechanical threshold. ED was interrupted in different time points (after one or two weeks) and, the mechanical hyperalgesia, evaluated 1, 3, 4, 5, 8, 9, 12, 15, 16 and 24 days after the first day of ED. Two repeated measures ANOVAs demonstrated that the groups that received ddC (■) were significantly different from the groups that received vehicle (o) in both panels: time $\times$ treatment interaction was (Panel A, $F_{9,90}=8.906$; $p<0.001$; Panel B, $F_{9,90}=5.304$; $p<0.001$), main effect of group was (Panel A, $F_{1,10}=18.810$; $p=0.001$; Panel B, $F_{1,10}=19.054$; $p=0.001$). $N=6$ paws for all groups.