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Independent Contributions of Alcohol and Stress Axis Hormones to Painful Peripheral Neuropathy

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Abstract

Painful small-fiber peripheral neuropathy is a debilitating complication of chronic alcohol abuse. Evidence from previous studies suggests that neuroendocrine mechanisms, in combination with other, as yet unidentified actions of alcohol, are required to produce this neuropathic pain syndrome. In addition to neurotoxic effects of alcohol, in the setting of alcohol abuse neuroendocrine stress axes release glucocorticoids and catecholamines. Since receptors for these stress hormones are located on nociceptors, at which they can act to cause neuronal dysfunction, we tested the hypothesis that alcohol and stress hormones act on the nociceptor, independently, to produce neuropathic pain. We used a rat model, which allows the distinction of the effects of alcohol from those produced by neuroendocrine stress axis mediators. We now demonstrate that topical application of alcohol and exposure to unpredictable sound stress, each alone, has no effect on nociceptive threshold. However, when animals that had previous exposure to alcohol were subsequently exposed to stress, they rapidly developed mechanical hyperalgesia. Conversely, sound stress followed by topical alcohol exposure also produced mechanical hyperalgesia. The contribution of stress hormones was prevented by spinal intrathecal administration of oligodeoxynucleotides antisense to β_2-adrenergic or glucocorticoid receptor mRNA, which attenuates receptor level in nociceptors, as well as by adrenal medullectomy. These experiments establish an independent role of alcohol and stress hormones on the primary afferent nociceptor in the induction of painful peripheral neuropathy.

Keywords

hyperalgesia; nociceptor; painfu peripheral neuropathy; alcohol neurotixicity; stress hormones

Small-fiber painful peripheral neuropathy is a devastating complication of alcohol abuse (Said, 1980, Koike et al., 2001, Koike et al., 2003, Zambelis et al., 2005). Although symptomatic therapy may provide some relief, in the majority of patients, response to treatment is inadequate. Development of successful therapies has been hampered by our lack of understanding of the cellular mechanisms underlying pathogenic effects of alcohol on the function of sensory neurons.

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While there is evidence that complications of alcohol abuse, including painful peripheral neuropathy, are due, at least in part, to activation of neuroendocrine stress axes (Adinoff et al., 1998, Errico et al., 2002, Gianoulakis et al., 2003, Thayer et al., 2006, Walter et al., 2006, Dina et al., 2008b) it has been difficult to parse out the individual contribution of alcohol and the stress axis mediators (i.e., catecholamines and glucocorticoids) to the pain reported in patients with alcoholic neuropathy.

We have previously established a model of painful peripheral neuropathy induced by "binge" alcohol consumption in the rat (Dina et al., 2006). This model is characterized by mechanical and thermal hyperalgesia, as well as hyperexcitability of primary afferent nociceptors (Dina et al., 2006, Chen and Levine, 2007). In addition, we also observed a significant contribution of the two major neuroendocrine axes in our model (Dina et al., 2008a, Dina et al., 2008b), which is in line with reports showing the dramatic role of sustained release of glucocorticoids and catecholamines triggered by alcohol consumption in the setting of binge drinking (Adinoff et al., 1998, Cerezo et al., 2002, Errico et al., 2002, Gianoulakis et al., 2003, Kiefer et al., 2006, Thayer et al., 2006, Walter et al., 2006), increased activity in the sympathetic nervous system implicated in some forms of neuropathic pain (Raja et al., 1995, Tracey et al., 1995, Hassantash et al., 2003, Singh et al., 2003), and exacerbation of pain by glucocorticoids in some animal models of peripheral neuropathy (Takasaki et al., 2005, Wang et al., 2006). The presence of adrenergic and glucocorticoid receptors on sensory neurons (Smith et al., 1991, Bowles et al., 2003, Hucho et al., 2006) coupled with the persistent increased plasma concentrations of glucocorticoids and catecholamines in alcoholics, which is further enhanced during alcohol withdrawal (Cerezo et al., 2002, Errico et al., 2002, Gianoulakis et al., 2003, Kiefer et al., 2006, Thayer et al., 2006, Walter et al., 2006), led us to evaluate the role of these hormones in alcohol-induced neuropathic pain. In the present study, to assess the action of alcohol on the primary afferent nociceptor, independent of its effect on the neuroendocrine stress axes, a model of painful peripheral neuropathy, developed by topical local application of a neurotoxic agent to sensory innervation of the skin (Levine et al., 1986) was employed. We report that alcohol and stress play independent roles in the induction of painful peripheral neuropathy; while neither alone was able to reproduce the alcohol-induced painful neuropathy observed in the binge-drinking rat (Dina et al., 2006), it took both together to produce this pain syndrome.

2. Experimental Procedures

2.1. Animals

Experiments were performed on adult male Sprague Dawley rats (200–250 g; Charles River, Hollister, CA, USA). Animals were housed three per cage, under a 12-h light/dark cycle, in a temperature and humidity controlled environment. Food and water were available *ad libitum*. All behavioral nociceptive testing was performed between 10:00 am and 4:00 pm. Rats were acclimatized to the experimental area and behavioral procedures prior to the experiment. To acclimatize rats to the testing environment, they were brought to the experimental area, in their home cages, and left in their cages for 15–30 min, after which they were placed in a restrainer (cylindrical transparent acrylic tubes that have openings on their sides, to allow extension of the hind legs from the restrainer, for nociceptive testing). Rats were left in the restrainer for another 15–30 min before nociceptive testing was started. Baseline mechanical nociceptive threshold, determined prior to experiments, was defined as the mean of three readings taken at 5-min intervals. All experimental protocols were approved by the UCSF Committee on Animal Research and conformed to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

2.2. Nociceptive testing

The nociceptive flexion reflex was quantified with an Ugo Basile Analgesymeter® (Stoelting, Chicago, IL), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw. No ciceptive threshold was defined as the force, in grams, at which the rat withdrew its hind paw. Hyperalgesia was defined as a decrease in mechanical nociceptive threshold, here presented as percent change from baseline. This acclimatization procedure consistently results in baseline paw withdrawal thresholds of 117.6 ± 4.8 g (N=96 paws) for the body weight range for the rats used in this study. Each paw was treated as an independent measure; both paws of the same rat received the same treatment. Each experiment was performed on separate groups of rats.

2.3. Reagents

In this study, for topical alcohol applications, we used ethyl alcohol (proof 190, Gold Shield Chemical Company, Hayward, CA, USA).

2.4. Experimental protocols

2.4.1. Topical alcohol protocol—Topical treatment with alcohol was performed daily on the dorsum of the hind paw for 4 consecutive days. A small alcohol-soaked cotton ball (80 μl) was put in contact with the skin and held in place with a plastic cover in order to limit evaporation. Each paw received three 90 s applications of alcohol or control (distilled water), with 30 s inter-application intervals, during which the soaked cotton balls were replaced.

2.4.2. Sound stress protocol—Chronic unpredictable sound stress was produced as described previously and used in our laboratory (Strausbaugh et al., 2003, Khasar et al., 2005). Groups of three animals were placed 25 cm from a speaker in a 12 × 15 × 9.5 inch wire mesh cage inside a 22 × 22 × 28 inch sound-insulated box. Sound pulses were emitted as pure tones at three frequencies (11, 15, and 19 kHz); amplitudes varied from 20 to 110 dB independently for each frequency. The sound exposure protocol was initiated immediately after placing rats in a wire mesh cage and terminated 30 min later, when rats were returned to their home cages. Over the 30 min period, a 5 or 10 s tone was presented every minute at random times during the minute. This sound stress protocol was performed on days 1, 3, and 4, and nociceptive tests were performed at 24 h intervals after the exposure to this stressor. Of note, this sound stress protocol does not produce changes in nociceptive threshold (Khasar et al., 2005, Khasar et al., 2009).

2.4.3. Interaction between topical alcohol and stress—To evaluate the effects of the interaction between topical application of alcohol and exposure to unpredictable sound stress on the cutaneous mechanical threshold, we used different combinations of both protocols described above. In the experiments shown in figures 2, 4, and 5A, alcohol was applied on skin for 4 consecutive days and, on the 5th day, the sound stress protocol was started. In experiments 3 and 5B, animals were submitted to sound stress protocol (3 sessions over 4 days) and, 24 h after the last session, alcohol applications were performed (for 4 days).

2.5. Antisense to the β_2-adrenergic and glucocorticoid receptor

β_2-adrenergic receptor—To investigate whether epinephrine acting at β_2-adrenergic receptors on sensory neurons plays a role in the hyperalgesia induced by the interaction between topical alcohol and stress, β_2-adrenergic receptor oligodeoxynucleotide (ODN)-antisense (AS) was used (Dina et al., 2008b). The ODN-AS sequence, 5′-AAAGGCAGAAGGATG TGC-3′ (Invitrogen Life Technologies, Carlsbad, CA, USA),

was directed against a unique region of the rat β_2-adrenergic receptor sequence (GeneBank accession number NM_012492). The ODN-mismatch (MM) sequence 5′-**AAAGGCAGAAGGATGTGC**-3′ was designed by mismatching six bases (denoted by bold typeface) of the AS sequence.

Glucocorticoid receptor—The role of glucocorticoids in the same experimental model of hyperalgesia was also investigated by using ODN-AS against glucocorticoid receptor mRNA (Engelmann et al., 1998, Wang et al., 2004, Wang et al., 2006, Dina et al., 2008b). The sequence 5′-TGGAGTCCATTGGCAAAT-3′ was used to decrease the expression of glucocorticoid receptors in the rat sensory neuron (Engelmann et al., 1998, Wang et al., 2004, Wang et al., 2006) and, as control, an ODN-MM of the same sequence but with five bases switched (shown in the bold typeface: 5′-TGAAGTTCAGTGTCAAAT-3′) was used.

2.5.1. Preparing and injecting ODNs—The β_2-adrenergic receptor or glucocorticoid receptor ODNs-As or MM, reconstituted in nuclease-free 0.9% Nacl, was administered intrathecally at a dose of 2 μg/μ1 in a volume of 20 μ1 for 8 consecutive days, starting 3 days before the sound stress protocol started and continued until the last sound stress session. As described previously (Alessandri-Haber et al., 2003), rats were anesthetized with isoflurane (2.5% in O_2), and the ODN injected using a microsyringe with a 30-gauge needle, inserted into the subarachnoid space, between the L_4 and L_5 vertebrae. In the experiment in which both receptors were knocked-down, each AS or MM was administered separately in different periods of the day, in order to avoid a possible interaction between the ODNs.

2.6. Adrenal medullectomy

In order to evaluate the contribution of the sympathoadrenal axis in our model of hyperalgesia induced by the interaction of topical alcohol and stress, adrenomedullectomy (ADMX) was performed 5 weeks before the topical applications (or the sound stress protocol, depending on the experiment) started. To enucleate the adrenal gland (i.e., removing the adrenal medulla), under isoflurane anesthesia (2.5% isoflurane in O_2), the adrenal gland was located through an incision in the lateral abdominal wall. Then the adrenal gland was exposed and the encapsulated adrenal medulla enucleated (Wilkinson et al., 1981, Khasar et al., 1998, Khasar et al., 2005, Dina et al., 2008b). Rats then received drinking water (*ad libitum*) containing 0.5% sodium chloride for the frist week after surgery. The long postsurgical period was employed to allow hypothalamic-pictuitary-adrenal axis function to recover.

2.7. Statistics

The dependent variable in the cutaneous threshold experiments was change in withdrawal threshold on the dorsum of the hind paw, represented as percentage change from the pre-treatment baseline threshold, or as actual mechanical threshold in grams. Group data are represented as mean ± SEM. Statistical significance was determined by one or two-way repeated measures ANOVA or by Student's t-test (as noted in the result section or figure legend). Importantly, paw withdrawal threshold values observed before and after the topical applications of alcohol (i.e., after 4 days) were not significantly different: average paw withdrawal threshold before and after topical alcohol: 117.4 ± 0.5 g and 118.8 ± 0.6g, respectively (paired Student's t-test, p=0.1313, N=72); in the same way, the sound stress protocol also did not induce significant changes in the mechanical threshold in hind paws (average paw withdrawal thresholds before and after sound stress sessions: 118.1 ± 0.9 g and 118.2 ± 0.8 g, respectively (paired p Student's t-test, p=0.9489, N=24)). ODN-AS treatments did not induce significant changes in the mechanical nociceptive threshold (data not shown).

3. Results

3.1. Effect of local application of alcohol

Since alcohol can be absorbed through the skin following topical application (Beskitt and Sun, 1997), to determine if its effect alone on the primary afferent nociceptor is sufficient to induce pain, we employed a method that we have previously developed to establish that other neurotoxins can induce painful peripheral neuropathy (Levine et al., 1986), i.e., topical application under an occlusive dressing, soaked with alcohol. In contrast to exposure to other neurotoxic substances (Levine et al., 1986), in this experiment we observed that the topical application of alcohol, for up to 5 min, daily for 4 days, failed to elicit sensitization to mechanical stimulation (Fig. 1, panel A).

3.2. Effect of sound stress

We have previously shown that sound stress, which can enhance inflammatory mediator-induced hyperalgesia, alone does not produce a change in cutaneous mechanical nociceptive threshold (Khasar et al., 2005, Khasar et al., 2009). In the present experiments, we confirmed that sound stress alone failed to produce cutaneous mechanical hyperalgesia (Fig. 1, panel B).

3.3. Effect of alcohol followed by sound stress

To test the hypothesis that combined topical alcohol exposure and sound stress are required to produce mechanical hyperalgesia, rats treated with alcohol were then exposed to sound stress. While neither alone produced hyperalgesia (Fig. 1), even after the first exposure to the sound stress we observed decrease in the mechanical threshold in the paws previously treated with topical alcohol. Moreover, subsequent exposures to sound stress significantly increased the intensity of the hyperalgesia (Fig. 2).

3.4. Effect of sound stress followed by alcohol

Conversely, when animals that had been exposed to sound stress were administered alcohol, topically, they also developed mechanical hyperalgesia, which increased after each additional application of alcohol (Fig. 3).

3.5. Effect of antisense to the β_2-adrenergic and glucocorticoid receptors

To determine if the mediators of both stress axes – sympathoadrenal and hypothalamic-pituitary-adrenal – contribute to the hyperalgesia induced by the combined exposure to topical alcohol and sound stress, and whether or not the action of the stress hormones are at their receptors on the primary afferent nociceptor, rats were administered, intrathecally, ODN-AS to either the β_2-adrenergic (Fig. 4, panel A) or glucocorticoid (Fig. 4, panel B) receptor mRNA. Compared to rats treated with ODN-MM to the β_2-adrenergic receptor or glucocorticoid receptor mRNA, the AS to either receptor markedly attenuated the hyperalgesia induced by alcohol followed by sound stress (Fig. 4, panels A and B). In addition, the co-administration of ODN-AS to both receptors completely eliminated the development of mechanical hyperalgesia (Fig. 4, panel C).

3.6. Adrenal medullectomy

Since adrenal medullectomy prevents painful peripheral neuropathy induced by oral alcohol consumption (Dina et al., 2008b), we next determined if the source of the catecholamines involved in the contribution of sound stress to alcohol-induced hyperalgesia is the adrenal medulla. In this experiment we investigated if adrenal medullectomy would also prevent the effect of sound stress in rats that had been treated with topical alcohal. We report that adernal medullectomy markedly reduced the hyperalgesia induced by sound stress in rats

pretreated with alcohal, topicallly (Fig. 5, panel A). Moreover, adernal medullectomy also prevented the hyperalgesia induced by topical application of alcohal in rats previously submitted to sound stress(Fig. 5, panel B).

4. Discussion

It is well established that stress exacerbates the effects of alcohol (Liu and Weiss, 2002, Morrow et al., 2009, Prendergast and Mulholland, 2012, Vendruscolo et al., 2012), including those on the peripheral nervous system (Dina et al., 2008a, Dina et al., 2008b). In studies of a model of alcohol-induced painful peripheral neuropathy we have previously shown that adrenal medullectomy can completely prevent and reverse mechanical hyperalgesia produced by binge drinking (Dina et al., 2008b). We interpreted this finding to suggest that stress hormones are permissive for a neurotoxic effect of alcohol on the peripheral nervous system, to produce a painful peripheral neuropathy. In the present set of experiments we further tested this hypothesis by administering alcohol in the absence of unpredictable sound stress, a form of psychological stress that alone does not affect nociceptive threshold (Khasar et al., 2005, Khasar et al., 2009), and the two together. As hypothesized, while the isolated exposure to either topical alcohol or stress alone had no effect on nociceptive threshold, their combined administration produced robust mechanical hyperalgesia. Importantly, this study provides evidence for the *independent* effects of alcohol and neuroendocrine stress axis mediators to produce a painful peripheral neuropathy, a more direct test than our previous observation that adrenal medullectomy eliminates painful peripheral neuropathy induced in a model of binge drinking (Dina et al., 2008b).

The present experiments also provide evidence that the mechanism of action of the stress axis hormones is at their cognate receptors, the β_2-adrenergic receptor for catecholamines, and the glucocorticoid receptor for glucocorticoids (Smith et al., 1991, Bowles et al., 2003, Hucho et al., 2006), on the primary afferent nociceptor. While the mechanism by which alcohol contributes to painful peripheral neuropathy remains to be established, ethanol has been shown to have several effects on neuronal function (Diamond and Messing, 1994, Monforte et al., 1995, Ortiz-Plata et al., 1998). Amongst these, are effects mediated by protein kinase C (PKC) (Coe et al., 1996, Pandey, 1996, Gerstin et al., 1998), in particular the calcium independent, novel isoform, PKCε (Gordon et al., 1997, Dina et al., 2000). Importantly, in this regard, we have shown that the mechanical hyperalgesia in alcohol-induced painful peripheral neuropathy in the rat is PKCε mediated (Gordon et al., 1997, Dina et al., 2000, Dina et al., 2006). Given the model developed in the present experiments, it should be possible to determine if PKCε mediates the alcohol and/or stress hormone contribution to alcohol-induced painful peripheral neuropathy.

Two established cellular effects of alcohol that might contribute to the interaction between alcohol and stress hormones to produce painful neuropathy are: 1) enhancing oxidative stress (Nordmann et al., 1987, Rouach et al., 1987, Rouach et al., 1997, Sun et al., 2001, Chen et al., 2008, Crews and Nixon, 2009, Liu et al., 2009, Luo, 2009) and other mitochondrial functions that interact with mitochondrial bioenergetics (Marin-Garcia et al., 1995, Manfredi and Beal, 2000, Ramachandran et al., 2001, de la Monte and Wands, 2002, Ravagnan et al., 2002, Jaatinen et al., 2003, Jaatinen and Rintala, 2008), and 2) neuroplastic changes in the primary afferent nociceptor, produced by binge alcohol consumption (Weise et al., 1985, Yokoyama et al., 1991, Spahn et al., 1995, Dina et al., 2000, Dina et al., 2006) which can create a catecholaminergic phenotype in nociceptors that could provide a positive feedback loop contributing to alcohol induced painful peripheral neuropathy (Dina et al., 2008a, Dina et al., 2008b). Additional studies will be required to elucidate whether these effects of alcohol on neuronal function, primarily established on central nervous system

neurons, or as yet to be described effects of alcohol, mediates the interaction between stress hormones and alcohol to produce painful peripheral neuropathy.

A caveat with respect to our finding that alcohol and stress alone do not produce painful peripheral neuropathy is that we might have been using a sub neuropathic exposure to alcohol and stress hormones such that the combination reached a threshold level, able to produce a model of a painful peripheral neuropathy. However, while some forms of stress (e.g., water avoidance stress) can alone produce a decrease in nociceptive threshold (Green et al., 2011), we have not found that sound stress can produce mechanical hyperalgesia (Khasar et al., 2005, Khasar et al., 2009), as well as longer topical exposures to alcohol, which, alone, also has no effect on nociceptive threshold (data not shown). Thus, we propose that alcohol exposure requires additional, independent, exposure to stress hormones released from the neuroendocrine stress axes, to produce alcoholic painful peripheral neuropathy.

An alternative approach going forward would be to attempt to induce alcoholic painful peripheral neuropathy *in vitro*, by addition of alcohol and stress axis hormones to cultures of dorsal root ganglion neurons. While in vitro studies of the effect of alcohol (Chandler et al., 1993a, Chandler et al., 1993b, Maldve et al., 2004, Mameli et al., 2005, Kelm et al., 2007, 2008, Mameli et al., 2008, Theile et al., 2009, McCool, 2011), and neuroendocrine stress axis hormones (Schmidt et al., 2001, Dai et al., 2004, Brunton et al., 2007, Duvarci and Pare, 2007, Daftary et al., 2009, Chen et al., 2010, Hu et al., 2010, Stranahan et al., 2010, Yuen et al., 2011) on neuronal function have been performed, their combined effect on neuronal function remains to be established. Also, these previous studies have been performed mainly on neurons derived from the central nervous system. And, while, the combined local administration of alcohol to the peripheral nervous system, with and without stress axis hormones, may provide a model system to study the independent effects of stress, and alcohol, a considerable number of parametric studies will be required to establish an *in vitro* model of the combined effects of alcohol, catecholamines and glucocorticoids on nociceptor function.

In summary, prior studies have established a role of neuroendocrine stress axis mediators from the sympathoadrenal and hypothalamic-pituitary-adrenal stress axes, to both the addictive (Adinoff et al., 1998, Gianoulakis, 1998, Adinoff et al., 2005, Devaud et al., 2006, Morrow et al., 2006, Richardson et al., 2008, Li et al., 2011, Evans et al., 2012, Prendergast and Mulholland, 2012, Vendruscolo et al., 2012) and neurotoxic (Silva et al., 2002, Patterson-Buckendahl et al., 2004, Patterson-Buckendahl et al., 2005, Dina et al., 2008a) effects of alcohol. However, prior experimental models have not allowed a distinction between the individual impact of alcohol and stress hormones. In this study we have developed an experimental approach that has allowed us to examine their independent impact. The results of the present study will allow future experiments to explore the mechanisms mediating the contribution of alcohol and stress axis hormones, an important question in our understanding of the neurobiology of alcohol and alcohol abuse.

Acknowledgments

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List of abbreviations

ADMX	adrenomedullectomy
ODNAS	oligodeoxynucleotide antisense

ODNMM	oligodeoxynucleotide mismatch
PKCε	protein kinase C epsilon
SEM	standard error of the mean
SS	sound stress

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Highlights

- Stress plays a role in the toxic effects of alcohol
- Alcohol abuse activates neuroendocrine stress axes
- The individual contributions of alcohol and stress hormones can be isolated
- The site of action of both alcohol and stress hormones is the nociceptor.

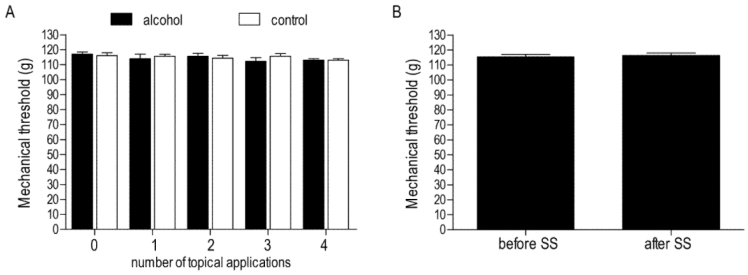

Figure 1. Neither topical application of alcohol nor sound stress alone induce changes in nociceptive threshold

Rats were submitted either to topical application of alcohol or sound stress. Mechanical thresholds were evaluated, by the Randall-Selitto paw-withdrawal test, before and 24 h after each alcohol/distilled water application (panel A) or before and after sound stress protocol (panel B). **Panel A:** Alcohol-(black bars) or distilled water-(white bars) soaked cotton balls were applied daily for 4 consecutive days (3 × 90 s applications, with 30 s intervals to replace the cotton balls) to the dorsum of the hind paw. Average paw withdrawal threshold before and after treatment was 117.0 ± 1.6 g and 113.0 ± 1.0 g, respectively, for the alcohol group, and 116.0 ± 2.0 g and 113.0 ± 1.0 g, respectively, for the vehicle group. Two-way repeated measures ANOVA showed no significant changes in the mechanical threshold (p=0.4298) or vehicle applications (p =0.2726). No difference was observed between the groups after treatments (p= 0.6567); **Panel B:** Rats were submitted to the sound stress protocol, i.e., 3 sessions of unpredictable sound exposures over 4 days (sessions on days 1, 3 and 4). Average paw withdrawal threshold before and after sound exposure was 115.3 ± 1.6 g and 116.3 ± 1.7 g, respectively. Paired Student's t-test showed no significant change in the mechanical threshold after sound stress exposures (p=0.5805). N=6 per group in all cases.

Figure 2. Topical application of alcohol followed by sound stress induces a decrease in nociceptive threshold

Mechanical nociceptive threshold was evaluated before and after the 4th application of alcohol (3 × 90 s daily applications, with 30 s intervals) to the dorsum of the hind paw (before the first exposure to sound stress). Subsequent readings were taken daily until the last sound stress session, and 4 and 7 days later (N=18 paws). Although no difference was observed in the mechanical threshold after the alcohol applications (average paw withdrawal thresholds before and after applications were 119.1 ± 0.9 g and 119.1 ± 1.6 g, respectively, paired Student's t-test, p=1.0000), repeated measures ANOVA showed changes in the mechanical threshold during the experiment (p<0.0001), with significant difference from baseline threshold (***p <0.05 in all cases) from day 2 to day 11 after alcohol treatment.

Figure 3. Topical application of alcohol in animals previously submitted to sound stress induces a decrease in nociceptive threshold

Mechanical nociceptive threshold was evaluated before and after the sound stress protocol. Alcohol was applied, after the 3rd sound exposure, to the dorsum of the hind paw for 4 consecutive days (3 × 90 s daily applications, with 30 s intervals), and the readings were taken daily, 24 h after each application, and 3, 7, 11, 14 and 18 days after the last alcohol application. (N=6 paws). No significant difference was observed in the mechanical threshold after sound exposures (average paw withdrawal thresholds before and after 3 sound sessions was 118.3 ± 1.9 g and 117.3 ± 1.3 g, respectively, paired Student's t-test, p=0.5177). However, repeated measures ANOVA showed changes in the mechanical threshold during the experiment (p<0.0001), with significant difference from baseline from day 2 to day 15 after sound exposures (***p<0.05 in all cases).

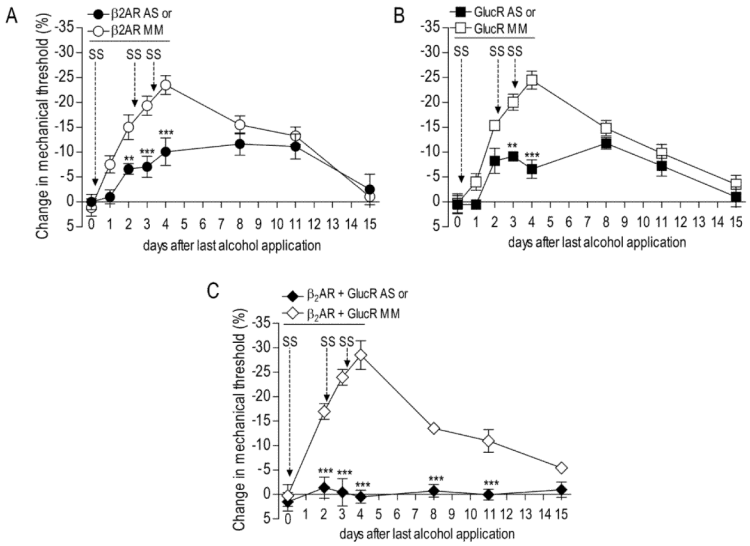

Figure 4. Hyperalgesia induced by interaction of topical application of alcohol and sound stress (SS) is dependent on β_2-adrenergic- and glucocorticoid-receptors in the sensory neuron

Rats were treated with ODN-AS or MM against β_2-adrenergic-(Panel A) or glucocorticoid-(Panel B), or both (Panel C) receptors for 3 days before sound stress (SS) sessions; ODN treatments continued until the last session of SS. Alcohol was applied daily to the dorsum of the hind paws for 4 days before SS was started; no change in the mechanical threshold was observed (data not shown). Mechanical thresholds were evaluated on days 0 (before SS), 1, 2, 3, 4 (during SS sessions) and 4, 7 and 11 days after the last SS session. (N=6 per group). **Panel A:** Average paw withdrawal thresholds before and after 3 injections (immediatel protocol) of β_2-adrenergic receptor ODN-AS (●) or MM (○) were 117.3±1.3 g and 120.0 ± 1.8 g, respectively, for the AS, and 120.6 ± 0.4 g and 121.3 ± 2.1 g, respectively, for the MM group. Paired Student's t-test showed no effect of the ODN treatment, during this period of time, on the mechanical threshold: p=0.3370 for the AS and p=0.7497 for the MM groups (N=6). Repeated measures ANOVA showed significant group time interaction for both AS and MM groups (p <0.0001), with significant difference between the groups on days 2 (***p<0.001); **Panel B:** Average paw withdrawal thresholds before and after 3 injections (immediately before SS protocol) of glucocorticoid receptor ODN-AS (■) or MM (□) were 115.6 ± 2.0 g and 118.6 ± 2.1 g, respectively, for the AS, and 119.6 ± 2.0 g and 120.3 ± 2.2 g, respectively, for the MM group. Paired Student's t-test showed no effect of the ODN treatment, during this period of time, on the mechanical threshold: p=0.0756 for the AS and p=0.8645 for the MM groups (N=6). Repeated measures ANOVA showed a significant group time interaction for both AS and MM groups (<0.0001), with significant difference between the groups on days 3 (**p<0.01) and 4 (***p<0.001); **Panel C**:Average paw withdrawal thresholds before and after 3 injections (immediately before SS protocol) of both β_2-adrenergic and glucocorticoid receptor ODN-AS (◆) or MM (?) were 118.0 ± 2.1 g and 119.6 ± 2.0 g, respectively, for the AS, and 115.3 ± 1.8 g and 115.6 ± 3.5 g, respectively, for the MM group. Paired Student's t-test showed no effect of the ODNs, in this period of time, on the mechanical threshold: p=0.4968 for the AS and p=0.9031 for the MM groups (N=6). Repeated measures ANOVA showed a significant group time interaction for both AS and MM groups (p<0.0001) with significant difference between the groups on days 2, 3, 4, 8 and 11 (***p<0.001 in all cases).

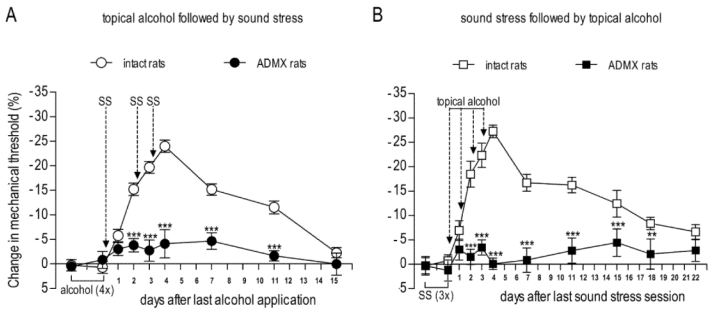

Fig. 5. Adrenal medullectomy prevents development of mechanical hyperalgesia induced by the interaction between alcohol and stress

Rats were submitted to adrenal medullectomy 5 weeks before the experiments. **Panel A:** alcohol was applied on the dorsum of the hind paw for 4 consecutive days. The sound stress protocol begun on the 5th day, with sessions on the 1st, 3rd and 4th day post-alcohol applications. Mechanical thresholds were evaluated before sound stress (day 0), and on days 1, 2, 3, 4 (during sound stress protocol), 7, 11 and 15 (N=6 per group); average paw withdrawal thresholds before and after alcohol applications were 119.1 ± 0.9 g and 119.1 ± 1.6 g, respectively, for the control (intact rats, group ○), and 120.6 ± 0.4 g and 119.6 ± 2.0 g, respectively, for the adrenal medullectomized rats (ADMX, group ●) (Paired Student's t-test showed no significant difference in the mechanical threshold after the alcohol applications: p =1.0000 for the control (N=18) and p = 0.6560 for the ADMX group (N=6)). Repeated measures ANOVA showed a significant group time interaction in the control group (<0.0001), with significant change in the mechanical threshold from day 2 to day 11 after alcohol treatment (p <0.05 in all cases). However, no significant difference was observed in the ADMX group during the experiment (p=0.1790). Comparison of both groups showed difference on days 2, 3, 4, 7 and 11 (Two-way ANOVA, *** <0.001 in all cases); **Panel B:** Alcohol was applied, for 4 consecutive days, on the dorsum of the hind paws of rats previously submitted to the sound stress protocol. Mechanical thresholds were evaluated before alcohol applications (day 0), and on days 1, 2, 3, 4 (during alcohol treatment), 7, 11, 15, 18 and 22 (N=6 per group). Average paw withdrawal thresholds before and after sound stress protocol were 118.3 ± 1.9 g and 117.3 ± 1.3 g, respectively, for the control (intact rats, group □) and 120.3 ± 2.2 g and 121.6 ± 1.5 g, respectively, for the ADMX group (■) (Paired Student's t-test showed no significant difference in the mechanical threshold after the sound exposures: p= 0.5177 (N=6) for the control and p= 0.6320 for the ADMX group (N=6)). Repeated measures ANOVA showed a significant group time interaction in the control group (p<0.0001), with significant change in the mechanical threshold from day 2 to day 15 after sound stress exposures (p<0.05 in all cases). However, no significant difference was observed in the ADMX group during the experiment (p= 0.2097). Comparison of both groups showed difference on days 2, 3, 4, 7, 11, 15 (Two-way ANOVA, ***p<0.001) and 18 (**p<0.01).