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RESEARCH PAPER

β_2-Adrenoceptor agonists alleviate neuropathic allodynia in mice after chronic treatment

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Background and purpose: Antidepressants are a first-line treatment against neuropathic pain. We previously demonstrated that β_2-adrenoceptors are necessary for antidepressants to exert their anti-allodynic action. The aim of the present study was to assess whether β_2-adrenoceptor agonists could be sufficient to alleviate neuropathic allodynia.
Experimental approach: We used a murine model of neuropathy induced by unilateral sciatic nerve cuffing in C57BL/6J mice. We previously demonstrated that this animal model is sensitive to chronic, but not to acute, treatment with antidepressant drugs, which is clinically relevant. The mechanical allodynia was evaluated using the von Frey filaments.
Key results: We showed that chronic but not acute treatment with the β-adrenoceptor agonists, bambuterol, isoprenaline, fenoterol, salbutamol, salmeterol, terbutaline or ritodrine suppressed mechanical allodynia. We confirmed that the action of these β-adrenoceptor agonists was mediated through β_2-adrenoceptors by blocking it with intraperitoneal or intrathecal, but not intracerebroventricular or intraplantar, injections of the antagonist ICI118551. We also showed that chronic treatments with the β-adrenoceptor antagonists, propranolol or ICI118551 did not suppress the allodynia.
Conclusions and implications: Our data show that chronic treatment with β-adrenoceptor agonists has the same antiallodynic properties as treatments with antidepressant drugs. This study was, however, conducted in an animal model, and a clinical validation will be required to confirm the value of the present findings in patients.
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Keywords: pain; neuropathy; antidepressant; nortriptyline; β_2-adrenoceptor; allodynia; terbutaline

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; ICI118551, (±)-1-[2,3-(Dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; i.c.v, intracerebroventricular; i.t, intrathecal; SSRI, specific serotonin reuptake inhibitor; TCA, tricyclic antidepressant

Introduction

Neuropathic pain results from damage or abnormal function of the central or peripheral nervous system (Merksey and Bogduk, 1994). It is generally a chronic and disabling condition, and it is challenging to treat (Attal *et al.*, 2006). Either tricyclic antidepressant drugs (TCAs) or anticonvulsant drugs are clinically recommended as first-line treatments for neuropathic pain (Attal *et al.*, 2006; Moulin *et al.*, 2007). However, in both cases, these drugs have drawbacks related to their side effects or to their poor efficacy in some patients.

The precise mechanism by which TCAs relieve neuropathic pain remains poorly understood. TCAs have no acute antalgic action against neuropathic pain, and a sustained treatment is necessary to their therapeutic action (Sindrup *et al.*, 2005; Micó *et al.*, 2006). These drugs primarily increase the extracellular concentrations of noradrenaline and 5-HT by blocking the reuptake sites of these amines. The downstream action of the amines leading to the therapeutic action is still unknown. However, the specific 5-HT reuptake inhibitors (SSRIs) are clinically less efficient than TCAs in treating neuropathic pain (Attal *et al.*, 2006; Moulin *et al.*, 2007). This suggests that the noradrenergic component of antidepressant drugs might play a critical role.

Recently, we used a murine model of peripheral neuropathic pain (Benbouzid *et al.*, 2008a) to study the role of adrenoceptors in the antiallodynic action of antidepressant drugs. We showed that mechanical allodynia in this model is sensitive to chronic but not to acute TCA treatment, and is resistant to SSRI treatment (Benbouzid *et al.*, 2008b,c). We demonstrated that neither α_2-adrenoceptors, β_1-adrenoceptors nor β_3-adrenoceptors (nomenclature follows

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Alexander *et al.*, 2008) are critical for antidepressant drug action against neuropathic pain (Yalcin *et al.*, 2009a,b). On the contrary, the absence or the blockade of β_2-adrenoceptors totally suppressed the effect of chronic nortriptyline, desipramine, venlafaxine or reboxetine treatments on mechanical allodynia (Yalcin *et al.*, 2009a,b).

Previous results thus demonstrated that stimulation of β_2-adrenoceptors was necessary for antidepressant drug action against neuropathic pain. The aim of the present study was to evaluate whether β_2-adrenoceptor stimulation could be sufficient to induce an antiallodynic effect. While it is established that β_2-adrenoceptor agonists have no acute intrinsic analgesic property, the consequence of a chronic treatment has not been evaluated yet in the context of neuropathic pain. We thus studied the effect of acute and chronic treatments with non-selective β-adrenoceptor agonists, such as bambuterol and isoprenaline, and with different β_2-adrenoceptor agonists, such as fenoterol, salbutamol, salmeterol, ritodrine and terbutaline. We show that all these agonists suppress neuropathic allodynia after chronic but not acute treatment, an effect that reproduces the results obtained with the TCA nortriptyline. Anti-allodynic action of β-adrenoceptor agonists was reversed by intraperitoneal or intrathecal, but not intracerebroventricular or intraplantar, co-treatment with the β_2-adrenoceptor antagonist ICI118551. Our data provide evidence that chronic β_2-adrenoceptor stimulation can relieve neuropathic allodynia in a murine model of peripheral neuropathy.

Methods

Animals

All animal care and experimental procedures were performed in accordance with the guidelines for animal experimentation of the International Association for the Study of Pain (IASP) and the European Communities Council Directive 86/6609/EEC. The animal facilities are legally registered for animal housing and experimentation (veterinary Animal House Agreement B67-482-1). The scientists in charge of the experiments possess the French certificate authorizing experimentation on living animals, delivered by the governmental veterinary office. All experiments used adult male C57BL/6J mice (n = 242 mice for all the studies; 6 weeks old upon arrival, Charles River, L'Arbresle, France). The mice were group housed 4–5 per cage. They were maintained under standard conditions in the animal facilities, with an ambient temperature of 21 ± 1°C and a 12 h light–dark cycle (lights on 06:00–18:00). Food and water were available *ad libitum*. The experiments started after 2 weeks of habituation to the animal facilities. All behavioural studies were performed during the light period.

Sciatic nerve cuffing model

Neuropathic pain was induced by the insertion of a polyethylene cuff around the main branch of the right sciatic nerve. This model, characterized in both rats (Mosconi and Kruger, 1996; Pitcher *et al.*, 1999) and mice (Cheng *et al.*, 2002; Benbouzid *et al.*, 2008a), has been successfully used by various research groups. It provided a better understanding of the pathogenesis and mechanism of peripheral neuropathic pain and its treatments (Cheng *et al.*, 2002; Coull *et al.*, 2005; Holdridge and Cahill, 2007). The cuff model induces alterations in fibre-size spectrum of peripheral nerves and an anterograde Wallerian degeneration that are similar to those produced with the chronic constriction injury model, but with the advantage of being more consistent (Mosconi and Kruger, 1996). This consistency (due to the standardized cuffs) allows the number of animals per group to be reduced, which is of ethical importance. Moreover, we previously showed that neuropathic allodynia in this model displays the critical feature of being highly sensitive to chronic (but not acute) antidepressant treatment (Benbouzid *et al.*, 2008b,c; Yalcin *et al.*, 2009a,b), which is in agreement with clinical observations. These features led us to use this model for addressing the involvement of adrenoceptors in antidepressant action (Yalcin *et al.*, 2009a,b) and for the present study on action of β-adrenoceptor agonists.

Before surgery, the mice were assigned to different experimental groups so that the baseline for mechanical nociceptive threshold and the body weight were equivalent between the groups. The surgery was carried out under ketamine/xylazine anaesthesia (ketamine: 17 mg·mL^{-1}, xylazine: 2.5 mg·mL^{-1}, given i.p., 4 mL·kg^{-1}) (Centravet, Taden, France). The common branch of the right sciatic nerve was exposed, and a 2 mm section of split PE-20 polyethylene tubing (Harvard Apparatus, Les Ulis, France) was placed around it (Cuff group) (Benbouzid *et al.*, 2008a). Sham-operated mice underwent the same surgical procedure without implantation of the cuff (Sham group).

Drug treatments

The drug treatment began 14 days after the surgical procedure in cuff-implanted and sham-operated mice. To evaluate the acute effects of adrenoceptor agonists, the mice were tested before and 30, 60 and 90 min after the first drug administration. Independent groups of mice were used to test each of the compounds.

During chronic treatment, the mice received two injections per day of either terbutaline hemisulphate (0.05 mg·kg^{-1}, 0.125 mg·kg^{-1}, 0.25 mg·kg^{-1}, 0.5 mg·kg^{-1} or 5 mg·kg^{-1}, i.p.) (Zarkovic *et al.*, 2000), salbutamol hemisulphate (2 mg·kg^{-1}, i.p.) (Goldschmidt *et al.*, 1984), fenoterol hydrobromide (0.7 mg·kg^{-1}, i.p.) (Ryall *et al.*, 2004), salmeterol xinafoate (1 mg·kg^{-1}, i.p.) (Ryberg and Johansson, 1995), ritodrine hydrochloride (10 mg·kg^{-1}, i.p.) (Serradeil-Le Gal *et al.*, 2004), isoprenaline hydrochloride (0.5 mg·kg^{-1}, i.p.) (Thornhill and Desautels, 1984) or bambuterol (0.5 mg·kg^{-1}, i.p.) (Rosenborg *et al.*, 2000). Nortriptyline hydrochloride (5 mg·kg^{-1}, i.p.) was used as reference drug (Benbouzid *et al.*, 2008b,c). Propranolol was used as a (non-selective) β-adrenoceptor antagonist (5 mg·kg^{-1}, i.p.) (Yalcin *et al.*, 2007), metoprolol was used as a specific β_1-adrenoceptor antagonist (10 mg·kg^{-1}) (Yalcin *et al.*, 2009a), and ICI118551 was used as a specific β_2-adrenoceptor antagonist (2 mg·kg^{-1}) (Yalcin *et al.*, 2007). The doses were chosen based on either rodent or human studies. For terbutaline, a range of doses were examined to assess whether lower doses could also be efficient. The mice received the injections

twice a day (morning and evening). To evaluate the effect of the long-term treatment, each assay of mechanical sensitivity thresholds was performed before the morning injection (i.e. at least 15 h following the previous evening injection), as previously described (Benbouzid *et al.*, 2008b,c; Yalcin *et al.*, 2009a,b).

After 3 weeks of treatment with β-adrenoceptor agonists, the β_2-adrenoceptor antagonist ICI118551 (2 mg·kg^{-1}) was co-administered with the agonists. This co-treatment was done using a previously validated procedure (Yalcin *et al.*, 2009a,b), which allowed limiting the number of i.p. injections to the mice. We aimed at testing whether the anti-allodynic action was indeed mediated by β_2-adrenoceptor stimulation. The co-injections with the antagonist lasted 3–4 days.

In a separate set of experiments, intrathecal (i.t.) injections of the antagonist ICI118551 (3 μg in 10 μL) were performed under halothane anaesthesia after 3 weeks of treatment with salbutamol hemisulphate (2 mg·kg^{-1}, i.p. twice a day). The i.t. injections were done just before the i.p. injections of salbutamol. Briefly, a 27-gauge needle connected to a 50 μL Hamilton syringe was inserted between the L_5 and L_6 vertebrae, into the sub-arachnoidal space. The needle placement was checked by the elicitation of a tail flick movement. For technical reasons, such procedure can be done only twice on the same mouse. We thus did the two i.t. injections on the same day, before the morning and the evening treatments with salbutamol. The mice were then tested for mechanical sensitivity in the following morning, that is 40 h following the last injection of salbutamol alone. This procedure is similar to the one previously used to assess the effect of i.t. ICI118551 on the antiallodynic action of antidepressant drugs (Yalcin *et al.*, 2009b).

Intraplantar injection of ICI118551 (3 μg in 10 μL) were performed under halothane anaesthesia after 3 weeks of treatment with terbutaline (0.125 mg·kg^{-1}, i.p. twice a day). Each intraplantar injection in the right hindpaw of the mice was made just before i.p. injection of terbutaline. A 29-gauge injection needle punctured the plantar skin and was placed in the subcutaneous space just proximal to the footpads. Two intraplantar injections were done on the same day, before the morning and the evening injection of terbutaline. Mice were tested for mechanical sensitivity in the following morning, i.e. 40 h following the last injection of terbutaline alone.

Intracerebroventricular (i.c.v.) injections of the ICI118551 (10 μg in 2.5 μL) were performed using stereotaxic surgery under halothane anaesthesia after 3 weeks of treatment with salbutamol hemisulphate (2 mg·kg^{-1}, i.p. twice a day). Anesthetized mice were mounted on a stereotaxic frame (Kopf Instruments) connected to the anesthetic apparatus. The skull was exposed, and a hole was drilled above the target injection site during the first i.c.v. injection procedure. The coordinates relative to Bregma for the 3rd ventricle were: anteroposterior –0.4 mm, lateral +1.0 mm, dorsoventral –2.2 mm below the dura. A 5.0 μL Hamilton syringe with a 33-gauge needle was used to inject 2.5 μL over 7.5 min. The syringe was left in place for an additional 5 min to ensure diffusion of the injected solution. The surgical procedure was optimized to last 25 min only. Similar to the i.t. injection experiment, we did two i.c.v. injections on the same day, before the morning and evening treatments with salbutamol. Mice were then tested for mechanical sensitivity in the following morning. The dose of ICI118551 (10 μg) has been shown to be effective for blocking β_2-adrenoceptor-related behaviour following i.c.v. injection in rodents (Funada *et al.*, 1994). After completion of the behavioural tests, the mice were killed by rapid decapitation, and the brains were dissected out, rapidly frozen on dry ice, and stored at –20°C. The brains were sectioned at 40 μm thickness on a cryostat, and the coronal sections were processed for cresyl-violet staining to control for the injection needle placement. This procedure is similar to the one previously used to assess the effect of i.c.v. ICI118551 on the anti-allodynic action of antidepressant drugs (Yalcin *et al.*, 2009b).

Measurement of mechanical allodynia

We showed in a previous characterization of the sciatic nerve cuffing model in C57BL/6J mice that the hyperalgesia to a hot thermal stimulus lasted only 3 weeks while the mechanical allodynia remained stable over 2 months (Benbouzid *et al.*, 2008a). While heat hyperalgesia is usually present in inflammatory pain, it is not a clinical feature of neuropathic pain. We thus focused the present work on mechanical allodynia. The mechanical threshold of hindpaw withdrawal was evaluated using von Frey filaments (Bioseb, Chaville, France) as previously described (Benbouzid *et al.*, 2008a) and is expressed in grams.

The mice were placed in clear Plexiglas boxes (7 cm × 9 cm × 7 cm) on an elevated mesh screen. They were allowed to acclimatize for 15 min before testing. The filaments were applied perpendicularly to the plantar surface of each hindpaw in a series of ascending forces (0.07 grams to 8 grams), with sufficient force to slightly bend the filament. Brisk withdrawal was considered as a positive response. In the absence of a response, a filament of next-greater force was applied. Each filament was tested five times per paw, and the threshold was defined as three or more withdrawals observed out of the five trials. The effect of drug injections was evaluated before (pre-test) and at different time points (post-tests or time course) following the considered drug injections.

Statistical analysis

During the von Frey experiments, none of the animals reached the upper cut-off without a response. Similarly, none of the animals responded to the lower cut-off. Most data are thus expressed as mean ± SEM. Statistical analysis were performed with STATISTICA 7.1 (Statsoft, Tulsa, OK, USA) using multi-factor analysis of variance (ANOVA). The surgery procedure (Sham or Cuff) and the treatments (saline vs. drug injections) were taken as between-group factors. When needed, the paw laterality (left vs. right) and/or the time of measurement (either time-course or pre-injection vs. post-injection data) were taken as within-subject factors (with repeated measures on the same animal). Interaction between factors was also tested. When appropriate, the Duncan test was used for *post hoc* comparisons. The significance level was set at $P < 0.05$.

For the terbutaline dose-response experiments, two other ways to present data were also used. Data were expressed as

percentage of recovery, i.e. percentage of the nociceptive threshold measured before surgery and calculated on the 3rd week of treatment in order to emphasize the fact that treatments totally suppressed the allodynia without affecting Sham mice. Data were also expressed as area under the curve (AUC) over the period of treatment, and a dose-response curve with Hill coefficient was adjusted to data with SigmaPlot (SPSS Inc., Chicago, IL, USA), using the formula $AUC = a + b/(1 + (EC_{50}/Dose)^n)$ where '*a*' is the theoretical value for the dose 0, '*b*' the amplitude of the response and '*n*' the Hill coefficient.

Similarly, two other ways to present data were used to compare drugs. As for terbutaline dose response, data were expressed as percentage of recovery. For a more precise analysis of therapeutic onsets and drug comparison, we also presented the time course of cumulated AUC of mechanical nociceptive threshold percent change, using the pretreatment values as reference. For this last data presentation, homogeneity of the experimental conditions is critical. We thus presented it for drugs that were tested by the same experimenter and with the same time course of results, i.e. the short-acting drug salbutamol, the long-acting drugs fenoterol and salmeterol, and the very long-acting drug bambuterol.

Materials

All drugs were obtained from Sigma-Aldrich, St Quentin Fallavier, France. They were dissolved in 0.9% NaCl except for ritodrine, which was dissolved in a 0.9% NaCl solution containing 0.3% ascorbic acid.

Results

Chronic, but not acute, treatment with terbutaline alleviates neuropathic allodynia

The unilateral cuff implantation induced a lasting ipsilateral mechanical allodynia, which was evaluated using von Frey filaments (group × time × paw interaction, $F_{3,48} = 14.18$, $P < 0.001$; *post hoc*: from day1, 'cuff, right paw' < all other groups at $P < 0.0001$) (Figure 1A). We studied the consequence of a longer-term terbutaline treatment (0.5 mg·kg^{-1}, i.p.), with injections twice a day (Figure 1B). This chronic treatment with terbutaline suppressed the cuff-induced allodynia after 10 days compared with saline treatment (group × time × treatment interaction, $F_{9,117} = 10.63$, $P < 0.001$; *post hoc*: cuffTer > cuffSal at $P < 0.0001$ on post-surgery days 25–39). The treatment had, however, no effect on the paw withdrawal threshold of Sham mice or on the contralateral paw withdrawal threshold of Cuff mice.

To determine the lowest effective dose of terbutaline, a range of doses of terbutaline was studied with independent groups of mice for each dose (Figure 2). We observed an antiallodynic action of terbutaline at 5 mg·kg^{-1} (Figure 2A) ($F_{11,77} = 18.67$, $P < 0.001$; *post hoc*: cuffTer < shamTer at $P < 0.0001$ on post-surgery days 2–24 only), 0.25 mg·kg^{-1} (Figure 2B) ($F_{13,91} = 6.16$; $P < 0.05$; *post hoc*: cuffTer < shamTer at $P < 0.002$ on post-surgery days 1–25), and 0.125 mg·kg^{-1} (Figure 2C) ($F_{12.84} = 11.76$; $P < 0.05$; *post hoc*: cuffTer < shamTer at $P < 0.0001$ on post-surgery days 1–22 only). No significant effect of the

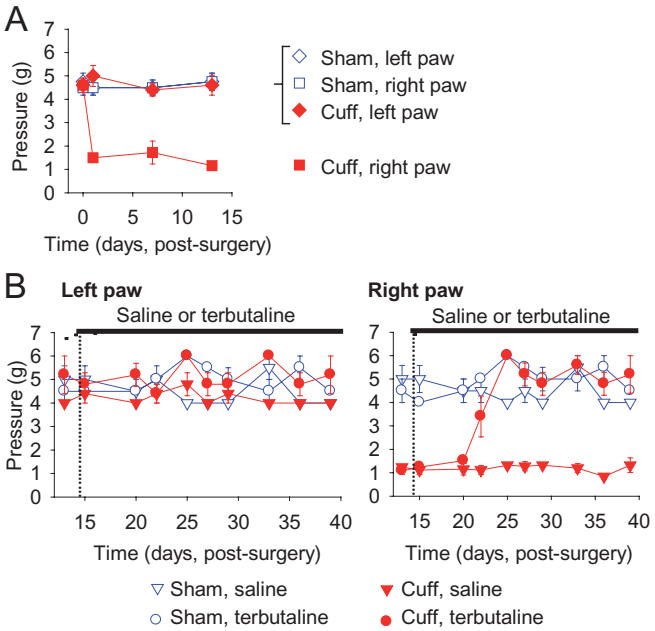

Figure 1 Chronic terbutaline treatment alleviates neuropathic allodynia. (A) Cuff implantation induced a sustained mechanical allodynia (n = 8–10 per group). (B) Chronic treatment with terbutaline (0.5 mg·kg^{-1}, i.p.) suppressed the cuff-induced mechanical allodynia. The same treatment had no effect on the contralateral hindpaw sensitivity in Cuff mice or on hindpaw sensitivity in Sham mice. The mechanical sensitivity, shown as pressure (g), was evaluated using von Frey filaments. The neuropathic allodynia was induced by the unilateral insertion of a polyethylene cuff around the main branch of the right sciatic nerve (Cuff, n = 5 per group). The control groups (Sham, n = 4 per group) underwent the same surgical procedure without cuff implantation. Data are expressed as mean ± SEM. Left paw = contralateral to the surgery; right paw = ipsilateral to the surgery.

chronic treatment was present at 0.05 mg·kg^{-1} (Figure 2D) or after saline treatment (Figure 2E). Similar results were observed with data presented as percent of recovery (Figure 2F) or as AUC (Figure 2G). A non-linear regression analysis ($r = 0.96$) gave $EC_{50} = 0.078$ with a Hill coefficient of 39.9 (Figure 2H), but these values should be considered with caution, as sharp changes in the responses were observed between 0.05 and 0.125 mg·kg^{-1}, which is a very narrow range for *in vivo* studies. For all the doses, the treatments had no influence on the withdrawal threshold for the hindpaw contralateral to the surgical procedure (Figure 2F). The anti-allodynic action of terbutaline was thus not related to a non-specific or generalized analgesia.

To evaluate terbutaline efficacy, we used the TCA nortriptyline (5 mg·kg^{-1}, i.p. twice a day) as a reference compound (Figure 2F). This TCA is used clinically against neuropathic pain (Attal *et al.*, 2006; Moulin *et al.*, 2007). As previously published (Benbouzid *et al.*, 2008b,c; Yalcin *et al.*, 2009a), chronic but not acute nortriptyline treatment relieved neuropathic allodynia, and the time course of the therapeutic effect was similar to the one observed with terbutaline (group × time × treatment interaction, $F_{11,143} = 4.04$, $P < 0.0001$; *post hoc*: cuffNor > cuffSal at $P < 0.0001$ on post-surgery days 25–34) (data not shown).

We also confirmed that the antiallodynic action of terbutaline was specifically related to the stimulation of

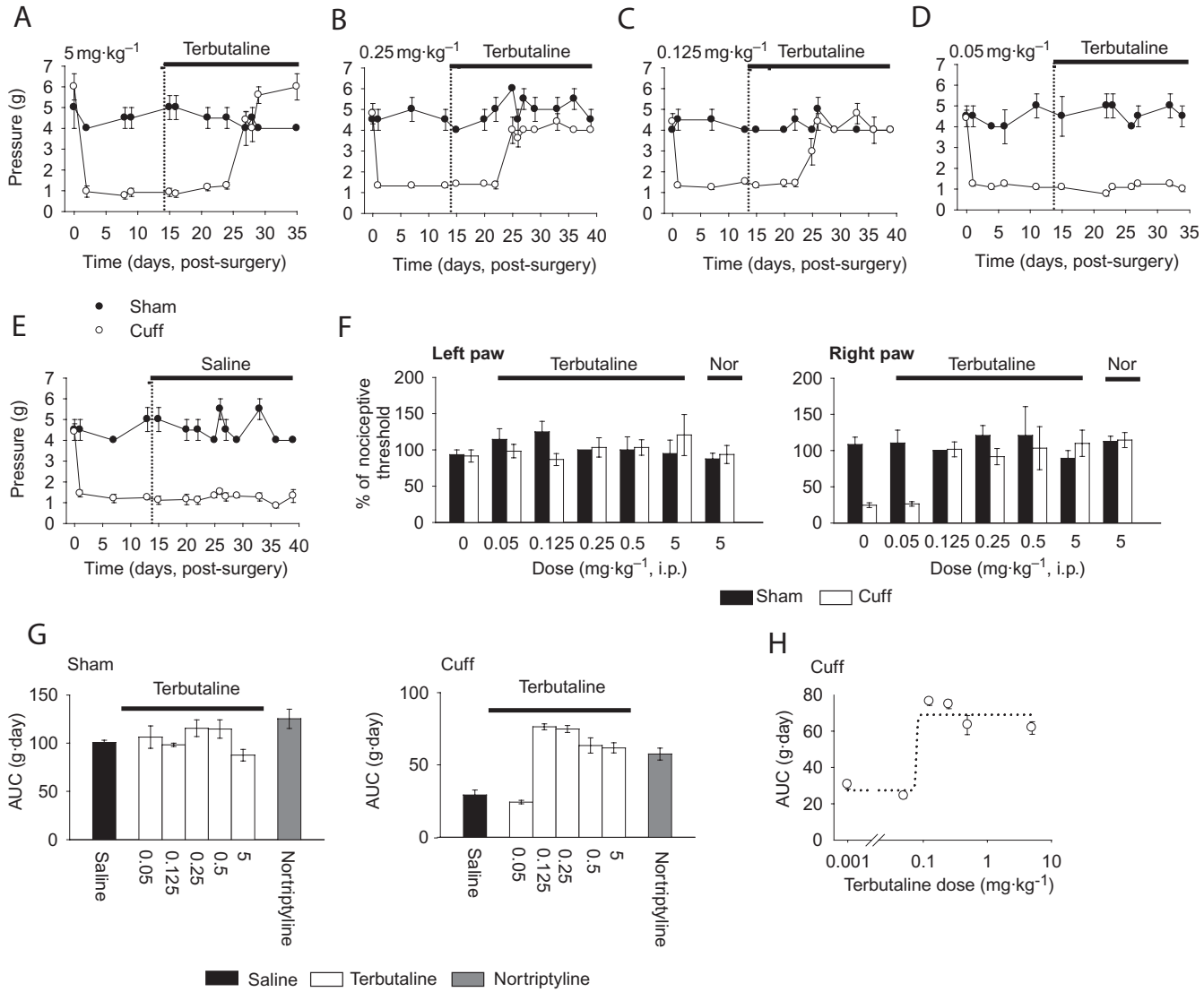

Figure 2 Effects of a range of doses of terbutaline on neuropathic allodynia. The chronic treatments with terbutaline 5 mg·kg^{-1} (A), 0.25 mg·kg^{-1} (B) or 0.125 mg·kg^{-1} (C) suppressed the cuff-induced mechanical allodynia. Treatment with 0.05 mg·kg^{-1} terbutaline (D) or with a 0.9% NaCl solution (E) had no effect on the neuropathic allodynia. (F) Effect of chronic terbutaline treatment on the left hindpaw (left graph) and on the right hindpaw (right graph) of Sham and Cuff mice. These data in Figure 2F are presented as percentage of the nociceptive threshold measured before surgery, and were calculated on the 3rd week of treatment. Results using the tricyclic antidepressant (TCA) nortriptyline (5 mg·kg^{-1}, i.p.; $n = 4$ per group) are presented as reference control. (G) Area under the curve (AUC) from post-surgical day 14 (first day of treatment) until the end of the treatment, for terbutaline (0. 0.05, 0.125, 0.25 and 5 mg·kg^{-1}), and nortriptyline (5 mg·kg^{-1}), for sham mice (left graph) and cuff mice (right graph). (H) Non-linear regression analysis of AUC for terbutaline (0. 0.05, 0.125, 0.25 and 5 mg·kg^{-1}) for Cuff animals. Sham = control groups ($n = 4$ per group); Cuff = neuropathic groups ($n = 5$ per group). Left paw = contralateral to the surgery; right paw = ipsilateral to the surgery.

β_2-adrenoceptors. For this purpose, the mice chronically treated with saline or with terbutaline received a co-administration of the β_2-adrenoceptor antagonist ICI118551 (Figure 3). This co-treatment suppressed the anti-allodynic action of terbutaline at 0.5 mg·kg^{-1} (Figure 3A) (group × time × treatment interaction for the right paw: $F_{3,42} = 8.39$, $P < 0.001$; *post hoc*; cuffTer < sham at $P < 0.0001$ on day 3 of co-treatment with ICI118551) and at 0.125 mg·kg^{-1} (Figure 3B) (group × time × treatment interaction for the right paw: $F_{3,78} = 7.74$ $P < 0.0001$; *post hoc*: cuffTer < sham at $P < 0.0001$ on day 3 of co-treatment with ICI118551). However, ICI118551 had no effect on the withdrawal threshold for the hindpaw contralateral to the surgical procedure, or on the paw withdrawal threshold in Sham mice or in saline-treated mice. In order to eliminate a possible implication of β_1-adrenoceptors in the antiallodynic effect of terbutaline, we gave repeated co-injections of terbutaline (0.125 mg·kg^{-1}) with the selective β_1-adrenoceptor antagonist metoprolol (10 mg·kg^{-1}) for 5 days. This procedure did not modify the anti-allodynic effect of terbutaline (Figure 3C). In a set of mice chronically treated with terbutaline (0.125 mg·kg^{-1}, twice a day for at least 3 weeks), we interrupted the treatment in order to evaluate the delay before spontaneous relapse. This delay was similar to that observed after systemic co-injections with

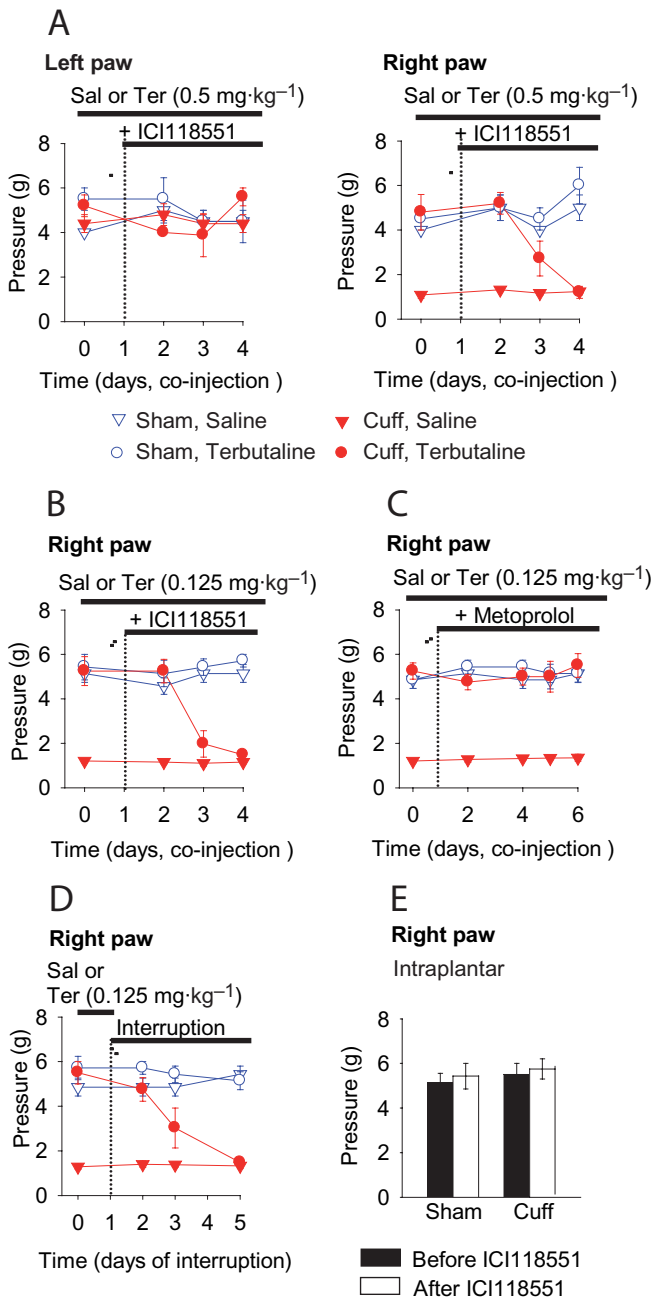

Figure 3 Effect of co-administration of ICI118551 with terbutaline. After 3 weeks of 0.9% NaCl injections, or of 0.5 mg·kg^{-1} terbutaline treatment (A) or 0.125 mg·kg^{-1} terbutaline treatment (B), the β_2-adrenoceptor antagonist ICI118551 (2 mg·kg^{-1}) was co-administered over 4 days. This co-administration suppressed the anti-allodynic action of chronic terbutaline treatment without affecting the mechanical thresholds of the left hindpaw (presented for terbutaline 0.5 mg·kg^{-1} only), and without affecting the mechanical thresholds of the right hindpaw for Sham mice or for saline-treated Cuff mice. (C) Effect of chronic β_1-adrenoceptor antagonist co-treatment on the right hindpaw of Sham and Cuff mice chronically treated with either 0.9% NaCl or 0.125 mg·kg^{-1} terbutaline. (D) Effect of treatment interruption on the nociceptive threshold of the right hindpaws of Sham and Cuff animals chronically treated with either 0.9% NaCl or 0.125 mg·kg^{-1} terbutaline. (E) Effect of repeated intraplantar injections of ICI118551. After 3 weeks of terbutaline treatment (0.125 mg·kg^{-1}, i.p. twice a day), the mice received two intraplantar injections of the β_2-adrenoceptor antagonist ICI118551 in the right hindpaw on the same day, before the morning and before the evening treatments with terbutaline. They were tested for mechanical sensitivity on the following morning. The intraplantar administration of ICI118551 did not affect terbutaline action. Data are expressed as mean ± SEM. Symbols are the same for graphs A–D. Sham = control groups (n = 4–7 per group); Cuff = neuropathic groups (n = 5–8 per group). Left paw = contralateral to the surgery; right paw = ipsilateral to the surgery.

ICI118551 (Figure 3D) (group × time × treatment interaction for the right paw: $F_{3,78} = 3.28$, $P < 0.05$; *post hoc*: cuffTer<sham at $P < 0.001$ on day 3 of withdrawal).

To assess whether peripheral terminals from primary afferents could be involved in the anti-allodynic action of chronic terbutaline (0.125 mg·kg^{-1}, twice a day for at least 3 weeks), we performed repeated intraplantar injections of ICI118551. Our results show no effect of these intraplantar injections on the antiallodynic action of terbutaline (Figure 3E).

Acute injection of β-adrenoceptor agonists has no effect on mechanical allodynia

To assess the acute effects of β-adrenoceptor agonists on allodynia, we tested in separate sets of mice the non-selective β-adrenoceptor agonist bambuterol (0.5 mg·kg^{-1}) and the selective β_2-adrenoceptor agonists salbutamol (2 mg·kg^{-1}), terbutaline (5 mg·kg^{-1}), fenoterol (0.7 mg·kg^{-1}) and salmeterol (1 mg·kg^{-1}). The acute injections were given 2 weeks after surgery, and the mechanical thresholds were evaluated before the injections (0 min) and 30, 60 and 90 min after. The acute injection of these β-adrenoceptor agonists had no significant influence on the mechanical withdrawal response of the hindpaw (Supporting Information Figure S1).

Chronic treatment with β-adrenoceptor agonists alleviates neuropathic allodynia

We then generalized the data obtained with chronic terbutaline treatment to other clinically used β-adrenoceptor agonists (Figure 5A–F). We screened six agonists: bambuterol (0.5 mg·kg^{-1}), salbutamol (2 mg·kg^{-1}), salmeterol (1 mg·kg^{-1}), fenoterol (0.7 mg·kg^{-1}), isoprenaline (0.5 mg·kg^{-1}) and ritodrine (10 mg·kg^{-1}). With each of these drugs, the ipsilateral cuff-induced allodynia disappeared after a sustained treatment: ritodrine ($F_{12,84} = 8.55$; $P < 0.0001$; *post hoc*: cuffRit < shamRit at $P < 0.0001$ on days 1–22), salbutamol ($F_{10,70} = 7.51$; $P < 0.0001$; *post hoc*: cuffSalb < shamSalb at $P < 0.001$ on days 2–24 only), isoprenaline ($F_{11,77} = 4.73$; $P < 0.0001$; *post hoc*: cuffIso < shamIso at $P < 0.05$ on days 1–26 only), fenoterol ($F_{11,66} = 5.81$; $P < 0.0001$; *post hoc*: cuffFen < shamFen at $P < 0.01$ on days 2–24 only), salmeterol ($F_{11,66} = 9.39$; $P < 0.0001$; *post hoc*: cuffSalm < shamSalm at $P < 0.01$ on days 2–28 only) and bambuterol ($F_{10,70} = 4.26$; $P < 0.001$; *post hoc*: cuffBam < shamBam at $P < 0.001$ on post-surgery days 2–24 only). Similar results were observed with data presented as percent of recovery (Figure 5G). No significant effect of the β-adrenoceptor agonists was observed on the contralateral hindpaw of the Cuff mice and on both hindpaws of Sham mice (Figure 5G). We observed no time course difference between the short-acting drug salbutamol, the long-acting

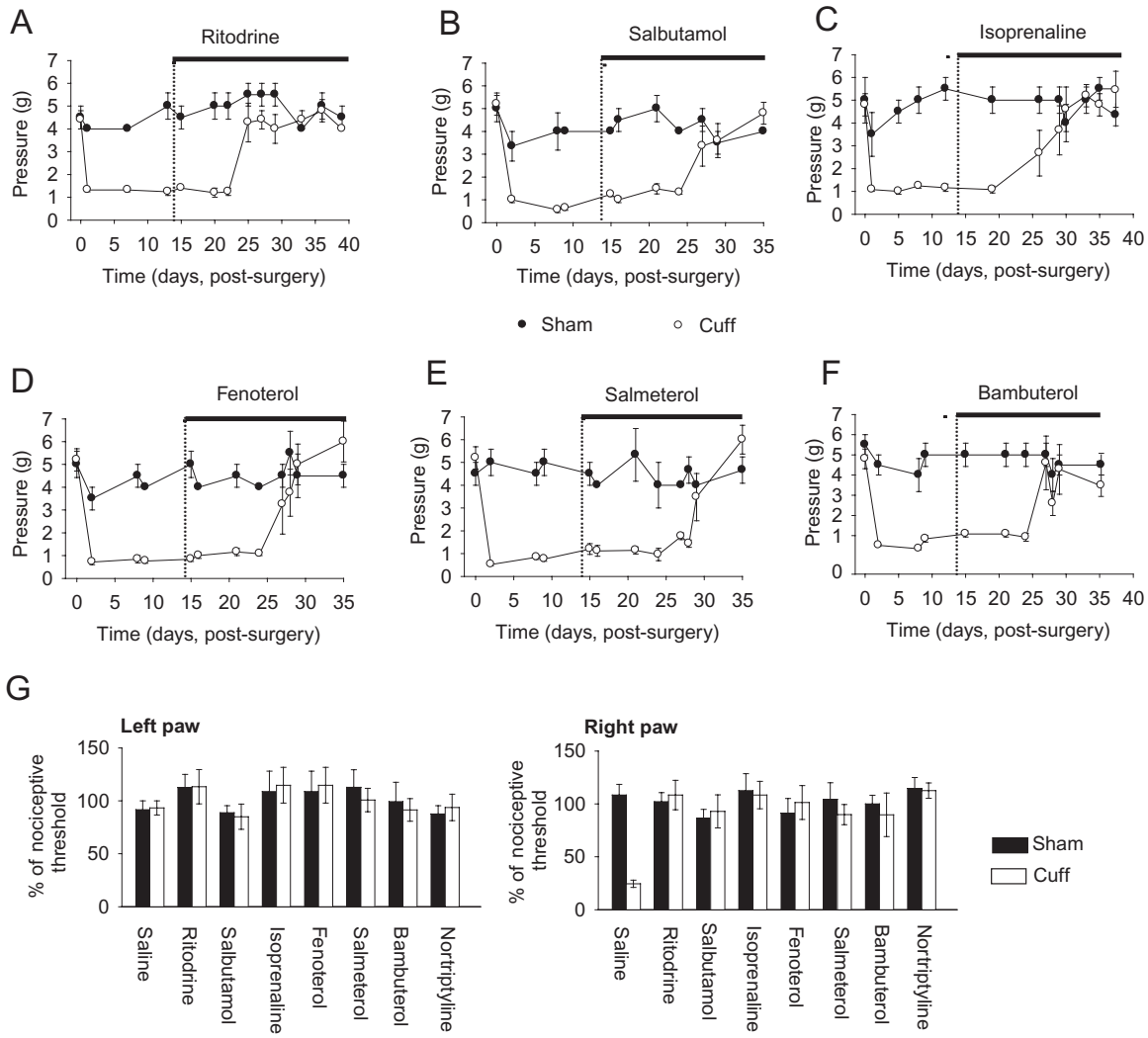

Figure 4 Effect of chronic treatment with β-adrenoceptor agonists on neuropathic allodynia. The chronic treatments with the β-adrenoceptor or β_2-adrenoceptor agonists ritodrine (10 mg·kg^{-1}, i.p.) (A), salbutamol (2 mg·kg^{-1}, i.p.) (B), isoprenaline (0.5 mg·kg^{-1}, i.p.) (C), fenoterol (0.7 mg·kg^{-1}, i.p.) (D), salmeterol (1 mg·kg^{-1}, i.p) (E) or bambuterol (0.5 mg·kg^{-1}, i.p.) (F), suppressed the cuff-induced mechanical allodynia. (G) Effect of chronic β-adrenoceptor or β_2-adrenoceptor agonist treatment on the left hindpaw (left graph) and on the right hindpaw (right graph) of Sham and Cuff mice. Data in Figure 4G are presented as percentage of the nociceptive threshold measured before surgery, and are calculated on the 3rd week of treatment. The agonists are presented in order according to their known half-life. Results using the tricyclic antidepressant (TCA) nortriptyline (5 mg·kg^{-1}, i.p.; $n = 4$ per group) are presented as a reference control. The general procedures are identical to those described in Figure 1. Data are expressed as mean ± SEM. Sham = control groups ($n = 4$ per group); Cuff = neuropathic groups ($n = 5$ per group). Left paw = contralateral to the surgery; right paw = ipsilateral to the surgery.

drugs fenoterol and salmeterol, and the very long-acting drug bambuterol using the AUC of percent change in mechanical nociceptive threshold (Supporting Information Figure S2).

The β_2-adrenoreceptor antagonist ICI118551 blocked the antiallodynic effects of β-adrenoceptor agonists

After chronic treatment with the different agonists, we co-administered the β_2-adrenoceptor antagonist ICI118551 intraperitoneally (Figure 4). We observed that this antagonist ICI118551 blocked the antiallodynic effect of ritodrine (group × time interaction: $F_{1,7} = 36.06$; $P < 0.001$), salbutamol (group × time interaction: $F_{1,7} = 27.77$; $P < 0.01$), isoprenaline (group × time interaction: $F_{1,7} = 7.75$, $P < 0.05$), fenoterol (group × time interaction: $F_{1,7} = 24.97$; $P < 0.01$), salmeterol (group × time interaction: $F_{1,6} = 17.46$; $P < 0.01$) and bambuterol (group × time interaction: $F_{1,7} = 12.38$; $P < 0.01$). No significant effect of this co-treatment was observed on the contralateral hindpaw of Cuff mice or on the right or left hindpaws of Sham mice.

Intrathecal but not intracerebroventricular administration of ICI118551 suppressed the anti-allodynic action of salbutamol

We injected ICI118551 i.t. at lumbar level (3 μg in 10 μL) in animals chronically treated with salbutamol (Figure 6A). This antagonist treatment was without effect on Sham mice, but it reversed the antiallodynic effect of systemically injected salbutamol in Cuff mice (Figure 6A; group × time interaction: $F_{1,7} = 13.47$, $P < 0.01$). In contrast, i.c.v. injections of

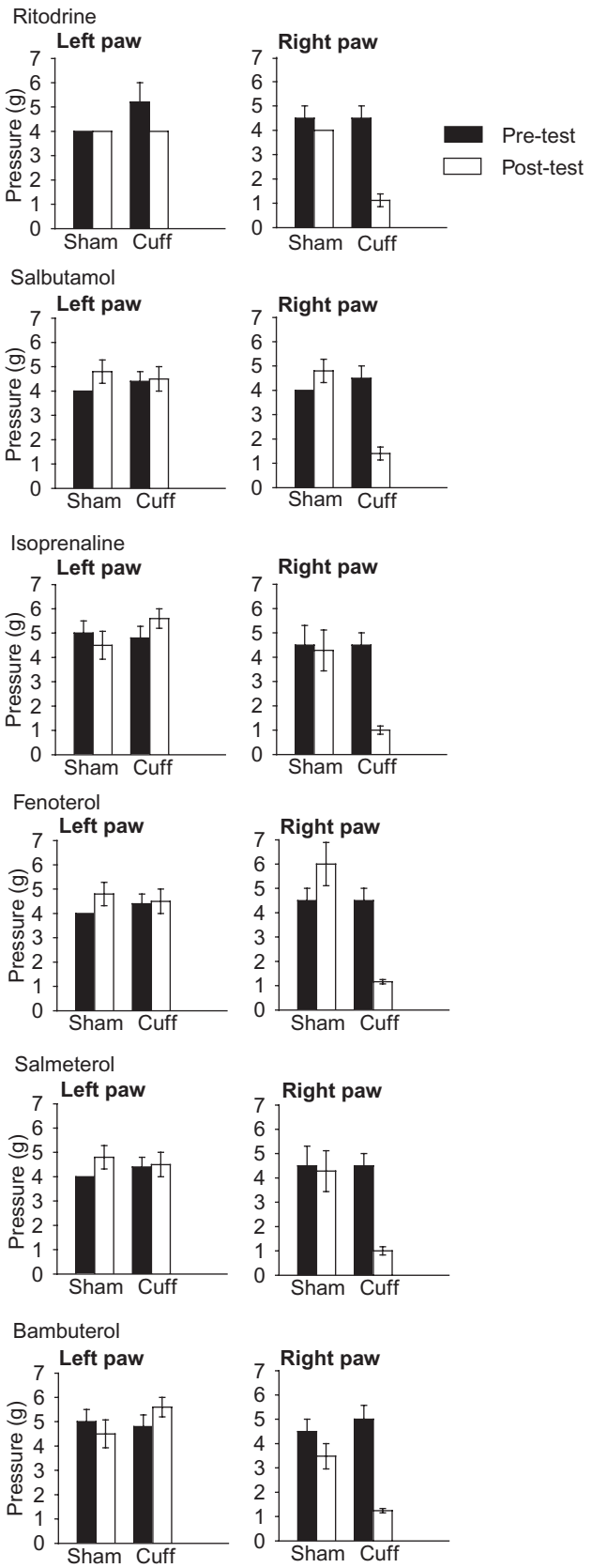

Figure 5 Effect of ICI118551 co-administration with β-adrenoceptor agonists. After 3 weeks of ritodrine (10 mg·kg^{-1}, i.p.), salbutamol (2 mg·kg^{-1}, i.p.), isoprenaline (0.5 mg·kg^{-1}, i.p.), fenoterol (0.7 mg·kg^{-1}, i.p.), salmeterol (1 mg·kg^{-1}, i.p) or bambuterol (0.5 mg·kg^{-1}, i.p.) treatments, the β_2-adrenoceptor antagonist ICI118551 (2 mg·kg^{-1}) was co-administered over 3 days. This co-administration suppressed the anti-allodynic action of each β-adrenoceptor agonist without affecting the mechanical threshold of the left hindpaw and without affecting the mechanical threshold of Sham mice right hindpaw. The results are presented before the co-administration (i.e. after 3 weeks of treatment) (pre-test), and after 3 days of co-administration with ICI118551 (post-test). The general procedures are identical to those described in Figure 1. Data are expressed as mean ± SEM. Sham = control groups (n = 4 per group); Cuff = neuropathic groups (n = 5 per group). Left paw = contralateral to the surgery; right paw = ipsilateral to the surgery.

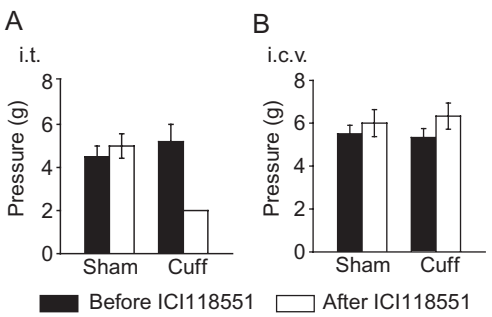

Figure 6 Effect of intrathecal (i.t.) or intracerebroventricular (i.c.v.) administration of ICI118551 on the anti-allodynic action of salbutamol. After 3 weeks of salbutamol treatment (2 mg·kg^{-1}, i.p. twice a day), the mice received two i.t. (A) or i.c.v. (B) injections of the β_2-adrenoceptor antagonist ICI118551 on the same day, before the morning and before the evening treatments with salbutamol. They were tested for mechanical sensitivity on the following morning. Only i.t. administration of ICI118551 resulted in the reversal of the effects of salbutamol and re-emergence of neuropathic allodynia. Data for the right paw (ipsilateral to the surgery) are expressed as mean ± SEM. Sham = control groups (n = 4–5 per group); Cuff = neuropathic groups (n = 5–6 per group).

ICI118551 (10 μg in 2.5 μL) did not modify the antiallodynic effect of salbutamol (Figure 6B).

Chronic treatment with β-adrenoceptor antagonists alone did not alleviate neuropathic allodynia

To assess the consequences of repeated β-adrenoceptor blockade on allodynia, we tested the β-adrenoceptor antagonist propranolol (5 mg·kg^{-1}, twice a day) and the β_2- adrenoceptor antagonist ICI118551 (2 mg·kg^{-1}, twice a day) on separate sets of mice. Contrary to treatment with β_2-adrenoceptor agonists, chronic treatment with propranolol or with ICI118551 did not suppress the cuff-induced allodynia (Supporting Information Figure S3).

Discussion and conclusions

Using a murine neuropathic pain model, we studied the actions of β-adrenoceptor agonists on neuropathic allodynia. Allodynia was suppressed by chronic, but not acute, treatment with β-adrenoceptor agonists. This was observed with

various compounds already used clinically against other pathologies. Studies with terbutaline revealed a therapeutic effect with doses as low as 0.125 $mg \cdot kg^{-1}$. Using a β_2-adrenoceptor antagonist, we demonstrated that the anti-allodynic effect of β-adrenoceptor agonists is mediated by β_2-adrenoceptors are neither supraspinal nor at the peripheral terminals. Moreover, we showed that chronic treatments with β- or β_2-adrenoceptor antagonists had no antiallodynic action. These findings suggest that β_2-adrenoceptor agonists might offer a therapeutic potential for neuropathic pain relief.

TCAs are among the first-line drugs for neuropathic pain management, and the poor efficacy of SSRIs (Attal *et al.*, 2006) suggested a prominent role for the noradrenergic system in relief of neuropathic pain. Both β_1- and β_2-adrenoceptors have been implicated in the acute action of TCA on thermal sensitivity (Micó *et al.*, 1997). α_2-Adrenoceptors were also implicated in the acute action of antidepressant drugs in naïve animals or in acute visceral pain models (Gray *et al.*, 1999; Ghelardini *et al.*, 2000; Ozdoğan *et al.*, 2004). However, these studies did not specifically address the pathology for which the antidepressant drugs are prescribed, nociceptive parameters alleviated in patients, or treatment regimens clinically used. Using a model of peripheral neuropathy, which is sensitive to chronic but not acute antidepressant treatment, we previously demonstrated that β_2-adrenoceptors, but not α_2-, β_1- or β_3-adrenoceptors, were necessary for various antidepressant drugs to alleviate mechanical allodynia after chronic treatment (Yalcin *et al.*, 2009a,b). This provided new insights into the mechanism by which these drugs alleviate neuropathic pain. In the present work, we demonstrated that β_2-adrenoceptor stimulation is sufficient for the anti-allodynic action, and we confirmed that β_1-adrenoceptors do not contribute to this action.

β_2-Adrenoceptors have been already implicated in nociception and pain. They are expressed within the nociceptive system (Nicholson *et al.*, 2005), and genetic studies revealed an association between variants of the β_2-adrenoceptor gene and a chronic musculoskeletal pain disorder (Diatchenko *et al.*, 2006). There is, however, no consensus as to whether β_2-adrenoceptor influence is pro-nociceptive or anti-nociceptive. Adrenaline can induce a mechanical hyperalgesia and sensitize dorsal root ganglia by acting on β-adrenoceptors (Khasar *et al.*, 1999), and a β_2-adrenoceptor antagonist decreased allodynia or hyperalgesia in models of inflammatory pain (Parada *et al.*, 2003; Nackley-Neely *et al.*, 2007). However, reduced acetic acid-induced abdominal constrictions were observed following acute isoprenaline administration (Bentley and Starr, 1986). In our model of neuropathic pain, we showed that chronic blockade of β-adrenoceptors did not affect allodynia. We also showed that an acute administration of β-adrenoceptor agonists did not affect the mechanical sensitivity thresholds. We observed this lack of effect both in control and in neuropathic mice. It confirms that β-adrenoceptor agonists are not acute analgesics. β-Adrenoceptor agonists also have vascular properties that can explain their action on neurovascular deficits in diabetes (Cotter and Cameron, 1998). These peripheral vascular effects are, however, different from the anti-allodynic action observed in our model of neuropathy, as we could block the action of β-adrenoceptor agonists by i.t. delivery of a specific β_2-adrenoceptor antagonist.

Even though the therapeutic onset of antidepressant drugs is faster against neuropathic pain than against depression, a sustained treatment is necessary (Sindrup *et al.*, 2005; Micó *et al.*, 2006; Benbouzid *et al.*, 2008a). Similarly, the onset of action of β-adrenoceptor agonists against neuropathic allodynia requires a sustained treatment. As observed with antidepressant drugs (Yalcin *et al.*, 2009b), i.t. but not i.c.v. blockade of β_2-adrenoceptors suppressed the anti-allodynic action of a β-adrenoceptor agonist. Using a similar protocol, intraplantar blockade of β_2-adrenoceptors did not affect the effects of β-adrenoceptor agonists in our model. The β_2-adrenoceptors responsible for this anti-allodynic action are thus neither supraspinal nor at the level of peripheral terminals. These findings suggest that similar mechanisms are likely to be recruited by antidepressant drugs and by β-adrenoceptor agonists to relieve allodynia. This hypothesis is further supported by similar delays to relapse after either spontaneous treatment withdrawal or ICI118551 co-treatment whether the drug is a β-adrenoceptor agonist (present results) or an antidepressant (Yalcin *et al.*, 2009a,b).

The side effects of TCAs led clinicians to titrate the treatments, starting with low doses and adjusting them until pain is controlled. It was proposed that sustained treatments result in a progressive increase in blood levels until therapeutic levels are reached, which makes the drug half-life critical. Our results do not support such a hypothesis. Indeed, similar delays are observed with short half-life molecules, such as salbutamol and ritodrine (half-life around 3 h), or longer half-life molecules, such as fenoterol or bambuterol (half-life range: 6–17 h). Moreover, the lowest effective dose of terbutaline was 40 times lower than the highest dose, but the therapeutic delay remained similar. This delay is thus not simply related to pharmacokinetics, but the antiallodynic action of antidepressant drugs and of β-adrenoceptor agonists requires a secondary molecular change initiated by β_2-adrenoceptor stimulation.

About 10 days of treatment with a β-adrenoceptor agonist are necessary to reverse the neuropathic allodynia, while it only takes 2 to 3 days of co-treatment with ICI118551 for allodynia to reappear. The long delay for therapeutic onset suggests that β-adrenoceptor agonists act via long-term molecular and neural changes, similarly to antidepressant drugs. Once the animals responded to β_2-adrenoceptor stimulation, the delay to relapse after antagonist co-treatment was shorter, but similar to the delay of spontaneous relapse after treatment withdrawal. This is also what is observed with antidepressant drugs (Yalcin *et al.*, 2009a). This suggests that two levels of changes may be implicated in the anti-allodynic action of both β-adrenoceptor agonists and antidepressant drugs. A first change induced by the treatment may be required to make the system competent, while a second change (also recruited by adrenoceptors) may be necessary to maintain the therapeutic effect. This second (shorter-term) change can be reversed in 2–3 days. Based on knowledge of antidepressant action against depression, we can suspect that the action against neuropathic pain will also imply complex molecular changes. Such downstream molecular changes are, at present, considered as the main explanation for antidepres-

sant action against depression. This complex mechanism implicates changes in chromatin structure, in transcription factor recruitment and in gene expression (Tsankova *et al.*, 2007; Wallace *et al.*, 2009). It also involves the recruitment of neurotrophins, stimulation of dendritic arborization and spines, as well as neurogenesis (Nestler *et al.*, 2002; Coyle and Duman, 2003). The action of antidepressant drugs against neuropathic allodynia was less studied, but we demonstrated that the recruitment of an opioid tone on δ-opioid receptors is necessary to mediate the final anti-allodynic action of nortriptyline (Benbouzid *et al.*, 2008c). Although our data provide evidence for action against neuropathic pain by β-adrenoceptor agonists, further studies will be necessary to fully describe the complex molecular mechanism underlying this anti-allodynic action.

Antidepressant or anticonvulsant drugs are the most efficient treatments against neuropathic pain. Their clinical use is however limited by their side effects. TCAs can induce sedation, dry mouth, constipation, blurred vision, urinary retention, dizziness, tachycardia, cardiovascular risks, memory impairments, delirium, orthostatic hypotension, weight gain, excessive perspiration or sexual dysfunction (McQuay *et al.*, 1996; Maizels and McCarberg, 2005; Sindrup *et al.*, 2005). Side effects of anticonvulsant drugs include somnolence, dizziness, generalized fatigue, ataxia and peripheral edema (Attal *et al.*, 2006). Gabapentin, widely prescribed against neuropathic pain, has a high risk of viral infections and fever (>10%). Carbamazepine has a risk of hepatitis-anaplastic effects or hyponatremia by affecting liver enzymes, blood cells, platelets and sodium levels (Attal *et al.*, 2006). Due to side effects, 10–30% of the patients stop anticonvulsant drug treatments (Morello *et al.*, 1999; Dworkin *et al.*, 2003; 2007). Similar rates of treatment drop-outs are observed due to side effects of antidepressant drugs (Sindrup *et al.*, 2005).

β-Adrenoceptor agonists could offer a therapeutic improvement by reducing side effects at therapeutic doses. They are commonly used against respiratory disorders such as asthma or chronic obstructive pulmonary diseases. They present major side effects at high doses, including cardiac hypertrophy and necrosis. These effects are well studied in experimental conditions within research laboratories, but they are not present at therapeutic doses in clinical conditions. At therapeutic doses, the potential side effects include increased heart rate, palpitations or tremors (Furukawa and Lokewick, 2007), which can be decreased with the most selective β_2-drugs or by lowering the doses. These side effects are mild enough for β-adrenoceptor agonists to be prescribed for respiratory difficulties in children. Our experiments with terbutaline revealed a therapeutic efficacy at a dose of 0.125 $mg \cdot kg^{-1}$, which is compatible with doses clinically used for respiratory conditions. This suggests that β-adrenoceptor agonists could be effective against neuropathic pain at doses already used in other pathological conditions in patients.

In conclusion, β_2-adrenoceptor stimulation is not only necessary for the anti-allodynic action of antidepressant drugs, but it is also sufficient to relieve allodynia in a model of neuropathic pain. Effective doses of terbutaline were close to those of other β-adrenoceptor agonists in current clinical use. Potentially, β_2-adrenoceptor agonists could thus offer a therapeutic alternative to TCAs for neuropathic pain management. It is important to note that our study was conducted in an animal model and the conclusions now require clinical validation.

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Conflicts of interest

The CNRS have protected data presented within this manuscript by a patent (BF0800273) for which Dr Choucair-Jaafar, Dr Yalcin, Professor Freund-Mercier and Dr Barrot are among the co-inventors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Acute effect of β-adrenoceptor agonists on the mechanical nociceptive threshold and on neuropathic allodynia. The acute injection of 0.9% NaCl, of the non-selective β-adrenoceptor agonist bambuterol (0.5 mg·kg^{-1}, i.p) or of the selective β_2-adrenoceptor agonists salbutamol (2 mg·kg^{-1}, i.p.), terbutaline (5 mg·kg^{-1}, i.p.), fenoterol (0.7 mg·kg^{-1}, i.p) and salmeterol (1 mg·kg^{-1}, i.p) had no effect on the mechanical threshold for hindpaw withdrawal and on the neuropathic allodynia. The nociceptive mechanical threshold was measured before and 30, 60 and 90 min after the first administration of the agonists. The general procedures are identical to those described in Figure 1. Data are expressed as mean ± SEM. Symbols are the same for all graphs. Sham = control groups (n = 4 per group; except for bambuterol, n = 5); Cuff = neuropathic groups (n = 5 per group; except for bambuterol, n

= 7). Left paw = contralateral to the surgery; Right paw = ipsilateral to the surgery.

Figure S2 Time course of cumulated area under the curve of mechanical nociceptive threshold percent change. In Sham (left graph) and Cuff (right graph) animals, the results are presented for the short-acting drug salbutamol, the long-acting drugs fenoterol and salmeterol, and the very long-acting drug bambuterol. The pretreatment values were used as reference to calculate the percent change. We observed no time-course difference between salbutamol, fenoterol, salmeterol and bambuterol treatments. Sham = control groups (n = 4 per group); Cuff = neuropathic groups (n = 5 per group).

Figure S3 Effect of chronic treatment with the β-adrenoceptor antagonist propranolol or with the β_2-adrenoceptor antagonist ICI118551. The chronic treatment with propranolol (5 mg·kg^{-1} twice a day, i.p.) (A) or with ICI118551 (2 mg·kg^{-1} twice a day, i.p.) (B) did not suppress the cuff-induced allodynia. The general procedures are identical to those described in Figure 1. Data are expressed as mean ± SEM. Sham = control groups (n = 4 per group); Cuff = neuropathic groups (n = 5 per group). Left paw = contralateral to the surgery; right paw = ipsilateral to the surgery.