Attenuation by baclofen of nicotine rewarding properties and nicotine withdrawal manifestations

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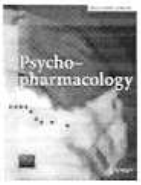

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Attenuation by baclofen of nicotine rewarding properties and nicotine withdrawal manifestations

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Abstract

Rationale: Nicotine is a major active ingredient in tobacco and plays a major role in tobacco addiction. In rodents, repeated nicotine administration produces behavioral responses related to its addictive properties, such as reinforcing effects and physical dependence.

Objectives: The aim of the present study was to evaluate the possible role of $GABA_B$ receptor in responses induced by repeated nicotine administration in Swiss Webster mice.

Results: Nicotine hydrogen tartrate salt (0.5 mg/kg, s.c.) administration induced rewarding properties in the conditioning place preference test. The $GABA_B$ receptor agonist, baclofen (3 mg/kg, i.p.) abolished the rewarding properties induced by nicotine hydrogen tartrate salt (0.5 mg/kg, s.c.). In addition, naloxone-precipitated nicotine withdrawal induced somatic manifestations, anxiety-like effects in the elevated plus maze test and dysphoric manifestations in the conditioned place aversion paradigm. Baclofen (2 and 3 mg/kg; i.p.) prevented the somatic manifestations and the anxiety-like effects associated with naloxone-precipitated nicotine withdrawal but not the dysphoric manifestations.

Conclusions: These results showed that nicotine rewarding properties and negative aspects of nicotine withdrawal, such as anxiety-like effects and somatic manifestations, can be modulated by the $GABA_B$ receptor activity. This study now reveals a novel possible application of baclofen to develop new therapeutic strategies to achieve smoking cessation.

Keywords: Nicotine, Baclofen, $GABA_B$ receptors, Reward, Withdrawal, Mice.

Introduction

Nicotine is a major active ingredient in tobacco smoke and plays a major role in tobacco addiction (Stolerman and Jarvis, 1995). Nicotine produces its pharmacological responses by acting on nicotinic acetylcholine receptors (nAChRs) that are mainly located at a presynaptic level (Dani 2001).

The activation of these receptors by nicotine increases the release of several neurotransmitters, including dopamine, noradrenaline, serotonin, acetylcholine, glutamate and GABA (in Picciotto and Corrigall 2002). The varied properties of the nAChRs can result in different patterns of neurotransmitter release following acute and chronic nicotine administration, accounting for the complex behavioral effects of nicotine (in Picciotto et al. 2002).

People smoke to experience the psychoactive effects of nicotine, like mild euphoria, increased energy, heightened arousal, reduced stress and anxiety and improve cognitive function, such as concentration, reaction time and task performance (Pomerleau 1986; Gilbert et al., 1989). In rodents, acute nicotine administration modifies a large number of physiological processes such as locomotion, nociception, anxiety, learning and memory, as well as repeated nicotine administration produces several behavioral responses directly related to its addictive properties, including rewarding effects and physical dependence (Decker 1995).

Preclinical studies support the idea that a number of GABAergic drugs, particularly the $GABA_B$ receptor agonist baclofen, decreases the abstinence signs from different drugs, such as ethanol (File et al. 1991), barbitone (Sandoval and Palermo-Neto 1985) and morphine (Diaz et al. 2001). Baclofen also reduces craving for several addictive drugs, including heroin, alcohol, and nicotine (Colombo et al. 2000; Corrigall et al. 2000; Spano et al. 2007). In addition, baclofen antagonizes nicotine-, cocaine-, and morphine-induced dopamine release in the nucleus accumbens (NAc) of rat (Fadda et al. 2003). Interestingly, baclofen prevents nicotine rewarding properties in rats (Paterson et al. 2004; Le Foll et al. 2008) and the activation of $GABA_B$ receptor in the ventral tegmental area (VTA) attenuates the rewarding properties of psychostimulants and nicotine (Cousins et al. 2002). Our laboratories have extensively explored the behavioral and neurochemical effects of nicotine addiction. Indeed, we have previously observed an interaction between the GABAergic and nicotinic cholinergic systems, given that baclofen prevented the somatic manifestation and

neurochemical changes induced by mecamylamine-precipitated nicotine withdrawal in mice (Varani et al. 2011). On the other hand, the $GABA_B$ antagonist, 2-OH-saclofen, blocked the anxiolytic- and anxiogenic-like effects induced by nicotine (Varani and Balerio 2012). Finally, we recently showed that acute behavioral responses to nicotine (antinociception, hypolocomotion and anxiety-like effects) and nicotine withdrawal syndrome are modified in $GABA_{B1}$ knockout mice (Varani et al. 2012).

Taken together, these results suggest that several behavioral responses of nicotine related to its addictive properties can be modulated through the activity of $GABA_B$ receptors. The aim of the present study was to clarify the behavioral effects of baclofen on two important responses related to nicotine dependence: nicotine rewarding properties and the emotional and somatic manifestation of nicotine withdrawal. Several behavioral responses induced by repeated nicotine administration were evaluated in Swiss Webster mice pretreated with baclofen. Nicotine reinforcing properties were investigated using the place preference paradigm. In addition, the somatic manifestations and the anxiety-like effects induced by naloxone-precipitated nicotine withdrawal were evaluated. Finally, the conditioned place aversion paradigm was used to evaluate the aversive motivational state associated with nicotine withdrawal.

Materials and methods

Animals

Male Swiss Webster mice obtained from Bioterio Central (Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina) weighing 22-24 g were housed five per cage, acclimated to the laboratory conditions according to local regulation (SENASA, 2002) (12-h light: 12-h dark cycle, 21 ± 0.5 °C room temperature, 65 ± 10 % humidity) and manipulated for three days prior to the experiment for handling habituation. Food and water were available *ad libitum*. Behavioral tests and animal care were conducted in accordance with the standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC and 2001-486/EEC) and approved by the local ethical committee. All experiments were performed with the investigators being blind to the treatment conditions.

Drugs

(-)-Nicotine hydrogen tartrate salt ([-]-1-methyl-2-[3-pyridyl]pyrrolidine), naloxone hydrochloride (Sigma-Aldrich, USA) were dissolved in physiological saline (0.9% NaCl) and administered by the subcutaneous route. Nicotine doses used were calculated as nicotine hydrogen tartrate salt. (±) baclofen (Novartis, Basel, Switzerland) was dissolved in physiological saline (0.9% NaCl) and administered by intraperitoneal route. Nicotine, naloxone and baclofen were administered in a volume of 10 mL/kg. Nicotine doses are reported as nicotine hydrogen tartrate salt (1 mg/kg of nicotine hydrogen tartrate salt equals to 0.35087 mg/kg nicotine free base).

Place preference paradigm

In a different set of animals, the rewarding effects of nicotine hydrogen tartrate salt (0.5 mg/kg, sc) were evaluated by using the conditioned place preference paradigm, as previously described (Maldonado et al. 1997; Matthes et al. 1996). The apparatus consisted of two main square conditioning compartments separated by a triangular central division. During the preconditioning phase, each mouse was placed in the middle of the central division and had free access to both compartments of the conditioning apparatus for 18 min, with the time spent in each compartment recorded by computerized monitored software (Vision Robot®, Argentina). Treatments were counterbalanced between compartments in order to use an unbiased procedure. No initial place preference or aversion for the different compartments was observed in any of the experiments. In the conditioning phase, saline and nicotine hydrogen tartrate salt conditioning sessions were conducted daily over the next 4 days. Each day, animals were injected with saline and placed into corresponding chamber for 20 min. Four hours later, animals were injected with nicotine hydrogen tartrate salt (0.5 mg/kg, s.c.) and placed in the opposite chamber for 20 min. Control animals received saline every session in both chambers. The test phase was conducted exactly as for the pre-conditioning phase, i.e. free access to both compartments during 18 min, and the time spent in each compartment recorded. The time in the central area was proportionally shared and added to the time value of each compartment as previously described (Maldonado et al. 1997; Valverde et al. 1996). A score value was calculated for each mouse as the difference between the time spent in the drug-paired compartment during the testing and pre-conditioning

phases. Baclofen (1, 2 and 3 mg/kg, i.p.) or vehicle was administered 45 min before nicotine injection during the conditioning phase to evaluate their effect on the acquisition of the place preference.

Nicotine dependence and withdrawal

Induction of nicotine dependence

Mice were implanted subcutaneously with Alzet osmotic minipumps (Model, 2001) (Alzet®, Cupertino, CA), under brief diethyl ether anaesthesia. The minipumps contained saline or nicotine hydrogen tartrate salt solutions and delivered a constant subcutaneous flow at a rate of 1 μL/h. The concentration of nicotine hydrogen tartrate salt was adjusted to compensate for differences in the body weight of the subjects. Thus, average-weight mouse received a dose of approximately 25 mg/kg/day nicotine hydrogen tartrate salt over 6 days. Two different set of mice were used to evaluate the somatic signs and the anxiety-like effects associated with naloxone-precipitated nicotine withdrawal.

Naloxone-precipitated nicotine withdrawal: somatic signs

Nicotine withdrawal syndrome was precipitated 6 days after minipump implantation by injection of the μ-opioid receptor antagonist naloxone (3 mg/kg, s.c.). The somatic signs of withdrawal were visually recorded by one observer during a period of 15 min before and 30 min after naloxone injection, as previously reported (Balerio et al. 2004). The number of wet dog shakes, front paw tremors, writhes and scratches was counted. Body tremor, ptosis, teeth chattering, genital licks, and piloerection were scored 1 for appearance or 0 for non-appearance within each 5 min time. The locomotor activity over 5 min periods was rated 0, 1, or 2 (0 for inactivity, 1 for low activity, and 2 for normal activity). A global withdrawal score was calculated for each animal by giving each individual sign a relative weight, as previously reported (Castañé et al. 2002). Baclofen (1, 2 and 3 mg/kg, i.p.) or vehicle were administered 45 min before naloxone injection.

Naloxone-precipitated nicotine withdrawal: anxiety-like effects

Other set of nicotine-dependent mice received a naloxone (3 mg/kg, s.c.) injection in order to precipitate the nicotine withdrawal and were immediately placed in the elevated plus-maze test. The elevated plus-maze

(Pellow et al., 1985; File et al., 1992) consisted of a black plastic apparatus with fours arms (16×5 cm) set in a cross from a neutral central square (5×5 cm). Two opposite arms were delimited by vertical walls (closed arms), while the other two opposite arms had unprotected edges (open arms). The maze was elevated 30 cm above the ground and illuminated from the top (100 lx). At the beginning of the 15-min observation session, each mouse was placed in the central neutral area, facing one of the open arms. The total number of visits to the closed and open arms, and the cumulative time spent in the open and closed arms were then observed on a monitor through a video camera system (Vision Robot, Buenos Aires, Argentina). An arm visit was recorded when the mouse moved both forepaws and the head into the arm, as we previously described (Balerio et al. 2005). Baclofen (1, 2 and 3 mg/kg, i.p.) or vehicle were administered 45 min before naloxone injection.

Conditioned place aversion induced by naloxone-precipitated nicotine withdrawal

In a different set of animals, the place conditioning paradigm was used to evaluate the effect of baclofen (2 mg/kg) on the dysphoric manifestations associated with nicotine withdrawal. The apparatus consisted of two main square conditioning compartments separated by a triangular neutral area (Maldonado et al. 1997). The time spent by the mouse in each compartment was recorded by computerized monitoring software (Videotrack®, View Point, France). In order to induce nicotine dependence in animals, mice were implanted subcutaneously with osmotic minipumps (Alzet®, Model 2002, Cupertino, CA) under brief diethyl ether anaesthesia. The minipumps contained saline or nicotine hydrogen tartrate salt and delivered a constant flow in a rate of 0.5 μL/h. The concentration of nicotine will be adjusted to compensate for differences in the body weight of the mice, and average-weight mouse received a dose of approximately 10 mg/kg/day nicotine hydrogen tartrate salt over 14 days. In the present study, naloxone (0.12 mg/kg, s.c.) was used to precipitate the aversive manifestations of nicotine withdrawal.

Five days after minipumps implantation, the pre-conditioning phase was performed. Each animal was placed in the neutral area, allowing to freely explore the conditioning compartments, and the time spent in each compartment was recorded during 18 min. Animals did not show any initial preference or aversion for the two compartments. Mice were distributed in the different treatment groups counterbalancing the drug-paired

compartments. In the conditioning phase, animals received baclofen (2 mg/kg, i.p.) or vehicle 45 min before the withdrawal induction by the administration of naloxone (0.12 mg/kg, s.c.) or saline (control group) on days 7, 9, 11 and 13 after minipump implantation. Then, they were immediately confined in the withdrawal-paired compartment by using guillotine doors matching walls for 30 min. On days 8, 10, 12 and 14 after minipump implantation, all mice received vehicle 45 min before saline injection, and were then immediately confined in the saline-paired compartment for 30 min. The post-conditioning phase was conducted on day 18 after minipump implantation, i.e., four days after the end of the nicotine infusions. This phase was performed as the pre-conditioning phase, i.e., animals were placed in the neutral area and had free access to the conditioning compartments during 18 min. The time spent in the central area was proportionally distributed and added to the time spent in each compartment, as previously reported (Maldonado et al. 1997). A score was calculated for each mouse as the difference between the post-conditioning and pre-conditioning time spent in the drug-paired compartment.

Statistical analysis

The score values of nicotine place conditioning experiments, the somatic signs and the anxiety-like behavior associated with nicotine withdrawal were analyzed using two-way ANOVA with chronic treatment (saline or nicotine) and acute treatment (vehicle or baclofen) as between-subjects factors of variation, followed by corresponding *post-hoc* test when appropriate (Fisher's test). For the place aversion conditioning experiments, score values were compared using one way ANOVA for each chronic treatment (saline and nicotine) followed by Fisher´s *post-hoc* test.

Results

Baclofen blocked nicotine rewarding properties

We investigated the effect of baclofen (1, 2 and 3 mg/kg, ip) on the acquisition of nicotine hydrogen tartrate salt (0.5 mg/kg, sc) rewarding properties in the place conditioning paradigm (Fig 1). Baclofen pre-treatment (3 mg/kg) blocked the rewarding properties induced by nicotine (Fig. 4). Two-way ANOVA revealed a significant effect of nicotine treatment [$F_{(1,102)}$=92.855, $p<0.001$], baclofen pre-treatment [$F_{(3,102)}$=9.188,

p<0.001] and interaction between these two factors [$F_{(3,102)}$=11.621, p<0.001]. Subsequent *post-hoc* (Fisher) analysis revealed a significant effect of nicotine treatment in the vehicle (p<0.001) and baclofen (1 and 2 mg/kg) (p<0.001) pre-treated mice, but not in those mice which were pre-treated with 3 mg/kg of baclofen. *Post-hoc* also showed a significant blockade in the rewarding properties induced by nicotine in the baclofen (p<0.001) (3 mg/kg, ip) but not in the vehicle pre-treated animals (Fig. 1).

Baclofen prevented the somatic expression of nicotine withdrawal

The following somatic signs were significantly revealed in the different experimental groups: paw tremor, body tremor, teeth chattering, genital licks, wet-dog shakes, scratches, ptosis, piloerection and locomotor activity (see Table 1 for two-way ANOVA and Fisher´s *post-hoc* test). Comparisons between saline and nicotine treated groups receiving acute vehicle pre-treatment (Fisher´s *post-hoc* test) showed that nicotine withdrawal produced a significant incidence of paw tremor (p<0.001), body tremor (p<0.001), teeth chattering (p<0.001) and wet-dog shakes (p<0.05) (Fig. 2). In addition, baclofen (3 mg/kg) pre-treatment significantly decreased paw tremor (p < 0.001), body tremor (p < 0.001), teeth chattering (p < 0.001) and wet-dog shakes (p < 0.05) in nicotine withdrawal mice in comparison to mice pre-treated with vehicle (Fig. 2).

The analysis of the global withdrawal score (see Table 1 for two-way ANOVA and Fisher´s *post-hoc* test) confirmed that baclofen (3 mg/kg) significantly prevented the severity of nicotine withdrawal. The severity of nicotine withdrawal was significant in vehicle (p<0.001) but not in baclofen pre-treated mice, in comparison to nondependent mice. In addition, the global withdrawal score was significantly reduced in nicotine dependent mice pre-treated with baclofen (3 mg/kg) in comparison to those pre-treated with vehicle (p<0.001) (Fig. 2).

Baclofen prevented the anxiety-like effects associated with nicotine withdrawal

We investigated the effect of baclofen (1, 2 and 3 mg/kg) on anxiety-like behavior induced by naloxone-precipitated nicotine withdrawal in the elevated plus maze test (Fig 3). Baclofen pre-treatment (3 mg/kg) blocked the anxiety-like behavior induced by naloxone-precipitated nicotine withdrawal (Fig. 3). The two-

way ANOVA revealed a significant effect of nicotine treatment [$F_{(1,66)}$=29.173, p<0.001], baclofen pre-treatment [$F_{(3,66)}$=9.409, p<0.001] and interaction between these two factors [$F_{(3,66)}$=10.050, p<0.001] for the percentage of entries into the open arms. Comparisons between saline and nicotine treated groups (Fisher´s *post-hoc* test) showed that the percentage of entries into the open arms was significantly decreased (p<0.001) in mice receiving acute vehicle pre-treatment (Fig. 3A). In addition, the decreased percentage of entries into the open arms induced by nicotine withdrawal was significantly prevented in baclofen (2 and 3 mg/kg) (p<0.001) pre-treated mice, but not in those mice which were pre-treated with baclofen 1 mg/kg (Fig. 3A). On the other hand, two-way ANOVA revealed a significant effect of nicotine treatment [$F_{(1,66)}$=25.549, p<0.001], baclofen pre-treatment [$F_{(3,66)}$=5.321, p<0.01] and interaction between these two factors [$F_{(3,66)}$=5.869, p<0.001] for the percentage of time spent in the open arms. Comparisons between saline and nicotine treated groups (Fisher´s *post-hoc* test) showed that the percentage of time spent in the open arms was significantly decreased (p<0.001) in mice receiving acute vehicle pre-treatment (Fig. 3B). In addition, the decreased percentage of time spent in the open arms induced by nicotine withdrawal was significantly prevented in baclofen (2 mg/kg, p<0.05 and 3 mg/kg, p<0.001) pre-treated mice, but not in those mice which were pre-treated with baclofen 1 mg/kg (Fig. 3B).

Baclofen did not modify the dysphoric manifestations of nicotine withdrawal

The effect of baclofen (2 mg/kg, i.p.) pre-treatment on the dysphoric manifestations of naloxone-precipitated nicotine withdrawal was analyzed (Fig. 4). Time spent in the withdrawal paired compartment during pre-test was similar in the different experimental groups, which ensures the use of an unbiased procedure (one-way ANOVA, [$F_{(5,74)}$ = 0.1243, p = 0.987], data not shown). No abnormal behavior was observed during the conditioning phase after the administration of naloxone (0.12 mg/kg). Fisher's *post hoc* test revealed a significant decrease in the score of vehicle and baclofen pre-treated nicotine-dependent animals conditioned to naloxone-precipitated withdrawal as compared to vehicle pre-treated nicotine-dependent animals conditioned with saline (p<0.05). No significant changes were observed when comparing vehicle or baclofen pre-treated mice, revealing that conditioned place aversion induced by naloxone in nicotine treated mice was not modified by baclofen pre-treatment (Fig. 4).

Discussion

The effects of baclofen administration have been evaluated on the chronic effects of nicotine. Our results demonstrate that acute baclofen administration prevented rewarding properties induced by nicotine. Baclofen also prevented the somatic signs and anxiety-like effects, but not the dysphoric manifestations associated with naloxone-precipitated nicotine withdrawal.

Acute rewarding effects of drugs of abuse are responsible for the initiation of the addictive process (in Koob and Le Moal 2001). Nicotine acute rewarding properties were investigated in mice by using the place conditioning paradigm. Nicotine hydrogen tartrate salt (0.5 mg/kg, s.c.) induced a significant place preference, as expected (Berrendero et al. 2002, 2005; Castañé et al. 2002, 2006; Trigo et al. 2009) and baclofen did not produce any intrinsic response in this paradigm, in agreement with previous studies (Heinrichs et al. 2010). In the present study, baclofen (3 mg/mg, i.p.) blocked the nicotine conditioned place preference, suggesting that $GABA_B$ receptor activity would be involved in the rewarding effects of nicotine. In agreement, baclofen prevents the nicotine rewarding properties in the place conditioning paradigm in rats (Le Foll et al. 2008). Moreover, baclofen has been reported to reduce nicotine rewarding effects in the rat self-administration paradigm (Corrigall et al. 2000; Fattore et al. 2002; Paterson et al. 2004). In addition, a recent report showed that baclofen blocked the reinstatement of extinguished nicotine place preference in mice (Fattore et al. 2009). Therefore, $GABA_B$ receptor activation would modulate nicotine self-administration, the reinstatement of extinguished nicotine place preference as well as the acquisition of nicotine place preference in mice. The effect of baclofen here observed could be related to its capacity to activate $GABA_B$ receptors located postsynaptically in dopaminergic neurons of the VTA (Laviolette and van der Kooy 2004), causing a decrease of dopamine release in the NAc (Fadda et al. 2003), leading to the blockade of nicotine rewarding effects (Cousins et al. 2002).

Importantly, acute baclofen administration prevented the somatic expression of naloxone-precipitated nicotine withdrawal, as revealed by the significant decrease of relevant somatic signs, such as paw-tremors, body tremor, teeth chattering and wet-dog-shakes. Nicotine dependence was induced by using minipumps delivering a constant rate of nicotine hydrogen tartrate salt solution over a 6-day period and the μ-opioid

receptor antagonist naloxone (3 mg/kg, s.c.) precipitated a withdrawal syndrome, as previously reported (Balerio et al. 2004; Biala et al. 2005). Naloxone did not modify somatic signs by itself, in agreement with previous studies (Diaz et al. 2001; Luo et al. 2012; Mori et al. 2013), In addition, naloxone administration has been widely used to precipitate withdrawal in nicotine-dependent rodents (Adams and Cicero 1998; Carboni et al. 2000; Malin et al. 1993), and also induces somatic signs of nicotine withdrawal in chronic smokers (Krishnan-Sarin et al. 1999), presumably by a possible blockade of nAChRs (Tome et al. 2001). Conversely, human clinical trials showed that another opioid antagonist (naltrexone) decreases nicotine withdrawal signs in chronic smokers (Epperson et al. 2010; Toll et al. 2010; King et al. 2012), which support the possible effect of naloxone on nAChRs (Tome et al. 2001). Together, these effects reveal the role of the endogenous opioid system in nicotine abstinence (Houdi et al. 1998; Isola et al. 2002; Berrendero et al., 2002, 2005). The present study revealed that baclofen pre-treatment prevented the somatic manifestations of naloxone-precipitated nicotine withdrawal, suggesting that $GABA_B$ receptors would be involved in the control of nicotine withdrawal signs. Similarly, we have previously shown that baclofen prevented the somatic signs of nicotine withdrawal precipitated in mice by the selective nicotinic receptor antagonist mecamylamine (Varani et al. 2011), and this nicotine withdrawal syndrome was abolished in $GABA_{B1}$ knockout mice (Varani et al. 2012). The effects of baclofen in preventing naloxone-precipitated nicotine withdrawal signs were not due to changes in locomotion since no significant differences between saline-saline and saline-baclofen groups were revealed (see table 1 for locomotor activity sign), in agreement with previous studies (Diaz et al. 2001; Varani et al. 2011; Villas Boas et al. 2012). In addition, baclofen did not induce motor incoordination or sedation, as confirmed by the rota-rod test (Balerio and Rubio 2002). Therefore, our results suggest the involvement of $GABA_B$ receptors on naloxone-precipitated nicotine withdrawal syndrome, which could imply the existence of a possible interaction between the opioid, GABAergic and nicotine neurotransmitter systems (in Berrendero et al. 2010; Kenny and Markou 2001 and Markou 2008). The prevention of nicotine withdrawal by baclofen here evidenced could be related to its ability to modulate opioid cells directly connected with dopaminergic in the striatum (Ma et al. 2012; Miura et al. 2007), a brain area involved in nicotine withdrawal expression (Natividad et al. 2010; Rada et al. 2001; Varani et al. 2011). However, further studies would be required to support this mechanistic explanation.

Several studies in animals (Cheeta et al. 2001; Irvine et al. 2001) and humans (Parrott and Garnham 1998) have revealed that nicotine withdrawal results in an increased anxiety-like effect, which has been proposed as an affective aspect of nicotine withdrawal. Our results showed that naloxone-precipitated nicotine withdrawal increased the anxiety-like effects in the elevated plus maze. Similarly, it has been reported that mecamylamine-precipitated (Jackson et al. 2008; Rehni et al. 2012) or spontaneous nicotine withdrawal induce a reduction in exploration of the open arms of the elevated plus maze in mice (Abreu-Villaça et al. 2008; Jackson et al. 2009; Manhães et al. 2008). Our results revealed that naloxone alone did not change the elevated plus maze responses when compared to control mice, in accordance to previous reports (Hodgson et al. 2008). Interestingly, baclofen pre-treatment prevented nicotine abstinence-induced anxiety-like effects on the elevated plus maze test, without any intrinsic effect of baclofen on anxiety levels (Dalvi and Rodgers 1996; Varani and Balerio 2012). Therefore, our study revealed that the anxiety-like effects induced by nicotine withdrawal could be pharmacologically minimized by the activation of $GABA_B$ receptors. The effects of baclofen in preventing the anxiety-like responses associated with naloxone-precipitated nicotine withdrawal were not due to changes in locomotion since no significant differences were revealed on the locomotor responses after the different treatments (see table 1 for locomotor activity sign).

Finally, the effects of baclofen pre-treatment were evaluated on the aversive manifestations associated with nicotine withdrawal. The dose of baclofen (2 mg/kg) used in these set of experiments was selected taking into account the results obtained in the experiments of somatic manifestations and the anxiety-like effects associated to naloxone-precipitated nicotine withdrawal. The administration of naloxone precipitated dysphoric manifestations of nicotine withdrawal in nicotine dependent mice, as previously described for mice (Balerio et al. 2004) and rats (Ise et al. 2000; Watkins et al. 2000), indicating that the opioid system is particularly important in the negative emotional states associated with nicotine withdrawal (in Kenny and Markou 2001). In our experimental conditions, no somatic manifestations of nicotine withdrawal were observed during the conditioning phase in mice receiving this low dose of naloxone (0.12 mg/kg), in agreement with previous studies (Balerio et al. 2004). These aversive manifestations of nicotine withdrawal were not modified by the pre-treatment with baclofen (2 mg/kg), which had no intrinsic effect as previously reported (Chester and Cunningham 1999). Therefore, the aversive states produced during naloxone-

precipitated nicotine withdrawal cannot be modulated by the activation of $GABA_B$ receptors, at least in our experimental conditions.

In conclusion, we provide herein pharmacological evidence of the specific involvement of $GABA_B$ receptors in the regulation of rewarding properties induced by nicotine. Interestingly, the present results also indicate that baclofen administration prevents the expression of somatic signs and the anxiety-like effects associated with naloxone-precipitated nicotine withdrawal in mice, suggesting an interaction between the opioid, GABAergic and cholinergic systems. The elucidation of this interaction provides a further step towards a better understanding of the complex nicotine behavioral responses in mice. All together, these data underline the interest of $GABA_B$ receptor as a potential target for the development of novel therapeutics approaches for nicotine addiction (Markou et al. 2004; Franklin et al. 2009). In addition, based on the available preclinical and clinical studies we suggested that baclofen could be a promising compound for the treatment of nicotine addiction. This study now reveals a novel possible application of baclofen to develop new therapeutic strategies to achieve smoking cessation.

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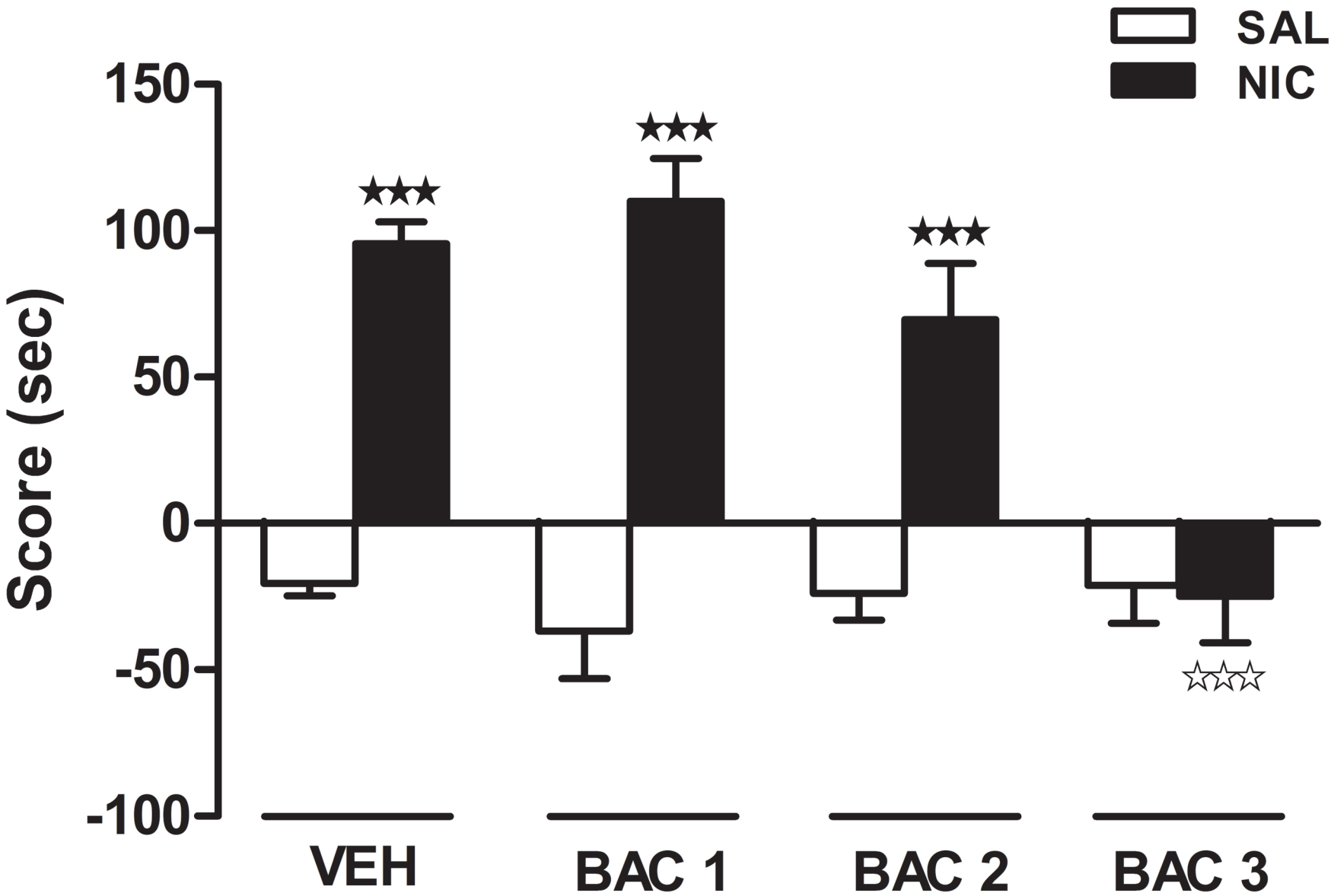
Figures Legends

Fig 1. Baclofen (3 mg/kg, ip) blocked the rewarding properties induced by nicotine. The effect of baclofen (BAC; 1, 2 and 3 mg /kg; i.p.) on the rewarding properties induced by nicotine hydrogen tartrate salt (NIC; 0.5 mg/kg, s.c.) in the place conditioning paradigm was investigated in mice. Results are represented as the difference between post-conditioning and pre-conditioning time spent in the drug-paired compartment. BAC or vehicle (VEH,0.9% NaCl) were administered 45 min before NIC or saline (SAL, 0.9% NaCl) injection during the conditioning phase. Data are expressed as mean ± SEM (n = 12-18 mice for each group). Statistical analysis was performed using two-way ANOVA with chronic treatment (SAL or NIC) and acute treatment (VEH or BAC) as between-subjects factors of variation, followed by Fisher´s *post-hoc* test. ★★★ $p < 0.001$ when compared to VEH group. ☆☆☆ $p < 0.001$ comparisons between similar groups receiving SAL or NIC.

Fig 2. Baclofen (3 mg/kg, ip) prevented the somatic expression of naloxone-precipitated nicotine withdrawal. Counted (wet-dog shakes and paw tremor) and checked (body tremor, genital licks, ptosis, teeth chattering and piloerection) somatic signs of withdrawal were observed during 30 min immediately after naloxone (3 mg/kg, s.c.) administration. Baclofen (BAC; 1, 2 and 3 mg /kg; i.p.) or vehicle (VEH, 0.9% NaCl) were administered 45 min before withdrawal. A global withdrawal score was calculated for each animal as described in the methods. Data are expressed as mean ± SEM (n = 10-14 mice for each group). Statistical analysis was performed using two-way ANOVA with chronic treatment (saline, SAL, 0.9% NaCl or nicotine hydrogen tartrate salt, NIC, 25 mg/kg/day) and acute treatment (VEH or BAC) as between-subjects factors of variation, followed by Fisher´s *post-hoc* test. ★ $p < 0.05$, ★★★ $p < 0.001$ when compared to VEH group. ☆ $p < 0.05$, ☆☆☆ $p < 0.001$ comparisons between similar groups receiving chronic SAL or NIC.

Fig 3. Baclofen (3 mg/kg, ip) prevented the anxiety-like effects associated with naloxone-precipitated nicotine (NIC) withdrawal. Percentage of entries into (A) and time spent in the open arms of withdrawal were observed during 15 min immediately after naloxone (3 mg/kg, s.c.) administration. Baclofen (BAC; 1, 2 and 3 mg /kg; i.p.) or vehicle (VEH, 0.9% NaCl) were administered 45 min before withdrawal. Data are expressed as mean ± SEM (n = 8-10 mice for each group). Statistical analysis was performed using two-way ANOVA with chronic treatment (saline, SAL, 0.9% NaCl or nicotine hydrogen tartrate salt, NIC, 25 mg/kg/day) and acute treatment (VEH or BAC) as between-subjects factors of variation, followed by Fisher´s *post-hoc* test. ★★ $p < 0.01$, ★★★ $p < 0.001$ when compared to VEH group. ☆☆ $p < 0.01$, ☆☆☆ $p < 0.001$ comparisons between similar groups receiving chronic SAL or NIC.

Fig 4. Baclofen (BAC, 2 mg/kg, i.p.) did not modify the dysphoric manifestations of naloxone-precipitated nicotine (NIC). Naloxone (NLX, 0.12 mg/kg, s.c.) induced nicotine withdrawal place aversion score and was calculated as the difference between the time (s) spent in the withdrawal associated compartment during the post-conditioning and the pre-conditioning phase (see Materials and methods for details). Data are expressed as mean ± SEM (n = 10–14 mice for each group). ★ $p < 0.05$ compared to vehicle-saline (VEH-SAL) group (Fisher's *post hoc* test).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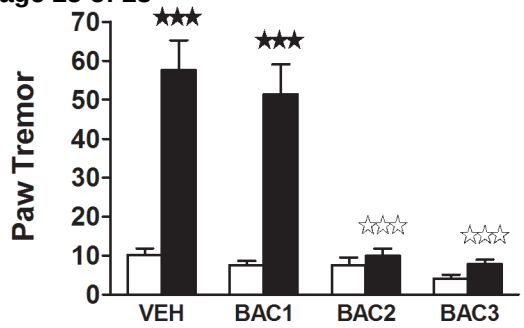
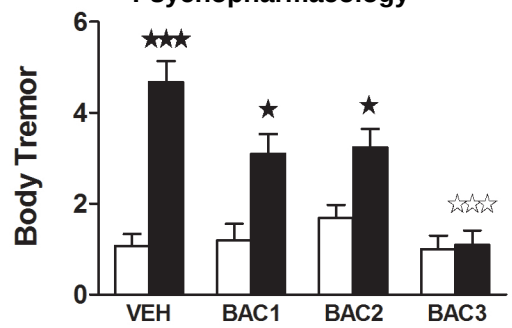
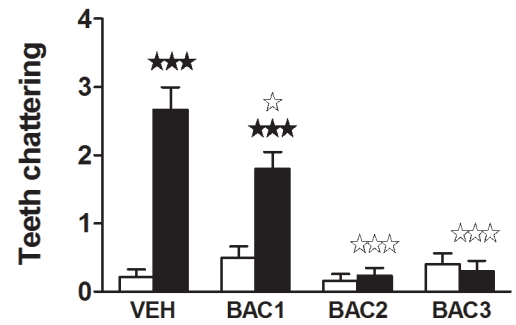
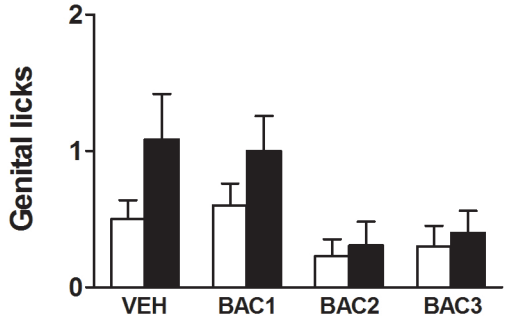
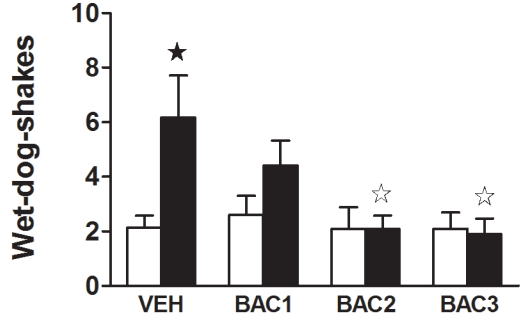
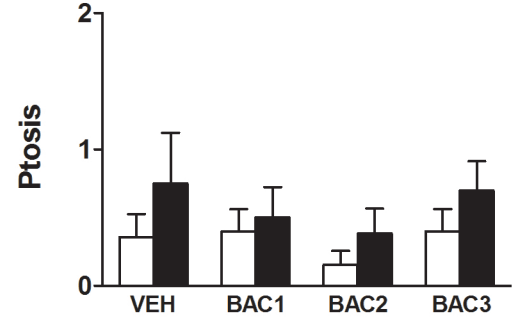


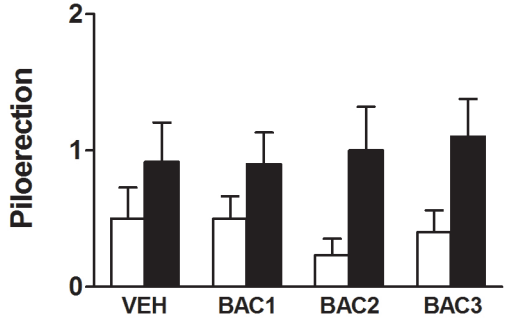


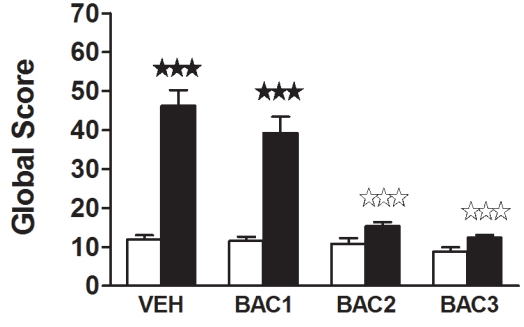






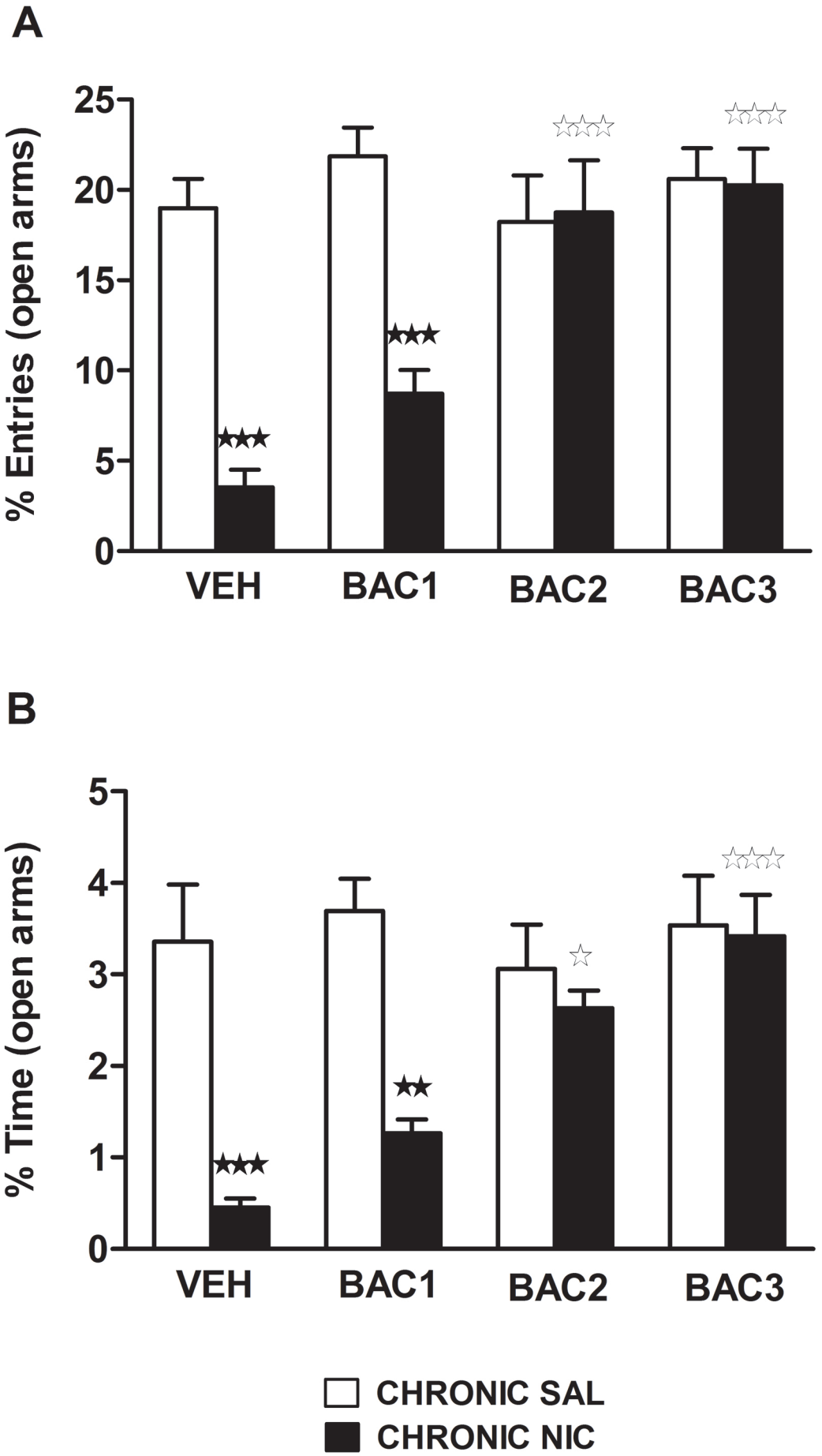






CHRONIC SAL

CHRONIC NIC


A
% Entries (open arms)
25
20
15
10
5
0
VEH
BAC1
BAC2
BAC3
B
% Time (open arms)
5
4
3
2
1
0
VEH
BAC1
BAC2
BAC3
CHRONIC SAL
CHRONIC NIC

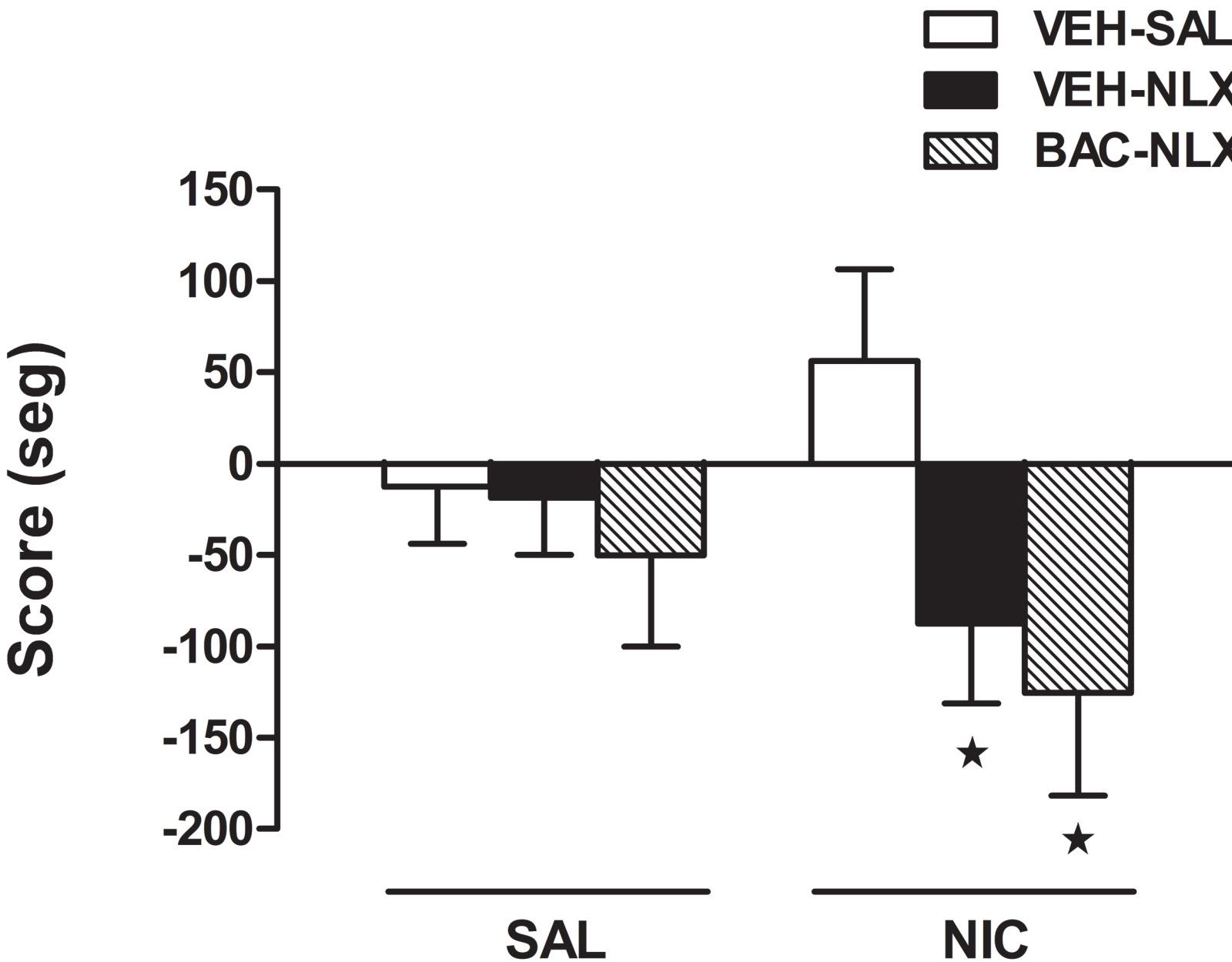

VEH-SAL
VEH-NLX
BAC-NLX
Score (seg)
150
100
50
0
-50
-100
-150
-200
SAL
NIC

Table 1 Baclofen prevents the somatic expression of naloxone-precipitated nicotine withdrawal

	Two-way ANOVA						Fisher´s *post-hoc test*						
	Chronic treatment		Acute treatment		Interaction		Sal-Sal vs Nic-Sal	Sal-BAC$_1$ vs Nic-BAC$_1$	Nic-Sal vs Nic-BAC$_1$	Sal-BAC$_2$ vs Nic-BAC$_2$	Nic-Sal vs Nic-BAC$_2$	Sal-BAC$_3$ vs Nic-BAC$_3$	Nic-Sal vs Nic-BAC$_3$
Sign	F(1,84)	*P-value*	F(3,84)	*P-value*	F(3,84)	*P-value*	*P-value*	*P-value*	*P-value*	*P-value*	*P-value*	*P-value*	*P-value*
Paw tremor	74.3	< 0.001	25.8	< 0.001	19.6	< 0.001	< 0.001	< 0.001	NS	NS	< 0.001	NS	< 0.001
Body tremor	47.8	< 0.001	8.8	< 0.001	7.9	< 0.001	< 0.001	< 0.05	NS	< 0.05	NS	NS	< 0.001
Teeth chattering	49.0	< 0.001	22.2	< 0.001	21.4	< 0.001	< 0.001	< 0.001	< 0.05	NS	< 0.001	NS	< 0.001
Genital licks	4.1	NS	4.1	< 0.01	0.8	NS	-	-	-	-	-	-	-
Wet-dog shakes	5.6	< 0.05	3.4	< 0.05	2.9	< 0.05	< 0.05	NS	NS	NS	< 0.05	NS	< 0.05
Scratches	10.6	< 0.01	2.1	NS	1.8	NS	-	-	-	-	-	-	-
Ptosis	2.8	NS	0.8	NS	0.2	NS	-	-	-	-	-	-	-
Piloerection	11.2	< 0.001	0.1	NS	0.3	NS	-	-	-	-	-	-	-
Locomotor activity	0.9	NS	0.3	NS	0.3	NS	-	-	-	-	-	-	-
Global score	127.3	< 0.001	35.1	< 0.001	27.1	< 0.001	< 0.001	< 0.001	NS	NS	< 0.001	NS	< 0.001

Two-way ANOVA with chronic treatment (saline or nicotine) and acute treatment (saline or baclofen) as between-subjects factors, followed by corresponding *post-hoc* test when appropriate (Fisher's test). To simplify the table, the comparison between Sal-Sal and Sal-Bac is not shown, since it was not significant in any case. See Materials and methods for details.